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Designing effective *Wolbachia* release programs for mosquito and arbovirus control

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Abstract

Mosquitoes carrying endosymbiotic bacteria called *Wolbachia* are being released in mosquito and arbovirus control programs around the world. Open field releases of *Wolbachia*-infected male mosquitoes have achieved over 95% population suppression, while the replacement of populations with *Wolbachia*-infected females is self-sustaining and can greatly reduce local dengue transmission. Despite many successful interventions, significant questions and challenges lie ahead. *Wolbachia*, viruses and their mosquito hosts can evolve, leading to uncertainty around the long-term effectiveness of a given *Wolbachia* strain, while few ecological impacts of *Wolbachia* releases have been explored. *Wolbachia* strains are diverse and the choice of strain to release should be made carefully, taking environmental conditions and the release objective into account. Mosquito quality control, thoughtful community awareness programs and long-term monitoring of populations are essential for all types of *Wolbachia* intervention. Releases of *Wolbachia*-infected mosquitoes show great promise, but existing control measures remain an important way to reduce the burden of mosquito-borne disease.

A brief introduction to *Wolbachia*

Wolbachia are a group of Gram-negative bacteria that live inside the cells of insects and other arthropods. *Wolbachia* are normally maternally inherited, where females that carry *Wolbachia* transmit the infection to their offspring (Newton et al., 2015). On rare occasions, *Wolbachia* can spread between species through routes such as predation and parasitism (Sanaei et al., 2020)**.** *Wolbachia* are best known for their effects on host reproduction. *Wolbachia* may increase the proportion of females produced by their host by killing or feminizing male offspring (Hurst and Jiggins, 2000). *Wolbachia* can also trigger asexual reproduction in females, removing the need for males entirely (Huigens and Stouthamer, 2003, Ma and Schwander, 2017). More commonly, *Wolbachia* induce cytoplasmic incompatibility, where males infected with *Wolbachia* do not produce viable offspring with females that are not infected

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(Shropshire et al., 2020). These effects on reproduction aid the spread of *Wolbachia* through populations by making the females that carry and transmit *Wolbachia* relatively more fit.

Wolbachia can also provide direct host fitness benefits (Zug and Hammerstein, 2015). For instance, *Wolbachia* can increase host lifespan or fecundity through nutritional provisioning (Brownlie et al., 2009, Ju et al., 2020) or altering programmed cell death (Fast et al., 2011, Guo et al., 2018). Furthermore, *Wolbachia* can protect their hosts against natural pathogens, including viruses (Teixeira et al., 2008, Hedges et al., 2008). The dramatic effects of *Wolbachia* on their hosts are being used to control mosquito populations through releases of *Wolbachia*-infected mosquitoes into the environment (Figure 1B).

Effects of natural and artificial *Wolbachia* infections in mosquitoes

Wolbachia are found naturally in about 30% of mosquito species (Inacio da Silva et al., 2021). Although *Wolbachia* are absent from many important vectors including *Aedes aegypti* (Gloria-Soria et al., 2018, Ross et al., 2020c) and most *Anopheles* species (Chrostek and Gerth, 2019, Walker et al., 2020), *Wolbachia* can be introduced artificially through a process called transinfection (Hughes and Rasgon, 2014). To transfer *Wolbachia* to a new host, cytoplasm or tissue homogenate from a *Wolbachia*-infected insect is injected into mosquito embryos using a fine needle. Stably infected lines can be generated by selecting for *Wolbachia*-infected females each generation. Both natural and artificial *Wolbachia* infections can have effects on that are useful for mosquito control programs, including cytoplasmic incompatibility, pathogen blocking and host fitness costs (Figure 1B).

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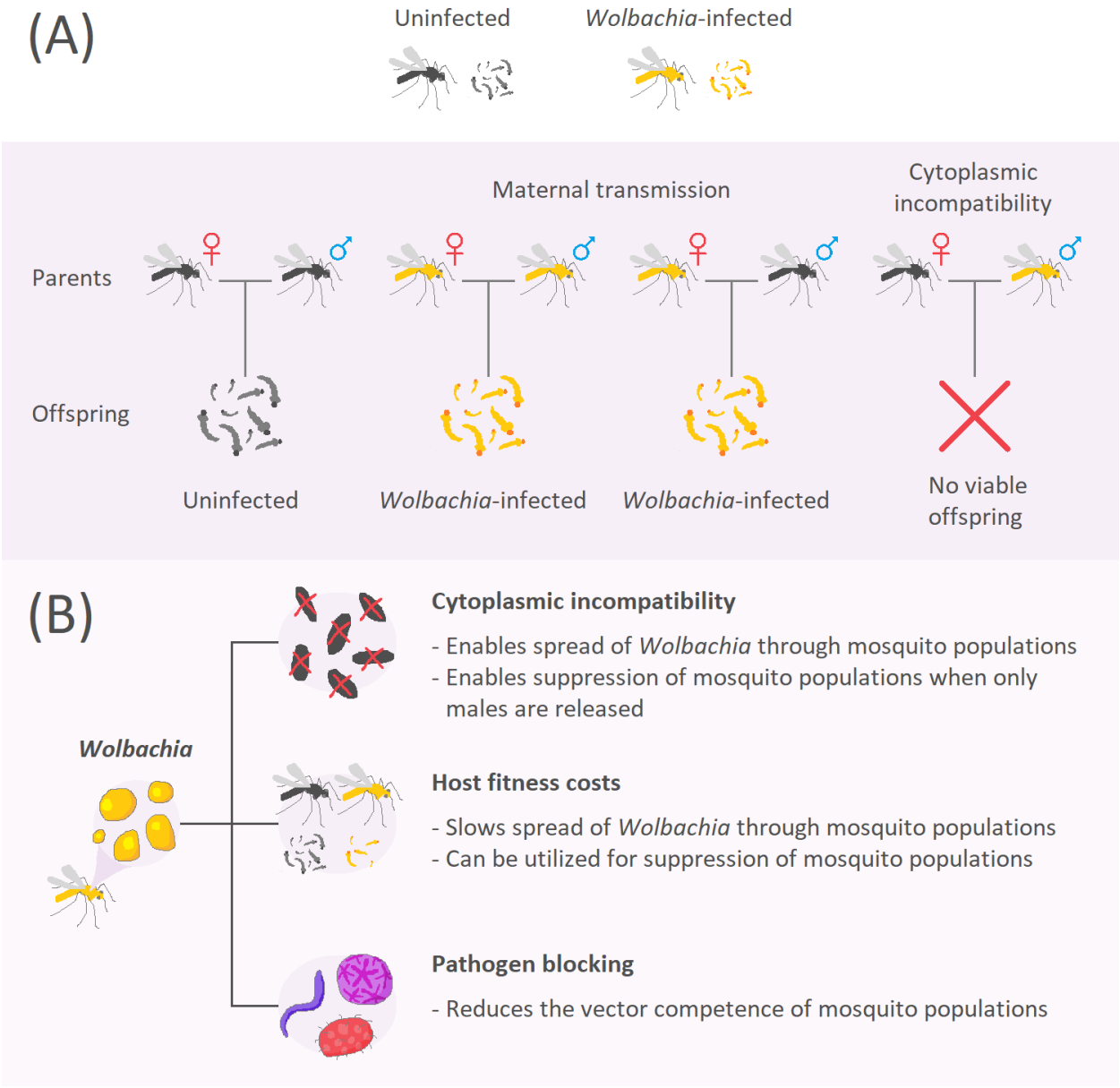

Figure 1. *Wolbachia* transmission and effects in mosquitoes. (A) *Wolbachia* are maternally inherited and cause cytoplasmic incompatibility. *Wolbachia*-infected females transmit *Wolbachia* to their offspring, regardless of whether males are *Wolbachia*-infected or uninfected. Uninfected females that mate with *Wolbachia*-infected males have fewer or no viable offspring due to cytoplasmic incompatibility. (B) *Wolbachia* have three key effects in mosquitoes that can be utilized to reduce pathogen transmission or mosquito population sizes.

