UJEMI PEARLS

Using *Wolbachia* infected mosquitos to combat arboviral transmission: from molecular mechanisms to global application

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SUMMARY The global disease burden inflicted by arboviruses such as dengue, Zika, and chikungunya viruses represents a pressing public health crisis. These mosquito-borne diseases co-circulate in the common *Aedes aegypti* vector, leading to co-morbidities and mortalities, often in low-income countries. Despite the prevalence of arboviruses worldwide, no reliable antivirals or vaccines are currently available. Furthermore, existing strategies for vector biocontrol, such as insecticide dissemination, have resulted in resistance development in mosquitos and can lead to toxic bioaccumulation within ecosystems. *Wolbachia* is an obligate intracellular bacterium that has co-evolved over 200 million ears to live in endosymbiosis with 60-70% of insects. *Wolbachia* transmission between insects is exclusively limited to vertical transmission between females and their offspring. As a result, *Wolbachia* has evolved mechanisms to promote its own transmission and persistence within insect populations. These mechanisms include preventing live births between *Wolbachia*-infected males and uninfected females. Moreover, *Wolbachia* alters the insect immune system and lipid homeostasis to protect against invasion from other pathogens, rendering mosquitos remarkably resistant to arboviral infection. Recently, these mechanisms have been exploited as novel techniques to combat arboviral transmission. By releasing *Wolbachia*-infected mosquitos to areas afflicted by arboviral outbreaks, researchers have hoped to curtail new infections by rendering *A. aegypti* an ineffective vector (Fig. 1). This article will first provide an in-depth review of the molecular mechanisms underpinning *Wolbachia*-mediated arboviral resistance in insects. Next, it will discuss how this strategy is currently being harnessed, and critically analyze the cost, safety, scalability, efficacy, and long-term impacts of these field releases. Thorough understanding of this technique will help expand and improve future deployments, as well as inform novel drug development using *Wolbachia* metabolites.

INTRODUCTION

Globally, mosquito-borne arboviruses constitute a burgeoning public health crisis. Arboviruses such as dengue (DENV), Zika (ZIKV) and chikungunya (CHKV) annually infect over 100 million people worldwide (1). Arboviral infections incur a diverse range of symptoms, and often become fatal. DENV can induce severe dengue, a condition characterized by severe abdominal pain and blood loss (2). ZIKV infection can cause neonatal microcephaly in pregnant women and sterility in men, while CHKV infection can result in rheumatic disorders (3,4). While pervasive in low-income countries, arboviral infections have spread to developed countries, such as DENV in Australia, ZIKV in the US, and West Nile virus (WNV) in Canada (5)**.** Many arboviruses share a geographic distribution and are co-transmitted through the same insect vector: the *Aedes aegypti* mosquito. As a result, this epidemiological synergy leads to frequent co-infections, exacerbating clinical outcomes (6) . In a 2018 study, Zaidi *et al* found that 9 of 24 CHKV-infected Mexican atients were also co-infected with DENV(6). With the exception of Yellow fever virus, no effective vaccines or antivirals against arboviruses exist despite the prevalence of disease. (7). As a result, novel mechanisms of viral biocontrol are needed to curtail this global health burden. Further, these methods must be compatible with the

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limited financial resources available in developing countries where arboviral diseases are most prevalent.

