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TITLE: Living in the endosymbiotic world of *Wolbachia*: A centennial review

Short Title: The *Wolbachia* World

Authors: Rupinder Kaur[1,2*], J. Dylan Shropshire[1,2], Karissa L. Cross[1,2], Brittany Leigh[1,2], Alexander J. Mansueto[1,2], Victoria Stewart[1,2], Sarah R. Bordenstein[1,2], and Seth R. Bordenstein[1,2,3,4*]

[1]Department of Biological Sciences, Vanderbilt University, Nashville, TN 37235, USA
[2]Vanderbilt Microbiome Initiative, Vanderbilt University, Nashville, TN 37235, USA
[3]Department of Pathology, Microbiology, and Immunology, Vanderbilt University, Nashville, TN 37235, USA
[4]Vanderbilt Institute for Infection, Immunology, and Inflammation, Vanderbilt University Medical Center, Nashville, TN 37235, USA

***Correspondence to:**
Rupinder Kaur, Nashville, TN, 37235, email: rupinder.kaur@vanderbilt.edu
Seth Bordenstein, Nashville, TN, 37235, email: s.bordenstein@vanderbilt.edu

Abstract

The most widespread intracellular bacteria in the animal kingdom are maternally-inherited endosymbionts of the genus *Wolbachia*. Their prevalence in arthropods and nematodes worldwide and stunning arsenal of parasitic and mutualistic adaptations make these bacteria a biological archetype for basic studies of symbiosis and applied outcomes for curbing human and agricultural diseases. Here, we conduct a summative, centennial analysis of living in the *Wolbachia* world. We synthesize literature on *Wolbachia*'s host range, phylogenetic diversity, genomics, cell biology, and applications to filarial, arboviral, and agricultural diseases. We also review the mobilome of *Wolbachia* including phage WO and its essentiality to hallmark phenotypes in arthropods. Finally, the *Wolbachia* system is an exemplar for discovery-based science education using biodiversity, biotechnology, and bioinformatics lessons. As we approach a century of *Wolbachia* research, applications, and education, the interdisciplinary science and knowledge

from this symbiosis stand as a model for consolidating and teaching the integrative rules of endosymbiotic life.

Keywords: *Wolbachia*, phage WO, cytoplasmic incompatibility, male killing, feminization, parthenogenesis, evolution, vector control

ORCID ID: Rupinder Kaur (https://orcid.org/0000-0002-2132-8107),
J. Dylan Shropshire (https://orcid.org/0000-0003-4221-2178),
Karissa L. Cross (http://orcid.org/0000-0001-5618-7642),
Brittany Leigh (https://orcid.org/0000-0001-5668-9925),
Alexander J. Mansueto (https://orcid.org/0000-0003-0936-7362),
Victoria Stewart (https://orcid.org/0000-0002-2475-1648),
Sarah Bordenstein (https://orcid.org/0000-0001-6092-1950),
Seth R. Bordenstein (https://orcid.org/0000-0001-7346-0954)

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Introduction

The central role of microbial symbiosis in the origin of eukaryotic cell, species formation, ecological interactions, and the activities of animal and plant biology across the biosphere is increasingly clear (Bordenstein and Theis, 2015; Corliss and Margulis, 1972; López-García et al., 2017; McFall-Ngai, 2008; Raina et al., 2018). The intimate relationships between the microbiological and macrobiological worlds are important for generating evolutionary adaptations, assisting host health and performance, and causing disease (Hurst, 2017; Kiers and West, 2015). The most prevalent symbiotic microbe in the animal world, wherein rules of interspecies engagement and imperatives for research may form, is the Alphaproteobacterium *Wolbachia pipientis* (Taylor et al., 2018). First described as a *Rickettsia*-like organism in the gonads of various insects including *Culex pipiens* mosquitoes nearly a century ago (Hertig and Wolbach, 1924), *W. pipientis* was named in 1936 by entomologist Dr. Marshall Hertig in recognition of his Ph.D. advisor, Dr. Simeon Burt Wolbach and the *C. pipiens* mosquito (Hertig, 1936). These gram-negative, maternally-transmitted bacterial endosymbionts belong to the Anaplasmataceae family within the order Rickettsiales (**Figure 1a**). Since *W. pipientis* is currently the only member of its genus, it is conventionally referred to as *Wolbachia* (Newton and Slatko, 2019).

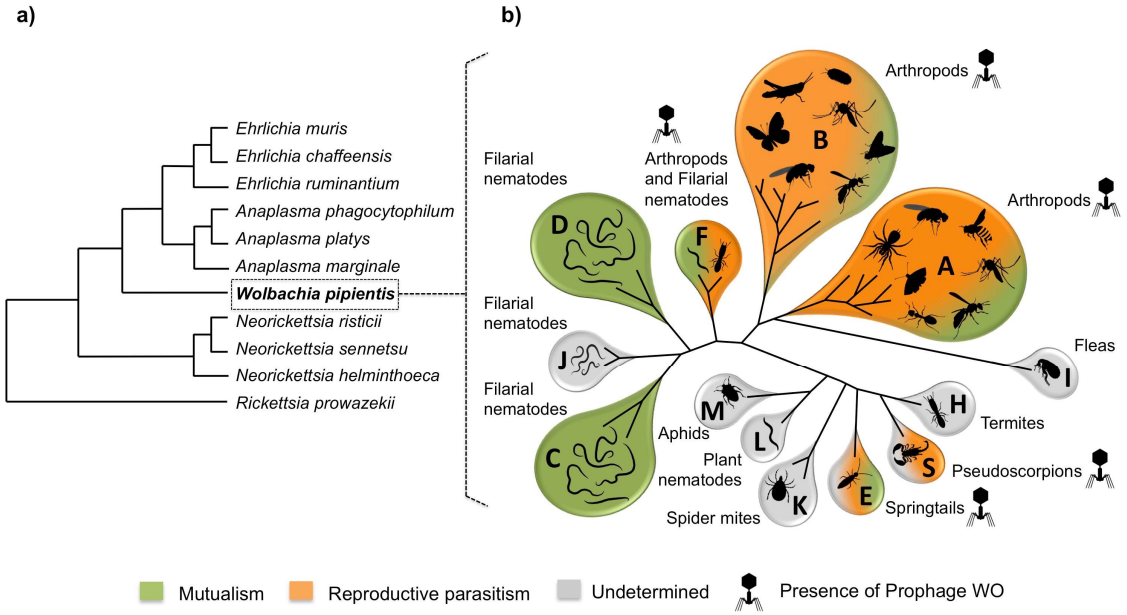

Figure 1. Phylogeny and evolution of *Wolbachia*. Schematic representation of a) phylogenetic relationships of *Wolbachia* and members of the family Anaplasmataceae, with *Rickettsia* shown as an

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outgroup, and b) an unrooted 16S rRNA-based consensus phylogenetic tree of the major and well-established *Wolbachia* supergroups. The phylogenetic positions of the supergroups are currently tentative based on previously-published single gene and multigene analyses. Colors correspond to different patterns of host-*Wolbachia* associations across the supergroups. Supergroup E is multicolor labeled since the evidence for *Wolbachia*-associated phenotypes in the host are suggestive but inconclusive. A phage symbol represents *Wolbachia* supergroups with genomic evidence of intact or relic prophage WO presence. Specific examples of phage-association and reproductive phenotypes are listed in **supplemental table 2**.

In the 1990's, entomologists and microbiologists revealed at least 16% of arthropod species harbor *Wolbachia* (O'Neill et al., 1992; Werren et al., 1995). As costs for molecular biology techniques plummeted, surveys and detailed investigations of *Wolbachia* surged in a stunning variety of hosts including insects, spiders, mites, and terrestrial isopods, as well as filarial and plant nematodes (Brown et al., 2016; Hilgenboecker et al., 2008; Scholz et al., 2020; Werren et al., 2008). Current estimates suggest ~50% of arthropod and several filarial nematode species harbor *Wolbachia* (Lefoulon et al., 2016; Weinert et al., 2015). Since arthropods represent ~85% of all animal species, tens of millions of species with *Wolbachia* are estimated to occur.

A century in the making, the knowledge in this review serves as a summative gateway to six major topics that underpin the biology of one of the greatest endosymbioses in the biosphere: (i) Genome evolution and diversity; (ii) Mobile elements including temperate phage as a hotspot for genetic novelty and key functions, transposons, and a putative plasmid; (iii) Cell biology spanning tissue tropism and transmission routes; (iv) Hallmark phenotypes, genes, and mechanisms crossing the continuum of symbiotic functions; (v) Diverse and fundamental impacts on host counter-adaptations, extinction, and speciation; and (vi) Translational applications for positive outcomes on human health and agriculture. We also highlight the worldwide impact of *Wolbachia* research on science outreach via Discover the Microbes Within! The *Wolbachia* Project (Lemon et al., 2020). Finally, throughout this centennial review, we highlight future research directions to improve our understanding of living in the *Wolbachia* world.

Wolbachia diversity, evolutionary history, and genomics

Diversity

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Wolbachia genetic diversity was initially characterized using the 16S rRNA gene (O'Neill et al., 1992) and the more variable surface protein gene *wsp* (Zhou and Neill, 1998). However, the slow evolutionary rate of 16S rRNA and extensive *wsp* recombination posed subsequent challenges to resolving *Wolbachia* strains and phylogenies (Werren and Bartos, 2001). Therefore, a multilocus sequence typing (MLST) system comprised of a set of five or more conserved housekeeping genes was established as a standard for *Wolbachia* classification (Baldo et al., 2006). MLST studies supported the discoveries of new phylogenetic lineages, and further classification of *Wolbachia* genus into genetically-distinct, monophyletic lineages named "supergroups" (Zhou et al., 1998). More recently, with the aid of genome sequences, the utility of the MLST loci has been found to insufficiently type *Wolbachia* relative to better-performing single copy loci (Bleidorn and Gerth, 2018). To date, *Wolbachia* are subdivided into at least 17 possible phylogenetic supergroups (named A-F, H-Q, and S, **Figure 1b**) (Glowska et al., 2015; Lefoulon et al., 2020; Wang et al., 2016). Notably, the vast majority of *Wolbachia* genome sequences are from strains of the A and B supergroups, and some supergroups are represented by a single strain only (**Supplementary table 1**).

Supergroups A and B mostly occur in arthropods and often function as reproductive parasites (Lo et al., 2007; Vandekerckhove et al., 1999) that manipulate host reproduction to increase their own spread through the matriline. Conversely, supergroups C and D are obligate mutualists that enhance the fertility and development in filarial nematodes (Bandi et al., 1998). Supergroup E *Wolbachia* infect primitively wingless arthropods, the springtails (Czarnetzki and Tebbe, 2004; Vandekerckhove et al., 1999), although its phylogenetic position is still under investigation because marked divergence from other *Wolbachia* supergroups prevents confident, phylogenetic placement (Bordenstein et al., 2009; Brown et al., 2016). Supergroup F *Wolbachia* associate with both arthropods (termites) and filarial nematodes (*Mansonella ozzardi)* (Casiraghi et al., 2006). Supergroup H, I, J, and K occur in termites (Bordenstein and Rosengaus, 2005), fleas (Gorham et al., 2003), filarial nematodes (Casiraghi et al., 2004) and spider mites (Ros et al., 2009), respectively. Supergroup L *Wolbachia* occur in the plant-parasitic nematode *Pratylenchus penetrans* (Haegeman et al., 2009), and supergroup M occurs in aphids (Brown et al., 2016). Supergroup S is in pseudoscorpions (Lefoulon et al.,

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2020), and a putative Supergroup T may occur in the bedbugs of *Cimex hemipterus* (Laidoudi et al., 2020).