Cytoplasmic incompatibility

Most *Wolbachia* infections in mosquitoes cause cytoplasmic incompatibility, where uninfected females

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that mate with *Wolbachia*-infected males produce fewer (or no) viable offspring (Figure 1A). Female mosquitoes carrying *Wolbachia* are compatible with both *Wolbachia*-infected and uninfected males. Cytoplasmic incompatibility provides *Wolbachia*-infected females with a reproductive advantage, allowing *Wolbachia* to spread through mosquito populations. This advantage is frequency-dependent (Hoffmann and Turell, 1997); when *Wolbachia* is at a high frequency in the population, uninfected females have a high likelihood of mating with *Wolbachia*-infected males and becoming infertile. When *Wolbachia* is at a low frequency, cytoplasmic incompatibility provides little advantage because most uninfected females will mate with uninfected males.

Cytoplasmic incompatibility is key to all *Wolbachia*-based mosquito control programs (Figure 1B). It can drive *Wolbachia* through mosquito populations, replacing the native population with mosquitoes that are less capable of transmitting viruses. When only *Wolbachia*-infected male mosquitoes are released, cytoplasmic incompatibility can reduce the fertility of wild female mosquitoes and their overall population size. Cytoplasmic incompatibility can occur between mosquitoes carrying different *Wolbachia* strains; when the infertility occurs in both directions this is called bidirectional incompatibility (Joubert et al., 2016, Atyame et al., 2014). By using *Wolbachia* infections with different patterns of cytoplasmic incompatibility it is possible to replace or crash mosquito populations even if they already carry *Wolbachia* (Zheng et al., 2019, Fu et al., 2010).

The strength of cytoplasmic incompatibility (in other words, the proportion of eggs hatching from a cross between a *Wolbachia*-infected male and an uninfected female) depends on the *Wolbachia* strain and mosquito species. Most characterized *Wolbachia* infections in mosquitoes cause strong or complete cytoplasmic incompatibility (Fraser et al., 2017, Atyame et al., 2011, Dobson et al., 2001) but some do not cause any (Ant et al., 2018, Wu, 2020). The strength of cytoplasmic incompatibly is also related to the density of *Wolbachia* inside the mosquito, which can be influenced by environmental conditions. The *w*Mel strain in *Aedes aegypti* shows complete cytoplasmic incompatibility under standard laboratory conditions but weakens when mosquitoes experience high temperatures (Ross et al., 2017) or hatch from quiescent eggs (Lau et al., 2021). For some natural *Wolbachia* infections, cytoplasmic incompatibility weakens as males age, likely corresponding to a decline in *Wolbachia* density (Kittayapong et al., 2002).

There are no known cases of *Wolbachia* causing feminization, male killing or asexual reproduction in mosquitoes. However, *Wolbachia* strains with these effects could be useful for driving *Wolbachia* into mosquito populations and strains causing these effects could plausibly be introduced from other insects.

Pathogen blocking

Wolbachia are renowned for their ability to protect their hosts against and interfere with the transmission of pathogens. *Wolbachia*-induced pathogen protection was first discovered in *Drosophila*, where flies carrying *Wolbachia* had higher survival compared to uninfected flies when challenged with RNA viruses (Teixeira et al., 2008, Hedges et al., 2008) or the fungus *Beauveria bassiana* (Panteleev et

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al., 2007). In mosquitoes, *Wolbachia* infections can interfere with the replication and transmission of human pathogens (Table 1), insect-specific viruses (Schnettler et al., 2016) and non-human animal pathogens including the filarial nematode *Brugia pahangi* (Kambris et al., 2009, Andrews et al., 2012) and rodent malaria parasites (Joshi et al., 2017). How *Wolbachia* can block pathogen transmission is not fully understood, but there appear to be multiple mechanisms involved (Terradas and McGraw, 2017, Lindsey et al., 2018), including immune up-regulation (Rances et al., 2012) and interactions with host lipids (Geoghegan et al., 2017, Koh et al., 2020, Manokaran et al., 2020).

The pathogen blocking effects of some *Wolbachia* strains form the basis of mosquito release programs to reduce dengue transmission (Figure 1B). When natural *Aedes aegypti* populations carry the *w*Mel or *w*AlbB *Wolbachia* strains at a high frequency, this reduces the vector competence of the population, resulting in fewer dengue cases (Ryan et al., 2019, Nazni et al., 2019, Indriani et al., 2020). *Wolbachia* infections do not always provide protection against pathogens, and the strength of blocking depends on the pathogen (Teixeira et al., 2008), insect host (Lu et al., 2012), *Wolbachia* strain (Osborne et al., 2009, Ferguson et al., 2015) and environmental conditions (Murdock et al., 2014, Chrostek et al., 2020, Mancini et al., 2020a, Caragata et al., 2013). The strength of virus blocking may also depend on the novelty of the *Wolbachia*-mosquito association (Terradas and McGraw, 2017); *Wolbachia* strains that show weak blockage in their native host can block much more effectively when transferred to a new host (Lu et al., 2012). However, some *Wolbachia* strains like *w*Pip (originating from *Culex quinquefasciatus*) do not block, even in novel hosts (Moretti et al., 2018b, Fraser et al., 2020).

Some *Wolbachia* infections can increase the probability of pathogen infection or transmission by mosquitoes (Hughes et al., 2012, Dodson et al., 2014) and there is a risk that releases of *Wolbachia*-infected mosquitoes could increase rather than prevent disease. At low virus doses, the *w*Mel strain in *Ae. aegypti* can increase the susceptibility of mosquitoes to dengue infection (King et al., 2018). Importantly, the *Wolbachia* strains in *Ae. aegypti* that are being released into the field, including *w*Mel, have an overall suppressive effect on dengue transmission, both in laboratory (Ferguson et al., 2015, Carrington et al., 2017, Flores et al., 2020) and field trials (O'Neill et al., 2018, Ryan et al., 2019, Nazni et al., 2019, Indriani et al., 2020). However, given that pathogen blocking by *Wolbachia* is rarely complete, it is important to consider this variability when planning releases of *Wolbachia*-infected mosquitoes.

Table 1. *Wolbachia* infections can block human pathogens transmitted by mosquitoes. Blocking is defined by a reduction in pathogen titer, infection rate or transmission rate in *Wolbachia*-infected mosquitoes compared to uninfected controls. Note that cases where *Wolbachia* infections have no effect or enhance pathogen infection are not included here, but see Pan et al. (2017) and Caragata and Moreira (2017).