In the last decade, research has highlighted the ability for *Wolbachia* to limit arboviral spread (8-11). *Wolbachia* is a bacterium that naturally lives in obligate endosymbiosis with 60% of insects, including terrestrial arthropods and nematodes (10). From reductively evolving to live within the cytoplasm of insect cells over a predicted evolutionary period of 200 million years, *Wolbachia* contains a genome of only 1.4 kb (12,13). Thus, the bacterium has incomplete essential amino acid biosynthesis pathways and typically parasitizes insects for amino acids, which then become primary energy sources for *Wolbachia* (14). While globally distributed, *Wolbachia* is non-pathogenic to mammals (9). *Wolbachia* is exclusively spread through insect vertical transmission and cannot be transmitted through insect bites (9). Interestingly, *A. aegypti* infected with *Wolbachia* (W+) become markedly resistant to DENV, CHKV, and ZIKV infection (10,11). *Wolbachia*-infected mosquitos fail to harbour infectious virions in their saliva after a bloodmeal artificially spiked with these arboviruses (11). Thus, W+ mosquitos become incapable of intraspecific transmission of arboviruses, rendering them unproductive vectors (11). Furthermore, matings between W+ males and W- females result in stillborn larvae (11). These observations have suggested release of W+ mosquitos into a native population is a promising, natural mechanism of arboviral biocontrol. Field releases in Brazil, Singapore, and Australia have effectively limited epidemics of ZIKV, CHKV, and DENV respectively (10,11,15) . Despite preliminary success, further research is required to elucidate the underlying mechanism of *Wolbachia*-mediated arboviral resistance. These mechanisms will inform how field releases can be better executed for maximum effectiveness in different climates and epidemiological landscapes. Finally, cost, scalability, and long-term ecological impact must be assessed to determine the feasibility of implementation in low-income countries, as well as the efficacy of combating emerging outbreaks in developed nations. This article will review the current scientific literature to better understand these questions in order to show that release of W+ mosquitos can safely, efficaciously, and cost-effectively limit arboviral spread worldwide by rendering *A. aegypti* an unproductive vector.

RESEARCH QUESTIONS

While field releases of W+ mosquitos have successfully curtailed arbovirus outbreaks, the mechanisms by which *Wolbachia* induces arboviral resistance in *A. aegypti* are inadequately elucidated. Focusing on DENV, ZIKV and CHKV, this article aims to better understand these anti-arboviral molecular mechanisms in order to improve the efficacy and safety of this novel technique. Whether or not these mechanisms can be generalized to additional mosquito species and arboviruses, such as WNV and Japanese encephalitis virus (JEV) will also be investigated. Currently, successful field releases have been limited to smaller, localized communities with low population density (15). In order to assess whether this

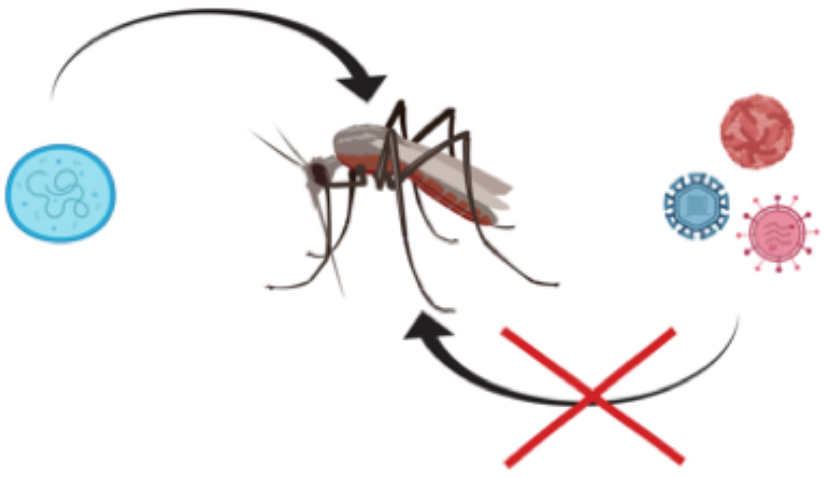


FIG. 1 The *A. aegypti* mosquito is the natural vector for numerous arboviruses, such as DENV, ZIKV, and CHKV. The arbovirus is transmitted from the human to the mosquito following a blood meal containing infectious virions. The arbovirus then replicates in the insect saliva and midgut, and progeny are transmitted when the mosquito bites another human host. Interestingly, mosquitos infected with the obligate intracellular bacterium *Wolbachia* can no longer transmit arboviruses. This mechanism of rendering the vector incompetent is a promising new method of arboviral biocontrol.

