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Review

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

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SUMMARY

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 reproductive 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, the interdisciplinary science of this symbiosis stands as a model for consolidating and teaching the integrative rules of endosymbiotic life.

INTRODUCTION

The central roles of microbial symbiosis in the origin of the eukaryotic cell, species formation, ecological interactions, and animal and plant activities across the biosphere are increasingly clear (Bordenstein and Theis, 2015; McFall-Ngai, 2008). 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*, first described nearly a century ago as a *Rickettsia*-like organism in the gonads of various insects including *Culex pipiens* mosquitoes (Hertig and Wolbach, 1924). 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*.

In the 1990s, 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, a stunning variety of hosts were identified, including insects, spiders, mites, and terrestrial isopods, as well as filarial and plant nematodes. Current estimates suggest ~50% of arthropod and several filarial nematode species harbor *Wolbachia* (Lefoulon et al., 2016; Weinert et al., 2015).

A century in the making, the knowledge in this review serves as a summative gateway to six major topics underpinning the biology of one of the greatest endosymbioses: (1) genome evolution and diversity, (2) mobile elements including temperate phage as a hotspot for genetic novelty and key functions, transposons, and a putative plasmid, (3) cell biology spanning tissue tropism and transmission routes, (4) hallmark phenotypes, genes, and mechanisms crossing the continuum of symbiotic functions, (5) fundamental impacts on host counter-adaptations, extinction, and speciation, and (6) 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, we note future research directions to improve our understanding of living in the *Wolbachia* world.

WOLBACHIA DIVERSITY, EVOLUTIONARY HISTORY, AND GENOMICS

Diversity

Wolbachia genetic diversity was initially characterized using sequences for the 16S rRNA gene (O'Neill et al., 1992) and the more variable surface protein gene *wsp* (Zhou et al., 1998). Due to the slow evolutionary rate of 16S rRNA and extensive *wsp* recombination that posed challenges to resolving *Wolbachia* strains and phylogenies, a multilocus sequence typing (MLST) system comprising conserved housekeeping genes was established as a standard for *Wolbachia* classification (Baldo et al., 2006). *Wolbachia* are subdivided into at least 17 possible phylogenetic supergroups (named A–F, H–Q, and S, detailed in Figure 1B). 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 (Table S1). Overall,

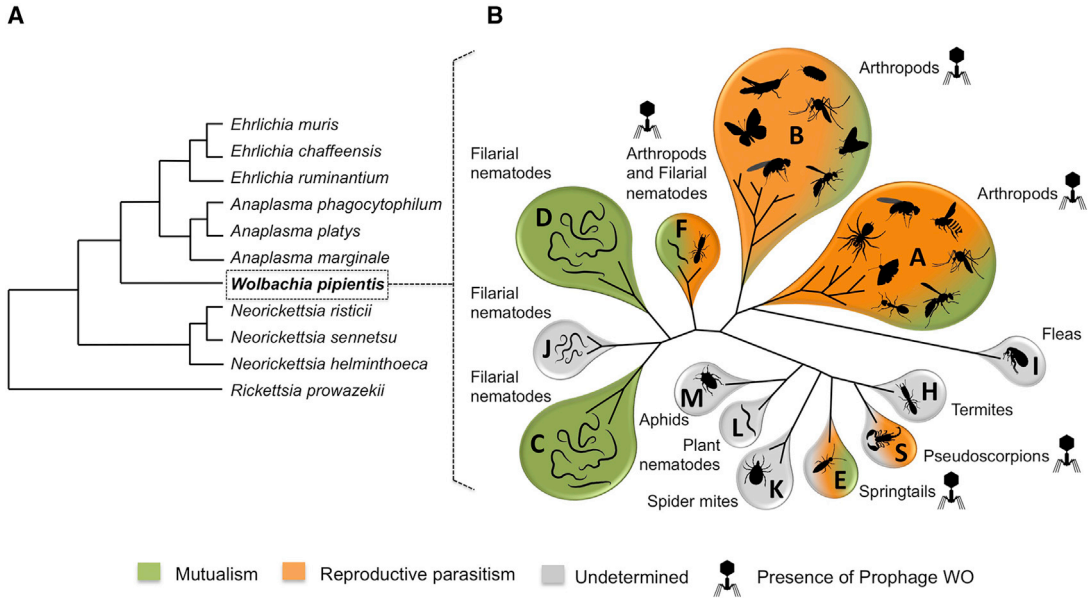

Figure 1. Phylogeny and evolution of *Wolbachia*

(A and B) Schematic representation of (A) phylogenetic relationships of *Wolbachia* and members of the family Anaplasmataceae, with *Rickettsia* shown as an 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 Table S2.

our understanding of *Wolbachia*'s genetic diversity is still developing. Recent advances in target enrichment protocols (Hotopp et al., 2017; Kent and Bordenstein, 2010) and whole-genome typing methods with an improved set of MLST loci (Bleidorn and Gerth, 2018) can be used across diverse arthropod species and environments to discover and delineate *Wolbachia* genomic diversity and phylogenetic classification.

