Chapter 2
Field Trials of Gene Drive Mosquitoes: Lessons from Releases of Genetically Sterile Males and *Wolbachia*-infected Mosquitoes

John M. Marshall and Váleri N. Vásquez

Abstract The discovery of CRISPR-based gene editing and its application to homing-based gene drive has been greeted with excitement, for its potential to control mosquito-borne diseases on a wide scale, and concern, for the invasiveness and potential irreversibility of a release. At the same time, CRISPR-based gene editing has enabled a range of self-limiting gene drive systems to be engineered with much greater ease, including (1) threshold-dependent systems, which tend to spread only when introduced above a certain threshold population frequency, and (2) temporally self-limiting systems, which display transient drive activity before being eliminated by virtue of a fitness cost. As these CRISPR-based gene drive systems are yet to be field-tested, plenty of open questions remain to be addressed, and insights can be gained from precedents set by field trials of other novel genetics-based and biological control systems, such as trials of *Wolbachia*-transfected mosquitoes, intended for either population replacement or suppression, and trials of genetically sterile male mosquitoes, either using the RIDL system (release of insects carrying a dominant lethal gene) or irradiation. We discuss lessons learned from these field trials and implications for a phased exploration of gene drive technology, including homing-based gene drive, chromosomal translocations, and split gene drive as a system potentially suitable for an intermediate release.

Keywords Trial · Gene drive · Mosquitoes · Genetically sterile males · *Wolbachia*

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2.1 Introduction

The discovery of CRISPR and its application as a gene editing tool has enabled gene drive systems to be engineered with much greater ease (Doudna and Charpentier 2014; Champer et al. 2016). Recent attention has focused on homing-based drive systems and their potential to control mosquito-borne diseases on a wide scale, either by spreading disease-refractory genes (Gantz et al. 2015) or by spreading genes that confer a fitness load or sex bias thereby suppressing mosquito populations (Hammond et al. 2016; Kyrou et al. 2018). However, there is growing awareness of the invasiveness of homing-based drive systems (Noble et al. 2018) and interest in alternatives that could be confined to partially isolated populations and remediated—properties that are well aligned to the conduct of field trials (Marshall and Akbari 2018).

In addition to homing-based gene drive, the increased ease of gene editing has advanced the entire field of gene drive, including systems that, by design, limit their spread in space and time (Marshall and Akbari 2018). Such systems would ideally be capable of enacting local population control by: (a) effectively spreading into populations to the extent required to achieve the desired epidemiological or ecological effect and (b) being recallable from the environment in the event of unwanted consequences, public disfavor, or the end of a trial period. Two varieties of these systems have been recently engineered: (1) threshold-dependent systems that tend to spread when introduced above a certain population frequency (Akbari et al. 2013; Buchman et al. 2018) and (2) temporally self-limiting systems that display transient drive activity before being eliminated by virtue of a fitness cost (Gould et al. 2008; Li et al. 2020).

In this chapter, we discuss considerations for field trials of gene drive systems, with a specific focus on confinement and reversibility criteria, and lessons learned from other genetics-based and biological control systems (Table 2.1). We pay special attention to reciprocal chromosomal translocations (Buchman et al. 2018), as an example of a threshold-dependent system that is confineable and reversible, and then extend our consideration to CRISPR-based homing gene drive systems and temporally self-limiting systems, such as split gene drive (Li et al. 2020), which could be used as confineable and reversible intermediate systems in a development pathway of homing-based systems. While these gene drive systems are yet to be trialed in the wild, lessons can be learned from trials of several varieties of sterile male mosquitoes, specifically, those sterilized through radiation (sterile insect technique, SIT), transfection with *Wolbachia* (incompatible insect technique, IIT) (Zheng et al. 2019; Crawford et al. 2020), and release of insects carrying a dominant lethal (RIDL) gene (Harris et al. 2011; Carvalho et al. 2015), as well as releases of *Wolbachia*-infected mosquitoes for population replacement (Hoffmann et al. 2011). We begin by discussing trials of these systems and discuss threshold-dependent, self-limiting, and nonlocalized gene drive systems in this context.

Table 2.1 Genetics-based and biological mosquito control strategies and their potential to be confineable and reversible

Strategy	Variant	Mechanism of action	Confineable	Reversible
Sterile insect technique (SIT)	Ionizing radiation or chemosterilization	Offspring of released females and males are unviable	Yes	Yes
Wolbachia	Incompatible insect technique (IIT)	Offspring of released males are unviable	Yes, if no females released	Yes, if no females released
	Population replacement	Spreads through population due to cytoplasmic incompatibility	Yes, for moderate-to-high fitness costs	Possibly, for high fitness costs
Release of insects carrying a dominant lethal (RIDL) gene	Bisex RIDL (bi-RIDL)	Both female and male offspring having the RIDL allele are unviable	Yes	Yes
	Female-specific RIDL (fs-RIDL)	Only female offspring having the RIDL allele are unviable	Yes	Yes
Chromosomal translocations	CRISPR or other endonucleases	Translocation heterozygotes with unbalanced chromosome sets are unviable, leading to bistable dynamics	Yes	Yes
CRISPR-based gene drive	Homing-based drive systems	Bias inheritance by cleaving a target sequence and serving as a template for DNA repair, effectively turning a heterozygote into a homozygote	Potentially, but with difficulty	Potentially, but with difficulty
	Split gene drive systems	Components of drive system are split across two loci, leading to transient drive when they co-occur before being eliminated due to a fitness cost	Yes	Yes

2.2 Lessons from Releases of Genetically Sterile Male Insects

Releases of irradiated sterile male insects as a means of population suppression have been discussed since the early twentieth century (Klassen and Curtis 2005), and a transgenic version of this technology was the first transgenic mosquito product to be trialed in the field (Harris et al. 2011). As the first transgenic mosquito release, this intervention has come under high levels of scrutiny and serves as an important case study for potential releases of gene drive mosquitoes. The traditional SIT approach involves mass-rearing insects and applying a carefully calibrated amount of radiation such that their genetic material is mutated to render them sterile while still being able

to compete for female mates in the field (Knipling 1955). Upon release, sterile males (preferably the majority of released insects) seek out wild females, essentially wasting their reproductive potential as the females produce no or significantly less viable offspring. Consecutive releases over a sufficiently wide area result in less productive matings and a progressive reduction in insect population size over subsequent generations (Hendrichs and Robinson 2009).

The most widely celebrated application of SIT involved the use of ionizing radiation to eradicate the screwworm fly, *Cochliomyia hominivorax*, from North America—a program that began in 1957 following successful field trials on the Island of Curacao—and continues to this day to prevent reinvasion of the continent (Wyss 2000; Klassen and Curtis 2005). In this intervention, large-scale releases of sterilized insects led the screwworm fly population in the USA to crash within a decade. Subsequent releases progressively shifted the eradication zone southward, eventually covering all of North and Central America by 2001 (Robinson 2002).

The success of the screwworm SIT project motivated the application of SIT to a range of other insect pest species, including mosquito vectors of disease (Knipling 1968). Both irradiation and chemosterilization were initially explored for applications to mosquitoes, and in the 1960s and 1970s, large SIT field trials were conducted using chemosterilized *Culex quinquefasciatus* in India and *Anopheles albimanus* in El Salvador (Klassen and Curtis 2005). The trial in India was halted in the mid-1970s, following false accusations that the project was being used to collect data to engage in biological warfare (Nature 1975), highlighting the importance of effective community and political engagement for international biocontrol programs. Nevertheless, benefits of chemosterilization were demonstrated for this particular intervention due to reduced fitness costs as compared to irradiation.

