Focks, D. A., D. A. Dame, A. L. Cameron, and M. D. Boston. 1980. Predator-prey interaction between insular populations of *Toxorhynchites rutilus rutilus* and *Aedes aegypti*. Environmental Entomology 9: 37–42.

Hill, S., and C. R. Connelly. 2009. Southern house mosquito *Culex quinquefasciatus* Say (Insecta: Diptera: Culicidae). Publication Number EENY-457, University of Florida/Institute of Food and Agricultural Sciences Extension.

Kerzee, K. 2019. The whats and whys of mosquito abatement districts. Midwest Pesticide Action Center.

Kline, D. L. 2006. Mosquito population surveillance techniques. Technical Bulletin of the Florida Mosquito Control Association 6: 2–8.

(LCMCD) Lee County Mosquito Control District. 2019. Lehigh Acres, Florida, USA.

Lees, R. S., D. O. Carvalho, and J. Bouyer. 2021. Potential impact of integrating the Sterile Insect Technique into the fight against disease-transmitting mosquitoes, pp. 1081–1118. *In* V. A. Dyck, J. Hendrichs, and A. S. Robinson (eds.), Sterile Insect Technique – Principles and practice in Area-wide Integrated Pest Management. Second Edition. CRC Press, Boca Raton, Florida, USA.

McKenna, M. 2016. Disorganized mosquito control will make US vulnerable to Zika. February 29, 2016. National Geographic, Washington, DC, USA.

McDonald, P. T. 1977. Population characteristics of domestic *Aedes aegypti* (Diptera: Culicidae) in villages on the Kenya coast. II. Dispersal within and between villages. Journal of Medical Entomology 14: 49–53.

Muir, L. E., and B. H. Kay. 1998. *Aedes aegypti* survival and dispersal estimated by mark-release-recapture in northern Australia. American Journal of Tropical Medicine and Hygiene 58: 277–282.

Nayar, J. K., and C. R. Connelly. 2017. Mosquito-borne dog heartworm disease. SP486: Pests in and around the southern home. University of Florida/Institute of Food and Agricultural Sciences Extension.

Patterson, G. 2004. The mosquito wars: A history of mosquito control in Florida. University Press of Florida. Gainesville, Florida. 288 pp.

Provost, M. W. 1952. The dispersal of *Aedes taeniorhynchus*. I. Preliminary studies. Mosquito News 12(3): 174–190.

Provost, M. W. 1969. Man, mosquitoes and birds. The Florida Naturalist 41: 63–67.

Rey, J. R., and C. R. Rutledge. 2006. Mosquito control impoundments. Publication number ENY-648, University of Florida/Institute of Food and Agricultural Sciences Extension.

Rose, A., U. Kröckel, R. Bergbauer, M. Geier, and Á. E. Eiras. 2006. The BG-Sentinel, a novel mosquito trap for research and surveillance. Mitteilungen der Deutschen Gesellschaft für allgemeine und angewandte Entomologie 15: 345–348.

(WHO) World Health Organization. 2016. Monitoring and managing insecticide resistance in *Aedes* mosquito populations: Interim guidance for entomologists. Geneva, Switzerland.

Zettel, C., and P. Kaufman. 2019. Yellow fever mosquito *Aedes aegypti* (Linnaeus) (Insecta: Diptera: Culicidae). Publication number EENY-434, University of Florida/Institute of Food and Agricultural Sciences Extension.

Aedes aegypti CONTROL PROGRAMMES IN BRAZIL

H. R. C. ARAÚJO[1,3], D. O. CARVALHO[2] AND M. L. CAPURRO[1,3]

[1]Department of Parasitology, Institute of Biomedical Sciences, University of São Paulo, São Paulo, SP, Brazil; mlcapurro@gmail.com
[2]Joint FAO/IAEA Division, Insect Pest Control Laboratory, Seibersdorf, Austria; d.carvalho@iaea.org
[3]National Institute of Science and Technology in Molecular Entomology, Medical Biochemistry Institute, Federal University of Rio de Janeiro, Brazil

SUMMARY

Mosquito-borne diseases are among the most significant challenges facing societies around the world. In Brazil, current official epidemiological reports show increasing numbers of cases of mosquito-borne diseases, such as chikungunya, dengue, yellow fever and Zika, which are spreading to new areas of the country. Therefore, it can be stated that current methods used for the management of mosquito vectors in Brazil, established since 2002, have been ineffective. Thus, there is a necessity for readjustment or updating of the *Aedes aegypti* control programmes that are being applied in Brazil. As recommended by the World Health Organization (WHO), the best way to combat these pathogen vectors is an integrated approach where several convenient and compatible control techniques are combined to efficiently reduce or potentially eliminate a targeted insect vector population. In this manuscript, we updated a review published in 2015 by the same authors about *Aedes* control programmes in Brazil showing their basic concept and the principal components of *Aedes* integrated control programmes. Strategies such as public education, community engagement and responsibility; mechanical elimination of mosquito breeding habitats; the use of larvicides and adulticides; massive collection of eggs and adults using traps; and the reduction in the vector population through the promotion of sterility of mosquitoes by ionizing radiation, use of symbiont bacteria such as *Wolbachia,* or genetic modification, are discussed. The Brazilian experience to test and evaluate some of these technologies is described and compared with strategies to prevent and manage mosquito populations in other countries. It is concluded that there are new control methods that can be integrated on an area-wide basis to suppress mosquito populations successfully. Nevertheless, epidemiological studies are also needed to evaluate their impact on disease transmission, in addition to the proof-of-concept that they suppress mosquito populations.

Key Words: Mosquito control methods, community engagement, Projeto Aedes Transgênico, population suppression, integrated vector management (IVM), genetically modified mosquitoes, vector-borne diseases, Plano Nacional de Controle da Dengue (PNCD)

J. Hendrichs, R. Pereira and M. J. B. Vreysen (eds.), Area-Wide Integrated Pest Management: Development and Field Application, pp. 339–366. CRC Press, Boca Raton, Florida, USA.

1. INTRODUCTION

1.1. Aedes aegypti *Primary Vector of Arboviruses*

Aedes aegypti (L.), the yellow fever mosquito, is the primary mosquito vector of various arboviruses such as yellow fever (YFV; genus Flavivirus), dengue (DENV; genus Flavivirus), Zika (ZIKV; genus Flavivirus) and chikungunya (CHIKV, genus *Alphavirus*).

Yellow fever is endemic in tropical areas of Africa, as well as Central and South America. Symptoms include fever, headache, jaundice (origin of the name "yellow" fever), muscle pain, nausea, vomiting, and fatigue. A small proportion of patients develop severe symptoms and approximately half of those die within 7 - 10 days (WHO 2018).

Dengue is endemic in more than 100 countries and is one of the most serious public health problems in the world. Clinical manifestations of dengue virus infection include high fever (40°C) that can be accompanied by severe headache, pain behind the eyes, muscle and joint pains, nausea, vomiting, swollen glands or rash. It is estimated that worldwide, about 40% (2500 million people) of the human population is at risk of contracting dengue fever, and about 390 million people are each year becoming infected with the disease. In 2016, more than 2.38 million cases of dengue were reported in the Americas, of which 1.5 million cases occurred in Brazil, i.e. a threefold increase in cases as compared with 2014 (WHO 2009, 2016a).

A ZIKV infection brings complications and consequences such as microcephaly in babies and the Guillain-Barré syndrome, and their neurological complications are being intensively investigated. Symptoms are generally mild and include fever, rash, conjunctivitis, muscle and joint pain, malaise or headache that last for 2–7 days. However, most people with Zika virus infection do not develop symptoms. Since the 1960s, ZIKV disease has been reported in Africa, Asia, the Pacific islands, and the Americas, but since 2015, its geographic range has expanded rapidly (WHO 2016b). Currently, the ZIKV has been reported in more than 84 countries, territories or subnational areas in the world (WHO 2017a). Between 2015 and 2017, more than 200 000 confirmed autochthonous cases of ZIKV were reported in the countries and territories in the Americas, as well as 3323 confirmed cases of congenital syndrome associated with ZIKV infections. Of these ZIKV cases, a majority (134 057) were reported in Brazil (PAHO/WHO 2016).

CHIKV has been identified in over 60 countries in Asia, Africa, the Americas and Europe. The disease is characterized by fever and is frequently accompanied by joint pain, which is often very debilitating and lasts for a few days or weeks. In 2016, there were more than 150 000 laboratory confirmed cases of chikungunya fever in the Americas. Brazil reported 146 914 confirmed cases, followed by Argentina (322 confirmed cases) and Paraguay (38 confirmed cases) (PAHO/WHO 2014; WHO 2016c, 2017b).

All the above-mentioned viruses are transmitted by *Aedes* spp. when the female mosquitoes take a blood meal from a viremic human host and bites another non-viremic human host. These mosquitoes are distributed throughout tropical and subtropical territories, where they largely overlap, explaining their current scenario of co-infection (Furuya-Kanamori et al. 2016; Rückert et al. 2017).

1.2. Why Vector Control?

The co-distribution and/or co-transmission of vector-borne diseases pose a challenge for public health in endemic and epidemic regions of the world, in particular also in Latin America (Furuya-Kanamori et al. 2016; Rodriguez-Morales et al. 2016; Carrillo-Hernández et al. 2018; O Silva et al. 2018; Suwanmanee et al. 2018). More than 80 % of the global human population lives in areas where they are at risk of contracting at least one vector-borne disease and more than half lives in areas where they are at risk of contracting two or more of these diseases (PAHO 2016). Vector-borne diseases mainly affect poorer populations and impede economic development through direct and indirect medical and other costs such as loss of productivity and tourism (WHO 2017c).

Despite the emergence of new viruses transmitted by *Ae. aegypti*, dengue continues to be one of the most important public health problems in Brazil, considering the burden of disease and the great potential for evolution to death (Martelli et al. 2015; Araújo et al. 2017). Between 2013 and 2016, the cost of hospitalizations for dengue paid by Brazil's publicly funded health care system (known by the acronym SUS) was BRL 68.1 million (SHS 2017). In addition, dengue contributes to the loss of healthy years of life, affecting a large number of people from all age groups, causing some degree of disability during the infection period and deaths, mainly in children (Araújo et al. 2017). The application of remediation measures during epidemic periods can drastically reduce its cost through a more effective prevention programme using entomological surveillance, integrated with area-wide vector control strategies, resulting in the prevention of several diseases and increasing human population life quality in the target area.

In Singapore, for example, the haemorrhagic fever induced by dengue infection became a significant cause of death in the 1960s, affecting especially children. A vector control programme was implemented from 1968 to 1973, using data from entomological and epidemiological surveys to develop a strategy that was based on entomological surveillance, larval source reduction, public education, and law enforcement. The philosophy of the programme was to carry out vector control before the disease is detected as a means to reduce disease transmission. Singapore successfully controlled *Ae. aegypti* population and as a result, DENV infections were reduced and disease incidence remained low for a 15-year period. However, this success proved to be temporary, and the disease incidence increased again in the country in the 1990s (Ooi et al. 2006). The development of a local entomological index correlates the increase of new areas with breeding sites more supceptible to dengue transmission (Ong et al. 2019). In addition, cases of other arboviruses were reported, such as CHIKV in 2008 (Leo et al. 2009) and ZIKV in 2016 (Maurer-Stroh et al. 2016).

The reduction in the density of the *Ae. aegypti* population, the resurgence of DENV and appearance of other diseases transmitted by this vector seems like a paradox. However, it is speculated that several factors may have contributed to an increase in dengue incidence in Singapore: 1) decreased herd immunity after 30 years of low dengue exposure, 2) an increase in the proportion of adult infections, 3) virus transmission occurring outside houses, 4) the adoption of a reactive rather than a proactive approach to vector control, 5) the presence of asymptomatic persons, and 6) a continued introduction of the virus through increasing numbers of travelers returning from endemic areas (Ooi et al. 2006; Ooi and Gubler 2009). Moreover, peridomestic areas, where other competent vectors were present (*Aedes malayensis* (Colless) and *Aedes albopictus* (Skuse)), were not included in the vector management programme (Mendenhall et al. 2017), and hence the programme was not following area-wide principles.

In the 20th century, classic vector control strategies to reduce populations of mosquitoes that transmitted malaria, yellow fever and dengue temporarily reduced the impact of these diseases in several countries using mainly insecticides (NASEM 2016). However, the current distribution of vector-borne diseases in the world shows that these and other disorders are re-emerging and/or spreading to new areas. This means that the full potential for preventing disease transmission is not applied as it should. There are factors that contribute to the failures such as technical complexity, costs and logistic needs, complacency and environmental concerns about insecticides (Townson et al. 2005).

1.3. Aedes aegypti *Control in Brazil*

Control of *Ae. aegypti* in Brazil has been implemented according to the guidelines outlined in the National Plan for Dengue Control (in Portuguese, *Plano Nacional de Controle da Dengue* - PNCD) (MS/FNS 2002), which is aligned with the Integrated Management Strategy for Dengue Prevention and Control in the Region of the Americas (known by the abbreviation IMS-Dengue) approved in the Resolution CD44.R9 adopted by the 44th Directing Council of the Pan American Health Organization/World Health Organization (PAHO/WHO 2003; San Martín and Brathwaite-Dick 2007).

The main objective has been to promote a model for prevention and control of dengue that incorporates national and international experiences and emphasizes the need for change in previous models, including also the decentralisation of the vector control programme so that each municipality is responsible for the control with the support from the State Department of Health and the Ministry of Health (MS/FNS 2002; Tauil 2002; Brasil 2009; PAHO 2018). The main actions involve epidemiological surveillance, vector control, patient care, integration with primary health care, environmental sanitation actions, integrated health education actions, communication and social mobilization, training of human resources, social and political support and evaluation of the programme (MS/FNS 2002, 2009; Braga and Valle 2007; Araújo et al. 2015).

The strategies involve the participation of 'Community Health Agents' (CHA) that are responsible, together with the local community, to promote the mechanical (removal or elimination of potential breeding sites) and chemical control (insecticides) with the objective of guaranteeing the sustainability of the elimination of breeding sites by real estate owners, in an attempt to break the chain of transmission of dengue. Other actions also recommended by the Brazilian Ministry of Health include installations of screens on the doors and windows to prevent the entry of the adult mosquito, in addition to the use of predators or pathogens with potential to reduce the vector population (biological control). Among the available predators are fish and aquatic invertebrates, which eat the larvae and pupae, and pathogens that release toxins including bacteria, fungi and parasites (Zara et al. 2016).

Since the mid-1980s, temephos (organophosphate) has been the main insecticide used against larvae of *Ae. aegypti* in Brazil. However, since 2002, mosquito populations in half of the country have become resistant to temephos (Chediak et al. 2016). As a result, pyriproxyfen, an insect growth regulator that mimics a natural hormone and interrupts insect development, was introduced in 2014 for the suppression of *Ae. aegypti* larvae (MS 2014a). Since 2009, malathion (organophosphate insecticide) has been used to control adults, replacing the use of pyrethroids after the identification of high levels of knockdown resistance registered (Martins et al. 2009). Concentrations of the all insecticides currently used, as well as the applied bioassay protocols, are those recommended by the WHO (2013) and the Brazilian Ministry of Health (MS 2014b).

Despite the risk of favouring the rise and dispersal of resistant populations, and the consequent lack of alternative insecticides to the currently available malathion against *Ae. aegypti* adults, the Brazilian Ministry of Health intensified insecticide spraying against *Ae. aegypti,* as a response to the Zika and chikungunya epidemics. The reliance on a strategy that was mainly based on chemicals to bring the *Ae. aegypti* population under control gave the human population in Brazil a false conception of security (Augusto et al. 2016). The unprecedented spread of vector-borne diseases clearly highlights the challenges faced by everyone, not just the health agencies. Multiple control tactics will need to be used for the management of vector-borne diseases, and this will only be possible if an integrated vector management (IVM) approach is selected. An IVM approach was adopted in 2004 by WHO for all vector-borne diseases and involves a rational decision-making process for the optimal use of resources, to improve cost-effectiveness, ecological soundness, and sustainability of disease-vector control (WHO 2004, 2008, 2017c). The outcome of IVM is improved human capacity and strengthened infrastructure to increase the well-being, and not only protecting human population against disease. The WHO recommends integrated control of the mosquito vectors, mainly those of dengue. Control activities should target *Ae. aegypti* (or any of the other vectors depending on the evidence of transmission) in all its immature (egg, larva, and pupa) and adult stages (WHO 2017d). The critical components of *Aedes* integrated vector management programme in Brazil are illustrated in Fig. 1.

2. EDUCATION, COMMUNITY ENGAGEMENT AND RESPONSIBILITY

2.1. General Overview

Of primary importance in any IVM strategy is training of health personnel in community-based participation so that the local population can understand and hence participate in several aspects of vector control (Gubler and Clark 1996; Ulibarri et al. 2016). Vector control also requires national level support to provide strategic direction, technical expertise, and training, aside from the development of norms and indicators to monitor the progress of operational activities.

Figure 1. Integrated control programme for Aedes aegypti *populations in Brazil.*

The distribution and incidence of vector-borne diseases are determined by ecological factors, but they are also influenced by the behaviour of humans. Thus, vector control interventions that incorporate human population engagement are more likely to be successful as they offer the opportunity to take into account community problems (Townson et al. 2005).

The WHO has prepared and made available guidelines to assist national programmes with the design and implementation of social mobilization and communication strategies aimed at dengue fever prevention and control. The approaches to social mobilization are known by the initials "COMBI" (Communication-for-Behavioural-Impact) that integrate the participation of different members of the community, from households to political leaders. COMBI represents a set of marketing, education, communication, promotion, advocacy and mobilization approaches with the same goal, i.e. to ensure sustained community participation to combat *Ae. aegypti* and as such, to promote the health of community members (Parks and Lloyd 2004; Tapia-Conyer et al. 2012).

2.2. The Brazilian Perspective

In Brazil, the Municipal Health Secretariats have begun to manage and execute PNCD actions with the support of the States and the Ministry of Health, with most funding provided at the federal level. Engagement of the communities and education of the public in the control of *Ae. aegypti* does not mean to bombard people with information about mosquitoes or vector-borne diseases. In Brazil, it has happened that despite growing levels of public knowledge about mosquitoes and their control, many people are not taking the required basic actions such as the elimination of larval sources (Claro et al. 2006). Nevertheless, a study conducted in Ribeirão Petro (south-eastern Brazil) reveals the relevance of educational campaigns and educational health programmes using different types of media to reach different community levels to transmit the necessary information (Alves et al. 2016).

Caprara et al. (2015) developed an eco-health programme, based on community engagement, developing and distributing educational and informative material. They also promoted workshops for the community and developed activities to involve the community directly, such as mobilization of schoolchildren and the elderly, organization of meetings and active participation during campaigns to remove/relocate breeding sites. Although the overall result shows that there was still an increase in the mosquito population after the rainy season (which also corresponded to the end of the experiment), the non-treated site had a significantly higher increase in mosquito density compared to the treated area during the same period (Caprara et al. 2015).

The key to educational campaigns is achieving a long-term modification of the behavioural of the general public that must be conscious of its own actions and be responsible for the surrounding environment. In support, a recent Brazilian sanitary legislation allows the application of fines in the case of impediment or difficulty when implementing sanitary measures that aim at the prevention of the diseases and their dissemination (Brasil 2016).

2.3. Innovations and Experiences of Other Countries

Community engagement and information activities were performed in Brazil during the entire mosquito population suppression *Projeto Aedes Transgênico* (2010-2013 described in Section 7) that relied on the release of genetically modified mosquitoes (GMM) (Capurro et al. 2016). These activities, carried out before and during the mosquito release project, showed positive results and provided guidance for the design of similar public engagement plans in other regions or countries. This pioneering study in continental America showed that full transparency was crucial to make the public aware of all aspects of the mosquito release project, particularly in this case involving genetically modified organisms.

The work from Sommerfeld and Kroeger (2015) reviews community-based vector control interventions in different countries in Latin America that are fighting against dengue and using educational campaigns, chemical and non-chemical strategies, including new approaches such as waste management. The authors mention that these strategies involving the community require establishment of a prolonged interaction with control services, municipalities and other public actors, proving to be rewarding during the process and with excellent potentials for sustainability, however, they were time-consuming and costly at the beginning. The results of community participation programmes used in Mexico showed that continuity of these activities in long-term campaigns is a prerequisite to achieve the desired goals (Tapia-Conyer et al. 2012). However, governments are often reluctant to invest and support these initiatives, and consequently, these programmes are often relegated to serve as epidemiological projects during dengue outbreaks.

3. MECHANICAL CONTROL

3.1. General Overview

In general, mechanical control consists of the elimination of *Ae. aegypti* larval breeding sites from domestic and peridomestic areas, and the application of measures that prevent the contact between humans and the vector. The interventions include changing the environment through cleaning and removal of possible habitats suitable for any stage of *Ae. aegypti* and *Ae. albopictus* to prevent or minimize vector propagation. This entails covering water storage containers, disposing of non-biodegradable waste, and installing mosquito screens on windows, doors and other entry points, in addition to the use of mosquito bed nets. Local government agencies must take responsibility for the clean-up of public spaces and to eliminate illegal dumps and discarded tyres (Arunachalam et al. 2012; US-EPA 2017; WHO 2017e).

3.2. The Brazilian Perspective

Dengue is a disease that has ecological, biological and social factors involved in its transmission. The dynamics of *Ae. aegypti* breeding sites are closely linked to human behaviour; therefore, elimination of larval sites through household interventions is an efficient way to reduce the mosquito population. In Brazil, the removal of breeding sites is the responsibility of households. Periodically the community health agents, and the 'Endemic Disease Control Agents' (EDCA) visit houses looking for possible breeding sites, but they are mainly responsible for non-residential properties, and if necessary, integrate chemical (insecticide) application (Zara et al. 2016).

Chaebo and Medeiros (2017) investigated five conditions for an effective strategy for dengue control policy implementation through co-production, which they defined as the strategy for policy implementation resulting from technological, economic, and institutional influences. Initially the technical, economic, normative, cognitive and structural conditions were analysed and as a result they stated that technical, economic and normative conditions are interdependent, and changing one will change the others. In addition, the authors added two extra conditions to implement policy using co-production that they defined as cognitive and structural conditions. Including these conditions to the main study, the authors state:

> *"We believe, it is impossible to successfully undertake policy implementation via co-production unless users recognize that an important problem exists and are able and willing to undertake the necessary co-production actions"* (Chaebo and Medeiros 2017).

Unfortunately, besides the responsibilities of authorities, the communities often wait for the public vector control services to carry out the task of controlling mosquito breeding sites. In some cases, communities are fully aware of the threats leaving breeding sites and their responsibility to eliminate them, but they are not involved in the programme, and this paradigm needs to change. In integrated vector control, the householders must be stimulated to interact with vector control staff and to ensure appropriate interpersonal communication (Arunachalam et al. 2010).

3.3. Innovations and Experiences of Other Countries

The effect of encouraging community members to eliminate *Ae. aegypti* breeding sites showed a favourable impact in studies carried out in the Caribbean (Rosenbaum et al. 1995), Latin America (Tapia-Conyer et al. 2012), Thailand (Suwannapong et al. 2014), Pakistan (Zahir 2016), USA (Healy et al. 2014), and many other parts of the world (Spiegel et al. 2002; Kay and Nam 2005; Vanlerberghe et al. 2009; Sanchez et al. 2012). There is a consensus among health authorities that this measure is an essential component of environmentally sustainable mosquito control programmes. A recent mathematical model for dengue control developed by Carvalho et al. (2019) confirms that, even though the combination of mechanical and chemical approaches is the most suitable one instead of using them separately, it is still insufficient to eliminate disease transmission completely.

4. MASS-TRAPPING

4.1. General Overview

Different models of traps are available to monitor *Ae. aegypti* and *Ae. albopictus* populations, and they can generate baseline data that are essential to guide control operations. They can also be included in the entomological surveillance to improve mosquito population density prediction prior to epidemic periods (Honório et al. 2009; Degener et al. 2014). An increased number of deployed traps can be used to reduce the target mosquito population, i.e. the gravid females are attracted to the oviposition traps (ovitrap) and are killed when making contact with the oviposition substrate that is impregnated with insecticides, or lethal ovitraps, collecting eggs that are subsequently killed by an insecticide-treated ovistrip (Paz-Soldan et al. 2016).

According to a review on mass-trapping interventions for suppression of urban *Aedes* by Johnson et al. (2017), successful deployment is achieved with a high area coverage (>80%), a pre-intervention and/or additional source reduction, the direct involvement of community members for sustainability, and the use of new-generation traps (such as the Autocidal Gravid Ovitrap – AGO, or Gravid *Aedes* Trap – GAT) to outcompete remaining water-holding containers.

In areas where *Ae. albopictus* co-exists with *Ae. aegypti*, eggs or larvae collected in ovitraps need to be taken to the laboratory for species identification at the larval stage or maintained until adult emergence. In those areas, the AGO is a good alternative to monitor mosquito populations (Caputo et al. 2015). These traps are simple, specific and efficient for gravid females, and their integration with other chemical or biological control methodologies can contribute significantly to decrease mosquito populations. However, their use is laborious, which is a disadvantage for deployment over large areas of action. Combining mass-trapping of adults, with the use of larvicides, can have a more significant impact on *Ae. aegypti* populations than using each of these methods alone (Regis et al. 2008).

4.2. The Brazilian Perspective

Mass-trapping is not currently used for mosquito control in any vector control programme in the country; however, several works have assessed the effect of lethal ovitraps, and the results were promising in several situations (Regis et al. 2008, 2013). A modified ovitrap containing *Bacillus thuringiensis israelensis* (*Bti* – see next Section) that kills any larvae developing inside was evaluated in north-eastern Brazil. The *Bti*-treated trap can safely remain in the field for up to two months and during that time can collect more than 7000 eggs/trap (Regis et al. 2008), of course depending on the initial population density.

Deployment in urban areas of ovitraps treated with the pyrethroid deltamethrin reduced the density of the adult female population by 40% (Perich et al. 2003). The study involved the placement of 10 ovitraps/residence (five inside and five outside) for 12 weeks in two municipalities in Rio de Janeiro, and the sampling of 30 houses per intervention neighbourhood. The authors mentioned that although lethal ovitraps were not designed to be a control method to be used alone, their results show that lethal ovitraps could provide an inexpensive, simple, environmentally benign way to be integrated into vector control strategies (Perich et al. 2003).

Sticky ovitraps with an adhesive strip, rather than an insecticide-treated oviposition surface that traps the ovipositing females when they land, have been used for surveillance in areas with high mosquito insecticide resistance. A study was conducted for 17 months to suppress mosquito populations in the Amazon region through a mass-trapping system using sticky ovitraps. The authors conclude that this intervention alone was not able to show mosquito population suppression, and they indicate as probable reasons a lack of buffer zones, which allowed mosquito migration from other areas, the lack of an area-wide approach due to the small size of the treated area, and insufficient collection efficacy of the trap or inadequate number of traps/household (Degener et al. 2015).

4.3. Innovations and Experiences of Other Countries

Like the Brazilian experience, *Ae. aegypti* populations were significantly reduced in Thailand when lethal permethrin-treated ovitraps were deployed in conjunction with other interventions such as source reduction, use of screen covers, and biological control. In this case, they also evaluated the impact on dengue transmission and the proportion of DENV IgG–IgM positives in the treated areas, which were reduced from 13.46% to 0%, whereas those from untreated areas increased from 9.43 to 19.15% (Kittayapong et al. 2008).

A previous study using lethal ovitraps, also in Thailand, showed a 49-80% reduction in the mosquito population in an experiment over 30 weeks (Sithiprasasna et al. 2003). In Cairns, Australia, the acceptance by households of a mass-trapping scheme allowed the comparison of different types of lethal ovitraps in three separate trials. The results suggest that a high trap density can collapse a mosquito population over time (Ritchie et al. 2009).

The AGO, baited with an attractant and containing an adhesive card placed inside the trap entrance that serves as an autocidal oviposition substrate, was developed by the Centers for Disease Control and Prevention (CDC) to catch gravid *Ae. aegypti* females (Mackay et al. 2013). The AGOs placed in 85% of residences in four communities of two municipalities in Puerto Rico between November 2015 and February 2016 to control *Ae. aegypti* mosquitoes significantly reduced the prevalence of CHIKV IgG antibodies in participating communities without any other control tactic used (Lorenzi et al. 2016).

5. LARVAL CONTROL

5.1. General Overview

Larvicides are biocides used against immature mosquito stages and their use fits well within environment-friendly management strategies (except in emergency situations). Larval control can minimize the need for widespread use of insecticides to kill adult mosquitoes. Larvicides are used by vector control staff to treat water-holding structures and containers in public places, whereas the general citizen is supposed to do the same to treat fountains, septic tanks, pots and pools on private properties. The use of larvicides should be restricted to containers that are not used for drinking, and that cannot be covered, dumped or removed (CDC 2017a). Widely used is *Bti,* a bacterium marketed commercially as a biological larvicide to control insects relevant to public health. It is safe for humans, but when ingested by mosquito larvae, lethal endotoxins proteins are produced during the bacterium sporulation, killing the larvae before reaching adulthood (Federici et al. 2007; Ibrahim et al. 2010). An alternative is the auto-dissemination approach, that can be augmented by the release of males which were tainted with pyriproxyfen, a juvenile hormone analogue, and who will contaminate females during mating or directly the larval habitats (Bouyer and Lefrançois 2014).

5.2. The Brazilian Perspective

In Brazil, *Bti* is used since 2002 when resistance to the organophosphate larvicide, temephos, was observed (Suter et al. 2017). In those cases, *Bti* can be used alone or in association with different chemical larvicides such as pyriproxyfen (MS/FNS 2009; Suter et al. 2017). Recent bioassays with Brazilian populations of *Ae. aegypti* and *Ae. albopictus* that have been exposed for many years to insecticides, in particular *Bti*, showed that both species are equally susceptible to *Bti*, suggesting that the same application rates may be used where the species co-exist (Suter et al. 2017).

A study in Manaus (northern Brazil), using pyriproxyfen, showed not only mosquito mortality, but also that adult emergence was reduced more than 10 times (Abad-Franch et al. 2015). They concluded that this approach is very promising to complement current mosquito control strategies, which heavily rely on the difficult task of detecting vector breeding sites and therefore perform poorly.

In some contexts, however, the application of larvicides by public health services can be complicated. Many *Aedes* breeding sites are small, sheltered and difficult to locate (cryptic habitats). Therefore, depending entirely on breeding site treatment or removal is complex, requiring a combined strategy. Therefore, auto-dissemination methods are an alternative to overcome these limitations, as they rely on the oviposition behaviour of adult mosquitoes and their attraction to breeding sites. The auto-dissemination method to control *Aedes* mosquitoes requires artificial adult resting sites (dissemination stations) to which adult females are attracted and where they are contaminated with pyriproxyfen when entering the station and then contaminate breeding sites with lethal levels of pyriproxyfen (Caputo et al. 2012; Unlu et al. 2017).

5.3. Innovations and Experiences of Other Countries

In a study carried out in Thailand, about 61.8% of water containers were treated with *Bti* and temephos, and the rate of positive containers (with larvae) was reduced from 13.8% in untreated areas to 3.7% in treated areas ($P < 0.001$) showing the combined approach of *Bti* and insecticide were effective in achieving the result in the target area (Arunachalam et al. 2010).

The autodissemination approach was tested in the USA with pyriproxyfen-treated males and showed, in combination with another insecticide, a decline in the *Ae. albopictus* adult population by around 74-78% (Unlu et al. 2018). In a similar approach using only pyriproxyfen, the male mosquitoes were shown to be vehicles of insecticide in areas with low mosquito densities to intoxicate potential breeding sites before the seasonal emergence of the target population (Mains et al. 2015). These males can also contaminate the females, increasing even more the affected breeding sites, interrupting the development of immature offspring. On the other hand, a study only using pyriproxyfen conducted in Florida showed that there was no apparent pupal mortality during the study period (Lloyd et al. 2017).

In Southeast Asia, larvivorous fish, e.g. from the genus *Gambusia*, that feed on mosquito larvae are often used in pots that decorate houses and terraces (Araújo et al. 2015) (Fig. 2). This practice is also employed as a non-insecticidal method to control malaria vectors in India and Africa (Kamareddine 2012; Kant et al. 2013; Walshe et al. 2013). However, the use of fish to control mosquito larvae is feasible and effective only in breeding sites that are easily identified and in those as observed in Asian culture (Chandra et al. 2008).

Studies carried out in Mexico have shown that larvivorous fish can reduce larval and pupal numbers in household water containers, but there was no evidence of a reduction in DENV infection (Morales-Pérez et al. 2017). In villages of Karnataka, South India, the introduction of fish, e.g. the guppy *Poecilia reticulata* (Peters) and *Gambusia affinis* (Baird & Girard), combined with information, education and communication campaigns, had a significant impact on the density of the *Aedes* population and decreased the prevalence of chikungunya (Ghosh et al. 2011). This method is harmless to humans and exhibits minimal risks of mosquito resistance. Besides, the fish are cheap to produce in most cases, saving resources that could serve for other needs. However, in some cases, these invasive fish can be negative effects on biodiversity (El-Sabaawi et al. 2016).

Figure 2. Larvivorous fish in water containers in Southeast Asia (Araújo et al. 2015).

6. ADULT CONTROL

6.1. General Overview

Adulticides are intended to impact a significant number of infected adult mosquitoes in a short time through surface (indoor) and/or spatial (outdoor) treatments with insecticides of residual or low residual activity. The indoor residual spraying (IRS) consists of the application of long-acting chemical insecticides on the walls or others surfaces of houses in a given area using backpack sprayers. A recent review using seven databases evaluated the effectiveness of indoor spraying of insecticides and showed the effect on adult mosquitoes is high immediately after application (Samuel et al. 2017). These spraying activities are usually carried out by staff of the vector control programmes, but the general public can also buy commercial adulticides for use in their homes.

Space spraying is recommended only in emergency situations when people in a large area are at risk of infection, or mosquito densities are very high. The insecticides can be applied by backpack sprayers, trucks or airplanes. When cases of the disease are detected in the early stages of an epidemic, emergency space spraying can reduce disease transmission quickly. However, applying other vector control measures such as larviciding or environmental modification help provide longer-term control as a part of an integrated mosquito management programme (CDC 2017b; MS 2017a; WHO 2017b, 2017f).

6.2. The Brazilian Perspective

In 2016, the Brazilian Government published law No. 13.301 of July 27, 2016 that allows the incorporation of adult vector control mechanisms through aerial spraying upon approval of sanitary authorities and scientific evidence on the efficacy of the measure (MS 2017b). However, in the same year, the Oswaldo Cruz Foundation (FIOCRUZ) issued a technical note stating that there are risks to human health related to the spraying of a neurotoxic product such as malathion in urban areas. They considered that it not only posed a threat to the environment and the population's health, but it is also of little efficacy in the combat of *Ae. aegypti*, which in its adult stage lives mainly within the domiciles (FIOCRUZ 2016).

6.3. Innovations and Experiences of Other Countries

The Florida Keys Mosquito Control District used aerial sprays with insecticide (naled) and bacterial larvicides to reduce *Ae. aegypti* populations in urban areas of Key West, Florida, USA (CDC 2017c; Pruszynski et al. 2017). The aerial applications of *Bti* caused a significant decrease in adult female populations throughout the summer because, in Key West, larvae of this mosquito develop in micro-containers around human habitations. The advantage of aerial spraying of larvicide is the area-wide coverage over and around urban areas achieved in a short period and in the case of Key West, the aerial application of larvicide was effective in controlling the *Ae. aegypti* outbreak (Pruszynski et al. 2017).

7. POPULATION SUPPRESSION INTEGRATING THE STERILE INSECT TECHNIQUE, THE INCOMPATIBLE INSECT TECHNIQUE, AND GENETICALLY MODIFIED MOSQUITOES

To improve the Brazilian dengue vector control programme, it is mandatory to use the principles of IVM to minimise financial and personnel requirements and be able to cover the target geographic area to be treated with the chosen vector control methods. Furthermore, improved monitoring and evaluation tools for vector control should be developed and applied, and relevant training must be performed based on necessity (Horstick et al. 2010).

As described above, suppression of disease-transmitting mosquito populations is still mainly based on insecticides (larvicides and adulticides). A reduction in mosquito densities is the most reliable method to decrease pathogen-host contact, which will reduce the probability of humans becoming infected. Nevertheless, the long-term use of chemical compounds has selected for mosquito populations resistant to them, resulting in the increase of the number of cases in endemic areas and the spread of diseases transmitted by these insects into entirely new areas (Campos et al. 2015; Díaz et al. 2015; Zanluca et al. 2015; Luksic et al. 2017).

Other population suppression approaches are therefore under development and evaluation, and these could be integrated into the currently used IVM approaches. These methods have the benefit that they can reduce vector populations in a target area, without causing the selection of resistance as promoted by insecticides (Bourtzis et al. 2016). They have in common the release of sterilized male insects (because male mosquitoes do not blood-feed and therefore do not transmit diseases), and the monitoring of the sterile and wild male populations in the target area (Lees et al. 2014). These males must be mass-reared to achieve the required numbers to promote suppression of the target population. After release, an efficient monitoring system is needed to be able to follow the vector population fluctuation and if required, to adjust male production and release rates (Hood-Nowotny et al. 2006; Vreysen 2021).

The first of these approaches is the Sterile Insect Technique (SIT), which uses an ionizing radiation source (gamma or X-ray) to sterilize the mass-reared males that will be released into the open field in numbers 10-100 times larger than the wild-type population. The high sterile to wild male overflooding ratios increase the probability of a mating of a wild virgin female with a sterile male (Vreysen et al. 2014; Dyck et al. 2021). For more than 50 years, the SIT has proven to be an effective control tactic to suppress agricultural insect pests such as moths, fruit flies, screwworm and tsetse flies (Hendrichs and Robinson 2009; Klassen et al. 2021). With support from the international scientific community through the International Atomic Energy Agency (IAEA) and the Food and Agricultural Organization of the United Nations (FAO), several countries like Brazil, Cuba, Italy, France (La Réunion), Mauritius, Mexico, Thailand, USA and others have or are initiating pilot trials against mosquitoes on a small to medium scale as a proof-of-concept (Lees et al. 2021).

A similar approach is the release of males that are infected with symbionts that cause sterility without the use of ionizing radiation. The intracellular bacterium, *Wolbachia*, is a symbiont that is sexually transmitted and maternally inherited and can promote cytoplasmic incompatibility in embryos when the father is infected with a particular strain but not the mother (Sinkins 2004). This approach is called the Incompatible Insect Technique (IIT) (Zabalou et al. 2009) and is already under evaluation in several countries like USA (Mains et al. 2016), China, and in French Polynesia for *Aedes polynesiensis* (O'Connor et al. 2012). A related approach under evaluation in Australia, Brazil (Niteroi and Rio de Janeiro), Colombia (Bello and Medellín), and Indonesia (Yogyakarta) through the Eliminate Dengue and other campaigns (De Barro et al. 2011; Maciel-de-Freitas et al. 2012; Flores and O'Neill 2018) involves the release of both *Wolbachia*-infected males and females, resulting in population replacement by substituting the original population with a *Wolbachia*-infected population, this approach takes advantage of *Wolbachia*'s capacity to block pathogen transmission to the human host (Van den Hurk et al. 2012; Frentiu et al. 2014; Dutra et al. 2016).

The release of genetically modified mosquitoes (GMM) is the third population suppression alternative that is under evaluation, and so far, some programmes or trials have demonstrated success in reducing the mosquito population in the target areas Carvalho et al. (2015). This transgenic approach, which requires regulatory approvals and involves other issues, has a broad range of possibilities to interfere and trigger mosquito population suppression or population replacement by blocking disease transmission, due to its potential to manipulate, exclude or include new features at the genomic level of the target mosquito species (Handler 2002; Travanty et al. 2004; Catteruccia et al. 2009).

Brazil is one of the best locations to test and evaluate these new technologies, due to its diverse environments and extensive prevalence of arboviruses. Since 2002, Brazil has been implementing the PNCD to control dengue transmission and related diseases, such as chikungunya, Zika, and yellow fever. However, the efforts and strategies that are combined in the PNCD cannot entirely prevent disease dissemination; on the contrary, the number of reported cases only increases every year (Pessanha et al. 2009; SS-PE 2015; MS 2017c). Therefore, the inclusion of new technologies cannot alone change the vector density and transmission situation if their deployment is not carefully planned according to the specific characteristics and needs of each target area and taking advantage of the best characteristic of each of the technologies. Thus, it is necessary to combine and better apply where appropriate all these techniques as part of effective IVM approaches (Horstick et al. 2010; Bourtzis et al. 2016; Van den Berg et al. 2012).

7.1. Two-step Male Release Strategy – Integration of Techniques

Several models on the use of these inovative technologies, such as GMM, the IIT, the SIT and others, predict that it will take several seasons to suppress a targetted mosquito population, and even when achieving it, some virus transmission can still occur (Andraud et al. 2012; Chen and Hsieh 2012; Okamoto et al. 2013; Ndii et al. 2015). The IVM approaches can be improved by applying the suppression methods more effectively and based on mosquito biology. Models combining several techniques demonstrate the advantages of targeting different developmental stages and integrating different ways to suppress a population.

A two-step strategy was proposed to reduce mosquito populations and then block efficiently disease transmission (Carvalho et al. 2014). A first step involves the integration of any methods which have a significant impact decreasing the target vector population, such as the use of larvicides and adulticides, educational campaigns, breeding site elimination, the release of sterile males, which also can be carrying pyriproxyfen to suppress a population. Once the population has been suppressed, this should be followed by a second step, which could involve releases targeting population substitution (for example *Wolbachia*-infected females or GMM), in order to disrupt disease transmission entirely. The idea is first to reduce the mosquito population to extremely low levels, and then to substitute this residual population by one that is no longer able to transmit viruses, thereby obtaining the advantage of this low-cost combination strategy that can be implemented as part of IVM over larger areas (Fig. 3).

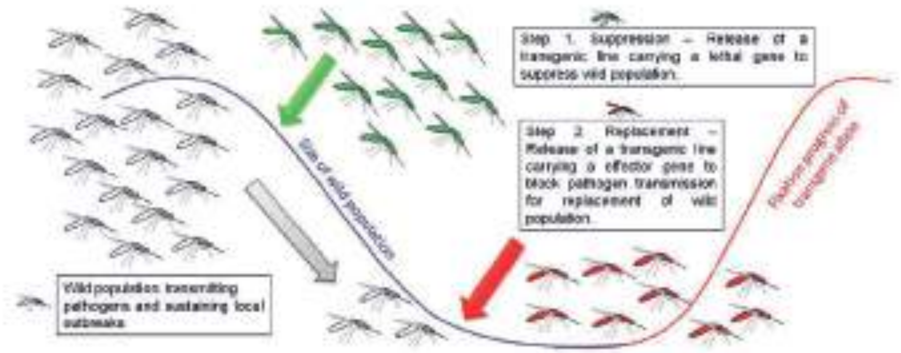

Figure 3. A schematic diagram of two-step male release strategy (Carvalho et al. 2014) using as an example a genetically modified mosquito (GMM) strain for population suppression and replacement.

Recently, some studies proposed transgenic constructions able to block the virus transmission with low impact on the overall mosquito fitness (Jupatanakul et al. 2017; Buchman et al. 2019). Strategies using RNA interference or RNA-based strategies targeting critical virus RNA's were already developed and tested under laboratory conditions and their fitness evaluated (Franz et al. 2006, 2014; Buchman et al. 2019). In addition, other strains targeting malaria parasites have also been developed for population replacement, entirely blocking the parasite transmission (Kokoza et al. 2010).

Nevertheless, it is a common evolutionary fact that without a stable gene drive mechanism such systems alone may not be enough to replace the population successfully, and over time will be displaced (James 2005). However, the use of new gene editing techniques, such as CRISPR-Cas9, provide an easier way not only to create strains for population suppression, but also for population replacement (Häcker and Schetelig, this volume). They may also eventually overcome the issue of gene drive resistance mechanisms that emerge in field populations (Champer et al. 2018). This resistance can originate from the drive itself, when cleavage is repaired and it changes the sequence of the target site, so that it can no longer be recognized, becoming resistant to future conversion.

A model provides that more than 100 generations are needed for the wild-type population to reach 50% of resistant alleles and the use of CRISPR-Cas9 can be an efficient way to provide stable strains for vector control programmes without the accumulation of genetical instability (Unckless et al. 2017). It is a matter of time for the availability and developing state of art of the gene drive technologies to provide further information on their behaviour in the genome and their ecological impact and long-term effects.

Further discussion among the scientific community, stakeholders and population regarding the advantages, risk assessment, and regulatory issues of using them are needed (Carter and Friedman 2016; Häcker and Schetelig, this volume; Nielsen, this volume).

7.2. *Open Field Release Using the OX513A* Ae. aegypti *Transgenic Line and its Evaluation*

Brazil and other countries have initiated the field assessment of the impact of some of these new technologies as part of IVM approaches. The first continental GMM release to suppress an *Ae. aegypti* mosquito population was carried out between 2011 to 2015 in two different cities, Juazeiro and Jacobina in the state of Bahia, in north-eastern Brazil. This *Projeto Aedes Transgênico* aimed at evaluating various aspects of a full IVM programme by using the OX513A transgenic line developed by the commercial company Oxitec Ltd. (Lee et al. 2009). Before the release of the genetically modified male mosquitoes in Brazil, several regulatory steps, as described by Carvalho and Capurro (2015), had to be performed. The most crucial approval was provided by the Brazilian National Committee of Biosafety, which regulates all research projects and products directly and indirectly involving genetically modified organisms, including a public review of the project that had no vote against it.

