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Evolutionary Ecology of *Wolbachia* Releases for Disease Control

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Abstract

Wolbachia is an endosymbiotic *Alphaproteobacteria* that can suppress insect-borne diseases through decreasing host virus transmission (population replacement) or through decreasing host population density (population suppression). We contrast natural *Wolbachia* infections in insect populations with *Wolbachia* transinfections in mosquitoes to gain insights into factors potentially affecting the long-term success of *Wolbachia* releases. Natural *Wolbachia* infections can spread rapidly, whereas the slow spread of transinfections is governed by deleterious effects on host fitness and demographic factors. Cytoplasmic incompatibility (CI) generated by *Wolbachia* is central to both population replacement and suppression programs, but CI in nature can be variable and evolve, as can *Wolbachia* fitness effects and virus blocking. *Wolbachia* spread is also influenced by environmental factors that decrease *Wolbachia* titer and reduce maternal *Wolbachia* transmission frequency. More information is needed on the interactions between *Wolbachia* and host nuclear/mitochondrial genomes, the interaction between invasion success and local ecological factors, and the long-term stability of *Wolbachia*-mediated virus blocking.

Keywords

transinfections; fitness costs; dengue; biocontrol; vector suppression; vector replacement

1. INTRODUCTION

Wolbachia is a genus of common intracellular bacterial endosymbionts, prevalent in insects and other arthropods. Recent estimates suggest that approximately one-half of insect species are infected (148), with infection incidence perhaps lower for aquatic insects (120). Infection frequencies in natural populations are variable, ranging from near 100% to very rare, both within and among species (121). *Wolbachia* is particularly common in some insect orders, such as Diptera and Hemiptera, but lower in others, such as Ephemeroptera (mayflies) (120). Most *Wolbachia* infections have been detected with molecular probes, and few have been characterized for any phenotypic effects, including cytoplasmic incompatibility (CI)—

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increased embryo mortality when *Wolbachia*-infected males mate with uninfected females or with females carrying an incompatible *Wolbachia* infection (74). Disease applications depend on *Wolbachia*'s abilities to induce CI and suppress the growth of disease-causing microbes within its hosts.

1.1. Wolbachia

Like mitochondria, *Wolbachia* is normally maternally inherited—but *Wolbachia* transmission is often imperfect. Some *Wolbachia* infections show near-perfect maternal transmission (79), but others show transmission rates as low as 80% (96). High rates of maternal transmission likely reflect tissue tropism, particularly strong associations with ovary tissues (135). Discordance between the phylogenies of *Wolbachia* and its hosts or mitochondrial DNA (mtDNA) haplotypes, which occurs across insect orders and within species, demonstrates horizontal transmission (100). Intraspecific examples include herbivory-based transmission in whiteflies (76) and horizontal transmission in *Trichogramma* (63). However, the most extensively sampled species, especially of *Drosophila* and *Culex*, reveal near-complete concordance of intraspecific mitochondrial and *Wolbachia* phylogenies, suggesting that horizontal transmission is generally rare, at least in these taxa (9, 140).

Wolbachia is known for reproductive manipulations (101), especially CI (58). CI produces a frequency-dependent fitness advantage for *Wolbachia*-infected females that can drive the spread of *Wolbachia* within and among populations. When *Wolbachia* produces deleterious fitness effects, CI can drive spatial spread only once the local *Wolbachia* frequency exceeds a threshold value over a sufficiently large area (15, 122, 139). Despite the importance of CI in driving frequency increases, once *Wolbachia* has established in a host species, selection on *Wolbachia* variants within that host species focuses on enhancing host fitness and ensuring reliable maternal transmission (137), with essentially no selection to intensify or maintain CI, even with population subdivision and local density effects (51). These patterns of selection promote the evolution of mutualistic phenotypes. One such mutualistic effect, critical for many applications, is the suppression of viruses and other microbes within infected host individuals, first demonstrated in laboratory settings (91, 131).

Studies of *Wolbachia* transinfections are being explored through microinjection into new hosts to inhibit the spread of vector-borne diseases in two ways: as agents of population suppression (analogous to sterile-male release) and as agents of population replacement (Table 1). Both rely on CI, and they are not mutually exclusive, given that suppression can facilitate replacement (59) and replacement can reduce or even crash populations owing to deleterious *Wolbachia* effects (113). The suppression approach was first applied on a field population in 1967 (Table 1). Male-only releases are used to effectively sterilize resident females; analogous sterile-male releases have controlled many agricultural invertebrate pests, such as true fruit flies (34). Males were traditionally sterilized by radiation; now some releases use *Wolbachia*-infected males subjected to low-dose radiation, which leaves males fertile but sterilizes females that might be accidentally released (155).

Replacement in a natural population was first successfully implemented in 2011 near Cairns, Australia (55) (Table 1), and preliminary data suggest that this intervention has significantly

reduced dengue transmission (111). This approach uses CI to drive *Wolbachia* variants that inhibit disease transmission, even when they might reduce host fitness, into a vector population. Other *Wolbachia*-based strategies, such as suppressing populations using *Wolbachia* with environment-dependent deleterious effects, have been proposed (81, 107). Replacement is undertaken under permissive conditions, in which the transinfection is relatively benign but the population would crash when the *Wolbachia* becomes severely deleterious. Because of their pervasiveness in nature and lack of genetic modification, *Wolbachia* variants are regarded as biocontrol agents. This contributes to greater public acceptance of *Wolbachia*-based strategies than of genetically modified organisms (89), with fewer risks identified (95).

Although releases of multiple *Wolbachia* variants, involving both suppression and replacement, are now being undertaken around the world, there are challenges in their application (Figure 1). Some revolve around the long-term stability of *Wolbachia* effects, which depends on a tripartite system of host, *Wolbachia*, and other microbes, including those that cause disease, interacting within a domestic landscape. Other issues involve the feasibility of widespread releases, especially of those undertaken by public health authorities with less quality control than scientific trials.

Our aim is to explore these challenges within an evolutionary and ecological genetic context. How do we identify useful *Wolbachia* variants, likely to have desirable phenotypic effects for alternative strategies, and maintain stability in the longer term? Which ecological and evolutionary issues need to be considered to ensure effective release programs, minimizing the number of releases required and maximizing spread? Is there evidence for interactions between *Wolbachia* and host genomes that might threaten a *Wolbachia* strategy or enhance its effectiveness? What environmental effects need to be considered in a successful *Wolbachia* strategy, and do these effects place limits on suitable variants for different contexts? We link findings from natural *Wolbachia* infections to applications in disease vector organisms. This requires understanding the similarities and differences between natural infections and human-introduced transinfections.