A significant advancement in SIT technology was ushered with specific DNA changes introduced by the RIDL construct (Thomas et al. 2000; Alphey 2002). Insects sterilized through mutagens are subject to a myriad of random genetic mutations, which are invariably associated with significant fitness costs. In theory, releases of insects carrying (in homozygous form) a dominant lethal gene (RIDL) have essentially the same population impact as SIT—i.e., offspring of released males are unviable—although in a more controlled way that has potential for smaller associated fitness costs. Benedict and Robinson (2003) argued that a transgenic version of SIT should be the first application of transgenic mosquitoes in the wild (as it was), both for enhanced efficacy and for biosafety features—i.e., lethality-inducing transgenes should be quickly eliminated from the environment, causing the intervention to be reversible within a few generations. Quick elimination of transgenes also leads to confinement, since released mosquitoes can only travel so far in a few generations.

Sterile insect approaches based on genetic engineering present more opportunities than those based on mutagenesis, as genes and their associated traits can be modified in a more precise way. The original RIDL strain in *Aedes aegypti*, OX513A, causes lethality in both female and male offspring (bi-RIDL) (Thomas et al. 2000); however, an alternative construct was engineered soon after that only causes female offspring to be inviable (female-specific RIDL, or fs-RIDL). This allows the

population-suppressing trait to persist for a few more generations through the male line, while continuing to suppress the female population, which is effective since only female mosquitoes bite and transmit diseases to humans. Furthermore, the introduced trait is late acting, affecting the development of wing muscles in adult females (Fu et al. 2010). This has the benefit that viable population reduction is not seen until the adult stage, delaying the reduction in larval density and hence maintaining high larval mortality rates for longer due to density-dependent competition of larvae in breeding sites (Black et al. 2011).

The first field trials of *Ae. aegypti* mosquitoes having the RIDL construct were conducted using the OX513A strain in Malaysia and the Cayman Islands. In Malaysia, Oxitec Ltd. and the Institute for Medical Research, Malaysia, worked closely with the Malaysian government in conducting a risk assessment. Releases were carried out in an uninhabited area to assess the mortality and dispersal characteristics of released RIDL mosquitoes; however, negative reactions were encountered from nongovernmental organizations and the media, preventing a trial from being conducted in an inhabited area where the impact on wild *Ae. aegypti* populations could be assessed (Enserink 2011).

In the Cayman Islands, Oxitec Ltd. worked with the local Mosquito Research and Control Unit (MRCU), initially conducting smaller releases over the course of 4 weeks to assess the fitness of genetically modified (GM) sterile males relative to wild males and subsequently conducting a population suppression field trial over the course of several months, again using the OX513A *Ae. aegypti* strain. In a lab cage study, GM sterile males were found to be more or less of equal competitiveness in mating with wild females, and the lethality trait was found to be effective in all crosses between GM sterile males and wild females (Harris et al. 2011). Subsequent field releases over a 4-week period found that GM males successfully mated with wild females in the field and fertilized their eggs resulting in unviable offspring; however, the field competitiveness of the GM males was estimated at ~56% that of wild males, albeit with a very wide 95% confidence interval of 3.2–197% (Harris et al. 2011).

The subsequent suppression field trial in the Cayman Islands was carried out across three contiguous areas on Grand Cayman island (denoted areas A, B, and C) over a period of 23 weeks (Harris et al. 2012). The initial goal had been to achieve a 10:1 GM-to-wild male ratio by releasing across all three areas (55 ha in total); however, production limitations led the actual achieved ratio to be significantly less (~2:1 GM-to-wild males), and a subsequent release in areas A and B still only achieved a ratio of ~5:1 GM-to-wild males. The third phase of the release was carried out solely in area A, achieving a release ratio of ~25:1 GM-to-wild males and demonstrating the benefit of a smaller trial area. Another benefit of the area A release was that area C served as a control and area B served as a buffer region. Significant population reduction was seen in this phase, with an 80% reduction in the mean ovitrap index in area A relative to areas B and C over the last 7 weeks of the release period (Harris et al. 2012).

Releases of GM sterile males in the Cayman Islands faced some controversy (Nightingale 2010; Enserink 2010); however, the major criticisms concerned the

manner in which information about the trials was disseminated, rather than the conduct of the trials themselves. The releases did abide by national regulations, in particular, a draft biosafety bill that had yet to become law, the MRCU obtained a permit from the Cayman Islands Department of Agriculture, and a risk analysis and environmental impact assessment were carried out. The degree of community engagement was questioned; however, with several groups complaining, they had not been given details of the releases in advance (Enserink 2010).

Subsequent releases in Brazil followed a much more transparent approach. From the outset, a joint project was agreed, the Projeto Aedes Transgênico (PAT), between the University of São Paulo and Oxitec Ltd. to explore the potential use of GM sterile male *Ae. aegypti* as a form of urban mosquito control in terms of its social, technical, and operational dimensions. The project was launched by Moscamed, a Brazilian not-for-profit organization dependent on the Brazilian Ministry of Agriculture. The project enjoyed significant support in its early years as the government and public were aware of dengue outbreaks caused by this mosquito, and governmental support showed that they were being proactive in using the latest technology to control these outbreaks. The PAT worked closely with the Brazilian regulatory system to obtain required permits for field activities and adopted a vigorous community engagement campaign including school presentations, public events, interviews on TV and radio, house visits, and involvement of the community in trap monitoring and surveillance (de Campos et al. 2017).

The most well-documented trial of GM sterile male *Ae. aegypti* in Brazil was carried out in the Itaberaba suburb of the city of Juazeiro in Bahia, Brazil. This site had generally low socioeconomic indicators and relied on stored water to a large extent, providing breeding sites for mosquitoes and leading to relatively high dengue transmission. Similar to the Cayman Islands, the study area was divided into treatment areas A and B and a control area, with treatment eventually being restricted to area A in order to maintain sufficiently high release ratios. A Moscamed mass-rearing facility was built specifically for the project, producing millions of GM sterile males over the course of the study. Releases began with a "range finder" phase lasting a little over a month, which allowed the release requirements to be calibrated and estimates of parameters such as male mating competitiveness to be refined. GM male mating competitiveness was estimated to be ~3.1% that of wild males (95% CI: 2.5–3.6%), suggesting that releases for the "suppression" phase would need to be increased ninefold in order to achieve the target of 50% of mating events involving a GM sterile male (Carvalho et al. 2015).

The GM sterile male field trial in Brazil was successful, achieving a ~95% reduction in mosquito density at the release site, albeit with large release requirements of ~140,000 mosquitoes per week over a 5.5 ha control site for ~3 months (Carvalho et al. 2015). Enthusiasm for the GM sterile male approach was initially raised when the Zika outbreak began in 2015; however, an unexpected complication arose as untrue claims began to circulate in social media linking the Zika outbreak to past releases of the GM mosquitoes (de Campos et al. 2017). This draws attention to the importance of an enduring community engagement effort as well as political engagement and stakeholder messaging.