Pathogen	***Wolbachia* strains (mosquito host species)**	**Selected references**
Dengue virus	*w*AlbB (*Aedes aegypti*)	Bian et al. (2010)
	*w*MelPop (*Aedes aegypti*)	(Moreira et al., 2009a, Ferguson et al., 2015)

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	*w*Mel (*Aedes aegypti*)	(Walker et al., 2011, Carrington et al., 2017)
	*w*MelCS (*Aedes aegypti*)	(Fraser et al., 2017)
	*w*Ri (*Aedes aegypti*)	(Fraser et al., 2017)
	*w*Au (*Aedes aegypti*)	(Ant et al., 2018)
	*w*Mel/*w*AlbB (*Aedes aegypti*)	(Joubert et al., 2016)
	*w*Mel (*Aedes albopictus*)	(Blagrove et al., 2012)
	*w*Mel/*w*Pip (*Aedes albopictus*)	(Moretti et al., 2018b)
	*w*AlbA/*w*AlbB (*Aedes albopictus*)	(Mousson et al., 2012)
	*w*Au/*w*AlbA/*w*AlbB (*Aedes albopictus)*	(Mancini et al., 2020b)
	*w*AlbB (*Aedes polynesiensis*)	(Bian et al., 2013b)
Zika virus	*w*AlbB (*Aedes aegypti*)	(Ant et al., 2018)
	*w*Mel (*Aedes aegypti*)	(Dutra et al., 2016, Aliota et al., 2016a)
	*w*AlbA (*Aedes aegypti*)	(Chouin-Carneiro et al., 2020)
	*w*Au (*Aedes aegypti*)	(Ant et al., 2018)
	*w*AlbB (*Aedes albopictus*[1])	(Schultz et al., 2017)
	*w*Stri (*Aedes albopictus*[1])	(Schultz et al., 2017)
	*w*Au/wAlbA/wAlbB (*Aedes albopictus*)	(Mancini et al., 2020b)
Chikungunya virus	*w*MelPop (*Aedes aegypti*)	(Moreira et al., 2009a)
	*w*Mel (*Aedes aegypti*)	(van den Hurk et al., 2012, Aliota et al., 2016b)
	*w*Mel (*Aedes albopictus*)	(Blagrove et al., 2013)
	*w*AlbB (*Aedes albopictus*[1])	(Raquin et al., 2015)
	*w*Mel/*w*Pip (*Aedes albopictus*)	(Moretti et al., 2018b)
West Nile virus	*w*AlbB (*Aedes aegypti*)	(Joubert and O'Neill, 2017)
	*w*MelPop (*Aedes aegypti*)	(Hussain et al., 2013)
	*w*Pip (*Culex quinquefasciatus*)	(Glaser and Meola, 2010)
Yellow Fever virus	*w*MelPop (*Aedes aegypti*)	(van den Hurk et al., 2012)
	*w*Mel (*Aedes aegypti*)	(Rocha et al., 2019)
Sindbis virus	*w*AlbB (*Aedes aegypti*)	(Bhattacharya et al., 2020)
	*w*AlbB (*Aedes albopictus*[1])	(Ekwudu et al., 2020)
	*w*Stri (*Aedes albopictus*[1])	(Bhattacharya et al., 2020)
	*w*Mel (*Drosophila melanogaster*[2])	(Bhattacharya et al., 2017, Nainu et al., 2019)
Semliki forest virus	*w*AlbB (*Aedes aegypti*)	(Ant et al., 2018)
	*w*Mel (*Aedes aegypti*)	(Ant et al., 2018)
	*w*Au (*Aedes aegypti*)	(Ant et al., 2018)
	*w*Mel (*Drosophila melanogaster*[2])	(Rainey et al., 2016)

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Ross River virus	*w*AlbB (*Aedes albopictus*[1])	(Ekwudu et al., 2020)
Barmah Forest virus	*w*AlbB (*Aedes albopictus*[1])	(Ekwudu et al., 2020)
Mayaro virus	*w*Mel (*Aedes aegypti*)	(Pereira et al., 2018, Sucupira et al., 2020)
Plasmodium falciparum (human malaria parasite)	*w*AlbB[3] (*Anopheles gambiae*)	(Hughes et al., 2011)
	*w*MelPop[3] (*Anopheles gambiae*)	(Hughes et al., 2011)
	*w*AlbB (*Anopheles stephensi*)	(Bian et al., 2013a)

[1] mosquito cell line

[2] non-mosquito cell line model

[3] somatic (transient) *Wolbachia* infection

Host fitness costs

Wolbachia infections may alter the fitness of their hosts, with effects depending on the *Wolbachia* strain and insect host. Natural *Wolbachia* infections in mosquitoes tend to be benign (Rasgon and Scott, 2003, Baton et al., 2013) or even beneficial, with *Wolbachia*-infections conferring increased fertility or lifespans (Brelsfoard and Dobson, 2011, Dobson et al., 2002). In contrast, *Wolbachia* infections typically have harmful effects when transferred to novel mosquito hosts (Ross et al., 2019c). Most drastic are their effects on female fertility, with almost all *Wolbachia* transinfections reducing fecundity or egg hatch (Axford et al., 2016, Fraser et al., 2017, Ant et al., 2018). These negative effects become pronounced with age, such as when eggs are quiescent (Allman et al., 2020), or appear under poor nutritional conditions (Caragata et al., 2014). Consequently, the negative effects of *Wolbachia* strains are likely to be underestimated when evaluated under standard laboratory conditions.

Host fitness costs of *Wolbachia* can be utilized during mosquito release programs (Figure 1B). Before the discovery that *Wolbachia* could block pathogen transmission, a deleterious strain of *Wolbachia* called *w*MelPop (Min and Benzer, 1997) was introduced to *Ae. aegypti* mosquitoes. *w*MelPop shortens mosquito lifespan (McMeniman et al., 2009, Ross et al., 2020a) and reduces the blood feeding success of older females (Turley et al., 2009, Moreira et al., 2009b). These effects could indirectly reduce dengue transmission because *w*MelPop-infected mosquitoes are less likely to survive long enough to feed on an infected person, incubate the virus, then transmit it to a new host (Brownstein et al., 2003, Rasgon et al., 2003, Cook et al., 2008). Host fitness costs can also be used to suppress mosquito populations once a *Wolbachia* strain is established (Rašić et al., 2014, Ritchie et al., 2015). However, these same effects could prevent the establishment of *Wolbachia* in the population if they are too severe (Nguyen et al., 2015).

The use of *Wolbachia* for mosquito control

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Wolbachia-infected mosquitoes can be used in three main ways to control mosquito populations, with each approach having distinct objectives that rely on different *Wolbachia* traits (Figure 2). All types of *Wolbachia* intervention involve the release of *Wolbachia*-infected mosquitoes (either naturally occurring or transinfected) from the lab into the field. Unlike conventional vector control, *Wolbachia* releases target a single mosquito species. The released mosquitoes are intended to mate with wild mosquitoes and/or reproduce in the natural environment. Releases can either introduce *Wolbachia* infections into the target species, suppress populations of the target species, or both (Figure 2).