Evolutionary history

Sister genera to *Wolbachia* include *Ehrlichia*, *Anaplasma*, and *Rickettsia* endosymbionts. 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, 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 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. 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*, which, which provided an extended time frame 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 the evolutionary history and timing.

Genomics

The first sequenced *Wolbachia* genome, *w*Mel strain in *Drosophila melanogaster* from supergroup A, revealed a small 1.3 megabase (Mb) genome 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. pipiens* (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

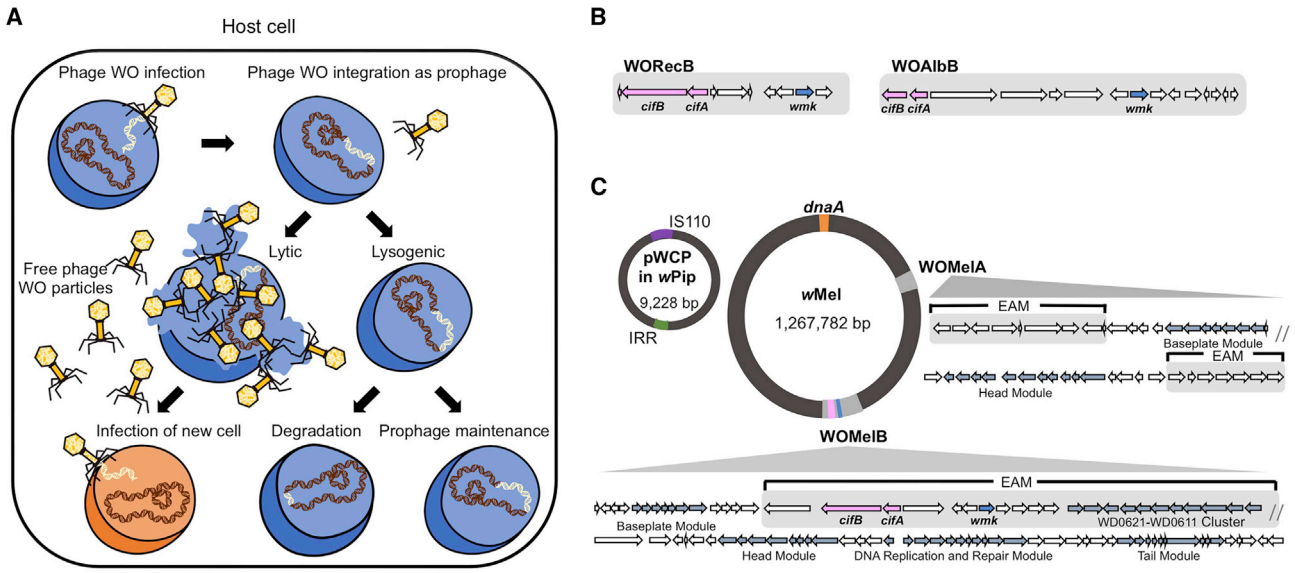

Figure 2. The tripartite symbiosis of bacteriophage WO, *Wolbachia*, and arthropods

(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 inability to form phage particles. In a process termed domestication, *Wolbachia* may retain selected, phage-associated genes for adaptive functions such as CI encoded by the *cifA* and *cifB* genes and MK 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 EAM. pWCP and phage WO are absent in the nematode *Wolbachia*.

(Table S1), and recently, over one thousand more *Wolbachia* genomes from different arthropod and nematodes species have been assembled (Scholz et al., 2020). Using this increasing array of *Wolbachia* genomes, comparative analyses identified strain-specific differences, significant genes involved in altering host biology, and evolutionary relationships at a genome level (Ishmael et al., 2009; LePage et al., 2017; Newton et al., 2016; Perlmutter et al., 2019; Rice et al., 2017).