While this is not an invasive technology, these releases of sterile male mosquitoes do provide lessons from which potential field trials of gene drive mosquitoes may learn. Releases both of chemosterilized *Cx. quinquefasciatus* in India and of GM sterile *Ae. aegypti* in Brazil highlight the crucial importance of an effective and sustained community engagement effort. This especially applies to technologies developed in the Global North and applied in the Global South, which provide much potential for community mistrust. Furthermore, releases of GM sterile *Ae. aegypti* in both the Cayman Islands and Brazil highlight the importance of choosing a study site in which the required release sizes can be achieved and in conducting a range finder release phase to refine release requirements. For threshold-dependent gene drive systems, this will be important to determine release sizes that exceed the threshold, while for nonlocalized gene drive systems, this will be important to determine release sizes that are expected to demonstrate population control within the timeframe of the trial.

2.3 Lessons from the *Wolbachia*-based Incompatible Insect Technique

A promising alternative to SIT and GM sterile male releases is IIT, in which male mosquitoes are released that are infected with a *Wolbachia* strain absent from the wild population, resulting in sterile matings with wild females that lack the *Wolbachia* strain due to a phenomenon referred to as cytoplasmic incompatibility (CI) (LePage et al. 2017) (Fig. 2.1). This strategy has proceeded with much less resistance than GM approaches in recent years and serves as a case study for potential releases of novel biological control technologies, particularly regarding the use of factory rearing facilities (Zheng et al. 2019; Crawford et al. 2020).

The first field trial of IIT was conducted in Burma (now Myanmar) in 1967. The technique was seen as an alternative to insecticide-based strategies given the growing insecticide resistance among target species, *Cx. pipiens fatigans*, a vector of lymphatic filariasis (LF) which had proliferated in Southeast Asia at the time (Laven 1967). Despite successful elimination of the vector species from that trial site, the approach has not been deployed operationally until recently due to concern that accidental releases of *Wolbachia*-infected fertile females could result in the *Wolbachia* strain spreading into the population, preventing further suppression efforts. This is because *Wolbachia* is maternally inherited, and in most cases, the only incompatible crosses are between infected males and uninfected females. In 2009 and 2010, however, subsequent trials were carried out in French Polynesia to suppress populations of *Aedes polynesiensis*, a primary vector of LF in the South Pacific (O'Connor et al. 2012). Results from those field experiments showed that (1) *Wolbachia*-transfected *Ae. polynesiensis* males successfully competed for mates following release and (2) the trial did not result in population replacement eventuating.

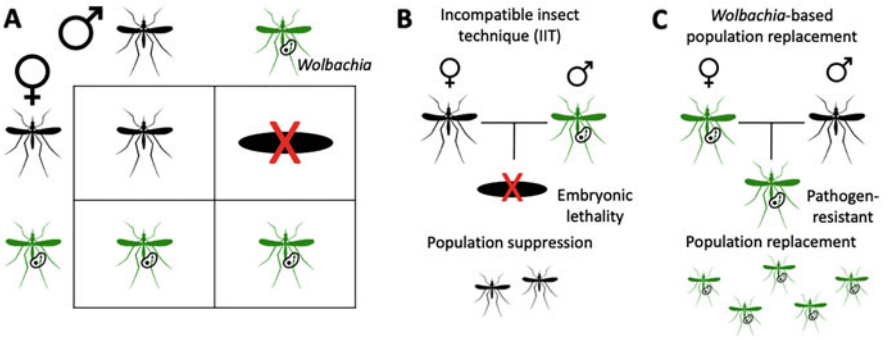

Fig. 2.1 (**a**) The use of *Wolbachia* as a means for both population suppression (incompatible insect technique, IIT) and population replacement hinges on the inheritance pattern in which crosses between *Wolbachia*-infected males and uninfected females produce unviable offspring due to cytoplasmic incompatibility (CI), while crosses involving *Wolbachia*-infected females produce *Wolbachia*-infected offspring due to *Wolbachia* being maternally inherited. (**b**) In IIT, *Wolbachia*-infected males are released into a wild population lacking that strain of *Wolbachia*. This leads to population suppression as mating events involving *Wolbachia*-infected males produce no viable offspring. (**c**) In *Wolbachia*-based population replacement, *Wolbachia*-infected females are included in the release. This leads to population replacement as CI biases inheritance in favor of *Wolbachia* when *Wolbachia*-infected females are present

In the last few years, two factory-scale IIT projects have moved forward to achieve community-scale mosquito population suppression: (1) an IIT program supplemented with sterilizing irradiation (also termed IIT-SIT) in Guangzhou, China (Zheng et al. 2019), and (2) an IIT program supplemented with factory-scale automation of production and sex sorting in Fresno, California (Crawford et al. 2020). The two projects represent different approaches to prevent population replacement: (1) through greatly reducing the fertility of any *Wolbachia*-infected females that may be accidentally released and (2) through using automation and machine learning to reduce the number of accidentally released *Wolbachia*-infected females effectively to zero.

In the IIT-SIT program in Guangzhou, *Aedes albopictus*, the main vector of dengue and other arboviruses in Guangzhou, were generated having an artificial triple *Wolbachia* infection (termed HC), through the addition of the *w*Pip *Wolbachia* strain to the native double infection of the *w*AlbA and *w*AlbB strains of *Wolbachia*. High levels of CI were confirmed such that matings of HC males with wild females produced no viable offspring and maternal transmission of the triple *Wolbachia* infection was confirmed, allowing efficient mass production of HC males. HC males were exposed to low-dose irradiation at the pupal stage to reduce the fecundity of any accidentally released HC females, and semi-field cage studies confirmed that the irradiated HC males effectively competed for mates leading to population suppression, without population replacement occurring due to released HC females. Furthermore, as an additional safety precaution, HC females were shown to be less competent at disease transmission than their wild counterparts (Zheng et al. 2019).

A trial carried out by the Wolbaki Biotech Company in 2016–2017 demonstrated the high degree of population suppression possible when factory rearing of mosquitoes is involved. Irradiated HC males were released on a weekly basis on two riverine islands within the jurisdiction of Guangzhou, with the ratio of released HC to wild males varying between 8.7:1 and 15.8:1 over the 38-week intervention period. Population suppression was highly successful, achieving a >94% reduction in the number of hatched eggs per ovitrap, as compared to control sites, and an 83–94% reduction in the number of wild adult females caught per trap. The success of the program also led to a significant increase in community support, with interviews suggesting 13% of residents were supportive prior to the intervention (notably, with 76% being neutral) and 54% were supportive following the intervention (Zheng et al. 2019).

The IIT program in Fresno, CA, showcased the role that large-scale, automated rearing and sex sorting of mosquitoes can play in increasing the scale of an IIT intervention. In this case, *Ae. aegypti*, the main arboviral vector through much of the Americas, was transfected with the *w*AlbB strain of *Wolbachia*, and sterility of crosses between infected males and wild females was confirmed. An automated larval rearing system was designed that, at maximum capacity, was able to produce almost 3 million pupae per week. A multistep sex-separation process was then designed that removed 95% of females at the pupal stage and the remainder at the adult stage based on a machine learning algorithm informed by photographic images as emerging adults walked down a narrow path. Estimates from the operation of this system suggested that a single *Wolbachia*-infected female mosquito would be released for every 900 million males, making the sex-sorting system near perfect (Crawford et al. 2020).

A trial carried out through a partnership between the Debug Project of Verily Life Sciences, MosquitoMate, and the Consolidated Mosquito Abatement District of Fresno County in 2018 demonstrated dramatic population suppression over an area nine times larger than that of the Guangzhou study. A total of more than 14 million *Wolbachia*-infected males were released as part of the study (an average of more than 78,000 per day), which led to a 96% reduction in the wild adult mosquito population; however, despite the large size of the releases, elimination was not achieved, likely due to inward migration of wild mosquitoes from neighboring untreated areas (Crawford et al. 2020). A public information campaign was conducted around the trial; however, formal documentation of this campaign is not yet available. A similar project is currently underway in Singapore through a partnership between Verily Life Sciences and the National Environment Agency of Singapore.