1.2. Dynamics of Transinfections Versus Natural *Wolbachia* Infections

Initial *Wolbachia* transinfections involved moving strains between *Drosophila* species (57, 62). However, over 20 *Wolbachia* variants have now been introduced into disease vector mosquitoes, in which they exhibit heterogeneous effects on fitness, reproduction, and virus interference (Supplemental Table 1). *Wolbachia* effects can change dramatically from native to novel hosts (145). In general, however, *Wolbachia* transinfections generated to date decrease host fecundity and viability (Figure 2) while causing CI and perhaps suppressing disease-causing microbes. The combination of CI, which provides a positive frequency-dependent fitness advantage to the *Wolbachia*, and deleterious effects on host fitness produces bistable frequency dynamics under which the *Wolbachia* infection frequency must exceed a threshold, the unstable equilibrium, denoted $\hat{p}$, for the infection frequency to tend to rise (26, 60, 138). Below $\hat{p}$, deleterious effects dominate and infection frequencies tend to decline. Occasionally, stochastic sampling effects, analogous to genetic drift, can move frequencies of fitness-decreasing *Wolbachia* infections above the unstable equilibrium,

leading to local establishment (65) and possibly spatial spread (15). Fitness variation will also arise among individuals owing to demographic effects (49).

Cumulative data point to a qualitative distinction between natural *Wolbachia* infections and human-mediated transinfections. The first example of *Wolbachia* spatial spread involved variant *w*Ri spreading northward through California *Drosophila simulans* at roughly 100 km/year (141, 142). When *Wolbachia* infection frequencies are low, CI has a negligible effect on frequency dynamics, which is dominated by the relative fitness of infected females, denoted F, and maternal transmission frequency, $1 - \mu$, where μ denotes the fraction of uninfected ova produced by in fected females. When $F(1 - \mu) < 1$, rare infections tend to be eliminated; but a sufficient level of CI can produce bistable dynamics. Prior to work by Weeks et al. (147), several studies demonstrated that *w*Ri-infected *D. simulans* produced fewer eggs than uninfected females in the laboratory, with typical estimates of relative fecundity near 0.9–0.95. Moreover, wild-caught *w*Ri-infected *D. simulans* typically produce 4–5% uninfected ova (24). These data indicated $F(1 - \mu) < 1$, so Turelli & Hoffmann (141, 142) conjectured that *w*Ri spread despite bistable dynamics. The hypothesis that fitness-decreasing *Wolbachia* might spread rapidly in nature motivated proposals for disease control via *Wolbachia*-based population replacement strategies (101). However, rapid spatial spread of *w*Ri and other natural *Wolbachia* infections (e.g., 12) is difficult to reconcile with the mathematical theory for bistable waves (discussed in Section 6.2.2), which anticipates the slow spatial spread, on the order of 100–200 m/year, observed for *Wolbachia* transinfections in Australian *Aedes aegypti* (122).

A more plausible view is that natural *Wolbachia* infections, unlike transinfections, often confer net fitness advantages, producing $F(1 - \mu) > 1$, and hence tend to increase when rare. This must be true for natural infections, such as *w*Au in *D. simulans*, that produce no detectable reproductive effects (or minimal effects) and are imperfectly maternally transmitted, yet spread in nature (58). These infections may represent an evolutionary progression from strong to weaker CI (90, 137); CI is not favored by selection on *Wolbachia* within host lineages, so it can be reduced as *Wolbachia* evolves to reduce costs to female hosts or increase maternal transmission (136), and it can be suppressed by host evolution. However, this progression may be a slow process, since at least for the *w*Ri infection there has been no change in CI under field or laboratory conditions across several decades (24) despite theoretical expectations.

2. *WOLBACHIA* AND VIRUS BLOCKING

Wolbachia-mediated protection in arbovirus vectors is an area of increasing interest, with over 50 studies of *Ae. aegypti* alone. Most studies demonstrate that *Wolbachia* reduces infection or transmission of pathogens but there are exceptions (e.g., 35, 45). The extent of virus blocking provided by transinfected *Wolbachia* depends on the *Wolbachia* variant and host species as well as on the nature of the virus (4, 39, 150). For the *w*Mel infection, which is the best studied and is now being released into the field, the extent of virus blocking is variable (25) and can differ between studies. Virus blocking depends on virus serotype, titer, mosquito genetic background, rearing conditions, and the method of infection; thus, methodological differences between studies may produce different outcomes (149).

Virus blocking has been linked to *Wolbachia* density (84), although some high-density variants do not seem to block, such as *w*AlbA when transferred to *Ae. aegypti* (4). Virus blocking may also depend on tissue distribution and effects on lipid pools needed for viral replication. In *Drosophila*, virus protection correlates with higher *Wolbachia* density in the head, gut, and Malpighian tubules (103), while in mosquitoes high densities in the midgut and salivary glands are thought to be important for dengue inhibition. Some variation in blocking levels may be related to viral dose. Recent data on *w*Mel (68) suggest low protection or even increased transmission when dengue dose is low, whereas protection appears stronger at high doses. This variability may still allow population-level reduction of viral transmission by *Wolbachia*, but variability must be considered when assessing overall effects and evolutionary dynamics (68).

Virus blocking may depend on the novelty of *Wolbachia*–host association. Multiple mechanisms are involved in blocking but novelty may be important for immune upregulation (78, 132). This is evident from the blocking caused by *Wolbachia* newly transferred to hosts, typified by *Ae. aegypti*, in which several *Wolbachia* variants block dengue virus (4, 91, 146). Conversely, blocking associated with native *Wolbachia* variants in *Aedes* species such as *Ae. albopictus* and *Ae. polynesiensis* appears weak or variable (16, 93), whereas novel *Wolbachia* transinfections in these species significantly block dengue and Zika viruses (16, 92). Virus blocking may relate to certain types of reproductive manipulations, but this needs further study particularly given that male-killing *Wolbachia* can also inhibit viruses, as demonstrated for *Drosophila pandora* (8).

Currently, there is little evidence that *Wolbachia*-based interventions will lead to increased disease virulence or will be rapidly circumvented by viruses (18), but this area requires more research. Across evolutionary time, virus blocking may evolve through changes in host genes, *Wolbachia* genes, or both. There may be a downregulation of virus blocking due to costs associated with mounting an immune response, which is countered by any ongoing selection for virus blocking in natural populations.

Virus blocking is not critical to *Wolbachia*; some natural variants that do not block viral transmission persist in host populations (86, 104). *Wolbachia* from natural populations is expected to block more effectively when arboviruses are detrimental—a condition met in assays of virus blocking in *Drosophila* where viruses often reduce longevity (52, 131). Apart from selection mediated through host fitness, virus blocking by *Wolbachia* should be favored if it increases *Wolbachia* transmission to offspring.