technology can be reliably implemented across diverse geographical and sociopolitical climates, this article will also investigate the cost-effectiveness and scalability of this method. For instance, a 2018 field release in Townsville, Australia to combat DENV reported a cost per protected person of approximately $18 CAD (15). While a relatively high cost compared to conventional insecticdes, this can be attributed to the preliminary nature of this research and that the field release also occurred over a small township of 187 000 individuals, with a low population density of 1061 individuals/km^2 (15). Careful analysis into the scalability of this technology, including rigorous mathematical modelling of mosquito-*Wolbachia* population dynamics, is required to accurately predict the necessary number of mosquitos released and the release schedule that confers adequate protection. A final limitation of this technique is that it is relatively new. As a result, this article will predict the long-term impacts on the environment, resistance development, and mosquito behaviour. Thorough risk-benefit analysis is necessary to seek government approval in at-risk countries where the technology has yet to be employed.

PROJECT NARRATIVE

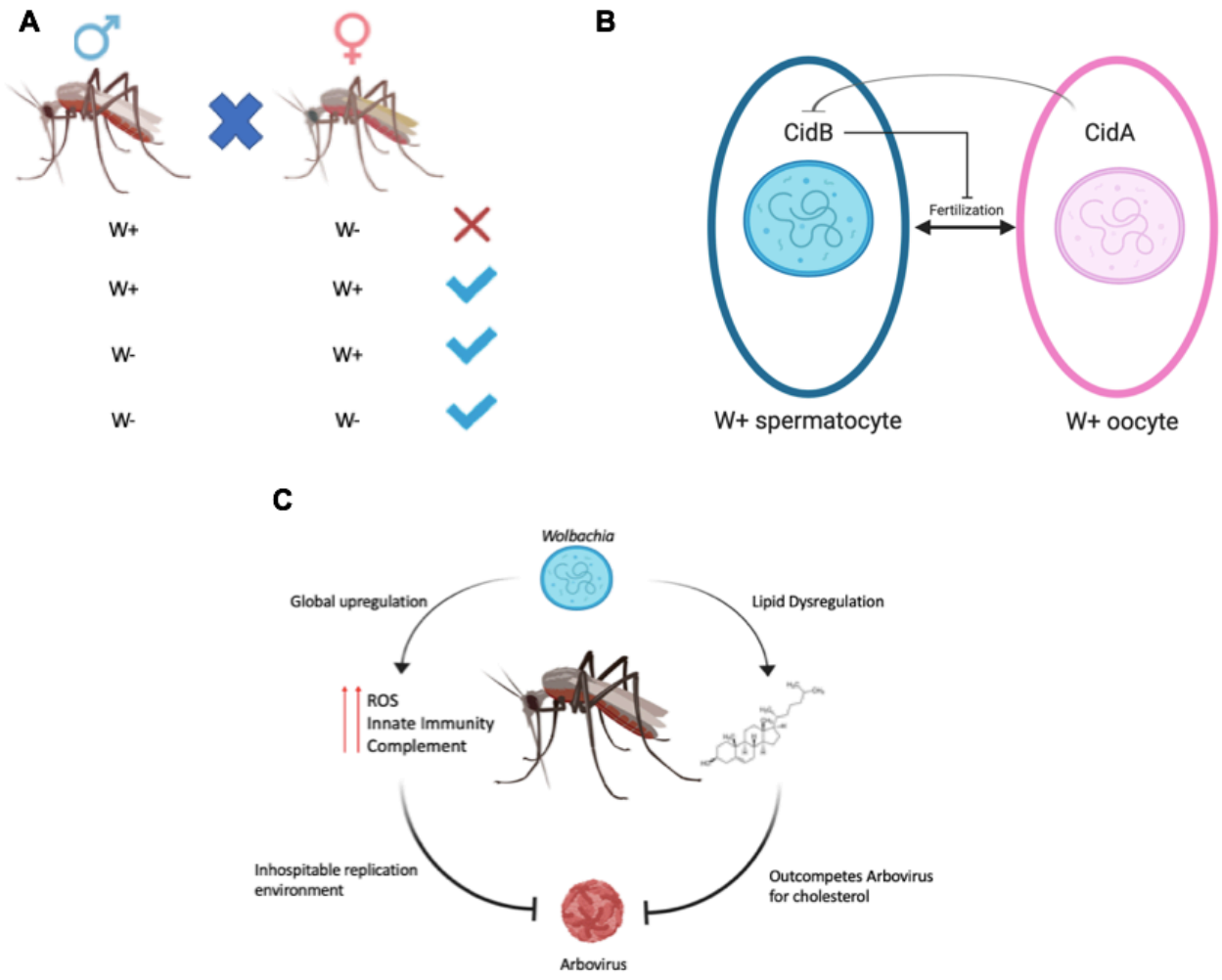
What are the molecular mechanisms by which *Wolbachia* limits arboviral spread and how generalizable are they between arboviruses? Currently, mosquito field releases have consisted of W+ males, or both W+ males and females (16) . The rationale behind using W+ males is based on the observation that when W+ males mate with W- females, nonviable progeny are produced, as shown in Fig. 2a. (9). In contrast, W- males can productively mate with W+ females (11) . This phenomenon has been termed cytoplasmic incompatibility (CI) and is proposed to be a natural mechanism by which *Wolbachia* promotes its dissemination throughout insect populations (17). *Wolbachia* spread is limited to vertical transmission between mothers and offspring (9). As a result, CI decreases the likelihood for W- females to produce viable offspring, consequently promoting *Wolbachia* spread throughout mosquito populations. This mechanism can potentially be repurposed as a natural approach to control mosquito population size. By releasing W+ males, CI will prevent W- females from reproducing successfully, consequently reducing the mosquito population. At the molecular level, CI is mediated by the *Wolbachia*-encoded *cidA*-*cidB* operon, a toxin-antidote system depicted in Fig. 2b. (18). CidB is a deubiquitinase that is provided by W+ males during fertilization (18). Expression of CidB results in defects during the first mitotic division of the fertilized egg, as well as blastoderm formation and segmentation (18) . While the cellular target of CidB is unknown, the enzyme induces infertility (18). However, W+ females express CidA during fertilization (18). CidA binds CidB with high affinity, leading to catalytic inactivation (18). As result, crosses between W+ parents produce viable offspring (9). While different *Wolbachia* strains express paralogous operons, binding affinity is highest between cognate toxin/antidote pairs (18). Hence, W+ mosquitos will reproduce most successfully with mates infected with the same *Wolbachia* strain.