Due to all apprehensions around genetically modified organisms in plants and other organisms, the *Projeto Aedes Transgênico* initiated a pioneering communication plan to create adequate public awareness regarding the use of this technology and its purpose. Emphasis was likewise placed on community engagement and stakeholder participation during the execution of the project. This experience can serve as a model for other initiatives using the same approach (Carvalho et al. 2015; Capurro et al. 2016).

In the initial phase, some quality parameters of these GMM males were assessed in the target area/environment, such as flight range and longevity under field conditions. Based on this first phase, an assessment was made of the number of sterile male mosquitoes to be released to achieve population suppression, and the data compared with those of the first trial in Grand Cayman Island (Harris et al. 2011, 2012). This range-finding process, consisting of six weeks of releases and the three following weeks for evaluation (around 2800 males/ha/week were released in this first phase), was helpful in optimizing the release number and mass-rearing process. It was also crucial for the next phase involving overflooding the target area in Juazeiro with male mosquitoes for suppression purposes because it provided and confirmed parameters to initiate this suppression phase (Carvalho et al. 2015).

After the 17 months release period, around 95% of population suppression was achieved in Juazeiro, based on an indirect evaluation using a monitoring system with ovitraps. Afterwards, the study kept track of the GMM and wild-type populations after the suppression effect. The outcome was that when the releases were discontinued, the wild mosquito population returned rapidly to pre-control levels within 17 weeks due to immigration and other factors, such the eclosion of eggs that remained unhatched during the release phase (Garziera et al. 2017).

The second part of *Projeto Aedes Transgênico* included procedures for ground shipment of pupae to the city of Jacobina, around 300 km from the mass-rearing facility. This step also included optimizing the monitoring system, increased community engagement and awareness activities, and improved efficiency of mass-rearing procedures and release methods. The *Projeto Aedes Transgênico* was terminated at the end of the contract with the Bahia State Health Department. Again, genetic monitoring of the GMM and wild-type populations continued post-suppression, indicating that portions of the transgenic strain genome became incorporated into the target population (Evans et al. 2019).

In parallel, as an independent initiative, Oxitec started a trial in Piracicaba and Juiz de Fora municipalities (in São Paulo state, south-eastern Brazil), following a similar approach and using the predetermined parameters established during the first two initials trials in the country (Paes de Andrade et al. 2016). So far these trials are service contracts directly performed with the municipalities without any support of the Brazilian Ministry of Health.

8. FINAL CONSIDERATIONS

Numerous activities are currently being integrated in Brazil to suppress *Ae. aegypti,* the vector of various arboviruses. Box 1 presents some important bullets summarizing the strengths, weaknesses, opportunities, and threats (SWOT) for the Brazilian vector control strategy.

Box 1. Strengths, weaknesses, opportunities, and threats of the Brazilian vector control strategy.

STRENGTHS	WEAKNESSES
• Strong research institutions • Decentralisation of the vector control • Existence of the National Plan for Dengue Control (PNCD) • Historical record of successful vector elimination • Reference research laboratories	• Insufficient budget / trained staff • Insufficient public mobilization resulting in low community commitment and household participation and acceptance • Limited time required for data analysis resulting in poor management • Lack of consistent and frequent control strategy application
OPPORTUNITIES	**THREATS**
• Possibility to test new techniques in different biomes and urbanization levels • Use of different combinations and methods for vector control for specific conditions • Learning from different models and previous experiences (including other countries)	• The continuous increase in reported arboviruses cases • Different vector species participating in disease transmission • Entry of new arboviruses promoting illness • Difficulty of treating increasing cases in big cities

PNCD activities and efforts are not enough to interfere with disease transmission by this vector. All have been recommended by WHO (Brasil 2009), however they have to be adapted to different levels of difficulty in different situations (for example, vector control in isolated small areas vs non-isolated, large and densely populated urban areas). Among the main reasons for the insufficient control are the low budget, lack of trained staff, insufficient insecticide application, insufficient public mobilization, and poor management. There is a need to increase vector control efforts all over the country, but at the same time to complement the adopted strategies with promising innovative approaches (Zara et al. 2016; Coelho 2012). There is the potential to exploit and include new methods as part of the IVM package in order to suppress more effectively and sustainably the mosquito populations and control disease transmission.

The current PNCD activities being performed should not be interrupted due to the advent of new technologies, but these can be validated and implemented as part of the IVM package. The range of approaches integrating new technologies is huge, and they have demonstrated that they can successfully contribute to mosquito population suppression and reduce disease transmission. In view of the proof-of-concept of these techniques (most of them carried out under Brazilian conditions), they are ready for the next step, which is their application as part of a long-term programme, not only to demonstrate their effect on mosquito populations, but also their impact on disease transmission.

9. REFERENCES

Abad-Franch, F., E. Zamora-Perea, G. Ferraz, S. D. Padilla-Torres, and S. L. B. Luz. 2015. Mosquito-disseminated pyriproxyfen yields high breeding-site coverage and boosts juvenile mosquito mortality at the neighborhood scale. PLoS Neglected Tropical Diseases 9 (4): e0003702.

Alves, A. C., A. L. dal Fabbro, A. D. Costa Passos, A. F. T. Mendes Carneiro, T. Martins Jorge, and E. Zangiacomi Martinez. 2016. Knowledge and practices related to dengue and its vector: A community-based study from southeast Brazil. Revista da Sociedade Brasileira de Medicina Tropical 49 (2): 222–226.

Andraud, M., N. Hens, C. Marais, and P. Beutels. 2012. Dynamic epidemiological models for dengue transmission: A systematic review of structural approaches. PLoS One 7: e49085.

Araújo, H. R. C., D. Carvalho, R. Ioshino, A. Costa-da-Silva, and M. Capurro. 2015. *Aedes aegypti* control strategies in Brazil: Incorporation of new technologies to overcome the persistence of dengue epidemics. Insects 6: 576–594.

Araújo, V. E. M., J. M. T. Bezerra, F. F. Amâncio, V. M. de A. Passos, and M. Carneiro. 2017. Aumento da carga de dengue no Brasil e unidades federadas, 2000 e 2015: Análise do Global Burden of Disease Study 2015. Revista Brasileira de Epidemiologia 20 (Suppl. 1): 205–216.

Arunachalam, N., S. Tana, F. Espino, P. Kittayapong, W. Abeyewickreme, K. T. Wai, B. K. Tyagi, A. Kroeger, J. Sommerfeld, and M. Petzold. 2010. Eco-bio-social determinants of dengue vector breeding: A multicountry study in urban and periurban Asia. Bulletin of the World Health Organization 88: 173–184.

Arunachalam, N., B. K. Tyagi, M. Samuel, R. Krishnamoorthi, R. Manavalan, S. C. Tewari, V Ashokkumar, A. Kroeger, J. Sommerfeld, and M. Petzold. 2012. Community-based control of *Aedes aegypti* by adoption of eco-health methods in Chennai City, India. Pathogens and Global Health 106: 488–496.

Augusto, L. G. S., A. M. Gurgel, A. M. Costa, F. Diderichsen, F. A. Lacaz, G. Parra-Henao, R. M. Rigotto, R. Nodari, and S. L. Santos. 2016. *Aedes aegypti* control in Brazil. The Lancet 387: 1052–1053.

Bourtzis, K., R. S. Lees, J. Hendrichs, and M. J. B. Vreysen. 2016. More than one rabbit out of the hat: Radiation, transgenic and symbiont-based approaches for sustainable management of mosquito and tsetse fly populations. Acta Tropica 157: 115–130.

Bouyer, J., and T. Lefrançois. 2014. Boosting the Sterile Insect Technique to control mosquitoes. Trends in Parasitology 30(6): 271–273.

Braga, I., and D. Valle. 2007. *Aedes aegypti*: Histórico do controle no Brasil. Epidemiologia e Serviços de Saúde 16: 113–118.

Brasil. 2009. Diretrizes nacionais para a prevenção e controle de epidemias de dengue. Ministerio da Saude, Secretaria de Vigilância em Saúde, and Departamento de Vigilância Epidemiológica. 1st ed. Série A. Normas e Manuais Técnicos. Brasilia, D. F., Brasil.

Brasil. 2016. Lei No 13.301, de 27 de junho de 2016. Dispõe sobre a adoção de medidas de vigilância em saúde quando verificada situação de iminente perigo à saúde pública pela presença do mosquito transmissor do vírus da dengue, do vírus chikungunya e do vírus da Zika; e altera a Lei no 6.437, de 20 de agosto de 1977. Diário Oficial da União, Brasilia, DF, 28 de junho de 2016. Seção 1, p. 1. ISSN 1677–7042

Buchman, A., S. Gamez, M. Li, I. Antoshechkin, H.-H. Li, H.-W. Wang, C.-H. Chen, M. J. Klein, J.-B. Duchemin, P. N. Paradkar, and O. S. Akbari. 2019. Engineered resistance to Zika virus in transgenic *Aedes aegypti* expressing a polycistronic cluster of synthetic small RNAs. Proceedings of the National Academy of Sciences of the United States of America 116 (9): 3656–3661.

Campos, G. S., A. C. O. Pinho, C. J. de Freitas, A. C. Bandeira, and S. I. Sardi. 2015. Dengue virus 4 (DENV-4) re-emerges after 30 years in Brazil: Cocirculation of DENV-2, DENV-3, and DENV-4 in Bahia. Japanese Journal of Infectious Diseases 68: 45–49.

Caprara, A., J. W. De Oliveira Lima, A. C. Rocha Peixoto, C. M. Vasconcelos Motta, J. M. Soares Nobre, J. Sommerfeld, and A. Kroeger. 2015. Entomological impact and social participation in dengue control: A cluster randomized trial in Fortaleza, Brazil. Transactions of the Royal Society of Tropical Medicine and Hygiene 109 (2): 99–105.

Capurro, M. L., D. O. Carvalho, L. Garziera, C. Pedrosa, I. Damasceno, I. Lima, B. Duarte, J. Fernandez-Virginio, R. S. Lees, and A. Malavasi. 2016. Description of social aspects surrounding releases of transgenic mosquitoes in Brazil. International Journal of Recent Scientific Research 7: 10363–10369.

Caputo, B., A. Ienco, D. Cianci, M. Pombi, V. Petrarca, A. Baseggio, G. J. Devine, and A. della Torre. 2012. The "auto-dissemination" approach: A novel concept to fight *Aedes albopictus* in urban areas. PLoS Neglected Tropical Diseases 6(8): e1793.

Caputo, B., A. Ienco, M. Manica, V, Petrarca, R. Rosà, and A. della Torre. 2015. New adhesive traps to monitor urban mosquitoes with a case study to assess the efficacy of insecticide control strategies in temperate areas. Parasites & Vectors 8: 134.

Carrillo-Hernández, M. Y., J. Ruiz-Saenz, L. Jaimes-Villamizar, S. Y. Gómez-Rangel, and M. Martínez-Gutierrez. 2018. Co-circulation and simultaneous co-infection of dengue, chikungunya, and Zika viruses in patients with febrile syndrome at the Colombian-Venezuelan border. BMC Infectious Diseases 18 (1): 61.

Carter, S. R., and R. M. Friedman. 2016. Policy and regulatory issues for gene drives in insects: Workshop report. J. Craig Venter Institute, San Diego, California, USA. 21 pp.

Carvalho, D. O., and M. L. Capurro. 2015. Community engagement, pp. 409–422. *In* J. Leonard (ed.), Genetic control of malaria and dengue. Elsevier, Amsterdam, The Netherlands.

Carvalho, D. O., A. L. Costa-da-Silva, R. S. Lees, and M. L. Capurro. 2014. Two step male release strategy using transgenic mosquito lines to control transmission of vector-borne diseases. Acta Tropica 132: 1–8.

Carvalho, D. O., A. R. McKemey, L. Garziera, R. Lacroix, C. A. Donnelly, L. Alphey, A. Malavasi, and M. L. Capurro. 2015. Suppression of a field population of *Aedes aegypti* in Brazil by sustained release of transgenic male mosquitoes. PLoS Neglected Tropical Diseases 9: e0003864.

Carvalho, S. A., S. O. da Silva, and I. D. C. Charret. 2019. Mathematical modeling of dengue epidemic: Control methods and vaccination strategies. Theory in Biosciences: 1–17.

Catteruccia, F., A. Crisanti, and E. A. Wimmer. 2009. Transgenic technologies to induce sterility. Malaria Journal 8 (Suppl. 2): S7.

(CDC) Centers for Disease Control and Prevention. 2017a. Integrated Mosquito Management - Zika virus. Atlanta, Georgia, USA.

(CDC) Centers for Disease Control and Prevention. 2017b. Integrated Mosquito Management for *Aedes aegypti* and *Aedes albopictus* mosquitoes. Atlanta, Georgia, USA.

(CDC) Centers for Disease Control and Prevention. 2017c. Information on aerial spraying - Zika virus. Atlanta, Georgia, USA.

Chaebo, G., and J. J. Medeiros. 2017. Conditions for policy implementation via co-production: The control of dengue fever in Brazil. Public Management Review 19 (10): 1381–1398.

Champer, J., J. Liu, S. Y. Oh, R. Reeves, A. Luthra, N. Oakes, A. G. Clark, and P. W. Messer. 2018. Reducing resistance allele formation in CRISPR gene drive. Proceedings of the National Academy of Sciences of the United States of America 115 (21): 5522–5527.

Chandra, G., I. Bhattacharjee, S. N. Chatterjee, and A. Ghosh. 2008. Mosquito control by larvivorous fish. The Indian Journal of Medical Research 127: 13–27.

Chediak, M., F. G. Pimenta, G. E. Coelho, I. A. Braga, J. B. P. Lima, K. R. L. Cavalcante, L. C. de Sousa, M. A. V. de Melo-Santos, M. L. G. Macoris, A. P. de Araújo, C. F. J. Ayres, M. T. M. Andrighetti, R. Gonçalves de Gomes, and R. N. C. Guedes. 2016. Spatial and temporal country-wide survey of temephos resistance in Brazilian populations of *Aedes aegypti*. Memorias Do Instituto Oswaldo Cruz 111: 311–321.

Chen, S. C., and M. H. Hsieh. 2012. Modeling the transmission dynamics of dengue fever: Implications of temperature effects. Science of the Total Environment 431: 385–391.

Claro, L. B. L., H. Kawa, L. T. Cavallini, and M. L. Garcia Rosa. 2006. Community participation in dengue control in Brazil. WHO Regional Office for South-East Asia. Dengue Bulletin 30: 214–222.

Coelho, G. E. 2012. Challenges in the control of *Aedes aegypti*. Revista do Instituto de Medicina Tropical de São Paulo 54 (Suppl. 18): S13–14.

De Barro, P. J., B. Murphy, C. C. Jansen, and J. Murray. 2011. The proposed release of the yellow fever mosquito, *Aedes aegypti* containing a naturally occurring strain of *Wolbachia pipientis*, a question of regulatory responsibility. Journal of Consumer Protection and Food Safety 6: 33–40.

Degener, C. M., T. M. F. de Ázara, R. A. Roque, C. T. Codeço, A. Araújo Nobre, J. J. Ohly, M. Geier, and Á. E. Eiras. 2014. Temporal abundance of *Aedes aegypti* in Manaus, Brazil, measured by two trap types for adult mosquitoes. Memórias Do Instituto Oswaldo Cruz 109: 1030–1040.

Degener, C. M., T. M. F. de Ázara, R. A. Roque, S. Rösner, E. S. Oliveira Rocha, E. G. Kroon, C. T. Codeço, A. Araújo Nobre, J. J. Ohly, M. Geier, and A. E. Eiras. 2015. Mass trapping with MosquiTRAPs does not reduce *Aedes aegypti* abundance. Memórias do Instituto Oswaldo Cruz 110 (4): 517–527.

Díaz, Y., J.-P. Carrera, L. Cerezo, D. Arauz, I. Guerra, J. Cisneros, B. Armién, A. M. Botello, A. B. Araúz, V. Gonzalez, Y. López, L. Moreno, S. López-Vergès, and B. A. Moreno. 2015. Chikungunya virus infection: First detection of imported and autochthonous cases in Panama. The American Journal of Tropical Medicine and Hygiene 92: 482–485.

Dutra, H. L. C., M. N. Rocha, F. B. S. Dias, S. B. Mansur, E. P. Caragata, and L. A. Moreira. 2016. *Wolbachia* blocks currently circulating Zika virus isolates in Brazilian *Aedes aegypti* mosquitoes. Cell Host & Microbe 19: 771–774.

Dyck, V. A., J. Hendrichs, and A. S. Robinson (eds.). 2021. Sterile Insect Technique – Principles and practice in Area-Wide Integrated Pest Management. Second Edition. CRC Press, Boca Raton, Florida, USA. 1200 pp.

El-Sabaawi, R. W., T. C. Frauendorf, P. S. Marques, R. A. Mackenzie, L. R. Manna, R. Mazzoni, D. A. Phillip, M. L. Warbanski, and E. Zandonà. 2016. Biodiversity and ecosystem risks arising from using guppies to control mosquitoes. Biology Letters 12: 20160590.

Evans, B. R., P. Kotsakiozi, A. L. Costa-da-Silva, R. S. Ioshino, L. Garziera, M. C. Pedrosa, A. Malavasi, J. F. Virginio, M. L. Capurro, and J. R. Powell. 2019. Transgenic *Aedes aegypti* mosquitoes transfer genes into a natural population. Scientific Reports 9: 13047.

Federici, B. A., H.-W. Park, D. K. Bideshi, M. C. Wirth, J. J. Johnson, Y. Sakano, and M. Tang. 2007. Developing recombinant bacteria for control of mosquito larvae. Journal of the American Mosquito Control Association 23 (Suppl. 2): 164–175.

(FIOCRUZ) Oswaldo Cruz Foundation. 2016. Nota Técnica No. 4/2016/IOC-FIOCRUZ/DIRETORIA. Considerações técnicas sobre a aplicação aérea de inseticidas em área urbana.

Flores, H. A., and S. L. O'Neill. 2018. Controlling vector-borne diseases by releasing modified mosquitoes. Nature Reviews Microbiology 16(8): 508–518.

Franz, A. W. E., I. Sanchez-Vargas, Z. N. Adelman, C. D. Blair, B. J. Beaty, A. A. James, and K. E. Olson. 2006. Engineering RNA interference-based resistance to dengue virus type 2 in genetically modified *Aedes aegypti*. Proceedings of the National Academy of Sciences of the United States of America 103 (11): 4198–4203.

Franz, A. W. E., I. Sanchez-Vargas, R. R. Raban, W. C. Black IV, A. A. James, and K. E. Olson. 2014. Fitness impact and stability of a transgene conferring resistance to dengue-2 virus following introgression into a genetically diverse *Aedes aegypti* strain. PLoS Neglected Tropical Diseases 8 (5): e2833.

Frentiu, F. D., T. Zakir, T. Walker, J. Popovici, A. T. Pyke, A. van den Hurk, E. A. McGraw, and S. L. O'Neill. 2014. Limited dengue virus replication in field-collected *Aedes aegypti* mosquitoes infected with *Wolbachia*. PLoS Neglected Tropical Diseases 8: e2688.

Furuya-Kanamori, L., S. Liang, G. Milinovich, R. J. S. Magalhaes, A. C. A. Clements, W. Hu, P. Brasil, F. D. Frentiu, R. Dunning, and L. Yakob. 2016. Co-distribution and co-infection of chikungunya and dengue viruses. BMC Infectious Diseases 16: 84.

Garziera, L. , M. C. Pedrosa, F. A. Souza, M. Gomez, M. B. Moreira, J. F. Virginio, M. Capurro, and D. O. Carvalho. 2017. Effect of interruption of over-flooding releases of transgenic mosquitoes over wild population of *Aedes aegypti*: Two case studies in Brazil. Entomologia Experimentalis et Applicata 164: 327–339.

Ghosh, S. K., P. Chakaravarthy, S. R. Panch, P. Krishnappa, S. Tiwari, V. P. Ojha, R. Manjushree, and A. P. Dash. 2011. Comparative efficacy of two poeciliid fish in indoor cement tanks against chikungunya vector *Aedes aegypti* in villages in Karnataka, India. BMC Public Health 11: 599.

Gubler, D. J., and G. C. Clark. 1996. Community involvement in the control of *Aedes aegypti*. Acta Tropica 61: 169–179.

Handler, A. M. 2002. Prospects for using genetic transformation for improved SIT and new biocontrol methods. Genetica 116: 137–149.

Harris, A. F., D. Nimmo, A. R. McKemey, N. Kelly, S. Scaife, C. A. Donnelly, C. Beech, W. D. Petrie, and L. Alphey. 2011. Field performance of engineered male mosquitoes. Nature Biotechnology 29: 1034–1037.

Harris, A. F., A. R. McKemey, D. Nimmo, Z. Curtis, I. Black, S. A. Morgan, M. N. Oviedo, R. Lacroix, N. Naish, N. I. Morrison, A. Collado, J. Stevenson, S. Scaife, T. Dafa'alla, G. Fu, C. Phillips, A. Miles, N. Raduan, N. Kelly, C. Beech, C. A. Donnelly, W. D. Petrie, and L. Alphey. 2012. Successful suppression of a field mosquito population by sustained release of engineered male mosquitoes. Nature Biotechnology 30: 828–830.

Healy, K., G. Hamilton, T. Crepeau, S. Healy, I. Unlu, A. Farajollahi, and D. M. Fonseca. 2014. Integrating the public in mosquito management: Active education by community peers can lead to significant reduction in peridomestic container mosquito habitats. PLoS One 9: e108504.

Hendrichs, J., and A. S. Robinson. 2009. Sterile Insect Technique, pp. 953–957. *In* V. H. Resh, and R. T. Cardé (eds.), Encyclopedia of insects. Second Edition. Academic Press, Burlington, Massachussetts, USA.

Honório, N. A., C. T. Codeço, F. C. Alves, M. A. F. M. Magalhães, and R. Lourenço-De-Oliveira. 2009. Temporal distribution of *Aedes aegypti* in different districts of Rio de Janeiro, Brazil, measured by two types of traps. Journal of Medical Entomology 46: 1001–1014.

Hood-Nowotny, R., L. Mayr, and B. Knols. 2006. Use of carbon-13 as a population marker for *Anopheles arabiensis* in a Sterile Insect Technique (SIT) context. Malaria Journal 5(1): 6.

Horstick, O., S. Runge-Ranzinger, M. B. Nathan, and A. Kroeger. 2010. Dengue vector-control services: How do they work? A systematic literature review and country case studies. Transactions of the Royal Society of Tropical Medicine and Hygiene 104(6): 379–386.

Ibrahim, M. A., N. Griko, M. Junker, and L. A. Bulla. 2010. *Bacillus thuringiensis*. Bioengineered Bugs 1: 31–50.

James, A. A. 2005. Gene drive systems in mosquitoes: Rules of the road. Trends in Parasitology 21 (2): 64–67.

Johnson, B. J., S. A. Ritchie, and D. M. Fonseca. 2017. The state of the art of lethal oviposition trap-based mass interventions for arboviral control. Insects 8(1): 5.

Jupatanakul, N., S. Sim, Y. I. Angleró-Rodríguez, J. Souza-Neto, S. Das, K. E. Poti, S. L. Rossi, N. Bergren, N. Vasilakis, and G. Dimopoulos. 2017. Engineered *Aedes aegypti* JAK/STAT pathway-mediated immunity to dengue virus. PLoS Neglected Tropical Diseases 11 (1): e0005187.

Kamareddine, L. 2012. The biological control of the malaria vector. Toxins 4(9): 748–767.

Kant, R., S. Haq, H. C. Srivastava, and V. P. Sharma. 2013. Review of the bioenvironmental methods for malaria control with special reference to the use of larvivorous fishes and composite fish culture in central Gujarat, India. Journal of Vector Borne Diseases 50: 1–12.

Kay, B., and V. S. Nam. 2005. New strategy against *Aedes aegypti* in Vietnam. The Lancet 365 (9459): 613–617.

Kittayapong, P., S. Yoksan, U. Chansang, C. Chansang, and A. Bhumiratana. 2008. Suppression of dengue transmission by application of integrated vector control strategies at sero-positive GIS-based foci. The American Journal of Tropical Medicine and Hygiene 78: 70–76.

Klassen, W., C. F. Curtis, and J. Hendrichs. 2021. History of the Sterile Insect Technique, pp. 1–44. *In* V. A. Dyck, J. Hendrichs, and A. S. Robinson (eds.), Sterile Insect Technique – Principles and practice in Area-Wide Integrated Pest Management. Second Edition. CRC Press, Boca Raton, Florida, USA.

Kokoza, V., A. Ahmed, S. Woon Shin, N. Okafor, Z. Zou, and A. S. Raikhel. 2010. Blocking of *Plasmodium* transmission by cooperative action of Cecropin A and Defensin A in transgenic *Aedes aegypti* mosquitoes. Proceedings of the National Academy of Sciences of the United States of America 107 (18): 8111–8116.

Lee, H. L., H. Jokob, W. A. Naznia, and S. S. Vasanc. 2009. Comparative life history parameters of transgenic and wild strains of *Ae. aegypti* in the laboratory. Dengue Bulletin 33: 103–114.

Lees, R. S., B. Knols, R. Bellini, M. Q. Benedict, A. Bheecarry, H. C. Bossin, D. D. Chadee, J. Charlwood, R. K. Dabiré, L. Djogbenou, A. Egyir-Yawson, R. Gato, L. C. Gouagna, M. M. Hassan, S. A. Khan, L. L. Koekemoer, G. Lemperiere, N. C. Manoukis, and J. R. L. Gilles. 2014. Review: Improving our knowledge of male mosquito biology in relation to genetic control programmes. Acta Tropica 132 (Suppl.): S2–S11.

Lees, R. S., D. Carvalho, and J. Bouyer. 2021. Potential impact of the integration of the Sterile Insect Technique in the fight against disease-transmitting mosquitoes, pp. 1081–1118. *In* V. A. Dyck, J. Hendrichs, and A. S. Robinson (eds.), Sterile Insect Technique – Principles and practice in Area-Wide Integrated Pest Management. Second Edition. CRC Press, Boca Raton, Florida, USA.

Leo, Y. S., A. L. P. Chow, L. K. Tan, D. C. Lye, L. Lin, and L. C. Ng. 2009. Chikungunya outbreak, Singapore, 2008. Emerging Infectious Diseases 15: 836–837.

Lloyd, A. M., M. Farooq, A. S. Estep, R.-D. Xue, and D. L. Kline. 2017. Evaluation of pyriproxyfen dissemination via *Aedes albopictus* from a point-source larvicide application in northeast Florida. Journal of the American Mosquito Control Association 33 (2): 151–155.

Lorenzi, O. D., C. Major, V. Acevedo, J. Perez-Padilla, A. Rivera, B. J. Biggerstaff, J. Munoz-Jordan, S. Waterman, R. Barrera, and T. M. Sharp. 2016. Reduced incidence of chikungunya virus infection in communities with ongoing *Aedes aegypti* mosquito trap intervention studies — Salinas and Guayama, Puerto Rico, November 2015–February 2016. Morbidity and Mortality Weekly Report 65(18): 479–480.

Luksic, B., N. Pandak, E. Drazic-Maras, S. Karabuva, M. Radic, A. Babic-Erceg, L. Barbic, V. Stevanovic, and T. Vilibic-Cavlek. 2017. First case of imported chikungunya infection in Croatia, 2016. International Medical Case Reports Journal 10: 117–121.

Maciel-de-Freitas, R., R. Aguiar, R. V. Bruno, M. C. Guimarães, R. Lourenço-de-Oliveira, M. H. F. Sorgin, C. J. Struchiner, D. Valle, S. L O'Neill, and L. A. Moreira. 2012. Why do we need alternative tools to control mosquito-borne diseases in Latin America? Memorias Do Instituto Oswaldo Cruz 107: 828–829.

Mackay, A. J., M. Amador, and R. Barrera. 2013. An improved autocidal gravid ovitrap for the control and surveillance of *Aedes aegypti*. Parasites & Vectors 6(1): 225.

Mains, J. W., C. L. Brelsfoard, and S. L. Dobson. 2015. Male mosquitoes as vehicles for insecticide. PLoS Neglected Tropical Diseases 9(1): e0003406.

Mains, J. W., C. L. Brelsfoard, R. I. Rose, and S. L. Dobson. 2016. Female adult *Aedes albopictus* suppression by *Wolbachia*-infected male mosquitoes. Scientific Reports 6 (September): 33846.

Martelli, C. M. T., J. B. Siqueira, M. P. P. D. Parente, A. L. S. A. Zara, C. S. Oliveira, C. Braga, F. G. Pimenta Junior, F. Cortes, J. G. Lopez, L. R. Bahia, M. C. Ooteman Mendes, M. Q. Machado da Rosa, N. T. Siqueira Filha, D. Constenla, and W. V. Souza. 2015. Economic impact of dengue: Multicenter study across four Brazilian regions. Edited by M. S. Carvalho. PLoS Neglected Tropical Diseases 9 (9): e0004042.

Martins, A. J., J. B. Lima, A. A. Peixoto, and D. Valle. 2009. Frequency of Val1016Ile mutation in the voltage-gated sodium channel gene of *Aedes aegypti* Brazilian populations. Tropical Medicine & International Health 14: 1351–1355.

Maurer-Stroh, S., T.-M. Mak, Y.-K. Ng, S.-P. Phuah, R. G. Huber, J. K. Marzinek, D. A Holdbrook, R. T. C. Lee, L. Cui, and R. T. Lin. 2016. South-east Asian Zika virus strain linked to cluster of cases in Singapore, August 2016. Euro Surveillance 21(38): 30347.

Mendenhall, I. H., M. Manuel, M. Moorthy, T. T. M., Lee, D. H. W. Low, D. Missé, D. J. Gubler, B. R. Ellis, E. E. Ooi, and J. Pompon. 2017. Peridomestic *Aedes malayensis* and *Aedes albopictus* are capable vectors of arboviruses in cities. PLoS Neglected Tropical Diseases 11(6): e0005667.

Morales-Pérez, A., E. Nava-Aguilera, J. Legorreta-Soberanis, A. J. Cortés-Guzmán, A. Balanzar-Martínez, E. Harris, J. Coloma, V. M. Alvarado-Castro, M. V. Bonilla-Leon, L. Morales-Nava, R. J. Ledogar, A. Cockcroft, and N. Andersson. 2017. Where we put little fish in the water there are no mosquitoes: A cross-sectional study on biological control of the *Aedes aegypti* vector in 90 coastal-region communities of Guerrero, Mexico. BMC Public Health 17 (Suppl. 1): 433.

(MS) Ministério da Saúde. 2014a. Larvicidas. Brasilia, DF, Brasil.

(MS) Ministério da Saúde. 2014b. Aplicação espacial de inseticidas. Brasilia, DF, Brasil.

(MS) Ministério da Saúde. 2017a. Controle de vetores / Inseticidas e larvicidas. Brasilia, DF, Brasil.

(MS) Ministério da Saúde. 2017b. Zika virus in Brazil: The SUS Response. Brasilia, DF, Brasil.

(MS) Ministério da Saúde. 2017c. Monitoramento dos casos de dengue, febre de chikungunya e febre pelo vírus Zika até a Semana Epidemiológica 4, 2017. Brasilia, DF, Brasil.

(MS/FNS) Ministério da Saúde / Fundação Nacional de Saúde. 2002. Programa Nacional de Controle da Dengue (PNCD). Ministério da Saúde. Fundação Nacional de Saúde Brasilia, DF, Brasil.

(MS/FNS) Ministério da Saúde / Fundação Nacional de Saúde. 2009. Diretrizes nacionais para a prevenção e controle de epidemias de dengue. Secretaria de Vigilancia em Saúde. Brasilia, DF, Brasil.

(NASEM) National Academies of Sciences, Engineering, and Medicine. 2016. Global health impacts of vector-borne diseases: Workshop summary. National Academies Press. Washington DC, USA. 396 pp.

Ndii, M. Z., R. I. Hickson, D. Allingham, and G. N. Mercer. 2015. Modelling the transmission dynamics of dengue in the presence of *Wolbachia*. Mathematical Biosciences 262: 157–166.

O'Connor, L., C. Plichart, A. C. Sang, C. L. Brelsfoard, H. C. Bossin, and S. L. Dobson. 2012. Open release of male mosquitoes infected with a *Wolbachia* biopesticide: Field performance and infection containment. Edited by Jason L. Rasgon. PLoS Neglected Tropical Diseases 6 (11): e1797.

Okamoto, K. W., M. A. Robert, A. L. Lloyd, and F. Gould. 2013. A reduce and replace strategy for suppressing vector-borne diseases: Insights from a stochastic, spatial model. PLoS One 8(12): e81860.

Ong, J., X. Liu, J. Rajarethinam, G. Yap, D. Ho, and L. Ching Ng. 2019. A novel entomological index, *Aedes aegypti* breeding percentage, reveals the geographical spread of the dengue vector in Singapore and serves as a spatial risk indicator for dengue. Parasites & Vectors 12 (1): 17.

Ooi, E.-E., and D. J. Gubler. 2009. Dengue in Southeast Asia: Epidemiological characteristics and strategic challenges in disease prevention. Cadernos de Saúde Pública 25 (Suppl. 1): S115–S124.

Ooi, E.-E., K.-T. Goh, and D. J. Gubler. 2006. Dengue prevention and 35 years of vector control in Singapore. Emerging Infectious Diseases 12(6): 887–893.

O Silva, M. M., L. B. Tauro, M. Kikuti, R. O. Anjos, V. C. Santos, T. S. F. Gonçalves, I. A. D. Paploski, P. S. S. Moreira, L. C. J. Nascimento, G. S. Campos, A. I. Ko, S. C. Weaver, M. G. Reis, U. Kitron, and G. S. Ribeiro. 2018. Concomitant transmission of dengue, chikungunya and Zika viruses in Brazil: Clinical and epidemiological findings from surveillance for acute febrile illness. Clinical Infectious Diseases 68 (8): 1353–1359.

Paes de Andrade, P., F. J. L. Aragão, W. Colli, O. A. Dellagostin, F. Finardi-Filho, M. H. Hirata, A. de Castro Lira-Neto, M. Almeida de Melo, A. L. Nepomuceno, F. G. da Nóbrega, G. D. de Sousa, F. H. Valicente, and M. H. B. Zanettini. 2016. Use of transgenic *Aedes aegypti* in Brazil: Risk perception and assessment. Bulletin of the World Health Organization 94(10): 766–771.

(PAHO) Pan American Health Organization. 2016. Vector borne diseases in the region of the Americas (2013–2016). Washington, DC, USA.

(PAHO) Pan American Health Organization. 2018. Integrated management strategy for dengue prevention and control in the region of the Americas. Washington, DC, USA.

(PAHO/WHO) Pan American Health Organization / World Health Organization. 2003. 44th Directing Council. 22-26 September 2003. Washington, DC, USA.

(PAHO/WHO) Pan American Health Organization / World Health Organization. 2014. Number of reported cases of chikungunya fever in the Americas by country or territory 2013–2014. Washington, DC, USA.

(PAHO/WHO) Pan American Health Organization / World Health Organization. 2016. Zika suspected and confirmed cases reported by countries and territories in the Americas, 2015–2017, cumulative cases. Washington, DC, USA.

Parks, W., and L. Lloyd. 2004. Planning social mobilization and communication for dengue fever prevention and control: A step-by-step guide. UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases. World Health Organization. Geneva, Switzerland. 138 pp.

Paz-Soldan, V. A., J. Yukich, A. Soonthorndhada, M. Giron, C. S. Apperson, L. Ponnusamy, C. Schal, A. C. Morrison, J. Keating, and D. M. Wesson. 2016. Design and testing of novel lethal ovitrap to reduce populations of *Aedes* mosquitoes: Community-Based Participatory Research between Industry, Academia and Communities in Peru and Thailand PLoS One 11 (8): e0160386

Perich, M. J., A. Kardec, I. A. Braga, I. F. Portal, R. Burge, B. C. Zeichner, W. A. Brogdon, and R. A. Wirtz. 2003. Field evaluation of a lethal ovitrap against dengue vectors in Brazil. Medical and Veterinary Entomology 17: 205–210.

Pessanha, J. E. M., W. T. Caiaffa, C. C. César, and F. A. Proietti. 2009. Avaliação do plano nacional de controle da dengue. Cadernos de Saúde Pública 25(7): 1637–1641.

Pruszynski, C. A., L. J. Hribar, R. Mickle, and A. L. Leal. 2017. A large scale biorational approach using *Bacillus thuringiensis israelensis* (strain AM65-52) for managing *Aedes aegypti* populations to prevent dengue, chikungunya and Zika transmission. PLoS One 12(2): e0170079.

Regis, L., A. M. Monteiro, M. A. V. de Melo-Santos, J. C. Silveira Jr, A. F. Furtado, R. V. Acioli, G. M. Santos, M. Nakazawa, M. S. Carvalho, P. J. Ribeiro Jr, and W. V. de Souza. 2008. Developing new approaches for detecting and preventing *Aedes aegypti* population outbreaks: Basis for surveillance, alert and control system. Memorias Do Instituto Oswaldo Cruz 103: 50–59.

Regis, L. N., R. V. Acioli, J. C. Silveira Jr, M. A. V. Melo-Santos, W. V. Souza, C. M. N. Ribeiro, J. C. S. da Silva, A. M. Vieira Monteiro, C. M. F. Oliveira, R. M. R. Barbosa, C. Braga, M. A. Benedetti Rodrigues, M. Gomes N. M. Silva, P. J. Ribeiro Jr., W. H. Bonat, L. C. de Castro Medeiros, M. Sa Carvalho, and A. F. Furtado. 2013. Sustained reduction of the dengue vector population resulting from an integrated control strategy applied in two Brazilian cities. PLoS One 8(7): e67682.

Ritchie, S. A., L. P. Rapley, C. Williams, P. H. Johnson, M. Larkman, R. M. Silcock, S. A. Long, and R. C. Russell. 2009. A lethal ovitrap-based mass trapping scheme for dengue control in Australia: I. Public acceptability and performance of lethal ovitraps. Medical and Veterinary Entomology 23 (4): 295–302.

Rodriguez-Morales, A. J., W. E. Villamil-Gómez, and C. Franco-Paredes. 2016. The arboviral burden of disease caused by co-circulation and co-infection of dengue, chikungunya and Zika in the Americas. Travel Medicine and Infectious Disease 14 (3): 177–179.

Rosenbaum, J., M. B. Nathan, R. Ragoonanansingh, S. Rawlins, C. Gayle, D. D. Chadee, and L. S. Lloyd. 1995. Community participation in dengue prevention and control: A survey of knowledge, attitudes, and practice in Trinidad and Tobago. The American Journal of Tropical Medicine and Hygiene 53(2): 111–117.

Rückert, C., J. Weger-Lucarelli, S. M. Garcia-Luna, M. C. Young, A. D. Byas, R. A. Murrieta, J. R. Fauver, and G. D. Ebel. 2017. Impact of simultaneous exposure to arboviruses on infection and transmission by *Aedes aegypti* mosquitoes. Nature Communications 8: 15412.

Samuel, M., D. Maoz, P. Manrique, T. Ward, S. Runge-Ranzinger, J. Toledo, R. Boyce, and O. Horstick. 2017. Community effectiveness of indoor spraying as a dengue vector control method: A systematic review. PLoS Neglected Tropical Diseases 11(8): e0005837.

San Martín, J. L., and O. Brathwaite-Dick. 2007. La estrategia de gestión integrada para la prevención y el control del dengue en la región de las Américas. Revista Panamericana de Salud Pública 21: 55–63.

Sanchez, L., J. Maringwa, Z. Shkedy, M. Castro, N. Carbonell, and P. Van der Stuyft. 2012. Testing the effectiveness of community-based dengue vector control interventions using semiparametric mixed models. Vector-Borne and Zoonotic Diseases 12(7): 609–615.

(SHS) Secretariat of Health Surveillance. 2017. Health Brazil 2015/2016. An analysis of health situation and the epidemic caused by Zika virus and other diseases transmitted by *Aedes aegypti*. Ministry of Health of Brazil. Braslia, DF, Brazil. 132 pp.

Sinkins, S. P. 2004. *Wolbachia* and cytoplasmic incompatibility in mosquitoes. Insect Biochemistry and Molecular Biology 34(3): 723–729.

Sithiprasasna, R., P. Mahapibul, C. Noigamol, M. J. Perich, B. C. Zeichner, B. Burge, S. L. Norris, J. W. Jones, S. S. Schleich, and R. E. Coleman. 2003. Field evaluation of a lethal ovitrap for the control of *Aedes aegypti* (Diptera: Culicidae) in Thailand. Journal of Medical Entomology 40(4): 455–462.

Sommerfeld, J., and A. Kroeger. 2015. Innovative community-based vector control interventions for improved dengue and chagas disease prevention in Latin America: Introduction to the Special Issue. Transactions of the Royal Society of Tropical Medicine and Hygiene 109 (2): 85–88.

Spiegel, J., A. Yassi, and R. Tate. 2002. Dengue in Cuba: Mobilisation against *Aedes aegypti*. The Lancet Infectious Diseases 2(4): 207–208.

(SS-PE) Secretaria de Saúde de Pernambuco (2015). Boletim epidemiológico arboviroses transmitidas pelo *Aedes aegypti*. Pernambuco, Brasil.

Suter, T., M. M. Crespo, M. F. de Oliveira, T. S. A. de Oliveira, M. A. V. de Melo-Santos, C. M. F. de Oliveira, C. F. J. Ayres, R. M. Rodrigues Barbosa, A. P. Araújo, L. N. Regis, E. Flacio, L. Engeler, P. Müller, and M. H. N. L. Silva-Filha. 2017. Insecticide susceptibility of *Aedes albopictus* and *Ae. aegypti* from Brazil and the Swiss-Italian border region. Parasites & Vectors 10(1): 431.

Suwannapong, N., M. Tipayamongkholgul, A. Bhumiratana, C. Boonshuyar, N. Howteerakul, and S. Poolthin. 2014. Effect of community participation on household environment to mitigate dengue transmission in Thailand. Tropical Biomedicine 31: 149–158.

Suwanmanee, S., P. Surasombatpattana, N. Soonthornworasiri, R. Hamel, P. Maneekan, D. Missé, and N. Luplertlop. 2018. Monitoring arbovirus in Thailand: Surveillance of dengue, chikungunya and Zika virus, with a focus on coinfections. Acta Tropica 188: 244–250.

Tapia-Conyer, R., J. Méndez-Galván, and P. Burciaga-Zúñiga. 2012. Community participation in the prevention and control of dengue: The *patio limpio* strategy in Mexico. Pediatrics and International Child Health 32 (Suppl. 1): 10–14.

Tauil, P. L. 2002. Aspectos críticos do controle do dengue no Brasil. Cadernos de Saúde Pública 18 (June): 867–871.

Townson, H., M. B. M. Nathan, M. Zaim, P. Guillet, L. Manga, R. Bos, and M. Kindhauser. 2005. Exploiting the potential of vector control for disease prevention. Bulletin of the World Health Organization 83(12): 942–947.

Travanty, E. A., Z. N. Adelman, A. W. Franz, K. M. Keene, B. J. Beaty, C. D. Blair, A. A. James, and K. E. Olson. 2004. Using RNA interference to develop dengue virus resistance in genetically modified *Aedes aegypti*. Insect Biochemistry and Molecular Biology 34(7): 607–613.

Ulibarri, G., B. Betanzos, M. Betanzos, and J. J. Rojas. 2016. Control of *Aedes aegypti* in a remote Guatemalan community vulnerable to dengue, chikungunya and Zika virus: Prospective evaluation of an integrated intervention of web-based health worker training in vector control, low-cost ecological ovillantas, and community engagement. F1000Research 5: 598.

Unckless, R. L., A. G. Clark, and P. W. Messer. 2017. Evolution of resistance against CRISPR/Cas9 gene drive. Genetics 205(2): 827–841.

Unlu, I., D. S. Suman, Y. Wang, K. Klingler, A. Faraji, and R. Gaugler. 2017. Effectiveness of autodissemination stations containing pyriproxyfen in reducing immature *Aedes albopictus* populations. Parasites & Vectors 10(1): 139.

Unlu, I., G. M. Williams, I. Rochlin, D. Suman, Y. Wang, K. Chandel, and R. Gaugler. 2018. Evaluation of lambda-cyhalothrin and pyriproxyfen barrier treatments for *Aedes albopictus* (Diptera: Culicidae) management in urbanized areas of New Jersey. Journal of Medical Entomology 55 (2): 472–76.

(US-EPA) United States Environmental Protection Agency. 2017. Success in mosquito control: An integrated approach. Washington, DC, USA.

Van den Berg, H., C. M. Mutero, and K. Ichimori. 2012. Guidance on policy-making for Integrated Vector Management. Department of Control of Neglected Tropical Diseases, World Health Organization, Geneva, Switzerland. 11 pp.