While neither a transgenic nor invasive technology, these IIT releases do provide lessons regarding the scale of releases that can be achieved when investment is made into automated rearing and sex-sorting facilities. Release requirements for low-threshold gene drive mosquitoes will be orders of magnitude lower than those for sterile male releases, and hence a facility capable of producing tens of millions of mosquitoes, such as the one designed by Verily Life Sciences, would be capable of achieving control over a much greater spatial scale than for IIT. The technological

capacity for sex sorting is also encouraging given that male mosquitoes don't bite or transmit diseases to humans and hence may also be preferable for gene drive mosquito releases. The IIT releases enjoyed much less resistance from communities and regulatory agencies than GM sterile male releases, despite acting through a similar mechanism, highlighting issues that trials of gene drive mosquitoes will likely also face and must invest in.

2.4 Lessons from *Wolbachia*-based Population Replacement

A second approach to the use of *Wolbachia* to control mosquito-borne disease transmission is to intentionally include *Wolbachia*-infected females in a release. In IIT, care is taken to only release *Wolbachia*-infected males, as CI causes matings between *Wolbachia*-infected males and wild females to be sterile; however, CI-induced sterility, combined with the fact that *Wolbachia* is maternally inherited, provides an inheritance bias in favor of *Wolbachia* when *Wolbachia*-infected females are also present (Turelli and Hoffmann 1991) (Fig. 2.1). For *Wolbachia* strains that also block pathogen transmission, this can be used to drive the pathogen-blocking trait into the mosquito population (Moreira et al. 2009). This strategy has advanced significantly over the last decade (Hoffmann et al. 2011) and, like IIT, has faced much less resistance than GM strategies. It serves as an interesting case study for potential releases of transgenic population replacement technologies, as it has faced many of the non-GM issues that future gene drive programs will face.

The first *Wolbachia* population replacement program was carried out by the Eliminate Dengue project (now known as the World Mosquito Program) in the communities of Yorkeys Knob and Gordonvale in Queensland, Australia (Hoffmann et al. 2011). In this program, *Ae. aegypti*, the main vector of dengue and other arboviruses in Queensland, was transfected with the *w*Mel strain of *Wolbachia* from *Drosophila melanogaster*, a strain that has been shown to (1) block dengue transmission, (2) have a small associated fitness cost, and (3) be capable of driving into a small field cage (Walker et al. 2011). *Wolbachia* displays threshold properties in the presence of a fitness cost such that releases above a certain population frequency tend to spread, while releases below that frequency tend to be eliminated. The exact value of the threshold is determined by the point at which the inheritance bias induced by CI outweighs the fitness cost associated with the infection and has been estimated at ~20–30% for the *Wolbachia* strain used in this release (Hoffmann et al. 2011; Hancock et al. 2019).

The releases in Yorkeys Knob and Gordonvale were a clear success—after 10 weekly releases of 11,000–22,000 *Wolbachia*-infected *Ae. aegypti* per week, the *Wolbachia* infection reached near fixation in both populations within 3 months, despite a tropical storm postponing one of the releases in Gordonvale (Hoffmann et al. 2011) (Fig. 2.2). The finer details of this program provide an excellent example of how gene drive systems may be successfully trialed in the future. To begin, they highlight the importance of a detailed monitoring effort and adaptive release

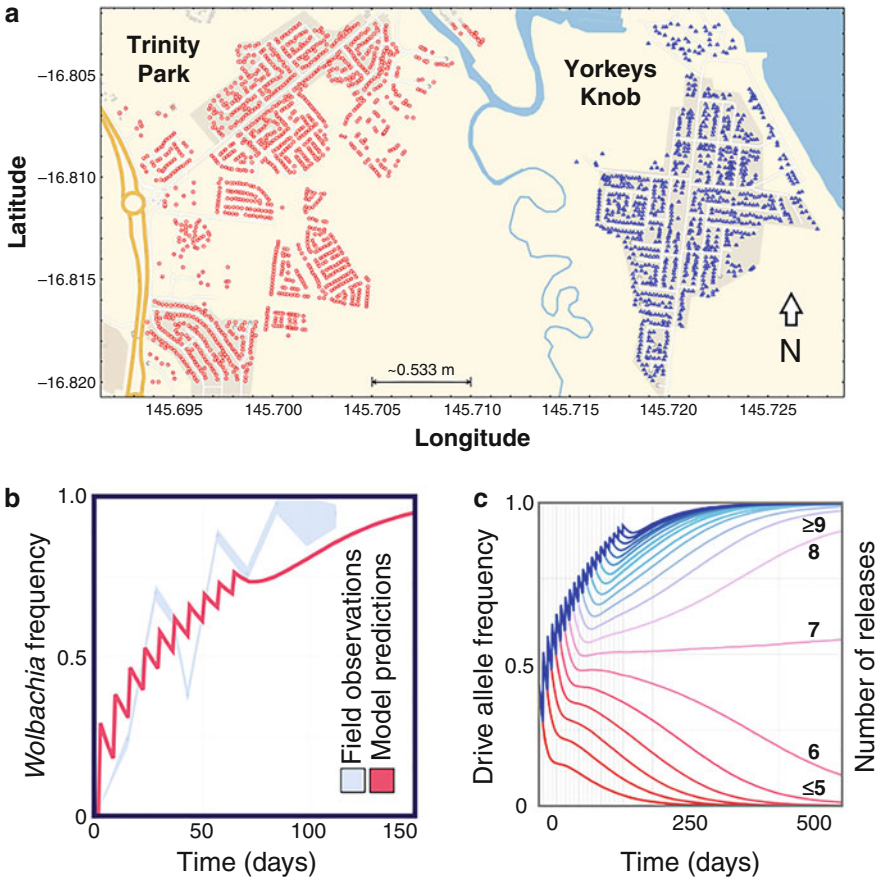

Fig. 2.2 (**a**) Landscape of Yorkeys Knob and Trinity Park in Queensland, Australia, where field trials of *Wolbachia*-based population replacement for *Aedes aegypti* were carried out and where trials of reciprocal chromosomal translocations were simulated. (**b**) Blue lines depict data for *Wolbachia* frequency over time from the *Wolbachia* population replacement field trial conducted in Yorkeys Knob in 2011 (Hoffmann et al. 2011), with line thickness representing 95% binomial confidence intervals around observed proportions. Red lines depict simulated data for an analogous release scheme consisting of 20 *Wolbachia*-infected mosquitoes per household at a coverage of 30% over 10 weeks, demonstrating good agreement with field data (Sánchez et al. 2020). (**c**) Translocation frequency over time for a given number of weekly releases of 20 adult male *Ae. aegypti* mosquitoes homozygous for the translocation per household with the intent of population replacement in Yorkeys Knob. Results are depicted for a coverage of 50%, at which seven or more releases result in the translocation being driven into the population (Sánchez et al. 2020). Due to the 50% threshold property of translocations, the same release scheme for wild types can be used to remediate translocations from the population

protocol. The releases in Yorkeys Knob and Gordonvale were accompanied by a network of 29 Biogents Sentinel mosquito traps that monitored *Wolbachia* infection frequency at the block level. Heterogeneity in *Wolbachia* infection frequency was

observed, and releases were supplemented in areas where *Wolbachia* frequency was low.