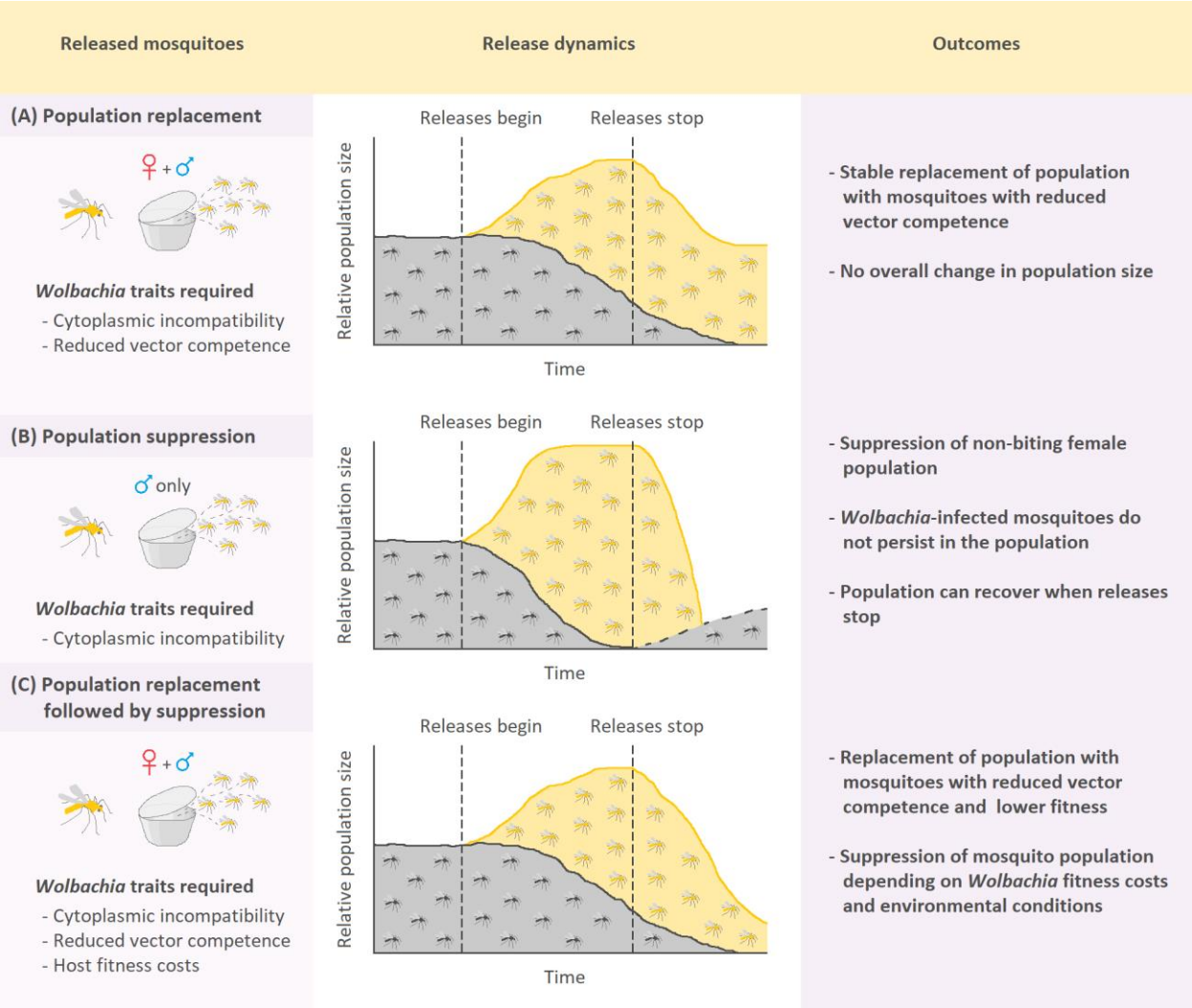

Figure 2. Three ways to use *Wolbachia* for mosquito and mosquito-borne pathogen control. (A) 'Population replacement' involves releasing *Wolbachia*-infected mosquitoes with the aim of spreading *Wolbachia* through the population to reduce mosquito vectorial capacity. (B) 'Population suppression' involves releasing only male mosquitoes that cause cytoplasmic incompatibility to reduce the fertility of wild mosquitoes and temporarily crash the population. (C) 'Population replacement followed by suppression' involves releasing *Wolbachia*-infected mosquitoes that cause substantial host fitness costs

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with the aim of directly reducing the vector competence of mosquitoes as well as reducing the population size. In the 'Release dynamics' column, yellow and gray shaded regions show the proportion of *Wolbachia*-infected and wild mosquitoes respectively in the population.

Population replacement

Population replacement programs (Figure 2A) use cytoplasmic incompatibility to spread *Wolbachia* through a mosquito population to reach a stable high frequency, with the overall goal of reducing the vectorial capacity of mosquitoes. This approach requires a *Wolbachia* strain that directly blocks human pathogen transmission (Table 1) or indirectly reduces mosquito vectorial capacity, such as by shortening adult lifespan (McMeniman et al., 2009). Alternatively, the *Wolbachia* strain could be coupled with an anti-pathogen transgene (Curtis and Sinkins, 1998, Buchman et al., 2020).

Population replacement programs involve the release of both male and female *Wolbachia*-infected mosquitoes. Both sexes contribute to *Wolbachia* proliferation; *Wolbachia*-infected females transmit *Wolbachia* to their offspring, while *Wolbachia*-infected males mate with wild females and induce cytoplasmic incompatibility, increasing the relative fitness of *Wolbachia*-infected females. Once *Wolbachia*-infected mosquitoes reach a threshold frequency in the population, *Wolbachia* can spread by itself without further releases of mosquitoes. The threshold frequency depends on the fidelity of *Wolbachia* maternal transmission and cytoplasmic incompatibility, as well as the host fitness costs of *Wolbachia* infection (Caspari and Watson, 1959, Hoffmann and Turell, 1997). For the *w*Mel strain in *Ae. aegypti* which has relatively low host fitness costs, the threshold frequency is estimated to be 20-30% (Hoffmann et al., 2011). If the frequency of *Wolbachia* in the population is below this point, the infection will likely be lost without further releases of *Wolbachia*-infected mosquitoes.

The number of *Wolbachia*-infected mosquitoes required for successful population replacement depends on the native mosquito population density and the size of the release area. Releases can be timed so that the mosquito population is at its lowest point; alternative mosquito control efforts prior to releases can aid population replacement (Hoffmann et al., 2011). Population replacement releases can involve hundreds of thousands to millions of mosquitoes. The first *Wolbachia* population replacement program in Cairns, Australia involved the weekly release of ~30,000 *Wolbachia*-infected *Ae. aegypti* for 10 weeks in two suburbs with an estimated population size of ~20,000 *Ae. aegypti* (Hoffmann et al., 2011, Ritchie et al., 2013). Releases and monitoring (i.e. regular *Wolbachia* screening of wild mosquitoes) should continue until the frequency of *Wolbachia* is well above the threshold for several weeks.

Population replacement is irreversible; once a *Wolbachia* infection has established it may persist indefinitely. The *w*Mel strain of *Wolbachia* has remained at high frequencies in Cairns for a decade (Ryan et al., 2019) with other programs also showing persistence of *Wolbachia* across multiple years (Tantowijoyo et al., 2020, Ahmad et al., 2021). In continuous populations, where an area with *Wolbachia*-infected mosquitoes is adjacent to an area with uninfected mosquitoes, *Wolbachia* can continue to spread outside the release area (Schmidt et al., 2017). The rate of spread depends on *Wolbachia* traits such as host fitness costs (Turelli and Barton, 2017) but also the environment;

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heterogeneity in mosquito population densities (Hancock et al., 2019) and landscape features that inhibit mosquito dispersal (Schmidt et al., 2018) can both slow spread. In *Ae. aegypti*, *w*Mel spreads at a rate of 100-200 meters per year (Schmidt et al., 2017) or less (Tantowijoyo et al., 2020), making it possible to contain *Wolbachia* within a local area. Consequently, releases across most of the target location may be required for complete *Wolbachia* coverage. Once established, *Wolbachia* infections can only drop out of the population if incomplete maternal transmission and/or immigration of uninfected mosquitoes push the *Wolbachia* frequency below the threshold required for spread. This irreversibility means that population replacement can provide ongoing protection from dengue without the need for further intervention.

Population suppression

Population suppression programs (Figure 2B) rely on the release of *Wolbachia*-infected males to induce cytoplasmic incompatibility with wild females, making them infertile. *Wolbachia*-based population suppression (also known as the incompatible insect technique) is like the sterile insect technique (Benedict, 2021), but uses cytoplasmic incompatibility rather than irradiation as the method of reducing female fertility. Population suppression programs require accurate sex sorting so that only male mosquitoes are released. Sex sorting is typically achieved through mechanical separation at the pupal stage, followed by visual inspection of the adults to remove any remaining females (O'Connor et al., 2012, Mains et al., 2016). Recent releases have also used automated processes including machine learning to allow for larger-scale and more efficient sex sorting (Crawford et al., 2020).