Wolbachia's pangenome is subdivided into a typical core of genes shared by all sequenced strains and a variable accessory set of genes that contribute to considerable genome size variation (size range: 1.2–1.8 Mb, complete genomes only, Table S1). Strictly vertically inherited *Wolbachia* that are mutualistic in nematodes have a 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 mobile elements such as bacteriophage WO, transposons, and plasmids (see mobilome section below). Using microarray-based comparative genomic hybridization, a large number of accessory and rapidly evolving genes were identified, especially in prophage WO regions among closely-related *Wolbachia* supergroup A strains (Ishmael et al., 2009). Understanding the functional and adaptive significance of changes in genome size and accessory gene number remains a nascent, but exciting effort to ultimately resolve the general direction of evolution in *Wolbachia* genome size, along with the selective pressures and host-*Wolbachia* interaction processes that drive these changes.

MOBILOME: THE MASTER MANIPULATOR PHAGE WO, TRANSPOSONS, AND A PUTATIVE PLASMID

Phage WO

Phage 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; Table S2). The known majority occurs and frequently exchanges within the A and B supergroups in arthropods. 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). When phage WO enters the lytic lifecycle to copy and disperse itself, the particles assemble into standard phage structures including an icosahedral head with a short tail (Bordenstein et al., 2006; Masui et al., 2001). 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 most likely not capable of producing particles (Figure 2B). Relic phage can however be advantageous for the bacteria because their remaining genes can still produce protein products, such as prophage WO-encoded accessory genes for cytoplasmic incompatibility (*cif*) and male killing (*wmk*) (Beckmann et al., 2017; LePage

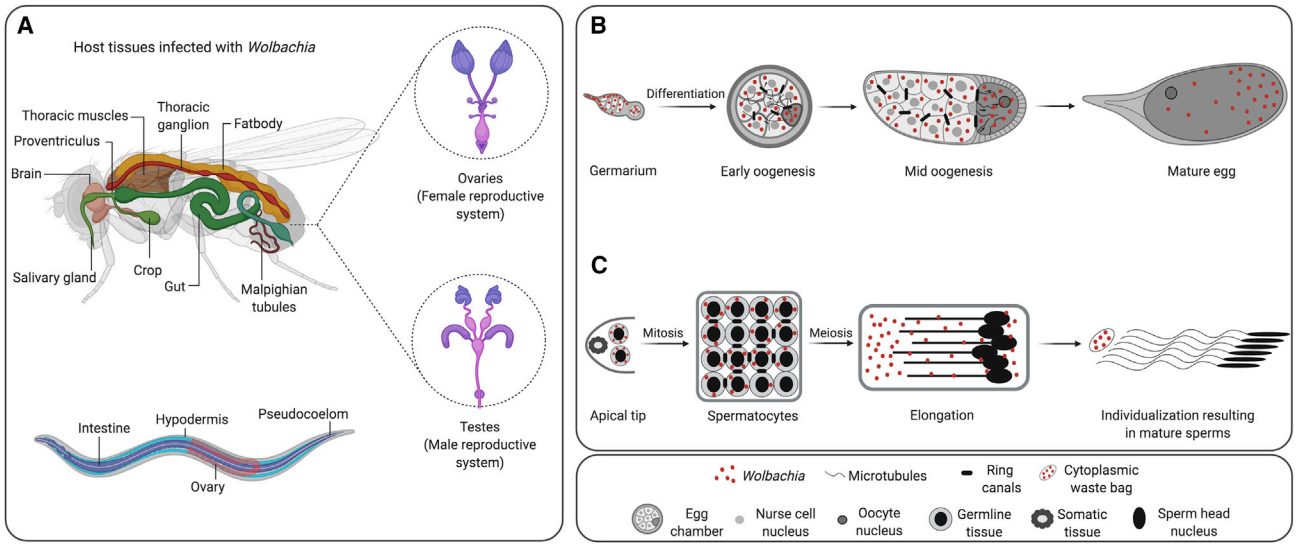

Figure 3. *Wolbachia* tissue tropism in arthropods and nematodes

(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 elongation and individualization. During individualization, excess cytoplasmic components including *Wolbachia* are removed from the mature sperm into a cytoplasmic waste bag.

et al., 2017; Perlmutter et al., 2019) found in the eukaryotic association module (EAM) (Figure 2C).

Phage WO's EAM constitutes approximately 30%–70% of the phage genome. Its genomic composition varies across prophage WO variants and encompasses proteins with domains such as ankyrins, a latrotoxin C-terminal domain, which is a crucial component of the black widow spider venom, and NACHT domains that signal programed 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). Thus, two-way transfer of DNA between phages and animal hosts is an area ripe for further investigation and discovery.