Van den Hurk, A. F., S. Hall-Mendelin, A. T. Pyke, F. D. Frentiu, K. McElroy, A. Day, S. Higgs, and S. L. O'Neill. 2012. Impact of *Wolbachia* on infection with chikungunya and yellow fever viruses in the mosquito vector *Aedes aegypti*. PLoS Neglected Tropical Diseases 6(11): e1892.

Vanlerberghe, V., M. E. Toledo, M. Rodríguez, D. Gomez, A. Baly, J. R. Benitez, and P. Van der Stuyft. 2009. Community involvement in dengue vector control: Cluster randomised trial. BMJ 338: b1959.

Vreysen, M. J. B., K. Saleh, F. Mramba, A. Parker, U. Feldmann, V. A. Dyck, A. Msangi, and J. Bouyer. 2014. Sterile insects to enhance agricultural development: The case of sustainable tsetse eradication on Unguja Island, Zanzibar, using an Area-Wide Integrated Pest Management approach. PLoS Neglected Tropical Diseases 8(5): e2857.

Vreysen, M. J. B. 2021. Monitoring sterile and wild insects in Area-Wide Integrated Pest Management programmes, pp. 485–528. *In* V. A. Dyck, J. Hendrichs, and A. S. Robinson (eds.), Sterile Insect Technique – Principles and practice in Area-wide Integrated Pest Management. Second Edition. CRC Press, Boca Raton, Florida, USA.

Walshe, D. P., P. Garner, A. A. Abdel-Hameed Adeel, G. H. Pyke, and T. Burkot. 2013. Larvivorous fish for preventing malaria transmission. Cochrane Database of Systematic Reviews 10 (12): CD008090.

(WHO) World Health Organization. 2004. Global strategic framework for Integrated Vector Management. Geneva, Switzerland.

(WHO) World Health Organization. 2008. Position statement on Integrated Vector Management. Geneva, Switzerland.

(WHO) World Health Organization. 2009. Dengue guidelines for diagnostics, treatment, prevention and control. Geneva, Switzerland.

(WHO) World Health Organization. 2013. WHO recommended insecticides for indoor residual spraying against malaria vectors. Geneva, Switzerland.

(WHO) World Health Organization. 2016a. Dengue and severe dengue. Geneva, Switzerland.

(WHO) World Health Organization. 2016b. Zika Strategic Response Plan Quarterly. Geneva, Switzerland.

(WHO) World Health Organization. 2016c. Number of reported cases of Chikungunya fever in the Americas in 2016. Geneva, Switzerland.

(WHO) World Health Organization. 2017a. Situation report: Zika virus, microcephaly, Guillain-Barré Syndrome, 10 March 2017. Situation Report. Geneva, Switzerland.

(WHO) World Health Organization. 2017b. Chikungunya. Geneva, Switzerland.

(WHO) World Health Organization. 2017c. Global vector control response 2017-2030. Geneva, Switzerland.

(WHO) World Health Organization. 2017d. Dengue control strategies. Geneva, Switzerland.

(WHO) World Health Organization. 2017e. Environmental management. Geneva, Switzerland.

(WHO) World Health Organization. 2017f. Chemical control. Geneva, Switzerland.

(WHO) World Health Organization. 2018. Yellow fever.

Zabalou, S., A. Apostolaki, I. Livadaras, G. Franz, A. S. Robinson, C. Savakis, and K. Bourtzis. 2009. Incompatible Insect Technique: Incompatible males from a *Ceratitis capitata* genetic sexing strain. Entomologia Experimentalis et Applicata 132: 232–240.

Zahir, A. 2016. Community participation, dengue fever prevention and practices for control in Swat, Pakistan. International Journal of Maternal and Child Health and AIDS 5: 39–45.

Zanluca, C., V. C. A. de Melo, A. L. P. Mosimann, G. I. V. dos Santos, C. N. D. dos Santos, and K. Luz. 2015. First report of autochthonous transmission of Zika virus in Brazil. Memórias Do Instituto Oswaldo Cruz 110: 569–572.

Zara, A. L. S. A., S. M. Santos, E. S. Fernandes-Oliveira, R. G. Carvalho, and G. E. Coelho, 2016. *Aedes aegypti* control strategies: A review. Epidemiologia e Serviços de Saúde 25(2): 391–404.

COMBINING THE INCOMPATIBLE AND STERILE INSECT TECHNIQUES FOR PEST AND VECTOR CONTROL

L. A. BATON[1], D. ZHANG[2], Y. LI[2] AND Z. XI[1,2]

[1]*Department of Microbiology and Molecular Genetics, Michigan State University, East Lansing, Michigan 48824, USA; xizy@msu.edu*
[2]*Key Laboratory of Tropical Disease Control of the Ministry of Education, Sun Yat-sen University - Michigan State University Joint Center of Vector Control for Tropical Diseases, Zhongshan School of Medicine, Sun Yat-Sen University, Guangzhou, Guangdong 510080, China*

SUMMARY

The Incompatible Insect Technique (IIT) is a Sterile Insect Technique (SIT)-related approach that uses the reproductive parasitism caused by infection with maternally-inherited bacterial endosymbionts to make released males reproductively incompatible with the wild-type females of the target population. The most common and widespread of such endosymbionts is *Wolbachia*, which is found throughout many insect orders, and often causes cytoplasmic incompatibility (CI), a form of conditional sterility where the fertilized eggs of females not infected with the same *Wolbachia* strain as the males with which they are mated undergo embryonic death. An advantage of IIT is that the incompatibility induced by *Wolbachia* often has either no or only minor effects on the quality of infected males. In addition, such endosymbionts can also have other desirable phenotypic effects on their hosts, such as reducing the ability of target species to act as disease vectors, thus allowing the undesirable sex(es) to be tolerated among the sterile insects to be released. However, an inherent problem with IIT, which has so far restricted its operational use, is that, unlike SIT, the accidental release of endosymbiont-infected females may prevent further population suppression by causing unintended population replacement, whereby the original target population is replaced with individuals infected with the same endosymbiont strain as the released males. A solution to this problem, at least for the majority of insects whose females are more sensitive than males to radiation, is to combine IIT with SIT, such that all endosymbiont-infected individuals destined for release are also first subjected to low-dose radiation, which completely sterilizes any contaminant females without affecting the incompatibility or quality of the irradiated males. Here, we discuss the biology and general theoretical principles underlying the use of IIT alone, and the rationale and necessity of combining IIT with SIT, as well as the logistical problems encountered, and technological developments required, for the mass-production and release of irradiated endosymbiont-infected individuals as part of area-wide integrated pest control programmes. We primarily illustrate our discussion with examples involving mosquitoes, for which the majority of the relevant research has been conducted, including the first open-release field trial of combined IIT/SIT application against the important arboviral vector *Aedes albopictus* (Skuse). However,

J. Hendrichs, R. Pereira and M. J. B. Vreysen (eds.), Area-Wide Integrated Pest Management: Development and Field Application, pp. 367–404. CRC Press, Boca Raton, Florida, USA.

the combined IIT/SIT approach should be broadly applicable to a wide range of other insect pests and vectors, and so of interest to entomologists in general.

Key Words: Aedes, *Wolbachia,* combined IIT/SIT, cytoplasmic incompatibility, symbiosis, arboviruses, mosquitoes, radiation, vector-borne diseases

1. INTRODUCTION

The irradiation-based Sterile Insect Technique (SIT) has been successfully used to suppress the populations of a number of insect pests and vectors of agricultural and veterinary importance (Dyck et al. 2021). However, the application of the SIT against other important groups of insects, especially the mosquito vectors of human pathogens, has, so far, been limited (Benedict and Robinson 2003; Dame et al. 2009; Bourtzis et al. 2016; Scott and Benedict 2016). There are various reasons for this, and they have been debated, but the development and implementation of the SIT for such insects continues, and is still an active and productive area of research, and it is hoped that, with further investigation and optimization, the SIT can be successfully and operationally deployed against mosquitoes in the not so distant future (Alphey et al. 2010; Bourtzis et al. 2016; Lees et al. 2015, 2021).

Concurrently, however, other approaches have also been, and continue to be, explored (Alphey 2014; Scott and Benedict 2016). One alternative approach is the Incompatible Insect Technique (IIT), which uses infection with maternally-inherited prokaryotic endosymbionts, such as the alpha-proteobacterium *Wolbachia*, to suppress host populations. Like other sterile-male-based methods of population suppression, these endosymbionts make the released males reproductively incompatible with wild-type females in the target population.

In this chapter, we outline the biological and theoretical basis of endosymbiont-mediated IIT and argue why it is an attractive alternative to the SIT for some groups of insects. However, in the absence of a perfect sex separation system, IIT application has a fundamental constraint necessitating its combination with the SIT. Our discussion primarily concerns mosquitoes, but there is an increasingly large research literature on endosymbionts causing reproductive parasitism in a wide range of insect hosts, which readers are encouraged to explore for themselves.

2. THE STERILE INSECT TECHNIQUE (SIT)

Historically, there have been a number of laboratory tests and pilot field trials of irradiation-based SIT against mosquitoes (Benedict and Robinson 2003; Dame et al. 2009). Despite this, the SIT against mosquitoes has not yet been deployed operationally on a larger scale. Various reasons have been given for this, such as the failure to effectively optimize the timing and magnitude of the irradiation dose used on mass-reared individuals, the high rate of intrinsic increase of these insects and the failure to release sufficient males, an inability to efficiently and cost-effectively mass-produce and/or release them, a lack of knowledge regarding the basic biology and ecology of the target species, and inadequate methods of sex separation (Dame et al. 2009; Scott and Benedict 2016).

More recently, interest in using the SIT against mosquitoes has been revived, and the positive results of small-scale field trials (Bellini et al. 2007, 2013b) support the notion that the SIT is feasible against mosquitoes. In addition, over the past decade, the Food and Agriculture Organization of the United Nations (FAO) and the International Atomic Energy Agency (IAEA), in response to increasing requests from their member states, have increased their efforts to explore and disseminate the possibilities of integrating the SIT within AW-IPM approaches to manage better mosquito populations (Bourtzis et al. 2016; Lees et al. 2013, 2015; 2021).

2.1. Reduced Quality of Irradiation-sterilized Insects

A requirement of the SIT is that radiation-based sterilisation does not have serious adverse effects on male competitiveness or overall quality (Knipling 1955). Such effects may derive either from the direct deleterious effects of radiation itself, or indirectly through the mass-rearing and handling procedures and ambient conditions required for the administration of radiation (Bourtzis and Robinson 2006; Bakri et al. 2021). Insects vary in their radio-sensitivity, with some species being inherently more sensitive to the effects of irradiation, such that irradiation doses inducing high levels of male sterility often also have appreciable negative effects on male quality (e.g. mating competitiveness and survival) for some insect species (Bakri et al. 2005; Helinski et al. 2009). For mosquitoes, the process of irradiation has frequently been reported to reduce male competitiveness and survival (Arunachalam and Curtis 1985; Dame et al. 2009; Helinski et al. 2009; Oliva et al. 2012; Maïga et al. 2014; Yamada et al. 2014a, 2014b; Zhang et al. 2016; Zheng et al. 2019). However, whether such negative effects are due to the radiation itself, or the conditions and procedures under which the radiation is administered is often unclear (Scott and Benedict 2016; Yamada et al. 2019). The latter can impose significant fitness costs independent of the effects of radiation itself, which can reduce their quality for use in the SIT.

Many pest/vector species are fragile, with complex holometabolous life cycles, complicating their handling and irradiation, especially under the conditions of mass-rearing and mass-release required for the SIT. Different life cycle stages may also vary in their radio-sensitivity (e.g. late pupae versus adults), and careful timing of irradiation can help to minimize radiation-induced damage, as well as maximize sex-specific differences in radio-sensitivity, which is important for sterilizing contaminant females without adversely affecting male quality (see Section 4.2) (Andreasen and Curtis 2005; Helinski et al. 2006; Brelsfoard et al. 2009; Balestrino et al. 2010; Ndo et al. 2014; Zhang et al. 2015b).

Regardless of whether the adverse effects of irradiation are direct or indirect – and whether or not it might be possible in future to ameliorate such effects through optimization of irradiation protocols and development of better technology – for some groups of insects there is currently a necessary trade-off between sterility and quality, such that, as higher irradiation doses increase male sterility, they simultaneously decrease male quality (Helinski and Knols 2008; Balestrino et al. 2010; Bellini et al. 2013a). In many instances, intermediate irradiation doses can be identified that provide an optimal balance between male sterility and quality (Parker and Mehta 2007; Helinski et al. 2009). Consequently, the use of the SIT may not be precluded

(Bellini et al. 2007, 2013b; Scott and Benedict 2016), although its overall efficiency may be reduced, and its cost-effectiveness decreased, through necessitating larger numbers of insects to be produced and released during area-wide control programmes. For target insects with very high reproductive potential, like some mosquito species (Alphey et al. 2010), the problem of trading-off residual fertility against male quality could be particularly acute, because population can rebound easily through those survived eggs as seeds. With low residual fertility, the number of emerging adults in the wild may be relatively high as the low number of hatching eggs is compensated by low competition for resources among surviving larvae, and hence higher survival rates during the development. Therefore, minimum levels of sterility are necessarily required to overcome the intrinsic growth of the target population (Barclay 2021).

2.2. Imperfect Sex Separation

Another problem for the implementation of the SIT, as well as all other sterile-insect-based methods, against insects like mosquitoes where adult females (and not males) of the target species are the pests/vectors, is the absence of perfect sex separation methods (Gilles et al. 2014). Thus, the release of females, even as relatively small numbers of contaminant individuals, is considered unacceptable for SIT applications to control those pests/vectors due to the risk of increased crop destruction, parasitism or pathogen transmission. In other instances, where either males or both sexes act as pests/vectors, the release of any sterile individuals has to be carefully managed, e.g. feeding tsetse males with trypanocidal drugs before their release or using strains with enhanced vector refractoriness (Kariithi et al. 2018). New methods are, therefore, needed that either completely remove any females from among the insects to be released, or reduce the ability of target species to act as pests/vectors, thus allowing the undesirable sex(es) to be tolerated among the sterile insects to be released.

3. THE INCOMPATIBLE INSECT TECNIQUE (IIT)

3.1. Wolbachia, *Cytoplasmic Incompatibility and Population Suppression*

The IIT is an analogue of the SIT, using infection with naturally-occurring maternally-inherited bacterial endosymbionts that cause reproductive parasitism – instead of radiation – to make released males reproductively incompatible with females of the target field population (Bourtzis et al. 2014; Scott and Benedict 2016; Xi and Joshi 2016). *Wolbachia* is the most common and widespread of such endosymbionts (Werren et al. 2008), being found throughout many insect orders, i.e. it is estimated to infect between approximately 48 to 57% of all terrestrial arthropods (Hilgenboecker et al. 2008; Zug and Hammerstein 2012; Weinert et al. 2015). One of the manipulations of host reproduction caused by *Wolbachia* is cytoplasmic incompatibility (CI), a form of conditional sterility whereby the fertilized eggs of females not infected with the same *Wolbachia* strain as the males with which they are mated, undergo embryonic death (Sinkins 2004; Werren et al. 2008; Hurst and Frost 2015). In contrast, *Wolbachia*-infected females produce off-spring normally, whether mated with uninfected males or with males infected with the same *Wolbachia* strain.

The level of CI induced can vary considerably between *Wolbachia* strains: some strains do not cause CI – or any other reproductive manipulation – while others cause either partial or complete CI that either only kills some or all embryos, respectively. In general, *Wolbachia* and other similar endosymbiotic reproductive parasites are only maternally-inherited, but the level of transmission from mother-to-offspring can vary considerably. In mosquitoes, native *Wolbachia* infections typically exhibit very high levels (~100%) of both CI and maternal transmission (Sinkins 2004; Baton et al. 2013), while these characteristics are often markedly lower (<50%) and more variable in other Diptera, such as well-studied drosophilids.

As CI prevents uninfected females – or those infected with a different incompatible *Wolbachia* strain – from having off-spring, infected females leave more off-spring. The consequence is that, over succeeding generations, uninfected females can be driven to extinction as the number of infected females increases, potentially resulting in complete replacement of the original uninfected host population with *Wolbachia*-infected individuals (Caspari and Watson 1959; Fine 1978). The speed and extent with which population replacement occurs, i.e. replacing the original uninfected host population with *Wolbachia*-infected individuals, primarily depends on the level of CI-induced and the rate of endosymbiont maternal transmission, as well as whether or not the endosymbiont has any fitness costs or benefits for its hosts. When CI is complete, maternal transmission is perfect, and the endosymbiont has no fitness costs, complete population replacement is expected, and, at least theoretically, is predicted to be very rapid: occurring in about 100 generations, from a very low (~1%) initial proportion. If the initial proportion is higher (e.g. >10%), population replacement could occur in <10 generations (Caspari and Watson 1959; Fine 1978).

When CI is partial, and/or maternal transmission imperfect, and/or there are fitness costs, endosymbiont-infected individuals either will go extinct, or will only partially if not completely replace uninfected individuals if they constitute a certain proportion of the host population, known as the invasion threshold of the endosymbiont. During the process of population replacement, the size of the uninfected part of the host population is reduced due to the inhibition of reproduction by uninfected females as a result of mating with incompatible infected males (Dobson et al. 2002a). This creates a positive feedback-loop that increases the relative proportion of infected individuals in the population, as well as creating vacant niche space to be filled, and thereby accelerates and drives the rate of both replacement of uninfected individuals and the degree of population suppression (as uninfected females are increasingly more likely to mate with infected males). It is this naturally-occurring mechanism of host population suppression that is exploited by IIT. However, as only males should be released during IIT application, the subsequent population replacement by endosymbiont-infected individuals that occurs in natural systems does not occur during target population suppression, as there are no infected females to maternally-transmit the endosymbiont to the next generation. The consequence is target population elimination.

An alternative strategy for vector control which is currently being extensively investigated and actively implemented – and which we do not discuss further here – involves intentionally releasing endosymbiont-infected females in order to deliberately trigger population replacement (Sinkins et al. 1997; Iturbe-Ormaetxe et al. 2011; Bourtzis et al. 2014; Xi and Joshi 2016). As described in Section 3.4.2 below, some *Wolbachia* infections can reduce vector competence for vector-borne pathogens, such that population replacement with such endosymbiont variants would reduce or prevent pathogen transmission by a vector population. However, we regard the aim of population suppression as preferable to population replacement, because it can be guaranteed to completely prevent any future pathogen transmission (endosymbiont-mediated reduction in vector competence may not be complete, and/or may be lost over time due to the evolution of resistance by the transmitted pathogen and/or changes in the vector-endosymbiont association), and is likely to have greater public acceptance due to male-only releases, the reduction of nuisance biting, and the possibility of vector eradication (Zheng et al. 2019).

3.2. A Brief History of the IIT

CI was first observed in the mosquito *Culex pipiens* L. (Marshall and Staley 1937; Marshall 1938; Roubaud 1941), shortly after the independent discovery of the endosymbiont *Wolbachia* in the same mosquito species (Hertig and Wolbach 1924; Hertig 1936). However, it was not until more than three decades later that the causal link between *Wolbachia* and CI was hypothesized, and then empirically proven through curing mosquitoes of their bacterially-induced CI by antibiotic treatment (Yen and Barr 1971, 1973).

The notion of using CI for suppression of vector populations was developed during the 1960s – that is, prior to the realization that maternally-inherited endosymbionts cause CI – as part of a World Health Organization (WHO)-sponsored programme instigated and led by the German entomologist Hannes Laven (WHO 1964; Pal 1966; Knipling et al. 1968; Laven 1971; Davidson 1974). In unpublished studies, it was first shown, using cage experiments, that the release of incompatible males at an initial 1:1 ratio with target-compatible males, could eradicate a stable target population in only 3 or 4 generations (Pal 1966; Laven 1967, 1971).

Consequently, a small-scale open-release pilot trial in the field was undertaken in a relatively isolated rural village (Okpo) near Rangoon in Myanmar (Burma) against the local vector of filariasis, *Culex quinquefasciatus* Say (Laven 1967, 1971). This trial was a resounding success, effectively eliminating the local mosquito population by the end of the 12-week intervention period, although there were some reservations about the significance, and general applicability, of this "proof-of-principle" demonstration (Laird 1967; Barr 1970; Weidhaas and Seawright 1976).

Subsequently, a larger-scale joint WHO / Indian Council of Medical Research (ICMR)-backed project to further investigate the feasibility of using the IIT was established in the 1970s in India (Grover and Sharma 1974; Pal 1974), resulting in a number of studies characterizing the incompatibility, mating competitiveness and vector competence of endosymbiont-infected mosquitoes (Subbarao et al. 1974, 1977; Grover et al. 1976; Singh et al. 1976; Curtis 1977; Krishnamurthy 1977; Thomas and Singh 1977; Curtis and Reuben 2007), as well as the first attempts of combining the IIT with genetic modification (Laven and Aslamkhan 1970; Krishnamurthy and Laven 1976; Curtis 1977). The results of the field trials were less convincing than before with only partial population suppression (<70%) achieved, apparently due to unexpected high levels of immigration of previously inseminated females from the areas surrounding the release sites (Brooks et al. 1976; Curtis 1977; Curtis et al. 1982).

During this latter period, with the discovery of CI in tephritid flies (the European cherry fruit fly *Rhagoletis cerasi* L., Boller and Bush 1974), and pyralid moths (the almond moth *Cadra cautella* (Walker), Brower 1976), there was interest for using CI to suppress other pest insects (Russ and Faber 1979; Neuenschwander et al. 1983; Blümel and Russ 1989; Boller 1989), with the term "IIT" being coined (Boller et al. 1976), and several promising laboratory studies and semi-field trials undertaken (Brower 1979, 1980; Ranner 1990).

During the 1980s, interest in the IIT (and the SIT) for mosquitoes waned (Scott and Benedict 2016), partly due to the premature termination of the joint WHO/ICMR project (Anonymous 1975; Curtis and Reuben 2007), but also because of doubts about the practical feasibility and economics of rearing large numbers of mosquitoes, as well as the possibility/sustainability of population suppression/elimination in the presence of immigration from outside control areas (Sinkins et al. 1997; Scott and Benedict 2016).

From the 1990s to the present, a new generation of researchers and their academic descendants have given fresh impetus to investigating the use of *Wolbachia* for pest and vector control (Iturbe-Ormaetxe et al. 2011; Bourtzis et al. 2014; Xi and Joshi 2016), resulting in a renewed interest in the IIT and its operational deployment (O'Connor et al. 2012; Mains et al. 2016, 2019; Zheng et al. 2019). The IIT has been under consideration for controlling the Mediterranean fruit fly *Ceratitits capitata* (Wiedemann) (Zabalou et al. 2004, 2009), the olive fruit *fly Bactrocera oleae* Rossi (Apostolaki et al. 2011), and the spotted wing drosophila *Drosophila suzukii* (Matsumura) (Cattel et al. 2018; Nikolouli et al. 2018), as well as tsetse flies (*Glossina* spp.) (Alam et al. 2011; Bourtzis et al. 2016). However, the development and implementation of the IIT was, and remains, the most advanced for mosquitoes, with open-release field trials planned or already recently undertaken for the arboviral and/or filarial vector species *Aedes aegypti* L. in Australia, Mexico, Singapore, and the USA (Xi and Manrique-Saide 2018; Yeung 2018; Corbel et al. 2019; Mains et al. 2019); *Aedes albopictus* (Skuse) in China and the USA (Mains et al. 2016; Zheng et al. 2019); and *Aedes polynesiensis* Marks in French Polynesia (Brelsfoard et al. 2008; O'Connor et al. 2012); as well as for *Cx. quinquefasciatus* on the four islands in the south-western Indian Ocean (La Réunion, Mauritius, Grande Glorieuse and Mayotte) (Atyame et al. 2011, 2015).

3.3. Generating and Characterizing Novel Endosymbiont Infections

In order to control a target pest or vector species using the IIT, it is necessary to have incompatible individuals for mass-rearing and release. The simplest method for obtaining such individuals is to collect them from the field. This is possible, for example, if different geographic populations of a target species naturally possess different incompatible endosymbiont infections (Brower 1976; Chen et al. 2013). This was the origin of the incompatible individuals used for the first IIT trials against *Cx. quinquefasciatus* (Laven 1967, 1971), as well as other pest insects. However, there are several problems that require attention.

3.3.1. Introgressing the Nuclear Genome of the Target Population

The first problem is that individuals from one geographic area may not be well-adapted to another location and may have lower mating competitiveness compared to local males from the target population (Barr 1966). This problem can be solved by using backcrosses to introgress the nuclear genome of the target population into the incompatible colony to be used for releases (Barr 1966; Krishnamurthy 1977), which is achieved by mating males from the target population to females from the incompatible colony. The process is repeated but using the daughters of each cross instead. As *Wolbachia* and other endosymbiotic reproductive parasites are maternally inherited, the outcome is a new line that possesses the cytoplasmic organelles (mitochondria and endosymbionts) of the original incompatible line, but now with the nuclear genome of the target population.

This laborious technique is still used to create *Wolbachia*-infected lines (Atyame et al. 2011), and it remains a fundamental method for matching the genetic background of released individuals to the target population in the field. This technique was also used more recently to transfer through inter-specific introgression a naturally occurring *Wolbachia* infection into a target species (*Ae. polynesiensis*) from a closely related non-target sister species (*Aedes riversi* Bohart & Ingram) (Brelsfoard et al. 2008).

3.3.2. Generating Artificial Wolbachia *Infections through Transinfection*

The second – and more significant – problem with relying on naturally-occurring endosymbiont infections is that it limits the availability and diversity of incompatible individuals, and, therefore, the insect species that are amenable to control using the IIT. Although endosymbionts are widespread among arthropods, many important pest and vector species, such as the mosquito *Ae. aegypti*, as well as the many mosquito species in the *Anopheles* genus of malaria vectors, are thought not to be naturally infected with *Wolbachia* (Bourtzis et al. 2014).

Even in those species that are infected with *Wolbachia*, there is often no intra-specific geographic variation in the endosymbionts and their mating compatibilities (as might be expected given the nature of their reproductive parasitism), and closely-related infected sister taxa capable of inter-specific interbreeding may not exist. The many intra-strain mating types observed in *Cx. pipiens*, which enable the IIT against this target species (Laven 1967; Atyame et al. 2011), are apparently atypical (Bourtzis et al. 2014). Although host species are not infrequently superinfected with two or more endosymbiont strains, these are often found throughout their geographic range (e.g. *Ae. albopictus*) (Bourtzis et al. 2014).

The ability to generate artificial *Wolbachia* infections in the laboratory through transinfection between individuals within the same or different host species was, therefore, a major breakthrough (Boyle et al. 1993; Braig et al. 1994). The application of these techniques has since provided renewed impetus to the use of endosymbionts for pest and vector control (Xi et al. 2005b; Hughes and Rasgon 2014), enabling the first open-release in the field of artificially-transinfected *Wolbachia*-infected mosquitoes for IIT application (Mains et al. 2016).

For mosquitoes, transinfection can be achieved either through embryonic (Xi and Dobson 2005) or intra-thoracic microinjection of adults (Ruang-areerate and Kittayapong 2006). However, in order to establish stable germline infections, the former method is regarded as the most efficient, due to the low likelihood of the somatic infections resulting from the latter colonizing the gonads and being maternally transmitted (Hughes and Rasgon 2014). For other insects, inoculation of larval or pupal stages has also been occasionally reported, as has transfer through co-rearing and predation.

Xi et al. (2005b) successfully established the first artificial *Wolbachia* infection of mosquitoes through embryonic microinjection of the cytoplasm from endosymbiont-infected donor eggs (Xi and Dobson 2005), and since then, a number of different artificial germline *Wolbachia* infections have been established in mosquitoes, including *Ae. aegypti* (Xi et al. 2005b; McMeniman et al. 2009; Walker et al. 2011; Ant and Sinkins 2018), *Ae. albopictus* (Xi et al. 2005a; Xi et al. 2006; Suh et al. 2009; Calvitti et al. 2010; Fu et al. 2010; Blagrove et al. 2012; Ant and Sinkins 2018; Moretti et al. 2018b; Zheng et al. 2019), and *Ae. polynesiensis* (Andrews et al. 2012), as well as the malaria vector *Anopheles stephensi* Liston (Bian et al. 2013).

Artificial transfer of CI-inducing *Wolbachia* has now also been achieved in a number of different insect groups, including other Diptera (Drosophilidae and Tephritidae), as well as Lepidoptera, Hemiptera and Coleoptera (Hughes and Rasgon 2014).

Transinfection allows both naturally uninfected, as well as already infected, target species to be artificially infected with *Wolbachia*. In the latter instance, pre-existing native endosymbiont infections can either be first removed by antibiotics or moderately high temperature (Yen and Barr 1973; Portaro and Barr 1975; Dobson and Rattanadechakul 2001), and then replaced with a different strain of *Wolbachia* (Suh et al. 2009; Calvitti et al. 2010; Andrews et al. 2012).

Alternatively, novel superinfections can be generated by adding new artificial infestations of *Wolbachia* strains to the endosymbiont strains already present in the target populations (Fu et al. 2010; Joubert et al. 2016; Ant and Sinkins 2018; Zheng et al. 2019). Establishing superinfections (especially triple infections) may be trickier than replacing pre-existing endosymbiont infections (due to competitive interactions and/or incompatibilities between different *Wolbachia* strains, e.g. Ant and Sinkins 2018), but come with the added benefit of higher endosymbiont densities and broader somatic tissue distributions, which is thought to be of importance for altering pest/vector status (see Section 3.4.2) (Moretti et al. 2018b; Zheng et al. 2019), but not necessarily for the induction of CI.

3.3.3. Selection of Endosymbionts for Transinfection

The potentially unconstrained ability to transfer any *Wolbachia* strain between any host species raises the issue of selecting which endosymbiont strains to transinfect (Hoffmann et al. 2015). So far, only a relatively limited number of *Wolbachia* strains from well-studied hosts have been tried (Hughes and Rasgon 2014), but *Wolbachia* is an ancient, phenotypically diverse, and vast bacterial clade spread across phylogenetically-distant host taxa, with potentially more strains (i.e. millions) than infected host species (due to the occurrence of superinfection) (Werren et al. 2008).

Although the characteristics of endosymbionts after host transfer can sometimes be unpredictable, and depend upon host background (Hoffmann et al. 2015), the behaviour of a given *Wolbachia* strain in one host generally provides a reasonable "rule-of-thumb" for predicting its behaviour in other hosts, especially if those hosts are phylogenetically-related, enabling some guidance in the selection of endosymbiont strains to be transferred.

For example, the unusually virulent *Wolbachia* strain *w*MelPop (Min and Benzer 1997), generally retains its pathogenicity, whether present in closely or more distantly related dipteran hosts. Similarly, native *Wolbachia* infections in mosquito species generally have the same characteristics when transferred to new mosquito hosts (see below).

An important exception to this pattern seems to be that novel *Wolbachia* infections often have higher endosymbiont strain-specific densities and/or broader somatic tissue distributions, which are associated with host fitness and other phenotypic effects (Hoffmann et al. 2015; Xi and Joshi 2016; Ant and Sinkins 2018).

3.3.4. Characterization of New Host-endosymbiont Associations

Once endosymbiont-infected individuals have been found from the field or generated de novo in the laboratory, their host-endosymbiont association needs to be thoroughly characterized to determine if it is suitable for IIT application. The basic requirements for an endosymbiont to be used for the IIT are to induce CI, have favourable levels of maternal transmission, and, in general, to have low fitness costs. CI is required to generate the male incompatibility that enables sterilisation of wild-type females in the target population. Stable maternal transmission is required to ensure that males can cause CI, and to enable their efficient mass-production.

If males are not infected, they cannot induce CI, and if maternal transmission is low then many uninfected individuals will be produced in each generation. As there is currently no method to separate the uninfected from the infected individuals, their presence during factory rearing requires more individuals to be mass-produced, and more males to be released, for a given level of target population suppression. In addition, if maternal transmission is unstable, it can result in self-incompatibility between superinfected individuals, compromising colony maintenance and preventing mass-production of appropriately infected individuals (Ant and Sinkins 2018).

Low fitness costs of *Wolbachia* infection are required to enable efficient mass-production of large numbers of factory-reared individuals for release, as well as to ensure the mating competitiveness of the released males.

Many, although not all, of the artificially infected mosquito lines have been shown to have these characteristics, inducing high levels (~100%) of CI, when the transinfected males mate with wild-type females, causing high levels (~100%) of stable maternal inheritance, and having no or only low fitness costs (Section 3.4.1) (Xi et al. 2005b; Bian et al. 2010, 2013; Calvitti et al. 2010, 2012; Blagrove et al. 2012, 2013; Joshi et al. 2014; Zheng et al. 2019).

3.4. Advantages of Using Endosymbionts

The fundamental difference between the IIT and the SIT is the sterilizing procedure: infection with CI-inducing endosymbionts in the former, and irradiation in the latter (Bourtzis and Robinson 2006). Other aspects of the IIT and the SIT tend to be common to all sterile-male-based methods (Alphey et al. 2010; Bourtzis et al. 2016; Dyck et al. 2021), although the use of endosymbionts entails some specific considerations (Section 5).

As discussed above (Section 2.1), there are potentially direct and/or indirect harmful effects associated with irradiation-based sterilisation, which can both be circumvented by using CI-inducing endosymbionts. Although the initial introduction of a novel endosymbiont strain into a target species is not trivial, requiring considerably more effort, time and specialist skill than administering a single dose of irradiation (Hughes and Rasgon 2014), it only needs to be done once. As CI-inducing endosymbionts are maternally-inherited, once stably introduced into the germline of a target species, incompatibility is self-perpetuating and maintained across generations, so that there is no need for repeated rounds of sterilisation – with their associated economic and biological costs – within and across generations, as is the case for irradiation-based sterilisation. The use of radiation also entails various logistical and bureaucratic requirements (e.g. infrastructure and regulatory frameworks), which are not necessary when using endosymbiont infection. In addition, use of CI-inducing endosymbionts allows greater flexibility with regard to the life cycle stages of the target species that can be released (Bourtzis and Robinson 2006), while the SIT is often restricted by the life cycle stage at which irradiation is optimally performed.

Another overlooked advantage of the IIT is that the released individuals are conveniently "tagged" by their endosymbiont infections: there is no need to additionally mark released insects using chemical dyes – which may impose fitness costs (Curtis et al. 1982) – in order to track them during control programmes (Bourtzis and Robinson 2006). Identification of infected or sterile males, or their sperm, can be done by PCR (O'Connor et al. 2012; Juan-Blasco et al. 2013; Mains et al. 2016, 2019; Zheng et al. 2019). In addition, *Wolbachia* may have beneficial effects on larval development, such as promoting faster development, and thus lower rearing costs (Zhang et al. 2015a; Puggioli et al. 2016).

Some potential disadvantages of using endosymbionts, other than the major one of accidental female release resulting in unintended population replacement (see Section 3.5), are that incompatibility may decline with increasing adult male age (Tortosa et al. 2010), and with male sperm depletion following multiple mating (Bourtzis and Robinson 2006). So far, the decline in male incompatibility with age, which occurs with some native *Wolbachia* infections (Singh et al. 1976; Krishnamurthy et al. 1977; Calvitti et al. 2015), has not been reported for artificial infections (Moretti and Calvitti 2013), possibly due to the higher endosymbiont densities of the latter (Calvitti et al. 2015). Sperm depletion also affects the SIT, and, again, may occur during native *Wolbachia* infections, but it has been reported to have no effect for artificial endosymbiont infections (Turley et al. 2013).

3.4.1. Cytoplasmic Incompatibility without Male Fitness Costs

A widely-perceived advantage of the male incompatibility caused by CI-inducing endosymbionts, such as *Wolbachia*, is that it often has either no or only minor effects on male quality (Pal 1966; Laven 1974; Boller et al. 1976; Brower 1976; Sinkins et al. 1997; Scott and Benedict 2016). Although *Wolbachia* infections can be highly virulent (Min and Benzer 1997; McMeniman et al. 2009; Suh et al. 2009; Rasgon 2012), this is apparently atypical. In general, in their co-evolved native hosts, maintained under field conditions, these endosymbionts are thought to more commonly reside in the commensal to mutualist region of the spectrum of symbiosis (if their parasitic and "spiteful" reproductive manipulations are not considered) (Xi and Joshi 2016). CI-inducing endosymbionts can be expected to have been optimized over many millennia of natural selection to specifically induce sterility, while minimizing any harmful effects on male quality, as this would reduce their capacity to invade host populations (Segoli et al. 2014).

Consistent with this theoretical understanding, native *Wolbachia* infections of mosquitoes have generally been reported to have no effects on male quality (Dobson et al. 2002b; Calvitti et al. 2009; Baton et al. 2013). Although several studies have reported reduced mating competitiveness of field released incompatible males (~30 to 70%) (possibly due to the possession of a sterility-inducing chromosomal translocation in one study, and the use of chemical marker dyes in another) (Grover et al. 1976; O'Connor et al. 2012), the majority of studies have shown both native and artificially infected males to have mating competitiveness equal to that of wild-type males in both laboratory and field settings (Brower 1978; Curtis et al. 1982; Arunachalam and Curtis 1985; Blagrove et al. 2013; Moretti and Calvitti 2013; Joshi et al. 2014; Segoli et al. 2014; Atyame et al. 2015; Axford et al. 2016; Puggioli et al. 2016; Zhang et al. 2016; Zheng et al. 2019), with some even suggesting increased mating competitiveness for incompatible males (Puggioli et al. 2016; Moretti et al. 2018b).

In comparison to other aspects of endosymbiotic reproductive parasites, the effects of CI-inducing endosymbionts on the individual components of male fitness, such as sperm competition, are relatively under-studied, although the highly virulent *w*MelPop strain has been found to have no effect on insemination rates, or sperm quantity and viability (Turley et al. 2013). However, the mating competitiveness studies described above imply that the individual components of male fitness are generally unaffected by *Wolbachia* infection.

Many studies have found that *Wolbachia* infection has no effect on male longevity, while some studies have even found that artificial *Wolbachia* infections significantly increase adult male survival, which might increase the efficiency of incompatible males in IIT programmes (Blagrove et al. 2013; Joshi et al. 2014). A few studies have also compared irradiation-based sterilisation with endosymbiont-induced incompatibility, but these have not always used the most appropriate comparison (i.e. uninfected and irradiated individuals compared to non-irradiated *Wolbachia*-infected individuals with the same genetic background) (Atyame et al. 2016; Puggioli et al. 2016; Zhang et al. 2016; Zheng et al. 2019). In addition, standardized protocols need to be developed to enable robust comparison between these different sterilisation methods (Bourtzis et al. 2016).

3.4.2. Other Useful Phenotypes Enabling Tolerance of the Undesired Sex(es)

In addition to causing incompatibility, endosymbiotic reproductive parasites like *Wolbachia* can also have a range of other phenotypic effects on their invertebrate hosts, including effects on both adult male and female fitness. One of the most important phenotypic effects is that *Wolbachia* can inhibit viral pathogens (Hedges et al. 2008; Teixeira et al. 2008), and artificially-transinfected mosquitoes have been shown to often strongly inhibit or completely block a variety of vector-borne pathogens, especially arboviruses, including dengue, chikungunya, Mayaro, West Nile, yellow fever, and Zika viruses, and to a lesser extent filaria and malaria parasites (Kambris et al. 2009; Moreira et al. 2009; Bian et al. 2010, 2013; Glaser and Meola 2010; Andrews et al. 2012; van den Hurk et al. 2012; Aliota et al. 2016; Dutra et al. 2016; Joshi et al. 2017; Pereira et al. 2018; Zheng et al. 2019). As well as direct effects on pathogen infection, endosymbionts might also indirectly reduce pathogen transmission, for example by reducing the survival of their adult female vectors (Brownstein et al. 2003; Rasgon et al. 2003; Cook et al. 2008).

In contrast, native *Wolbachia* infections tend to have less predictable effects on vector-borne pathogens and have been reported to inhibit, enhance or have no effect upon them (Curtis et al. 1983; Dutton and Sinkins 2005; Bian et al. 2010; Blagrove et al. 2012; Graham et al. 2012; Baton et al. 2013; Bourtzis et al. 2014; Zélé et al. 2014). If possible, any released insects should be lower pests/vectors than the target population (Laven and Aslamkhan 1970; Thomas and Singh 1977). Given the potentially variable effects of endosymbiont infection on pest/vector status, this aspect of target species biology should be thoroughly characterized prior to releasing *Wolbachia*-infected insects (e.g. Zheng et al. 2019).

The ability of *Wolbachia* to reduce the ability of target species to act as pests or vectors, enables the release of the sex(es) which are pests/vectors to be tolerated, compensating for imperfect sex separation (Section 2.2) (Moretti et al. 2018b; Zheng et al. 2019), and could be an additional means to make the release of male pests/vectors more tolerable (e.g. tsetse flies) (Bourtzis et al. 2016; Kariithi et al. 2018). The ability of CI-inducing endosymbionts to reduce the ability of insects to act as pests/vectors also provides an important fail-safe during IIT implementation, given the high probability of accidental female release during operational programmes (Section 3.5), as unintended population replacement would then reduce pathogen transmission, while target population suppression would not be achieved (Zheng et al. 2019).

3.5. The Problem of Unintended Population Replacement

An inherent and significant problem with the IIT, in the absence of perfect sex separation methods, which has been recognized since the idea of IIT use was first conceived, and has so far prevented its operational use, is that, unlike SIT, the accidental release of *Wolbachia*-infected females may prevent further population suppression by causing unintended population replacement (Barr 1966; Pal 1974; Curtis 1977).

When the IIT is deployed, release of incompatible males will prevent reproduction by the wild-type females of the target population, and the target population will become suppressed. However, if any *Wolbachia*-infected females are accidentally released along with the released males, the former may successfully begin to reproduce in the field, because they are compatible with the released males infected with same *Wolbachia* strain, as well as the wild-type males in the original target population. Consequently, after an initial period of population suppression, during which the reproduction of wild-type females is inhibited, the original target population may become replaced by a population with *Wolbachia*-infected individuals (Section 3.1).

Whether population replacement actually occurs will depend upon a number of parameters, such as the number of females accidentally released, and the characteristics of the *Wolbachia* infection used to make released males incompatible with the target population. In principle, a single female could trigger population replacement, if her *Wolbachia* infection causes high levels of CI, has high maternal transmission, and no fitness costs (Section 3.1). In reality, stochastic and population density-dependent processes mean that a single female is unlikely to leave surviving off-spring, even if her *Wolbachia* infection has no invasion threshold. Just how many released females are required to inevitably trigger population replacement is unknown, and difficult to quantify accurately in the absence of relevant empirical data. However, the risk of population replacement will clearly increase as the original uninfected target population is suppressed, because this will inevitably increase the relative proportion of any accidentally-released *Wolbachia*-infected females (and their descendants) in relation to the wild-type individuals of the original target population, increasing the likelihood that the former passes its invasion threshold (Section 3.1).

Furthermore, if the original target population is eliminated, rather than merely suppressed, there will be a vacant niche that will be filled, by default, by any accidentally released females (Curtis 1977), which are now more likely to be able to establish a field population, as any inhibitory stochastic and density-dependent processes related to intra-specific competition with wild-type uninfected individuals will now be relaxed (Berryman et al. 1973; Weidhaas and Seawright 1976; Dobson et al. 2002b). Whether population replacement occurs will then mostly depend on the characteristics of the *Wolbachia* infection used to induce incompatibility (i.e. the *Wolbachia*-infected individuals might go extinct if the *Wolbachia* infection imposes severe fitness costs, but otherwise they would be expected to persist, given the general characteristics of the *Wolbachia* strains so far used for IIT application – see Sections 3.1 and 3.4).

Laboratory cage experiments and mathematical modelling both indicate that inundative releases of incompatible males, contaminated with some females, facilitates *Wolbachia* invasion and population replacement (Hancock et al. 2011; Bian et al. 2013; Moretti et al. 2018a; Zheng et al. 2019). In addition, artificial *Wolbachia* infections, with the requisite characteristics, have been able to invade and persist in field populations (Hoffmann et al. 2011, 2014). These observations reinforce the notion that the risk of unintended population replacement following accidental female release is real, and not merely a “hypothetical” or purely academic concern.

Some researchers have claimed that the risk of unintended population replacement can be minimized by releasing *Wolbachia*-infected insects that are bidirectionally incompatible with their wild-type target field population (Sharma et al. 1979; Calvitti et al. 2012; Bourtzis et al. 2014). Bidirectional CI occurs when the target population is infected with its own native *Wolbachia* strain, which causes incompatibility when wild-type field males mate with released females infected with a different *Wolbachia* strain that is used for IIT. The reproduction of any accidentally released females, therefore, will be prevented if they mate with the wild-type field males. This contrasts with unidirectional CI, which occurs when the target field population is either uninfected or infected with *Wolbachia* strain(s) that are not incompatible with the *Wolbachia* strain used for IIT, enabling any accidentally released females to successfully reproduce when mated wild-type field males.

However, explicit mathematical modelling and laboratory cage experiments indicate that bidirectional CI will only provide protection against population replacement if the frequency of the released *Wolbachia* strain does not exceed its own invasion threshold (Dobson et al. 2002a; Moretti et al. 2018a). Although this is unlikely to happen for bidirectional CI at low or intermediate levels of target population suppression, this is not, in general, the intended endpoint of sterile-male-based methods, which aim for high levels of population suppression, if not population elimination, at which point any accidentally-released bidirectionally incompatible females will exceed their invasion threshold. In practice, therefore, bidirectional CI does not provide appreciably greater protection from population replacement than unidirectional CI, if the aim is either population elimination or merely to reduce target population densities below that which causes a pest/vector problem (i.e. high levels of population suppression).