Although commonly accepted, the methodologies used to classify *Wolbachia* supergroups have infrequent discrepancies (Bleidorn and Gerth, 2018; Kaur et al., 2017). For instance, Supergroup G *Wolbachia* based on *wsp* sequences from Australian spiders (Rowley et al., 2004) was later determined to belong to supergroup B (Baldo and Werren, 2007), and supergroup R *Wolbachia* based on 16S rRNA and MLST genes from cave spiders (Wang et al., 2016) likely belongs to supergroup A (Gerth, 2016). In summary, our understanding of *Wolbachia*'s genetic diversity is still developing, and the occurrence of unresolved or single, long branches within the *Wolbachia* tree of life necessitates more data and analyses. Indeed, both target enrichment protocols (Hotopp et al., 2017; Kent and Bordenstein, 2011) and whole-genome typing methods (Gerth and Bleidorn, 2016) can be used across diverse arthropod species and environments to discover and delineate *Wolbachia* genomic diversity and phylogenetic classification.

Evolutionary history

Wolbachia belong to the family Anaplasmataceae, order Rickettsiales, and class Alphaproteobacteria (Dumler et al., 2001) (**Figure 1a**). Sister genera to *Wolbachia* include *Ehrlichia, Anaplasma,* and *Rickettsia* endosymbionts (Duron et al., 2008). *Rickettsia*, like *Wolbachia*, are reproductive parasites that can cause parthenogenesis in parasitoid wasps (Giorgini et al., 2010) and male killing in ladybird beetles and leaf miners (Lawson et al., 2001; Werren et al., 1994). However, unlike these related genera, *Wolbachia* have not been discovered in vertebrates.

The utility of the *Wolbachia* model to assess directional shifts in the evolution of endosymbiont genome sizes (reduction versus expansion), host ranges (arthropods versus nematodes), and phenotypes (mutualism versus parasitism) has not gone unnoticed. While various studies have attempted to phylogenetically determine these evolutionary transitions by rooting the *Wolbachia* tree with the closest sister genera (Bordenstein et al., 2009; O'Neill et al., 1992), severe systematic errors associated with the extensive divergence between *Wolbachia* and its closest relatives cause long-branch attraction artifacts that lead to either gross overestimates of the root or ambiguous results for the rooted phylogenetic tree (Bordenstein et al., 2009). Defining a confidently rooted *Wolbachia* tree is paramount

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for answering questions such as: What is *Wolbachia*'s original host? What is the direction of ecological and evolutionary changes in host ranges, genome sizes and mobile element acquisitions? Does genome evolution increase or decrease in size over time? Rooting the *Wolbachia* tree remains one of the major goals for the field and will require more suitable and closely-related outgroups.

Moreover, dating the major divergence events between *Wolbachia* supergroups is an emerging area of research. For example, by extrapolating observed substitutions in the *ftsZ* gene (a rapidly-evolving, cell-cycle gene), the last common ancestor of arthropod supergroups A and B was estimated to be 58–67 million years ago (Ma) (Werren et al., 1995). With a similar approach, supergroups A and B were estimated to diverge from nematode supergroups C and D ~100 Ma (Bandi et al., 1998). Genome-scaled *Wolbachia* datasets were calibrated using putative codivergence times with hosts from the bee genus *Nomada* (Hymenoptera, Anthophila) and provided an extended timeframe of ~200 Ma for the divergence event splitting supergroups A and B (Gerth and Bleidorn, 2016). Notably, this older dating suggests an evolutionary alignment of the origin of *Wolbachia* in arthropods with the diversification of many insect lineages in which *Wolbachia* occur. Investigation of *Wolbachia*'s evolutionary history remains a central research focus, and genomic data from more host taxa as well as alternative calibration priors may help pinpoint *Wolbachia's* evolutionary history and timing.

Genomics

The first sequenced genome in the genus was from supergroup A *w*Mel strain in *Drosophila melanogaster*, which revealed a small 1.3 megabase (Mb) genome stunningly littered with mobile genetic elements (Wu et al., 2004). The genome sequences from *w*Bm of *Brugia malayi* (Foster et al., 2005), *w*Pip of *C. pipens* (Klasson et al., 2008), *w*Ri of *D. simulans* (Klasson et al., 2009), and *w*AlbB of *Aedes albopictus* (Mavingui et al., 2012) followed thereafter. Twenty-six complete *Wolbachia* genomes are available to date (**Supplementary table 1**), and recently, a new analysis expanded this number by assembling over one thousand more *Wolbachia* genomes from different arthropod and nematodes species (Scholz et al., 2020). Using this increasing array of *Wolbachia* genomes, comparative analyses have identified strain-specific differences, significant genes involved in altering host biology, and evolutionary relationships at a genome level (Brownlie et al., 2007;

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Cerveau et al., 2011; Ioannidis et al., 2007; Ishmael et al., 2009; LePage et al., 2017; Newton et al., 2016; Perlmutter et al., 2019; Rice et al., 2017; Salzberg et al., 2005; Sutton et al., 2014).

Wolbachia's pangenome is subdivided into a typical core genome of genes shared by all sequenced strains and an accessory set of genes not present in all genomes (Hogg et al., 2007; Tettelin et al., 2008). For instance, the Octomom region is part of the *Wolbachia* accessory genome since it is variably present in *Wolbachia* strains and can be subjected to horizontal gene transfer between *Wolbachia* and host mosquitoes (Chrostek et al., 2013; Woolfit et al., 2009). The full Octomom region contains eight genes whose copy number is a driver of *Wolbachia*-mediated host pathogenesis (Chrostek and Teixeira, 2015; Duarte et al., 2020, but see Rohrscheib et al., 2016). Other important accessory genes or regions include Type IV secretion systems (T4SS) and Ankyrin repeat containing proteins (ANK). T4SS are a part of the transport system employed by the bacteria to carry out exchange of various biological molecules including DNA, DNA–protein complexes, and proteins across the cell envelope of the bacteria (Grohmann et al., 2018; Bhattacharya and Newton, 2019). ANKs are ~33 residue repeats that play a major role in protein–protein interactions in eukaryotic cells (Duron et al., 2007a; Walker et al., 2007). Using microarray-based comparative genomic hybridization, a large number of accessory and rapidly-evolving genes were identified, especially in prophage WO region (see Phage WO section below) among closely-related *Wolbachia* supergroup A strains (Ishmael et al., 2009). Prophage WO genes account for 21–87% of the absent or divergent genes in the genomes of this study, as phages often evolve faster than their bacterial genomes owing to rapid gene gains and losses, high rates of inter- and intragenic recombination, and frequent transfers between co-infecting *Wolbachia* (Bordenstein and Wernegreen, 2004; Gavotte et al., 2004, 2007; Kent and Bordenstein, 2010). These elevated genomic flux levels can bestow genetic and phenotypic diversity to *Wolbachia* and can contribute to its spread and success as one of the most abundant intracellular bacteria in animals. However, understanding the functional and adaptive significance of accessory and core genes in driving *Wolbachia* genome evolution remains largely a nascent, but exciting effort.

Genome size variation

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Theory predicts that relaxed selection from long-term associations and genomic redundancy between host and endosymbiont can cause a reduction in genome size of endosymbionts over evolutionary time (McCutcheon and Moran, 2012; Moran et al., 2008). The extent of the genome size reduction depends on whether the endosymbionts are strictly vertically-inherited or horizontally-transmitted to varying degrees (Newton and Bordenstein, 2011). Indeed, strictly vertically-inherited *Wolbachia* that are mutualistic in nematodes have a complete genome size ~30% smaller (size range: 0.96-1.1 Mb) than those that are parasitic in arthropods which occasionally host-switch and often uptake bacteriophage WO DNA (size range: 1.2-1.8 Mb, complete genomes only, **Supplementary table 1**). Arthropod *Wolbachia* also have many repetitive regions and unusually high numbers of mobile elements presumably leading to their genome expansion (Cerveau et al., 2011; Cordaux, 2008; Cordaux et al., 2008; Wu et al., 2004). Genomes of nematode *Wolbachia,* however, lack prophage WO and harbor few mobile elements, repetitive DNA, and pseudogenes (Foster et al., 2005). Despite their smaller genomes, nematode *Wolbachia* retain essential genes encoding enzymes for biosynthetic pathways used in the production of nutrients and metabolites that are needed by the nematode hosts, enforcing their mutualistic relationship (Bandi et al., 1998; Langworthy et al., 2000; Newton and Rice, 2020).

Among the arthropod *Wolbachia* genomes, *w*Fol from supergroup E in the parthenogenetic collembolan springtail *Folsomia candida* has the largest complete *Wolbachia* genome at 1.8 Mb, and notably over a quarter of the genome is comprised of prophage WO genes (Kampfraath et al., 2019). Despite having a large genome size, which is typical of non-obligatory *Wolbachia* in arthropods, *w*Fol appears obligate to its host since elimination by heat or antibiotic treatment renders the host eggs non-viable (Pike and Kingcombe, 2009), though off-target effects of these treatments may impact other beneficial symbionts as well. In the future, comparative phylogenomics and experimental approaches must be enhanced in order to identify the general direction of evolution in *Wolbachia* genome size, along with the selective pressures and host-*Wolbachia* interaction processes that drive these genome size changes.

Mobilome: The master manipulator phage WO, transposons, and a putative plasmid

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Phage WO

Phage WO particles in *Wolbachia* were first observed in electron micrographs of *C. pipiens* mosquito ovaries that showed “spheroidal or polyhedral structures” (Wright et al., 1978). Knowledge on this virus remained elusive until a partial prophage region of *Wolbachia*-infected *Teleogryllus taiwanemma* crickets (*w*Tai) was sequenced over 20 years later and formally named phage WO (Masui et al., 2000). Phage WO is temperate, and thus exists in two states: lytic phage particles and lysogenic prophages that are stably integrated into and replicate with the *Wolbachia* chromosome (**Figure 2a**). Prophage WO occurs in the genomes of at least five *Wolbachia* supergroups including A, B, E, F and S (Bordenstein and Wernegreen, 2004; Gerth et al., 2014; Kampfraath et al., 2019; Lefoulon et al., 2020; Vaishampayan et al., 2007) (**Figure 1b, Supplementary table 2**) with the known majority occurring and frequently exchanged within the highly studied A and B supergroups in arthropods (Bordenstein and Wernegreen, 2004; Wang et al., 2016). Prophage WO does not occur in filarial nematode supergroups C and D (Fenn and Blaxter, 2006; Foster et al., 2005).