Transposons

The genomes of *Wolbachia* harbor a high frequency of transposable elements, including many insertion sequences that can occupy up to 10% of the bacterial genome (Cordaux et al., 2008; Ling and Cordaux, 2010). Group II introns that are self-splicing, mobile ribozymes are also exceptionally 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). They also play roles in the evolution of *Wolbachia* genomes by disrupting genes such as *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 putative plasmid pWCP (plasmid of *w*Pip *Wolbachia* in *C. pipiens* mosquitoes) (Reveillaud et al., 2019) 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, its distribution across the *Wolbachia* phylogeny remains to be determined. While 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 (Ishmael et al., 2009). Additional extrachromosomal elements are likely to be discovered in the *Wolbachia* mobilome.

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. In filarial nematodes, *Wolbachia* also transmit transovarially, but they do not invade the male germline and may thus respond to signaling molecules in the female germline only (Foray et al., 2018; Landmann et al., 2012).

In *Drosophila* ovaries, as germline stem cells differentiate, *Wolbachia* randomly distribute via microtubules to inhabit both nurse cells and oocytes, through a series of cytoplasmic bridges known as ring canals (Ferree et al., 2005). *Wolbachia* navigate between the two sites 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. In *Drosophila*, mutants of actin regulatory proteins ablate this function (Newton et al., 2015) and in nematodes, 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) and reside within the cytoplasm of the developing spermatocytes. Following meiotic divisions, the 16 spermatocytes produce a cyst of 64 interconnected spermatids, which are elongated with a 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 (Riparbelli et al., 2007). This purging results in elimination of *Wolbachia* from the mature sperm that typically does not transfer to the embryo.

Somatic distribution of *Wolbachia* is restricted to the hypodermis, pseudocoelom, and intestine in both sexes of filarial nematodes. In arthropods, *Wolbachia* are found in various tissues such as the gut, malpighian tubules, fatbody, brain, and head and thoracic muscles among other tissues (Figure 3A) (Pietri et al., 2016). Although various studies have revealed the functional relevance of *Wolbachia* somatic infection (Pietri et al., 2016), understanding the significance and persistence of somatic cell localization to *Wolbachia* fitness remains largely unexplored.

Vertical and horizontal transmission

Wolbachia's success is attributable in part to efficient vertical transmission through the female germline. However, cases of imperfect maternal transmission have also been detected (Hague et al., 2020). Factors that impact vertical transmission include *Wolbachia* densities, ability to migrate into the oocyte, and interactions with other symbionts (Mouton et al., 2004). Indeed, low bacterial densities can result in stochastic loss of *Wolbachia* in developing oogonia, limiting transfer to the next generation. 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 led to the evolution of the wasp gene *Wds* that suppresses 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.

Phylogenomic analyses suggest numerous host-switching events among arthropods and ancient exchanges between arthropods and nematodes. Horizontal acquisition of *Wolbachia* occurs in the lab or field via cannibalism and predation of infected individuals (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). Collectively, diverse routes of horizontal transmission raise several intriguing questions such as: do *Wolbachia* have an extracellular stage that permits 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

The most prevalent and commonly described *Wolbachia*-induced reproductive phenotype in arthropods primarily occurs in two forms: unidirectional cytoplasmic incompatibility (CI) and bidirectional CI (Shropshire et al., 2020a). Unidirectional CI results in embryonic death in crosses between infected males and uninfected females (Figure 4A), whereas bidirectional CI results in embryonic death between males and females harboring reciprocally incompatible *Wolbachia* strains. 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 (Landmann et al., 2009).

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). Cif and Cif-like protein diversity has been qualified into five phylogenetic types (LePage et al., 2017; Lindsey et al., 2018; Martinez et al., 2021). Evolutionarily guided mutagenesis of conserved sites in the CifA protein identified functional roles in CI alone or both CI and rescue (Shropshire et al., 2020b). Similar analyses in CifB reveal protein-wide conserved sites that are crucial for CI induction (Beckmann et al., 2017; Shropshire et al., 2020b). Combinatorial, transgenic analyses of CI and rescue using *cif* type I and II homologs reveal that 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., 2021). Interestingly,