In addition, any released residual females are much more likely to mate with the males with which they are released, than with males in the field population (as the former are held together overnight in containers before their release, and afterwards released males vastly outnumber those in the field) (Zheng et al. 2019). Thus, the advantage from incompatible matings between released residual females and field males due to bi-directional CI may be negligible from a practical standing point.

Although several small-scale short-term field trials have reported the use of the IIT without the apparent consequence of population replacement (O'Connor et al. 2012; Mains et al. 2016, 2019; Zheng et al. 2019), it should be noted that in none of these instances was the target population sufficiently suppressed to enable the rapid replacement by any released *Wolbachia*-infected individuals which might occur as population elimination is approached (i.e. within the ~6 month time-scale of the reported results of these field trials). These studies also involved the release of relatively small numbers of mosquitoes using manual sex separation, which ensured lower female contamination rates than when using mechanical sex separation. However, manual separation is impractical for medium- to large-scale area-wide applications (Pal 1974; Brelsfoard et al. 2008; Zheng et al. 2019). As such, the above described small-scale field trials were probably unlikely to have released enough *Wolbachia*-infected females to enable establishment of *Wolbachia*-infected field populations.

4. COMBINED IIT/SIT APPLICATION

4.1. The Solution to an "Intractable" Problem

In order to solve the inevitable problem of accidental female release, the second series of IIT field trials undertaken in India during the 1970s (Section 3.2) combined the IIT with genetic modification of the released insects, such that they carried a chromosomal translocation that induced semi-sterility when they mated amongst themselves (Laven and Aslamkhan 1970; Brooks et al. 1976; Krishnamurthy and Laven 1976; Curtis 1977; Curtis et al. 1982). Although this approach seemed to show promise in the laboratory, under field conditions it had little impact.

As an alternative, Curtis (1977), who described the problem of unintended population replacement following accidental female release during IIT implementation as "intractable", proposed, nonetheless, a practical, and, as it turns out, viable solution: combined IIT/SIT use. His solution was to exploit the fact that female insects are often more sensitive than males to radiation (Bakri et al. 2005, 2021), and to combine the IIT with the SIT, such that all incompatible individuals destined for field release were first subjected to low-dose radiation, which would completely sterilize any contaminant females without affecting the incompatibility or quality of the simultaneously irradiated males. The combination of the SIT and the IIT for mosquito control is shown in Fig. 1. Although some preliminary laboratory investigations were undertaken by Curtis and others (Sharma et al. 1979; Arunachalam and Curtis 1985; Shahid and Curtis 1987), the notion of using the combined IIT/SIT was neglected for several decades until its re-assessment in more recent times (Bourtzis and Robinson 2006; Brelsfoard et al. 2009; Zhang et al. 2015b, 2016; Kittayapong et al. 2018, 2019; Zheng et al. 2019; Kittayapong, this volume; Liew et al., this volume).

4.2. Optimum Irradiation Dose for Female Sterilisation

Combined IIT/SIT application requires that females are more sensitive to the effects of irradiation than males, and that this difference is sufficiently large to enable complete sterilisation of females without appreciably or only minimally impacting male quality (Curtis 1977). If this inherent biological condition is met, it is necessary to determine the optimum irradiation dose. At first thought, this might be considered to be the minimum irradiation dose required to completely sterilize females, under the assumption that higher doses of radiation would begin to negatively affect males, and thereby undermine the main rationale for using endosymbiont-induced CI.

Accordingly, a number of preliminary studies have characterized the relative susceptibility of females and males to irradiation, with the aim of identifying the minimum irradiation dose required to completely sterilize females (Sharma et al. 1979; Shahid and Curtis 1987; Arunachalam and Curtis 1985; Brelsfoard et al. 2009; Zhang et al. 2015b, 2016). These irradiation studies showed that, at least for mosquitoes, females are indeed more sensitive than males to irradiation, and that there are levels of irradiation that can completely sterilize females, without appreciably impacting on male quality. Importantly, these and other studies have also shown that the irradiation treatment used to completely sterilize females has no effect on the level of CI induction by the co-irradiated *Wolbachia*-infected *Ae. albopictus* males (Zheng et al. 2019).

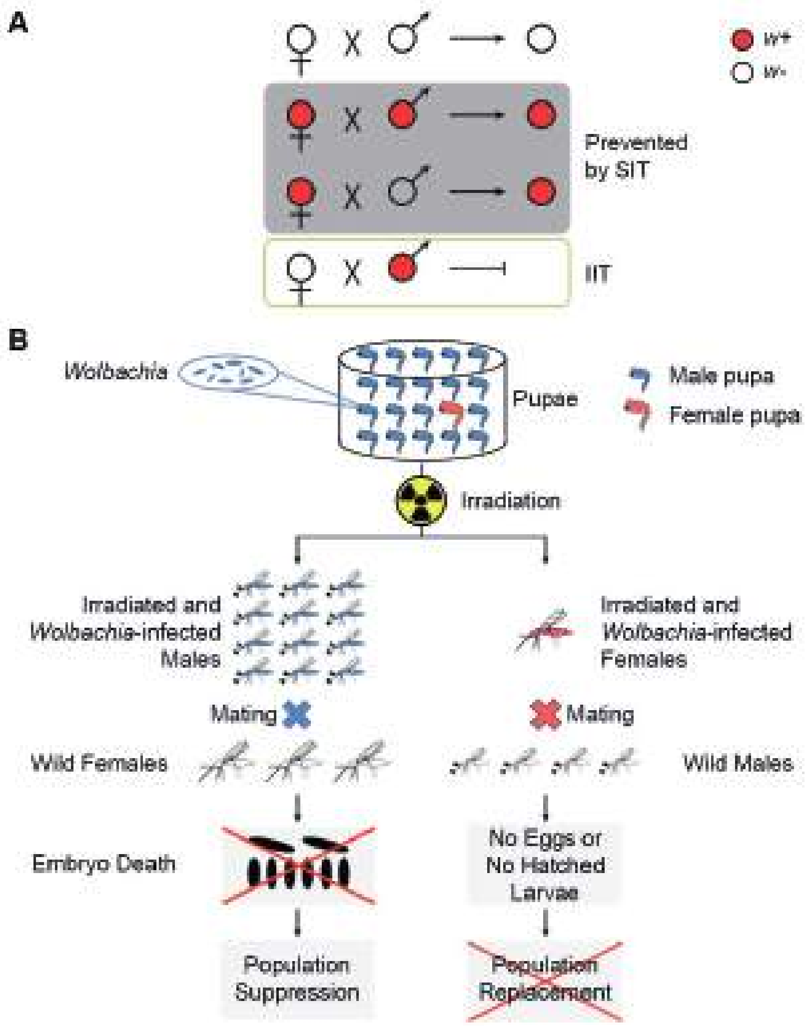

Figure 1. Schematic diagrams illustrating the combined IIT/SIT approach. (A) The four different types of crosses possible between wild-type uninfected and w*Pip-infected* Aedes albopictus*, and the role of irradiation in prevention of residual infected females from reproducing in the field. Red indicates* Wolbachia*-infected individuals (*W+*), while white indicates uninfected individuals (*W-*). (B) Illustration of production, irradiation, and release of* Wolbachia*-infected males with the residual females, respectively, and their mating with the wild population in the field.*

The optimal irradiation dose for the deployment of the combined IIT/SIT approach, however, is not necessarily the minimum irradiation dose for "complete" female sterilisation observed in laboratory studies, as the latter use small sample sizes, which defines a minimum detectable level of sterility, and different irradiation protocols (in particular, where there is no necessity to overcrowd pupae, as required during mass-irradiation, which may induce radio-protective hypoxia) (Yamada et al. 2019). It is vital for the IIT that any released females have no residual fertility, as this could render any current and future implementations using the same endosymbiont strain ineffectual. Consequently, combined IIT/SIT field releases involving millions of individuals need somewhat higher doses of irradiation than required in small-scale laboratory studies to ensure that all released females are fully sterilized (Yamada et al. 2019).

4.3. Sequential IIT/SIT Application

An alternative strategy related to the combined IIT/SIT releases to prevent population replacement resulting from accidental female release is a "sequential IIT/SIT" approach. This would involve initial IIT only releases, followed by SIT only releases (as opposed to the simultaneous combined IIT/SIT application in the same released individuals) (Atyame et al. 2016). The rationale here is that if the IIT is more efficient than the SIT because of the higher mating competitiveness and higher induced sterility of endosymbiont-infected males, large-scale only IIT releases can be used initially to suppress the target population, followed immediately by smaller-scale only SIT releases to eradicate – "mop-up" – any endosymbiont-infected individuals resulting from females inadvertently released during the initial phase of the IIT.

Whether a sequential IIT/SIT approach is preferable or superior to the combined IIT/SIT approach is not obvious and requires a careful quantitative comparison of the relative costs and benefits of the two strategies. Sequential IIT/SIT releases have the advantage that the males released during IIT application are not irradiated, maximizing their mating competitiveness, and removing the logistical costs/difficulties associated with large-scale irradiation (e.g. reduced male quality because of increased handling, etc.). However, these benefits would come with an increased risk of triggering population replacement in the first place (as many fertile females might now be released), the requirement to more carefully and rigorously monitor the target population to identify when/if population replacement occurs, and the risk of missing the optimal time window to switch to the SIT only releases (such that large-scale releases of relatively inefficient SIT, or other methods, would then be required).

Sequential IIT/SIT releases may be more convenient and effective in highly localized short-term programmes against geographically-restricted and low-abundance target populations, where, overall, relatively few incompatible males need to be initially released, and therefore the risk of accidental female release is inherently lower, while the combined IIT/SIT is likely to be more appropriate under the opposite conditions (i.e. area-wide long-term programmes against geographically-widespread and high-abundance target populations).

When sequential IIT/SIT releases are used, if irradiation-based sterilisation of any females released during the SIT step is not complete, then these individuals could also establish a field population. For this reason, it might be prudent *not* to use for the SIT releases the same endosymbiont-infected insect line used for the initial IIT releases, so as to maintain the effectiveness of the initial insect line originally used for IIT should it be required for this latter purpose again (i.e. in multiple alternate rounds of IIT and SIT application).

In a similar manner to that envisaged for the sequential IIT/SIT, only SIT releases could also be used as a fail-safe after combined IIT/SIT application, should the latter fail to prevent population replacement.

5. THE FIRST OPEN-RELEASE FIELD TRIAL OF COMBINED IIT/SIT

Despite the previous exploratory laboratory studies determining the possibility and optimal dose for differentially sterilizing females and males for use in the combined IIT/SIT approach (Section 4.2.), there had been no previous experimental or field evaluation of this combined strategy. Consequently, a project was initiated, involving collaboration between Sun Yat-sen University, Michigan State University and other partners, to develop and field test combined IIT/SIT releases against the important mosquito arboviral vector *Ae. albopictus.*

This project involved a series of stages (Figs. 2 to 4), as described below, including initial laboratory studies to generate and then characterize an incompatible artificially-*Wolbachia*-infected *Ae. albopictus* line, subsequent "proof-of-concept" semi-field trials of the combined IIT/SIT approach, and then finally an open-field trial to demonstrate the feasibility of area-wide application of combined IIT/SIT releases for the management of an *Ae. albopictus* population.

5.1. Generation and Characterization of Novel Wolbachia *Infection*

The first requirement was to create a novel *Wolbachia* infection in *Ae. albopictus* that would generate incompatibility with wild-type males in our study area. To do this, the *Wolbachia* strain *w*Pip was transferred by embryonic microinjection (Fig. 2) from its native mosquito host *Cx. pipiens* into *Ae. albopictus*, to generate the new mosquito line HC. This line had a similar nuclear genetic background to individuals from the area of our field trial in Guangzhou, China, but in addition a novel triple *Wolbachia* infection (the artificially-transinfected *w*Pip plus its two native *Wolbachia* strains) (Zheng et al. 2019). *w*Pip was chosen because in its native mosquito host it has characteristics appropriate for IIT: it causes complete CI, has perfect maternal transmission, and no appreciable fitness costs. Indeed, upon transfer to its new host, these properties were retained.

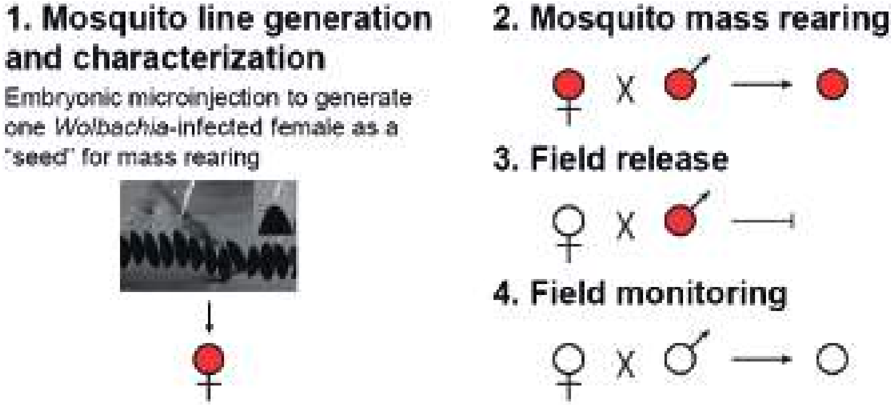

Figure 2. Overview of the IIT or combined IIT/SIT approach. First, a mosquito line with the novel Wolbachia *infection is generated by embryonic microinjection. The hallmark of success in this step is the generation of an infected female with ~100% maternal transmission efficiency to pass* Wolbachia *into their offspring (2.1). Then, the infected individuals are mass-produced in the factory (2.2), or also irradiated. The infected males are subsequently released into the field to induce sterility in the wild population (2.3). The density of the wild-type uninfected population is monitored to measure the effect of population suppression (2.4). Red indicates individuals carrying the novel* Wolbachia *infection, while white represents wild-type individuals.*

In laboratory studies, HC males caused complete CI when mated with wild-type females and had perfect maternal transmission (Zheng et al. 2019). There were also no differences between HC and wild-type *Ae. albopictus* in fecundity (number of eggs laid), fertility (egg hatch), larval/ pupal/ adult male or female survival, sex ratio or body size, although HC had a slightly faster larval development and adult emergence times (Zhang et al. 2015a). In addition, female HC had higher *Wolbachia* densities than wild-type females, and lower susceptibility to dengue and Zika virus infection, with both horizontal and vertical transmission of these arboviruses significantly reduced (Zheng et al. 2019). Although target population suppression/elimination was the aim of our field trial, the reduced vector competence of HC provided an important fail-safe should accidental female release and subsequently population replacement have occurred (Section 3.4.2) (Zheng et al. 2019).

5.2. Laboratory and Semi-field IIT/SIT Trials

A series of experimental laboratory studies were undertaken to characterize the HC-line further, and to confirm that it could be used for combined IIT/SIT releases. Laboratory cage experiments indicated that HC male mating competitiveness was equal to that of wild-type males (Zhang et al. 2016; Zheng et al. 2019). They also showed that HC females could cause population replacement, when seeded into cages containing wild-type individuals, with the speed of this population replacement being enhanced when excess HC males (4:1 ratio with wild-type males) were simultaneously released (Zheng et al. 2019). As these experiments demonstrated the potential of accidentally released HC females to trigger population replacement during IIT application, the minimum irradiation dose (28 Gy) necessary to completely sterilize HC females was identified. This dose caused extensive damage to the ovaries, and hence prevented egg-laying as well as the establishment of *w*Pip-infected individuals in a small laboratory cage population (Zhang et al. 2015b). This irradiation dose also did not affect mating competitiveness or survival of HC males, nor did it reduce CI induction (Zhang et al. 2016).

A subsequent semi-field trial was undertaken in field cages, which simulated the accidental release of HC females, in order to provide the first "proof-of-concept" that combined IIT/SIT application could prevent unintended population replacement (Zheng et al. 2019). Replicate control and experimental wild-type populations were established in large cages, into which irradiated excess HC males were repeatedly released (5:1 ratio with wild-type males), each time together with sufficient irradiated HC females to mimic a 2.0% contamination rate of the released HC males. Successful eradication of the wild-type populations occurred in all three of the field cages, without the occurrence of population replacement by the released *w*Pip-infected HC mosquitoes, demonstrating that the combined IIT/SIT strategy works. Having demonstrated experimentally that the combined approach works, an open-release field trial was implemented in Guangzhou, China.

5.3. Mass-production for Field Release

In order to produce sufficient numbers of irradiated-incompatible males for open-release during our combined SIT/IIT field trial, it was necessary to optimize rearing protocols and to develop new equipment to enable factory-scale mass-rearing and pupal irradiation (Fig. 3). Artificially-*Wolbachia*-infected mosquitoes do not require special rearing conditions and can be reared using the same protocols as those used for uninfected/wild-type individuals. However, some care should be given to ensure that *Wolbachia*-infected mosquitoes are not exposed to high temperatures or antibiotics (e.g. via their larval food or adult blood meals), as this could potentially remove their endosymbionts. In addition, larval rearing conditions may affect *Wolbachia* density, and, hence, possibly the level of CI expression, as well as maternal transmission of the endosymbiont (Puggioli et al. 2016). Consequently, it should be confirmed during the early stages of mass-production that the rearing conditions used do not adversely affect either the reproductive incompatibility or the quality of the males produced (Zhang et al. 2017, 2018).

For mass-rearing of larval mosquitoes, many rearing trays are required, thus several units for holding and storing large numbers of trays in order to improve space utilization have been developed at the FAO/IAEA Insect Pest Control Laboratory (IPCL) in Seibersdorf, Austria, and at the Wolbaki Institute of Biological Sciences in Guangzhou, China (Balestrino et al. 2012, 2014a; Zhang et al. 2017). The first generation "Wol-unit" holds 40 larval rearing trays, while only occupying 0.68 m^2 of floor space, and enables simultaneous rearing of 264 000 larvae, generating up to 89 000 male pupae per rearing cycle (Zhang et al. 2017).

Figure 3. The different stages of the first combined IIT/SIT field trial against Ae. albopictus *in Guangzhou, China. Photographs illustrating the nine different stages of the combined IIT/SIT field trial. In stage 1-3, artificially-triply-*Wolbachia*-infected adults, eggs and larvae were mass-produced in the mosquito rearing factory. In stage 4, a Fay-Morlan sorter was used for sex separation of pupae, followed by stage 5 with the Wolbaki® X-ray irradiator custom-made for the field trial to enable pupal irradiation. In stage 6, the sex-separated males were packed into buckets for mass-release. After quality control of emerged adult males by manual checking for contaminant adult females (stage 7), those buckets were delivered by vehicle to release sites (stage 8), as shown in the satellite images of the control and release sites (map data: Google, DigitalGlobe). Field populations were monitored through samples collected each week for diagnosis of* wPip *infection using PCR (stage 9).*

The "FAO/IAEA-unit" (Balestrino et al. 2012) holds 50 larval rearing trays, covers 0.94 m^2 ground area, has a capacity to hold 900 000 larvae, and can generate 314 000 male pupae per rearing cycle (Zhang et al. 2017). The second generation "Wol-unit 2.0" is based on the FAO/IAEA-unit and holds 100 larval rearing trays, covers 1.2 m^2 ground area, has a capacity to hold up to 1.5 million larvae, and can generate 550 000 male pupae per rearing cycle. A comparison between these three larval rearing units for *Ae. albopictus* is summarized in Table 1. The Wol-unit 2.0 is recommended for medium to large-scale applications as it requires relatively less space and enables more male pupae to be generated per unit.

Table 1. Comparison between three larval rearing units for production of one million Aedes albopictus *males*

Parameter	Wol-unit	FAO/IAEA-unit	Wol-unit 2.0
Number of trays per unit	40	50	100
Number of larvae reared per unit (10^5)	2.64	9.0	15.0
Number of male pupae acquired per unit (10^5)	0.89	3.14	5.5
Dimensions per unit (m, L * W * H)	0.97 × 0.70 × 1.85	0.78 × 1.2 × 2.10	1.41 x 0.84 x 2.1
Ground area per unit (m^2)	0.68	0.94	1.2
Quantity (unit)	11.2	3.2	1.9
Total space (m^2)	7.6	3.0	2.3
Labour - Adding water	Manual operation	Semi-automatic operation	Semi-automatic operation
Labour - Pupae/Larvae collection	Manual operation	Semi-automatic operation	Semi-automatic operation
Labour - Cleaning	Manual operation	Semi-automatic operation	Semi-automatic operation
Price	Low	High	Medium
Application	Small size factory	Medium size factory	Medium/Large size factory

For holding of adult mosquitoes, a suitable cage structure is important to maximize egg production. A prototype mass-production cage based on a design originally used for Mediterranean fruit flies, had been previously developed at the IPCL that allowed sugar and blood-feeding, as well as a simplified egg collection system that minimized the risk of mosquito escapes (Balestrino et al. 2014b; Mamai et al. 2017). However, we found that the egg production of *Ae. albopictus* was quite low, i.e. an average of ~16 eggs per female per blood meal. As rearing density seems to be the main factor causing low egg production (Balestrino et al. 2014b), the cage height was reduced and, together with the addition of ATP to the blood meal, we were able to increase egg production to an average of ~70 eggs per female given two blood meals (Zhang et al. 2018). The modified mass-production cage and mass-rearing protocol described currently enables the Wolbaki factory to produce 10 million *Ae. albopictus* eggs every 15 days (Zhang et al. 2018).

Male and female pupae of mosquitoes in the genera *Aedes* and *Culex* can be separated on the basis of size differences by using sieves or glass separators, although the traditional equipment is laborious to use (McCray 1961; Focks 1980; Balestrino et al. 2014a). An automated glass separator has been developed at Wolbaki to reduce manual operation and improve sex separation efficiency.

5.4. Irradiation of Pupae for Release

An irradiator specific for mosquito pupae was required for our field trial. Gamma rays have been the most common type of radiation used for insect sterilisation, because of their high energy and penetration (Bakri et al. 2021). However, the use of gamma rays is challenging because of regulatory, logistical and economic issues, related to safety, security, recycling, transportation, storage and initial cost. Consequently, in the past decade the use of X-rays has been suggested as a potential alternative to gamma rays (Mastrangelo et al. 2010; Ndo et al. 2014; Yamada et al. 2014a; FAO/IAEA 2017).

For insect sterilisation, a dose uniformity ratio (DUR: the maximum dose divided by the minimum dose) below 1.2 is required, in order to ensure a uniform dose is given to the irradiated individuals (Yamada et al. 2019). Dose uniformity is required to ensure that males do not receive unnecessarily high doses of radiation, which might needlessly reduce their quality, and is important for the combined IIT/SIT, where it is vital that *all* contaminant females are sufficiently irradiated to ensure complete sterilisation. However, the X-ray irradiators currently available on the market with the recommended DUR are not suitable for larger-scale applications using mosquitoes, because either only a small number of pupae can be simultaneously irradiated (RS 2000, Biological System Irradiator, RadSource, Georgia, USA), or they require relatively frequent replacement of the costly X-ray tube and are inconvenient for pupal irradiation (RS 2400) (Yamada et al. 2014a).

Consequently, Wolbaki in cooperation with the FAO/IAEA, developed a new X-ray irradiator – “the Wolbaki irradiator” – specifically designed for pupal irradiation, which meets the technical requirements and large-scale processing capacity required for our field trial. The irradiator is equipped with a ray tube at a 40-degree angle, and with a maximum power of 4.5 kW. At a horizontal distance of 30 cm from the radiation source, the dose rate is measured at 3.2 Gy/min through a 0.3 mm copper filter. A rotary table for holding canisters is set up for horizontal rotation during exposure. Two separated canisters, with a total loading capacity of one litre male pupae, can be vertically swapped at half target dose. The DUR is reduced to 1.07 by rotating and swapping during exposure.

As described above, the optimum irradiation dose for sterilizing of contaminant females for field release is likely to be appreciably higher than that indicated by a naïve interpretation of laboratory data based on very small sample sizes. Accordingly, we erred on the side of caution, and chose an irradiation dose of 45 Gy to ensure the success of our field trial (i.e. no fertile contaminant females released).

5.5. Open-Release in the Field and Entomological Surveillance

The open-release field trial was undertaken over a 2 to 3-year period (2016-2018) on two residential islands in Guangzhou, with each release site having its own control sites (Fig. 3) (Zheng et al. 2019). The field trial started a year earlier in Release Site 1 (2014 compared to 2015 for Release Site 2), with an initial pilot test of the IIT only in Release Site 1 during 2015, and a test of the combined IIT/SIT strategy being performed simultaneously in both sites during 2016 and 2017.

In the years prior to the male HC releases, base-line entomological surveys were carried out in both sets of control and release sites to confirm their suitability for the field trial (2014 for Site 1 and 2015 for Site 2; Fig. 4).

For the pilot test of IIT only, non-irradiated HC mosquitoes, from which the females had been removed by a combination of mechanical and manual sex sorting, were released during the mosquito breeding season (March to October). Initially, males were released throughout the entire area of Release Site 1, and the target field population was suppressed by as much as 55% (March to May; Fig. 5). However, as the mosquito breeding season peaked (late May to early June), the level of population suppression diminished, as it was not possible to release sufficient numbers of *Wolbachia*-infected males throughout the entire release site in order to attain the critical overflooding ratio. This was due to the labour-intensive checks required to manually remove contaminant females from the released males, a rate-limiting step which constrained how many sex-sorted mosquitoes could be produced per week (given the number of staff available for our field trial). Therefore, in an attempt to achieve the critical overflooding ratio for the remainder of the IIT trial in 2015 (mid-June to October), we reduced the treated area within Release Site 1 in which males were released (Fig. 4B), and subsequently expanded it following the "rolling carpet" approach (Dyck et al. 2021), so that the local density of released males would be increased, without the need to release a larger number of males overall.

After reducing the size of the release area, population suppression within the area of continuing releases was striking and significant, whereas very high mosquito densities were found in the immediately neighbouring area of Release Site 1 without continued releases (Fig. 4B), as well as within the control site (Fig. 5). These observations demonstrate the feasibility of using the IIT only for mosquito population suppression, and its potential for population elimination, if technological developments can be made that enable the large-scale mass-production – at a reasonable cost-effectiveness – of sufficient numbers of incompatible males lacking appreciable female contamination.

A trial of the combined IIT/SIT approach was then subsequently undertaken, in which irradiated HC mosquitoes were released. In this instance, females were removed from the released mosquitoes using mechanical separation only, resulting in a higher level of female contamination, but which could be tolerated as the residual females were sterilized by irradiation. As manual checks for contamination were no longer used or required, it was possible to release much larger numbers of male mosquitoes (>10-fold) for the combined IIT/SIT approach than for the IIT alone, and so HC releases could be undertaken throughout the entire area of both release sites for the entire duration of the two-year combined IIT/SIT field trial.

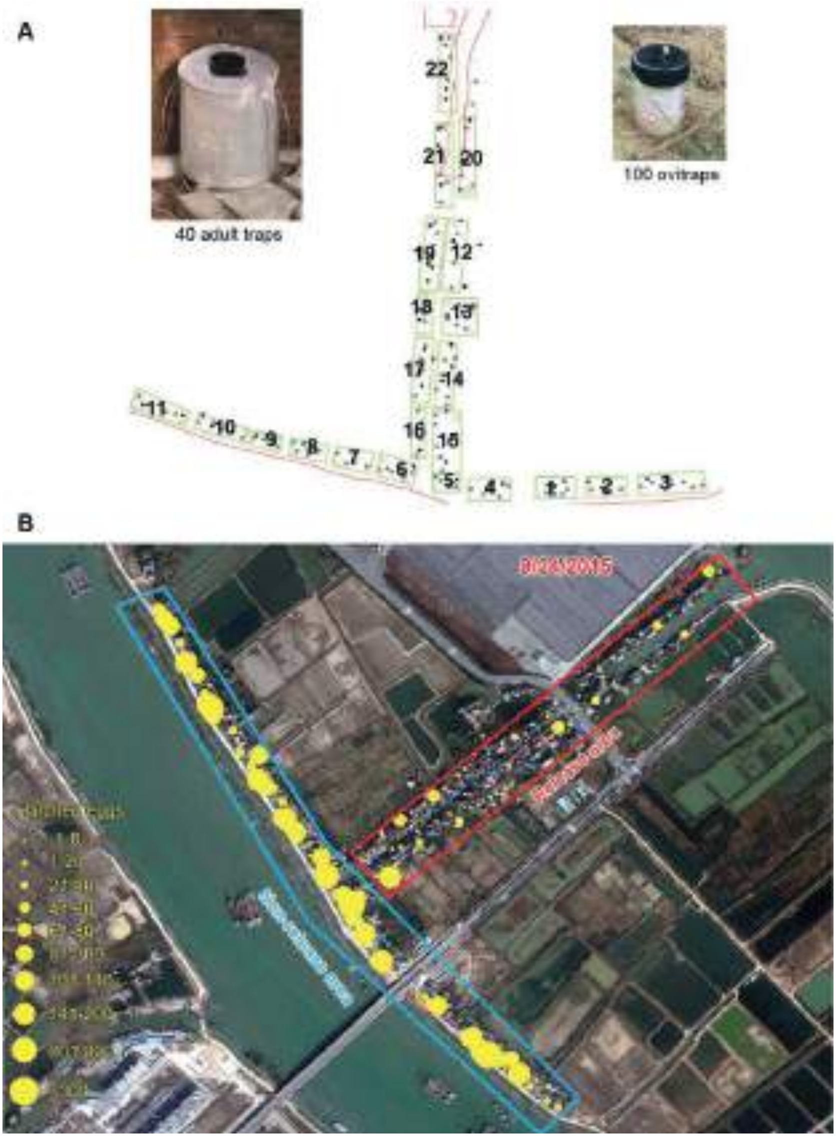

Figure 4. Monitoring and comparison of egg hatch rates during IIT and combined IIT/SIT application against Ae. albopictus *in Release Site 1 in Guangzhou, China. (A) Schematic diagram illustrating the division of Release Site 1 into 22 zones (green boxes), and the location of the ovitraps and adult-collecting Biogents BG-Sentinel traps that were used weekly to monitor* Aedes albopictus *populations during the field trial. (B) Satellite image showing the non-release (blue box; zones 1 to 11) and release (red box; zones 12 to 22) areas within Release Site 1 during the IIT only phase of the field trial in September 2015 (Map data: Google, DigitalGlobe). Yellow circles indicate ovitrap locations, with areas proportional to the number of hatched eggs collected in each for that week in September 2015.*

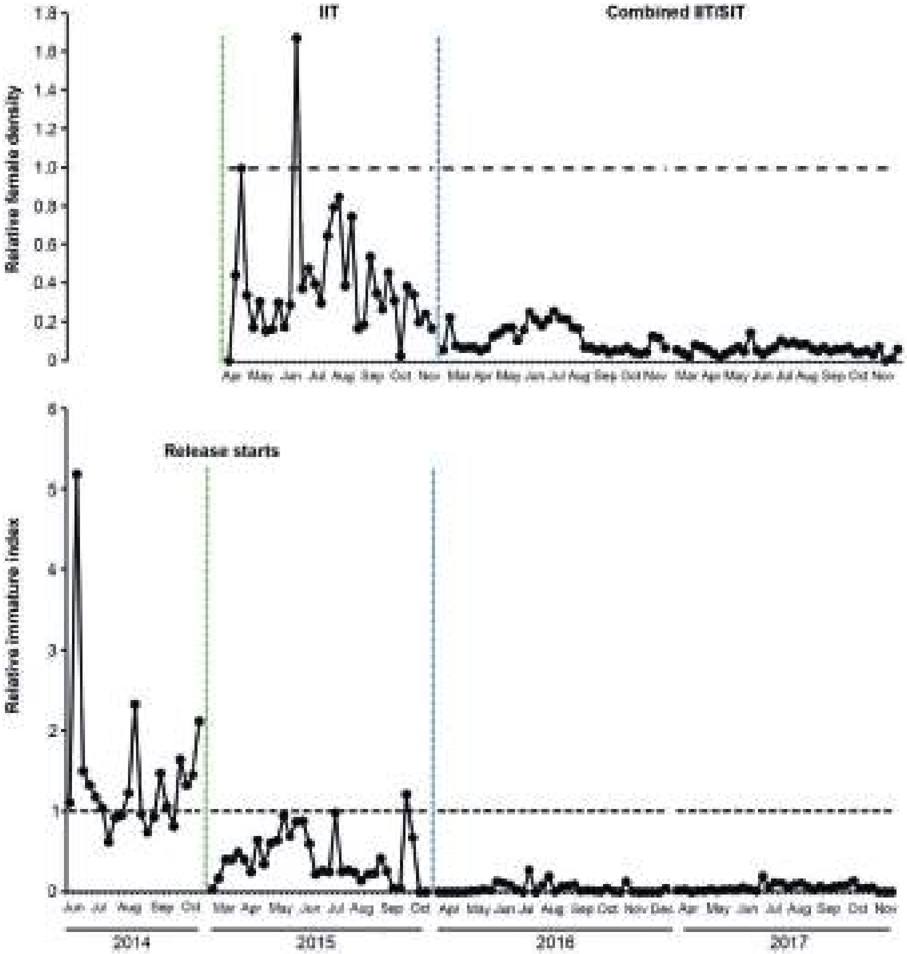

Figure 5. Suppression of Aedes albopictus *in Release Site 1 during the field trial in Guangzhou, China. The solid black lines indicate, respectively, the densities of wild-type adult females (A) and larvae (B) collected in Release Site 1 standardized by dividing by the corresponding number of individuals collected in Control Site 1 (see stage 9 in Figure 3). In 2014, baseline data were collected during the "pre-release" period before any compatible males were released. In 2015, IIT only releases were undertaken. In 2016 and 2017, combined IIT/SIT releases were performed. The horizontal black dashed lines indicate the relative level of larvae/adults in Control Site 1. The vertical green dashed line indicates the onset of IIT-only releases of incompatible males, while the vertical blue dashed line indicates onset of combined IIT/SIT releases of irradiated incompatible males.*

Overall, during the mosquito breeding seasons of 2016 and 2017, over 197 million factory-reared irradiated HC males were released using buckets from which adults emerged (Fig. 3). On average, 0.2-0.3% of the released insects were contaminant females. The sterile to wild male overflooding ratio was estimated at between 8.7:1 to 15.8:1, which resulted in the near-elimination of wild-type adult female *Ae. albopictus* from both release sites, i.e. > 94% reduction in egg hatch and up to 94% reduction in the apparent density of wild adult females (Fig. 5). The failure to eliminate completely the target populations in our release sites appeared to have been due to a low level of immigration.

Importantly, we found no evidence of population replacement during the three-year period of our field trial: throughout the period of male releases, we carefully monitored not only the wild-type target population, but also used PCR to screen collected larvae for the *w*Pip *Wolbachia* strain infecting released HC individuals. Although we did find a very low level of *w*Pip-positive larvae (0.87%, 16/1844 ovitrap samples), confirming the potential risk of population replacement, their collection was spatially and/or temporally-isolated, and they did not seem to constitute a viable breeding population. The field population did not increase in size after its initial suppression, nor was there a delayed rebound increase in egg hatch over time, which would have been expected, as a result of compatible matings becoming more frequent, if *w*Pip-infected mosquitoes had established in the field.

Overall, these observations demonstrate that the combined IIT/SIT approach can (i) suppress and effectively eliminate mosquito vector populations, and (ii) provide protection against the risk of population replacement resulting from the accidental release of fertile compatible endosymbiont-infected females.

6. FUTURE AREA-WIDE COMBINED IIT/IIT RELEASES

Despite our successful field trial, doubts about the area-wide implementation of the combined IIT/SIT persist (Armbruster 2019). As with other sterile-male-based methods, concerns include the affordability and sustainability of large-scale mass-release programmes. We believe that with optimization of the protocols used in our field trial, the combined IIT/SIT approach can be both affordable and sustainable for lower-income countries, and have an important and leading role as part of area-wide integrated pest control programmes (see Supplementary Information to Zheng et al. 2019).

To completely remove the risk of population replacement and to obtain population suppression or elimination, pupae were irradiated with a relatively high dose in the mass-rearing facility (Zheng et al. 2019). In addition, the current design of the X-ray irradiator and canister require a large number (up to 200 000) of pupae to stay in an overcrowded condition for an extended period (up to 15 min). Both result in a negative impact on male mating competitiveness and reduced cost-effectiveness of combined IIT/SIT releases. Thus, efforts will be made to optimize the approach for radiation exposure and to further improve the design of the X-ray irradiator.

As with any sterile-male-based method, large-scale area-wide deployment of the combined IIT/SIT approach would benefit from the development of improved and/or new methods/technologies to facilitate the efficient mass-production and mass-release of sufficient incompatible males to achieve population suppression/elimination. Many of these requirements are not unique to combined IIT/SIT releases (Alphey et al. 2010; Bourtzis et al. 2016), and, as such, we do not review them here in detail, other than to indicate how improvements in sex separation might impact the combined IIT/SIT approach.

Although the development of perfect sex separation methods is highly desirable (Gilles et al. 2014), the existence of such methods would negate the need and necessity for combined IIT/SIT, enabling IIT to be conducted without the risk of accidental female release resulting in unintended population replacement, and enabling the SIT to be conducted without risk of increased pest/vector activity. However, as described by Franz et al. (2021), even the best genetic sexing systems available are not perfect under large-scale operational programmes. In addition, where CI-inducing endosymbionts and irradiation reduce the ability of insects to act as pests/vectors (Sections 3.4.2), the low levels of contaminant females is less problematic.

Improvements in sex separation are likely to have their greatest impact on combined IIT/SIT releases by enabling the application of this method to target species for which there are currently either insufficient sex separation methods to enable mass-releases, or they are not available (e.g. *Anopheles* mosquitoes, which cannot be easily separated on the basis of size).

7. CONCLUSIONS

The combined IIT/SIT strategy integrates the strengths of the IIT with those of the SIT, and in so doing overcomes the current technological limitations of each approach. It can be used as an environment-friendly biopesticide to meet the current need for a novel solution to suppress mosquito populations and their transmitted diseases. Our successful field trial demonstrates the feasibility of area-wide application of combined IIT/SIT releases for *Aedes* mosquitoes.

8. REFERENCES

Alam, U., J. Medlock, C. Brelsfoard, R. Pais, C. Lohs, S. Balmand, J. Carnogursky, A. Heddi, P. Takac, A. Galvani, and S. Aksoy. 2011. *Wolbachia* symbiont infections induce strong cytoplasmic incompatibility in the tsetse fly *Glossina morsitans*. PLoS Pathogens 7: e1002415.

Aliota, M. T., S. A. Peinado, I. D. Velez, and J. E. Osorio. 2016. The *w*Mel strain of *Wolbachia* reduces transmission of Zika virus by *Aedes aegypti*. Scientific Reports 6: 28792.

Alphey, L. 2014. Genetic control of mosquitoes. Annual Review of Entomology 59: 205–224.

Alphey, L., M. Benedict, R. Bellini, G. G. Clark, D. A. Dame, M. W. Service, and S. L. Dobson 2010. Sterile-insect methods for control of mosquito-borne diseases: An analysis. Vector-Borne and Zoonotic Diseases 10: 295–311.

Andreasen, M. H., and C. F. Curtis. 2005. Optimal life stage for radiation sterilisation of *Anopheles* males and their fitness for release. Medical and Veterinary Entomology 19: 238–244.

Andrews, E. S., P. R. Crain, Y. Fu, D. K. Howe, and S. L. Dobson. 2012. Reactive oxygen species production and *Brugia pahangi* survivorship in *Aedes polynesiensis* with artificial *Wolbachia* infection types. PLoS Pathogens 8: e1003075.

Anonymous. 1975. Oh New Delhi, Oh Geneva. Nature 256: 355–357.

Ant, T. H., and S. P. Sinkins. 2018. A *Wolbachia* triple-strain infection generates self-incompatibility in *Aedes albopictus* and transmission instability in *Aedes aegypti*. Parasites & Vectors 11: 295.

Apostolaki, A., I. Livadaras, A. Saridaki, A. Chrysargyris, C. Savakis, and K. Bourtzis. 2011. Transinfection of the olive fruit fly *Bactrocera oleae* with *Wolbachia:* Towards a symbiont-based population control strategy. Journal of Applied Entomology 135: 546–553.

Armbruster, P. A. 2019. Tiger mosquitoes tackled in a trial. Nature 572 (7767): 39–40.

Arunachalam, N., and C. F. Curtis. 1985. Integration of radiation with cytoplasmic incompatibility for genetic control in the *Culex pipiens* complex (Diptera: Culicidae). Journal of Medical Entomology 22: 648–653.

Atyame, C. M., N. Pasteur, E. Dumas, P. Tortosa, M. L. Tantely, N. Pocquet, S. Licciardi, A. Bheecarry, B. Zumbo, M. Weill, and O. Duron. 2011. Cytoplasmic incompatibility as a means of controlling *Culex pipiens quinquefasciatus* mosquito in the islands of the south-western Indian Ocean. PLoS Neglected Tropical Diseases 5: e1440.

Atyame, C. M., J. Cattel, C. Lebon, O. Flores, J. S. Dehecq, M. Weill, L. C. Gouagna, and P. Tortosa. 2015. *Wolbachia*-based population control strategy targeting *Culex quinquefasciatus* mosquitoes proves efficient under semi-field conditions. PLoS One 10: e0119288.

Atyame, C. M., P. Labbé, C. Lebon, M. Weill, R. Moretti, F. Marini, L. C. Gouagna, M. Calvitti, and P. Tortosa. 2016. Comparison of irradiation and *Wolbachia* based approaches for sterile-male strategies targeting *Aedes albopictus.* PLoS One 11: e0146834.

Axford, J. K., P. A. Ross, H. L. Yeap, A. G. Callahan, and A. A. Hoffmann. 2016. Fitness of *w*AlbB *Wolbachia* infection in *Aedes aegypti:* Parameter estimates in an outcrossed background and potential for population invasion. The American Journal of Tropical Medicine and Hygiene 94: 507–516.

Bakri, A., N. Heather, J. Hendrichs, and I. Ferris. 2005. Fifty years of radiation biology in entomology: Lessons learned from IDIDAS. Annals of the Entomological Society of America 98: 1–12.

Bakri, A., K. Mehta, and D. R. Lance. 2021. Sterilizing insects with ionizing radiation, pp. 355–398. *In* V. A. Dyck, J. Hendrichs, and A. S. Robinson (eds.), Sterile Insect Technique – Principles and practice in Area-Wide Integrated Pest Management. Second Edition. CRC Press, Boca Raton, Florida, USA.

Balestrino, F., M. Q. Benedict, and J. R. L. Gilles. 2012. A new larval tray and rack system for improved mosquito mass rearing. Journal of Medical Entomology 49: 595–605.

Balestrino, F., A. Puggioli, J. R. L. Gilles, and R. Bellini. 2014a. Validation of a new larval rearing unit for *Aedes albopictus* (Diptera: Culicidae) mass rearing. PLoS One 9: e91914.

Balestrino, F., I. A. Puggioli, R. Bellini, D. Petric, and J. R. L. Gilles. 2014b. Mass production cage for *Aedes albopictus* (Diptera: Culicidae). Journal of Medical Entomology 51: 155–163.

Balestrino, F., A. Medici, G. Candini, M. Carrieri, B. Maccagnani, M. Calvitti, S. Maini, and R. Bellini. 2010. Gamma ray dosimetry and mating capacity studies in the laboratory on *Aedes albopictus* males. Journal of Medical Entomology 47: 581–591.

Barclay, H. J. 2021. Mathematical models for using sterile insects, pp. 201–244. *In* V. A. Dyck, J. Hendrichs, and A. S. Robinson (eds.), Sterile Insect Technique – Principles and practice in Area-Wide Integrated Pest Management. Second Edition. CRC Press, Boca Raton, Florida, USA.

Barr, A. R. 1966. Cytoplasmic incompatibility as a means of eradication of *Culex pipiens* L. Proceedings and Papers of the Annual Conference of the California Mosquito Control Association 34: 32–35.

Barr, A. R. 1970. Partial compatibility and its effect on eradication by the incompatible male method. Proceedings and Papers of the Annual Conference of the California Mosquito Control Association (Thirty-Seventh Annual Conference, Los Angeles, 27-29th January 1969) 37: 19–24.

Baton, L.A., E. C. Pacidônio, D. da Silva Gonçalves, and L. A. Moreira. 2013. *w*Flu: Characterization and evaluation of a native *Wolbachia* from the mosquito *Aedes fluviatilis* as a potential vector control agent. PLoS One 8: e59619.

Bellini, R., M. Calvitti, A. Medici, M. Carrieri, G. Celli, and S. Maini. 2007. Use of the sterile insect technique against *Aedes albopictus* in Italy: First results of a pilot trial, pp. 505–515. *In* M. J. B. Vreysen, A. S. Robinson, and J. Hendrichs (eds.), Area-wide control of insect pests: From research to field implementation. Springer, Dordrecht, The Netherlands.