While W+ males can curtail mosquito populations by means of CI, pairing their release with W+ females may be even more effective at limiting arboviral spread (9). This is due to the observation that W+ mosquitos, regardless of gender, are remarkably resistant to arbovirus infection (7,8,10,17,19,20). Notably, W+ mosquitos contain markedly reduced levels of infectious virions in their saliva, abdomen, and thorax, following DENV, ZIKV, or CHKV infection (10,11). This resistance is controlled by the ability of *Wolbachia* to render the host environment unfavourable for viral replication (Fig. 2c.). First, *Wolbachia* infection generates a global pro-inflammatory state in mosquitos (19). Transcriptomic analyses have shown general upregulation of pro-inflammatory markers in W+ mosquitos, including genes such as LRIM1, TEP1, and CEC1, all of which are involved in host antimicrobial immune responses (19). *Wolbachia* can additionally create a hostile environment to arboviruses by inducing generation of reactive oxygen species (20). While detrimental to the host, this capability could have allowed *Wolbachia* to persist in insect populations at the exclusion of other symbionts. Aside from generating a pro-inflammatory state, recent research has also highlighted the ability of *Wolbachia* to compete with certain arboviruses for cholesterol (21). In particular, DENV utilizes host cholesterol during its replication cycle to mediate viral budding (22). *Wolbachia* is proposed to incorporate cholesterol into its cell membrane

in lieu of lipopolysaccharide (22). This direct competition can reduce the replicative success of arboviruses like DENV that require cholesterol to propagate. Immune upregulation has also been shown to protect W+ mosquitos from non-arboviral pathogens as well, such as the malaria-inducing *Plasmodium* or filarial worms (10,19).