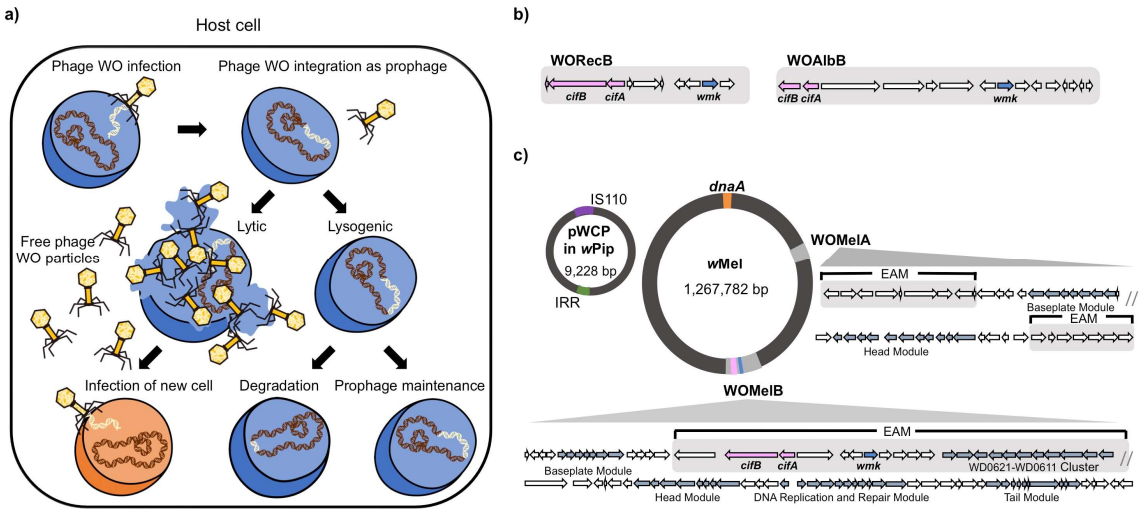

Figure 2. The tripartite symbiosis of bacteriophage WO, *Wolbachia*, and arthropods. a) Schematic representation of two phage WO life cycles – lytic and lysogenic - in *Wolbachia* cells (blue and orange) living in endosymbiosis within an arthropod host cell. During the lytic cycle, phage WO particles are produced and can exit and enter *Wolbachia* cells to establish new infections, while in the lysogenic cycle, prophage WO is stably integrated into the *Wolbachia* chromosome. b) Relic prophage WO regions, such as in the phage regions of WORecB and WOAlbB, likely arose by invasion of a fully intact phage WO, chromosome integration, and erosion over time that led to loss of

structural genes and the ability to form phage particles. In a process termed domestication, *Wolbachia* can retain selected phage-associated genes for adaptive functions such as cytoplasmic incompatibility encoded by the *cifA* and *cifB* genes and male killing encoded by the candidate gene *wmk*. c) Genomic maps of the newly-discovered pWCP plasmid in the *w*Pip *Wolbachia* strain of mosquitoes and the *w*Mel chromosome from flies with its two integrated prophages, WOMelA and WOMelB. Prophage regions are highlighted in light gray on the *Wolbachia* chromosome, phage structural genes are in dark gray, and key reproductive parasitism genes, *cifA;cifB* (pink) and *wmk* (blue), are highlighted in their respective colors within the eukaryotic association module (EAM). pWCP and phage WO are absent in the nematode *Wolbachia*.

When phage WO enters the lytic lifecycle to copy and disperse itself (**Figure 2a**), the particles assemble into standard phage structures including an icosahedral head ~20-45nm in diameter, often with a short tail (Bordenstein et al., 2006; Masui et al., 2001; Sanogo and Dobson, 2006). The WO head harbors a dsDNA genome that varies in size and content (Kent et al., 2011) and undergoes inversion upon integration, allowing for practical discrimination of WO in its prophage versus active phage form within a single sample (Bordenstein and Bordenstein, 2016). During lysis, phage WO particle production corresponds with canonical features of bacterial lysis including the breakage of cell membranes and degradation of DNA, leading to *Wolbachia* death and an acute lytic event that releases numerous WO progenies at once (Bordenstein et al., 2006; Masui et al., 2001). Relic prophage WO, defined as truncated or mutated prophages in *Wolbachia* genomes, are also common and are presumably not capable of producing particles (**Figure 2b**). Recombination events within bacterial genomes and genomic deletions can also result in fragmentation of intact prophages that make them inactive (i.e. unable to produce viral particles) and fully domesticated as relic prophages (Canchaya et al., 2004; Metcalf et al., 2014). This domestication can be advantageous for the bacteria because although a relic prophage may not produce particles, its remaining genes may still produce protein products, such as prophage WO-encoded accessory genes for cytoplasmic incompatibility and male killing (Beckmann et al., 2017; LePage et al., 2017; Perlmutter et al., 2019).

Phage WO is a interesting virus because it may have contend not only with escaping bacterial cell membranes, but also the encapsulating arthropod-derived membranes that surround the bacterial cell (Bordenstein and Bordenstein, 2016; Kent and Bordenstein, 2010). It has been hypothesized that this “two-fold cell membrane challenge” may exert a unique set of selective pressures on phage biology and evolution (Bordenstein and Bordenstein, 2016). The novel composition of putative lysis genes includes a prevalent patatin gene (Kent and Bordenstein,

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2010) typically found in potato tubers known to have insecticidal activity (Strickland et al., 1995) as well as an ankyrin repeat protein and hypothetical protein which are located downstream from the tail module. Such enzymes may lyse the cell membrane in a novel manner and aid in the navigation of phage WO across diverse cell membranes. Moreover, a suite of EAM genes is predicted to or directly interact with eukaryotic hosts, and many of these genes contain domains with putative or validated eukaryotic interactions and motifs with eukaryotic protease cleavage sites. Depending upon the strain, phage WO's EAM constitutes approximately 30-70% of the phage genome (Bordenstein and Bordenstein, 2016) and contains the *cif* and *wmk* genes (**Figure 2c**) (Beckmann et al., 2017; LePage et al., 2017; Perlmutter et al., 2019). Genes within the EAM may also be eukaryotic-like in length (i.e., ~3x larger than the typical size of phage genes) and polyvalent such that they consist of multiple domains within a single coding region (Bordenstein and Bordenstein, 2016; Lindsey et al., 2018a). Combining different domains forged one of the largest bacteriophage genes to date, sized at 14.3 Kb (Bordenstein and Bordenstein, 2016).

Genes found within the EAM vary across prophage WO variants and encompass proteins with domains such as ankyrins that are predicted to interact with animal proteins, a latrotoxin C-terminal domain, which is a crucial component of the black widow spider venom, and NACHT domains that signal programmed cell death (Bordenstein and Bordenstein, 2016). Though the origin of EAM genes remains to be fully resolved, evidence suggests multiple, previously-undescribed horizontal gene transfer events between arthropod hosts and phage WO (Bordenstein and Bordenstein, 2016). The direction of transfer is not necessarily unidirectional, with WO genes identified in uninfected *Chorthippus parallelus* grasshoppers (Funkhouser-Jones et al., 2015) and, more recently, EAM pseudogenes in the terrestrial isopod *Trachelipus rathkei* (Russell et al., 2020). Two-way transfer of DNA between phages and animal hosts is fairly novel, and the mechanism of how it occurs is ripe for investigation and discovery.

Transposons

The genomes of *Wolbachia* harbor a high frequency of transposable elements, including many insertion sequences (IS) that can occupy 10% of the bacterial genome in some cases (Cordaux, 2008; Ling and Cordaux, 2010; Wu et al., 2004). Group II introns that are self-splicing, mobile ribozymes are also exceptionally

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abundant in *Wolbachia* relative to both endosymbiotic and free-living bacterial genomes; they can be involved in *Wolbachia* genomic rearrangements and extensive horizontal gene transfers (Leclercq et al., 2011). Transposable elements generally cause a large amount of bacterial genome variability and have proven very useful for discriminating very closely related strains of *Wolbachia* (Duron et al., 2005; Kaur et al., 2017; Siguier et al., 2014). They also play roles in the evolution of *Wolbachia* genomes by inserting into and disrupting genes such as the *Wolbachia* surface protein *wspB* (Sanogo et al., 2007) and by potentially assisting the movement of *cifA* and *cifB* genes within or between *Wolbachia* genomes (Cooper et al., 2019).

Putative plasmid

The mobilome of *Wolbachia* not only includes phage WO and various transposons (Cerveau et al., 2011) but also a recently characterized and putative plasmid pWCP (plasmid of *w*Pip *Wolbachia* in *C. pipiens* mosquitoes) (Reveillaud et al., 2019). pWCP is a circular element spanning 9.23 kb of DNA with 15 genes including an IS110 transposable element and an intergenic repeat region (IRR) (**Figure 2c**). pWCP is present in natural populations and from publicly available metagenomes of *C. pipiens* from multiple countries, suggesting this extrachromosomal element is a natural member of the *w*Pip mobilome. As pWCP is the first putative *Wolbachia* plasmid discovered to date, it represents an expansion of extrachromosomal genomic information whose distribution across the *Wolbachia* phylogeny remains to be determined. While fairly novel in *Wolbachia*, plasmids are common in the closely related *Rickettsia* genus (El Karkouri et al., 2016) and contain genes that are often present in prophage WO genomes (Gillespie et al., 2018; Ishmael et al., 2009).

Cell biology of *Wolbachia*

Tissue tropism

Wolbachia inhabit the cells of both reproductive and somatic tissues in arthropods and nematodes (**Figure 3a**). The main transmission route for *Wolbachia* is vertical through female's ovaries to developing eggs. Although males harbor *Wolbachia*, they do not transmit the bacteria except in rare cases as demonstrated in

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Drosophila flies (Hoffmann et al., 1998) and *Nasonia* hybrid wasps (Chafee et al., 2011). In filarial nematodes, *Wolbachia* also transmit transovarially, however they do not invade the male germline and may thus respond to signaling molecules in the female germline (Foray et al., 2018; Landmann et al., 2012). Indeed, in uninfected *Drosophila* females, *Wolbachia* injected into the abdomen hemolymph can remarkably target the somatic stem cell niche of the ovary via unknown *Wolbachia*-encoded factors to ensure maternal colonization and transmission (Frydman et al., 2006; Toomey et al., 2013). Similar tropism occurs in the nematode *Brugia malayi*, suggesting an evolutionarily conserved mechanism for *Wolbachia* invasion into the gonads (Landmann et al., 2012). There are, however, instances such as in mosquitoes and moths where a somatic *Wolbachia* infection never stably established in reproductive tissues (Hughes et al., 2011; Kageyama et al., 2008).