Bellini, R., F. Balestrino, A. Medici, G. Gentile, R. Veronesi, and M. Carrieri. 2013a. Mating competitiveness of *Aedes albopictus* radio-sterilized males in large enclosures exposed to natural conditions. Journal of Medical Entomology 50: 94–102.

Bellini, R., A. Medici, A. Puggioli, F. Balestrino, and M. Carrieri. 2013b. Pilot field trials with *Aedes albopictus* irradiated sterile males in Italian urban areas. Journal of Medical Entomology 50: 317–325.

Benedict, M. Q., and A. S. Robinson. 2003. The first releases of transgenic mosquitoes: An argument for the sterile insect technique. Trends in Parasitology 19: 349–355.

Berryman, A. A., T. P. Boo'co, and L. C. Dickmann. 1973. Computer simulation of population reduction by release of sterile insects: II. The effects of dynamic survival and multiple mating, pp. 31–43. *In* Computer models and application of the Sterile-Male Technique: Proceedings of a Panel, Vienna, 13-17 December 1971, organized by the Joint FAO/IAEA Division of Atomic Energy in Food and Agriculture, International Atomic Energy Agency, Vienna, Austria.

Bian, G., Y. Xu, P. Lu, Y. Xie, and Z. Xi. 2010. The endosymbiotic bacterium *Wolbachia* induces resistance to dengue virus in *Aedes aegypti*. PLoS Pathogens 6: e1000833.

Bian, G., D. Joshi, Y. Dong, P. Lu, G. Zhou, X. Pan, Y. Xu, G. Dimopoulos, and Z. Xi. 2013. *Wolbachia* invades *Anopheles stephensi* populations and induces refractoriness to *Plasmodium* infection. Science 340: (6133) 748–751.

Blagrove, M. S. C., C. Arias-Goeta, A.-B. Failloux, and S. P. Sinkins. 2012. *Wolbachia* strain *w*Mel induces cytoplasmic incompatibility and blocks dengue transmission in *Aedes albopictus*. Proceedings of the National Academy of Sciences of the USA 109: 255–260.

Blagrove, M. S. C., C. Arias-Goeta, C. Di Genua, A.-B. Failloux, and S. P. Sinkins. 2013. A *Wolbachia* *w*Mel transinfection in *Aedes albopictus* is not detrimental to host fitness and inhibits Chikungunya virus. PLoS Neglected Tropical Diseases 7: e2152.

Blümel, S., and K. Russ. 1989. Manipulation of races, pp. 387–389. *In* A. S. Robinson and G. Hooper (eds.), Fruit flies: Their biology, natural enemies and control. World Crop Pests, Volume 3B. Elsevier, Amsterdam, The Netherlands.

Boller, E. F. 1989. Cytoplasmic incompatibility in *Rhagoletis cerasi*, pp. 69–74. *In* A. S. Robinson and G. Hooper (eds.), Fruit flies: Their biology, natural enemies and control. World Crop Pests, Volume 3B. Elsevier, Amsterdam, The Netherlands.

Boller, E. F., and G. L. Bush. 1974. Evidence for genetic variation in populations of the European cherry fruit fly, *Rhagoletis cerasi* (Diptera: Tephritidae) based on physiological parameters and hybridization experiments. Entomologia Experimentalis et Applicata 17: 279–293.

Boller, E. F., K. Russs, V. Vallo, and G. L. Bush. 1976. Incompatible races of European cherry fruit fly, *Rhagoletis cerasi* (Diptera: Tephritidae), their origin and potential use in biological control. Entomologia Experimentalis et Applicata 20: 237-247.

Bourtzis, K. and A. S. Robinson. 2006. Insect pest control using *Wolbachia* and/or radiation, pp. 225–246. *In* K. Bourtzis and T. A. Miller (eds.), Insect symbiosis. Volume 2. CRC Press, Boca Raton, USA.

Bourtzis, K., R. S. Lees, J. Hendrichs, and M. J. B. Vreysen. 2016. More than one rabbit out of the hat: Radiation, transgenic and symbiont-based approaches for sustainable management of mosquito and tsetse fly populations. Acta Tropica 157: 115–130.

Bourtzis, K., S. L. Dobson, Z. Xi, J. L. Rasgon, M. Calvitti, L. A. Moreira, H. Bossin, R. Moretti, L. A. Baton, G. L. Hughes, P. Mavingui, and J. R. L. Gilles. 2014. Harnessing mosquito-*Wolbachia* symbiosis for vector and disease control. Acta Tropica 132 (Supplement): S150–S163.

Boyle, L., S. L. O'Neill, H. M. Robertson, and T. L. Karr. 1993. Interspecific and intraspecific horizontal transfer of *Wolbachia* in *Drosophila*. Science 260: (5115) 1796–1799.

Braig, H. R., H. Guzman, R. B. Tesh, and S. L. O'Neill. 1994. Replacement of the natural *Wolbachia* symbiont of *Drosophila simulans* with a mosquito counterpart. Nature 367: (6462) 453–455.

Brelsfoard, C. L., Y. Séchan, and S. L. Dobson. 2008. Interspecific hybridization yields strategy for South Pacific filariasis vector elimination. PLoS Neglected Tropical Diseases 2: e129.

Brelsfoard, C. L., W. St Clair, and S. L. Dobson. 2009. Integration of irradiation with cytoplasmic incompatibility to facilitate a lymphatic filariasis vector elimination approach. Parasites & Vectors 2: (1) 38.

Brooks, G. D., C. F. Curtis, K. K. Grover, B. S. Krishnamurthy, P. L. Rajagopalan, L. S. Sharma, V. P. Sharma, D. Singh, K. R. P. Singh, M. Yasuno, M. A. Ansari, T. Adak, H. V. Aggarwal, C. P. Batra, R. K. Chandrahas, P. R. Malhotra, P. K. B. Menon, R. Menon, S. Das, R. K. Razdan, and V. Vaidyanathan. 1976. A field trial on control of *Culex pipens fatigans* Wied. by release of males of a strain integrating cytoplasmic incompatibility and a translocation. WHO/VBC/76.635. World Health Organization, Geneva, Switzerland.

Brower, J. H. 1976. Cytoplasmic incompatibility: Occurrence in a stored product pest *Ephestia cautella*. Annals of the Entomological Society of America 69: 1011–1015.

Brower, J. H. 1978. Propensity of interstrain mating in cytoplasmically incompatible strains of the almond moth. Journal of Economic Entomology 71: 585–586.

Brower, J. H. 1979. Suppression of laboratory populations of *Ephestia cautella* (Walker) (Lepidoptera: Pyralidae) by release of males with cytoplasmic incompatibility. J. Stored Products Research 15: 1–4.

Brower, J. H. 1980. Reduction of almond moth populations in simulated storages by the release of genetically incompatible males. Journal of Economic Entomology 73: 415–418.

Brownstein, J. S., E. Hett, and S. L. O'Neill. 2003. The potential of virulent *Wolbachia* to modulate disease transmission by insects. Journal of Invertebrate Pathology 84: 24–29.

Calvitti, M., R. Moretti, D. Porretta, R. Bellini, and S. Urbanelli. 2009. Effects on male fitness of removing *Wolbachia* infections from the mosquito *Aedes albopictus*. Medical and Veterinary Entomology 23: 132–140.

Calvitti, M., R. Moretti, E. Lampazzi, R. Bellini, and S. L. Dobson. 2010. Characterization of a new *Aedes albopictus* (Diptera: Culicidae)-*Wolbachia pipientis* (Rickettsiales: Rickettsiaceae) symbiotic association generated by artificial transfer of the *w*Pip strain from *Culex pipiens* (Diptera: Culicidae). Journal of Medical Entomology 47: 179–187.

Calvitti, M., R. Moretti, A. R. Skidmore, and S. L. Dobson. 2012. *Wolbachia* strain *w*Pip yields a pattern of cytoplasmic incompatibility enhancing a *Wolbachia*-based suppression strategy against the disease vector *Aedes albopictus*. Parasites & Vectors 5: 254.

Calvitti, M., F. Marini, A. Desiderio, A. Puggioli, and R. Moretti. 2015. *Wolbachia* density and cytoplasmic incompatibility in *Aedes albopictus*: Concerns with using artificial *Wolbachia* infection as a vector suppression tool. PLoS One 10: e0121813.

Caspari, E., and G. S. Watson. 1959. On the evolutionary importance of cytoplasmic sterility in mosquitoes. Evolution 13: 568–570.

Cattel, J., K. Nikolouli, T. Andrieux, J. Martinez, F. Jiggins, S. Charlat, F. Vavre, D. Lejon, P. Gibert, and L. Mouton. 2018. Back and forth *Wolbachia* transfers reveal efficient strains to control spotted wing drosophila populations. Journal of Applied Ecology 55: 2408–2418.

Chen, L., C. Zhu, and D. Zhang. 2013. Naturally occurring incompatibilities between different *Culex pipiens pallens* populations as the basis of potential mosquito control measures. PLoS Neglected Tropical Diseases 7: e2030.

Cook, P. E., C. J. McMeniman, and S. L. O'Neill. 2008. Modifying insect population age structure to control vector-borne disease. Advances in Experimental Medicine and Biology 627: 126–140.

Corbel, V., C. Durot, N. L. Achee, F. Chandre, M. B. Coulibaly, J. P. David, G. J. Devine, I. Dusfour, D. M. Fonseca, J. Griego, W. Juntarajumnong, A. Lenhart, S. Kasai, A. J. Martins, C. Moyes, L. C. Ng, J. Pinto, J. F. Pompon, P. Muller, K. Raghavendra, D. Roiz, H. Vatandoost, J. Vontas, and D. Weetman. 2019. Second WIN International Conference on "Integrated approaches and innovative tools for combating insecticide resistance in vectors of arboviruses". 1-3 October 2018, Singapore. Parasites & Vectors 12 (1): 331.

Curtis, C. F. 1977. Testing systems for the genetic control of mosquitoes, pp. 106–116. *In* J. S. Packer and D. White (eds.), XV International Congress of Entomology, Entomological Society of America, College Park, Maryland, USA.

Curtis, C. F., and R. Reuben. 2007. Destruction in the 1970s of a research unit in India on genetic control of mosquitoes and a warning for the future management of transgenic research. Antenna 31: 214–216.

Curtis, C. F., G. D. Brooks, M. A. Ansari, K. K. Grover, B. S. Krishnamurthy, P. K. Rajagopalan, L. S. Sharma, V. P. Sharma, D. Singh, K. R. P. Singh, and M. Yasuno. 1982. A field trial on control of *Culex quinquefasciatus* by release of males of a strain integrating cytoplasmic incompatibility and a translocation. Entomologia Experimentalis et Applicata 31: 181–190.

Curtis, C. F., D. S. Ellis, P. E. Doyle, N. Hill, B. D. Ramji, L. W. Irungu, and H. Townson. 1983. Susceptibility of aposymbiotic *Culex quinquefasciatus* to *Wuchereria bancrofti*. Journal of Invertebrate Pathology 41: 214–223.

Dame, D. A., C. F. Curtis, M. Q. Benedict, A. S. Robinson, and B. G. Knols. 2009. Historical applications of induced sterilisation in field populations of mosquitoes. Malaria Journal 8 (Supplement 2): S2.

Davidson, G. 1974. Genetic control of insect pests. Academic Press, London, UK. 168 pp.

Dobson, S. L., and W. Rattanadechakul. 2001. A novel technique for removing *Wolbachia* infections from *Aedes albopictus* (Diptera: Culicidae). Journal of Medical Entomology 38: 844–849.

Dobson, S. L., C. W. Fox, and F. M. Jiggins. 2002a. The effect of *Wolbachia*-induced cytoplasmic incompatibility on host population size in natural and manipulated systems. Proceedings of the Royal Society, Series B: Biological Sciences 269: (1490) 437–445.

Dobson, S. L., E. J. Marsland, and W. Rattanadechakul. 2002b. Mutualistic *Wolbachia* infection in *Aedes albopictus*: Accelerating cytoplasmic drive. Genetics 160: 1087–1094.

Dutra, H. L. C., M. N. Rocha, F. B. S. Dias, S. B. Mansur, E. P. Caragata, and L. A. Moreira. 2016. *Wolbachia* blocks currently circulating Zika virus isolates in Brazilian *Aedes aegypti* mosquitoes. Cell Host and Microbe 19: 771–774.

Dutton, T. J., and S. P. Sinkins. 2005. Filarial susceptibility and effects of *Wolbachia* in *Aedes pseudoscutellaris* mosquitoes. Medical and Veterinary Entomology 19: 60–65.

Dyck, V. A., J. Hendrichs, and A. S. Robinson (eds.). 2021. Sterile Insect Technique – Principles and practice in Area-Wide Integrated Pest Management, Second Edition. CRC Press, Boca Raton, Florida, USA. 1200 pp.

(FAO/IAEA) Food and Agriculture Organization / International Atomic Energy Agency. 2017. Technical specification for an X-ray system for the irradiation of insects for the sterile insect technique and other related technologies. Joint FAO/IAEA Programme of Nuclear Techniques in Food and Agriculture, Vienna, Austria. 11 pp.

Fine, P. E. M. 1978. On the dynamics of symbiote-dependent cytoplasmic incompatibility in culicine mosquitoes. Journal of Invertebrate Pathology 31: 10–18.

Focks, D. A. 1980. An improved separator for the developmental stages, sexes, and species of mosquitoes (Diptera: Culicidae). Journal of Medical Entomology 17: 567–568.

Franz, F., K. Bourtzis, and C. Cáceres. 2021. Practical and operational genetic sexing systems based on classic genetic approaches in fruit flies, an example for other species amenable to large-scale rearing for the Sterile Insect Technique, pp. 575–604. *In* V. A. Dyck, J. Hendrichs, and A. S. Robinson (eds.), Sterile Insect Technique – Principles and practice in Area-Wide Integrated Pest Management. Second Edition. CRC Press, Boca Raton, Florida, USA.

Fu, Y., L. Gavotte, D. R. Mercer, and S. L. Dobson. 2010. Artificial triple *Wolbachia* infection in *Aedes albopictus* yields a new pattern of unidirectional cytoplasmic incompatibility. Applied and Environmental Microbiology 76: 5887–5891.

Gilles, J. R. L., M. F. Schetelig, F. Scolari, F. Marec, M. L. Capurro, G. Franz, and K. Bourtzis. 2014. Towards mosquito sterile insect technique programmes: Exploring genetic, molecular, mechanical and behavioural methods of sex separation in mosquitoes. Acta Tropica 132 (Supplement): S178–S187.

Glaser, R. L., and M. A. Meola. 2010. The native *Wolbachia* endosymbionts of *Drosophila melanogaster* and *Culex quinquefasciatus* increase host resistance to West Nile virus infection. PLoS One 5: e11977.

Graham, R. I., D. Grzywacz, W. L. Mushobozi, K. Wilson, and D. Ebert. 2012. *Wolbachia* in a major African crop pest increases susceptibility to viral disease rather than protects. Ecology Letters 15: 993–1000.

Grover, K. K., and V. P. Sharma. 1974. The present status of the work on induced sterility mechanisms for control of mosquitoes *Culex pipiens fatigans* and *Aedes aegypti* at the WHO/ICMR Research Unit on Genetic Control of Mosquitoes. The Journal of Communicable Diseases 6: 91–97.

Grover, K. K., C. F. Curtis, V. P. Sharma, K. R. P. Singh, K. Dietz, H. V. Aggarwal, R. K. Razdan, and V. Vaidyanathan. 1976. Competitiveness of chemosterilised males and cytoplasmically incompatible translocated males of *Culex pipiens fatigans* Wiedemann (Diptera, Culicidae) in the field. Bulletin of Entomological Research 66: 469–480.

Hancock, P. A., S. P. Sinkins, and H. C. J. Godfray. 2011. Strategies for introducing *Wolbachia* to reduce transmission of mosquito-borne diseases. PLoS Neglected Tropical Diseases 5: e1024.

Hedges, L. M., J. C. Brownlie, S. L. O'Neill, and K. N. Johnson. 2008. *Wolbachia* and virus protection in insects. Science 322: (5902) 702.

Helinski, M. E. H., and B. G. J. Knols. 2008. Mating competitiveness of male *Anopheles arabiensis* mosquitoes irradiated with a partially or fully sterilizing dose in small and large laboratory cages. Journal of Medical Entomology 45: 698–705.

Helinski, M. E. H., A. G. Parker, and B. G. J. Knols. 2006. Radiation-induced sterility for pupal and adult stages of the malaria mosquito *Anopheles arabiensis*. Malaria Journal 5: 41.

Helinski, M. E. H., A. G. Parker, and B. G. J. Knols. 2009. Radiation biology of mosquitoes. Malaria Journal 8 (Supplement 2): S6.

Hertig, M., 1936. The rickettsia, *Wolbachia pipientis* (gen. et sp.n.) and associated inclusions of the mosquito, *Culex pipiens*. Parasitology 28: 453–486.

Hertig, M., and S. B. Wolbach. 1924. Studies on rickettsia-like micro-organisms in insects. The Journal of Medical Research 44: 329–374.

Hilgenboecker, K., P. Hammerstein, P. Schlattmann, A. Telschow, and J. H. Werren. 2008. How many species are infected with *Wolbachia*? A statistical analysis of current data. FEMS Microbiology Letters 281: 215–220.

Hoffmann, A. A., P. A. Ross, and G. Rasic. 2015. *Wolbachia* strains for disease control: Ecological and evolutionary considerations. Evolutionary Applications 8: 751–768.

Hoffmann, A. A., B. L. Montgomery, J. Popovici, I. Iturbe-Ormaetxe, P. H. Johnson, F. Muzzi, M. Greenfield, M. Durkan, Y. S. Leong, Y. Dong, H. Cook, J. Axford, A. G. Callahan, N. Kenny, C. Omodei, E. A. McGraw, P. A. Ryan, S. A. Ritchie, M. Turelli, and S. L. O'Neill. 2011. Successful establishment of *Wolbachia* in *Aedes populations* to suppress dengue transmission. Nature 476: (7361) 454–457.

Hoffmann, A. A., I. Iturbe-Ormaetxe, A. G. Callahan, B. L. Phillips, K. Billington, J. K. Axford, B. Montgomery, A. P. Turley, and S. L. O'Neill. 2014. Stability of the *w*Mel *Wolbachia* infection following invasion into *Aedes aegypti* populations. PLoS Neglected Tropical Diseases 8: e3115.

Hughes, G. L., and J. L. Rasgon. 2014. Transinfection: A method to investigate *Wolbachia*-host interactions and control arthropod-borne disease. Insect Molecular Biology 23: 141–151.

Hurst, G. D. D., and C. L. Frost. 2015. Reproductive parasitism: Maternally inherited symbionts in a biparental world. Cold Spring Harbor Perspectives in Biology 7: a017699.

Iturbe-Ormaetxe, I., T. Walker, and S. L. O'Neill. 2011. *Wolbachia* and the biological control of mosquito-borne disease. EMBO Reports 12: 508–518.

Joshi, D., M. J. McFadden, D. Bevins, F. Zhang, and Z. Xi. 2014. *Wolbachia* strain *w*AlbB confers both fitness costs and benefit on *Anopheles stephensi*. Parasites & Vectors 7: 336.

Joshi, D., X. Pan, M. J. McFadden, D. Bevins, X. Liang, P. Lu, S. Thiem, and Z. Xi. 2017. The maternally inheritable *Wolbachia w*AlbB induces refractoriness to *Plasmodium berghei* in *Anopheles stephensi*. Frontiers in Microbiology 8: 366.

Joubert, D. A., T. Walker, L. B. Carrington, J. T. De Bruyne, D. H. Kien, N. T. Hoang, N. V. Chau, I. Iturbe-Ormaetxe, C. P. Simmons, and S. L. O'Neill. 2016. Establishment of a *Wolbachia* superinfection in *Aedes aegypti* mosquitoes as a potential approach for future resistance management. PLoS Pathogens 12: e1005434.

Juan-Blasco, M., A. Urbaneja, V. San Andrés, P. Castañera, and B. Sabater-Muñoz. 2013. Improving the sterile sperm identification method for its implementation in the area-wide sterile insect technique program against *Ceratitis capitata* (Diptera: Tephritidae) in Spain. Journal of Economic Entomology 106: 2541–2547.

Kariithi, H. M., I. K. Meki, D. I. Schneider, L. De Vooght, F. M. Khamis, A. Geiger, G. Demirbaş-Uzel, J. M. Vlak, I. A. Ince, S. Kelm, F. Njiokou, F. N. Wamwiri, I. I. Malele, B. L. Weiss, and A. M. M. Abd-Alla. 2018. Enhancing vector refractoriness to trypanosome infection: Achievements, challenges and perspectives. BMC Microbiology 18 (Supplement 1): 179.

Kambris, Z., P. E. Cook, H. K. Phuc, and S. P. Sinkins. 2009. Immune activation by life-shortening *Wolbachia* and reduced filarial competence in mosquitoes. Science 326: (5949) 134–136.

Kittayapong, P., N. Kaeothaisong, S. Ninphanomchai, and W. Limohpasmanee. 2018. Combined sterile insect technique and incompatible insect technique: Sex separation and quality of sterile *Aedes aegypti* male mosquitoes released in a pilot population suppression trial in Thailand. Parasites & Vectors 11 (Supplement 2): 657.

Kittayapong, P., S. Ninphanomchai, W. Limohpasmanee, C. Chansang, U. Chansang, and P. Mongkalangoon. 2019. Combined sterile insect technique and incompatible insect technique: The first proof-of-concept to suppress *Aedes aegypti* vector populations in semi-rural settings in Thailand. PLoS Neglected Tropical Diseases 13: e0007771.

Knipling, E. F. 1955. Possibilities of insect control or eradication through the use of sexually sterile males. Journal of Economic Entomology 48: 459–462.

Knipling, E. F., H. Laven, G. B. Craig, R. Pal, J. B. Kitzmiller, C. N. Smith, and A. W. A. Brown. 1968. Genetic control of insects of public health importance. Bulletin of the World Health Organization 38: 421–438.

Krishnamurthy, B. S. 1977. Evaluation of the D3 cytoplasmic incompatible strain of *Culex pipiens fatigans* in laboratory and field cages. The Indian Journal of Medical Research 65 (Supplement): 13–20.

Krishnamurthy, B. S., and H. Laven. 1976. Development of cytoplasmically incompatible and integrated (translocated incompatible) strains of *Culex pipiens fatigans* for use in genetic control. Journal of Genetics 62: 117–129.

Krishnamurthy, B. S., C. F. Curtis, S. K. Subbarao, K. R. P. Singh, R. K. Chandrahas, and T. Adak. 1977. Further studies on the effect of aging and mating history of males on cytoplasmic incompatibility in *Culex pipiens fatigans.* Journal of Genetics 63: 31–37.

Laird, M. 1967. Eradication of *Culex pipiens fatigans* through cytoplasmic incompatibility. Nature 216: (5122) 1358.

Laven, H. 1967. Eradication of *Culex pipiens fatigans* through cytoplasmic incompatibility. Nature 216: (5113) 383–384.

Laven, H. 1971. Une expérience de lutte génétique contre *Culex pipiens fatigans* Wied. 1828. Annales de Parasitologie Humaine et Comparée 46 (3, Supplément): 117–148.

Laven, H. 1974. Genetic control of mosquitoes, pp. 19-26. *In* Proceedings Tall Timbers Conference on Ecological Animal Control by Habitat Management, Number 5. Tall Timbers Research Station, Tallahassee, Florida, USA.

Laven, H., and M. Aslamkhan. 1970. Control of *Culex pipiens pipiens* and *C. p. fatigans* with integrated genetical systems. Pakistan Journal of Science 22: 303–312.

Lees, R. S., D. Carvalho, and J. Bouyer. 2021. Potential impact of the fight against disease-transmitting mosquitoes using the sterile insect technique, pp. 1081–1118. *In* V. A. Dyck, J. Hendrichs, and A. S. Robinson (eds.), Sterile Insect Technique – Principles and practice in Area-Wide Integrated Pest Management. Second Edition. CRC Press, Boca Raton, Florida, USA.

Lees R. S., B. Knols, R. Bellini, M. Q. Benedict, A. Bheecarry, H. C. Bossin, D. D. Chadee, J. Charlwood, R. K. Dabiré, L. Djogbenou, A. Egyir-Yawson, R. Gato, L. C. Gouagna, M. M. Hassan, S. A. Khan, L. L. Koekemoer, G. Lemperiere, N. C. Manoukis, and J. R. L. Gilles. 2013. Review: Improving our knowledge of male mosquito biology in relation to genetic control programmes. Acta Tropica 13 (Supplement): S2–S11.

Lees, R. S., J. R. L Gilles, J. Hendrichs, M. J. B. Vreysen, and K. Bourtzis. 2015. Back to the future: The Sterile Insect Technique against mosquito disease vectors. Current Opinion in Insect Science 10: 156–162.

Maïga, H., D. Damiens, A. Niang, S. P. Sawadogo, O. Fatherhaman, R. S. Lees, O. Roux, R. K. Dabiré, G. A. Ouedraogo, F. Tripet, A. Diabate, and J. R. L. Gilles. 2014. Mating competitiveness of sterile male *Anopheles coluzzii* in large cages. Malaria Journal 13: 460.

Mains, J. W., C. L. Brelsfoard, R. I. Rose, and S. L. Dobson. 2016. Female adult *Aedes albopictus* suppression by *Wolbachia*-infected male mosquitoes. Scientific Reports 6: 33846.

Mains, J. W., P. H. Kelly, K. L. Dobson, W. D. Petrie, and S. L. Dobson. 2019. Localized control of *Aedes aegypti* (Diptera: Culicidae) in Miami, FL, via inundative releases of *Wolbachia*-infected male mosquitoes. Journal of Medical Entomology 56: 1296–1303.

Mamai, W., N. S. Bimbile-Somda, H. Maiga, J. G. Juarez, Z. A. I. Muosa, A. B. Ali, R. S. Lees, and J. R. L. Gilles. 2017. Optimization of mosquito egg production under mass rearing setting: Effects of cage volume, blood meal source and adult population density for the malaria vector, *Anopheles arabiensis*. Malaria Journal 16: 41.

Marshall, J. F. 1938. The British Mosquitoes. William Clowes and Sons, Limited, London and Beccles, UK. 341 pp.

Marshall, J. F., and J. Staley. 1937. Some notes regarding the morphological and biological differentiation of *Culex pipiens* Linnaeus and *Culex molestus* Forskål (Diptera, Culicidae). Proceedings of the Royal Entomological Society of London, Series A: General Entomology 12: 17–26.

Mastrangelo, T., A. G. Parker, A. Jessup, R. Pereira, D. Orozco-Davila, A. Islam, T. Dammalage, and J. Walder. 2010. A new generation of x ray irradiators for insect sterilisation. Journal of Economic Entomology 103: 85–94.

McCray, E. M. 1961. A mechanical device for the rapid sexing of *Aedes aegypti* pupae. Journal of Economic Entomology 54: 819.

McMeniman, C. J., R. V. Lane, B. N. Cass, A. W. C. Fong, M. Sidhu, Y.-F. Wang, and S. L. O'Neill. 2009. Stable introduction of a life-shortening *Wolbachia* infection into the mosquito *Aedes aegypti*. Science 323: (5910) 141–144.

Min, K.-T., and S. Benzer. 1997. *Wolbachia*, normally a symbiont of *Drosophila*, can be virulent, causing degeneration and early death. Proceedings of the National Academy of Sciences of the USA 94: 10792–10796.

Moreira, L.A., I. Iturbe-Ormaetxe, J. A. Jeffery, G. Lu, A. T. Pyke, L. M. Hedges, B. C. Rocha, S. Hall-Mendelin, A. Day, M. Riegler, L. E. Hugo, K. N. Johnson, B. H. Kay, A. E. McGraw, A. F. van den Hurk, P. A. Ryan, and S. L. O'Neill. 2009. A *Wolbachia* symbiont in *Aedes aegypti* limits infection with dengue, Chikungunya, and Plasmodium. Cell 139: 1268–1278.

Moretti, R., and M. Calvitti. 2013. Male mating performance and cytoplasmic incompatibility in a *w*Pip *Wolbachia* trans-infected line of *Aedes albopictus* (*Stegomyia albopicta*). Medical and Veterinary Entomology 27: 377–386.

Moretti, R., G. A. Marzo, E. Lampazzi, and M. Calvitti. 2018a. Cytoplasmic incompatibility management to support Incompatible Insect Technique against *Aedes albopictus*. Parasites & Vectors 11 (Supplement 2): 649.

Moretti, R., P.-S. Yen, V. Houé, E. Lampazzi, A. Desiderio, A.-B. Failloux, and M. Calvitti. 2018b. Combining *Wolbachia*-induced sterility and virus protection to fight *Aedes albopictus*-borne viruses. PLoS Neglected Tropical Diseases 12: e0006626.

Ndo, C., H. Yamada, D. D. Damiens, S. N'do, G. Seballos, and J. R. L. Gilles. 2014. X-ray sterilisation of the *An. arabiensis* genetic sexing strain 'ANO IPCL1' at pupal and adult stages. Acta Tropica 131: 124–128.

Neuenschwander, P., K. Russ, E. Höbaus, and S. Michelakis. 1983. Ecological studies on *Rhagoletis cerasi* L. in Crete for the use of the incompatible insect technique, pp. 41–51. *In* R. Calvalloro (ed.), Fruit flies of economic importance. Proceedings of the CEC/IOBC International Symposium. Athens, Greece, 16-19 November 1982. A. A. Balkema, Rotterdam, The Netherlands.

Nikolouli, K., H. Colinet, D. Renault, T. Enriquez, L. Mouton, P. Gibert, F. Sassu, C. Caceres, C. Stauffer, R. Pereira, and K. Bourtzis. 2018. Sterile insect technique and *Wolbachia* symbiosis as potential tools for the control of the invasive species *Drosophila suzukii*. Journal of Pest Science 91: 489–503.

O'Connor, L., C. Plichart, A. C. Sang, C. L. Brelsfoard, H. C. Bossin, and S. L. Dobson. 2012. Open release of male mosquitoes infected with a *Wolbachia* biopesticide: Field performance and infection containment. PLoS Neglected Tropical Diseases 6: e1797.

Oliva, C. F., M. Jacquet, J. R. L. Gilles, G. Lemperiere, P.-O. Maquart, S. Quilici, F. Schooneman, M. J. B. Vreysen, and S. Boyer. 2012. The sterile insect technique for controlling populations of *Aedes albopictus* (Diptera: Culicidae) on Réunion Island: Mating vigour of sterilized males. PLoS One 7: e49414.

Pal, R. 1966. Genetic control of vectors of disease with special reference to *Culex pipiens fatigans*. WHO/Vector Control/66.198. World Health Organization, Geneva, Switzerland.

Pal, R. 1974. WHO/ICMR programme of genetic control of mosquitoes in India, pp. 73–95. *In* R. Pal and M. J. Whitten (eds.), The use of genetics in insect control. Elsevier / North-Holland Publishing Company, Amsterdam, The Netherlands.

Parker, A., and K. Mehta. 2007. Sterile insect technique: A model for dose optimization for improved sterile insect quality. Florida Entomologist 90: 88–95.

Pereira, T. N., M. N. Rocha, P. H. F. Sucupira, F. D. Carvalho, and L. A. Moreira. 2018. *Wolbachia* significantly impacts the vector competence of *Aedes aegypti* for Mayaro virus. Scientific Reports 8: 6889.

Portaro, J. K., and A. R. Barr. 1975. "Curing" *Wolbachia* infections in *Culex pipiens*. Journal of Medical Entomology 12: 265.

Puggioli, A., M. Calvitti, R. Moretti, and R. Bellini. 2016. *w*Pip *Wolbachia* contribution to *Aedes albopictus* SIT performance: Advantages under intensive rearing. Acta Tropica 164: 473–481.

Ranner, H. 1990. Untersuchungen zur Biologie und Bekämpfung der Kirschfruchtfliege, *Rhagoletis cerasi* L. (Diptera, Trypetidae) - V. Versuche zur Bekämpfung der Kirschfruchtfliege mit Hilfe der Incompatible Insect Technique (IIT). Pflanzenschutzberichte 51: 1–16.

Rasgon, J. L. 2012. *Wolbachia* induces male-specific mortality in the mosquito *Culex pipiens* (LIN strain). PLoS One 7: e30381.

Rasgon, J. L., L. M. Styer, and T. W. Scott. 2003. *Wolbachia*-induced mortality as a mechanism to modulate pathogen transmission by vector arthropods. Journal of Medical Entomology 40: 125–132.

Roubaud, É. 1941. Phénomènes d'amixie dans les intercroisements de Culicides du groupe *pipiens*. Comptes Rendus Hebdomadaires des Séances de l'Académie des Sciences 212: 257–259.

Ruang-areerate, T., and P. Kittayapong. 2006. *Wolbachia* transinfection in *Aedes aegypti*: A potential gene driver of dengue vectors. Proceedings of the National Academy of Sciences of the USA 103: 12534–12539.

Russ, K., and B. Faber. 1979. The possible use of IIT to control *Rhagoletis cerasi* L., the European cherry fruit fly in Austria, pages 38–39. *In* IOBC-WPRS Bulletin, Volume 2, Proceedings of a Joint Meeting of the Working Groups "Biological Control of Olive Pests", "Genetic Control of *Rhagoletis cerasi*", "Genetic Control of *Ceratitis capitata*" and "Genetic Methods of Pest Control". 15-20 May 1978. Sassari, Sardinia, Italy.

Scott, M. J., and M. Q. Benedict. 2016. Concept and history of genetic control, pp. 31–54. *In* Z. N. Adelman (ed.), Genetic control of malaria and dengue. Academic Press, London, UK.

Segoli, M., A. A. Hoffmann, J. Lloyd, G. J. Omodei, and S. A. Ritchie. 2014. The effect of virus-blocking *Wolbachia* on male competitiveness of the dengue vector mosquito, *Aedes aegypti*. PLoS Neglected Tropical Diseases 8: e3294.

Shahid, M. A., and C. F. Curtis. 1987. Radiation sterilisation and cytoplasmic incompatibility in a "tropicalized" strain of the *Culex pipiens* complex (Diptera: Culicidae). Journal of Medical Entomology 24: 273–274.

Sharma, V. P., S. K. Subbarao, T. Adak, and R. K. Razdan. 1979. Integration of gamma irradiation and cytoplasmic incompatibility in *Culex pipiens fatigans* (Diptera: Culicidae). Journal of Medical Entomology 15: 155–156.

Singh, K. R. P., C. F. Curtis, and B. S. Krishnamurthy. 1976. Partial loss of cytoplasmic incompatibility with age in males of *Culex fatigans*. Annals of Tropical Medicine and Parasitology 70: 463–466.

Sinkins, S. P. 2004. *Wolbachia* and cytoplasmic incompatibility in mosquitoes. Insect Biochemistry and Molecular Biology 34: 723–729.

Sinkins, S. P., C. F. Curtis, and S. L. O'Neill. 1997. The potential application of inherited symbiont systems to pest control, pp. 155–175. *In* S. L. O'Neill, A. A. Hoffmann, and J. H. Werren (eds.), Influential passengers: Inherited microorganisms and arthropod reproduction. Oxford University Press, Oxford, UK.

Subbarao, S. K., C. F. Curtis, K. R. P. Singh, and B. S. Krishnamurthy. 1974. Variation in cytoplasmic crossing type in population of *C. p. fatigans* Wied. from Delhi area. The Journal of Communicable Diseases 6: 80–82.

Subbarao, S. K., C. F. Curtis, B. S. Krishnamurthy, T. Adak, and R. K. Chandrahas. 1977. Selection for partial compatibility with aged and previously mated males in *Culex pipiens fatigans* (Diptera: Culicidae). Journal of Medical Entomology 14: 82–85.

Suh, E., D. R. Mercer, Y. Fu, and S. L. Dobson. 2009. Pathogenicity of life-shortening *Wolbachia* in *Aedes albopictus* after transfer from *Drosophila melanogaster*. Applied and Environmental Microbiology 75: 7783–7788.

Teixeira, L., A. Ferreira, and M. Ashburner. 2008. The bacterial symbiont *Wolbachia* induces resistance to RNA viral infections in *Drosophila melanogaster*. PLoS Biology 6: e2.

Thomas, V., and K. R. P. Singh. 1977. Comparative susceptibility to *Wuchereria bancrofti* of *Culex pipiens fatigans* Delhi strain and of strains cytoplasmically incompatible with it. The Indian Journal of Medical Research 65 (Supplement): 102–106.

Tortosa, P., S. Charlat, P. Labbé, J.-S. Dehecq, H. Barré, and M. Weill. 2010. *Wolbachia* age-sex-specific density in *Aedes albopictus*: A host evolutionary response to cytoplasmic incompatibility? PLoS One 5: e9700.

Turley, A. P., M. P. Zalucki, S. L. O'Neill, and E. A. McGraw. 2013. Transinfected *Wolbachia* have minimal effects on male reproductive success in *Aedes aegypti*. Parasites & Vectors 6: 36.

van den Hurk, A. F., S. Hall-Mendelin, A. T. Pyke, F. D. Frentiu, K. McElroy, A. Day, S. Higgs, and S. L. O'Neill. 2012. Impact of *Wolbachia* on infection with Chikungunya and yellow fever viruses in the mosquito vector *Aedes aegypti*. PLoS Neglected Tropical Diseases 6: e1892.

Walker, T., P. H. Johnson, L. A. Moreira, I. Iturbe-Ormaetxe, F. D. Frentiu, C. J. McMeniman, Y. S. Leong, Y. Dong, J. Axford, P. Kriesner, A. L. Lloyd, S. A. Ritchie, S. L. O'Neill, and A. A. Hoffmann. 2011. The *w*Mel *Wolbachia* strain blocks dengue and invades caged *Aedes aegypti* populations. Nature 476: (7361) 450–453.

Weidhaas, D. E., and J. Seawright. 1976. Comments on the article "Genetic control of mosquitoes" (Laven, 1974), pp. 211–220. *In* Proceedings Tall Timbers Conference on Ecological Animal Control by Habitat Management, Number 6. Tall Timbers Research Station, Tallahassee, Florida, USA.

Weinert, L. A., E. V. Araujo-Jnr, M. Z. Ahmed, and J. J. Welch. 2015. The incidence of bacterial endosymbionts in terrestrial arthropods. Proceedings of the Royal Society, Series B: Biological Sciences 282: (1807) 20150249.

Werren, J. H., L. Baldo, and M. E. Clark. 2008. *Wolbachia*: Master manipulators of invertebrate biology. Nature Reviews Microbiology 6: 741–751.

(WHO) World Health Organization. 1964. Genetics of vectors and insecticide resistance. Report of a WHO Scientific Group meeting held in Geneva from 5 to 9 August 1963. Number 268. Geneva, Switzerland. 40 pp.

Xi, Z., and S. L. Dobson. 2005. Characterization of *Wolbachia* transfection efficiency by using microinjection of embryonic cytoplasm and embryo homogenate. Applied and Environmental Microbiology 71: 3199–3204.

Xi, Z., and D. Joshi. 2016. Genetic control of malaria and dengue using *Wolbachia*, pp. 305–333. *In* Z. N. Adelman (ed.), Genetic control of malaria and dengue. Academic Press, London, UK.

Xi, Z., and P. Manrique-Saide. 2018. Scale up from field trial to operation: The combined IIT/SIT approach to eliminate the primary dengue mosquito vectors in China and Mexico. Reunión Nacional del Programa de Enfermedades Transmitidas por Vectores. Los Cabos, Baja California Sur, Mexico.

Xi, Z., J. L. Dean, C. C. H. Khoo, and S. L. Dobson. 2005a. Generation of a novel *Wolbachia* infection in *Aedes albopictus* (Asian tiger mosquito) via embryonic microinjection. Insect Biochemistry and Molecular Biology 35: 903–910.

Xi, Z., C. C. H. Khoo, and S. L. Dobson. 2005b. *Wolbachia* establishment and invasion in an *Aedes aegypti* laboratory population. Science 310: (5746) 326–328.

Xi, Z., C. C. H. Khoo, and S. L. Dobson. 2006. Interspecific transfer of *Wolbachia* into the mosquito disease vector *Aedes albopictus*. Proceedings of the Royal Society, Series B: Biological Sciences 273 (1592): 1317–1322.

Yamada, H., A. G. Parker, C. F. Oliva, F. Balestrino, and J. R. L. Gilles. 2014a. X-ray-induced sterility in *Aedes albopictus* (Diptera: Culicidae) and male longevity following irradiation. Journal of Medical Entomology 51: 811–816.

Yamada, H., M. J. B. Vreysen, J. R. L. Gilles, G. Munhenga, and D. D. Damiens. 2014b. The effects of genetic manipulation, dieldrin treatment and irradiation on the mating competitiveness of male *Anopheles arabiensis* in field cages. Malaria Journal 13: 318.

Yamada, H., H. Maiga, J. Juarez, D. De Oliveira Carvalho, W. Mamai, A. Ali, N. S. Bimbile-Somda, A. G. Parker, D. Zhang, and J. Bouyer. 2019. Identification of critical factors that significantly affect the dose-response in mosquitoes irradiated as pupae. Parasites & Vectors 12: (1) 435.

Yen, J. H., and A. R. Barr. 1971. New hypothesis of the cause of cytoplasmic incompatibility in *Culex pipiens* L. Nature 232: (5313) 657–658.

Yen, J. H., and A. R. Barr. 1973. The etiological agent of cytoplasmic incompatibility in *Culex pipiens*. Journal of Invertebrate Pathology 22: 242–250.

Yeung, J. 2018. Australian experiment wipes out over 80% of disease-carrying mosquitoes. CNN Health. 10th July 2018.

Zabalou, S., M. Riegler, M. Theodorakopoulou, C. Stauffer, C. Savakis, and K. Bourtzis. 2004. *Wolbachia*-induced cytoplasmic incompatibility as a means for insect pest population control. Proceedings of the National Academy of Sciences of the USA 101: 15042–15045.

Zabalou, S., A. Apostolaki, I. Livadaras, G. Franz, A. S. Robinson, C. Savakis, and K. Bourtzis. 2009. Incompatible insect technique: Incompatible males from a *Ceratitis capitata* genetic sexing strain. Entomologia Experimentalis et Applicata 132: 232–240.

Zélé, F., A. Nicot, A. Berthomieu, M. Weill, O. Duron, and A. Rivero. 2014. *Wolbachia* increases susceptibility to *Plasmodium* infection in a natural system. Proceedings of the Royal Society, Series B: Biological Sciences 281: (1779) 20132837.

Zhang, D., X. Zheng, Z. Xi, K. Bourtzis, and J. R. L. Gilles. 2015a. Combining the sterile insect technique with the incompatible insect technique: I - Impact of *Wolbachia* infection on the fitness of triple- and double-infected strains of *Aedes albopictus*. PLoS One 10: e0121126.

Zhang, D., R. S. Lees, Z. Xi, J. R. L. Gilles, and K. Bourtzis. 2015b. Combining the sterile insect technique with *Wolbachia*-based approaches: II - A safer approach to *Aedes albopictus* population suppression programmes, designed to minimize the consequences of inadvertent female release. PLoS One 10: e0135194.

Zhang, D., R. S. Lees, Z. Xi, K. Bourtzis, and J. R. L. Gilles. 2016. Combining the sterile insect technique with the incompatible insect technique: III - Robust mating competitiveness of irradiated triple *Wolbachia*-infected *Aedes albopictus* males under semi-field conditions. PLoS One 11: e0151864.

Zhang, D., M. Zhang, Y. Wu, J. R. L. Gilles, H. Yamada, Z. Wu, Z. Xi, and X. Zheng. 2017. Establishment of a medium-scale mosquito facility: Optimization of the larval mass-rearing unit for *Aedes albopictus* (Diptera: Culicidae). Parasites & Vectors 10: 569.

Zhang, D., Y. Li, Q. Sun, X. Zheng, J. R. L. Gilles, H. Yamada, Z. Wu, Z. Xi, and Y. Wu. 2018. Establishment of a medium-scale mosquito facility: Tests on mass production cages for *Aedes albopictus* (Diptera: Culicidae). Parasites & Vectors 11: 189.

Zheng, X., D. Zhang, Y. Li, C. Yang, Y. Wu, X. Liang, Y. Liang, X. Pan, L. Hu, Q. Sun, X. Wang, Y. Wei, J. Zhu, W. Qian, Z. Yan, A. G. Parker, J. R. L. Gilles, K. Bourtzis, et al. and Z. Xi. 2019. Incompatible and sterile insect techniques combined eliminate mosquitoes. Nature 572: 56–61.

Zug, R., and P. Hammerstein. 2012. Still a host of hosts for *Wolbachia*: Analysis of recent data suggests that 40% of terrestrial arthropod species are infected. PLoS One 7: e38544.