The implications of understanding these mechanisms are twofold. Firstly, field releases of male and/or female W+ mosquitos aimed to widely disseminate *Wolbachia* in native mosquitlo populations may create evolutionary pressures on arboviruses, promoting mutations that allow persistence in W+ mosquitos. By defining the molecular mechanisms, we can better predict the likelihood of resistance development. For instance, discovery of the *cidA-cidB* operon revealed that CI is the result of bacterial effectors targeting the host. Since these proteins do not interact with the arbovirus, resistance development is unlikely. Development of mosquito resistance to CI is also unlikely, as *Wolbachia* and arthropods have co-evolved together over millions of years leading to horizontal transfer of *Wolbachia* genes into the mosquito genome (12,23) . This highly-evolved endosymbiotic relationship indicates that resistance in mosquito populations is unlikely to arise. Moreover, while he mechanisms which prevent arboviruses from producing infectious progeny in W+ insects

FIG. 2 (A) *Wolbachia* can alter the mating patterns of infected insects by inducing CI. When male W+ mosquitos mate with W- females, stillborn offspring are produced. This suggests release of W+ males into mosquito reservoirs could naturally control population levels without the need for insecticides. **(B)** The *cidA-cidB* operon regulates CI by acting as a toxin/antidote system. CidB is a deubiqutinase expressed in W+ spermatocytes that induces sterility upon fertilization. However, expression of CidA in W+ oocytes will inhibit CidB through direct binding. **(C)** W+ mosquitos of both genders become poor arboviral vectors. *Wolbachia* can induce global upregulation of the insect immune system and outcompete arboviruses for lipids. This creates an inhospitable environment for arboviral replication within the insect.