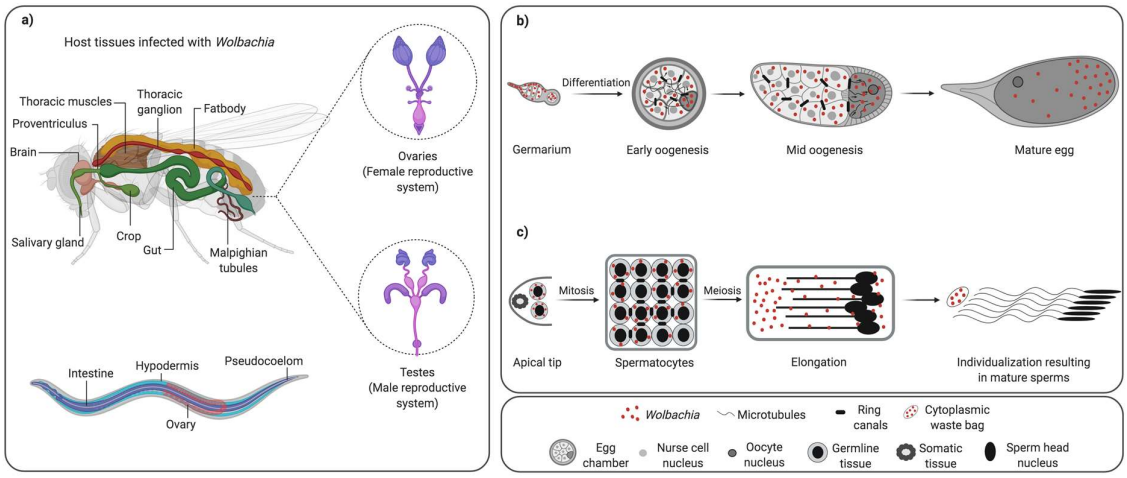

Figure 3. *Wolbachia* tissue tropism in arthropods and nematodes. a) Somatic and reproductive tissues with *Wolbachia* are labeled in the arthropod host *Drosophila* and nematode host *Brugia malayi*. b) During *Drosophila* oogenesis, *Wolbachia* (red) are present in germline stem cells where cell differentiation gives rise to a *Wolbachia*-infected egg chamber composed of an oocyte and nurse cells that interconnect by ring canals. During early oogenesis, *Wolbachia* utilize microtubules to move into the oocyte. They localize to the posterior pole during mid oogenesis and remain throughout development in the mature egg. c) During *Drosophila* spermatogenesis, *Wolbachia* are present in the germline stem cells, which divide mitotically to give rise to 16 interconnected spermatocytes with unevenly distributed *Wolbachia*. The spermatocytes then undergo meiosis to create a 64-cell cyst of interconnected spermatids that undergo differentiation and individualization. During individualization, excess cytoplasmic components including *Wolbachia* are removed from the mature sperm into a cytoplasmic waste bag.

As germline stem cells differentiate in *Drosophila* ovaries (**Figure 3b**), *Wolbachia* randomly distribute via microtubules through a series of cytoplasmic bridges known as ring canals to inhabit both nurse cells and oocytes (Ferree et al.,

2005). *Wolbachia* navigate between the two as the mature eggs develop, ultimately localizing to the posterior end of the oocyte to ensure germline-based transmission (**Figure 3b**) (Serbus et al., 2008). Posterior enrichment of *Wolbachia* in *D. melanogaster,* as well as in filarial nematode *B. malayi,* relies on microtubule-based motor proteins such as dynein and kinesin (Ferree et al., 2005; Landmann et al., 2014). *Wolbachia* also utilize the actin cytoskeleton to facilitate their maternal transmission, and *Drosophila* mutants of actin regulatory proteins ablate this function (Newton et al., 2015; Newton and Sheehan, 2015). *Wolbachia* in filarial nematodes utilize actin microtubule network as well for their movement (Landmann et al., 2012; Melnikow et al., 2013), however, the actin association is weak during somatic to germline transmission of the symbiont (Landmann et al., 2012). A *Wolbachia* protein named toxic manipulator of oogenesis (TomO) interacts with *nanos* mRNA in *Drosophila* ovaries to enhance the maintenance and proliferation of germline stem cells (Ote et al., 2016). In the male germline of *D. melanogaster* and *D. simulans*, *Wolbachia* localize and concentrate at the apical tip of the testes during early spermatogenesis (**Figure 3c**) (Clark et al., 2002; Riparbelli et al., 2007) and reside within the cytoplasm of the developing spermatocytes. Following meiotic divisions, the 16 spermatocytes produce a cyst of 64 interconnected spermatids, which undergo a morphological transformation involving elongation of the basal body-derived axoneme and decrease in nuclear volume. Spermatids then undergo an individualization process that strips the ring canals, cytoplasm, *Wolbachia,* and other unnecessary organelles into a waste bag for eventual degradation (**Figure 3c**) (Clark et al., 2002; Riparbelli et al., 2007). This purging results in elimination of *Wolbachia* from the mature sperm that typically does not transfer to the embryo.

As aforementioned, *Wolbachia* can occur in a variety of somatic tissues. In filarial nematodes, the somatic distribution of *Wolbachia* is restricted to the hypodermis, pseudocoelom, and intestine in both sexes (**Figure 3a**) (Ferri et al., 2011; Jiang et al., 2011; Landmann et al., 2012). In arthropods, *Wolbachia* are found in digestive and metabolic tissues such as gut, malpighian tubules, fatbody, and head and thoracic muscles among other tissues (**Figure 3a**) (Andersen et al., 2012; Chevalier et al., 2011; Dobson et al., 1999; Faria and Sucena, 2013; Hughes et al., 2011; Pietri et al., 2016; Zouache et al., 2009). Notably, this ability to inhabit somatic tissues appears to be a general tropism of *Wolbachia*. For instance, an artificial infection with *w*MelPop-CLA strain from *D. melanogaster* into naturally uninfected *A.*

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aegypti mosquitoes resulted in *Wolbachia* distribution into the fat body, anterior midgut, muscles, and nervous tissues (Moreira et al., 2009). Similarly, upon introgression of *Wolbachia* between *Nasonia* parasitoid wasp species, there is a marked 100-fold increase in *Wolbachia* densities and expanded tissue tropism into various somatic tissues including malpighian tubules, head, thorax, and abdomen (Chafee et al., 2011). *Wolbachia* also inhabit the brain of *Drosophila* (Albertson et al., 2013) and may play a role in modulating host behavior (Pietri et al., 2016; Strunov et al., 2017). Moreover, various biological and environmental factors such as temperature, host diet, and microbiota composition can impact *Wolbachia* replication and titer distribution across host tissues (López-Madrigal and Duarte, 2020), and titers can modulate the intensity of *Wolbachia*-induced phenotypes (Murdock et al., 2014; Reynolds et al., 2003). Although studies have revealed the functional relevance of *Wolbachia* somatic infection (Pietri et al., 2016), understanding the significance and persistence, if any, of somatic cell localization to *Wolbachia* fitness remains largely unexplored.

Vertical transmission

Wolbachia's success is attributable in part to efficient vertical transmission through the female germline. However, cases of imperfect maternal transmission have been detected that may prevent *Wolbachia* from reaching an optimum transmission frequency to spread and establish in wild populations (Jaenike, 2009; Rasgon et al., 2003; Turelli and Hoffmann, 1995). Factors that impact vertical transmission include *Wolbachia* densities, ability to migrate into the oocyte, and interactions with other symbionts (Guo et al., 2018; Mouton et al., 2004; Veneti et al., 2004). Indeed, low bacterial densities can result in stochastic loss of *Wolbachia* in developing oogonia (Werren, 2005), limiting transfer to the next generation (Jaenike, 2009). However, high densities in excess of what is required for successful maternal transmission can lead to a reduced lifespan in flies (Min and Benzer, 1997) and fecundity in wasps (Chafee et al., 2011). Selection for host responses can lead to the evolution of genes that suppress the maternally-transmitted densities of *Wolbachia* (Funkhouser-Jones et al., 2018). Therefore, a balance of vertical transmission of *Wolbachia* with optimum densities is important for a long-term, stable endosymbiosis (Funkhouser-Jones et al., 2018; López-Madrigal and Duarte, 2020)

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Horizontal transmission

Although transmission within species is predominantly vertical through the matriline, *Wolbachia* rarely exhibit corresponding phylogenies with their hosts, indicating frequent, horizontal transmission on an evolutionary timescale (Ahmed et al., 2013; Raychoudhury et al., 2009; Sanaei et al., 2020). Phylogenomic analyses suggest numerous host-switching events among arthropods and infrequent instances of host-switching between arthropods and nematodes (Gerth and Bleidorn, 2016; Gerth et al., 2014; Scholz et al., 2020; Turelli et al., 2018). The consequence is an incredibly wide range of hosts in which *Wolbachia* reside.

Horizontal acquisition of *Wolbachia* occurs in the lab or field via cannibalism and predation of infected individuals (Brown and Lloyd, 2015; Le Clec'h et al., 2013), parasitism (Heath et al., 1999), hybrid introgression (Raychoudhury et al., 2009; Turelli et al., 2018), and shared ecological niches (Li et al., 2017). *Wolbachia*-infected parasitoid wasps of the whitefly nymph *Bemisia tabaci* even use their mouthparts and ovipositors to transmit *Wolbachia* to their uninfected hosts (Ahmed et al., 2015). Additionally, two species of woodlice acquire stable *Wolbachia* infections by cannibalism within the same species (*Armadillidium vulgare*) and by predation between species (*A. vulgare* and *Porcellio dilatatus dilatatus*) (Le Clec'h et al., 2013). In the *Nasonia* parasitoid wasp species complex, there are 11 different *Wolbachia* strains spanning A and B *Wolbachia* supergroups, five of which were acquired via horizontal transmission from other insects including a likely predator-prey exchange, three as a result of hybrid introgression between species, and the rest as a result of codivergence with their hosts after acquisition (Raychoudhury et al., 2009). Collectively, diverse routes of horizontal transmission raise several intriguing questions such as: do *Wolbachia* have an extracellular stage that permits such high rates of horizontal transmission, how often do *Wolbachia* move between hosts, and how often are the host-switching events successful?

Wolbachia **phenotypes: Genes and mechanisms**

Cytoplasmic incompatibility

CI occurs in a diverse range of arthropod species where the most prevalent and commonly described *Wolbachia*-induced reproductive phenotype primarily occurs in two forms: unidirectional CI and bidirectional CI (Shropshire et al., 2020a).

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One-way or unidirectional CI results in embryonic death in crosses between infected males and uninfected females as well as between individuals where one strain can rescue another strain but the other strain does not reciprocate the rescue (**Figure 4a**) (Perrot-Minnot et al., 1996). Two-way, or bidirectional, CI also results in embryonic death between males and females harboring reciprocally incompatible *Wolbachia* strains (Bourtzis and Miller, 2003; Duron and Weill, 2006).

At the cytological level, male and female pronuclei in CI embryos often fail to synchronize at the initial stage of mitosis. Embryos experience a delay in male nuclear envelope breakdown and histone H3 phosphorylation (a histone modification that is required for the initiation of mitosis), which then delays the activity of Cdk1, a key kinase that drives the cell into mitosis (Tram and Sullivan, 2002). As a result, female chromosomes separate normally during anaphase, whereas male chromosomes often undergo chromatin-bridging defects and improper segregation, resulting in embryonic arrest during early embryogenesis (Bonneau et al., 2018; Landmann et al., 2009). However, some *Wolbachia* strains cause CI later in embryogenesis, and it remains unknown if late stage defects are caused by or independent of early mitotic abnormalities (Callaini et al., 1996; Duron et al., 2007b; LePage et al., 2017).