There is currently little information on whether natural *Wolbachia* infections effectively block viruses in the field, or on any interactions between blocking and host fitness or maternal transmission. For *w*Mel in *Drosophila*, the evidence for such blocking looks weak so far; in flies isolated from the same field population in which the *Wolbachia* infection was either present or absent, there was no association between *Wolbachia* and either viral load overall or the presence/absence of specific viruses (126). This is an area where additional data, particularly for disease vectors, are needed.

3. FITNESS EFFECTS OF *WOLBACHIA*

Fitness is an important determinant of invasion success for deliberate releases of mosquitoes; severe fitness costs can prevent transinfected *Wolbachia* from establishing even when reproductive effects are strong (98), whereas positive fitness effects increase the rates of establishment and spread of *Wolbachia*. Laboratory experiments indicate that native *Wolbachia* infections can provide fitness benefits such as fecundity benefits or changes in life cycles (71, 158) as well as virus protection. As noted above, positive effects—largely uncharacterized—can explain the persistence of non-CI *Wolbachia* in natural populations and the spread of CI-causing *Wolbachia* from low frequencies (72, 90). However, the overall impact of *Wolbachia* infections on host fitness is often poorly understood for natural infections, particularly under field conditions. Fitness effects observed in laboratory studies are often insufficient to explain the dynamics of *Wolbachia* in natural populations (71, 90); thus, many fitness effects are likely undescribed.

Wolbachia introduced experimentally into mosquitoes for disease suppression has diverse effects on fitness, with a mix of costs and benefits depending on the variant (Figure 2). Fitness effects in novel hosts can be hard to predict since densities and tissue distributions can change dramatically upon interspecific transfer. For example, the *w*AlbA/*w*AlbB superinfection in *Ae. albopictus* provides an overall fitness benefit, but when *w*AlbB alone is transferred to *Ae. aegypti* it induces fitness costs (Figure 2*a*). Experimentally generated *Wolbachia* infections in *Ae. aegypti* tend to affect fertility most severely, while effects on other traits are less clear (Figure 2*b*). Fitness effects depend strongly on the environmental context; costs are often not apparent in standard laboratory assays but can be exacerbated under stressful conditions such as when larvae are held under competitive conditions (115) or when adults or eggs are aged (88, 154).

Evaluating the fitness of *Wolbachia*-infected individuals is challenging because confounding factors affect experimental outcomes. Yet fitness parameters, often from preliminary studies, are used in mathematical models aimed at informing the choice of *Wolbachia* variants for disease control (152). For variants introduced into *Ae. aegypti*, studies of the same trait sometimes produce conflicting results (Supplemental Data Set 1). Most studies compare the fitness of *Wolbachia*-infected and uninfected populations by removing *Wolbachia* with tetracycline treatment, but this can cause off-target effects (77). Fitness effects can also vary depending on genomic background (108) and may change over time with laboratory culture (22, 87). In *Ae. aegypti*, fitness can differ between replicate populations after only a few generations, making it difficult to attribute fitness effects to *Wolbachia* infection alone, particularly if lines differ in inbreeding levels (116). Most studies measure fitness under laboratory conditions, so traits important for disease control, such as host-seeking behavior and biting ability, are rarely measured under realistic contexts.

Fitness effects on mating are particularly important when considering releases aimed at population suppression. Most data, including those from experiments on *Ae. aegypti* in tent enclosures (125), suggest that the mating competitiveness of *Aedes* males is unaffected by *Wolbachia* (Supplemental Data Set 1). There is also no evidence for *Wolbachia*-related assortative mating in *Ae. aegypti* (19) or *Culex pipiens* (10). However, *Wolbachia*-related

assortative mating has been documented in other systems including *D. melanogaster* (69) and spider mites (144).

From a disease perspective, a key question is whether virus blocking is mechanistically associated with deleterious fitness effects (making population replacement harder for variants with strong virus blocking). In *Ae. aegypti*, transinfected *w*MelPop and *w*Au provide strong blocking (4, 146) but induce large fitness costs. In *Drosophila*, infections providing strong blocking tend to occur at a higher density and have higher fitness costs (29, 84). But there are exceptions to these patterns; *w*Au has a high density and strong virus protection with little impact on fitness in *D. simulans* (85), suggesting that virus blocking and fitness costs are not always linked. Further, multiple strains present in the same individual might impose additional fitness costs but also additional blocking potential. In *Ae. aegypti* doubly infected with *w*Mel and *w*AlbB, extra protection seems to be provided without additional fitness costs (67).

Theory predicts that *Wolbachia* should evolve toward mutualism or at least toward reduced costs (137). Evolutionary changes leading to increased fitness have been documented for *D. simulans* infected with *w*Ri (147), and laboratory selection can produce rapid changes in *Wolbachia*-associated phenotypic effects on host fitness (29) and nucleus-associated changes in *Wolbachia* effects on host fitness (23). A recently discovered *Wolbachia* strain limited to adult females (109) may also reflect ongoing *Wolbachia* and host evolution to reduce host costs but ensure maternal transmission. It is not clear whether these types of evolutionary shifts in fitness influence virus blocking by *Wolbachia*.

4. ENVIRONMENTAL EFFECTS ON *WOLBACHIA*

Frequencies of *Wolbachia* infections in natural populations are highly variable (121). In some insects, such as *Ae. albopictus*, *Wolbachia* infections are nearly fixed throughout their distribution (6), while in others there are strong geographic or climatic patterns. Frequencies of *Wolbachia* infection can vary clinally; for example, in *Drosophila melanogaster* (71) and *Curculio sikkimensis* (134) frequencies increase at lower latitudes. Similar patterns are observed across entire insect orders such as Lepidoptera (2), but patterns across arthropods as a whole are less clear (28). *Wolbachia* densities and frequencies can differ between locations (30, 136) and vary seasonally (130). These patterns may reflect environment-dependent selection on *Wolbachia* infections but could also result from direct environmental effects on *Wolbachia* or its maternal transmission.

Several environment-dependent fitness effects of *Wolbachia* have been documented. *Wolbachia* infections often reduce host viability during periods of dormancy such as in *Ae. aegypti* (88, 154) and *D. melanogaster* (71); this may reflect *Wolbachia* replication while the host is inactive. In *Ae. albopictus*, *Wolbachia* shifts from beneficial to detrimental when larvae are reared under nutritional stress (43). Nutrition also modulates the effects of *Wolbachia* on *Drosophila*, with both costs and benefits to fecundity depending on diet (17, 106). Temperature strongly influences fitness effects; the severity of life shortening by *w*MelPop in *D. melanogaster* is increased at higher temperatures (108), while *Wolbachia* can influence thermal tolerance in both natural (46) and transinfected (117) hosts.