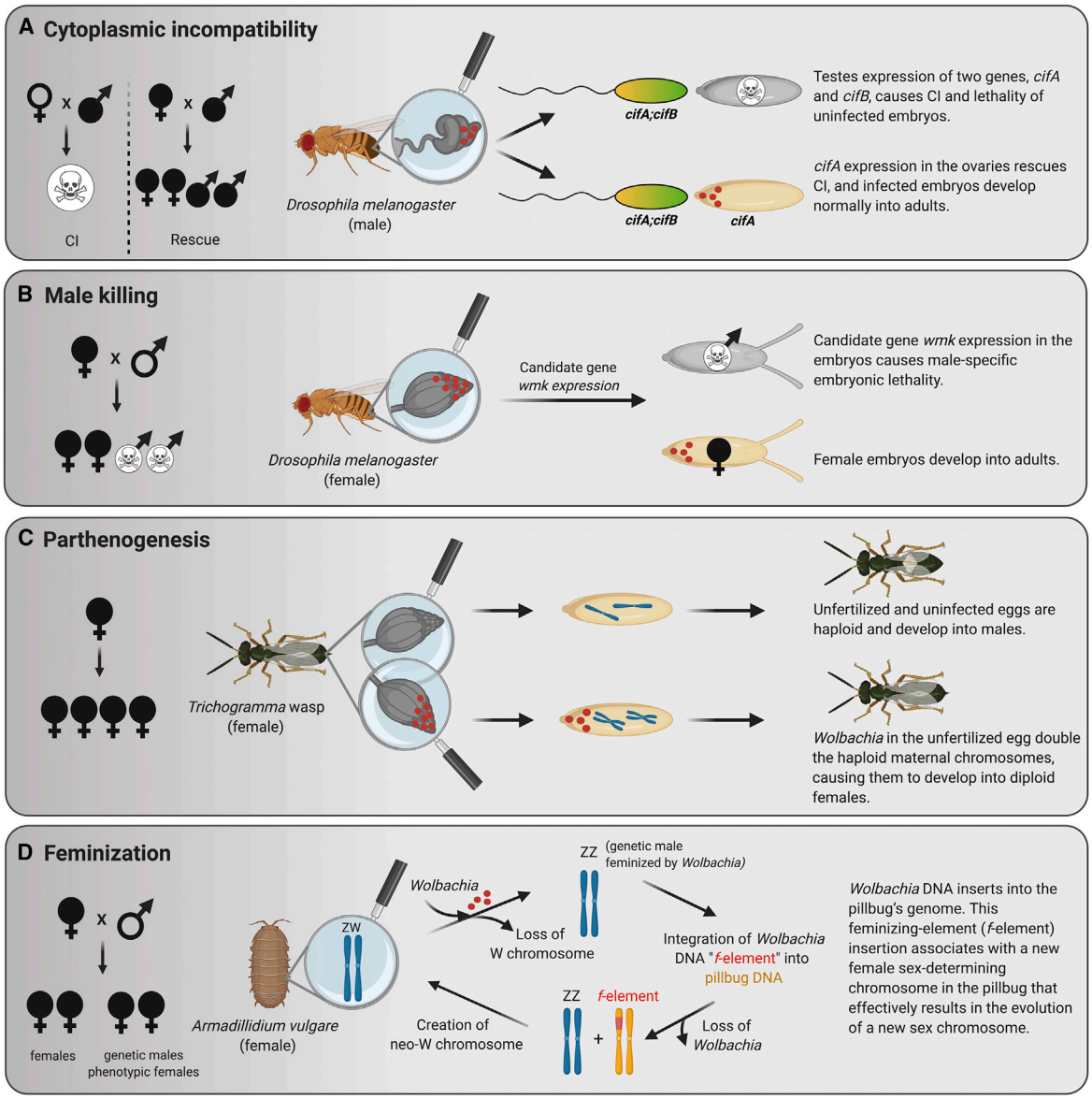

Figure 4. Reproductive phenotypes in arthropods

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 pill bug host genome that effectively resulted in the evolution of a new female sex-determining chromosome.

CI induction by co-expression of non-cognate *cifA* and *cifB* homologs support a similar mechanism of Cif-induced CI across the divergent lineages (Shropshire et al., 2021). Moreover, several host factors and genes linked to CI have been described (Shropshire et al., 2020a). Future 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 developing, infected males, resulting in female-biased sex ratios in the arthropod hosts (Figure 4B). *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* (Hurst and Jiggins, 2000). Various lines of evidence indicate that MK *Wolbachia* target developing males by hijacking the dosage compensation system that upregulates expression of male sex-chromosome-linked genes. 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).