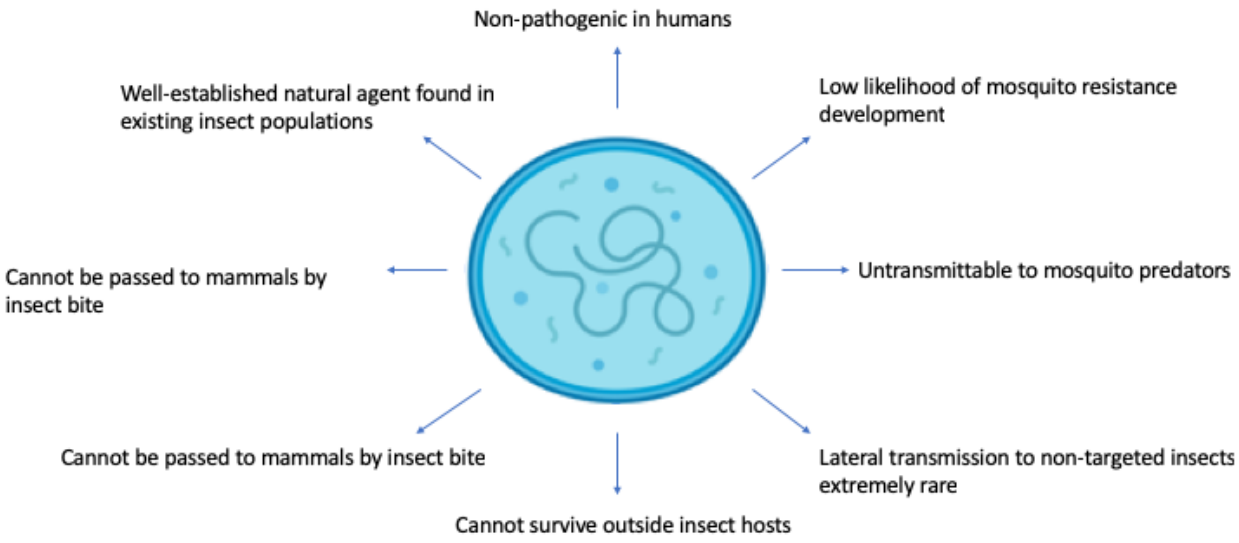


does may initially appear to exert some evolutionary pressure, understanding of this mechanism has allowed us to predict a low potential for resistance development. Unlike a direct-acting antiviral which targets a single viral pathway, *Wolbachia* infection leads to global upregulation of pro-inflammatory genes and lipid dysregulation in mosquitos. Thus, it is unlikely for arboviruses to develop resistance to these multifactorial effects.

How can W+ mosquitos be cost-effectively harnessed to combat arboviral transmission across diverse populations? While the mechanisms employed by *Wolbachia* to induce CI and generate a host environment inhospitable to viral persistence are effective at controlling arboviral populations *in vitro*, in order for this technology to widely implemented, we must address its cost-effectiveness, scalability, and reliability across diverse geographical environments. A 2018 article published in Gates Open Research profiled the successful deployment of W+ mosquitos in Townsville, Australia to combat DENV spread (15). The project began with public consultation from the local community regarding the W+ field releases. After general consent was obtained, the release process began by first obtaining wild mosquito eggs to generate a wildtype colony. These mosquitos were then backcrossed with a laboratory mosquito line infected with the wMel strain of *Wolbachia.* Of existing *Wolbachia* strains, wMel has been the most extensively studied in existing literature in the context of blocking arboviral spread. Progeny were reared over 3 generations over which sustained wMel infection was assessed by qPCR. Release of the wMel+ male and female mosquitos occurred over 4 stages and 28 months, with the first targeting known mosquito breeding grounds and the later covering remaining suburbs. These releases spanned the 190 km^2 area of Townsville and comprised approximately 4 million wMel+ mosquitos. Throughout, mosquito traps were used to allow monitoring of wMel persistence throughout the *A. aegypti* population by qPCR. As a result of this undertaking, only 4 cases of local DENV infection were reported in the 44-month window following the release of wMel+ mosquitos, compared to a median of 98 in preceding 44-month windows.

While the Townsville release reduced endemic DENV cases by 96%, there are potential difficulties with scaling this technique to different geographical and sociopolitical climates. One of the notable benefits of this technique is the proposed cost-effectiveness: rearing, releasing, and monitoring wMel+ mosquitos is inexpensive compared to other public health

FIG. 3 Our current understanding of the molecular mechanisms governing *Wolbachia*-mediated CI, immune upregulation, and lipid dysregulation allows us to predict the potential risks of this novel technique. Through millions of years of reductive evolution, *Wolbachia* has uniquely adapted to exclusively live within insects, suggesting a high degree of ecological and clinical safety. Thus, the mechanisms summarized above indicate that dissemination of W+ mosquitos is predicted to be a relatively risk-free and efficacious form of arboviral biocontrol.



measures, such as vaccination or antiviral administration programs (24) .This would enable use in low-income countries that shoulder the majority of the global arboviral disease burden. The Townsville release averaged a cost of approximately $18.22 CAD/person for each of the 4 release stages. In comparison, using insecticides to control arboviral spread costs approximately $4.00 CAD/person (25). This striking disparity should be of little concern however, as, the researchers attributed the high cost to the nature of the release itself, contending that it was more of an exploratory research activity instead of a public health operation. Thus, community engagement, thorough safety monitoring, and use of highly-trained personnel constituted the majority of costs. They recommend that future releases could reduce the frequency of *Wolbachia* monitoring and number of personnel involved, minimizing the cost to approximately $1 CAD/person. Another factor contributing to high cost per person is the low population density of Townsville, approximately 1061 people/km^2. Deployment in tropical cities with high risk of arboviral transmission and greater population density would significantly reduce the cost per person. Furthermore, the ability for *Wolbachia* to self-sustain in an insect population ensures the endeavor would be a one-time cost (9,16).