For population suppression to be effective, large numbers of *Wolbachia*-infected males need to be released over a sustained period. Suppression is easier to achieve when the mosquito population is low, such as during the dry season, because higher ratios of *Wolbachia*-infected males to wild males will increase the likelihood of female infertility (Chambers et al., 2011). Ratios of at least 5:1 (*Wolbachia*-infected to wild males) are thought to be required for suppression (Pagendam et al., 2020); a recent program that achieved greater than 95% suppression involved release ratios that exceeded 50:1 (Crawford et al., 2020). The released males must be able to survive under field conditions, inseminate wild females and induce cytoplasmic incompatibility, so unfit males will reduce the efficiency of population suppression. Although *Wolbachia* infections do not typically affect male mating success (Segoli et al., 2014), the fitness of released males could decrease as a consequence of mass-rearing, inbreeding or adaptation to laboratory conditions (Ross et al., 2019a, Qureshi et al., 2019).

Population suppression is substantially more labor-intensive than population replacement; it requires higher numbers of mosquitoes as well as accurate sex sorting. Population suppression is also temporary, requiring ongoing releases to maintain effectiveness. Elimination of a population is unlikely; *Wolbachia*-infected males may not reach all wild females in the population if coverage is incomplete or females exhibit mating preferences. Since *Aedes* eggs may undergo diapause or quiescence, fertile adults could appear for months after releases begin. Even if elimination is successful, the population can re-establish from immigrant mosquitoes if suitable habitat remains. Consequently, suppression is likely to be more

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effective in locations that are geographically isolated from other mosquito populations (O'Connor et al., 2012, Crawford et al., 2020).

Population suppression, even if temporary, is still an effective way to control mosquito populations and brings several advantages over population replacement. Population suppression may be perceived more favorably by the community because it can reduce nuisance biting by mosquitoes as well as the threat of disease. Because releases do not involve biting females, obtaining regulatory approval is also more straightforward (Murray et al., 2016). Furthermore, the *Wolbachia*-infected mosquitoes are not intended to persist in the population, which may ease potential concerns about irreversible changes to the ecosystem. Because direct pathogen blocking by females is not required, any *Wolbachia* strain that induces cytoplasmic incompatibility (even natural *Wolbachia* infections) can potentially be used for population suppression. This means that population suppression can target a much broader range of mosquito species, including mosquitoes where *Wolbachia* has no effect on pathogen transmission or mosquitoes that are more important as nuisance pests than vectors.

Population replacement followed by suppression

'Population replacement followed by suppression' aims to reduce the mosquito population size after the *Wolbachia* infection has already established in the population (Figure 2C). This strategy requires a *Wolbachia* strain that causes host fitness costs and involves the release of both males and females. Once the *Wolbachia* infection is at a high frequency, host fitness costs can reduce the size of the population by decreasing mosquito survival or fertility. If the *Wolbachia* infection is at an intermediate frequency in the population, cytoplasmic incompatibility can also contribute to population suppression by making some females infertile, particularly when incompatibility is bidirectional (Moretti et al., 2018a). Furthermore, *Wolbachia* strains that have pathogen blocking effects can further reduce the vectorial capacity of the remaining mosquitoes.

The extent of population suppression will depend on the nature and severity of the host fitness costs. When costs are triggered by environmental conditions, such as high temperatures or low rainfall, releases of *Wolbachia*-infected mosquitoes can be timed to facilitate the spread of *Wolbachia* when costs are low, followed by seasonal suppression when costs are high (Rašić et al., 2014, Ritchie et al., 2015). Most *Wolbachia* transinfections in mosquitoes reduce the viability of quiescent eggs (Allman et al., 2020), making it possible use these strains for population suppression during long dry seasons when rainfall is infrequent. *Aedes aegypti* carrying the *w*Mel strain of *Wolbachia* become partially self-incompatible at high temperatures (Ross et al., 2019b), which may decrease population sizes during the hottest times of the year. If host fitness costs occur regardless of conditions, ongoing suppression could be achieved without further mosquito releases if the *Wolbachia* infection remains in the population. If host fitness costs are too severe, stable population replacement may not be possible (Nguyen et al., 2015), but releases may still achieve temporary suppression.

Replacement followed by suppression has not yet been undertaken deliberately in the field, but semi-

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field trials demonstrate its potential (Ritchie et al., 2015). Some population replacement releases may have already achieved unintended suppression. Releases of *w*AlbB-infected *Ae. aegypti* in Malaysia decreased the size of the *Ae. aegypti* population in some locations (Nazni et al., 2019), possibly due to host fitness costs in mosquitoes hatching from quiescent eggs (Lau et al., 2021). Because all *Wolbachia* strains being used for population replacement have host fitness costs, observed decreases in dengue transmission could in part be explained by decreased vector population sizes. Population replacement releases should therefore include monitoring of mosquito abundance in addition to *Wolbachia* frequencies.

Field releases around the world

Population replacement

Mosquitoes carrying *Wolbachia* infections are now being released in open field trials (Figure 3). The first population replacement release was conducted in 2011, leading to establishment of the *w*Mel *Wolbachia* strain in two *Ae. aegypti* populations in Cairns, Australia (Hoffmann et al., 2011). Following this success, releases expanded to cover a total area of 150 km^2 throughout Australia's current *Ae. aegypti* distribution (O'Neill et al., 2018, Ryan et al., 2019). Although dengue is not endemic in Australia, imported dengue cases frequently result in local outbreaks. Today, local dengue transmission in Australia has almost completely been eliminated in locations where *Wolbachia* infections have established (O'Neill et al., 2018, Ryan et al., 2019).

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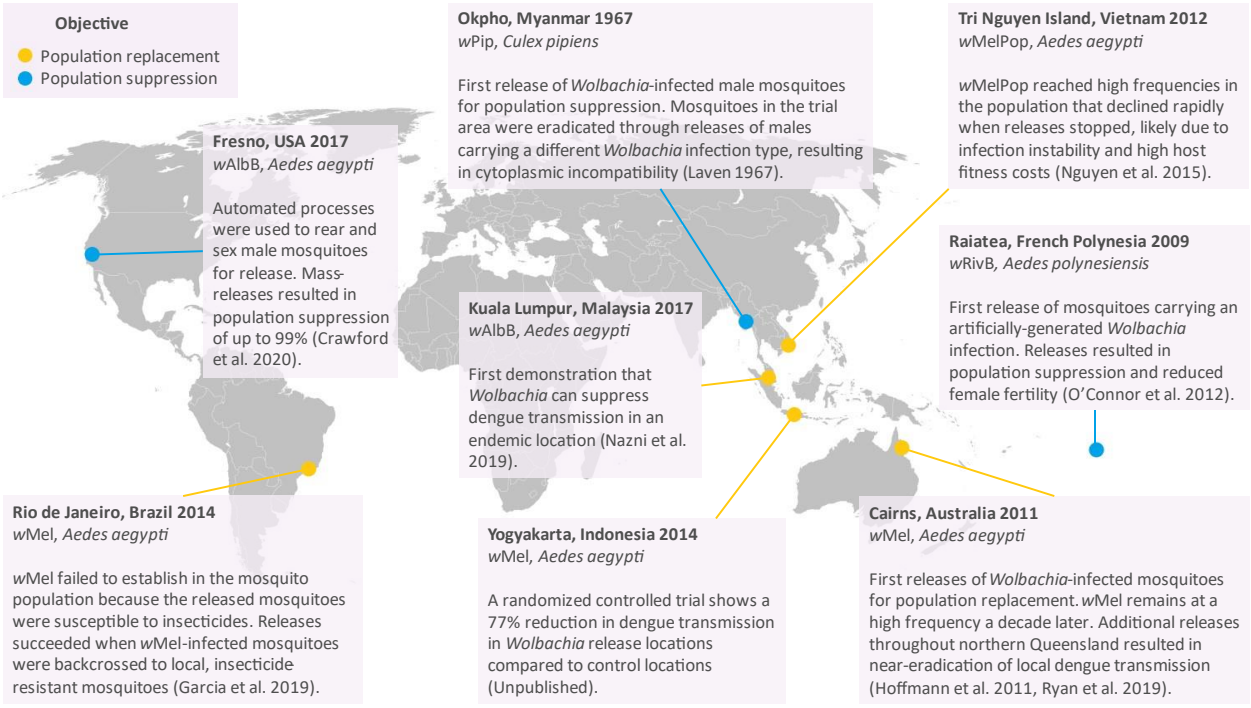

Figure 3. Milestone releases of *Wolbachia*-infected mosquitoes for population replacement or population suppression.