Environmental conditions associated with temperature and nutrition influence the reproductive effects of *Wolbachia* and may explain differences seen between field and laboratory populations (60). High temperatures can reduce rates of maternal transmission, CI, and male killing, with effects depending on host species (32, 77) and *Wolbachia* variants (118). In *Ae. aegypti*, *w*Au is more stable than *w*Mel under heat stress even though these variants are derived from closely related hosts (4). Temperature effects could influence the ability of *Wolbachia* transinfections to invade and persist in *Ae. aegypti* populations (117), but this will depend on the proportion of breeding sites exposed to high temperatures, which is likely to be highly variable. Critically, high temperatures could also influence viral disease blocking by *Wolbachia*, given the link between *Wolbachia* density and the strength of blocking dengue (80). There is some evidence that environmental conditions modulate *Wolbachia*-mediated pathogen blocking in other systems (21, 94). Simulated data for Malaysia suggest that high temperatures will reduce *Wolbachia*-mediated virus blocking (W.A. Nazni, unpublished data), but there is no evidence that *w*Mel has a reduced ability to block viruses under warm field conditions in Vietnam (25). Besides temperature and nutrition, *w*Mel *Wolbachia* density and host reproductive effects in *Ae. aegypti* are affected by low levels of antibiotics likely to be encountered in some mosquito breeding sites, although *w*AlbB appears unaffected by low levels of antibiotics (37).

5. EFFECTS OF HOST VARIATION

Components of hosts that need to be considered in *Wolbachia* releases include host nuclear genomes, other *Wolbachia* variants, other bacteria, and mtDNA. Pesticide resistance in release stocks should ideally match levels in target populations. If resistance levels are too low, field pesticide use can result in the failure of releases. This occurred in an area of Rio de Janeiro where *Wolbachia* releases led to intermediate frequencies of *w*Mel before the infection frequency dropped due to low levels of pyrethroid resistance (42). Because resistance alleles carry fitness costs, resistance decreased while release stocks were being developed. Field fitness may also be reduced through genetic adaptation to artificial rearing conditions, a phenomenon common to many reared insects, including mosquitoes (56). Maintaining the quality of release stocks is a key component for successful sterile release programs (129).

The impact of other *Wolbachia* infections carried by a host needs to be considered. For instance, *Ae. albopictus* generally carries *w*AlbA and *w*AlbB, so triple infections are needed for population replacement, whereas triply infected strains or novel strains with *w*AlbA and *w*AlbB removed but carrying another variant added are needed for suppression (20). Population replacement depends on the direction and strength of incompatibility. With bidirectional incompatibility, replacement will generally be difficult because the unstable equilibrium is likely to be near 50% (58). Such infections are unlikely to spread because they are easily stopped by barriers to dispersal and density-dependent effects that slow mosquito development rates (49). Unidirectional incompatibility facilitates invasion because the unstable point is typically lower.

In *Ae. aegypti*, natural *Wolbachia* infections are absent or at least geographically restricted. A recent global survey did not detect natural *Wolbachia* infections (44), but some studies

claim rare natural *Wolbachia* in some *Ae. aegypti* populations (53, 73). These studies are limited mostly to molecular detection, so it is unclear whether these infections could influence invasion of a CI-causing variant. However, it is important to monitor target release areas for natural *Wolbachia* infections.

Apart from native *Wolbachia*, interactions with other endosymbionts and the complex mosquito microbiome (53) could influence host fitness and indirectly affect *Wolbachia* invasion. Laboratory studies suggest that *Wolbachia* may substantially alter the gut microbiome in *D. melanogaster* even though *Wolbachia* is absent from the gut (127). However, in field populations of *Ae. aegypti*, *w*Mel shows relatively small effects on the microbiome of adults and none on larvae (11).

As *Wolbachia* invades, it may also introduce novel mtDNA variants (47, 64, 133, 143), so mtDNA–*Wolbachia* interactions may be relevant. Comparative genomics of mtDNA and *Wolbachia* within and between hosts can identify and help date horizontal or introgressive transfer of *Wolbachia* (31, 110, 140). There is no evidence for common horizontal transfer of *Wolbachia* in mosquitoes (9), and we are unaware of mtDNA–*Wolbachia* interactions that influence host or *Wolbachia* phenotypes, but this needs further testing.

6. POPULATION REPLACEMENT WITH *WOLBACHIA*

6.1. *Wolbachia* Invasion and Spread in Nature

Given the high incidence of *Wolbachia* across insects and mites, the bacterium is clearly successful in spreading within and among natural populations and species. If *Wolbachia* causes intense CI, local spread will be rapid once the frequency appreciably exceeds the unstable threshold frequency, expected to be near zero for natural infections but possibly 10–30% for many transinfections. The ultimate rapid local spread reflects the massive advantage that infected females have over uninfected females once infected males become common.

Only a few natural *Wolbachia* invasions have been documented. This is unsurprising since most cases of horizontal transmission of *Wolbachia* in nature are expected to fail and successful spatial spread can be rapid (72). Despite a few exceptions (63, 76), intraspecific horizontal transmission seems generally rare (31, 110, 140). Empirical examples of spatial spread occur with CI (12, 141), with male killing (61), and with no reproductive manipulation (72). *Wolbachia* variants that cause strong CI are usually at high frequencies in populations (71), whereas male-killing *Wolbachia* variants tend to persist at lower frequencies, but there are exceptions (27). *Wolbachia* spread can be blocked by other *Wolbachia*. In *Cx. pipiens* in Tunisia, two *Wolbachia* variants show clear spatial separation, with a sharp contact zone that has been stable for seven years; one variant causes strong CI in crosses with females carrying the other variant, while the reciprocal cross produces CI in only some crosses (10). As discussed in Section 6.2.4, dispersal barriers and spatial variation in population density probably contribute to contact zone stability. Stable but variable infection frequencies across populations also occur in *Drosophila* (71).

6.1.1. Field introductions.—In *Ae. aegypti*, successful field invasion of transinfections has been achieved for two variants, *w*Mel and *w*AlbB (Table 1). Both variants cause strong

CI in *Ae. aegypti*, and invasion was first established in small cages (146, 151). An additional variant, *w*MelPop, invaded field cages (146) but did not successfully invade field populations, likely because of strong deleterious fitness effects (98, 153).

For *w*Mel released in northern Australia, infection frequencies appear relatively stable after invasion (54, 102), although infection frequencies decreased in one area of Cairns following invasion, possibly because the release area was too small (122). High levels of maternal transmission have also been maintained in the field; however, one case of nontransmission has been detected (123). *w*Mel has maintained its ability to block dengue virus after one year in the field (40), but its effects on disease transmission will need to be monitored in case changes in bacterial density or host genomic background weaken virus suppression (114). This might occur if attenuation of negative fitness effects associated with *Wolbachia*, as occurred in *D. simulans* (147), is correlated with reduced virus blocking.