The genetic basis of *Wolbachia*-induced CI is governed by a Two-by-One genetic model in *D. melanogaster* (Shropshire and Bordenstein, 2019) whereby male expression of two genes, *cifA* and *cifB*, cause CI (Beckmann et al., 2017; LePage et al., 2017), and female expression of *cifA* rescues CI (Shropshire et al., 2018) (**Figure 4a**). These genes are encoded in the EAM of *Wolbachia's* prophage WO (Bordenstein and Bordenstein, 2016; LePage et al., 2017). Cif and Cif-like protein diversity has been qualified into five phylogenetic types (Bing et al., 2020; LePage et al., 2017; Lindsey et al., 2018; Martinez et al., 2020). CifA tends to evolve more slowly, is often less likely to be disrupted than CifB, and is under purifying selection in various regions (Martinez et al., 2020; Shropshire and Bordenstein, 2019). Recently, an evolutionary-guided mutagenesis study demonstrated region-specific roles of the CifA protein from *w*Mel *Wolbachia* in *D. melanogaster* whereby conserved amino acids in various, predicted domains or regions (Lindsey et al., 2018) play functional roles in CI alone or both CI and rescue (Shropshire et al., 2020b). Mutation analyses of Type I CifB protein from *w*Mel as well as *w*Pip reveal that numerous conserved sites across the proteins are crucial for phenotypic

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expression of CI (Beckmann et al., 2017; Shropshire et al., 2020b). Combinatorial, transgenic analyses of CI and rescue using *cif* Type I and II homologs reveal the *cifA* variants generally contribute to strong transgenic CI and interchangeable rescue, whereas *cifB* variants contribute to weak or no CI phenotypes (Shropshire et al., 2020c). Interestingly, unidirectional rescue of CI between the two Types and functional induction of CI upon co-expression of non-cognate *cifA* and *cifB* Types support a similar mechanism of CI across the divergent lineages (Shropshire et al., 2020c).

CifB harbors a putative core across the five phylogenetic types that include predicted or validated nuclease domains (Bing et al., 2020; LePage et al., 2017; Lindsey et al., 2018; Martinez et al., 2020). *In vitro* studies indicate that the C-terminal Ulp1 domain in Type I CifB from *w*Mel can act as a deubiquitinase cleaving polyubiquitin chains (Beckmann et al., 2017). A Type IV CifB homolog from *C. pipiens* mosquitoes acts as an *in vitro* nuclease. Mutating the catalytic sites prevents this activity as well as CI inducibility when expressed in *D. melanogaster* (Chen et al., 2019). However, it remains unknown if the deubiquitinase and nuclease activities are maintained *in vivo* and whether a singular mechanistic model of CI underpins the activity of these different domain functions (Beckmann et al., 2019a; Momtaz et al., 2020; Shropshire et al., 2019). Moreover, while a significant amount of work has been done to identify host genes linked to CI (Beckmann et al., 2019b; Biwot et al., 2020; Ju et al., 2017; Liu et al., 2014; Yuan et al., 2015; Zheng et al., 2011), cellular studies will be important to empirically determine their role in CI biology.

Male killing

Male killing (MK) is a form of reproductive parasitism in which *Wolbachia* selectively kill infected males often during embryogenesis, resulting in female-biased sex ratios in the arthropod hosts (**Figure 4b**) (Hurst and Frost, 2015; Hurst et al., 1999). *Wolbachia*-induced MK was first discovered in the ladybird species *Adalia bipunctata* and the butterfly species *Acraea encedon* (Hurst et al., 1999). In *Drosophila*, the earliest case of MK *Wolbachia* was reported in *D. bifasciata* (the obscura group of the subgenus *Sophophora*) (Hurst et al., 2000).

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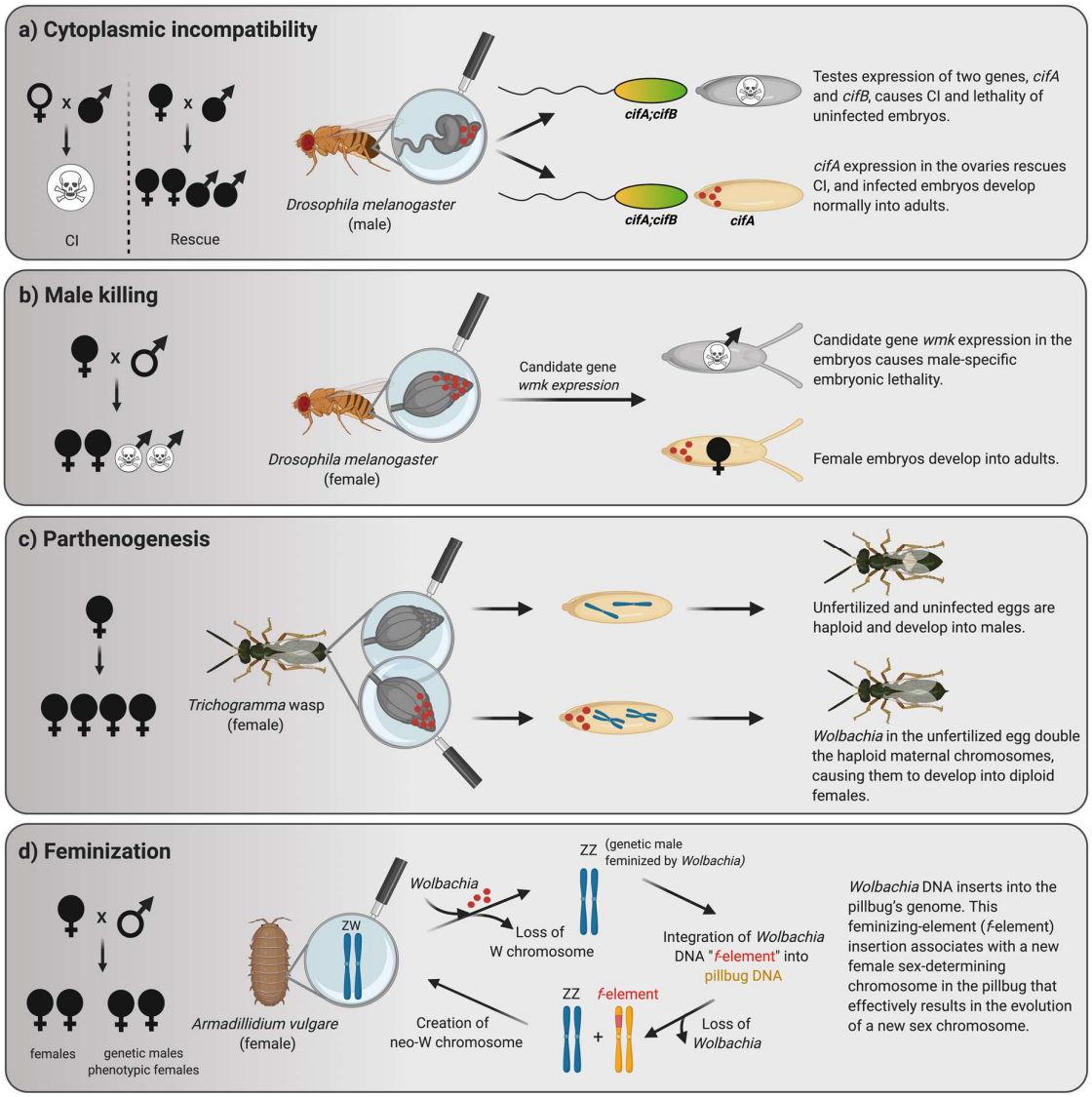

Figure 4. Reproductive phenotypes in arthropods. *Wolbachia* induce four, well-established reproductive parasitism phenotypes that assist its spread in a range of arthropod hosts. a) Cytoplasmic incompatibility (CI) causes embryonic death in crosses between infected males and uninfected females. In *D. melanogaster* males, expression of *Wolbachia* (red dots) prophage WO genes, cytoplasmic incompatibility factors (cif) *cifA* and *cifB* causes CI and results in embryonic death. Female expression of *cifA* in *Wolbachia*-infected eggs rescues the CI phenotype, leading to normal development of embryos. b) Male killing results in a female-biased sex ratio in various arthropods by selectively killing males. In *D. melanogaster,* transgenic expression of candidate gene *wmk* in the embryos causes partial, male-specific embryonic lethality. c) Parthenogenesis causes virgin mothers to produce all female offspring from their unfertilized eggs. In *Trichogramma* wasps, *Wolbachia* in the unfertilized eggs double the haploid set of maternal chromosomes, causing them to develop into diploid females. d) Feminization results in genetic males that phenotypically develop as females. The molecular mechanism of feminization is unknown; however, on an evolutionary scale, *Wolbachia* inserted a fragment of their DNA called '*f*-element' into the pillbug host genome that effectively results in the evolution of a new female-sex determining chromosome.

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Besides *Wolbachia*, *Spiroplasma poulsonii* is another MK symbiont found in various species of *Drosophila* (Haselkorn, 2010). In this bacterium, a plasmid-encoded gene, *Sp*AID, induces MK through abnormal apoptosis and neural defects during embryogenesis (Harumoto and Lemaitre, 2018). MK in *Spiroplasma* is linked to the misregulation of male dosage compensation (Cheng et al., 2016; Harumoto et al., 2016), a regulatory process that upregulates expression of X-linked genes in XY males to equilibrate their expression with the same genes expressed doubly in XX females (Lucchesi et al., 2005). In *D. melanogaster*, dosage compensation occurs through the action of a ribonucleoprotein complex, termed the MSL (Male-Specific Lethal) or DCC (Dosage Compensation Complex) that binds transcribed X-linked genes and modifies chromatin to elevate expression (Deng and Meller, 2006). Knockdown of genes encoding DCC subunits such as *msl-2* results in male-specific lethality in *D. melanogaster* (Conrad and Akhtar, 2012; Straub and Becker, 2011). Various lines of evidence indicate that MK *Wolbachia* may also hijack the dosage compensation system, although likely through a different method than *Spiroplasma* that mislocalize DCC components (Cheng et al., 2016). For instance, in the moth *Ostrinia furnacalis*, MK *Wolbachia* prevent dosage compensation by downregulating the Masculinizer gene required for both masculinization and dosage compensation in males (Fukui et al., 2015).

Comparative genomic, transgenic, and cytological approaches in *D. melanogaster* identified a candidate MK gene in the EAM region of *Wolbachia* prophage WO, termed *WO-mediated killing* (*wmk*) from *w*Mel *Wolbachia* (**Figure 4b**) (Perlmutter et al., 2019). *wmk* expression causes male-specific lethality during early embryogenesis and cytological defects typical of the pathology of MK, including chromatin-bridging, pyknotic nuclei and mitotic failure. Moreover, embryonic death is related to DNA damage associated with dosage compensation. The *wmk* gene occurs in all sequenced *Wolbachia* MK strains and is unique to *Wolbachia*, suggesting that the *Wolbachia* MK mechanism may have distinguishing phenotypic features compared to *Spiroplasma*. Questions for future research span whether or not the DCC is directly involved in *Wolbachia*-mediated MK, where the Wmk protein localizes, and how *wmk* sequence variation impacts MK.