A candidate MK gene was identified 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 linked 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 may have independent mechanisms in comparison with *Spiroplasma* MK. Questions for future research span whether or not the dosage compensation complex is directly involved in *Wolbachia*-mediated MK, where the Wmk protein localizes, and how *wmk* sequence variation impacts MK.

Parthenogenesis

Wolbachia-induced parthenogenesis occurs in several species of hymenopteran wasps, thysanopteran thrips, and trombidiformes mites (Ma and Schwander, 2017). In the absence of *Wolbachia*, these species typically exhibit arrhenotokous parthenogenesis due to their haplodiploid sex determination system. However, *Wolbachia*-infected 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 chromosomal endoduplication in which unfertilized eggs undergo diploidization in the first mitotic division as the haploid chromosome set is duplicated but fails to fully separate during anaphase. Mitotic divisions following this event are normal, resulting in the development of diploid, *Wolbachia*-infected, female offspring (Figure 4C).

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*. This 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 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). Recent work on the *T. pretiosum* epigenome showed *Wolbachia*-mediated altered splicing of various host genes involved in oocyte development, meiosis, and cell division (Wu et al., 2020). Functional genetic studies performing knockdown of target host gene expression will be useful to interrogate the contribution of particular proteins underpinning *Wolbachia*-induced parthenogenesis.

Feminization

Another sex ratio modification strategy used by *Wolbachia* is feminization, whereby genetic males morphologically develop into females (Figure 4D). The best-studied example of *Wolbachia*-induced feminization is the pillbug, *Armadillidium vulgare* (Rigaud et al., 1991). 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, developmentally forcing genetic males into morphological females (Bouchon et al., 2008). The *Wolbachia* strain *w*VulC 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*. 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 et al., 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. This phenotype was initially discovered in the *w*Mel strain of *Wolbachia* in *D. melanogaster* (Hedges et al., 2008; Teixeira et al., 2008). In mosquitoes, *Wolbachia* can limit the replication of viruses in various somatic tissues such as midgut and salivary glands, making them less capable of transmitting infection to humans (Ogunlade et al., 2021). *Wolbachia* can also confer resistance against bacteria, filarial nematodes, and the malaria parasite *Plasmodium* (Bourtzis et al., 2014), providing a broad range of pathogen protection.

Higher *Wolbachia* densities within hosts is often important for an effective antiviral response (Chrostek et al., 2013) with a few exceptions (Cattel et al., 2016; Kaur et al., 2020). At the cellular and molecular levels, *Wolbachia* can confer virus blocking by inhibiting viral binding, entry into the cell, and RNA replication in the early stages (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., 2020).

Certain host factors also play a role in *Wolbachia*-virus dynamics. Overexpression of methyltransferase gene, *aaDnmt2*, inhibits *Wolbachia* replication and promotes dengue virus replication 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, 2020). A comprehensive RNA sequencing (RNA-seq) analysis revealed that *prat2* gene involved in *de novo* synthesis of purine nucleotides upregulates in *Wolbachia*-infected flies; knockdown of *prat2* mRNA levels shows 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 that eventually result in *Wolbachia*-conferred pathogen-blocking phenotype.

Wolbachia can also upregulate the expression of genes involved in innate defense pathways and prime the insect innate immunity to block pathogen replication (Kambris et al., 2010). However, *Drosophila* species that are naturally infected with *Wolbachia* do not show an immune-priming phenotype yet confer antiviral activity (Wong et al, 2011), suggesting that such innate immune priming only occurs in hosts with artificially transinfected *Wolbachia* strains (Rancès et al., 2012) or recently introduced host-*Wolbachia* associations. Thus, it cannot explain the entirety of the effect.

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 *B. malayi* nematodes, *Wolbachia* are unable to perform *de novo* synthesis of several vitamins and cofactors such as coenzyme A, biotin, folate, etc. (Foster et al., 2005), rendering them metabolically dependent on their nematode hosts. On the other hand, elimination of *Wolbachia* via antibiotics impairs the normal development, fertility, and vitality of adult filarial worms (Coulibaly et al., 2009), suggesting host/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 genes required for heme metabolism and riboflavin essential for survival and fecundity of their host. Nevertheless, how transport, degradation and regulation of heme occurs within filarial hosts remains an open question.