COMBINED STERILE INSECT TECHNIQUE AND INCOMPATIBLE INSECT TECHNIQUE: CONCEPT, STUDY DESIGN, EXPERIENCE AND LESSONS LEARNED FROM A PILOT SUPPRESSION TRIAL IN THAILAND

P. KITTAYAPONG[1,2]

[1]Center of Excellence for Vectors and Vector-Borne Diseases, Faculty of Science, Mahidol University, 999 Phuttamonthon 4 Road, Nakhon Pathom 73170, Thailand
[2]Department of Biology, Faculty of Science, Mahidol University, 272 Rama VI Road, Bangkok 10400, Thailand; pkittayapong@gmail.com

SUMMARY

Climate change, rapid global transport and land use change leading to urbanization and agricultural intensification have facilitated disease emergence in vulnerable regions like Southeast Asia, and also the global expansion of vectors and vector-borne diseases into other regions like the Americas and Europe. Important vector-borne diseases, i.e. dengue, chikungunya, yellow fever, and Zika are transmitted by the major mosquito vector species, *Aedes aegypti* (L.) and *Aedes albopictus* (Skuse). Management of *Ae. aegypti* populations in countries endemic to these diseases, especially in Southeast Asia, is not sufficiently effective, resulting in high morbidity and mortality in the region. Insecticide resistance has become an important issue, causing failure in insecticide-based vector control. Innovative or alternative tools/approaches are needed to effectively reduce mosquito vector populations and consequently reduce the diseases they transmit. A trial integrating the environment-friendly Sterile Insect Technique (SIT) and the insect incompatible technique (IIT) was successfully carried out on a small-scale in a semi-rural setting in Thailand. In this chapter, we report on the design and methodology, as well as the experience and lessons learned from the baseline preparation and implementation of the pilot trial.

Key Words: *Aedes aegypti*, *Aedes albopictus*, SIT, IIT, *Wolbachia*, mosquito control, integrated vector management, vector-borne diseases

J. Hendrichs, R. Pereira and M. J. B. Vreysen (eds.), Area-Wide Integrated Pest Management: Development and Field Application, pp. 405–432. CRC Press, Boca Raton, Florida, USA.

1. INTRODUCTION

Vector-borne diseases are becoming increasingly a public health problem and globally a significant economic burden. According to the World Health Organization (WHO), about half of the world's people in over 100 countries are at risk of contracting dengue (WHO 2019a). Chikungunya, another viral disease transmitted to humans by mosquito vectors, was originally confined to Africa but has recently been spreading rapidly across the Indian Ocean, Europe, the Americas, Asia, and Oceania. In the last decade, outbreaks of Zika in several parts of the world epitomized the need for new and effective methodologies to manage mosquito populations vectoring these diseases.

With the number of dengue cases and the number of countries affected rising dramatically in recent years, the socio-economic impact of mosquito-transmitted diseases is enormous. The overall estimated annual economic burden of dengue in Southeast Asia was USD 950 million, with the average annual direct costs being USD 451 million and the indirect costs being USD 499 million (Shepard et al. 2013). In Thailand alone, a recent study estimated the mean economic cost of dengue at USD 135 million per annum (Shepard et al. 2013). In the absence of affordable and effective vaccines and drugs to combat dengue, chikungunya, and Zika, population control of mosquito vectors is the most effective way of managing these diseases. Most vector control strategies are insecticide-based, and their widespread use has resulted in increased insecticide resistance among the mosquitoes. Therefore, there is an urgent need for alternative novel approaches for vector control.

Aedes aegypti (L.), the yellow fever mosquito, is considered the main mosquito vector for dengue, chikungunya, and Zika in many parts of the world (Calvez et al. 2017; Kotsakiozi et al. 2017; Trewin et al. 2017). Attempts have been made to control this invasive species, but traditional mosquito control methods, such as insecticide applications and source reduction by eliminating larval breeding sites have been insufficient for suppressing this mosquito vector and reducing disease incidences (Fredericks and Fernandez-Sesma 2014; Trewin et al. 2017).

Several novel *Ae. aegypti* control methods, namely the Sterile Insect Technique (SIT) that is based on the release of irradiated sterile males (Dyck et al. 2021); the Incompatible Insect Technique (IIT), which depends on *Wolbachia*-induced cytoplasmic incompatibility (CI) by releasing *Wolbachia*-infected males (Bourtzis et al. 2014, Mains et al. 2016; Zheng et al. 2019); and the application of genetically modified mosquito strains, such as those carrying RIDL (Release of Insects carrying a Dominant Lethal) constructs (Thomas et al. 2000; Morrison et al. 2010; Carvalho et al. 2015), have recently been endorsed by the WHO to help contain the recent Zika virus outbreak (Zheng et al. 2015; Yakob et al. 2017; WHO 2019b).

Both the SIT and the IIT are based on the repeated inundated release of large numbers of high quality sterile male mosquitoes to compete with their wild male counterparts in mating with wild females in a target area, thus inducing female sterility, which results in a reduction in the target populations (Zheng et al. 2015; Mains et al. 2016; WHO 2017; Zhang et al. 2017; Zheng et al. 2019) and consequently a potential reduction or prevention of the transmission of mosquito-borne diseases.

As a component of area-wide integrated pest management (AW-IPM) programmes, the implementation of the SIT and the IIT depends on several important components, including mass-rearing, sex separation, sterilisation, transportation, release, and monitoring (Zhang et al. 2017; Nikolouli et al. 2018; Dyck et al. 2021). Hence, the number of released sterile males must significantly surpass the number of wild males in the release area to compensate for any negative effect associated with domestication, mass-rearing, storage, and their overall handling, so that they can compete with wild males for matings with wild females, allowing the introduction of sufficient sterility into the wild populations (Vreysen et al. 2007; Barnes et al. 2015; Nikolouli et al. 2018; Dyck et al. 2021).

The combination of *Wolbachia*-induced IIT and the SIT was applied together with initial source reduction to suppress natural populations of *Ae. aegypti* in a semi-rural village in Chachoengsao Province, eastern Thailand. Results of this pilot trial indicated successful reduction of local *Ae. aegypti* populations after 6 months of repeated releases of sterile males (Kittayapong et al. 2019).

In this chapter, we report on the study design and methodology of this pilot trial. In addition, experience and lessons learned from the baseline experiments and from the implementation of this small-scale pilot trial are discussed.

2. COMBINED SIT/IIT APPROACH: CONCEPT AND PROGRESS

2.1. Sterile Insect Technique (SIT) for Mosquito Control

The SIT is a method of insect pest control with a strong record of success against a wide range of agricultural pests and which potentially can work against mosquitoes (Dyck et al. 2021). The technique consists of repeated area-wide releases of large numbers of sterile males in the target area, where they will mate with native females. Eggs will be produced but they will not hatch. When adequate sterile to wild male overflooding ratios are maintained, the number of native insects decreases with each generation, potentially driving the native population to very low numbers or, under complete isolation, to local extinction.

The SIT has been successfully implemented in large-scale operations to control agricultural insect pests and to prevent losses in livestock or crops of economic importance. Because it has no environmental impact and its relatively unobtrusive means of deployment, the SIT had been well accepted, even in urban areas. This technique has been successfully proven for over 50 years and is cost-effective for the population control of some major agricultural and livestock pests (Vreysen et al. 2000; Dyck et al. 2021). For public health pests, the SIT has been the subject of extended research since the late-1950s. However, it has never reached an operational level (Dame et al. 2009), even though it is considered to be a highly sustainable and environment-friendly method with, so far, no negative effect on human health (Alphey et al. 2010).

The first experimental sterile mosquito releases were conducted by the United States Department of Agriculture (USDA) in southern Florida. A total of 32 000 sterile *Anopheles quadrimaculatus* Say males that emerged from pupae irradiated with 120 Gy were released for three months in 1959 and in 1960; this amount was increased to 300 000 released over a period of nine months (Weidhaas et al. 1962;

Dame et al. 1964, 2009). However, the project was considered not successful as insufficient sterility was induced in the wild population (Dame et al. 1964, 2009).

The Centers for Disease Control and Prevention (CDC) carried out a release trial in Pensacola, Florida with 110-180 Gy treated *Ae. aegypti*. Although 3.9 million sterile males were released over four months in 1960 and 6.7 million over six months in 1961, the project was considered a failure due to reduced sterile male competitiveness caused by the irradiation of the pupae (Morlan et al. 1962; Dame et al. 2009).

Between 1967 and 1974, the World Health Organization/Indian Council of Medical Research (WHO/ICMR) and the USDA released male *Culex quinquefasciatus* Say irradiated with 60-120 Gy in India and Florida, respectively. The daily release rate ranged between 9000 to 15 000 sterile males. Nevertheless, these studies confirmed previous laboratory findings that the somatic damage was greater when younger pupae were treated as compared with older pupae (Patterson et al. 1975, 1977; Dame et al. 2009).

In 1980, a total of 71 000 sterile *Culex tarsalis* Coquillett males, sterilized by 60 Gy irradiation at the adult stage, were released in California, USA and results showed that these sterile males were fully competitive. However, in 1981, 85 000 sterile males were released, but these sterile males were not capable of seeking out the wild females and transferring the sterile sperm (Reisen et al. 1982; Dame et al. 2009).

Mosquito releases have been carried out for numerous purposes related to SIT application, but most of them were directed at answering a specific research question without any anticipation of population suppression. However, a few suppression and/or elimination projects have been attempted, but only modest effects were observed on sterility of the oviposited eggs and reduction of the wild population density (Benedict and Robinson 2003).

More recently, several SIT pilot projects have been initiated to answer specific questions (Lees et al. 2021). The effect of irradiation on sexual maturation and mating success of males, and the sexual competitiveness of sterile versus wild males in the presence of wild females of *Aedes albopictus* (Skuse) were studied under semi-field conditions in La Réunion Island (Oliva et al. 2012). In Sudan, participation of irradiated *Anopheles arabiensis* Patton males in mating swarms during the evening after their release was demonstrated, but their competitiveness and achieving successful copulation in the field was not proven (Ageep et al. 2014).

In Mauritius, the Ministry of Health and Quality of Life has been developing an operational plan to assess the SIT for population reduction of *Ae. albopictus* to prevent and control chikungunya and dengue, and guidelines for site selection were developed with the beginning of population surveillance (Iyaloo et al. 2014).

The first successful SIT mosquito pilot project was initiated during the summer of 2004 in three small towns in northern Italy. Approximately 900-1600 irradiated *Ae. albopictus* pupae were released per hectare, per week, and this continued for five years. The trial induced up to 68% egg sterility in the target population, demonstrating the potential of sterile males to suppress populations of *Ae. albopictus* (Bellini et al. 2007, 2013; Lees et al. 2015).

To date, there have been no large operational mosquito SIT projects, but operational programmes should eventually become established and more efficient over time (Dame et al. 2009). Experimentation and preparation processes for SIT application tend to be longer-term. Also, it does not have immediate effects on vector numbers, but impacts the size of the wild population in the next generation. In addition, entomological surveillance of the vector population before and during releases is essential to monitor the impact of any releases (Dame et al. 2009; Alphey et al. 2010). Nevertheless, the SIT is robust in term of both efficacy and cost when used in combination with other compatible methods, resulting in successful and sustainable vector control. Apart from being an environmentally-sound biological control approach, the SIT can be easily integrated with other biological control strategies (parasitoids, predators, and pathogens) (Vreysen et al. 2007; Barnes et al. 2015; Nikolouli et al. 2018).

2.2. Wolbachia-*based Approach for Mosquito Control*

Wolbachia are intracellular endosymbionts belonging to Alpha-proteobacteria. They are found in many arthropods and nematodes, and the overall species infection rate is as high as 66% (Hilgenboecker et al. 2008). *Wolbachia* bacteria have attracted the interest of the scientific community because of their potential to block arbovirus infections in mosquitoes (Moreira et al. 2009; Bian et al. 2013), as well as their capacity to replace natural populations of insects through their CI properties (Turelli and Hoffmann 1995). *Wolbachia*-infected male insects are not compatible with their non-infected natural females, leading to a reduction in the egg hatch of *Wolbachia*-uninfected populations and then the replacement by *Wolbachia*-infected populations (Hoffmann et al. 2011). The benefit of CI has been widely recognized for mosquito vector control (Clark et al. 2002; Atyame et al. 2014; Altinli et al. 2018; Baton et al., this volume).

A few years after the development of *Wolbachia*-transinfected *Aedes* mosquitoes (Xi et al. 2005), open field releases of these mosquitoes were carried out to evaluate whether CI induced by *Wolbachia* and their antiviral ability could be used for population suppression in vector control programmes. An open field release of *Aedes polynesiensis* Marks fluorescent-marked males infected with *Wolbachia* was launched in French Polynesia in 2009. The study showed that *Wolbachia*-transinfected *Ae. polynesiensis* males were competitive under field conditions; and after 30 weeks of releases, the egg hatch rate was significantly reduced in the release area, resulting in a reduction of the density of the local mosquito population (O'Connor et al. 2012).

The feasibility of using *Wolbachia* triple infected *Ae. albopictus* as a biopesticide against natural *Wolbachia* double infected *Ae. albopictus* was demonstrated in Lexington, USA and Guangzhou, China. Both the egg hatch rate and the number of adult *Ae. albopictus* were significantly reduced following the release of *Wolbachia*-infected males in these trials (Mains et al. 2016; Zheng et al. 2019). In addition, IIT was demonstrated to successfully suppress natural populations of *Ae. aegypti* in South Miami, USA in order to prevent the Zika disease by releasing *w*AlbB *Wolbachia*-infected *Ae. aegypti* males (Mains et al. 2019).

Strict male release is required for IIT application to obtain vector suppression (O'Connor et al. 2012; Nikolouli et al. 2018; Baton et al., this volume). Indeed, the accidental release of females infected by *Wolbachia* may cause the replacement of the targeted population by a population carrying the *Wolbachia* infection, resulting in field populations being compatible with the released males. Therefore, IIT application requires the development of an efficient method for sex separation at mass-rearing scales, in order to strictly release only *Wolbachia*-infected males (O'Connor et al. 2012; Nikolouli et al. 2018). Different techniques like phenotypic sorting or genetic sexing methods based on classical genetic or molecular methods have been reported for separation or sexing methods (Gilles et al. 2014). However, these methods are not available for all target species, and some techniques involve the release of genetically modified organisms (GMOs), the use of which is of concern in the European Union, as they face public opposition. In addition to public acceptance, GMO releases also face regulation difficulties in some countries, including China and India (O'Connor et al. 2012; Nikolouli et al. 2018).

In Australia, a risk analysis was carried out before the first release of *Wolbachia*-transinfected *Ae. aegypti* male and female mosquitoes into the environment for the purpose of population replacement (Murray et al. 2016). The first release into the field of *Ae. aegypti* males and females infected with *w*Mel was approved and took place in 2011 near Cairns in north-eastern Australia. The study showed that *Wolbachia*-transinfected *Ae. aegypti* successfully invaded and completely replaced uninfected wild *Ae. aegypti* populations (Hoffmann et al. 2011). A follow-up study indicated that field *w*Mel-infected *Ae. aegypti* mosquitoes (F_1), collected one year following the field release, had very low levels of dengue virus replication and dissemination. The frequency of *w*Mel-infected *Ae. aegypti* remained at more than 90% in the mosquito populations for more than 3 years (Frentiu et al. 2014). The success of this first release has led to small- and large-scale releases of *w*Mel-transinfected *Ae. aegypti* in other countries, in order to evaluate the effectiveness of population replacement in controlling dengue disease in human populations (Joubert et al. 2016).

2.3. Development of the Combined SIT/IIT-based Approach

Much progress has been made in recent years towards developing the required technology and methodology to bring mosquito sterility to field application. Hence, pilot releases have begun in a number of sites around the world (Lees et al. 2015). Since the key mosquito disease vectors are all relatively amenable to colonization and rearing, and in many situations the natural population densities are low, the SIT, the IIT, or a combination of the two, are well suited for their management (Lees et al. 2015). The SIT/IIT combination could in principle be applied to any targeted species for which an adequate and highly effective sexing system is not available (Zhang et al. 2015a, 2015b, 2016; Nikolouli et al. 2018; Zheng et al. 2019). However, successful SIT/IIT programmes will also depend on having *Wolbachia* strains with good CI and maternal transmission phenotypes, apart from an effective sexing system.

As the female mosquitoes are more radiation sensitive than males, the minimum dose of radiation that leads to complete sterility in females, whilst not negatively affecting male mating competitiveness, has been identified (Zhang et al. 2015b, 2016, 2017; Nikolouli et al. 2018). As a result, any accidentally released *Wolbachia*-infected females are sterile, and the risk of population replacement is minimised (Lees et al. 2015; Bourtzis et al. 2014; Zhang et al. 2017; Nikolouli et al. 2018; Dyck et al. 2021). Integration of the low irradiation dose with CI when using the virus-resistant strains of *Wolbachia* also minimizes any potential disease transmission by accidentally released sterile females. This has proven to be an efficient strategy in programmes targeting population suppression of *Ae. albopictus* (Zhang et al. 2015a, 2015b, 2016; Nikolouli et al. 2018; Zheng et al. 2019) and *Ae. aegypti* (NEA 2019; Liew et al., this volume). As stated by the WHO, this combined SIT/IIT technology has potential for long-term control of *Ae. aegypti* and *Ae. albopictus* mosquito populations, and this approach is considered an effective and safe strategy for the management of mosquito populations (WHO 2017).

2.4. Combined SIT/IIT Pilot Trial in Thailand

A field application of the combined SIT/IIT approach to reduce a local *Ae. aegypti* population was first demonstrated on a small-scale pilot trial with a total study area of 2.19 km^2 in the Plaeng Yao District of Chachoengsao Province, in eastern Thailand. Using the direct microinjection method, two *Wolbachia* strains from *Ae. albopictus* collected from rubber plantations in Thailand were introduced into *Ae. aegypti*. This newly developed *Ae. aegypti* line produced progeny infected with the *Wolbachia* strains *w*AlbA and *w*AlbB, with maternal transmission efficiency as high as 85% after 6 generations (Ruang-areerate and Kittayapong 2006). For the combined SIT/IIT approach, the CI property of *Wolbachia* was used to sterilize natural *Ae .aegypti* mosquito vector populations, while radiation was used to avoid population replacement by assuring that no fertile females were accidentally released.

Once released into nature, *Wolbachia*-transinfected *Ae. aegypti* male mosquitoes not only induced sterility in the females of the natural populations, but any potential virus transmission was also blocked in case a few female mosquitoes were inadvertently present in the releases into nature (Moreira et al. 2009). As the SIT/IT method aims at developing a vector suppression tool that is environment-friendly, no propagation of released mosquitoes should happen in nature. To achieve this goal, sterility of the *Wolbachia*-transinfected male mosquitoes was ensured by exposing the males to an appropriate irradiation dose (Kittayapong et al. 2018).

3. STUDY DESIGN AND METHODOLOGY

3.1. Study Site Selection

Selection of the study site to assess SIT, IIT, or combined SIT/IIT application is important for the success of any pilot field trial, and general guidelines for site selection were considered using Mauritius as a case study (Iyaloo et al. 2014). However, due to the differences and uniqueness of any study site, local considerations on specific details are needed. For the ideal study site, the following general criteria should be considered:

a) geographically- or ecologically-isolated
b) targeted mosquito species are dominant
c) manageable size for surveillance and monitoring, and
d) good cooperation of the local government and local communities.

The study site selected for the pilot field trial of the SIT/IIT approach in Thailand was located in the Plaeng Yao District of Chachoengsao Province in the eastern part of the country, which is about 120 km southeast of Bangkok. Three study areas were selected: Nong Satit as the treatment area, Pleang Mai Daeng as the adjacent area, and Nong Sarika as the control area. The distance between the treatment and the control areas was approximately 12 km, whereas the distance between the treatment and the adjacent area was approximately 500-800 meters.

The study areas were located among rice and cassava fields, as well as rubber and other plantations, which formed an ideal partial barrier to the movement of *Ae. aegypti* mosquito vectors. The selected study site was considered a typical semi-rural village similar to most other villages in Thailand (Fig. 1). In addition, it met the general criteria for site selection as previously described.

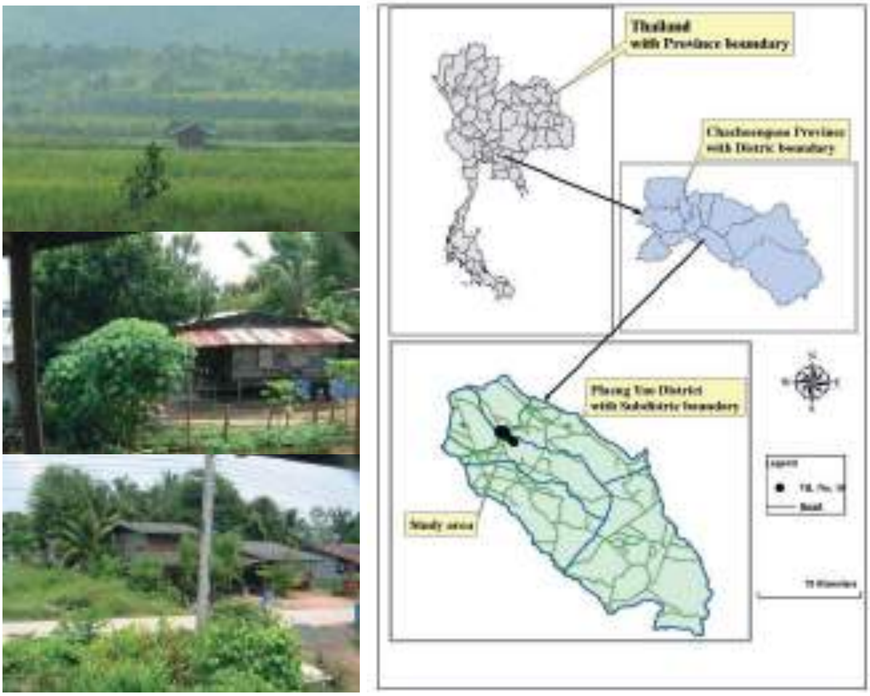

Figure 1. Maps and pictures showing the pilot treatment site located in Nong Satit Village (Village No. 10), Hua Sam Rong Sub-District, Plaeng Yao District, and surrounding areas in Chachoengsao Province, eastern Thailand, where sterile Aedes aegypti *male mosquitoes were released for the first time.*

3.2. Spatial Baseline Data Collection/Mapping of Study Sites

Spatial data obtained from a geographic information system (GIS), supplemented with 'ground truthing,' were used to characterize spatial distribution and patterns of households located at the study areas. Handheld Global Positioning System (GPS) sets were used to record all houses in the study site, and ArcMap software (ESRI, Redlands, California) was used to develop a GIS map (Chansang and Kittayapong 2007; Kittayapong et al. 2008). This GIS map was useful in determining the sampling households (Fig. 2).

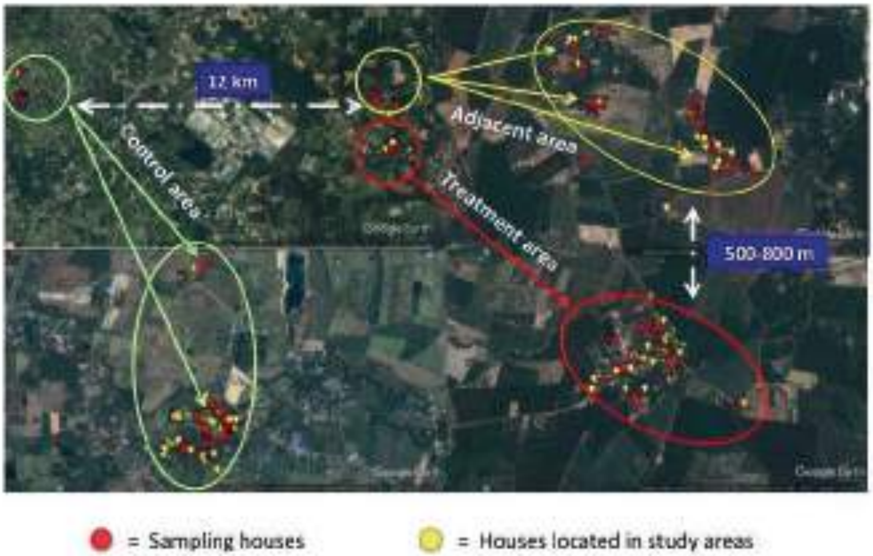

Figure 2. GIS map of the study site (upper left), including the treatment, adjacent, and control areas, and the sampling houses and other houses located in Pleang Yao District, Chachoengsao Province, eastern Thailand.

3.3. Community Engagement Strategy

Government authorities were officially informed about the project objectives and methodologies after the project had obtained institutional ethical approval. A community education campaign was organized for the communities in the study area, before implementation of the project, to raise awareness of vector-borne diseases and alternative vector control using combined SIT/IIT. Furthermore, the local leaders were invited to the meetings, and the key message delivered was that male mosquitoes cannot bite and do not feed on blood. In addition, sterile male mosquitoes in screened cages were brought to the community to demonstrate the key message.

The field release of sterile *Ae. aegypti* male mosquitoes was emphasized as an additional tool, in combination with other conventional control methods, to prevent vector-borne diseases. The message that other vector control measures, such as breeding container removal, could be applied prior to and during the release of sterile male mosquitoes was also emphasized, so that the community was not under the impression that they were fully protected from vector-borne diseases during the implementation of the sterile male releases. Routine classical vector control measures, i.e. fogging and source reduction, were applied to both treatment and control sites prior to the intervention.

3.4. Entomological Surveillance and Monitoring

Mosquito abundance and egg hatch rate of the targeted *Ae. aegypti* population were estimated in 60 and 90 households, respectively, in the treatment, adjacent, and control areas within the selected study site during the one-year baseline and six-month intervention. Mosquito abundance was determined using MosHouse sticky traps and MosVac portable vacuum aspirators (Go Green Co., Ltd., Nakhon Pathom, Thailand). MosHouse sticky traps (Fig. 3) were distributed to 60 households to sample adult mosquitoes in the treatment, adjacent, and control areas.

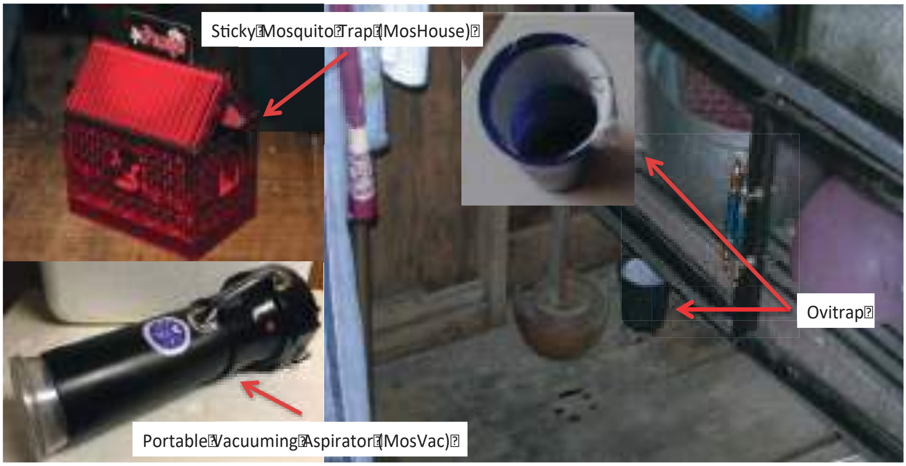

Figure 3. Picture showing the MosHouse sticky trap, the portable mosquito vacuuming aspirator, and the ovitrap that were used for surveillance and monitoring of natural Aedes aegypti *populations in the study site.*

The MosHouse traps were left in houses for one week before the sticky panels were collected and brought back to the laboratory to identify mosquito species and to determine relative mosquito abundance. In addition, the portable MosVac aspirators (Fig. 3) were used to collect resting mosquitoes in the same 60 households.

Sampled mosquitoes were killed by freezing and transported to the field laboratory station for species identification. The total number of *Ae. aegypti* males and females was recorded monthly for a total of two years to determine the dynamics of the different wild *Ae. aegypti* populations.

Ovitraps (Fig. 3) were distributed in 90 households to allow oviposition and then to collect *Ae. aegypti* eggs. The filter papers with eggs were collected on a weekly basis and brought back to the laboratory .The eggs that dried on the filter paper were counted and then hatched in water after 2-3 days. The number of eggs hatched was used to determine the sterility of natural *Ae. aegypti* populations.

4. EXPERIMENTS REQUIRED BEFORE THE PILOT TRIAL

4.1. Rearing of Wolbachia-infected Mosquitoes

Rearing is a crucial step for SIT/IIT implementation. Genomic adaptation to the mass-rearing environment, such as reduction in developmental time, life span, dispersal, and stress resistance, as well as early fertility and increased fecundity, is known to occur. This adaptation could make the individuals in the mass-rearing environment significantly different from the wild populations and affect the quality of the released male mosquitoes and hence, the efficacy of SIT/IIT applications (Nikolouli et al. 2018). Moreover, artificially *Wolbachia*-infected mosquito lines were observed to have increased larval mortality and decreased adult longevity when compared with aposymbiotic ones (Brelsfoard and Dobson 2011). Therefore, a strategy to maintain genetic diversity, biological quality, and competitiveness is required (Nikolouli et al. 2018). A high level of vigilance and consistent standardization of all processes, rearing conditions, and quality control needs to be maintained (Carvalho et al. 2014).

For the SIT/IIT trial in Thailand, mosquitoes were reared at the Center of Excellence for Vectors and Vector-Borne Diseases, Faculty of Science, Mahidol University at Salaya, Nakhon Pathom, Thailand and maintained in aluminium cages (40 x 40 x 40 cm) in a screened insectary at a temperature of 27 ± 2°C, a humidity of 75 ± 2%, and a photoperiod of L12:D12 (Kittayapong et al. 2018). Both male and female mosquitoes had access to a 10% sucrose solution, and females were fed with pig blood obtained from a qualified slaughterhouse.

The females were offered a blood meal for 3-4 consecutive days after mating using a Hemotek blood-feeding system (Hemotek Ltd., UK). Thereafter, plastic containers with the egg papers were placed inside the cages and were collected after 3-4 days. The eggs were dried and transferred to glass containers with screw-top covers filled with deionized water for egg hatching. After the eggs hatched into first-instar larvae, they were counted manually and transferred into plastic trays (32 cm x 42 cm x 5 cm), each containing about 2,000 larvae.

The larval diet had the following ingredients: mixed fish meal (Chanpongcharoen Kankaset Supplier, Thailand), pork liver powder, and yeast (*Saccharomyces cerevisiae*) (Cheese Powder Supplier, Thailand) at a ratio of 5:4:1 respectively. Each tray received 6.5 g of the diet every day. After 6-7 days, the developed pupae were placed inside plastic containers prior to sex separation.

4.2. Sex Separation before Sterilisation

Population suppression using the combined SIT/IIT approach requires release of a large number of male mosquitoes; therefore, an efficient system to separate the males from the females is essential to release only sterile males into the environment. Many studies have attempted to develop sex separation methods, based on biological, genetic, and transgenic approaches to support the application of the SIT for mosquito control. Sieving techniques were introduced in view of size differences between male and female pupae (Sharma et al. 1972; Bellini et al. 2007).

The development of genetic sexing strains (GSS), as well as other sex separation strategies, is currently under development and/or refinement, but none of them have so far succeeded in eliminating all females in order to achieve male-only releases for large-scale SIT or other applications (Benedict et al. 2009; Papathanos et al. 2009, 2018; Gilles et al. 2014).

Larval-pupal glass separators (Model 5412, John W. Hock, Co., Ltd., Gainesville, Florida, USA) were used to mechanically separate male and female pupae into different layers. The female pupae are larger in size and are collected in an upper layer between the two adjusted glass plates, while male pupae are drained into a receiving container placed below. Water circulation is supplied all along the process to push and wash the pupae down into the container. The female pupae are eventually flushed into a second receiving container, and the cycle of sex separation is complete.

In the experiments of sex separation, one litre of water that contained about 1500 to 2000 mixed male and female pupae were introduced each cycle into the system. One cycle took on average between 2 and 5 minutes, but it could take longer if the sample was mixed with larvae. After counting, the male pupae were transferred into a plastic cup and transported to the radiation source.

4.3. Appropriate Irradiation Dose for Male Sterilisation

Appropriate irradiation doses are different for different species of mosquitoes. Our preliminary studies showed that when *Wolbachia*-infected *Ae. aegypti* mosquitoes were irradiated at the pupal stage, an irradiation dose of 50 Gy was sufficient to obtain complete sterility in females, while males were fully sterilized with a dose of 70 Gy. *Wolbachia*-infected male pupae irradiated with 50 Gy could still produce some viable eggs when mated as adults with non-irradiated *Wolbachia*-infected females, the average percentage of egg hatch being 8%. However, egg hatch was zero when *Wolbachia*-infected males and females were irradiated with 70 Gy and then mated with non-irradiated *Wolbachia*-infected females and males, respectively (Kittayapong et al. 2018).

Since the *Wolbachia*-infected *Ae. aegypti* mosquitoes used in the pilot SIT/IIT trial in Thailand did not express complete CI (Ruang-areerate and Kittayapong 2006; Kittayapong et al. 2019), the complete sterility in these experiments was obtained through appropriate irradiation doses. If *Wolbachia* strains expressing strong CI are used, lower irradiation doses can be applied in order to obtain complete sterility of *Ae. aegypti* mosquitoes.

4.4. Mating Competitiveness and Release Ratio

Mating competitiveness of sterile male mosquitoes needs to be assessed before implementing a pilot SIT/IIT trial. In the past, many SIT trials were not successful in reducing natural mosquito populations due to the low competitiveness of sterile males after they were irradiated with too high doses (Dame et al. 2009). The advantage of the combined SIT/IIT approach is that lower dose radiation can be applied, as the sterility can also be induced in *Ae. aegypti* mosquitoes by the CI property of *Wolbachia* bacteria.

In the cage study, under controlled laboratory conditions, sterile *Ae. aegypti* males were evaluated for their mating competitiveness with wild males and females at different ratios (Fig. 4). Results indicated that a ratio of 10:1:1 or above was effective, as it reduced egg hatch significantly. The hatched eggs/total eggs of the 10:1:1 ratio experimental group was 3/619 (0.27 ± 0.65/103.17 ± 9.09). Complete sterility was observed with no egg hatch at a ratio of 20:1:1. Therefore, ratios between 10:1:1 and 20:1:1 were determined to be the optimal release ratios for the mass-reared sterile *Ae. aegypti* males, as they could compete with the wild males and induce near complete or complete sterility in the wild females. Our results also indicated that an irradiation dose of 70 Gy did not reduce mating competitiveness of the irradiated *Ae. aegypti* males (Kittayapong et al. 2019). In conclusion, our laboratory experiments demonstrated no significant difference in competitiveness of sterile males when compared to wild ones.

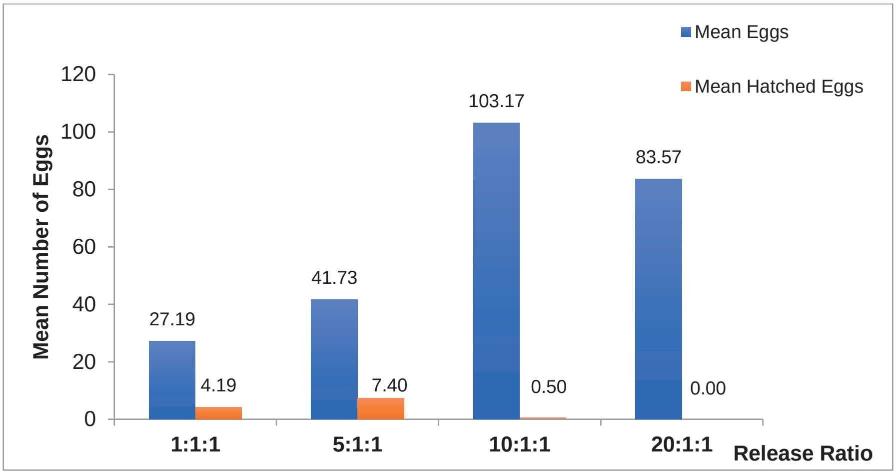

Figure 4. Mating competitiveness of sterile Aedes aegypti *males at different sterile male: wild male: wild female release ratios in cages under controlled laboratory conditions (modified from Kittayapong et al. 2019).*

4.5. Survival and Longevity of Sterile Males

Survival and longevity of the released sterile male mosquitoes are other parameters that may have an impact on the success of a SIT/IIT programme. The longer the sterile males can live, the higher the probability of mating with a wild female. In nature, both wild and sterile males should have shorter life spans than those kept under optimal controlled conditions. The same applies to wild and sterile females.

In our baseline experiments, carried out under controlled laboratory conditions, we observed that there was no significant difference in longevity between wild and sterile *Ae. aegypti* males (Fig. 5). On average, the wild males survived for 23.3 ± 0.9 days, while the sterile males survived for 23.8 ± 12.1 days. However, wild females lived significantly longer than sterile females, i.e. an average life span of 29.6 ± 1.0 days and 18.5 ± 9.8 days, respectively ($p = 0.000$) (Kittayapong et al. 2018).

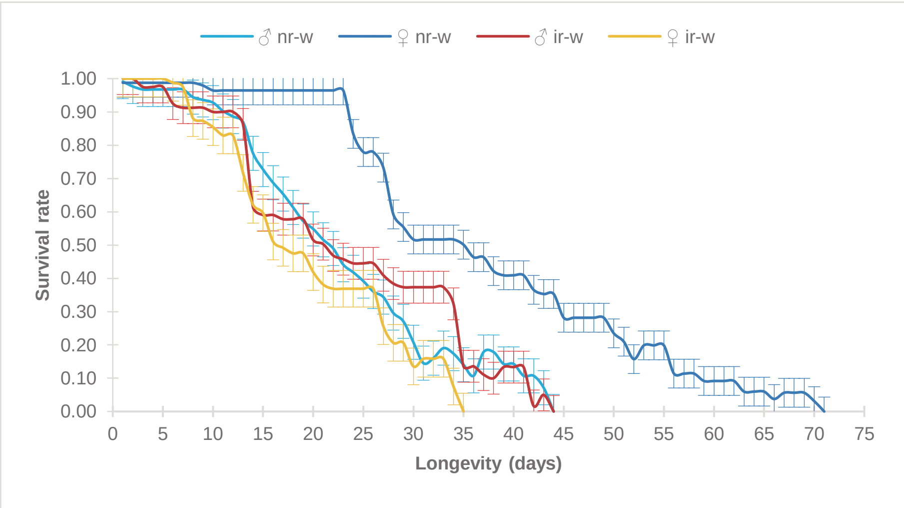

Figure 5. Mean longevity and survival rate of non-irradiated (nr) and irradiated (ir) Wolbachia-*infected* Aedes aegypti *male and female mosquitoes, after being sex-separated by using larval-pupal glass separators (modified from Kittayapong et al. 2018).*

5. EXPERIENCE AND LESSONS LEARNED FROM THE PILOT TRIAL

5.1. Community/Public/Stakeholder Engagement

Local stakeholders were identified. Engagement of local government authorities was initiated and followed up. Local government authorities coordinated with community leaders to initiate community engagement and facilitate community participation in implementing further on-site research activities, such as entomological surveillance and sterile male mosquito delivery using local health volunteers. Public education through media was carried out to raise awareness regarding vector-borne diseases and potential risk reduction using an alternative SIT/IIT approach.

From our experience, engagement of the community and public was more effective when it was initiated during times of epidemics. Our public engagement was initiated when both dengue and Zika were epidemic and people were aware of the consequences. Furthermore, we took advantage of related public events to draw the attention of the public. The first open release of sterile male mosquitoes was carried out on ASEAN Dengue Day, when the Ministry of Public Health of Thailand was an official host of regional activities (Fig. 6); hence representatives of many countries were present and witnessed the opening ceremony and first release of sterile males to fight dengue in Thailand.

In this pilot project, the general public was engaged through several national media reports, and TV news and radio programmes. A total of 109 media items, including a documentary, international news, national news, national radio, newspaper and online articles, and TV shows, were produced from January 2016 to February 2018 for public education of the sterile male release in Thailand.

Figure 6. Picture showing the first release of sterile Aedes aegypti *male mosquitoes on ASEAN Dengue Day at the study site in Pleang Yao District, Chachoengsao Province, eastern Thailand.*

A high number of views and sharing on social media, with the online articles published by reliable media publishers, was experienced (Kittayapong et al. 2019). Therefore, social media is an interesting additional channel to be used to communicate key messages to most of the general public. In addition, TV shows and documentaries on the topic of controlling the dengue vector through the SIT/IIT approach gained a lot of public attention in Thailand.

5.2. Handling and Transport of Sterile Mosquitoes for Open Field Release

Ideally, the radiation source used to sterilize the male mosquitoes should be in the same place as the rearing facility, while the subsequent transport to the release sites could be either as pupae or adults. In our case, male pupae had to be transported to a laboratory located 120 km from the rearing site for the irradiation treatment. *Wolbachia*-transinfected *Ae. aegypti* male mosquitoes were produced in the screened insectary at the Center of Excellence for Vectors and Vector-Borne Diseases, Faculty of Science, Mahidol University, Salaya Campus, Nakhon Pathom, and were then transported to the Thailand Institute of Nuclear Technology (TINT) in Nakhon Nayok for sterilisation at the pupal stage of 1-2 days old using a ^{60}Co source.

Sterile male pupae were transported weekly to the study sites using temperature-controlled containers. Our preliminary experiments showed 100% survival of the chilled sterile male pupae at temperatures between 8-12°C for up to 6 hours (Kittayapong et al. unpublished data).

Even though sterilisation at the pupal stage was shown to have no impact on mating competitiveness and the longevity of irradiated mosquitoes, it would be more practical to irradiate adult mosquitoes and then use the same containers for release. This would be less time-consuming, as the adult mosquitoes would not have to be transferred to the different release containers. Further experiments are needed to decide on the best temperature and container for chilling and transporting adults.

5.3. Management and Implementation of Sterile Male Release

A simple field laboratory station was set up in the city of Chachoengsao, 20 km from the release site in Plaeng Yao District. The research team worked with local workers to transfer male pupae to the release containers for adult emergence. Sterile *Ae. aegypti* males were provided with a 10% sucrose solution as food source after emergence for at least one day. They were then transported to the selected study sites, and the public health volunteers released the sterile mosquitoes at a rate of 100-200 per household per week. A total number of 437 980 sterile *Ae. aegypti* males, ranging from 9000 to 25 000 males per week, were released. The weekly releases were carried out only in the treatment area of the study site for a period of 24 weeks.

In this pilot trial, ULV fogging was used by local government staff to reduce the natural populations of *Ae. aegypti* to low densities before the releases of sterile male *Ae. aegypti* were initiated. The vector control activities by local government staff were conducted in both treatment and control areas. Public health volunteers provided assistance with the delivery of the sterile male mosquitoes to their respective households (Fig. 7).

Figure 7. Pictures showing activities related to the release of sterile males in the treatment community in Plaeng Yao District, Chachoengsao Province, eastern Thailand, by the health volunteers and homeowners under the supervision of the research team.

From our experience, frequent entering of private property caused some reluctance of a few homeowners to continue to cooperate. A visit of our staff together with the public health volunteers to these few houses was necessary to keep them cooperating. Therefore, the future planning of the release strategy should consider reducing disturbance of the privacy of homeowners. Open release using drones is recommended, especially in urban and crowded communities where intrusion into households is difficult. However, successful application of drone releases of sterile mosquitoes will need authorized and skilled operation.

5.4. Surveillance and Monitoring of Sterile Mosquitoes after Release

Both MosHouse sticky traps and MosVac portable vacuuming aspirators were used in the households of the study site for collecting both male and female mosquitoes of various species. MosHouse traps seemed to be more efficient in collecting *Ae. aegypti* females, while resting males were collected in higher numbers by using the MosVac portable vacuuming aspirators. When comparing the relative abundance of the *Ae. aegypti* mosquito populations, the average number of *Ae. aegypti* females sampled in the treatment area significantly decreased ($p < 0.05$) when compared to those in the control area, while those of males were not significantly different ($p > 0.05$), even though a large number of sterile males were released during the six-month intervention period in the treated areas (Table 1).

Table 1. Comparison of the mean numbers of Aedes aegypti *males and females collected by using MosHouse sticky traps and MosVac portable mosquito vacuuming aspirators in the treatment, adjacent, and control areas in Plaeng Yao District, Chachoengsao Province, eastern Thailand during the six-month intervention period (modified from Kittayapong et al. 2019)*

Variable	No. House	No. Mos-House traps	No. positive household (Mean ± SD)	Total mosquitoes (Mean ± SD)	Odds Ratio (total mos-quitoes)	95%CI	*P*
Males							
Control	20	20	15.83 ± 1.6	193 (32.17 ± 4.07)	1		
Adjacent	20	20	10.00 ± 3.4	92 (15.33 ± 6.31)	0.263	0.149-0.464	0.000*
Treatment	20	20	16.50 ± 2.9	137 (22.83 ± 6.55)	1.242	0.651-2.373	0.511
Females							
Control	20	20	17.00 ± 3.2	185 (30.83 ± 7.05)	1		
Adjacent	20	20	4.83 ± 1.83	35 (5.83 ± 2.64)	0.056	0.029-0.108	0.000*
Treatment	20	20	2.67 ± 1.75	16 (2.67 ± 1.75)	0.027	0.013-0.056	0.000*

* *Significant difference at* $p < 0.05$

It is possible that *Ae. aegypti* males mostly rested outside households where trapping and vacuuming activities took place (Table 1). The lower numbers of *Ae. aegypti* females in the treatment area compared to the control area indicate the effect of the sterile male releases that produced sterility in the wild *Ae. aegypti* females, resulting in a reduction in the numbers of *Ae. aegypti* female populations in nature by up to 97.30% (Fig. 8).