Parthenogenesis

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Wolbachia-induced parthenogenesis occurs in several species of hymenopteran wasps (Boivin et al., 2014; Meyer and Hoy, 2007; Zchori-Fein et al., 1992), thysanopteran thrips (Kumm and Moritz, 2008), and trombidiformes mites (Weeks et al., 2001). In the absence of *Wolbachia,* these species typically exhibit arrhenotokous parthenogenesis due to their haplodiploid sex-determination system (Heimpel and De Boer, 2008). However, in the presence of parthenogenesis-inducing (PI) *Wolbachia*, virgin mothers produce all female offspring from their unfertilized eggs instead of males (**Figure 4c**), thus switching from arrhenotokous to thelytokous parthenogenesis. *Wolbachia* in *Trichogramma* induce thelytoky by a mechanism of gamete duplication in which unfertilized eggs undergo diploidization in the first mitotic division as the haploid maternal gametes are duplicated but fail to separate during anaphase (**Figure 4c**). Mitotic divisions following this event are normal, resulting in the development of completely homozygous, *Wolbachia*-infected, female offspring (Stouthamer and Kazmer, 1994).

To identify the genetic basis of parthenogenesis, a comparative genomic analysis was performed using the *w*Uni strain of *M. uniraptor* and the closely related CI-inducing *w*VitA strain from *N. vitripennis* (Newton et al., 2016), which revealed significant rearrangements, protein truncations, and elevated substitution rates in the *w*Uni genome (Newton et al., 2016). Similarly, comparative genomics of the *w*Tpre genome of *T. pretiosum* determined truncations in 20% of the protein coding sequences (Lindsey et al., 2016), including in the genes subsequently identified to cause CI. Truncation or pseudogenization of genes in host-restricted endosymbionts commonly occurs during reductive genome evolution (McCutcheon and Moran, 2012). It has been hypothesized that truncation of CI genes might have resulted in the shift from CI to PI lifestyle of the *w*Tpre strain (Lindsey et al., 2016; Newton et al., 2016).

Due to the cytological defects imposed by *Wolbachia* on host chromosomes, it has been long thought that *Wolbachia* may modify host epigenomes (Harris and Braig, 2003; Negri et al., 2009; Zhang et al., 2013). Recent work on the *T. pretiosum* and *D. melanogaster* transcriptome and epigenome showed altered splicing of various host genes in response to *Wolbachia* (Wu et al., 2020; Lindsey et al., 2020). In *T. pretiosum,* differentially methylated genes spanned processes of oocyte development, meiosis, and cell division, consistent with the proposed role of parthenogenetic *Wolbachia* that disrupt chromosome segregation during oocyte

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mitotic divisions (Stouthamer and Kazmer, 1994). In *D. melanogaster*, differentially spliced genes spanned transcription and translation, cytoskeletal organization, nucleotide metabolic processes, and immune and stress responses. Functional genetic studies including knockdown of target gene expression in hosts or *Wolbachia* will be useful to interrogate the contribution of particular proteins to *Wolbachia*-induced parthenogenesis. Additionally, future research is required to understand the step-by-step molecular basis of *Wolbachia*-induced parthenogenesis (Lindsey and Stouthamer, 2017).

Feminization

Another sex ratio modification strategy used by *Wolbachia* is feminization, whereby genetic males morphologically develop into females (**Figure 4d**). *Wolbachia*-induced feminization was first described in isopods (Legrand et al., 1984), and the best-studied example is the pillbug, *Armadillidium vulgare* (Rigaud et al., 1991). In addition, it typically occurs in lepidopteran and hemipteran insects and spiders (Curry et al., 2015; Negri, 2012); thus, feminization is a rarer phenotype than CI and MK.

In *A. vulgare*, male sex differentiation is triggered by an androgenic hormone secreted by the androgenic gland. *Wolbachia* proliferate within the gland and may inhibit the secretion of androgenic hormone, thereby developmentally forcing genetic males into morphological females (Bouchon et al., 2008). In the moth *O. scapulalis, Wolbachia* inhibit the expression of male-type splice variants of the sex regulating gene *doublesex* (*dsx*) and upregulate female-type splice variants, leading to the production of more female progeny (Sugimoto and Ishikawa, 2012). In both cases, it remains unknown if *Wolbachia* directly interfere with androgenic hormone production or *dsx* splice variants, or indirectly by impacting upstream factors that influence these patterns. The *w*VulC strain feminizes its natural host *A. vulgare* as well as its artificially transinfected isopod host *Cylisticus convexus,* whereas the closely related and naturally occurring *w*Con strain causes CI in its native host *C. convexus*. Together, this indicates that variation in *Wolbachia* is associated with different forms of reproductive parasitism employed by *w*VulC and *w*Con (Badawi et al., 2015). Using comparative 'omics, two genes present in the *Wolbachia* prophage WO region (*w*Vul_*1408* and *w*Vul_*1821*) are of particular interest in feminization, since they are differentially expressed when *w*VulC is in either *A. vulgare* or *C. convexus* (Badawi

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et al., 2015; 2018). It remains unknown if these genes are responsible for *Wolbachia*-induced feminization or how/if they interact with host factors. Future work including functional genetic analyses will be necessary to determine the direct causative relationship of *Wolbachia*-induced feminization and to understand the complexity of its mechanism.

Pathogen resistance

Wolbachia can reduce pathogenic viral loads in various arthropod species. Known as 'pathogen blocking/resistance' or 'antiviral protection', the phenomenon was initially discovered in the *w*Mel strain of *Wolbachia* in *D. melanogaster* (Hedges et al., 2008; Teixeira et al., 2008) whereby *Wolbachia*-infected flies harbored lower viral loads and survived longer than uninfected ones. In mosquitoes, *Wolbachia* can limit the replication of viruses in various somatic tissues such as midgut and salivary glands among others, making them less capable of transmitting infection to humans (Aliota et al., 2016; Dutra et al., 2016; Pereira et al., 2018; Walker et al., 2011). *Wolbachia* can also confer pathogen resistance against bacteria, filarial nematodes, and the malaria parasite *Plasmodium gallinaceum* (Teixeira et al., 2008; Walker et al., 2011; Hughes et al., 2011; Kambris et al., 2010; Moreira et al., 2009), providing a broad range of pathogen protection. Subsequently, with a lens towards human health as well agricultural pest management programs, artificial *Wolbachia* infections are established in various arthropod species to limit the arboviral disease spread to humans (see below section on Applications of *Wolbachia* for curbing human diseases).

Higher *Wolbachia* densities within hosts is often important for an effective antiviral response (Chrostek et al., 2013; Martinez et al., 2014). However, there are certain exceptions where higher *Wolbachia* densities do not correlate with the extent of pathogen-blocking (Cattel et al., 2016; Kaur et al., 2020; Mousson et al., 2010). *Wolbachia*-mediated virus suppression is a cell-autonomous phenomenon (Nainu et al., 2019) whereby intracellular *Wolbachia* compete with viruses for space and/or cellular metabolic resources and exclude them from the same cell/tissue. For example, competition for cholesterol contributes to antiviral protection in *Drosophila melanogaster* (Caragata et al., 2013). However, *Wolbachia* densities and viral loads do not correlate in any particular tissue (Amuzu et al, 2016; Kaur et al, 2020), suggesting that *Wolbachia*-conferred pathogen blocking is a complex phenomenon

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(Lindsey et al., 2018b). From the mechanistic perspective, *Wolbachia* can inhibit viral binding, entry into the cell and RNA replication in the early stages (Rainey et al., 2016; Schultz et al., 2018; Lu et al., 2020). Such blocking reduces the production of progeny viruses from the same *Wolbachia*-infected cells and consequently limits virus dissemination and transmission (Bhattacharya et al., 2020a), thus contributing to the molecular mechanism/s of *Wolbachia*-conferred virus blocking.

At the whole organismal level, certain host factors may also play a role in *Wolbachia*-virus dynamics. The mosquito-specific microRNA aae-miR-2940 is highly induced in *A. aegypti* mosquitoes (Hussain et al., 2011), and overexpression of one of its target methyltransferase genes, *aaDnmt2,* inhibits *Wolbachia* replication and significantly promotes replication of dengue virus in the mosquito host (Zhang et al., 2013). In *Drosophila*, however, expression of *Dnmt2* is induced in the presence of *Wolbachia* and provides antiviral defense (Durdevic et al., 2013; Bhattacharya et al., 2017; 2020a; 2020b). A comprehensive RNAseq analysis revealed a tripartite interplay between *Wolbachia*, virus, and host at the interface of nucleotide metabolism, with *Wolbachia* and virus downregulating the pyrimidine and purine synthesis pathways, respectively (Lindsey et al., 2020). In particular, the *prat2* gene involved in *de novo* synthesis of purine nucleotides (Ji and Clark, 2006) was upregulated in *Wolbachia*-infected flies; and knockdown of *prat2* mRNA levels showed a *Wolbachia*-dependent impact on viral replication, with knockdown being pro-viral in the presence of *Wolbachia* and antiviral in the absence of *Wolbachia* (Lindsey et al., 2020). Functional metabolic and proteomic assays will be essential to connect transcriptomic changes to downstream events in the physiology of the host that eventually result in *Wolbachia*-conferred pathogen blocking phenotype.

Wolbachia can also upregulate the expression of genes involved in innate defense pathways, and thus prime the insect innate immune system to block pathogen replication (Bian et al., 2010; Kambris et al., 2010). However, this appears to only be the case in mosquitoes that have been artificially transinfected with *Wolbachia* strains (Rancès et al., 2012; 2013). *Drosophila* species that are naturally infected with *Wolbachia* do not show an immune-priming phenotype, yet still confer antiviral activity (Wong et al; 2011), suggesting that innate immune priming may occur only in recently introduced host-*Wolbachia* associations and that it cannot explain the entirety of the effect.

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Obligatory and facultative mutualisms

Obligate mutualisms between *Wolbachia* and their hosts, whereby both partners seem to benefit, are also common (Fenn and Blaxter, 2004). In nematodes, the complete genome of *Wolbachia* from the human filariid *Brugia malayi* revealed that *Wolbachia* are unable to perform *de novo* synthesis of several vitamins and cofactors such as coenzyme A, nicotinamide adenine dinucleotide, biotin, ubiquinone, folate, lipoic acid, and pyridoxal phosphate (Foster et al., 2005). Incomplete biochemical pathways in *Wolbachia* render them metabolically dependent on their nematode hosts. Various studies indicate that elimination of *Wolbachia* via antibiotics impairs the normal development, fertility, and vitality of adult filarial worms (Coulibaly et al., 2009; Taylor et al., 2010), suggesting hosts' dependency on *Wolbachia*. *Wolbachia* genome analysis from *B. malayi* (Foster et al., 2005) and the bovine tissular filariid *Onchocerca ochengi* (Darby et al., 2012) revealed that *Wolbachia* contain various intact metabolic pathways essential for survival and fecundity of their host. Indeed, *Wolbachia* carry the genes required for heme metabolism and riboflavin, whereas their filarial hosts do not (Darby et al., 2012; Strübing et al., 2010), indicating that *Wolbachia* may help filarial nematodes acquire and keep iron. Alternatively, heme from *Wolbachia* could be vital to nematode embryogenesis since ecdysteroid-like hormones, which require heme for their synthesis, control the molting and reproduction processes (Warbrick et al., 1993). Depletion of *Wolbachia* might halt production of these hormones and block embryogenesis. Nevertheless, how transport, degradation and regulation of heme occurs within filarial hosts remains an open question (Wu et al., 2009).