Population replacement programs are now operating in over 10 countries (http://worldmosquitoprogram.org, (Nazni et al., 2019). *Wolbachia* infections can invade and persist in natural populations (Garcia et al., 2019, Tantowijoyo et al., 2020) and reduce dengue transmission (Nazni et al., 2019, Indriani et al., 2020) in locations where dengue is endemic. Randomized controlled trials are also underway (Anders et al., 2018), with (unpublished) results from Yogyakarta, Indonesia indicating a 77% reduction in dengue transmission in locations where *Wolbachia*-infected mosquitoes have been released (https://www.worldmosquitoprogram.org/en/news-stories/media-releases/world-mosquito-programs-wolbachia-method-dramatically-reduces-dengue). Because *Wolbachia* infections remain in the population, population replacement is likely to provide long-term protection against dengue.

Not all population replacement releases have been successful. The *w*MelPop *Wolbachia* infection dropped out of natural populations after reaching high frequencies in two locations, likely because the host fitness costs of this strain are severe (Nguyen et al., 2015). Releases with *w*Mel in Rio de Janeiro, Brazil initially failed because insecticide-susceptible mosquitoes were being released into locations with heavy insecticide use, greatly reducing their fitness compared to the insecticide-resistant wild population (Garcia et al., 2019). *Wolbachia* may also drop out from some locations if immigration of uninfected mosquitoes is too high, requiring additional releases (Nazni et al., 2019). Although complete population replacement can be challenging, arbovirus transmission could still decrease if replacement is temporary or incomplete. In Niterói, Brazil, *w*Mel establishment was highly heterogeneous across

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different release regions, but dengue cases decreased even in sites *w*here *w*Mel was at a low to intermediate frequency (Pinto et al., 2021), possibly due to cytoplasmic incompatibility decreasing the relative population size of *Ae. aegypti*.

To date, population replacement programs have only been undertaken in *Ae. aegypti*, with almost all involving the *w*Mel *Wolbachia* strain. Although most research has focused on dengue, *Wolbachia* can block a variety of human pathogens in several vector species (Table 1), raising opportunities to expand release programs to target alternative mosquito hosts and human pathogens. Given the diversity of *Wolbachia* strains in nature, strains may exist that could more effectively block pathogen transmission and persist in populations.

Population suppression

Wolbachia-based population suppression has a long history. The first releases took place during the 1960s in *Culex pipiens* mosquitoes (Laven, 1967) before the discovery that cytoplasmic incompatibility was caused by infection with *Wolbachia* (Yen and Barr, 1973). *Culex pipiens* carry several related *Wolbachia* strains with diverse patterns of incompatibility (Atyame et al., 2014). Population suppression was achieved by releasing male mosquitoes from a population that carried a different *Wolbachia* strain to the target population, resulting in cytoplasmic incompatibility (Laven, 1967). A later study achieved suppression by releasing *Aedes polynesiensis* carrying a *Wolbachia* strain from *Aedes riversi,* a closely related species (O'Connor et al., 2012). These mosquitoes were generated by introducing the *Wolbachia* into an *Ae. polynesiensis* background through repeated backcrossing (Brelsfoard et al., 2008).

Population suppression programs experienced a resurgence in the 2010s after the first successful *Wolbachia* transinfections in mosquitoes (Xi et al., 2005, Xi et al., 2006, McMeniman et al., 2009), making population suppression with *Wolbachia* now possible for *Ae. aegypti* and *Ae. albopictus*. Recent studies show that releases of *Wolbachia*-infected males can directly reduce female fertility as well as population sizes (Caputo et al., 2020, Mains et al., 2016, Mains et al., 2019, Crawford et al., 2020). Although population suppression can reduce the incidence of biting by female mosquitoes (Zheng et al., 2019), no studies have linked reductions in mosquito populations to decreased disease incidence. Unfortunately, studies rarely include data from population monitoring after the release period, raising the question of how long suppression will last.

A common criticism of *Wolbachia*-based population suppression is that the accidental release of *Wolbachia*-infected females (which are fertile) could result in unintended population replacement. Although low levels of contamination during sex sorting are common, unintended population replacement has not yet been observed. Population replacement will only occur if the frequency of *Wolbachia*-infected females exceeds the threshold required for *Wolbachia* to spread, meaning that a low level of contamination is unlikely to pose a threat (Pagendam et al., 2020). Furthermore, if the target mosquito population carries a different *Wolbachia* strain, this will further reduce the probability of unintended replacement because the presence of bidirectional cytoplasmic incompatibility will

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increase the threshold frequency (Brelsfoard et al., 2008, Moretti et al., 2018a). To mitigate this risk, two recent population suppression programs irradiated all released mosquitoes to ensure that any released females are sterile (Zheng et al., 2019, Kittayapong et al., 2019). However, this added safeguard may come at the cost of reduced male mating competitiveness, requiring more males for the same level of suppression compared to *Wolbachia* infection alone.

Choosing the right *Wolbachia* strain for the job

Over 25 artificial *Wolbachia* infections have been generated in mosquitoes through transinfection, mainly in *Ae. aegypti* and *Ae. albopictus* (Ross et al., 2019c). These infections are diverse in their effects on pathogen blocking and host fitness costs, as well as their stability in different environments. *Wolbachia* superinfections, where more than one *Wolbachia* strain occupies a single mosquito, have also been generated, which can have cumulative effects (Joubert et al., 2016). For population suppression programs, the choice of *Wolbachia* strain is relatively simple: the best strain will induce complete cytoplasmic incompatibility with the target population and have as few host fitness costs as possible to increase the potential competitiveness of males. In contrast, ideal *Wolbachia* strains for population replacement do not exist, requiring a trade-off between *Wolbachia* infection stability, host fitness costs and pathogen blocking. The choice of *Wolbachia* strain must be made carefully.

A successful population replacement program relies on *Wolbachia* infections invading and persisting in natural mosquito populations and blocking pathogen transmission. Three different *Wolbachia* strains in *Ae. aegypti* have been released in population replacement programs, to varying levels of success (Table 2). *w*Mel is the strain of choice for the World Mosquito Program, who have been involved in most population replacement releases to date (https://www.worldmosquitoprogram.org). *w*Mel has relatively few host fitness costs, at least when tested under standard laboratory conditions (Ross et al., 2019c). *w*Mel reduces the transmission of several arboviruses (Table 1), although blocking is incomplete and can be quite variable when tested under realistic conditions (Carrington et al., 2017). *w*Mel's greatest strength is perhaps its reputation, with demonstrated success in multiple field trials. *w*Mel is used widely in basic research, including as a model for studying the mechanisms of pathogen blocking (Ford et al., 2019, Manokaran et al., 2020, Koh et al., 2020) and cytoplasmic incompatibility (LePage et al., 2017, Shropshire et al., 2018). A key limitation of *w*Mel is its instability at high temperatures (Ulrich et al., 2016, Ross et al., 2017). Although *w*Mel has successfully invaded multiple *Ae. aegypti* populations in the tropics (O'Neill et al., 2018, Ryan et al., 2019, Indriani et al., 2020), cytoplasmic incompatibility (Ross et al., 2019b) and dengue blocking (Mancini et al., 2020a) could weaken after *w*Mel is established. Instability at high temperatures could help to explain the slow spread of *w*Mel and fluctuating infection frequencies in some trial locations (Tantowijoyo et al., 2020, Ross et al., 2020b, Pinto et al., 2021), though direct comparisons between strains under field conditions have not yet been performed.