MosHouse sticky trap and MosVac portable vacuuming aspirators can be employed as tools for monitoring SIT, IIT, or combined SIT/IIT interventions, especially in view of their low cost and uncomplicated deployment. The advantage of the MosHouse traps as compared with the MosVac aspirators is that they can be placed either inside or around households without disturbing the homeowners. In addition, large numbers of the low-cost MosHouse sticky traps can be distributed in different locations in the study areas, resulting in better estimates of natural *Ae. aegypti* populations, compared to using a few high-cost traps placed in only a few locations.

As *Ae. aegypti* mosquitoes are more domestic, placing a few traps in a few locations could lead to a biased estimation of the total natural populations in the study areas. However, additional methods for collecting mosquitoes could be applied in combination to obtain more reliable data sets for entomological evaluation.

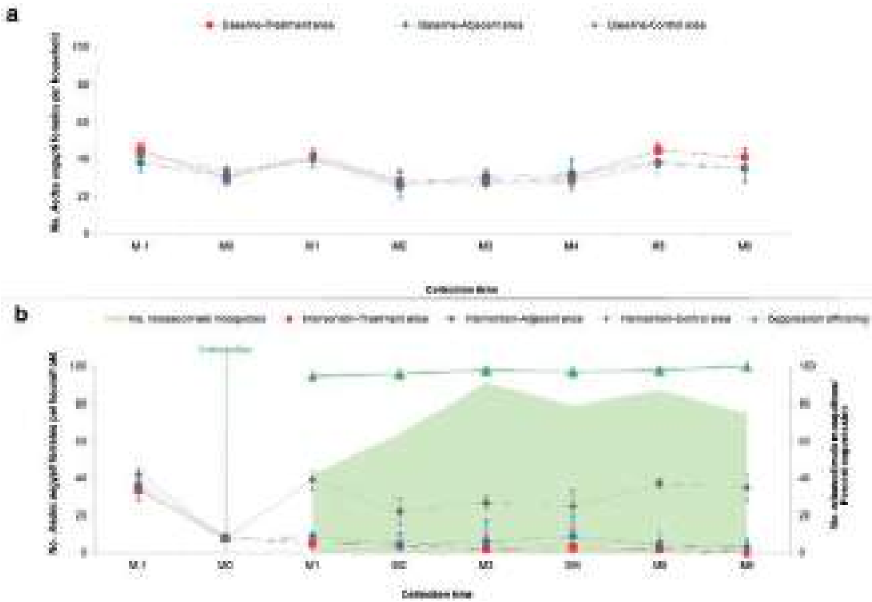

Figure 8. Mean numbers of Aedes aegypti *female mosquitoes collected per households in Pleang Yao District, Chachoengsao Province, Thailand during the baseline (Fig. 8a) and during the intervention (Fig. 8b) periods. Percent suppression efficiency in relation to the number of released sterile males per month is demonstrated in Fig. 8b (modified after Kittayapong et al. 2019).*

Weekly ovitrap data showed that the overall mean egg hatch was lowest in the treatment area, confirming the effectiveness of the sterile male release. The mean egg hatch for the treatment, adjacent, and control areas were 0.20 ± 0.10, 0.24 ± 0.14, and 0.41 ± 0.08 respectively; while those for the second twelve weeks were 0.18 ± 0.09, 0.25 ± 0.16, and 0.54 ± 0.11 respectively (Fig. 9).

There was a significant difference ($p < 0.05$) in mean egg hatch between the first and the second twelve weeks of sterile male releases (Table 2 and Table 3). The released sterile males seemed to show positive effects in reducing hatched eggs in the natural *Ae. aegypti* mosquito populations, in both the treatment area and the adjacent area, when compared to the households monitored in the control area (Fig. 9).

Except for a few outliers, egg hatch decreased to zero or near zero in most of the households monitored in the treatment area and adjacent area (Table 2). Since very low numbers of *Ae. albopictus* were found in this study area, especially in households, we could assume that the unhatched eggs were mostly from *Ae. aegypti* (Table 2 and Table 3).

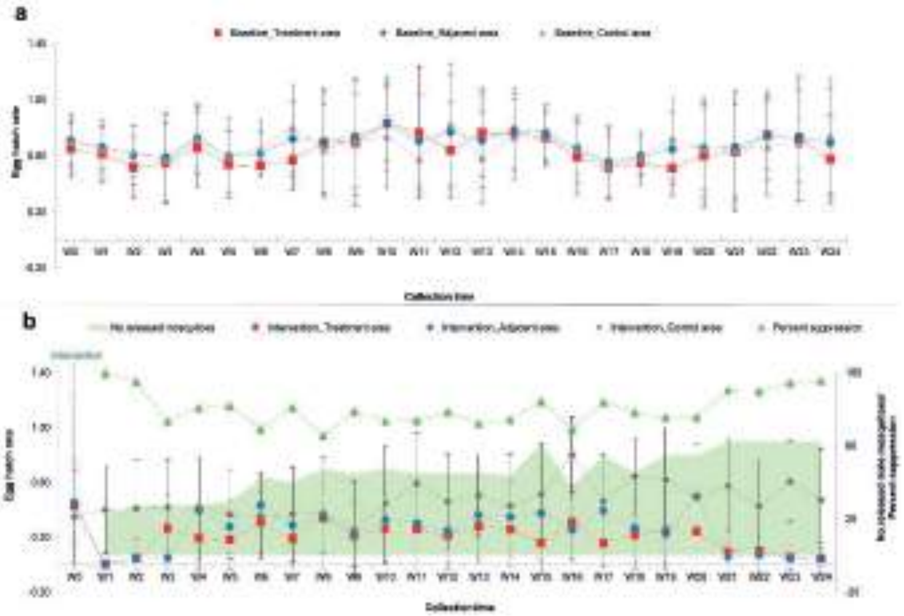

Figure 9. Mean egg hatch rate of natural Aedes aegypti *mosquito populations over time in the treatment, adjacent, and control areas of the study sites during the baseline (a) and during the intervention (b) periods. Percent suppression efficiency in relation to the number of released sterile males per week is demonstrated in Fig. 9b (modified after Kittayapong et al. 2019).*

5.5. Quality Control of Sterile Mosquito Production

Quality control to test for sterility of irradiated *Wolbachia*-infected *Ae. aegypti* males can be done through mating studies between mass-produced sterile males and untreated females from established mosquito colonies with no *Wolbachia* infection, originally collected from the same study site. Zero or near zero egg hatch was expected for each production lot. Mating tests performed during the 24-week open field trial in Plaeng Yao District, Chachoengsao Province, eastern Thailand indicated that the average numbers of hatched eggs in most production lots were quite low, i.e. 1.04 ± 2.18, which demonstrated that the irradiated *Wolbachia*-infected male mosquitoes were highly sterile (Kittayapong et al. 2018).

In addition, *Wolbachia* detection by PCR was conducted in 40 sterile *Ae. aegypti* males sampled from each of the production lots, and the results showed that the mean percentage of *Wolbachia* infection was 50.21 ± 0.49% in released males (Kittayapong et al. 2019).

Table 2. Progressive egg hatch rate of Aedes aegypti *mosquitoes in households in the treatment area, before and after 12 and 24 weeks of releases, when compared to those in households in the adjacent and the control areas*

No.	Before releases			After 12 weeks of releases			After 24 weeks of releases		
	W0			W12			W24		
	Control area	Adjacent area	Treatment area	Control area	Adjacent area	Treatment area	Control area	Adjacent area	Treatment area
1	0.60	0.00	0.00	0.11	0.29	0.10	1.00	0.00	0.00
2	0.00	0.25	0.28	0.37	0.00	0.00	0.43	0.00	0.00
3	0.09	0.81	0.38	0.59	0.00	0.44	0.80	0.00	0.16
4	0.29	0.23	0.64	0.87	0.00	0.00	0.27	0.05	0.38
5	0.39	0.34	0.00	0.26	0.46	0.57	0.68	0.00	0.00
6	0.00	0.52	0.48	0.43	0.00	0.02	0.00	0.09	0.00
7	0.00	0.00	0.03	0.96	0.19	0.25	0.15	0.00	0.00
8	0.74	0.68	0.10	0.36	0.17	0.00	0.60	0.00	0.00
9	0.00	0.94	0.67	1.00	0.34	0.00	0.32	0.04	0.07
10	0.73	0.95	0.87	0.81	0.28	0.00	0.77	0.00	0.00
11	0.22	0.52	0.64	0.00	0.00	0.26	0.44	0.00	0.07
12	0.94	0.31	0.39	1.00	0.16	0.54	0.44	0.18	0.00
13	0.67	0.26	0.18	0.56	0.08	0.00	0.00	0.15	0.00
14	0.94	0.20	0.39	0.47	0.00	0.61	0.20	0.00	0.04
15	0.00	0.35	0.00	0.94	0.84	0.39	0.00	0.01	0.00
16	0.12	0.94	0.00	0.00	0.22	0.00	0.00	0.00	0.00
17	0.00	0.09	0.00	0.31	0.67	0.00	0.50	0.00	0.00
18	0.94	0.53	0.00	0.18	0.18	0.50	0.30	0.14	0.10
19	0.19	0.28	0.74	0.95	0.29	0.00	0.94	0.00	0.00
20	0.09	0.80	0.37	0.94	0.59	0.50	0.60	0.03	0.46
21	0.00	0.20	0.74	0.47	0.42	0.23	0.00	0.13	0.00
22	0.94	0.00	0.21	0.17	0.26	0.50	1.00	0.07	0.00
23	0.00	0.23	0.00	0.45	0.14	0.16	0.00	0.27	0.00
24	0.21	0.62	0.29	0.33	0.26	0.84	0.70	0.00	0.07
25	0.13	0.47	0.00	0.34	0.04	0.00	1.00	0.15	0.00
26	0.00	0.82	0.00	1.00	0.39	0.00	0.00	0.07	0.00
27	0.00	0.80	0.71	1.00	0.00	0.26	0.31	0.00	0.00
28	0.00	0.00	0.55	0.00	0.37	0.00	1.00	0.03	0.00
29	0.00	0.41	0.00	0.31	0.49	0.00	0.10	0.00	0.00
30	0.33	0.94	0.00	0.00	0.25	0.25	1.00	0.13	0.00

High hatch rate (>0.50)

Moderate hatch rate (0.25-0.50)

Low hatch rate (0.01-0.24)

Zero hatch rate

Table 3. Statistical analysis of the egg hatch rate of Aedes aegypti *during the six-month (weeks 1-12 and weeks 13-24) intervention period in the treatment, adjacent, and control areas located in Plaeng Yao District, Chachoengsao Province, eastern Thailand (modified from Kittayapong et al. 2019)*

Variable	No. house	No. ovitrap	No. positive household (Mean ± SD)	Egg hatch rate (Mean ± SD)	Odds Ratio (Egg hatch rate)	95% CI	*P*
W1-W12							
Control	30	60	22.00 ± 0.43	0.41 ± 0.08	1		
Adjacent	30	60	24.50 ± 0.39	0.24 ± 0.14	1.620	0.679 – 3.862	0.277
Treatment	30	60	18.00 ± 0.50	0.20 ± 0.10	0.545	0.252 – 1.179	0.123
W13-W24							
Control	30	60	24.50 ± 0.39	0.54 ± 0.11	1		
Adjacent	30	60	19.00 ± 0.47	0.25 ± 0.16	0.388	0.168 – 0.897	0.027*
Treatment	30	60	12.50 ± 0.48	0.18 ± 0.09	0.160	0.070 – 0.368	0.000*

* *Significant difference at $p < 0.05$*

Our experience confirmed that an irradiation dose of 70 Gy is optimal to induce sterility in *Ae. aegypti* male mosquitoes. In our experiments, we also observed that *Ae. aegypti* females were more radio-sensitive and that a treatment with 50 Gy was sufficient to obtain complete female sterility. Therefore, accidentally released irradiated *Wolbachia*-infected *Ae. aegypti* females in our field trial, if any, were fully sterile after exposure to 70 Gy, and there was no danger of further propagation or *Wolbachia* establishment in the target population.

5.6. Female Contamination during SIT/IIT *Implementation*

Sustainable suppression of *Ae. aegypti* populations by integration of the SIT/IIT depends on the release of only sterile males. Hence, sex separation of mass-produced male and female mosquitoes is an important step, as female contamination could lead to an increase in disease transmission, although it is unlikely due to their *Wolbachia* infection. Inspection of female contamination was carried out weekly during the twenty-four weeks of the pilot field release of sterile *Ae. aegypti* males at the selected study site in Plaeng Yao District, Chachoengsao Province, eastern Thailand. Our results indicate a low percentage of female contamination among sterile males, i.e. 0.06 ± 0.10%, when *Ae. aegypti* pupae were separated through mechanical larval-pupal glass separators. Therefore, at least 99% of sterile males were purely separated from females (Kittayapong et al. 2018).

In this study, we also observed a significant difference in the percentage of female contamination during the first and the second 12-week periods of sterile male releases, i.e. 0.10 ± 0.13% vs 0.02 ± 0.02% ($p < 0.05$). The percentage of female contamination was remarkably reduced in the second twelve weeks of intervention. This was most likely due to the increasing skills of the technicians operating the mechanical sex separation machine. As such, we recommend hands-on training for operating technicians before project implementation to obtain a high efficiency in the manual sex separation process, and hence achieve the lowest possible female contamination during sterile male release.

5.7. Impact of SIT/IIT on the Environment and Ecosystem

The SIT/IIT approach for *Ae. aegypti* mosquito vectors was implemented using a two-step sterilisation process, combining the *Wolbachia*-induced IIT with the SIT using radiation to obtain sterile males. When these sterile males are systematically released into the target area, they can induce sterility in wild females after mating. Mated females lay eggs that cannot hatch, resulting in significant reduction in natural *Ae. aegypti* populations and subsequently, an "assumed" reduction in disease incidence that needs to be verified. In general, the SIT/IIT intervention is assessed to have little or no impact on the environment for the following reasons:

1. Mosquitoes released into the environment are irradiated males to ensure sterility. Also, any accidentally released females do not transmit disease if they are infected with pathogen-resistant *Wolbachia* strains. Thus, using both CI, the property of the *Wolbachia* endosymbiont that induces sterility in wild females, and radiation to sterilise the *Wolbachia*-infected mosquitoes, makes sure that they cannot become established in nature. These sterile mosquitoes have shorter life spans due to either the *Wolbachia* life-shortening effect or irradiation effect, and they will not survive in the natural environment longer than 2-3 weeks after release (Kittayapong et al. 2019). However, this means that the mosquitoes need to be released systematically into the target area to obtain the population reducing effect. Once the native mosquito population is at a low level, fewer sterile males can be released. In view of their short life span, there should be no residual mosquitoes left in the environment a few weeks after termination of the release activities.

2. As this method is species-specific, interfering only with the reproduction of the target population, it has no impact on beneficial insects or any other animals or humans, unlike chemical spraying which impacts the environment, affects non-target organisms, and can leave some residues.

3. The ecosystem will obviously experience a reduction of the *Ae. aegypti* vectors, and hence a reduction of available food for animals that feed on them. However, as there are over hundred species of mosquitoes in the tropical zone, together with the low biomass of the target population, other mosquito species should be able to serve in the food chain for some predators; therefore, the impact on the ecosystem in this regard should be very low or negligible.

Risk assessment on the use of *Wolbachia* for controlling mosquito vectors, both in terms of replacement and suppression approaches, was evaluated in the past, and a very low risk for the environment was reported (Popovici et al. 2010, Murray et al. 2016; NEA 2016).

6. CONCLUSIONS

The successful development and implementation of an operational SIT/IIT programme depends on several factors, and therefore these programmes require extensive and thorough planning based on available knowledge of the genetics, biology, and ecology of the target insect species. These include establishing and maintaining a *Wolbachia*-infected colony of the target species, understanding the field conditions and target population dynamics, assuring community participation, and assessing the potential side effects on humans and the environment.

The SIT is an environment-friendly method. Being species-specific and leaving no toxic residues, it has only minimal or no non-target impact, which has been demonstrated for over 50 years in large scale applications against agricultural pests. Moreover, it can be easily integrated with other biological control strategies. In terms of the IIT, it has already been proven successful in pilot field trials for suppressing *Aedes* mosquito vectors.

The pilot field trial of the combined SIT/IIT technology that was reported in this chapter represents the first clear proof-of-concept for the release of sterile male *Ae. aegypti* mosquitoes in Thailand (Kittayapong et al. 2019), one of the highly arboviral endemic countries in Southeast Asia. Our results show that the combined SIT/IIT approach for controlling mosquito vectors has potential for practical application as part of integrated vector management, working together with traditional control efforts to achieve better and more efficient outcomes (Zheng et al. 2019).

Potential large-scale application of this integrated SIT/IIT approach is possible through a commitment by the relevant vector control organizations, who should be informed of the technology, especially now that it has already been proven to work well in a pilot field trial.

7. REFERENCES

Ageep, T. B., D. Damiens, B. Alsharif, A. Ahmed, E. H. Salih, F. T. Ahmed, A. Diabaté, R. S. Lees, J. R. L. Gilles, and B. B. El Sayed. 2014. Participation of irradiated *Anopheles arabiensis* males in swarms following field release in Sudan. Malaria Journal 13: 484.

Alphey, L., M. Benedict, R. Bellini, G. G. Clark, D. A. Dame, M. W. Service, and S. L. Dobson. 2010. Sterile-insect methods for control of mosquito-borne diseases: An analysis. Vector Borne and Zoonotic Diseases 10(3): 295–311.

Altinli, M., F. Gunay, B. Alten, M. Weill, and M. Sicard. 2018. *Wolbachia* diversity and cytoplasmic incompatibility patterns in *Culex pipiens* populations in Turkey. Parasites & Vectors 11: 198.

Atyame, C. M., P. Labbé, E. Dumas, P. Milesi, S. Charlat, P. Fort, and M. Weill. 2014. *Wolbachia* divergence and the evolution of cytoplasmic incompatibility in *Culex pipiens*. PLoS One 9(1): e87336.

Bian, G., D. Joshi, Y. Dong, P. Lu, G. Zhou, X. Pan, Y. Xu, G. Dimopoulos, and Z. Xi. 2013. *Wolbachia* invades *Anopheles stephensi* populations and induces refractoriness to *Plasmodium* infection. Science 340: 748–751.

Barnes, B. N., J. H. Hofmeyr, S. Groenewald, D. E. Conlong, and M. Wohlfarter. 2015. The Sterile Insect Technique in agricultural crops in South Africa: A metamorphosis …. but will it fly? African Entomology 23(1): 1–18.

Benedict, M. Q., and A. S. Robinson. 2003. The first releases of transgenic mosquitoes: An argument for the sterile insect technique. Trends in Parasitology 19(8): 349–355.

Benedict, M. Q., B. G. J. Knols, H. C. Bossin, P. I. Howell, E. Mialhe, C. Caceres, and A. S. Robinson. 2009. Colonisation and mass rearing: Learning from others. Malaria Journal 8 (Supplement 2): S4.

Bellini, R., M. Calvitti, A. Medici, M. Carrieri, G. Celli, and S. Maini. 2007. Use of the Sterile Insect Technique against *Aedes albopictus* in Italy: First results of a pilot trial, pp. 505–515. *In* M. J. B. Vreysen, A. S. Robinson, and J. Hendrichs (eds.), Area-wide control of insect pests: From research to field implementation. Springer, Dordrecht, The Netherlands.

Bellini, R., A. Medici, A. Puggioli, F. Balestrino, and M. Carrieri. 2013. Pilot field trials with *Aedes albopictus* irradiated sterile males in Italian urban areas. Journal of Medical Entomology 50(2): 317–325.

Bourtzis, K., S. L. Dobson, Z. Xi, J. L. Rasgon, M. Calvitti, L. A. Moreira, H. C. Bossin, R. Moretti, L. A. Baton, G. L. Hughes, P. Mavingui, and J. R. L. Gilles. 2014. Harnessing mosquito–*Wolbachia* symbiosis for vector and disease control. Acta Tropica 132: S150–S163.

Brelsfoard, C. L., and S. L. Dobson. 2011. *Wolbachia* effects on host fitness and the influence of male aging on cytoplasmic incompatibility in *Aedes polynesiensis* (Diptera: Culicidae). Journal of Medical Entomology 48(5): 1008–1015.

Calvez, E., L. Guillaumot, D. Girault, V. Richard, O. O'Connor, T. Paoaafaite, M. Teurlai, N. Pocquet, V. M. Cao-Lormeau, and M. Dupont-Rouzeyrol. 2017. Dengue-1 virus and vector competence of *Aedes aegypti* (Diptera: Culicidae) populations from New Caledonia. Parasites & Vectors 10(1): 381.

Carvalho, D. O., D. Nimmo, N. Naish, A. R. McKemey, P. Gray, A. B. B. Wilke, M. T. Marrelli, J. F. Virginio, L. Alphey, and M. L. Capurro. 2014. Mass production of genetically modified *Aedes aegypti* for field releases in Brazil. Journal of Visualized Experiments 83: e3579.

Carvalho, D. O., A. R. McKemey, L. Garziera, R. Lacroix, C. A. Donnelly, L. Alphey, A. Malavasi, and M. L. Capurro. 2015. Suppression of a field population of *Aedes aegypti* in Brazil by sustained release of transgenic male mosquitoes**.** PLoS Neglected Tropical Diseases 9(7): e0003864.

Chansang, C., and P. Kittayapong. 2007. Application of mosquito sampling count and geospatial methods to improve dengue vector surveillance. American Journal of Tropical Medicine and Hygiene 77: 897–902.

Clark, M. E., Z. Veneti, K. Bourtzis, and T. L. Karr. 2002. The distribution and proliferation of the intracellular bacteria *Wolbachia* during spermatogenesis in *Drosophila*. Mechanisms of Development 111(1): 3–15.

Dame, D. A., D. B. Woodard, H. R. Ford, and D. E. Weidhaas. 1964. Field behavior of sexually sterile *Anopheles quadrimaculatus* males. Mosquito News 24: 6–14.

Dame, D. A., C. F. Curtis, M. Q. Benedict, A. S. Robinson, and B. G. Knols. 2009. Historical applications of induced sterilisation in field populations of mosquitoes. Malaria Journal 8 (Supplement 2): S2.

Dyck, V. A., J. Hendrichs, and A. S. Robinson (eds.). 2021. Sterile Insect Technique – Principles and practice in Area-Wide Integrated Pest Management. Second Edition. CRC Press, Boca Raton, Florida, USA. 1200 pp.

Fredericks, A. C., and A. Fernandez-Sesma. 2014. The burden of dengue and chikungunya worldwide: Implications for the southern United States and California. Annals of Global Health 80(6): 466–475.

Frentiu, F. D., T. Zakir, T. Walker, J. Popovici, A. T. Pyke, A. van den Hurk, E. A. McGraw, and S. L. O'Neill. 2014. Limited dengue virus replication in field-collected *Aedes aegypti* mosquitoes infected with *Wolbachia*. PLoS Neglected Tropical Diseases 8(2): e2688.

Gilles, J. R. L., M. F. Schetelig, F. Scolari, F. Marec, M. L. Capurro, G. Franz, and K. Bourtzis. 2014. Towards mosquito sterile insect technique programmes: Exploring genetic, molecular, mechanical and behavioural methods of sex separation in mosquitoes. Acta Tropica 132: S178–S187.

Hilgenboecker, K., P. Hammerstein, P. Schlattmann, A. Telschow, and J. H. Werren. 2008. How many species are infected with *Wolbachia*? A statistical analysis of current data. FEMS Microbiology Letters 281(2): 215–220.

Hoffmann, A. A., B. L. Montgomery, J. Popovici, I. Iturbe-Ormaetxe, P. H. Johnson, F. Muzzi, M. Greenfield, M. Durkan, Y. S. Leong, Y. Dong, H. Cook, J. Axford, A. G. Callahan, N. Kenny, C. Omodei, E. A. McGraw, P. A. Ryan, S. A. Ritchie, M. Turelli, and S. L. O'Neill. 2011. Successful establishment of *Wolbachia* in *Aedes* populations to suppress dengue transmission. Nature 476(7361): 454–457.

Iyaloo, D. P., K. B. Elahee, A. Bheecarry, and R. S. Lees. 2014. Guidelines to site selection for population surveillance and mosquito control trials: A case study from Mauritius. Acta Tropica 132: S140–S149.

Joubert, D. A., T. Walker, L. B. Carrington, J. T. De Bruyne, D. H. T. Kien, N. L. T. Hoang, N. V. V. Chau, I. Iturbe-Ormaetxe, C. P. Simmons, and S. L. O'Neill. 2016. Establishment of a *Wolbachia* superinfection in *Aedes aegypti* mosquitoes as a potential approach for future resistance management. PLoS Pathogens 12(2): e1005434.

Kittayapong, P., S. Yoksan, U. Chansang, C. Chansang, and A. Bhumiratana. 2008. Suppression of dengue transmission by application of integrated vector control strategies at sero-positive GIS-based foci. American Journal of Tropical Medicine and Hygiene 78: 70–76.

Kittayapong, P., N. Kaeothaisong, S. Ninphanomchai, and W. Limohpasmanee. 2018. Combined sterile insect technique and incompatible insect technique: Sex separation and quality of sterile *Aedes aegypti* male mosquitoes released in a pilot population suppression trial in Thailand. Parasites & Vectors 11 (Supplement 2): 657.

Kittayapong, P., S. Ninphanomchai, W. Limohpasmanee, C. Chansang, U. Chansang, and P. Mongkalangoon. 2019. Combined sterile insect technique and incompatible insect technique: The first proof-of-concept to suppress *Aedes aegypti* vector populations in semi-rural settings in Thailand. PLoS Neglected Tropical Diseases 13(10): e0007771.

Kotsakiozi, P., A. Gloria-Soria, A. Caccone, B. Evans, R. Schama, A. J. Martins, and J. R. Powell. 2017. Tracking the return of *Aedes aegypti* to Brazil, the major vector of the dengue, chikungunya and Zika viruses. PLoS Neglected Tropical Diseases 11(7): e0005653.

Lees, R. S., D. O. Carvalho, and J. Bouyer. 2021. Potential impact of integrating the Sterile Insect Technique into the fight against disease-transmitting mosquitoes, pp. 1081–1118. *In* V. A. Dyck, J. Hendrichs, and A. S. Robinson (eds.), Sterile Insect Technique – Principles and practice in Area-Wide Integrated Pest Management. Second Edition. CRC Press, Boca Raton, Florida, USA.

Lees, R. S., J. R. L. Gilles, J. Hendrichs, M. J. B. Vreysen, and K. Bourtzis, K. 2015. Back to the future: The sterile insect technique against mosquito disease vectors. Current Opinion in Insect Science 10: 156–162.

Mains, J. W., C. L. Brelsfoard, R. I. Rose, and S. L. Dobson. 2016. Female adult *Aedes albopictus* suppression by *Wolbachia*-infected male mosquitoes. Scientific Reports 6: 33846.

Mains, J. W., P. H. Kelly, K. L. Dobson, W. D. Petrie, and S. L. Dobson. 2019. Localized control of *Aedes aegypti* (Diptera: Culicidae) in Miami, FL, via inundative releases of *Wolbachia*-infected male mosquitoes. Journal of Medical Entomology 56(5): 1296–1303.

Murray, J. V., C. C. Jansen, and P. De Barro. 2016. Risk associated with the release of *Wolbachia*-infected *Aedes aegypti* mosquitoes into the environment in an effort to control dengue. Frontiers in Public Health 4: 43.

Moreira, L. A., I. Iturbe-Ormaetxe, J. A. Jeffery, G. J. Lu, A. T. Pyke, L. M. Hedges, B. C. Rocha, S. Hall-Mendelin, A. Day, M. Riegler, L. E. Hugo, K. N. Johnson, B. H. Kay, E. A. McGraw, A. F. van den Hurk, P. A. Ryan, and S. L. O'Neill. 2009. A *Wolbachia* symbiont in *Aedes aegypti* limits infection with dengue, chikungunya, and *Plasmodium*. Cell 139(7): 1268–1278.

Morlan, H. B., E. M. McCray, and J. W. Kilpatrick. 1962. Field tests with sexually sterile males for control of *Aedes aegypti*. Mosquito News 22: 295–300.

Morrison, N. I., G. Franz, M. Koukidou, T. A. Miller, G. Saccone, and L. S. Alphey. 2010. Genetic improvements to the Sterile Insect Technique for agricultural pests. Asia-Pacific Journal of Molecular Biology and Biotechnology 18(2): 275–295.

(NEA) National Environmental Agency, Singapore. 2016. Risk assessment for the use of male *Wolbachia*-carrying *Aedes aegypti* for suppression of the *Aedes aegypti* mosquito population. Risk Analysis Report of the National Environmental Agency, Singapore, 14 pp.

(NEA) National Environment Agency, Singapore. 2019. New NEA facility to boost production of male *Wolbachia-Aedes aegypti* mosquitoes to benefit more residents. 2 December 2019.

Nikolouli, K., H. Colinet, D. Renault, T. Enriquez, L. Mouton, P. Gibert, F. Sassu, C. Cáceres, C. Stauffer, R. Pereira, and K. Bourtzis. 2018. Sterile insect technique and symbiosis as potential tools for the control of the invasive *Drosophila suzukii*. Journal of Pest Science 91(2): 489–503.

O'Connor, L., C. Plichart, A. C. Sang, C. L. Brelsfoard, H. C. Bossin, and S. L. Dobson. 2012. Open release of male mosquitoes infected with a *Wolbachia* biopesticide: Field performance and infection containment. PLoS Neglected Tropical Diseases 6(11): e1797.

Oliva, C. F., M. Jacquet, J. R. L. Gilles, G. Lemperiere, P. O. Maquart, S. Quilici, F. Schooneman, M. J. B. Vreysen, and S. Boyer. 2012. The sterile insect technique for controlling populations of *Aedes albopictus* (Diptera: Culicidae) on Réunion Island: Mating vigour of sterilized males. PLoS One 7(11): e49414.

Papathanos, P. A., H. C. Bossin, M. Q. Benedict, F. Catteruccia, C. A. Malcolm, L. Alphey, and A. Crisanti. 2009. Sex separation strategies: Past experience and new approaches. Malaria Journal 8(2): S2–S5.

Papathanos, P. A., K. Bourtzis, F. Tripet, H. C. Bossin, J. F. Virginio, M. L. Capurro, M. C. Pedrosa, A. Guindo, L. Sylla, M. B. Coulibaly, F. A. Yao, P. S. Epopa, and A. Diabate. 2018. A perspective on the need and current status of efficient sex separation methods for mosquito genetic control. Parasites & Vectors. 11 (Supplement 2): 654.

Patterson, R. S., V. P. Sharma, K. R. P. Singh, G. C. LaBrecque, P. L. Seetharam, and K. K. Grover. 1975. Use of radio-sterilized males to control indigenous populations of *Culex pipiens quinquefasciatus* Say: Laboratory and field studies. Mosquito News 35: 1–7.

Patterson, R. S., R. E. Lowe, B. J. Smittle, D. A. Dame, M. D. Boston, and A. L. Cameron. 1977. Release of radiosterilized males to control *Culex pipiens quinquefasciatus* (Diptera: Culicidae). Journal of Medical Entomology 14: 299–304.

Popovici, J., L. A. Moreira, A. Poinsignon, I. Iturbe-Ormaetxe, D. McNaughton, and S. L. O'Neill. 2010. Assessing key safety concerns of a *Wolbachia*-based strategy to control dengue transmission by *Aedes* mosquitoes. Memórias do Instituto Oswaldo Cruz 105(8): 957–964.

Reisen, W. K., M. M. Milby, S. M. Asman, M. E. Bock, R. P. Meyer, P. T. McDonald, and W. C. Reeves. 1982. Attempted suppression of a semi-isolated *Culex tarsalis* population by the release of irradiated males: A second experiment using males from a recently colonized strain. Mosquito News 42: 565–575.

Ruang-areerate, T., and P. Kittayapong. 2006. *Wolbachia* transinfection in *Aedes aegypti*: A potential gene driver of dengue vectors. Proceedings of the National Academy of Science of the USA 103: 12534–12539.

Sharma, V. P., R. S. Patterson, and H. R. Ford. 1972. A device for the rapid separation of male and female mosquito pupae. Bulletin WHO 47(3): 429–43.

Shepard, D. S., E. A. Undurraga, and Y. A. Halasa. 2013. Economic and disease burden of dengue in Southeast Asia. PLoS Neglected Tropical Diseases 7(2): e2055.

Thomas, D. D., C. A. Donnelly, R. J. Wood, and L. S. Alphey. 2000. Insect population control using a dominant, repressible, lethal genetic system. Science 287(5462): 2474–2476.

Trewin, B. J., J. M. Darbro, C. C. Jansen, N. A. Schellhorn, M. P. Zalucki, T. P. Hurst, and G. J. Devine. 2017. The elimination of the dengue vector, *Aedes aegypti*, from Brisbane, Australia: The role of surveillance, larval habitat removal and policy. PLoS Neglected Tropical Diseases 11(8): e0005848.

Turelli, M., and A. A. Hoffmann. 1995. Cytoplasmic incompatibility in *Drosophila simulans*: Dynamics and parameter estimates from natural populations. Genetics 140(4): 1319–1338.

Vreysen M. J. B., J. Gerado-Abaya, and J. P. Cayol. 2007. Lessons from area-wide integrated pest management (AW-IPM) programmes with an SIT component: An FAO/IAEA perspective, pp. 723–744. *In* M. J. B. Vreysen, A. S. Robinson, and J. Hendrichs (eds.), Area-wide control of insect pests: From research to field implementation. Springer, Dordrecht, The Netherlands.

Vreysen, M. J. B., K. M. Saleh, M. Y. Ali, A. M. Abdulla, Z. R. Zhu, K. G. Juma, V. A. Dyck, A. R. Msangi, P. A. Mkonyi, and H. U. Feldmann. 2000. *Glossina austeni* (Diptera: Glossinidae) eradicated on the island of Unguja, Zanzibar, using the sterile insect technique. Journal of Economic Entomology 93(1): 123–135.

Weidhaas, D. E., C. H. Schmidt, and E. L. Seabrook. 1962. Field studies on the release of sterile males for the control of *Anopheles quadrimaculatus*. Mosquito News 22: 283–291.

(WHO) World Health Organization. 2017. Fifth Meeting of the Vector Control Advisory Group, Geneva, Switzerland, 2–4 November 2016. WHO/HTM/NTD/VEM/2017.02. Licence: CC BY-NC-SA 3.0 IGO. Geneva, Switzerland.

(WHO) World Health Organization. 2019a. Dengue and severe dengue. Geneva, Switzerland.

(WHO) World Health Organization. 2019b. Mosquito control: Can it stop Zika at source? Geneva, Switzerland.

Xi, Z., C. C. Khoo, and S. L. Dobson. 2005. *Wolbachia* establishment and invasion in an *Aedes aegypti* laboratory population. Science 310(5746): 326–328.

Yakob, L., S. Funk, A. Camacho, O. Brady, and W. J. Edmunds. 2017. *Aedes aegypti* control through modernized, integrated vector management. PLoS Currents Outbreaks 9: Jan 30.

Zhang, D., Zheng, X., Xi, Z., Bourtzis, K., and J. R. L. Gilles. 2015a. Combining the sterile insect technique with the incompatible insect technique: I. Impact of *Wolbachia* infection on the fitness of triple and double-infected strains of *Aedes albopictus*. PLoS One 10: e0121126.

Zhang, D., R. S. Lees, Z. Xi, J. R. L. Gilles, and K. Bourtzis. 2015b. Combining the sterile insect technique with *Wolbachia*-based approaches: II. A safer approach to *Aedes albopictus* population suppression programmes, designed to minimize the consequences of inadvertent female release. PLoS One 10: e0135194.

Zhang, D., R. S. Lees, Z. Xi, K. Bourtzis, and J. R. L. Gilles. 2016. Combining the sterile insect technique with the incompatible insect technique: III. Robust mating competitiveness of irradiated triple *Wolbachia*-infected *Aedes albopictus* males under semi-field conditions. PLoS One 11(3): e0151864.

Zhang, D., M. Zhang, Y. Wu, J. R. L. Gilles, H. Yamada, Z. Wu, Z. Xi, and X. Zheng. 2017. Establishment of a medium-scale mosquito facility: Optimization of the larval mass-rearing unit for *Aedes albopictus* (Diptera: Culicidae). Parasites & Vectors 10: 569.

Zheng, M. L., D. J. Zhang, D. D. Damiens, R. S. Lees, and J. R. L. Gilles. 2015. Standard operating procedures for standardized mass rearing of the dengue and chikungunya vectors *Aedes aegypti* and *Aedes albopictus* (Diptera: Culicidae): II. Egg storage and hatching. Parasites & Vectors 8: 348.

Zheng, X., D. Zhang, Y. Li, Y. Wu, X. Liang, Y. Liang, X. Pan, L. Hu, Q. Sun, X. Wang, Y. Wei, J. Zhu, W. Qian, Z. Yan, A. G. Parker, J. R. L. Gilles, K. Bourtzis, J. Bouyer, M. Tang, B. Zheng, J. Yu, J. Liu, J. Zhuang, Z. Hu, M. Zhang, J. Gong, X. Hong, Z. Zhang, L. Lin, Q. Liu, Z. Hu, Z. Wu, L. A. Baton, A. A. Hoffmann, and Z. Xi. 2019. Incompatible and sterile insect techniques combined eliminate mosquitoes. Nature 572: 56–61.

ECOLOGY, BEHAVIOUR AND AREA-WIDE CONTROL OF THE FLOODWATER MOSQUITO *Aedes sticticus*, WITH POTENTIAL OF FUTURE INTEGRATION OF THE STERILE INSECT TECHNIQUE

J. O. LUNDSTRÖM[1,2], M. L. SCHÄFER[2] AND P. KITTAYAPONG[3,4]

[1]*Zoonosis Research Center, Department of Medical Biochemistry and Microbiology, Uppsala University, Box 582, SE-75123 Uppsala, Sweden; jan.lundstrom@mygg.se*
[2]*Biologisk Myggkontroll, Nedre Dalälven Utvecklings AB, Vårdsätravägen 5, SE-75646 Uppsala, Sweden*
[3]*Center of Excellence for Vectors and Vector-Borne Diseases, Faculty of Science, Mahidol University, 999 Phuttamonthon 4 Road, Nakhon Pathom 73170, Thailand*
[4]*Department of Biology, Faculty of Science, Mahidol University, 272 Rama VI Road, Bangkok 10400, Thailand*

SUMMARY

The strategy of aerial control of the floodwater mosquito *Aedes sticticus* (Meigen) in the floodplains of River Dalälven, central Sweden, was developed to directly address specific larval breeding areas in temporary flooded wet meadows and swamps. Using the *Bti*-based larvicide VectoBac G®, a very strong reduction of larval abundance is achieved, resulting in a massive decrease of blood-seeking females that could otherwise spread from the wetlands to feast on blood from humans and animals within 5 km or more from the larval biotopes. However, there is also a political demand to reduce the usage of the control agent through hypothetical alternatives, such as cattle grazing and mowing of the meadows, as well as hydrological changes of the River Dalälven. An evaluation of these measures showed that they are either insufficient or unrealistic in reducing floodwater mosquito abundance. Thus, we searched for other potential population suppression methods. Using the criteria of efficacy, environmental neutrality and compatibility within an integrated suppression approach, we conclude that Sterile Insect Technique (SIT) and the Incompatibility Insect Technique (IIT) would qualify for a pilot-scale test of their feasibility for the integrated control of the floodwater mosquito *Ae. sticticus*. The SIT and the IIT are similar strategies involving the release of sterile males which mate with local fertile females and result in infertile eggs. Prerequisites for a sterile male strategy to control *Ae. sticticus* include: a laboratory colony of the species, a facility for mass-rearing of mosquitoes, the sterilisation of males, a transport strategy, a dispersal system, assay systems for several life stages, and a method capable of reducing the population of this superabundant

J. Hendrichs, R. Pereira and M. J. B. Vreysen (eds.), Area-Wide Integrated Pest Management: Development and Field Application, pp. 433–459. CRC Press, Boca Raton, Florida, USA.

species before commencing the sterile male release. One factor in favour of implementing the SIT or IIT against *Ae. sticticus* is that mating occurs in or near well-defined larval breeding areas with specific relation to flood events. Another factor in favour of the SIT or the IIT is the availability of existing methods to measure gender, larvae and egg abundance. Also, existing *Bti*-treatments can substantially lower the population size before sterile male release. Other prerequisites, like the successful colonization of *Ae. sticticus* will require more tests and adaptations of existing mosquito rearing protocols. A pilot study is suggested for an isolated study area, protected from reinvasion by *Ae. sticticus*-females and included in routine *Bti*-treatments.

Key Words: Aedes sticticus, Sweden, River Dalälven, floodplains, wetlands, *Bacillus thuringiensis israelensis* (*Bti*), larvicide, VectoBac G, SIT, Incompatibility Insect Technique (IIT)

1. INTRODUCTION

Sweden, located in the north of Europe, is a country where mosquitoes are pervasive. While mosquito abundances were assumed to be highest in the northernmost part of the country, mosquito diversity increases towards the south (Schäfer and Lundström 2001). Generally, snow pool mosquitoes, e.g. *Aedes communis* (De Geer) and *Aedes punctor* (Kirby) are the most common species found throughout the country. Nuisance by these univoltine mosquito species can be severe, but occurs mainly in spring and early summer, followed by rapidly declining numbers.

When people in the River Dalälven floodplains in central Sweden complained about mosquito problems in the 1980's and 1990's, they were not taken seriously and often met with the conventional wisdom that mosquito problems are much more severe in the north. For a long time, the actual nuisance mosquito species was unknown, since knowledge on the mosquito fauna in the River Dalälven region was insufficient. In a study from 1985, the floodwater mosquito *Aedes rossicus* Dolbeskin, Gorickaja and Mitrofanova was reported as the most abundant species (Jaenson 1986). Ten years later, researchers studying Sindbis virus in the area needed to use protective clothing due to the enormous abundance of mosquitoes, but no general identification of nuisance species was performed (Lundström et al. 1996).

In the summer of 2000, we studied mosquito species diversity in the central part of the region at Lake Färnebofjärden, which coincided with one of the worst mosquito nuisance years due to massive floods. Mosquito sampling with CDC miniature light traps baited with dry ice resulted in enormous numbers (up to 61 500 mosquitoes per trap and night) and the predominant species was *Aedes sticticus* (Meigen) (Schäfer et al. 2008). This can be compared to the maximum number of 4500 mosquitoes per trap and night (trap-night) from a wetland in northern Sweden (unpublished information).

The people of the River Dalälven floodplains were desperate, and children had to be transported away from the area by buses to be able to swim and play outside during their summer vacation. Media awakened and the mosquito-infected towns in the region became known in the whole country. The major breakthrough in the people's struggle to continue living in this area was a visit from the Minister of Environment, Mr. Kjell Larsson, who is still the only minister to experience massive floodwater mosquito nuisance. His words, "*You cannot have it like that*" became historic; and resulted in the development of the first professional mosquito control in Sweden.

The identification of the flood-water mosquito *Ae. sticticus* as the main cause of the horrendous nuisance made it the prime target species for control. Larviciding with

Bacillus thuringiensis israelensis (*Bti*) was the method of choice for its low environmental impact, efficacy, and practical application over large areas.

During recent years, political pressure has created a demand for alternative methods to control this superabundant day-active and long-range dispersing mosquito, motivating us to search for new, less intrusive mosquito control methods suitable for area-wide use in natural wetlands.

Below we describe development of our high-tech GIS-based strategy of direct *Bti*-based larval control (Section 2), the way forward for adapting SIT-based birth control for area-wide control of *Ae. sticticus* in natural wetlands (Section 5), and a section on perspectives (Section 6).

2. DEVELOPING AREA-WIDE CONTROL OF A*edes sticticus* IN NATURAL WETLANDS

2.1. The River Dalälven Floodplains

The River Dalälven covers a catchment area of approximately 29 000 km^2, originating in the mountains along the Swedish-Norwegian border and outflowing into the Baltic Sea (Fig. 1).