6.1.2. Multiple infections.—In addition to the release of hosts carrying one *Wolbachia* variant for population replacement or suppression, hosts carrying multiple *Wolbachia* variants are now being released (Table 1). Multiple infections may be useful in several contexts. First, a multiply infected host may be used to invade populations already naturally infected by one *Wolbachia* variant. In cases in which males carrying each variant cause CI with uninfected females and with females carrying the alternative variant, males carrying both variants are typically incompatible with females carrying only one variant, whereas females carrying both variants are compatible with doubly and singly infected males (58). This pattern is seen when *w*Ri from *D. simulans* is added to the natural infections *w*AlbA and *w*AlbB in *Ae. albopictus* (41). Second, double infections can be used after successful introduction of a singly infected strain fails to achieve its objectives, such as when it no longer effectively blocks arbovirus transmission (114).

Although many insects carry multiple *Wolbachia* variants [e.g., *Rhagoletis cerasi* carries at least five (7)], laboratory production of multiply infected strains capable of invading natural populations is not straightforward. In *Ae. albopictus*, which is usually naturally infected by *w*AlbA and *w*AlbB, adding *w*Mel produces a new strain that shows self-incompatibility likely because one of the native *Wolbachia* is lost from ovaries (5). By contrast, a strain of *Ae. aegypti* coinfected with *w*Mel and *w*AlbB appears relatively stable and self-compatible, and its males are incompatible with females singly infected with either *Wolbachia* variant (67). It remains to be seen whether multivariant transinfections are stable (and stably transmitted) in nature, given that natural double-infected species show occasional infection loss under field conditions. For instance, *Ae. albopictus* often exhibits a low frequency of singly infected or uninfected individuals in nature plus a high level of variation in relative densities of different *Wolbachia* variants (1). *Wolbachia* density variation is probably associated with environmental conditions, as discussed above.

6.1.3. Complications.—*Wolbachia* invasion has so far relied largely on releases in target areas with only low-rise buildings (55, 102, 122), but in many landscapes, building height varies (36), adding a vertical component to host distribution. *Wolbachia* releases must consider the effects of a three-dimensional urban landscape structure on the distribution of hosts in a target area. Nevertheless, in *Ae. aegypti*, invasion of a *w*AlbB transinfection has

succeeded in a relatively small area consisting of 18-story buildings (W.A. Nazni, unpublished data) likely because mosquito movement occurs primarily within rather than across buildings (66).

6.2. Theory Concerning Wave Initiation, Speed, Width, and Stopping with Bistable Dynamics

We focus on idealized models describing the spatial spread of transinfections that produce strong CI but induce fitness costs that lead to bistability. In these simple models the position of the unstable infection frequency, denoted $\hat{p}$, is determined by the rate of maternal transmission, fitness effects, and CI intensity (26, 58). More realistic models involving factors such as age structure (138), population dynamics (49), and sex-dependent effects cannot be fully described through the use of infection frequencies only. Nevertheless, idealized models that track temporal and spatial dynamics of infection frequencies suffice to illustrate key theoretical ideas (15) and seem to provide a useful first approximation for understanding actual transinfection releases (122). Easily described results are generally available only for continuous-time, continuous-space approximations. Hence, we focus on these results for their heuristic value but indicate how less idealized approximations that account for more realistic dispersal and local frequency dynamics alter the simplest predictions (139).

Although stochastic effects are needed to understand local spread of rare bistable variants (65) or spatial spread beyond barriers that would be expected to halt spread (48), we focus on deterministic models for simplicity.

6.2.1. Wave initiation.—In an isolated population, establishment of a transinfection with bistable dynamics requires that releases produce a transinfection frequency that exceeds the unstable point, $\hat{p}$. To replace mosquito populations in large continuous landscapes, release programs must introduce the transinfection in an area that is large enough to persist in the face of migration of uninfected individuals from the surrounding habitat. Barton and Turelli (15, 139) provide approximations for the minimum size of the release areas, and those predictions seem consistent with initial empirical releases (122). There are two key results. First, spatial spread can occur only if the unstable point, $\hat{p}$, is sufficiently small, with a maximum near 0.5. Second, the minimum size of a release area depends on both $\hat{p}$ and the average dispersal distance of the host, denoted σ. As discussed below, wave speed in a homogeneous environment is roughly proportional to $\left(\frac{1}{2} - \hat{p}\right)$ and this difference also governs the sensitivity of spread to environmental heterogeneity. Hence, transinfections should ideally have $\hat{p} < 0.3$ for spread. When this is satisfied, a fairly robust prediction is that release areas with a radius of at least 4σ should suffice to initiate spatial spread. For a vector such as *Ae. aegypti* with σ on the order of 100 m, release areas of roughly 0.5 km^2 should suffice. When more realistic dispersal models that are long tailed relative to a Gaussian (i.e., most individuals move short distances but a few go relatively far) are analyzed, the minimal release area is smaller (139).

Data from 2013 releases of *w*Mel-transinfected *Ae. aegypti* in Cairns seem broadly consistent with these predictions. Releases in areas of roughly 1 km^2 and 0.5 km^2 led to

spatial spread of the transinfection, but a release in a much smaller area (0.1 km^2) seemed to be collapsing before additional releases were undertaken. Releases across large areas require more resources depending on mosquito densities. For instance, invasion of an area with approximately 600 houses and an estimated density of 5–10 female mosquitoes per house was successful, after releases of approximately 10 *w*Mel females per house per week across 10 weeks (112). Releases in areas with strong density dependence in breeding sites are also predicted to occur much more slowly because of heterogeneity in mosquito fitness (49).

6.2.2. Wave speed.—If we assume complete CI, so that all embryos produced from incompatible matings die, an idealized model of spatial spread with bistable dynamics ($\hat{p} > 0$) in a homogeneous environment predicts that the transinfection will spread in all directions at a speed of

$$c = \sigma\left(\frac{1}{2} - \hat{p}\right) \quad 1.$$

per generation, where $\hat{p}$ is the unstable equilibrium and σ is the dispersal distance per generation (15). If CI is incomplete, wave speed is multiplied by $\sqrt{s_h}$, where s_h is the relative reduction in embryo viability induced by CI. Less idealized models, with fast local dynamics and long-tailed dispersal, predict slower spread, with plausible reductions on the order of 20–30% (139). Note that if σ is approximately 100 m and $\hat{p}$ is approximately 0.25, Equation 1 predicts spread at approximately 25 m per generation. Hence, with approximately 10 generations per year, we expect the transinfection to spread at roughly 250 m/year. Accounting for more realistic local dynamics and dispersal reduces the prediction to roughly 175–200 m/year. Note that Equation 1 predicts that wave speed should be relatively insensitive to slight decreases in fitness costs. For instance, decreasing fitness costs so that $\hat{p}$ is approximately 0.05 rather than 0.25 would less than double predicted speed.