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Table 2. *Wolbachia* strains are diverse in their effects. Comparison of the three *Wolbachia* strains in *Aedes aegypti* mosquitoes that have been that have been released in open field trials. For a comprehensive list of *Wolbachia* infections generated in mosquitoes, see table S1 of Ross et al. (2019c).

	Wolbachia strain		
	*w*AlbB	*w*MelPop	*w*Mel
Original host	*Aedes albopictus*	*Drosophila melanogaster*	*Drosophila melanogaster*
Reference for first transinfection	Xi et al. (2005)	McMeniman et al. (2009)	Walker et al. (2011)
Features	Cytoplasmic incompatibility, maternal transmission, pathogen blocking.	Cytoplasmic incompatibility, maternal transmission, pathogen blocking.	Cytoplasmic incompatibility, maternal transmission, pathogen blocking.
Key advantages	Heat and antibiotic resistant, with *Wolbachia* phenotypes likely to be stable in different environments (Ross et al., 2017, Endersby-Harshman et al., 2019). Host fitness costs when eggs are quiescent could be utilized for seasonal population suppression (Lau et al., 2021).	Strong pathogen blocking compared to other strains (Walker et al., 2011, Ferguson et al., 2015). Substantial host fitness costs could be effective for temporary population suppression (Ritchie et al., 2015).	Limited host fitness costs allow *w*Mel to invade natural populations with relative ease (Walker et al., 2011). Demonstrated success in multiple field trials around the world (worldmosquitoprogram.org).
Key disadvantages	*w*AlbB-infected females hatching from quiescent eggs become infertile. This fitness cost could reduce *Wolbachia* invasion and persistence in locations with long dry seasons (Lau et al., 2021).	Severe costs to host fitness, particularly when mosquitoes age, make stable population replacement unlikely (McMeniman and O'Neill, 2010, Yeap et al., 2011). Occasional maternal transmission instability, even under benign conditions (Ross et al., 2020a).	Instability at high temperatures could result in weakened cytoplasmic incompatibility, maternal transmission and pathogen blocking in tropical environments (Ross et al., 2017, Ross et al., 2019b, Mancini et al., 2020a).

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Field release outcomes	*w*AlbB reached stable, high frequencies in some release locations in a population replacement program in Malaysia (Nazni et al., 2019). *w*AlbB suppressed populations in Singapore, Australia and the USA through male-only releases (Crawford et al., 2020, Mains et al., 2019), unpublished).	*w*MelPop reached high frequencies in population replacement programs in Australia and Vietnam, but failed to persist (Nguyen et al., 2015).	*w*Mel reached stable, high frequencies in population replacement programs in several countries including Australia, Indonesia and Brazil (Hoffmann et al., 2011, Garcia et al., 2019, Tantowijoyo et al., 2020), worldmosquitoprogram.org).

*w*AlbB was the first *Wolbachia* strain to be introduced to *Ae. aegypti* (Xi et al., 2005) and is similar to *w*Mel in many ways; both strains induce complete cytoplasmic incompatibility and have similar effects on pathogen blocking (Joubert et al., 2016, Flores et al., 2020). However, *w*AlbB is relatively stable at high temperatures (Ross et al., 2017, Ant et al., 2018) and its effects on host fitness can differ to *w*Mel under some environmental conditions (Lau et al., 2021, Lau et al., 2020). When *w*AlbB-infected *Ae. aegypti* hatch from eggs that are more than a few weeks old, a substantial proportion of females that hatch are infertile (Lau et al., 2021). These traits could make *w*AlbB more effective *Wolbachia* than *w*Mel for reducing dengue transmission in hot environments, but population replacement with *w*AlbB could be more challenging in locations with a long dry season.

The *w*MelPop strain of *Wolbachia* is a variant of *w*Mel that imposes a suite of costly effects on mosquitoes, including dramatically reduced lifespan and fertility (McMeniman et al., 2009, Turley et al., 2009, Yeap et al., 2011). *w*MelPop is no longer being considered for field releases due to previous failures (Nguyen et al., 2015) but it remains a popular choice for basic research due to its strong and far-reaching effects on insect hosts.

Planning an effective *Wolbachia* release program

Releases of mosquitoes carrying *Wolbachia* aim to reduce the incidence of disease (or nuisance biting) in a target population, but many factors will influence these objectives (Figure 4). Anyone undertaking a *Wolbachia* release program should first decide on the approach, whether it be replacement, suppression, or a combination. The choice will depend on aspects of the target population including the incidence of disease, local expertise and facilities, as well as public perception. Population replacement is more cost-effective (Brady et al., 2020) but may have greater perceived risks (Murphy et al., 2010).

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Suppression could be preferred when nuisance biting is a key concern, when the release area is small and isolated, or when the costs associated with ongoing releases can be met. Population suppression is only possible in locations with the resources to mass-rear and sex mosquitoes, but population replacement can be done almost anywhere. Since sex-sorting is not required, mosquitoes can be mass-reared remotely, shipped as eggs to the release site, and hatched locally. This approach was recently used for *Wolbachia* replacement releases in the Pacific Islands (http://www.eliminatedengue.com/pi/Progress/view/news/1063/pg/1). However, because storage and transport can reduce *Wolbachia*-infected egg viability (Allman et al., 2020), remote mass-rearing may increase production requirements and produce lower quality mosquitoes.