The large geographical area of Townsville and the low population density could also have potentially contributed to the overall success of the pilot trial. Studies have indicated that mosquito-to-human arboviral transmission is more efficient in highly dense populations (26). This is due to increased frequency of infected hosts, enhancing rate of arboviral transmission through bloodmeals (26). Thus, it is possible the same strategy may not be as effective areas of high population density due to the increased transmission rate. This concern has been assuaged by subsequent pilot releases in Indonesia, Vietnam, Singapore, Brazil, Colombia, and the United States to combat DENV, as well as ZIKV and CHKV, all of which have been similarly successful despite targeting diverse population sizes (15,16,27-29). Mathematical modelling of *Wolbachia*-mosquito population dynamics would assist in predicting how many male/female W+ mosquitos would be needed to ensure *Wolbachia* persistence and confer arboviral resistance in a native insect community given the existing human population density (27). Socioculturally, the smaller population of Townsville and the status of Australia as a developed nation eased efforts to increase public awareness and obtain consent for the field releases. In developing nations, spreading public awareness and acquiring general consent from a local populace for a similar project may prove more difficult. In these instances, greater resources should be invested into public awareness campaigns to prevent ethical issues from potentially arising.

Finally, varying climates may also impact the degree to which *Wolbachia* can self-sustain in insect populations. *In vitro* studies have indicated that warmer temperatures may reduce the efficiency of *Wolbachia* vertical transmission, suggesting that warmer climates where arboviruses are endemic, as well as the onset of climate change, may limit the effectiveness of this method (30) . However, mosquitos have been shown to seek moderate microenvironments to reproduce, which are less harsh than ambient temperatures (9). This behaviour suggests that W+ mosquito release may be robust across different climates.

What is the long-term safety, efficacy, and ecological impact of this technique? In order to gain approval from government agencies and the general public, thorough analysis of this strategy must be conducted to ensure no immediate or long-term adverse effects. Existing methods of mosquito biocontrol, such as insecticide usage, have historically faced public opposition due toxic bioaccumulation in higher mammals and downstream ecological ramifications (31). Moreover, increasing mosquito resistance to commonly used insecticides such as permethrin and malathion has been documented (32). Since W+ mosquito release is a relatively new strategy, further experimentation and longitudinal studies must be conducted to accurately assess the long-term safety of this technique. However, using existing research on the *Wolbachia*-mosquito endosymbiotic relationship, we can predict the relatively safety of this technique. First, *Wolbachia* cannot be transmitted to humans by mosquito bites and is not pathogenic to mammals, thereby eliminating the risk of direct adverse effects on human populations (9). Another concern regarding *Wolbachia* dissemination is the potential for spread to non-mosquito populations. Interestingly, the filarial nematodes that cause onchocerciasis (river blindness) or elephantiasis are only

pathogenic if they are infected with *Wolbachia* (33). However, since *Wolbachia* cannot survive outside the insect host because it is an obligate intracellular endosymbiont, interspecific lateral transmission has been exceedingly rare (9) . Finally, *Wolbachia* is unable to be transmitted through predation of mosquitos (9). These characteristics are summarized in Fig. 3.