In the only field releases yet analyzed, *w*Mel-infected *Ae. aegypti* spread from two areas of Cairns at roughly 110 and 180 m/year (122). These data are broadly consistent with our approximations for bistable waves but orders of magnitude slower than the rates of spatial spread for natural *Wolbachia* infections in *D. simulans* (72) and *R. cerasi* (12) despite comparable dispersal behaviors of the dipteran hosts (122). Any factors that reduce dispersal, such as size-reducing high larval densities or increased adult densities associated with favorable habitats, will slow wave speed. A critical empirical question is whether natural populations of *Ae. aegypti* and other vectors are sufficiently homogeneous for Equation 1 to provide a useful approximation. Monitoring data from recent urban releases will determine the robustness of the apparent agreement of Equation 1 with field data from Cairns (122).

6.2.3. Wave width provides an estimate of average dispersal.—The explicit traveling-wave approximation that generates Equation 1 also provides a simple description for the shape of the advancing wave. Defining wave width as the inverse of the maximum slope of infection frequencies (38), both the simplest models and more realistic models predict that the traveling wave has a width of roughly (139)

$$w = 4\sigma/\sqrt{s_h}, \quad 2.$$

which becomes 4σ with complete CI, as in *Ae. aegypti*. From the shape of the wave, we expect infection frequencies to increase from approximately 0.18 to 0.82 over 3σ, with complete CI. This relationship allows us to approximate average dispersal in the field in locations where successful releases are undertaken. Because wave speed and minimal release areas depend on σ, initial estimates can inform future releases in comparable landscapes.

6.2.4. Wave stopping.—In addition to traveling much more slowly than natural *Wolbachia* infections, for which we expect $F(1-\mu) > 1$ and $\hat{p} = 0$, the spread of bistable transinfections is readily stopped by barriers to dispersal that effectively reduce migration in the direction of wave movement (14, 15). Conversely, spread is expected to accelerate from high-density to low-density locations because of asymmetry in migration. Once the unstable point approaches 0.3, spreading waves are predicted to be stopped by even relatively minor barriers, for instance, an increase in population density on the order of 2 or 3. Spreading into an area of higher population density is analogous to crossing a barrier that reduces migration (15). In Gordonvale, Australia, one of the initial release sites for *w*Mel-infected *Ae. aegypti* (55), a highway that interrupted housing for roughly 100 m sufficed to block the spread of *w*Mel for several years (139). The effects of roads on movement have also been documented through mark-release experiments (119) and through genetic relatedness comparisons (123). Vegetation may also act as a barrier to *Ae. aegypti* (122). High-density populations of conspecifics should prevent the spread of *Wolbachia* with deleterious effects due to density effects.

6.2.5. Release strategies and contribution of slow transinfection spread to area-wide control.—Turelli & Barton (139) address how to most efficiently transform large areas and the potential contributions of even slow spread as predicted by Equation 1 and its refinements. Given the tendency for transinfections to move down density gradients, initial releases should target areas of greatest disease vector density, which also tend to be hot spots of disease incidence. Even slow spatial spread of transinfections on the order of 10–20 m/month will lead to area-wide coverage over a few years, with releases covering only 20–30% of the target area. However, this idealized prediction ignores the practical problem that releases must be carried out in all areas that are isolated by effective barriers to dispersal. As field releases continue, we expect more useful predictions about barriers that stop transinfection spread (122).

7. VECTOR POPULATION SUPPRESSION WITH *WOLBACHIA*

The use of CI to suppress vector populations was initiated in successful local field trials by Laven (75) and is analogous to the release of sterile males (sterile insect technique), which has been effective in area-wide control of the screwworm (*Cochliomyia hominivorax*) and the Mediterranean fruit fly (*Ceratitis capitata*) (3, 70). Recently, CI-based suppression has been attempted in small areas around Guangzhou, China, with successful vector population reductions of 95% or higher (157). Varying levels of suppression in other areas have also

been achieved (Table 1). Unlike population replacement, suppression requires ongoing releases and is subject to reinvasion of disease vectors through long-distance dispersal, although it should be possible to reduce release rates once initial suppression has been successful (157).

Population suppression with *Wolbachia* depends on releasing only male hosts carrying *Wolbachia* variants that cause strong CI. The transinfected males must successfully compete with males from the natural target population for mates. Suppression is facilitated by the absence of multiple mating in host females (especially if the sperm from uninfected males are relatively more competitive), the absence of assortative mating associated with *Wolbachia*, and ecological factors that help released males find native females.

Data from natural populations suggest that *Wolbachia* rarely influences mating success, with a few exceptions (124). In transinfected populations, limited data indicate minimal effects on male mating success, as noted previously (see Supplemental Data Set 1). Assortative mating involving transinfections has been rarely tested. In *Ae. aegypti*, mating appears random with respect to *Wolbachia* infection, but the success of releases can be influenced by other forms of assortative mating. For instance, laboratory experiments indicate assortative mating for size (19), with small females preferentially mating with small males. This can influence field success of released males, which are typically larger than field mosquitoes and have a much greater variance in size.

Insecticide resistance may influence the success of released males, particularly when fogging targets adults. Other deleterious effects associated with inbreeding and adult male food might influence the success of infected males competing with males reared in nature. Although the impact of inbreeding on competitive male performance has not been rigorously tested, it is expected to decrease male fitness (116). Deleterious effects from feeding on particular blood sources might also carry over to the fitness of offspring (105) and are worth exploring further for effects on male mating success.

For releases to suppress populations, infected released males must find wild-type females. Releasing sufficient numbers of males at the right place for *Ae. aegypti* can be challenging because breeding sites producing males are often cryptic (e.g., 13). It may be possible to target the distribution of human hosts, which attract both male and female *Ae. aegypti* (50), but this remains to be tested. When suppression releases take place in complex urban landscapes with a mix of high-and low-rise buildings, different release strategies may be required. For high-rise buildings, males may need to be released on multiple floors, whereas dispersal of mosquitoes along thoroughfares by hand (157) or through engineered systems (https://debug.com/) may be adequate for only low-rise buildings. Suppression might still be achieved if enough males are released, even when there is a low likelihood of males effectively reaching mating sites, particularly given increasingly efficient production, sexing, and release technology (e.g., 156). However, a high density of (nonbiting) males might be unacceptable to residents when releases continue for some time, highlighting the need for ongoing community engagement (Figure 1).