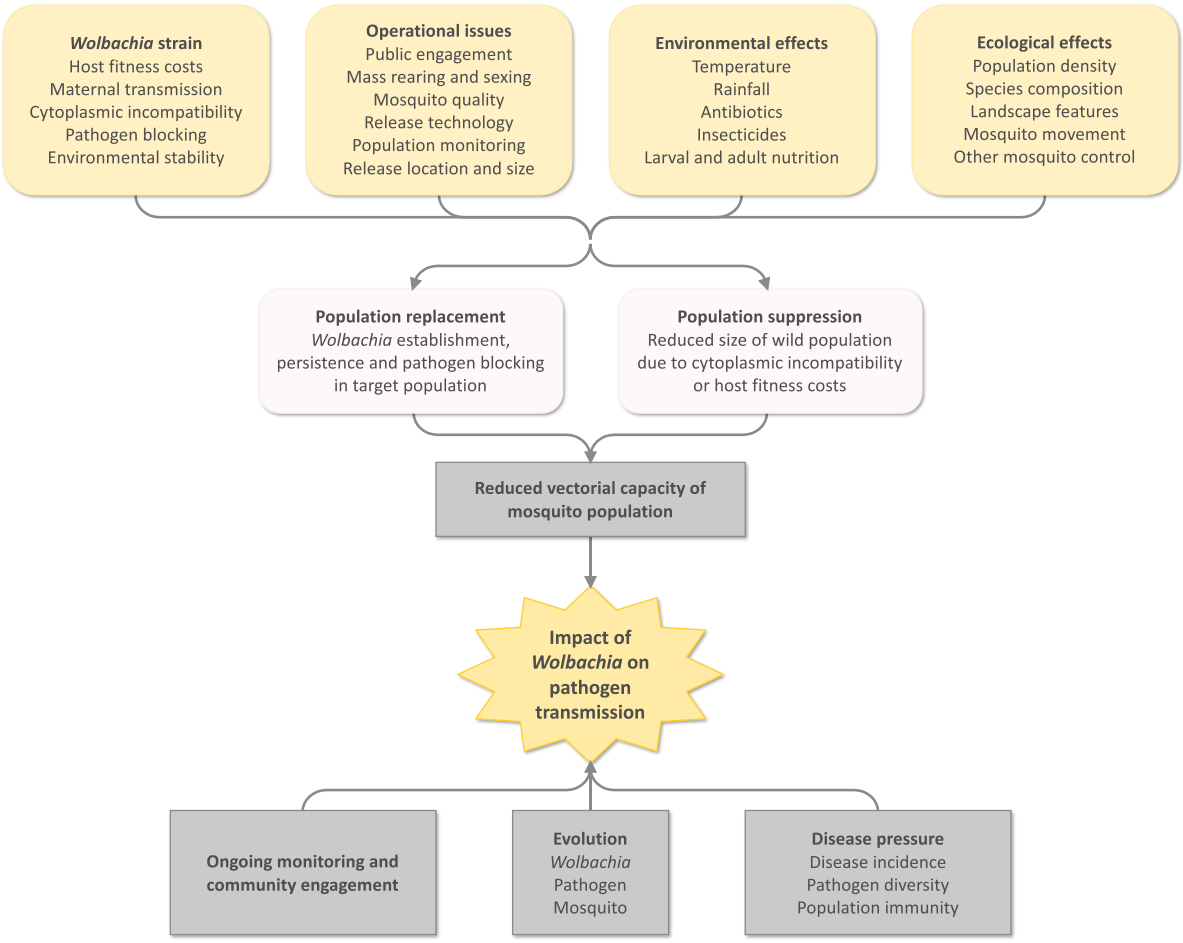

Figure 4. Factors affecting the success of *Wolbachia* releases. This figure has been modified and expanded from Ross et al. (2019c).

Controlling mosquito populations by releasing even more mosquitoes may seem counter-intuitive, especially when biting female mosquitoes are released. There are also potential risks unique to

Wolbachia releases, such as the possibility of *Wolbachia* spreading to non-target insects or enhancing, rather than suppressing, virus transmission. Community engagement programs are essential to ensure that the general public are informed and approve of releases (O'Neill et al., 2018, Costa et al., 2020). Engagement activities should clearly explain the *Wolbachia* technology and adequately address concerns from the community. Obtaining regulatory approval for *Wolbachia* releases is also necessary, but it is not enough. If the public oppose the technology or are unaware of the releases taking place, this will build mistrust and jeopardize future releases (Nading, 2014).

Successful population replacement or suppression requires more than just releasing as many mosquitoes as possible. Mosquitoes must be of high quality and released with adequate coverage of the target area. *Wolbachia* releases must consider the host fitness costs of the *Wolbachia* strain but also the fitness of the mosquitoes they inhabit. Mosquitoes that are inbred or lacking local genetic traits (such as insecticide resistance) are unlikely to perform well in the field (Ross et al., 2019a, Garcia et al., 2019). Backcrossing mosquitoes to the target population can improve fitness (Yeap et al., 2011), but the *Wolbachia* infection must also be maintained at a high frequency in release stocks. Mosquito genetic background can influence pathogen blocking by *Wolbachia* (Terradas et al., 2017, Ford et al., 2019) as well as host fitness costs (Ritchie et al., 2015, Carvalho et al., 2020), making it important to evaluate strains in local mosquitoes before releases take place. Handling conditions are also crucial, since rearing, storage, transport and release procedures can affect mosquito quality (Chung et al., 2018, Culbert et al., 2018).

Release planning should involve careful consideration of the local environment, including landscape features and climate. Gaps in *Wolbachia* coverage may occur in areas where mosquito dispersal is limited (Jasper et al., 2019) or where population densities are heterogeneous (Hancock et al., 2019), requiring additional targeted releases. *Wolbachia* spread may depend on the distribution and productivity of larval habitats, with slower spread expected when larval competition is high (Hancock et al., 2016) and when habitats are unsheltered and reach high temperatures (Ross et al., 2019b). Verticality is also an important consideration; population suppression programs in dense urban environments with high-rise apartments may be ineffective if mosquitoes are only released at ground level (https://www.straitstimes.com/singapore/male-wolbachia-carrying-mozzies-to-be-released-in-tampines-west-and-nee-soon-east-in-april). The nature of the local mosquito population can also affect release outcomes. In locations with multiple vector species, there is a risk that suppression of one species could increase the prevalence of others that occupy a similar niche, which could have unexpected consequences for pathogen transmission (Ritchie et al., 2018). Natural *Wolbachia* infections in the target mosquito population could also be an issue; if the resident *Wolbachia* strain is compatible with the release strain, this can prevent replacement or suppression (Ross et al., 2020c).

Releases of *Wolbachia*-infected mosquitoes for population replacement began only a decade ago, so how long will *Wolbachia* remain effective for? Over time, the phenotypic effects of *Wolbachia* are expected to weaken due to evolutionary changes in *Wolbachia*, mosquitoes or pathogens (Bull and Turelli, 2013). In *Ae. aegypti*, *Wolbachia* have so far remained stable, with no observed changes in cytoplasmic incompatibility, fitness costs or virus blocking (Hoffmann et al., 2014, Frentiu et al., 2014, Ross and Hoffmann, 2018, Ahmad et al., 2021, Gesto et al., 2020) and few changes in the *Wolbachia*

genome (Huang et al., 2020) after field releases. No studies have tested whether *Wolbachia* releases cause genetic changes in mosquito populations, but mosquitoes could plausibly adapt to *Wolbachia* infection (White, 2011). Because *Wolbachia* and mitochondria are both maternally inherited, mitochondrial variants associated with the release strain will spread alongside *Wolbachia*, reducing the native diversity (Yeap et al., 2016, Huang et al., 2020). Since pathogen blocking by *Wolbachia* is usually incomplete, there is a risk that pathogens could evolve to bypass *Wolbachia*-mediated blocking. Laboratory selection experiments have failed to generate *Wolbachia*-resistant virus strains (Koh et al., 2019, Martinez et al., 2019), but virus diversity is much greater in natural populations. There is also a risk that wild mosquitoes could evolve mating preferences against the released mosquitoes (Cator et al., 2020), decreasing the effectiveness of *Wolbachia* releases over time. With the potential for evolutionary changes, ongoing monitoring of *Wolbachia*, pathogen and mosquito populations is crucial to ensure that releases continue to achieve their objective (Ritchie et al., 2018). If a *Wolbachia* strain loses its effectiveness, releases of different *Wolbachia* strains might extent the longevity of disease suppression (Joubert et al., 2016), but this will depend on the mechanisms involved.

Conclusions

Releases of *Wolbachia*-infected mosquitoes can be an effective tool for disease control, with field trials showing tangible impacts on mosquito populations and dengue transmission. *Wolbachia* releases are best used in conjunction with existing vector control measures and are not intended to replace them (Ritchie and Johnson, 2017). Source reduction, surveillance and community awareness programs will continue to be effective ways to reduce mosquito-borne disease (Fonseca et al., 2013, Healy et al., 2014, Trewin et al., 2017, Forsyth et al., 2020) and public awareness programs for *Wolbachia* releases should continue to emphasize the importance of these measures. *Wolbachia* releases currently focus on a handful of mosquito species but there is great potential for *Wolbachia* to control other vectors, pests and diseases (Gong et al., 2020, Mateos et al., 2020, Kamtchum-Tatuene et al., 2016). Expansions of *Wolbachia* releases around the world will likely have great benefits, but releases must be supported by ongoing research, monitoring and community engagement.

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