Monitoring for unintended spread outside the study area was also conducted, and this did indeed reveal limited long-distance spread into a neighboring suburb from Yorkeys Knob and across a freeway from Gordonvale (Hoffmann et al. 2011). Although these migrants were expected to be lost due to being present at subthreshold levels, continued monitoring was important to confirm this. Continued monitoring was also conducted at the trials sites to confirm enduring intervention efficacy, and while the *Wolbachia* infection remained at near fixation for several years following the release, a low frequency of uninfected mosquitoes has also persisted, likely due to immigration (Hoffmann et al. 2014).

The releases in Yorkeys Knob and Gordonvale also highlight the importance of preparing for unexpected events. In addition to the tropical storm that affected both release sites and postponed one of the releases in Gordonvale, releases in a portion of Yorkeys Knob ceased two-thirds of the way into the intervention following a reported dengue case (Hoffmann et al. 2011). Although this dengue case likely originated elsewhere, a reactive insecticide intervention was carried out in surrounding households in agreement with local disease control protocols. Trials of mosquitoes with gene drive systems should make allowances for events such as these. Encouragingly, the *Wolbachia* infection continued to spread through the Yorkeys Knob *Ae. aegypti* population despite this, and no secondary dengue cases were documented following the reported case.

The Yorkeys Knob and Gordonvale releases provide an example of a successful community and regulatory engagement process. Community engagement was carried out over 2 years leading up to the releases and consisted of informal interviews, semi-structured in-depth interviews, qualitative and quantitative surveys, focus groups, historical research, and face-to-face presentations at community meetings (Hoffmann et al. 2011; McNaughton 2012). Issues explored through these activities included the sociopolitical context, lay knowledge of dengue fever and biological control programs, and acceptability of the project. Community members did raise concerns about a previous local biological control program—the introduction of the cane toad near Gordonvale in the 1930s. Largely seen as a failed biological control program, this was raised as a cautionary tale indicating the limits of scientific knowledge and the unpredictability of ecological interventions (McNaughton 2012).

The Queensland releases enjoyed substantial community support, with 85% of respondents viewing *Wolbachia* as an acceptable dengue prevention strategy in a March 2010 telephone survey (ahead of insecticides, at 66% acceptance) and 84% of respondents stating they would support a release that they were informed and updated about, that had regulatory oversight, and that was shown to be safe for people and the environment by a risk assessment carried out by Australia's Commonwealth Scientific and Industrial Research Organisation (CSIRO) (McNaughton 2012). The releases were ultimately approved by the Australian Pesticides and Veterinary Medicines Authority (APVMA) following risk assessments by CSIRO (Murphy et al. 2010) and the APVMA with support from the Federal Commonwealth Government's Department of the Environment, Water, Heritage and the Arts

(Marshall 2011). The World Mosquito Program is now exploring application of their technology beyond Australia, with active collaborations throughout Latin America, Asia, and Oceania.

In summary, key lessons from the *Wolbachia*-based population replacement strategy include the importance of (1) a detailed monitoring protocol to assess heterogeneity of spread at the field site, (2) an adaptive release scheme to supplement releases in areas of low *Wolbachia* frequency, (3) additional monitoring to assess levels of unintended spread to neighboring areas, and (4) preparing for unexpected events. The fact that *Wolbachia* infection has the potential to persist in the mosquito population for extended periods, and perhaps indefinitely, also emphasizes the need for a long-term, comprehensive, and multifaceted community engagement program.

2.5 Considerations for Trials with Reciprocal Chromosomal Translocations

Lessons from field trials of *Wolbachia*-based population replacement systems apply most closely to threshold-dependent gene drive systems, which are also expected to spread if released above a certain threshold frequency and to be eliminated if present below that frequency. One of the first of these systems to be proposed (Serebrovskii 1940; Curtis 1968), and perhaps currently one of the most promising (Sánchez et al. 2020), is reciprocal chromosomal translocations. These result from a mutual exchange between terminal segments of two nonhomologous chromosomes and produce a heterozygote reproductive disadvantage because, when translocation heterozygotes mate, several crosses result in unbalanced genotypes and hence unviable offspring. This produces a threshold frequency of 50%, which increases in the presence of a fitness cost (Curtis 1968). Early attempts to generate translocations through radiation-induced mutagenesis were abandoned due to high associated fitness costs (Laven et al. 1972; Lorimer et al. 1972); however, interest has been reignited as site-specific translocations have recently been generated using CRISPR (Lekomtsev et al. 2016; Jiang et al. 2016), and translocations generated in *D. melanogaster* using endonucleases were recently shown to drive in laboratory experiments with a threshold frequency of ~50% (Buchman et al. 2018).

A recent modeling study suggests that translocations represent one of the best systems to implement in field trials due to their symmetrical threshold properties and strong confinement potential. A key advantage of translocations is that releases required to introduce them into a population are of a similar magnitude to wild-type releases required to eliminate them once they have been introduced (Sánchez et al. 2020). Population replacement and reversion were modeled at the household level in the suburb of Yorkeys Knob, the site of the *Wolbachia* population replacement study, with low levels of migration modeled to the neighboring suburb of Trinity Park in Queensland, Australia (Fig. 2.2). Population replacement could be achieved in simulations with seven or more weekly releases of 20 *Ae. aegypti* males

homozygous for the translocations per household per week (a similar magnitude to that used in the *Wolbachia* population replacement trial at the same site) and for a coverage of 50% of the households in the community. Elimination could be achieved for the same release scheme using wild *Ae. aegypti* mosquitoes.

One benefit of translocations, and other underdominant systems that have a threshold in the absence of a fitness cost, is that their release threshold is more robust than that for *Wolbachia*, which only arises in the presence of a fitness cost. This property leads to translocations being more robustly confineable to a field site than a *Wolbachia* infection, since they are unlikely to exceed the release threshold in a neighboring population purely through migration, even if they spread to near fixation at the trial site. In the translocation modeling study in Yorkeys Knob and Trinity Park (Sánchez et al. 2020), it was considered unlikely that *Ae. aegypti* mosquitoes would travel from one suburb to another by their own flight, especially in numbers sufficient to exceed the release threshold there, and so "batch migration" was instead considered, in which several mosquitoes are carried, perhaps by a vehicle, from one suburb to another at once. Batch migration events were modeled as occurring between randomly chosen neighborhoods, and the number of daily migration events and effective number of adults carried per event were varied. Results from this modeling study made a strong case for the potential to confine translocations to the release site, as the number of daily migration events required for the translocation to exceed the threshold in the neighboring suburb exceeded those inferred from field data. Specifically, 3–4 daily migration events consisting of batches of ten adults were required for translocations to spillover to the neighboring suburb in simulations (Sánchez et al. 2020), while field data suggested 1–2 daily migration events consisting of batches of less than five adult females (Hoffmann et al. 2011).

Collectively, these modeling results for translocations are encouraging for the potential to conduct field trials of *Ae. aegypti* mosquitoes with translocations because (1) translocations could be introduced on a suburban scale and remediated through releases of non-disease-transmitting male mosquitoes with release sizes on the scale of what has been previously implemented and (2) spillover of translocations into neighboring suburbs is unlikely. Lessons for the conduct of field trials with translocations may be drawn from the field trials previously described in this chapter—most importantly, for *Wolbachia*-based population replacement. These lessons highlight the importance of a detailed monitoring effort, including outside the study area, and of an adaptive release protocol that can respond to heterogeneities in spread at the trial site. They also highlight the importance of preparing for unexpected events and for conducting a long-term and comprehensive community engagement program, given that translocations have the potential to persist in the environment long term. A comparison of the RIDL and IIT releases suggests that community engagement and regulatory requirements for translocations may be stricter than for those for *Wolbachia* due to the fact that mosquitoes with translocations, generated using CRISPR or other endonucleases, will be considered GM organisms. Finally, regarding the release protocol, including a range finder release

phase may help to refine fitness cost estimates and release requirements for translocations, as per a lesson from the RIDL field trial in Brazil.