8. CONCLUSIONS

Understanding the dynamics of natural *Wolbachia* infections has helped researchers identify factors that enhance and retard the rate of spread, inspired models to predict *Wolbachia* spread, and provided information on the likely long-term stability of both the infections and their phenotypic effects (18). Population studies of *Aedes* spp. have highlighted some of the challenges in using *Wolbachia* technology, including the heterogeneous distribution of breeding sites, the ability of some mosquitoes to disperse across large distances, the high levels of inbreeding depression, and the evolutionary potential to rapidly evolve pesticide resistance. The long-term stability of *Wolbachia*-based replacement interventions will depend on the ability of *Wolbachia* to continue to reduce local viral transmission under changing environmental conditions, and on the maintenance of a high frequency of *Wolbachia* in populations; both factors require ongoing monitoring. Suppression strategies require efficient ways to distribute infected males to areas where receptive females are common and to maintain highly competitive stocks for ongoing releases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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SUMMARY POINTS

1. Fitness effects associated with transinfections are variable, often measured under contrived circumstances poorly linked to field conditions, and applied arbitrarily in models.
2. Environmental effects need to be considered in release success and impact. As shown by natural infections, there is potential for *Wolbachia*–environment–host-genome interactions.
3. There is a contrast between natural (mutualistic) and transinfection (deleterious) invasion dynamics that makes the rapid spread observed for some natural infections implausible for transinfections.
4. Virus blocking is critical in population replacement releases, but host benefits associated with virus blocking by natural *Wolbachia* infections are poorly documented (and may not exist).
5. Theory should be expanded to incorporate three-dimensional landscapes and produce field-validated predictors of barriers likely to impede or stop spatial spread.
6. Releases must be followed by ongoing monitoring, given there is uncertainty around long-term outcomes including the stability of virus blocking and population replacement.
7. Artificial rearing conditions including surrogate blood sources may have negative effects on the fitness of released mosquitoes.

FUTURE ISSUES

1. Is there a trade-off between virus blocking and host fitness effects? This requires assessing trade-offs within and between multiple *Wolbachia* variants and host backgrounds.
2. Which *Wolbachia* variants are appropriate for different contexts? Field comparison of multiple variants is needed to assess invasion potential and virus blocking.
3. Are simple models for spatial spread applicable to different contexts? Models have been developed and tested near Cairns, Australia, but not in complex tropical environments.
4. Can we develop useful predictions to characterize barriers likely to halt spread?
5. What is the stability of transinfections and their impact on natural populations? Monitoring long-term stability of *Wolbachia* frequencies in different areas is required along with monitoring *Wolbachia* titer, virus blocking, and host fitness effects.
6. Do disease viruses evolve in response to *Wolbachia*? This requires monitoring viruses in endemic disease areas and assessing evolutionary forces affecting viral transmission through mosquito and human hosts.
7. How can genetic quality of mosquito release stocks be maintained? The effects of evolutionary adaptation to mass production conditions on field performance, particularly mating performance and human-host finding, need to be further assessed.
8. Which environmental conditions are optimal for mosquito releases? The impact of different blood sources, rearing conditions, and feeding methods needs to be assessed on release stocks, particularly for suppression.

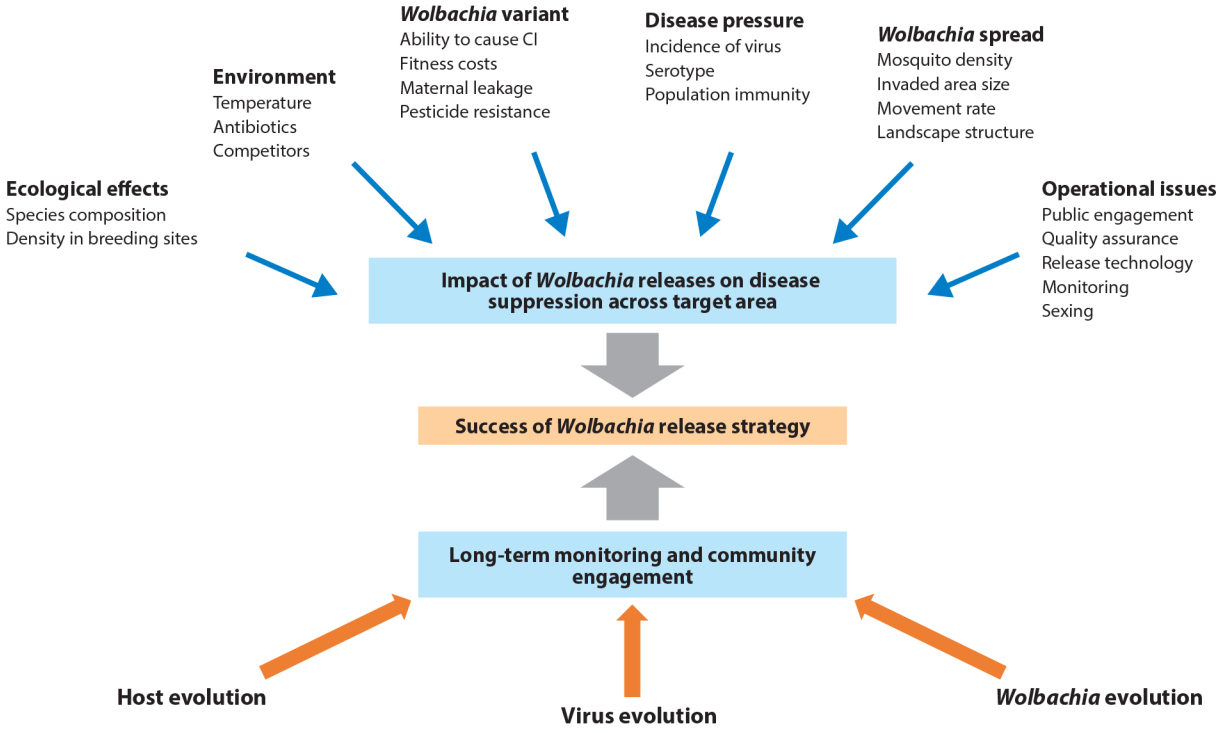

Figure 1.

Factors influencing the success of *Wolbachia* releases during and after the releases. *Wolbachia* population replacement and suppression are influenced by features of the *Wolbachia* variant, mosquito host factors, production issues, and environmental factors. Long-term disease suppression may be affected by evolutionary changes in the mosquito host, virus, or *Wolbachia* and by a public commitment to maintain surveillance and perform additional releases as required. Abbreviation: CI, cytoplasmic incompatibility.