In a few arthropods, *Wolbachia* can act as an obligate mutualist. 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). *Wolbachia* also enhance longevity, fitness, and fertility of flies and mosquitoes. 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

There is a genetic conflict between the biparental inheritance of host genes and the uniparental inheritance of maternally transmitted *Wolbachia* that increase their transmission through females. In response to the selective pressures exerted on hosts by *Wolbachia* functions, hosts may counteradapt and resist. For instance, *w*Mel *Wolbachia* cause variable levels of CI in their native host *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 long-term-associated hosts evolved resistance. Similarly, *Hypolimnas bolina* butterflies, *A. vulgare* pillbugs, and *T. kaykai* wasps evolved resistance to MK (Mitsuhashi et al., 2011), feminization (Rigaud and Juchault, 1992), and parthenogenetic *Wolbachia* (Stouthamer et al., 2001), respectively. While the mechanisms of host resistance to reproductive parasitism are currently unknown, their evolution is a legacy of the layers of selection and conflicts that exist between maternally transmitted *Wolbachia* and their biparentally inherited host genome.

Additionally in some lines of *A. vulgare* isopods, the "*f* element"—a horizontally transferred *Wolbachia* insert in the host isopod genome—is present on an autosome and responsible for female sex determination (Figure 4D) (Badawi et al., 2018; Cordaux and Gilbert, 2017). The *f* element contributes to feminization in the absence of *Wolbachia* and results in 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 is converted to symbiont-driven sex determination system 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

With marked impacts on gametogenesis and embryogenesis, *Wolbachia*-induced CI is a notable example whereby bacterial symbionts reduce nuclear gene flow between populations or incipient species in the absence of host genetic divergence or geographic isolation (Shropshire et al., 2020a). Moreover, *Wolbachia*-induced parthenogenesis or asexual reproduction can also cause RI because it effectively makes gene flow unnecessary (Shropshire and Bordenstein, 2016). 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). *Wolbachia* may also have subtle impacts on RI through environmental changes or host mate preference behaviors (Miller et al., 2010). Future work in additional systems can shed light on how common symbiont-assisted speciation is across the diversity of arthropods (Brucker and Bordenstein, 2012).

Gene transfers between host and *Wolbachia*

The intimate associations between host and intracellular *Wolbachia* frequently lead to lateral gene transfers (LGTs) between their genomes (Dunning Hotopp et al., 2007). In beetles, *Wolbachia* genomic fragments are transferred to the X chromosome and autosome of the host (Aikawa et al., 2009). The