In arthropods, there are a few cases whereby *Wolbachia* act as an obligate mutualist. For instance, in the parasitic wasp *Asobara tabida*, antibiotically cured females fail to develop eggs (Dedeine et al., 2001). In the bedbug *Cimex lectularius*, reduction or elimination of *Wolbachia* through antibiotic treatment renders abnormal development of eggs, which can be restored by dietary supplementation of Vitamin B and biotin (Nikoh et al., 2014), suggesting a coadaptive process similar to the nematode-*Wolbachia* association. *Wolbachia* also act as facultative mutualists whereby hosts benefit from the bacteria, but they do not depend on *Wolbachia* for survival or fecundity. For instance, *Wolbachia* increase the fecundity of *D. melanogaster* flies under varying levels of iron, suggesting that *Wolbachia* may be altering host iron metabolism as a nutritional mutualist (Brownlie et al., 2009).

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Wolbachia also enhance longevity, fitness and fertility of flies and mosquitoes (Fast et al., 2011; Fry and Rand, 2002; Dobson et al., 2002). It remains unclear what is the molecular mechanism behind facultative mutualism and how common this phenomenon is among other arthropods.

Evolutionary implications of host-*Wolbachia* associations

Wolbachia-driven evolution of host genomes

Since *Wolbachia* are maternally inherited, there is a natural conflict between the biparental inheritance of host genes (Fisher, 1958) and the uniparental inheritance of maternally-transmitted *Wolbachia* to favor females (Cordaux et al., 2011). In response to the selective pressures exerted on hosts by *Wolbachia*'s reproductive modifications, hosts can counteradapt and resist. For instance, wMel *Wolbachia* cause variable levels of CI in their native hosts of *D. melanogaster* but induce consistently strong CI when artificially introduced into other hosts such as *D. simulans* and *A. aegypti* (Poinsot et al., 1998; Walker et al., 2011), suggesting that hosts evolve resistance against strong CI-induction (Turelli, 1994). *Hypolimnas bolina* butterflies subject to male killing evolve resistance too (Mitsuhashi et al., 2011). Similar patterns of resistance occur in *A. vulgare* pillbugs (Rigaud and Juchault, 1992) and *T. kaykai* wasps (Stouthamer et al., 2001), which are infected with feminizing and parthenogenetic *Wolbachia,* respectively. While the mechanisms of host resistance to reproductive parasitism are currently unknown, their evolution peels back the layers of selection and conflicts that exist between sex ratio distorting *Wolbachia* in the cytoplasm and their biparentally inherited host genome.

Additionally, *Wolbachia*-induced feminization and parthenogenesis influence host sex-determination pathways and functions. *Wolbachia*-induced feminization occurs in insects (Hiroki et al., 2002) and isopods (Bouchon et al., 1998) with female heterogamety (ZW female/ ZZ male) or XX/X0 sex-determination. In ZW/ZZ systems, feminization is predicted to cause the loss and/or turnover of sex chromosomes since its function is redundant with the female sex-determination of *Wolbachia* (Cordaux and Gilbert, 2017; Cordaux et al., 2011). Indeed, in some lines of *A. vulgare* isopods, the *"f* element", which is a horizontally-transferred *Wolbachia* insert in the host isopod genome, is present on an autosome and is responsible for female sex-determination (**Figure 4d**) (Badawi et al., 2018). The *f* element contributes to

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feminization in the absence of *Wolbachia* and is responsible for the emergence of a novel W-like chromosome necessary for female heterogamety (**Figure 4d**) (Leclercq et al., 2016). In summary, feminizing *Wolbachia* may contribute to the turnover of sex chromosomes where a ZW/ZZ sex-determination system is converted to symbiont-driven sex-determination and then back to a ZW/ZZ-like system that may be dependent on a different set of mechanisms for sex-determination. It will be important to understand the nature and mechanisms of the *f* element, whether feminizing *Wolbachia* in ZW/ZZ systems commonly have this impact on the evolution of their host's sex-determination, and whether XX/X0 systems suffer comparable, cascading consequences.

Impact of Wolbachia *on host speciation*

In addition to genetic changes in the host genome, symbiont changes can also lead to speciation and reproductive isolation (RI) between two populations (Brucker and Bordenstein, 2012; Shropshire and Bordenstein, 2016). For instance, *Wolbachia*-induced CI can reduce nuclear gene flow between host individuals in the absence of host genetic divergence or geographic isolation (Hurst and Schilthuizen, 1998). Bidirectional CI restricts gene flow in both cross directions when populations or species harbor different, reciprocally incompatible *Wolbachia* strains. It is among the earliest forms of isolation evolved between *Nasonia* parasitoid wasp species that diverged ~0.25 mya (*N. giraulti* and *N. longicornis*), and it also prevents hybrids between the older species pair that diverged ~1 mya (Bordenstein et al., 2001). Since unidirectional CI only restricts gene flow in one direction, it is unlikely to contribute to speciation alone (Cooper et al., 2017). However, when unidirectional CI is coupled with RI in the reciprocal cross direction, it reduces gene flow, such as in the hybrid zone of North American populations of *Wolbachia*-infected *D. recens* and uninfected *D. subquinaria* mushroom-feeding flies (Jaenike et al., 2006; Shoemaker et al., 1999). Reciprocally, *D. subquinaria* females have strong mate-discriminating behaviors that prevent them from mating with infected *D. recens* males (Jaenike et al., 2006). In the *D. paulistorum* semi-species complex, *Wolbachia* are pathogenic in hybrids as over-replication triggers embryonic lethality and male sterility, and cause alterations in sexual behavior via selective mate avoidance in sympatry (Ehrman, 1968; Kernaghan and Ehrman, 1970; Miller et al., 2010; Schneider et al., 2019). Taken together, these and additional barriers may prevent hybridization between

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species (Jaenike et al., 2006). *Wolbachia* can also have subtle impacts on RI through environmental changes or host mate preference behaviors (Arnold et al., 2019; Hague et al., 2020; Truitt et al., 2019). Future work in additional systems will shed light on how common symbiont-assisted speciation is across the diversity of arthropods (Brucker and Bordenstein, 2012).

Wolbachia-induced parthenogenesis or asexual reproduction can also cause RI because it effectively makes gene flow unnecessary (Shropshire and Bordenstein, 2016). This form of RI is referred to as asexual speciation, and its effectiveness depends largely on how frequently or infrequently asexual-capable populations interbreed with sexual populations (Elias-Costa et al., 2019). If mating is frequent, then gene flow will continue and reduce the effect of parthenogenesis on RI. However, asexual reproduction may also be accompanied by the loss of sex-specific traits due to sexual degeneration or relaxed sexual selection (Gottlieb and Zchori-Fein, 2001; Stouthamer et al., 2010). Thus, degraded sexual behaviors and characteristics including fertilization and courtship can reinforce the evolution of RI between asexual and sexual lineages.

Gene transfers between host and Wolbachia

The intimate associations between host and intracellular *Wolbachia* frequently lead to lateral gene transfers (LGT) between their genomes (Dunning Hotopp et al., 2007), partly because *Wolbachia* LGTs to the nuclei of host gametes can become inherited to the next generation. One well-studied example is the *A. vulgare* pillbug with a large piece of the feminizing *Wolbachia* genome, noted as the "*f* element" above, inserted into a new female sex chromosome (Leclercq et al., 2016). In beetles, *Wolbachia* genomic fragments are transferred to the X chromosome and autosome of the host (Aikawa et al., 2009; Kondo et al., 2002). The transferred genes are, however, pseudogenized and likely derived from a single LGT (Nikoh et al., 2008). Several other cases of multiple *Wolbachia* insertions in the same host genome are also documented. For instance, in *Drosophila ananassae*, multiple copies of an entire *Wolbachia* genome occur in the fourth chromosome (Dunning Hotopp et al., 2007; Klasson et al., 2014). In the tsetse fly *Glossina morsitans morsitans*, two large *Wolbachia* genome insertions are in the host X and Y chromosomes (Brelsfoard et al., 2014). In two meadow grasshopper subspecies, *Chorthippus parallelus erythropus* and *C. parallelus parallelus*, recent and large gene

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transfers from two different *Wolbachia* supergroups (B and F) occur into the nuclear genome, revealing some inserts are subspecies-specific while others are present in both subspecies (Funkhouser-Jones et al., 2015). The functional significance of such LGTs, if any, remains largely unknown and in important topic of future investigation if the transferred genes retain function and contribute to host fitness phenotypes or processes such as speciation.

In addition to LGTs from *Wolbachia* to host, transfers in the other direction can occur. The EAM in *Wolbachia's* prophage WO is enriched with genes of putative arthropod origin including a snippet of DNA from the black widow latrotoxin gene (Bordenstein and Bordenstein, 2016). There is also an incident of a mosquito gene encoding salivary gland surface proteins that potentially transferred to *Wolbachia* (Woolfit et al., 2009). Collectively, LGT between *Wolbachia* and host are common and may have important consequences in providing adaptive novelty. More investigation is necessary to fully understand the how these transfers occur, are they functional, and what are the evolutionary consequences.

Applications of *Wolbachia* for curbing human diseases

Population Replacement Strategy (PRS)

PRS leverages two aspects of *Wolbachia* to curb mosquito-borne viral disease transmission - pathogen blocking and CI drive. *A. aegypti* mosquitoes, the vector of many human pathogenic viruses including Dengue, Chikungunya and Zika viruses, do not naturally carry *Wolbachia* (Kittayapong et al., 2000). Therefore, stable and heritable *Wolbachia* infections such as *w*AlbB from *A. albopictus* (Xi et al., 2005) and *w*MelPop and *w*Mel from *D. melanogaster* (McMeniman et al., 2009: Walker et al., 2011) have been successfully established in this species by artificial transinfection. Using CI-based drive, *Wolbachia*-infected mosquitoes released into the wild replace an otherwise uninfected population, reduce their vector competence, and curb the arboviral disease burden in humans (**Figure 5a**) (Flores and O'Neill, 2018; Joubert et al., 2016; Nazni et al., 2019; Tantowijoyo et al., 2020; Xi et al., 2005; Schmidt et al., 2017). Various studies have now demonstrated population replacement in several countries including Australia, Indonesia, Brazil, Colombia, Fiji, Kiribati, India, China, Mexico among others (Hoffmann et al., 2011; Schmidt et al., 2017; O'Neill et al., 2018; Garcia et al., 2019; Nazni et al., 2019; Zheng et al.,

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2019) and reduced cases of dengue transmission (Anders and Simmons, 2019; Nazni et al., 2019; Indriani et al., 2020; Ryan et al., 2020)