2.6 Considerations for Trials with CRISPR-based Gene Drive Systems

Finally, lessons from the field trials discussed here have implications for the spectrum of CRISPR-based gene drives, from those that are nonlocalized to those that are self-limiting. Recent attention has focused on CRISPR-based homing gene drives, for their ability to spread widely and their potential to control vector-borne diseases on a wide scale (Gantz et al. 2015; Kyrou et al. 2018); however, there are also threshold-dependent gene drive systems that can now be engineered using CRISPR, such as chromosomal translocations (Buchman et al. 2018) and various forms of underdominance (Akbari et al. 2013), as well as temporally self-limiting gene drive systems, such as split drive (Li et al. 2020), which display transient drive activity before being eliminated by virtue of a fitness cost. The CRISPR revolution has also enabled gene drive countermeasures to be engineered, such as homing-based drive remediation systems, ERACR (element for the reversal of the autocatalytic chain reaction) and e-CHACR (erasing construct hitchhiking on the autocatalytic chain reaction) (Gantz and Bier 2016; Xu et al. 2020).

CRISPR-based homing gene drive systems bias inheritance in their favor by cleaving a highly specific target sequence in the host genome and copying themselves to the cut chromosome through a mechanism known as homology-directed repair (Gantz and Bier 2015; Champer et al. 2016). For high homing efficiencies and low-to-moderate fitness costs, these systems are capable of driving into populations from arbitrarily low initial frequencies. This property allows them to spread widely, and hence they are considered "nonlocalized." For these gene drive systems, while we may learn from field trials of *Wolbachia*-based population replacement systems, the scale of their potential spread and impact leads to additional and unique challenges that we must carefully consider.

One way to manage the risks associated with the potential wide-scale spread of homing-based gene drive systems is for testing to proceed iteratively through multiple phases, with each phase involving a larger spatial scale and a higher degree of human or environmental exposure (James et al. 2018) (Fig. 2.3). In this phased release pathway, initial studies are to be conducted in contained laboratories and

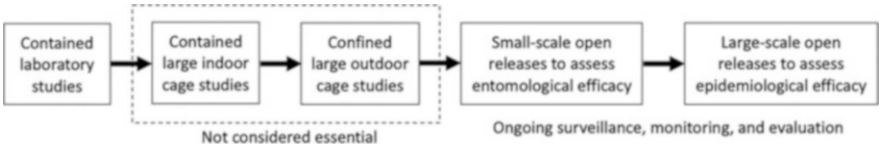

Fig. 2.3 Phased release pathway for CRISPR-based homing gene drive systems

insectaries, where product efficacy and safety are studied. Entering field-testing is a big decision, given the anticipated difficulty of remediating a homing-based gene drive system that is capable of spreading widely. Large outdoor cages present one option for moving beyond the laboratory; however, this is not considered essential since some mosquito behaviors, such as mating, and parameters, such as fitness, can only be meaningfully studied in the field. Furthermore, studies in outdoor cages must anticipate the possibility of escape occurring, and hence similar safety and efficacy criteria must be met before either outdoor cage studies or small-scale isolated releases are performed. Initial outdoor testing should be conducted at field sites within which the gene drive system is expected to be contained, for instance, on oceanic islands, following which open releases would be conducted on iteratively larger spatial scales (James et al. 2018).

Another consideration for trials of nonlocalized gene drive systems is that regulators are likely to require that a remediation plan be in place prior to field-testing (James et al. 2018). The chosen remediation strategy will depend on a number of factors, including the mode of action of the drive system and the scale and geography of the field site. A default remediation plan would be a large-scale insecticide-based campaign to eliminate the vector population at the field site. This would require an assessment of insecticide resistance in the local vector population prior to the gene drive trial. Failing this, releases of non-disease-transmitting male mosquitoes carrying a drive-resistant allele that restores the function of the gene targeted by the drive system are an attractive option, especially if the drive-resistant allele is sourced from a wild population.

Gene drive countermeasures such as ERACR and e-CHACR are another option for remediation. The ERACR system consists of a second homing system with a target site corresponding to the original drive system, essentially removing the original system as it homes into it, while utilizing the Cas9 of the original system and thus removing that as well (Gantz and Bier 2016; Xu et al. 2020). The e-CHACR system uses the Cas9 from the original homing system to home into a second site in the genome in addition to the site of the original drive system, thus driving itself into the population while removing the original system and its Cas9 in the process (Gantz and Bier 2016, Xu et al. 2020). Both of these systems hold promise; but they may not be the first choice for remediation efforts as they introduce additional transgenes into populations from which transgenes are trying to be removed.

Another potential phased release pathway is to precede the release of a nonlocalized gene drive system with a self-limiting one. Ideally, such a release would provide insights into the expected behavior of the nonlocalized system, and hence there should be strong resemblance between the two systems, to the extent possible. For a CRISPR-based homing gene drive system, one possibility is to begin with a trial of a split drive system, in which the Cas9 and guide RNA components are separated at different loci (Li et al. 2020). In the split drive system, transient drive activity occurs at the guide RNA locus when the Cas9 and guide RNA alleles co-occur in an organism; however, the Cas9 allele is gradually eliminated from the population due to its fitness cost, followed by the guide RNA if it also has a fitness cost. This transient drive activity also leads to spatial confinement, since a gene can

only disperse so far in a limited number of generations. Intermediate technologies also exist for other systems. For instance, a driving Y chromosome that spreads by cleaving the X chromosome at multiple sites during spermatogenesis is expected to spread on a wide scale (Galizi et al. 2014); however, if linked to an autosome, it is self-limiting, providing an opportunity for intermediate study in the field.

For self-limiting CRISPR-based gene drive systems that could be used as an intermediate system in a field trial, similar field trial considerations apply as for chromosomal translocations. Namely, the ability to confine the release to the trial site, and to remediate transgenes from the environment as needed, is a great strength. Furthermore, it is important to combine a detailed monitoring effort, both in and outside the trial site, with an adaptive release protocol to respond to heterogeneities in spread, and to make allowances for unexpected events. A range finder release phase may help to refine fitness cost estimates and release requirements.

For nonlocalized CRISPR-based gene drive systems, the potentially wide scale of spread and difficulty of remediation emphasize the need to monitor for the gene drive system both in and outside the field trial area. Additionally, a range finder release phase may help to predict release schemes capable of achieving population control within the desired timeframe. Finally, as the spatial scale of the release grows, lessons may be learned from the experience of the Fresno IIT trial regarding automated rearing and sex sorting of mosquitoes. Knowledge of the potential scale of mosquito production will allow us to set expectations for wide-scale vector-borne disease control.

As for all of the systems discussed in this chapter, effective community and regulatory engagement is essential prior to field trials of mosquitoes engineered with CRISPR-based gene drive systems; however, this is especially important for trials of nonlocalized gene drive systems. Mosquitoes engineered with these systems are GM organisms capable of spreading widely, potentially across international borders, and are often developed in the Global North for application in the Global South. Their potential to spread across international borders highlights the desirability of a multicountry or regional agreement on their release, especially when a country that shares a border with another is being considered for field trials. Indeed, such agreements may be required by the Cartagena Protocol on Biosafety, which governs the safe transfer, handling, and use of GM organisms (referred to as "living modified organisms" in the protocol), including their transboundary spread (Secretariat of the Convention on Biological Diversity 2000; Marshall 2010).