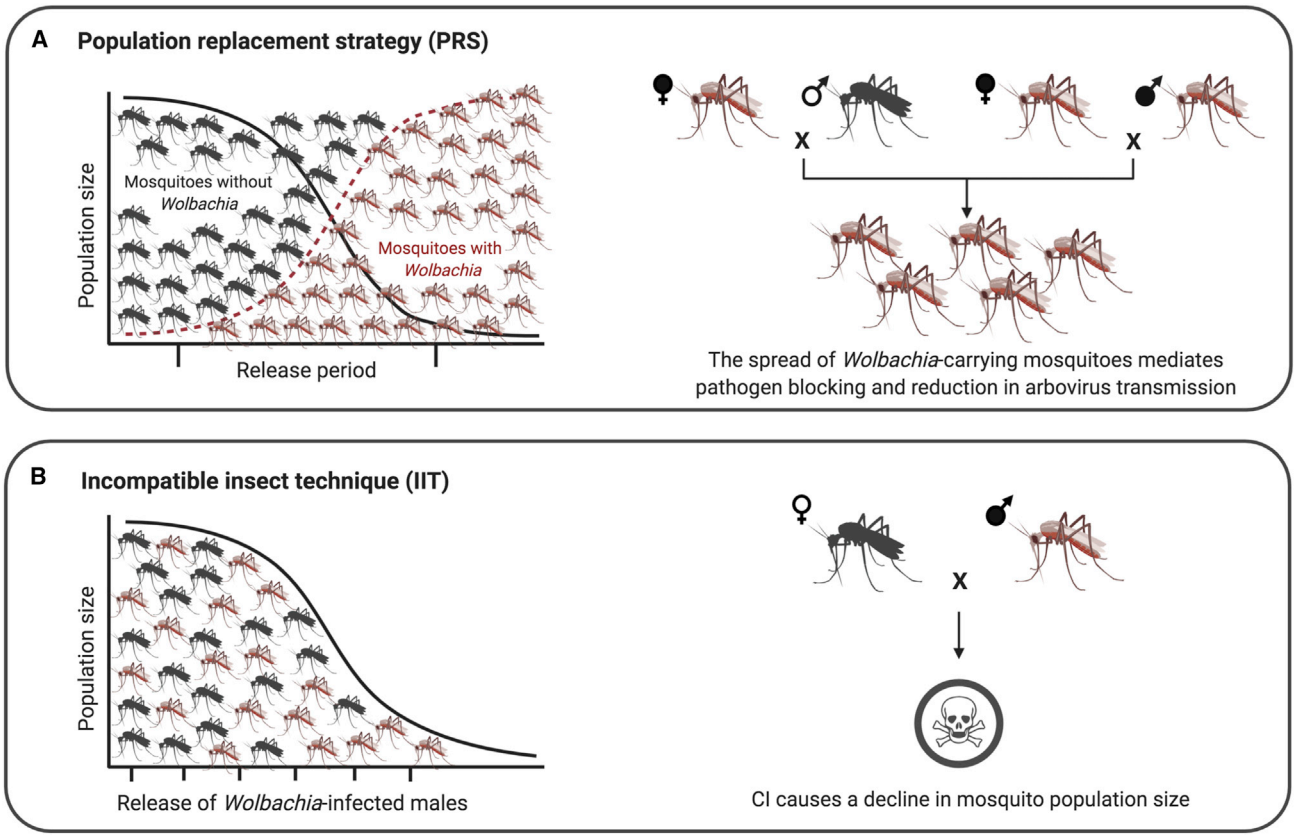

Figure 5. *Wolbachia*-mediated applications to control insect vectors
(A) Population replacement strategy commences with release of both male and female mosquitoes where CI-inducing *Wolbachia* spread throughout uninfected target populations, thus replacing the native species with pathogen-resistant, *Wolbachia*-infected mosquitoes that are 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).

transferred genes are, however, pseudogenized and likely derived from a single LGT. In *D. ananassae*, multiple copies of an entire *Wolbachia* genome occur in the fourth chromosome (Dunning Hotopp et al., 2007). 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 transfers from two different *Wolbachia* supergroups (B and F) occurred, revealing some inserts are subspecies specific while others are present in both subspecies (Funkhouser-Jones et al., 2015). The functional significance of LGTs, if any, is an important topic of future investigation, namely, to determine if the transferred genes impact phenotypes and contribute to host fitness and processes such as speciation or adaptation.

APPLICATIONS OF *WOLBACHIA* FOR CURBING HUMAN DISEASES

Population replacement strategy

Population replacement strategy (PRS) leverages two aspects of *Wolbachia* to curb mosquito-borne viral diseases—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). 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). Various studies have now demonstrated population replacement in several countries including Australia, Indonesia, Brazil, Colombia, Fiji, Kiribati, India, China, Mexico among others and reduced cases of dengue transmission (Flores and O'Neill, 2018; Joubert et al., 2016; Indriani et al., 2020; Nazni et al., 2019; Ryan et al., 2019). Furthermore, 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). 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 (Parry and Asgari, 2018).

Incompatible insect technique

Incompatible insect technique (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 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., 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* and protozoan vector *C. quinquefasciatus*.

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. 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 are not yet genetically editable (Rasgon et al., 2006); thus, efforts to investigate cellular factors required for *Wolbachia* growth and replication in the host-cell-free system are impending (Krafsur et al., 2020).

Genetic transformation of closely-related *Rickettsia* endosymbionts has been achieved using transposon mutagenesis (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 as well as bacterial integration sites 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 alter genomes and phenotypes. As such, discovery of the aforementioned plasmid pWCP in *w*Pip (Reveillaud et al., 2019) could enable development of a new genetic tool 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 can deplete *Wolbachia* by more than 99% (Johnston et al., 2014). However, lengthy treatments and restrictions against usage in children and pregnant women pose challenges to widespread implementations. 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 toward *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 trials (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

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 (Lemon et al., 2020). The project integrates genetics, entomology, evolution, molecular biology, and bioinformatics techniques to engage participants internationally. 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 end products of this lab research series are new discoveries of *Wolbachia* infections and DNA sequences, and the broad core goals of the project are to (1) engage students in nature and real-world research, (2) encourage international participation in the collection of new scientific data on bacterial endosymbionts (*Wolbachia*), (3) enhance student interest in science through an integrative lab series spanning biodiversity to molecular biology, and (4) 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 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 textbooks covering symbiosis. Several key questions for the future include: what are the biochemical and mechanistic bases of reproductive parasitism and pathogen blocking? Can *Wolbachia* be genetically manipulated to ultimately advance 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 this field, 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 educational outcomes.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.chom.2021.03.006.

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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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