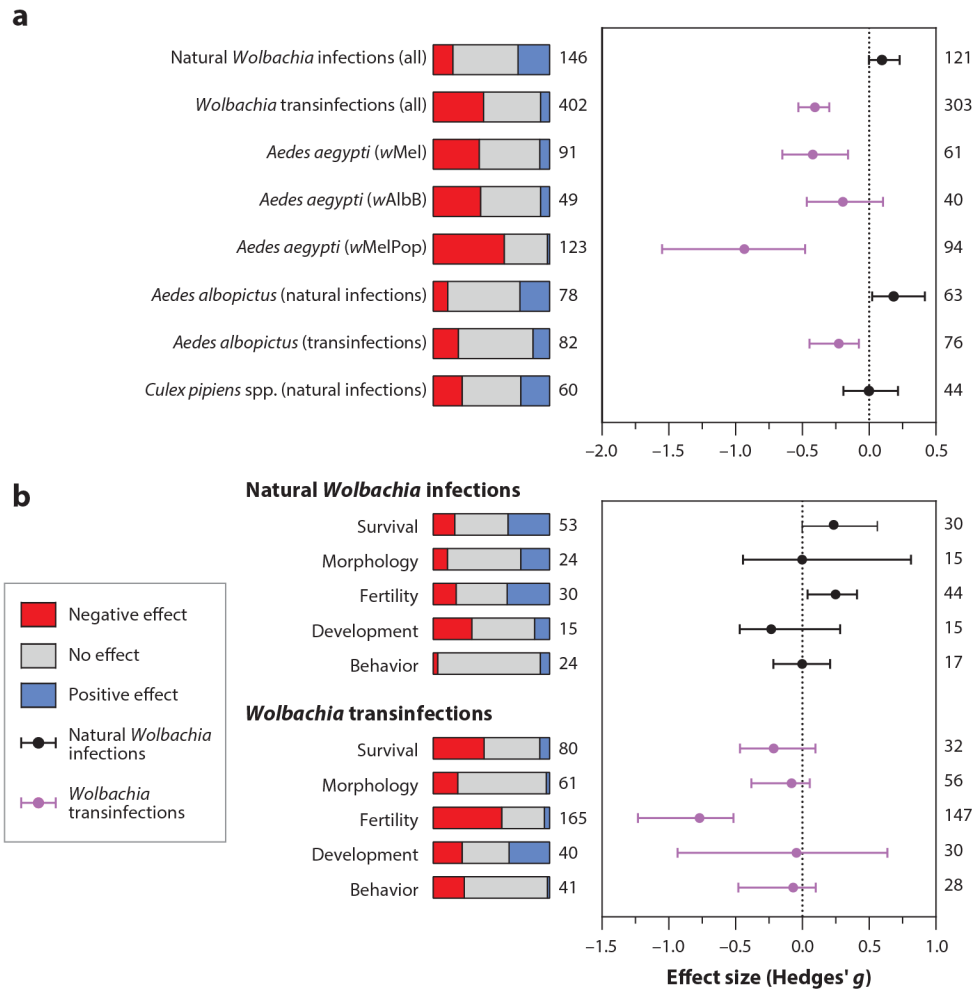

Figure 2.

Effects of *Wolbachia* infections on mosquito fitness traits. Data were extracted from 75 studies that compared the fitness of *Wolbachia*-infected mosquitoes with that of uninfected mosquitoes. Shaded bars represent the proportion of traits on which *Wolbachia* infections had a negative (*red*), positive (*blue*), or no statistically significant ($P > 0.05$) effect (*gray*) on fitness according to statistical tests by the authors. Magnitudes of fitness effects are expressed in terms of effect sizes (Hedges' *g*), where dots and error bars represent medians and 95% confidence intervals, respectively. Numbers to the right of the colored bars indicate the number of fitness estimates reported by the authors in each category. Numbers to the right of the effect-size chart are the number of estimated effects, often smaller than the number of fitness estimates because data needed to estimate effect sizes were not provided with all fitness estimates (an exception is fecundity for natural *Wolbachia* infections, where we were able to calculate some effect sizes, even when authors did not report statistical analyses of effects). Supplemental Data Set 1 provides the data and describes how they were compiled. Effects are shown separately for natural *Wolbachia* infections (*black error bars*) and *Wolbachia* transinfections (*purple error bars*). (*a*) Effects are separated by mosquito species and *Wolbachia* infection type (natural or transinfection). For *Aedes aegypti*, for which more data were available, effects are shown separately for three of the most-studied

Wolbachia variants. (*b*) Fitness effects are separated into different trait types for natural *Wolbachia* infections and transinfections.

Table 1

Field releases of *Wolbachia*-infected mosquitoes for population replacement and suppression interventions around the world

Mosquito species	*Wolbachia* variant	Objective	Location	First release	Outcome	Reference(s)
Culex pipiens fatigans	*w*Pip (Paris variant)	Suppression	Okpo (Burma)	February 1967	Eradication of mosquitoes in a small trial area	75
Culex quinquefasciatus	*w*Pip (Paris variant)	Suppression	Delhi (India)	August 1973	Reduction in population size and female fertility in release areas	33
Aedes polynesiensis	*w*Riv	Suppression	French Polynesia	December 2009	Reduction in population size and female fertility in release areas; releases are ongoing	99, 128
Aedes aegypti	*w*Mel	Replacement	Cairns and Townsville (Australia), Rio de Janeiro (Brazil), Yogyakarta (Indonesia), Tri Nguyen Island (Vietnam), and others	January 2011	Establishment of *w*Mel in release areas where it remains at a high frequency in most locations; releases are under way in several countries	42, 54, 55, 102, 122; https://www.worldmosquitoprogram.org/
Aedes aegypti	*w*MelPop	Replacement	Cairns (Australia) and Tri Nguyen Island (Vietnam)	January 2012	wMelPop reached high frequencies during active releases and then declined rapidly after releases stopped	98
Aedes albopictus	*w*Pip	Suppression	Kentucky, California, and New York (United States)	June 2014	Reduction in population size and female fertility in release areas	82; https://mosquitomate.com/?v=3.0
Aedes albopictus	*w*Pip/*w*AlbA/*w*AlbB	Suppression	Guangzhou (China)	April 2015	Population suppression of >95% in release areas	157
Aedes aegypti	*w*AlbB	Suppression	Singapore, Clovis and Fresno (California), South Miami and Stock Island (Florida), Innisfail (Australia)	August 2016	Variable population suppression achieved but averaged 95% in one area (Fresno)	83, 97; https://debug.com/
Aedes aegypti	*w*AlbB	Replacement	Kuala Lumpur (Malaysia)	February 2017	Establishment of wAlbB in three populations in which it remains at a high frequency after releases stopped	W.A. Nazni, unpublished data

Releases are listed chronologically.