2.7 Conclusion

The limitations of traditional insecticide-based strategies to control mosquito populations, and, in particular, the widespread emergence of insecticide resistance, have spurred interest in a variety of novel biological and genetics-based vector control strategies, including SIT, IIT, RIDL, *Wolbachia*-based population replacement, and CRISPR-based gene drive (Benelli et al. 2016). Trials of RIDL, IIT, and

Table 2.2 Significant field trials of novel biological and genetics-based mosquito control strategies

Method	Species	Location	Year	Outcome	Reference
SIT	*Anopheles quadrimaculatus*	Florida, USA	1962	Poor mating competitiveness	Weidhaas et al. (1962)
IIT	*Culex pipiens fatigans*	Burma (now Myanmar)	1967	Successful suppression	Laven (1967)
SIT	*Culex quinquefasciatus*	India	1971–1975	Modest suppression	Singh et al. (1975)
SIT	*Anopheles albimanus*	El Salvador	1971–1979	Significant suppression	Lowe et al. (1980)
RIDL	*Aedes aegypti*	Cayman Islands	2009	Small-scale suppression	Harris et al. (2011, 2012)
IIT	*Aedes polynesiensis*	French Polynesia	2009–2010	Demonstration of efficacy	O'Connor et al. (2012)
Wolbachia population replacement	*Ae. aegypti*	Queensland, Australia	2011	Successful population replacement	Hoffmann et al. (2011)
RIDL	*Ae. aegypti*	Juazeiro, Brazil	2012–2013	Community-scale suppression	Carvalho et al. (2015)
RIDL	*Ae. aegypti*	Jacobina, Brazil	2013	Suppression and resurgence	Garziera et al. (2017)
IIT	*Aedes albopictus*	Kentucky, USA	2014	Significant suppression	Mains et al. (2016)
IIT-SIT	*Ae. albopictus*	Guangzhou, China	2016–2018	Community-scale suppression	Zheng et al. (2019)
IIT	*Ae. aegypti*	California, USA	2018–2019	Community-scale suppression	Crawford et al. (2020)

Wolbachia over the last decade provide a series of case studies from which we may learn in preparing for field trials of CRISPR-based gene drive systems (Table 2.2).

There are challenges associated with gene drive technologies—notably, the controversies surrounding GM organisms and the potential for spread across international borders. However, these challenges are also a reason for promise as half of the world's population is at risk of vector-borne diseases, and genetic engineering provides new opportunities to interfere with pathogen transmission. In learning from recent field trials, we seek to move these technologies forward carefully and responsibly toward the eventual goal of global vector-borne disease control.

References

Akbari OS, Matzen KD, Marshall JM, Huang H, Ward CM et al (2013) A synthetic gene drive system for local, reversible modification and suppression of insect populations. Curr Biol 23:671–677

Alphey L (2002) Re-engineering the sterile insect technique. Insect Biochem Mol Biol 32:1243–1247

Benedict MQ, Robinson AS (2003) The first releases of transgenic mosquitoes: an argument for the sterile insect technique. Trends Parasitol 19:349–355

Benelli G, Jeffries CL, Walker T (2016) Biological control of mosquito vectors: past, present and future. Insects 7:52

Black WC, Alphey L, James AA (2011) Why RIDL is not SIT. Trends Parasitol 27:362–370

Buchman A, Ivy T, Marshall JM, Akbari OS, Hay BA (2018) Engineered reciprocal chromosome translocations drive high threshold, reversible population replacement in Drosophila. ACS Synth Biol 7:1359–1370

Carvalho DO, McKemey AR, Garziera L, Lacroix R, Donnelly CA et al (2015) Suppression of a field population of *Aedes aegypti* in Brazil by sustained release of transgenic male mosquitoes. PLoS Negl Trop Dis 9:e0003864

Champer J, Buchman A, Akbari OS (2016) Cheating evolution: engineering gene drives to manipulate the fate of wild populations. Nat Rev Genet 17:146–159

Crawford JE, Clarke DW, Criswell V, Desnoyer M, Cornel D et al (2020) Efficient production of male *Wolbachia*-infected *Aedes aegypti* mosquitoes enables large-scale suppression of wild mosquitoes. Nat Biotechnol 38:482–492

Curtis CF (1968) Possible use of translocations to fix desirable genes in insect pest populations. Nature 218:368–369

de Campos A, Hartley S, de Koning C, Lezaun J, Velho L (2017) Responsible innovation and political accountability: genetically modified mosquitoes in Brazil. J Respons Innov 4:5–23

Doudna JA, Charpentier E (2014) Genome editing. The new frontier of genome engineering with CRISPR-Cas9. Science 346:1258096

Enserink M (2010) GM mosquito trial alarms opponents, strains ties in Gates-funded project. Science 330:1030–1031

Enserink M (2011) GM mosquito release in Malaysia surprises opponents and scientists—again. Science Insider. Available online at: https://www.sciencemag.org/news/2011/01/gm-mosquito-release-malaysia-surprises-opponents-and-scientists-again

Fu G, Lees RS, Nimmo D, Aw D, Jin L et al (2010) Female-specific flightless phenotype for mosquito control. Proc Natl Acad Sci U S A 107:4550–4554

Galizi R, Doyle LA, Menichelli M, Bernardini F, Deredec A et al (2014) A synthetic sex ratio distortion system for the control of the human malaria mosquito. Nat Commun 5:3977

Gantz VM, Bier E (2015) The mutagenic chain reaction: a method for converting heterozygous to homozygous mutations. Science 348:442–444

Gantz VM, Bier E (2016) The dawn of active genetics. BioEssays 38:50–63

Gantz VM, Jasinskiene N, Tatarenkova O, Fazekas A, Macias VM et al (2015) Highly efficient Cas9-mediated gene drive for population modification of the malaria vector mosquito *Anopheles stephensi*. Proc Natl Acad Sci U S A 112:E6736–E6743

Garziera L, Pedrosa MC, de Souza FA, Gómez M, Moreira MB et al (2017) Effect of interruption of over-flooding releases of transgenic mosquitoes over wild populations of *Aedes aegypti*: two case studies in Brazil. Entomol Exp Appl 164:327–339

Gould F, Huang Y, Legros M, Lloyd AL (2008) A killer-rescue system for self-limiting gene drive of anti-pathogen constructs. Proc Biol Sci 275:2823–2829

Hammond A, Galizi R, Kyrou K, Simoni A, Siniscalchi C et al (2016) A CRISPR-Cas9 gene drive system targeting female reproduction in the malaria mosquito vector *Anopheles gambiae*. Nat Biotechnol 34:78–83

Hancock PA, Ritchie SA, Koenraadt CJM, Scott TW, Hoffmann AA et al (2019) Predicting the spatial dynamics of *Wolbachia* infections in *Aedes aegypti* arbovirus vector populations in heterogeneous landscapes. J Appl Ecol 56:1674–1686

Harris AF, Nimmo DD, McKemey AR, Kelly N, Scaife S et al (2011) Field performance of engineered male mosquitoes. Nat Biotechnol 29:1034–1037

Harris AF, McKemey AR, Nimmo DD, Curtis Z, Black I et al (2012) Successful suppression of a field mosquito population by sustained release of engineered male mosquitoes. Nat Biotechnol 30:828–830