While immediate risks are low, long-term ecological impact is more challenging to predict. For instance, *Wolbachia* is capable of altering mosquito mating behavior to promote its maternal transmission (17). These strategies include phenotypic feminization of male mosquitos and induction of parthenogenesis in females (the ability to reproduce without a male) (9,17) . These distortions to mating behavior may incur unforeseeable changes to insect ecosystems. Since lateral transfer of *Wolbachia* genes into the *A. aegypti* genome has also been described, there is the potential for genetically altering existing mosquito populations (23). However, these interdomain lateral transfer events are exceedingly rare and can result in pseudogenization, thus are unlikely to cause immediate functional effects. Lastly, most research utilizing W+ mosquitos to combat arboviral transmission has focused on DENV, ZIKV, and CHKV. Thus, it is possible that lesser studied arboviruses, such as Japanese encephalitis or Rift Valley fever viruses may be able to replicate differently in W+ mosquitos. A 2014 studied showed *Wolbachia* infection of mosquito hosts conversely enhanced WNV replication in thoracic segments by potentially downregulating the NFκB-like regulator REL1, thus cautioning against the use of this technique before thorough experimentation (34). However, the authors recognized that their use of transient *Wolbachia* infections rather than the maternal method of transmission employed in field releases may have influenced the WNV replication cycle. Another study showed that while higher WNV genomic RNA was detected in W+ mosquitos, the overall production of infectious virions was decreased (35). This suggests the resistance mechanisms conferred by *Wolbachia* may be generalizable to a broad range of arboviruses.

Finally, the long-term efficacy of this technique must be considered if it is to become a widely-implemented arboviral biocontrol strategy. While the technique benefits from a low likelihood of resistance development in arboviruses and poses no predictable risks to human populations or the environment, it should not replace efforts for direct acting antiviral development or vaccine discovery. Despite being highly effective at preventing local outbreaks, a protected population can still be susceptible to cases imported from untreated areas (15). Moreover, while population modelling can predict the necessary parameters to enable permanent establishment of W+ mosquitos in a given area, these mathematical predictions could be confounded by unforeseen factors (16). Thus, re-releases may be necessary to prevent further outbreaks. However, until effective antivirals or vaccinations are available for wide usage, this technique could rapidly and successfully curtail outbreaks of numerous arboviruses.

CONCLUSIONS

Field releases of W+ mosquitos could become a promising strategy to curtail arboviral outbreaks. Accomplishing this goal first requires a thorough understanding of the molecular mechanisms used by *Wolbachia* to limit arboviral transmission in mosquito populations. Discovery of cytoplasmic incompatibility, mediated by the *cidA-cidB* operon, demonstrates how release of W+ males into mosquito reservoirs could act as a novel biocontrol strategy, potentially replacing insecticide use. Given that *Wolbachia* generates an inhospitable environment for viral replication within mosquitos, release of W+ males/females could induce vector incompetence in a native mosquito population. Understanding of these underlying mechanisms will better inform the safety, efficacy, and long-term impact of using this technique. One of the principal benefits of this method is its relative cost-effectiveness and ease of deployment, making it an optimal anti-arboviral strategy in developing countries where such diseases are endemic. Since arboviruses typically co-circulate within the same vector, *Wolbachia*-induced vector incompetence may also limit arboviral co-morbidities. Field releases have the potential to be effectively and reliably scaled across different geographical regions of varying climate, population density, and land area. Finally, the current literature suggests field release of W+ mosquitos will not harm human populations nor the environment. *Wolbachia* is non-pathogenic in humans, cannot be

transmitted to mosquito predators or other insects, and is incapable of surviving outside insect hosts. The risk of resistance development in mosquitos is also low, as evidenced by years of *Wolbacha*-mosquito co-evolution. Similarly, arboviral adaptations that allow replication within insects are unlikely to evolve, given that *Wolbachia* does not target a single viral effector to prevent replication, but induces a multifactorial response through mosquito immunostimulation and lipid dysregulation. While further experimentation is required to validate the efficacy of this technique in diverse geographical and sociopolitical environments, preliminary field releases have been very promising. Future research could allow broader implementation, including combating West Nile Virus outbreaks in Ontario and Quebec and Japanese Encephalitis Virus in East Asia. This technique could be used to curtail arboviral outbreaks while antivirals and vaccines are still being developed. Such therapeutics could then be used in conjunction with W+ mosquito releases to effectively alleviate the global arbovirus disease burden through a multifaceted approach.

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