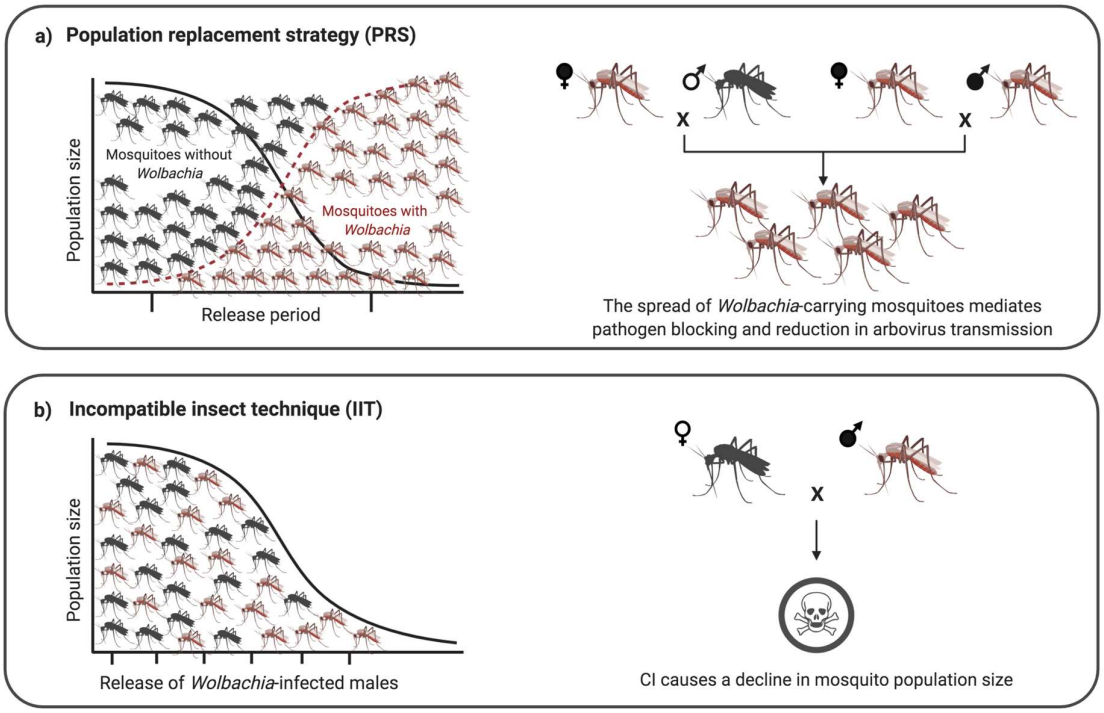

Figure 5. *Wolbachia*-mediated applications to control insect vectors. a) Population replacement strategy commences with release of both male and female mosquitoes where CI-inducing *Wolbachia* spread throughout uninfected target populations, thus replacing the native species with pathogen-resistant, *Wolbachia*-infected mosquitoes, no longer capable of transmitting disease. b) Incompatible insect technique entails release of CI-causing *Wolbachia*-infected male mosquitoes that do not produce viable embryos after mating with wild-type uninfected females, thus reducing the total number of disease-transmitting mosquitoes in natural populations. Figure is adapted from Yen and Failloux, 2020.

Not only in mosquitoes, the *w*Stri *Wolbachia* strain was recently stably introduced into the brown planthopper *Nilaparvata lugens*, a destructive agricultural pest, to inhibit the infection and transmission of rice tagged stunt virus that damages rice crops (Gong et al., 2020). Taken together, these studies show that PRS can drive *Wolbachia* to high frequencies and successfully reduce the vectoral capacity of arthropods. Notably, although at the early stages of development, there are efforts to extend the efficacy of PRS using vertically-transmitted, insect-specific viruses that, like *Wolbachia*, inhibit arboviral replication (Agboli et al., 2019; Parry and Asgari, 2018).

Incompatible Insect Technique (IIT)

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IIT relies on a key component of *Wolbachia*-induced CI, namely that *Wolbachia*-infected males cause embryonic lethality after mating with uninfected females (Laven, 1967). Thus, release of *Wolbachia*-infected males suppress the mosquito population size in the field (**Figure 5b**) as has been achieved in China (*w*Pip, *w*AlbB and *w*AlbB in *A. aegypti*), Italy, Singapore and America (*w*Pip in *A. albopictus* and *w*AlbB in *A. aegypti*) (Caputo et al., 2020; Mains et al., 2016; 2019; Puggioli et al., 2016; Zheng et al., 2019). Work is in progress to expand IIT to control other vectors and pests, including the fruit pest *Ceratitis capitata* (Kyritsis et al., 2019) and protozoan vector *C. quinquefasciatus* (Ant et al., 2020).

Genetic manipulation of Wolbachia

Genetic editing of *Wolbachia* would expand vector control efforts as insertion of genes into the genome for pathogen-blocking or increased penetrance of CI could serve an alternative or adjunct to current efforts (Iturbe-Ormaetxe and O'Neill, 2007; LePage and Bordenstein, 2013; Sinkins and Godfray, 2004; Sinkins and O'Neill, 2000). While some success has been reported using random mutagenesis to introduce genetic variation in the *Wolbachia* genome (Duarte et al., 2020), mutant identification and linkage with a *Wolbachia*-conferred phenotype remains a challenge. *Wolbachia* do not replicate outside of their host cells and not yet genetically editable (Rasgon et al., 2006), thus efforts are impending to investigate cellular factors required for *Wolbachia* growth and replication in the host cell-free system (Krafsur et al., 2020). The main challenges to achieve *Wolbachia* transformation involve (i) genetic constructs crossing the eukaryotic membrane, (ii) protecting DNA from cellular nucleases and degradation inside the eukaryotic cell, (iii) crossing the bacterial membrane, and (iv) stable and selectable incorporation into the *Wolbachia* genome.

Genetic transformation of closely related *Rickettsia* endosymbionts has been achieved using transposon mutagenesis technique (Qin et al., 2004). The same methodology can possibly be optimized for *Wolbachia* transformation in the future. Additionally, a metagenomic analysis of *Wolbachia*-encoded phage WO particles uncovered phage WO and *Wolbachia* attachment sequences and bacterial integration sites in the phage genome that are in development as a potential tool for gene insertions into *Wolbachia* (Bordenstein and Bordenstein, 2016). Moreover, plasmids are commonly used in genetic engineering and gene therapy research to

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alter genomes and phenotypes (Lorenz and Wackernagel, 1994). As such, discovery of aforementioned plasmid pWCP in *w*Pip (Reveillaud et al., 2019) could enable investigation of development of a new genetic tool in *Wolbachia* and applications for controlling arthropods or filarial nematode-associated animal and human diseases.

Anti-Wolbachia *drug therapy for curing filarial diseases*

Wolbachia occur in mutualistic relationships with many species of filarial nematode worms, which cause diseases such as river blindness, lymphatic filariasis, and heartworm (Slatko et al., 2014). Since elimination of *Wolbachia* can halt the worm's life cycle, *Wolbachia* serve as a compelling drug target for treating human filariasis (Taylor et al., 2005). A high throughput drug screening approach was undertaken by A-WOL (https://awol.lstmed.ac.uk), an international consortium assembled to identify anti-*Wolbachia* compounds. Several drug candidates were uncovered including doxycycline and minocycline that deplete *Wolbachia* by more than 99% (Johnston et al., 2014; Taylor et al., 2013). However, lengthy treatments and restrictions against usage in children and pregnant women pose challenges to widespread implementations (Mylonas, 2011). Recent testing of a new orally-available, antibiotic, tylosin A analog, A-1574083, showed robust anti-*Wolbachia* activity with a short treatment time frame of 7 to 14 days (Taylor et al., 2019). Thus, A-1574083 may provide clinical benefits with a shorter dosing regimen. Moreover, in order to enhance the drug specificity towards *Wolbachia*, a novel synthetic molecule, AWZ1066S, has been designed that confers minimal impact on the gut microbiota and shows superior efficacy to existing anti-*Wolbachia* therapies in preclinical human and mouse models of infection (David Hong et al., 2019). Overall, discovery of anti-filarial drugs has consistently shown the potential to be prophylactic, curative, and macrofilaricidal to reduce the disease pathology. Additionally, alternative methods may one day include phage WO therapy to specifically target *Wolbachia* in filarial infections, whereby phage WO particles or phage-derived enzymes could be developed to lyse *Wolbachia* cells with high specificity (Bordenstein et al., 2006).

Discover the Microbes Within! The *Wolbachia* Project: An archetype for student-driven and citizen scientist discoveries

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The multidisciplinary, biotechnology lab series “Discover the Microbes Within! The *Wolbachia* Project” (https://vu.edu/wolbachia) empowers students, teachers, and scientists to take ownership of their science, learn major concepts in biology, and make novel scientific contributions in a culture of excellence (Bordenstein et al., 2010; Lemon et al., 2020). The project integrates genetics, entomology, evolution, molecular biology and bioinformatics techniques to engage participants internationally in discovery, biotechnology, and microbial symbiosis. Implementation of the labs is facilitated by partnerships with the *Wolbachia* scientific community, online digital and social media resources, downloadable labs and lectures, a free loaner equipment program, and a DNA sequencing partnership. The lab modules are designed to be stand-alone and can either be incorporated into individual daily lesson plans addressing Next Generation Science Standards or used as a coherent unit progressively emphasizing the nature of a long-term science project throughout the school year.

The research end products of this lab series are new discoveries of *Wolbachia* infections and DNA sequences, and the broad core goals of the project are to: (i) Engage students in nature and real-world research; (ii) Encourage international participation in the collection of new scientific data on bacterial endosymbionts (*Wolbachia*); (iii) Enhance student interest in science through an integrative lab series spanning biodiversity to molecular biology; and (iv) Give students an idea of what it is like to be a scientist. As biologists and students continue to appreciate the dominance of the microbial and symbiotic worlds in the macrobiological biosphere, the story of *Wolbachia* will serve as a foundational blueprint for science education and how microbial symbiosis delivers fundamental textbook knowledge as well as human health applications.

Concluding remarks

Advances in multiomics, gene functional assays, and human health applications in the field have spurred rapid and fundamental insights that will undoubtedly be covered in future textbooks covering symbiosis. Several key questions for the future include: What is the biochemical and mechanistic basis of reproductive parasitism and pathogen blocking? Can *Wolbachia* be genetically manipulated to ultimately advance the systems for reductionist, functional studies of

Wolbachia gene products? Will *Wolbachia* studies and therapies continue to soften the global burden of human diseases such as filarial infections and arboviruses? What are the evolutionary trajectories of *Wolbachia* infections within and between species? Do *Wolbachia* promote speciation in numerous systems? Significant progress is now likely to be made in answering these questions, which makes the next century of *Wolbachia* research a more exciting and impactful one to the life sciences. What was once a curiosity to Hertig and Wolbach is now an archetype for microbial symbiosis and an exemplar for how basic science leads to positive, translational and education outcomes.

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Contributions

All authors contributed to the text of the article. R.K. and S.R.B. performed all of the editing and wrote the final version.

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Supplementary table legends

Supplementary table 1. List of *Wolbachia* genomes available. Supergroups, host names, and strain types have been listed for each *Wolbachia* strain sequenced, together with their genome sizes, IDs and genome assembly status.

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Supplemental Table 2. Patterns of phage association and reproductive phenotypes across *Wolbachia* supergroups. Regions of phage origin (ranging from intact phages to phage WO pseudogenes), corresponding EAM (eukaryotic association module) regions, presence of *cifA/cifB* genes, and reproductive phenotypes are listed for each *Wolbachia* strain. NR refers to data that, to the best of our knowledge, is not yet reported. Reproductive phenotypes are listed as CI (cytoplasmic incompatibility), MK (male-killing), P (parthenogenesis), and M (mutualism).