Hendrichs J, Robinson A (2009) Sterile insect technique. In: Resh VH, Carde RT (eds) Encyclopedia of insects, 2nd edn. Academic Press, London

Hoffmann AA, Montgomery BL, Popovici J, Iturbe-Ormaetxe I, Johnson PH et al (2011) Successful establishment of *Wolbachia* in *Aedes* populations to suppress dengue transmission. Nature 476:454–457

Hoffmann AA et al (2014) Stability of the wMel *Wolbachia* infection following invasion into *Aedes aegypti* populations. PLoS Negl Trop Dis 8:e3115

James S, Collins FH, Welkhoff PA, Emerson C, Godfray HCJ et al (2018) Pathway to deployment of gene drive mosquitoes as a potential biocontrol tool for elimination of malaria in sub-Saharan Africa: recommendations of a scientific working group. Am J Trop Med Hyg 98:1–49

Jiang J, Zhang L, Zhou X, Chen X, Huang G et al (2016) Induction of site-specific chromosomal translocations in embryonic stem cells by CRISPR/Cas9. Sci Rep 6:21918

Klassen W, Curtis CF (2005) History of the sterile insect technique. In: Dyck VA, Hendrichs J (eds) Sterile insect technique: principles and practice in area-wide integrated pest management. IAEA, Geneva, pp 3–36

Knipling EF (1955) Possibilities of insect control or eradication through the use of sexually sterile males. J Econ Entomol 48:459–462

Knipling EF (1968) The potential role of sterility for pest control. In: LeBrecque GC, Smith CN (eds) Principles of insect chemosterilization. Appleton-Century-Crofts, New York, pp 7–40

Kyrou K, Hammond AM, Galizi R, Kranjc N, Burt A et al (2018) A CRISPR-Cas9 gene drive targeting doublesex causes complete population suppression in caged Anopheles gambiae mosquitoes. Nat Biotechnol 36:1062–1066

Laven H (1967) Eradication of *Culex pipiens fatigans* through cytoplasmic incompatibility. Nature 216:383–384

Laven H, Cousserans J, Guille G (1972) Eradicating mosquitoes using translocations: a first field experiment. Nature 236:456–457

Lekomtsev S, Aligianni S, Lapao A, Bürckstümmer T (2016) Efficient generation and reversion of chromosomal translocations using CRISPR/Cas technology. BMC Genomics 17:739

LePage DP, Metcalf JA, Bordenstein SR, On J, Perlmutter JI et al (2017) Prophage WO genes recapitulate and enhance *Wolbachia*-induced cytoplasmic incompatibility. Nature 543:243–247

Li M, Yang T, Kandul NP, Bui M, Gamez S, Raban R, Bennett JB, Sánchez HM, Lanzaro GC, Schmidt H, Lee Y, Marshall JM, Akbari OS (2020) Development of a confinable gene-drive system in the human disease vector, *Aedes aegypti*. eLife 9:e51701

Lorimer N, Hallinan E, Rai KS (1972) Translocation homozygotes in the yellow fever mosquito, *Aedes aegypti*. J Hered 63:159–166

Lowe RE, Bailey DL, Dame DA, Savage KE, Kaiser PE (1980) Efficiency of techniques for the mass release of sterile male *Anopheles albimanus*. Am J Trop Med Hyg 29:695–703

Mains JW, Brelsfoard CL, Rose RI, Dobson SL (2016) Female adult *Aedes albopictus* suppression by *Wolbachia*-infected male mosquitoes. Sci Rep 6:33846

Marshall JM (2010) The Cartagena protocol and genetically modified mosquitoes. Nat Biotechnol 28:896–897

Marshall JM (2011) The Cartagena protocol in the context of recent releases of transgenic and Wolbachia-infected mosquitoes. Asia-Pacific J Mol Biol Biotechnol 19:93–100

Marshall JM, Akbari OS (2018) Can CRISPR-based gene drive be confined in the wild? A question for molecular and population biology. ACS Chem Biol 13:424–430

McNaughton D (2012) The importance of long-term social research in enabling participation and developing engagement strategies for new dengue control technologies. PLoS Negl Trop Dis 8: e1785

Moreira LA, Iturbe-Ormaetxe I, Jeffery JA, Lu G, Pyke AT et al (2009) A *Wolbachia* symbiont in *Aedes aegypti* limits infection with dengue, chikungunya, and plasmodium. Cell 139:1268–1278

Murphy B, Jansen C, Murray J, De Barro P (2010) Risk analysis on the Australian release of *Aedes aegypti* (L.) (Diptera: Culicidae) containing *Wolbachia*. CSIRO, Canberra

Nature (1975) Oh New Delhi, Oh Geneva (editorial). Nature 256:355–357

Nightingale K (2010) GM mosquito wild release takes campaigners by surprise. SciDev.Net. Available online at: https://www.scidev.net/global/policy/news/gm-mosquito-wild-release-takes-campaigners-by-surprise.html

Noble C, Adlam B, Church GM, Esvelt KM, Nowak MA (2018) Current CRISPR gene drive systems are likely to be highly invasive in wild populations. eLife. https://doi.org/10.7554/eLife.33423

O'Connor L, Pilchart C, Sang AC, Brelsfoard CL, Bossin HC, Dobson SL (2012) Open release of male mosquitoes infected with a *Wolbachia* biopesticide: field performance and infection containment. PLoS Negl Trop Dis 6:e1797

Robinson AS (2002) Mutations and their use in insect control. Rev Mutat Res 511:113–132

Sánchez HM, Bennett JB, Wu SL, Rašić G, Akbari OS et al (2020) Confinement and reversibility of threshold-dependent gene drive systems in spatially-explicit Aedes aegypti populations. BMC Biol 18:50

Secretariat of the Convention on Biological Diversity (2000) Cartagena protocol on biosafety to the convention on biological diversity. World Trade Center, Montreal

Serebrovskii AS (1940) On the possibility of a new method for the control of insect pests. Zool Zhurnal 19:618–630

Singh KRP, Patterson RS, LaBrecque GC, Razdan RK (1975) Mass rearing of *Culex fatigans*. J Commun Dis 7:31–53

Thomas DD, Donnelly CA, Wood RJ, Alphey LS (2000) Insect population control using a dominant, repressible, lethal genetic system. Science 287:2474–2476

Turelli M, Hoffmann AA (1991) Rapid spread of an inherited incompatibility factor in California *Drosophila*. Nature 353:440–442

Walker T, Johnson PH, Moreira LA, Iturbe-Ormaetxe I, Frentiu FD et al (2011) The *wMel Wolbachia* strain blocks dengue and invades caged *Aedes aegypti* populations. Nature 476:450–453

Weidhaas DE, Schmidt CH, Seabrook EL (1962) Field studies on the release of sterile males for the control of *Anopheles quadrimaculatus*. Mosq News 22:283–291

Wyss JH (2000) Screw-worm eradication in the Americas. In: Tan KH (ed) Area-wide control of fruit flies and other insect pests. Penerbit Universiti Sains Malaysia, Penang, pp 79–86

Xu XS, Bulger EA, Gantz VM, Klanseck C, Heimler SR et al (2020) Active genetic neutralizing elements for halting or deleting gene drives. Mol Cell 80:246–262

Zheng X, Zhang D, Li Y, Yang C, Wu Y et al (2019) Incompatible and sterile insect techniques combined eliminate mosquitoes. Nature 572:56–61