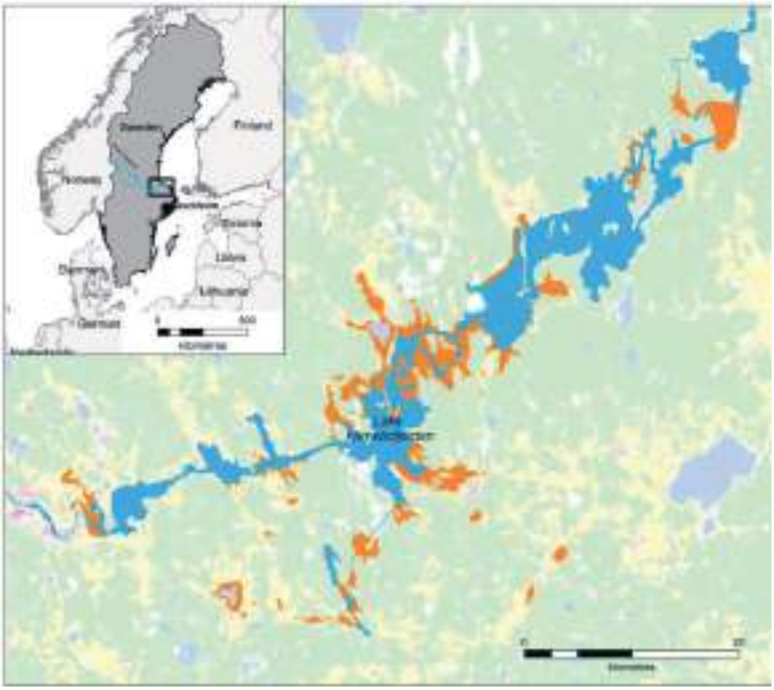

Figure 1. The location of the River Dalälven in central Sweden (inlet) and the floodplains with its many lakes in the lower part of River Dalälven. The areas with permit for mosquito control treatments by Bti *for 2019 are shown in orange.*

The River Dalälven, with its main branches Västerdalälven and Österdalälven, is partly regulated for production of hydro-electric power. In its lower part, the river forms a chain of lakes connected by rapids. In these floodplains, temporary flooded marshes, wet meadows and alder swamps cover several thousand hectares (ha) and most of this area is protected by both national regulations and EU-regulations. Water level fluctuations are most dramatic and frequent in Lake Färnebofjärden, which is protected as a National Park and contains several protected Nature Reserves and Natura 2000 areas. Flooding is induced by melting snow and/or heavy rainfall that causes increased waterflow in River Dalälven and other smaller watercourses in the floodplain area, and the water level can increase by 2.0 m or more in the Lake Färnebofjärden area. This is an area of enormous mosquito abundance and a hotspot for mosquito diversity (Schäfer et al. 2018).

2.2. Ecology and Behaviour of Aedes sticticus

Floodwater mosquitoes, in particular *Ae. sticticus* and *Aedes vexans* (Meigen), are the predominant mosquito species in areas influenced by large rivers or lakes with water level fluctuations in adjacent lowlands (Becker and Ludwig 1981; Merdic and Lovakovic 2001; Minar et al. 2001; Schäfer et al. 2008). These mosquito species oviposit their eggs on moist soil, into small depressions or in moss, which are subsequently flooded with rising water levels (Horsfall et al. 1973). The eggs are in diapause during autumn, winter and early spring, and remain viable for at least 4 years (Gjullin et al. 1950; Horsfall et al. 1973) but probably longer. When the eggs are flooded by shallow water, hatching of larvae is triggered by water temperature and decreasing oxygen level. After melting of the snow, water temperature needs to exceed about 8°C for eclosion of *Ae. sticticus* eggs (Becker et al. 2010), thus avoiding larval hatching during the cold seasons. Flowing water inundating the wetlands is oxygen-rich, but once the water in the inundated areas becomes stagnant, oxygen levels decrease due to bacterial degradation processes. This signals the appropriate time for larvae to hatch from the eggs. Newly hatched larvae no longer risk being carried away by flowing water, and the bacterial activity ensures adequate food supply (Becker et al. 2010).

The synchronised hatching of *Ae. sticticus* larvae after a flood results in massive amounts of larvae at about the same time, although not all eggs hatch during the same flood event. This so-called 'hatching-in-installment' ensures survival of the population in case the larval breeding site dries out before development to adults is completed (Wilson and Horsfall 1970; Becker 1989). The development of the larvae to pupae and emergence of adults is temperature-dependent (Trpis and Shemanchuk 1970; Becker 1989). The males emerge about one day before the females and need to rotate their hypopygium to be ready to mate. Females mate only once and store sufficient sperm in their spermathecae for fertilizing several egg batches (Becker et al. 2010). After mating, the females start searching for a blood meal to develop eggs. The blood-seeking *Ae. sticticus* females are known for their long-distance dispersal behaviour, covering distances of at least 10 km (Brust 1980; Sudarić Bogojević et al. 2011).

Floodwater mosquitoes are multivoltine and each flood during spring and summer can produce a new generation of mosquitoes. Together with their capability for mass-reproduction, this explains the enormous numbers and the lengthy occurrence of floodwater mosquito nuisance over several months in summer and fall. These mosquitoes cause an enormous nuisance affecting every aspect of living, working and visiting the mosquito-infested areas, as well as the health of the human and livestock populations, resulting in reduced property prices.

2.3. Area-wide Control of Ae. sticticus *using* Bti

In the summer of 2000, when people once again were attacked by horrendous numbers of floodwater mosquitoes, the desperate call for help to reduce mosquito nuisance became major and repetitive news in the media at all levels. Officials of one of the seven affected municipalities then made the decision to initiate professional mosquito control operations and the other six municipalities followed the lead.

It was rapidly clear that the only possible and realistic solution was larviciding using a *Bti*-product. In view of the multitude of protected areas in the River Dalälven floodplains and the high environmental awareness in Sweden, chemical control or the application of less specific control agents were excluded. When applied correctly, *Bti*-products are highly selective against target mosquitoes without any known negative effects on non-target organisms or the environment (Lundström et al. 2010a, 2010b; Caquet et al. 2011; Lagadic et al. 2013, 2016). We decided to use the ready-to-use product VectoBac G®, consisting of corn-cob granules coated with *Bti* attached to the granules with corn oil.

Successful application of VectoBac G® requires detailed knowledge on the ecology of the target species to direct the treatments to the correct sites at the appropriate time. Thus, the first step for the programme against *Ae. sticticus* was precise mapping of the larval breeding sites. Mapping in the field started in the autumn of 2000 using a high precision GPS, amounting to a total area of 1170 ha near the two most affected towns Österfärnebo and Tärnsjö. This method was based on vegetation as indicators for temporary flooded areas and was very labour-intensive.

To speed up the mapping process and get more precise information on the geographic extent of inundations, another approach was needed. We decided to develop a high-precision digital elevation model (DEM) based on laser-scanning of the relevant areas. In 2003, the entire lower part of the River Dalälven was covered by air-borne laser-scanning. The multitudinous point measurements of the laser-scanning were used to create a DEM with sufficient resolution to discern height with centimetre precision. Since then, we use modelling with this DEM to discern the shallow flooded areas harbouring *Ae. sticticus* larvae, and to prepare the polygons to precisely direct the VectoBac G® larval treatments.

The first mosquito control operation in Sweden was carried out in 2002 and covered in total 443 ha. In the beginning, only temporary flooded areas outside nature reserves, the national park and close to the towns of Österfärnebo and Tärnsjö were included (Fig. 2).

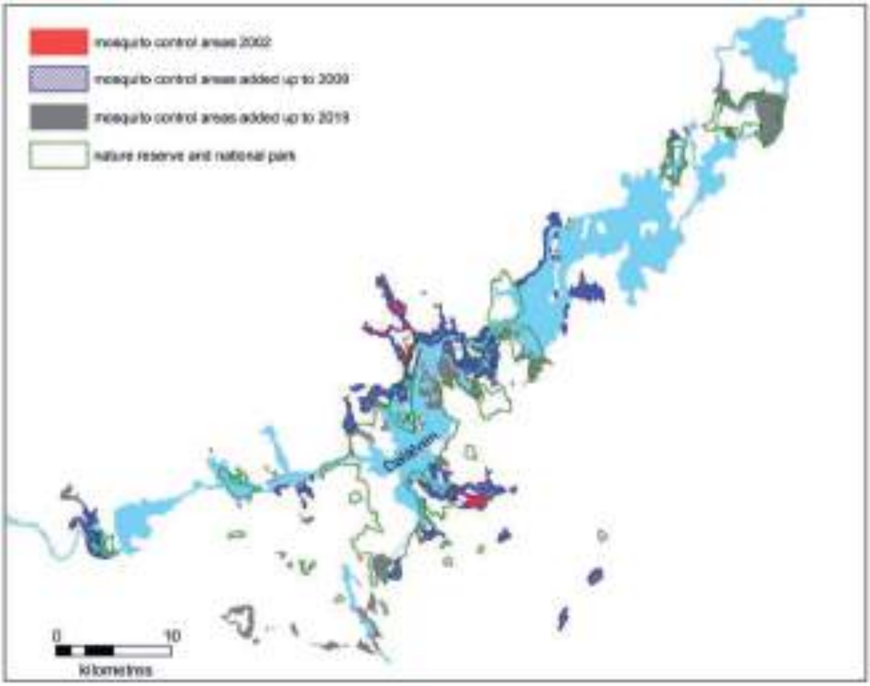

Figure 2. The gradual increase of areas with permissions for mosquito control based on dispersal of VectoBac G® granules by helicopter in natural wetlands, exemplified by the years 2002, 2009 and 2019. In 2009, some protected areas were finally included because of a case won at the Supreme Environmental Court of Sweden.

Over the years, we gradually increased the coverage of treatment areas, and another milestone was reached in 2009 with the first permission for treatments in protected areas (Fig. 2). This achievement however required a court case that was decided in favour of treatments at the Supreme Environmental Court of Sweden.

Since 2016, we have permission to treat more than 10 000 ha of swamps, marshes and meadows and the single largest treatment so far covered 4411 ha in May 2018. The need for VectoBac G® application varied between the years, from no application at all during some years when no floods took place, e.g. 2004 and 2017, to a maximum total of 9345 ha in 2015 (Fig. 3). In addition, the applied dosage of VectoBac G® was gradually reduced from 15-17 kg/ha during the first years to 11-13 kg/ha during recent years. This dose reduction was achieved by technical improvements regarding the helicopter application and navigation system used.

2.4. Routine Control Operations

From middle of April until end of August, the water flow fluctuations of the River Dalälven is followed seven days per week, and through collaboration with water regulation authorities we have access to a professional water flow prognosis. If there is an indication of rising water levels in the lakes of the floodplain, actual inundations are monitored in the field and the presence of newly hatched larvae of the target floodwater mosquitoes assessed. It is crucial to find the first-instar larvae of *Ae. sticticus* as early as possible to maximize the time window for *Bti*-treatments. During these first days, several two person teams visit selected sites to measure the abundance of mosquito larvae with a standard larval dipper and to map the waterline with a handheld high-precision GPS. These field-collected data provide the baseline for all decisions on treatments.

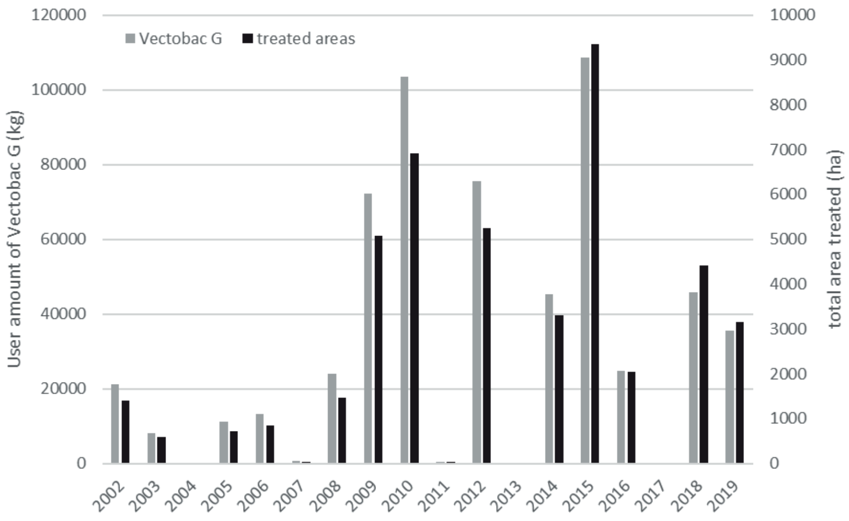

Figure 3. The amount of VectoBac G applied by helicopter in the River Dalälven floodplains for mosquito control and the total area treated per year for the years 2002 to 2019.

The GPS-points are transferred to a GIS-software and plotted on the DEM. The lakes in the floodplain are situated at different elevations, and the inundated areas are therefore modelled for each sub-area. As floodwater mosquito larvae are rare or absent in open deep water, the DEM is used to exclude those areas from treatments. The water depth limit for application depends on vegetation density and height as vegetation provides retreats for mosquito larvae in open waters. During spring floods, when there is little and low vegetation, areas up to 40 cm water depth are included in the applications, while during summer with plenty of vegetation, the limit is set at 60 cm water depth. In the GIS, the relevant areas are defined and prepared as polygons for the helicopter treatments.

Application of VectoBac G by helicopter can start approximately two to three days after detecting the first floodwater mosquito larvae, including the time needed to collect and analyse all necessary technical and biological information. Two sling buckets with rotating discs are calibrated for application of VectoBac G®. Using two buckets makes application very efficient, allowing for simple change of bucket for the helicopter pilot without landing (Fig. 4).

In the helicopter, a navigation system connected to a GPS with a high update-frequency reads the polygons as areas to be treated. The pilot prepares the appropriate flight routes with a defined distance (20-30m, exact distance is based on calibration results) between flight lines, guiding flight routes ensure complete coverage within each treatment area. All the VectoBac G®-applications are logged and transferred to the GIS for assessing the areas covered.

Figure 4. The use of two sling buckets, combined with change of bucket without landing the helicopter, allows for increased speed and reduced cost for aerial application of VectoBac G in the River Dalälven floodplains, central Sweden (credit J. O. Lundström).

All *Bti*-treatments should be completed before the mosquito larvae reach fourth instar. Therefore, especially during warm summer weeks, large floods require very rapid and efficient operations. Fortunately, there is almost 24 hours of daylight during summer in this part of Sweden. If necessary, the helicopter can apply VectoBac G®-from approximately 04:00 in the morning until 24:00 at night. These intensive 20 hrs working days require double crew on duty both on the ground and in the air.

In May 2018, a total area of 4411 ha was treated, including areas in seven municipalities along an approximately 100 km stretch of the River Dalälven, the largest mosquito control operation so far. Treatments were completed in 5 days with successful reduction of floodwater mosquito larvae. Currently, more than 1100 ha of natural wetlands can be treated by helicopter per day.

2.5. *Eighteen Years of* Bti-*based* Ae. sticticus *Control*

The goal for the floodwater mosquito control is to reduce mosquito abundance to less than 500 mosquitoes per trap-night to ensure that people in the River Dalälven floodplains can live normal lives during the short summer in Sweden. As mentioned, mosquito abundance in the area was extremely high before initiation of control and people were plagued by blood-seeking *Ae. sticticus* females, even in the centre of towns in the middle of the day. For example, in the centre of Österfärnebo village we measured 23 000 mosquitoes per CDC trap-night in August 2000 (Fig. 5). This measurement was before the first VectoBac G® application.

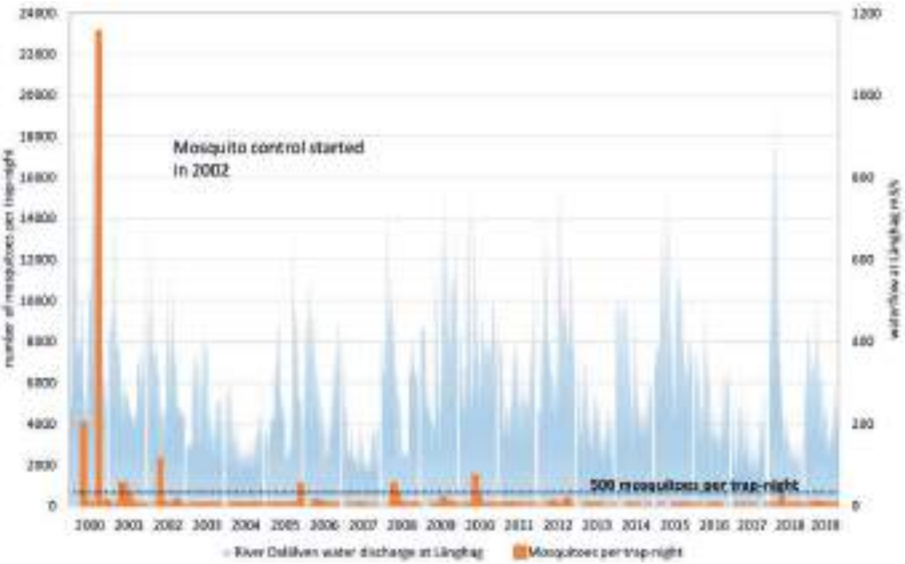

Figure 5. The number of mosquitoes per trap night in central Österfärnebo and River Dalälven water discharge at an upstream station from 2000 to 2019. The goal of mosquito control is to reduce mosquito abundance to less than 500 mosquitoes per trap night (reproduced with permission from Biologisk Myggkontroll 2019).

Since the start of mosquito control in 2002, mosquito abundance has been significantly reduced and since 2013, the number of mosquitoes collected in the trap in central Österfärnebo has been kept below 500 individuals per trap-night. This shows the effectiveness of successful *Bti*-applications and the results are greatly appreciated by both the local people and the visitors.

3. PREVIOUSLY SUGGESTED ALTERNATIVE CONTROL METHODS

Swedish authorities, including the Swedish Environmental Protection Agency (Swedish EPA), realize the importance of floodwater mosquito control and the need for *Bti*-treatments, but nevertheless want to reduce the usage of VectoBac G® in favour of alternative methods. However, the enormous abundance of mosquitoes causing the nuisance makes this a quite complicated task, since a very dramatic reduction is needed to reach the less than 500 mosquitoes per trap-night required by the locals and visitors alike.

For example, in Österfärnebo village a 97.8% reduction was required to reduce the 23 000 mosquitoes per trap-night to the acceptable abundance of 500 mosquitoes per trap-night. With respect to the impressive flight range of blood-seeking *Ae. sticticus* females, this means in practical terms that at least 90% of the *Ae. sticticus* larvae in at least 95% of the shallow flooded areas within 5 km around the village need to be controlled (Schäfer and Lundström 2014).

The alternative methods specifically suggested by the Swedish authorities are 1) changing the hydrology of the River Dalälven, and 2) increasing the mowing and grazing in the wet meadows (Lundqvist et al. 2013).

3.1. Changing the Hydrology of the River Dalälven

Changing hydrology for floodwater mosquito control requires stabilizing the water level to avoid floods and such a hydrological regime can cause a very strong reduction in mosquito abundance if fully implemented. However, nature conservationists request increased flood magnitude and duration in May and June, which is also the major larval production period for *Ae. sticticus*. Clearly, a single hydrological strategy will not be able to achieve the requirements for both a stable water level and increased flooding. In addition, this topic involves a very large number of stakeholders and many laws and regulations that control the water flow and water levels in different parts of the river. Furthermore, implementing the whole process would be very costly and time-consuming. Thus, reducing flood-water mosquito nuisance by hydrological changes of the River Dalälven, without reducing biological diversity, is a highly complex task that could be considered almost impossible (Hedström-Ringvall et al. 2017).

3.2. Increased Mowing and Grazing in the Wet Meadows

The suggestion of using mowing and grazing as alternative mosquito control methods originates from the general opinion that mosquito nuisance was less severe more than a century ago, when mowing and grazing of the floodplain meadows were common.

One study could show fewer mosquito larvae in areas with mowing and grazing than in areas without these methods, but this study was restricted to one year (Östman et al. 2015). In an unpublished study comparing the numbers of eggs in areas with and without mowing, no difference was found, and thus no long-term effect of mowing or grazing on the abundance of floodwater mosquitoes can be expected (Östman 2013).

We obtained similar results when comparing our own larval surveillance data over 15 years from areas with and without mowing and grazing Thus, although mowing and grazing sometimes might result in lower abundances of floodwater mosquito larvae, these measures cannot serve as reliable mosquito control methods. The potential reduction of larval abundance is too low and too unpredictable.

The search for effective alternatives to control *Ae. sticticus* in the River Dalälven floodplains should identify methods that could fulfil three main criteria: 1) ability to obtain very strong population suppression, 2) being environmentally neutral, and 3) suitable for large-scale application in natural swamps, marshes and wet meadows.

Evidently there are few mosquito control methods, other than larviciding with *Bti*-based products, capable of inducing such a strong population reduction without adding substances that might represent a distinct risk to the environment. In addition, Sweden is a member of the European Union and should comply with the biocide directive (EU 2012).

4. NEW METHODS FOR MOSQUITO CONTROL

Several potential new mosquito population suppression methods have surfaced in the last decades including the Sterile Insect Technique (SIT), the Incompatible Insect Technique (IIT), the Release of Insects carrying Dominant Lethality (RIDL), and genetically modifications based on CRISPR-Cas9 technology (Huang et al. 2017). The RIDL and CRISPR-Cas9 technologies have potential for strong population suppression, but the genetic modifications forming the strategic base for the methods will probably induce very strong counter-reactions from the general public, the Swedish EPA and other environmental protection authorities.

The SIT is a species-specific and environmentally safe method for area-wide management of insect pests which relies on repeated release of a large number of sterile male insects (Knipling 1955, 1979, 1998; Krafsur 1998; Dyck et al. 2021). The population reduction effect is achieved after sterile males are released and mate with the wild females, which will then lay infertile eggs. If a surplus of sterile males is regularly released on an area-wide basis over a sufficient time period, and they successfully mate with the local females, ultimately this will result in suppression or local elimination of the target insect population. The necessary ratio of released sterile males to local fertile males depends on the biology of the target species, the initial wild population density, the risk of reinfestation from neighbouring areas, the competitiveness of the released sterile males, and the complementary control operations that can be performed (Dame et al. 2009). The SIT has and is being used in successful area-wide integrated pest management programmes (AW-IPM) against the New World screwworm fly *Cochliomya hominivorax* (Coquerel), the Mediterranean fruit fly *Ceratitis capitata* (Wiedemann) and other tephritid flies, tsetse flies, the codling moth *Cydia pomonella* (L.), the false codling moth *Thaumatotibia leucotreta* (Meyrick) and the pink bollworm *Pectinophora gossypiella* (Saunders) (Lindquist et al. 1992; Vreysen et al. 2007; Dyck et al. 2021; Boersma, this volume; Nelson, this volume; Staten and Walters, this volume).

SIT field trials in the 1970's and 1980's demonstrated that it could also work against mosquitoes (Patterson et al. 1970; Lofgren et al. 1974; Benedict and Robinson 2003; Dame et al. 2009). In the last decade, the Joint FAO/IAEA Programme has been the main driver for development of the mosquito SIT package (Lees et al. 2014; Bourtzis et al. 2016). The focus is on three mosquito vector species of major medical importance: the arbovirus vectors *Aedes aegypti* L. and *Aedes albopictus* (Skuse), and the malaria vector *Anopheles arabiensis* Patton.

Major technical improvements for the SIT against the two *Aedes* species have already resulted in successful SIT pilot-scale field studies, paving the way for the development of the SIT as a full-scale mosquito population suppression method (Lees et al. 2021). Successful field trials of the SIT for suppressing populations of *Aedes* mosquitoes are recorded for *Ae. albopictus* in Italy (Bellini et al. 2013), while a SIT/IIT combination was shown successful in suppressing a population of *Ae. albopictus* in China (Zhang et al. 2016; Zheng et al. 2019) and suppressing a population of *Ae. aegypti* in Thailand (Kittayapong et al. 2019).

The IIT relies on symbiotic bacteria of the genera *Wolbachia*, inherited in insects, and that can manipulate the reproductive system of their host insects (Kittayapong et al. 2002; Werren et al. 2008). The incompatibility of sperm from a *Wolbachia*-infected male that fertilizes the eggs of a non-infected female, or of a female that is infected with another *Wolbachia* strain, can be used for population suppression by the IIT. The technique was first developed in 1967 against the lymphatic filariasis vector *Culex pipiens fatigans* in Burma, and the IIT was shown capable of eliminating the local mosquito vector population (Laven 1967). More recent positive results have been obtained in field experiments with the IIT against *Aedes polynesiensis* Marks 1954, *Culex pipiens quinquefasciatus* Say 1823, *Ae. albopictus* and *Ae. aegypti* (Atyame et al. 2011, 2015; Moretti and Calvitti 2012; O´Connor et al. 2012; Ritchie et al. 2015; Mains et al. 2016; Strugarek et al. 2019).

The control action of both the SIT and the IIT relies on providing a surplus of sexually active males that upon mating with the local females cause infertility of their eggs. These eggs cannot hatch to larvae, thus precluding development of new mosquito generations and over time the local population declines and perhaps, if isolated, is even locally eliminated. With the SIT, this is achieved by the release of sexually active mosquito males that have been sterilized by radiation. With the IIT, infertile eggs are the consequence of incompatibility between released sexually active *Wolbachia*-transfected males mating with local females that either are uninfected by *Wolbachia* or are infected with a different *Wolbachia*-strain.

We consider the SIT and the IIT as interesting to evaluate as part of an integrated approach for area-wide population suppression of our target species, the floodwater mosquito *Ae. sticticus.* Being environmentally neutral, both the SIT and the IIT could, after population pre-treatment with VectoBac G, potentially meet the criteria of inducing a high level of population suppression, although they have not been tested against a floodwater mosquito species.

5. PREREQUISITES FOR A STERILE MALE STRATEGY TO CONTROL *Aedes sticticus*

Applying the SIT or the IIT for *Ae. sticticus* control requires a laboratory colony of the species, a facility for mass-rearing of mosquitoes, the sterilisation of males, a transport strategy, a dispersal system, monitoring systems for several life stages, and a method capable of reducing the population of this superabundant species before commencing the sterile male release.

Differences in ecology and behaviour will demand a partially different SIT strategy for *Ae. sticticus* than for more commonly considered species *Ae. aegypti* or

Ae. albopictus (Lees et al. 2021). The latter two have continuous reproduction during a major part of the year and larval habitats are small, cryptic and widely dispersed. SIT-based control of these species requires that sterile males are released at least once a week over many months over mosquito habitat in domestic and rural areas. In contrast, *Ae. sticticus* larval sites are well-defined temporary flooded areas with synchronised batches of larvae during a flood event. Thus, SIT-based control requires very focused release of males in conjunction with flood events. The synchronised emergence of *Ae. sticticus* in relation to floods indicate that the release of sterile males during this emergence period could be sufficient to induce a high percentage of egg infertility in local females. However, it is probably a safer strategy to continue with weekly releases of sterile males for an additional 3-4 weeks after each emergence.

Several supporting factors necessary for successful SIT or IIT application are already well established for *Ae. sticticus*, while other factors need to be dealt with. As shown on previous pages, an efficient method for large-scale larval suppression is available that can significantly reduce population size before sterile male release. One factor in favour of implementing the SIT or the IIT against *Ae. sticticus* is that mating occurs in or near well-defined larval breeding areas with specific relation to flood events. Another factor in favour of the SIT or the IIT is the availability of existing methods to measure gender, larvae and egg abundance. The following pages provide details on the major factors that need to be addressed to develop and test the SIT or the IIT against *Ae. sticticus* in Sweden.

5.1. Egg Storage and Hatching

The eggs of the floodwater mosquito *Ae. sticticus* range from 0.610 to 0.645 mm in length and from 0.180 to 0.215 mm in width (Gjullin et al. 1950). The eggs are extremely hardy and remain viable for several years (Gjullin et al. 1950; Trpis and Horsfall 1967), allowing for a long shelf-life and stockpiling of eggs during industrial mass-production year-round. Eggs could be stored at 4°C for long time periods.

The eggs will not hatch in clean tap water but hatch readily in a willow-leaf infusion or when amino acids are added to the water (Gjullin et al. 1950). A reduction in dissolved oxygen is the main hatching stimulus for the eggs (Gjullin et al. 1950) with increased eclosion when the hatching media is a nutrient rich broth (Trpis and Horsfall 1967). Hatching of eggs can occur at 8°C, but the hatching is more efficient and better synchronised at higher temperatures with optimum of about 21°C (Trpis and Horsfall 1967). Eggs of *Ae. sticticus* may have to be exposed to a period of winter before hatching (Horsfall and Trpis 1967).

5.2. Larval Rearing

The development of *Ae. sticticus* larvae depends on temperature, diet, larval density and water depth (Trpis and Horsfall 1969). Water temperatures of 8ºC to 32ºC were tested, and 21ºC was considered the optimum rearing temperature with maximum percentage maturing in the shortest time interval. At 25ºC larval development was accelerated by 1-2 days, but mortality increased.

The larvae were fed liver yeast suspended in water, and for a pan with 30 larvae in 1700 ml of water the optimal yield was achieved when the larvae were provided 110 mg of dry yeast equivalent per pan per day. Feeding every second day required doubled amount of food and feeding every third days resulted in increased mortality. The density of larvae, reared in 1700 ml of water at 25ºC, influenced the developmental time. Pupation began and was completed on day 6 in pans with 30 larvae, while pupation occurred days 7 to 10 in pans with 60 larvae and on days 8 to 13 in pans with 90 larvae. Water depth was also important, especially at higher temperature both development and survival were best in very shallow water.

5.3. Adult Rearing and Mating

The rearing of adult *Ae. sticticus* may require relatively large cages of 1.0 x 2.0 x 2.5 m (5 m^3) to maintain a normal mating behaviour of the laboratory reared males. Mating of *Ae. sticticus* occurs in damp and shady areas among trees and bushes, but they are not forming any obvious swarms. The actual triggers of mating activity are unknown, and this is of course a potential obstacle when trying to establish a laboratory colony. Experience from colonization of other mosquito species showed that a combination of natural light cycle and a sufficiently sizeable cage triggered mating (Kuhn 2002; Lundström et al. 1990). Small cage size can induce a problem if mating couples split when not in the air, but this is not a problem with *Ae. sticticus*.

Photo documentation of a mating *Ae. sticticus* couple in the field show that they continue the sexual activity even after landing on the rubber boot of the observer (Fig. 6).

Figure 6. A mating couple of the floodwater mosquito Aedes sticticus*, that initiated mating in the air and continued after they landed on the photographer's rubber boot, in the Valmbäcken alder swamp in July 2015 (credit J. O. Lundström).*

Our strategy for colonization of *Ae. sticticus* will initially focus on evaluating the mating in relatively large cages with simulated natural dusk and dawn periods. If not successful, forced copulation could be used for a few generations. Experience from colonization of the floodwater mosquito *Ae. vexans* could provide additional suggestions (Kuhn 2002). The colonization of *Ae. vexans* was based on mosquitoes released in a walk-in cage of 5 m^3 and simulated dusk and dawn periods. Once the colony was established, *Ae. vexans* adapted to mating in smaller cages with a 1.4 m^3 volume. However, such changes in behaviour could be a disadvantage for the laboratory reared males in the competition for mating with wild females. We are also aware that the close ecological similarities between these two floodwater *Aedes* species of the northern hemisphere is no guarantee that colonization of *Ae. sticticus* will be successful.

The adult mosquitoes require a food regime with constant access to 10% sugar solution (males and females) while the females in addition need to be provided blood approximately once a week for egg production. Our practical experience is that Swedish *Ae. sticticus* readily feed on bovine blood heated to 38ºC and provided from a membrane feeder. The initial unsuccessful trials to colonize *Ae. sticticus* have shown that there is no need for a specific egg substrate, since the females readily deposit the eggs on moist paper. However, many details concerning larval and adult rearing and mating will have to be optimized before efficient rearing and mass-production of high-quality males will be possible.

5.4. Sex Separation

The male pupae of *Ae. sticticus* are smaller than the female pupae, allowing for mechanical size-based sex separation in the pupal stage. The Fay-Morlan separator, a mechanical sex separation method (Fay and Morlan 1959; Sharma et al. 1972; Focks 1980), is the standard method for sex separation of *Ae. aegypti* and *Ae. albopictus* pupae. The sieves method to separate male and female pupae is also commonly used and both methods are potential options for *Ae. sticticus* sex separation.

The eventual contamination with some female mosquitoes among the sterile mosquito males, is a serious problem. The blood-seeking females could cause nuisance, which may cause public aversion that severely reduces the perceived effect of the control strategy. In addition, females mixed with the released sterile males could also divert some mating away from the target native females, thereby reducing the effect of the SIT intervention. Therefore, an efficient and secure method of separating males from females in the mass-production process is imperative to the success of the strategy.

5.5. Sterilisation by Ionizing Radiation

Sterilisation of male insects for the SIT can be done by ionizing radiation or by chemical treatment (Bakri et al. 2021). Sterilisation by ionizing radiation that randomly destroys fractions of DNA in the male gonads was the first tested method. Sterilisation by X-ray or gamma radiation from a ^{60}Co radiation chamber is nowadays the standard method of sterilizing male insects for SIT application. This is an extremely reliable method, suitable for industrial-scale insect sterilisation.

The use of gamma radiation for sterilisation of mosquitoes was first tested against *Ae. albopictus* in Italy (Bellini et al. 2013) and has later been more generally applied as the mosquito sterilisation method. However, the use of ^{60}Co requires special security measures, adequate regulation in the country, radiation protection protocols, and the initial investment is high. More recently, X-ray machines suitable for mass-sterilisation of male insects have been developed and are becoming available (Yamada et al. 2014; Bakri et al. 2021). The X-ray equipment is cheaper, requires no regulation in the country, and requires less security than the gamma radiation equipment. Also, X-ray is commonly used for medical purposes making it probably the least controversial method for sterilizing male *Ae. sticticus*.

5.6. Wolbachia *and* Aedes sticticus

The *Wolbachia* bacterial symbiont can be used to induce sterility through mating incompatibility (Bourtzis 2008; Werren et al. 2008; Rasić et al. 2014). Recently, the United States Environmental Protection Agency (US-EPA) has approved the release of *Wolbachia pipientis* transfected male *Ae. albopictus* (*w*Pip strain; ZAP males) for population suppression in the District of Columbia, and in 20 states of the USA (US-EPA 2017; Waltz 2017).

The use of *Wolbachia* for IIT implementation is dependent on knowledge about the eventual occurrence of natural infection in the target populations, as it requires that the wild female is either free of any *Wolbachia* bacteria symbionts or carries another strain of the bacteria than the infected and released males.

A preliminary study of the occurrence of *Wolbachia* was carried out with *Ae. sticticus* samples collected during the regular annual mosquito surveillance programme in the River Dalälven floodplains by Biologisk Myggkontroll (Schäfer et al. 2018). *Wolbachia*-specific PCR screening, as reported in Kittayapong et al. (2000), was carried out in 279 mosquitoes of 17 mosquito species for naturally occurring infections with this bacterial symbiont (Table 1). A total of 7 out of 17 species (41.2%) contained the *Wolbachia* symbiont. However, the PCR results indicated that the 20 *Ae. sticticus* individuals screened were free of the *Wolbachia* symbiont, indicating that the species is probably free from *Wolbachia* infection.

Based on this information, there is potential for using *Wolbachia*-transfected *Ae. sticticus* males for population suppression. This would require the establishment of a *Wolbachia*-transfected *Ae. sticticus* strain that could be produced in large numbers for male-only release. The use of a local mosquito strain as well as a local *Wolbachia* strain might also make it easier to receive the necessary permits from the authorities.

5.7. Transport

The production of sterile male *Ae. sticticus* could either be done in Sweden, or in another country within reach for timely delivery to the suggested pilot study area in the River Dalälven floodplain. Any decision on the location of such a production unit will require an evaluation of the costs and reliability for production and delivery, as well as the logistics for delivery, in relation to the reaction time from low-level maintenance production to full-scale production for release. Also, it has to be guaranteed that the long-distance shipment is not detrimental to the quality of the insects. A key factor is the availability of a provider with the knowledge and drive to perform the sterile male production.

Table 1. The occurrence of the bacterial symbiont Wolbachia *in mosquito species collected in the wetlands of the River Dalälven floodplains, central Sweden*

Species	No. Tested	No. *Wolbachia* positive specimen	Percent positive
Aedes annulipes (Meigen)	32	0	0
Aedes cantans (Meigen)	15	0	0
Aedes cinereus (Meigen)	30	5	16.67
Aedes communis (De Geer)	20	0	0
Aedes diantaeus (Howard, Dyar & Knab)	19	0	0
Aedes intrudens (Dyar)	18	0	0
Aedes punctor (Kirby)	20	0	0
***Aedes sticticus* (Meigen)**	20	0	0
Aedes vexans (Meigen)	20	0	0
Culiseta alaskensis Ludlow	13	0	0
Culiseta bergrothi Edwards	15	8	53.33
Culiseta morsitans (Theobald)	5	4	80.00
Culiseta ochroptera (Peus)	2	0	0
Culex pipiens L./ *torrentium* Martini	4	4	100.00
Coquillettidia richiardii Ficalbi	10	10	100.00
Anopheles maculipennis sl (Meigen)	22	9	40.91
Anopheles claviger (Meigen)	14	6	42.86

A recent SIT pilot study in Heidelberg, Germany, relied on sterile male *Ae. albopictus* produced in Italy and the transport time from the production unit to field release was 24 h by car (R. Bellini, personal communication). More recently, this sterile male transport is done by DHL delivery by air, shortening transport time and increasing reliability. Transport by air allows rapid long-distance transport between mosquito factory and the field release area.

Insects are poikilotherms and thus have about the same body temperature as their surrounding environment. This is reflected in slower activity at low temperature and increasing activity with rising temperature within certain temperature limits. Thus, it is possible to chill mosquitoes and thereby make them less active and less vulnerable to physical damage during transport. Since the chilling reduces all life processes, it allows for packing of very large number of insects in a small volume as long as they are in a chilled state.

The technique of chilling mosquitoes for long-distance transport has been tested for *Anopheles arabiensis*, *Ae. aegypti* and *Ae. albopictus* and the conclusion is that there is large variability in responses (Culbert et al. 2017, 2019). Apparently, the reaction to chilling needs to be established for each species and may even have to be evaluated for the specific population (Culbert et al. 2019). Although the northern floodwater mosquito *Ae. sticticus* is already used to an environment where immobilization by chilling is inevitable, there is an obvious need to test the species for optimal transportation temperature before practical use.

5.8. Male Mating Quality

Since mating success is central for the SIT and the IIT, there is a need to evaluate male mating quality on a regular basis. This is normally done using walk-in field cages to sufficiently frequently test for mating competition and success. This argues in the direction of creating large and efficient production units, supplemented with capacity for adequate male quality evaluation. Such facilities need capacity for timely delivery of quality sterile males for release.

5.9. Male Dispersal

Ground release of *Ae. sticticus* males in large and inaccessible natural wetland areas is not possible within a limited timeframe. Aerial release is the main alternative as it is rapid and allows high precision; it is also the least disturbing for vegetation and animals alike. The release should preferable be done by either helicopter or drone (unmanned aerial vehicle, see Benavente et al., this volume), but probably not by airplane, as the male mosquitoes are fragile and may become damaged if speed of the dispersing aircraft is too high.

The dispersal of *Ae. sticticus* males, in conjunction with mating, is important information when deciding on the male release strategy. Our preliminary observations provide evidence of *Ae. sticticus* mating in the same general area as the larval habitat. The synchronised hatching of *Ae. sticticus* eggs during a flood, the likewise synchronised development of larvae to pupal stage, and the emergence of males about one day before females provide opportunities for males to encounter emerging females without searching over extensive areas. Furthermore, the temporary flooded areas are very humid and thereby favourable environment for mosquitoes that are sensitive to desiccation.

Mating of *Ae. sticticus* was only observed in the shade under deciduous trees and bushes. The present level of biology knowledge indicates that the mating in this species occurs in the shaded terrestrial parts of the temporary flooded wet meadows and swamps. This information indicates that it is possible to develop a remote assessment method for locating actual mating areas. Release of sterile *Ae. sticticus* males could be concentrated in the defined mating environment and thus optimize impact in relation to time and costs.

5.10. Monitoring of Ae. sticticus *Males and Females*

The ability of released *Ae. sticticus* males to survive and disperse in the target areas, and the actual abundance of sterile males relative to native fertile males, are crucial to follow before, during and after the release. Discrimination of released sterile males from native males of the same species will require a marking system for released males, for example fluorescent powder or dye (Pal 1947; Verhulst et al. 2013). However, marking with fluorescent dye is not always recommended for mosquitoes as a coloured mosquito might have negative impact on the human population acceptance of the technique. Johnson et al. (2017) evaluated a new internal marking technique for mosquitoes using rhodamine B, showing that the marking remained after sugar-feeding and was visible for lifetime in *Ae. aegypti.* However, the authors recommend that small-scale mark-release-recapture experiments be performed to obtain more accurate estimates of male survival and mark persistence prior to adoption as an operational assessment (FAO/IAEA 2019).

Mosquito males are more difficult to collect than the females, and there is no trapping system available for specific sampling of *Ae. sticticus* males. As no obvious way of attracting and sampling males is available, we decided to test a more general strategy using the MosVac, a portable battery-operated, aspirator (Go Green Co., Ltd., Bangkok, Thailand).

The test was done in the Valmbäcken alder swamp, at the edge of the frequently flooded lake Färnebofjärden and in conjunction with a flood event, to make sure that males would occur in the study area. The test was carried out in June 2015, on the day we expected emergence of adult *Ae. sticticus* to commence. Only males were caught during the first sampling event, while on the following consecutive sampling days a mixture of males and females were collected (Fig. 7).

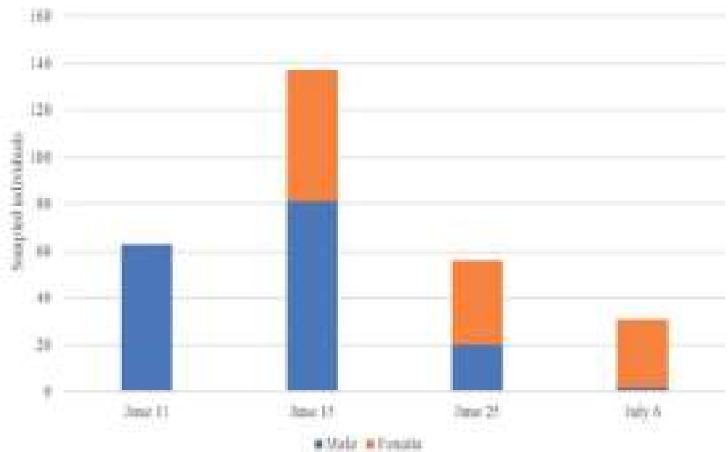

Figure 7. Abundance of male and female Aedes sticticus *measured by vacuuming grassy and bushy areas of the Valmbäcken alder swamp, River Dalälven floodplains, with a MosVac aspirator. The first sampling was done June 11, 2015, coinciding with the first day (D1) of adult emergence after the flood in May. Sampling was repeated June 15 (D5), June 25 (D15) and July 6 (D26).*

Only a few male *Ae. sticticus* were collected 26 days after initiation of emergence, when the male to female ratio was heavily distorted towards female dominance in the sample.

These preliminary data show that the MosVac aspirator was able to sample sufficient numbers of males for evaluating a pilot SIT study against *Ae. sticticus*. It also indicated that male abundance was reduced more rapidly than female abundance, with almost no males left approximately four weeks after adult emergence. Our intention is to carry out more detailed evaluations of male *Ae. sticticus* sampling strategy using MosVac aspirators, and other potential male sampling methods, to develop a standardized protocol for evaluation of sterile male releases.

Monitoring of female mosquito abundance is routinely done by Biologisk Myggkontroll (2019) in about 40 trap sites spread over the whole floodplain of the River Dalälven. Sampling is performed in all trap sites for one night every second week from spring to fall using CDC-traps baited with carbon dioxide as an attractant. This sampling will provide information on the relative abundance of the blood-seeking *Ae. sticticus* females that cause the nuisance.

5.11. Measuring the Abundance of Ae. sticticus *Larvae During Floods*

The *Ae. sticticus* larval abundance is routinely measured by dipping with a white plastic dipper on a long shaft. A large amount of data on larval abundance before and after each mosquito control operation in the River Dalälven floodplains are available since 2002. Such background data are very useful when evaluating the effect of the SIT on an *Ae. sticticus* population, because a subsequent reduction of larval abundance is expected if sufficient numbers of native females have mated with sterile males, resulting in infertile eggs.

Furthermore, as the egg bank of *Ae. sticticus* remains viable for many years (Gjullin et al. 1950), it will be useful for a SIT- or IIT-based intervention to continue measuring the larval abundance as a proxy for the abundance of fertile eggs. Declining abundance of larvae over the years will show a real population reduction. This will make it possible to observe if the actual population is reduced or even locally eliminated.

5.12. Potential IPM Strategy: Combination of Other Tools

The high population density of *Ae. sticticus* in the River Dalälven floodplains, without efficient control, is of a magnitude that would make it extremely costly and almost impossible to solely rely on SIT- or IIT-based control. However, the area-wide use of aerial dispersal of VectoBac G against the larvae is highly efficient and already induces about a 95%-99% reduction in blood-seeking female abundance (Schäfer and Lundström 2014). Such pre-treatment of the pilot area, reducing the target species population to a fraction, will make it possible to decrease the required number of sterile males to be released substantially, thereby boosting both the economics of the releases and their population suppression effect.

5.13. A Suggested Pilot Study of SIT or IIT Application Against Aedes sticticus

Before implementing new area-wide population suppression methods against *Ae. sticticus*, there is an obvious need for a pilot study of the techniques to be integrated against the target population in its natural setting. The results of the pilot study will provide guidance for evaluating efficacy and will also provide guidance on whether and how to proceed when expanding into AW-IPM programmes using the SIT or the IIT.

Two flood-prone and extremely productive areas for *Ae. sticticus* have been selected as suggested pilot study areas (Fig. 8). Former lakes Hallsjön and Karbosjön are located close to the village of Huddunge and have been subject to *Bti*-based mosquito control since 2005. All known floodwater mosquito breeding sites within flight distance are included in the routine *Bti*-treatments, thus the study areas are protected from massive reinvasion of *Ae. sticticus* and would function as isolated populations.

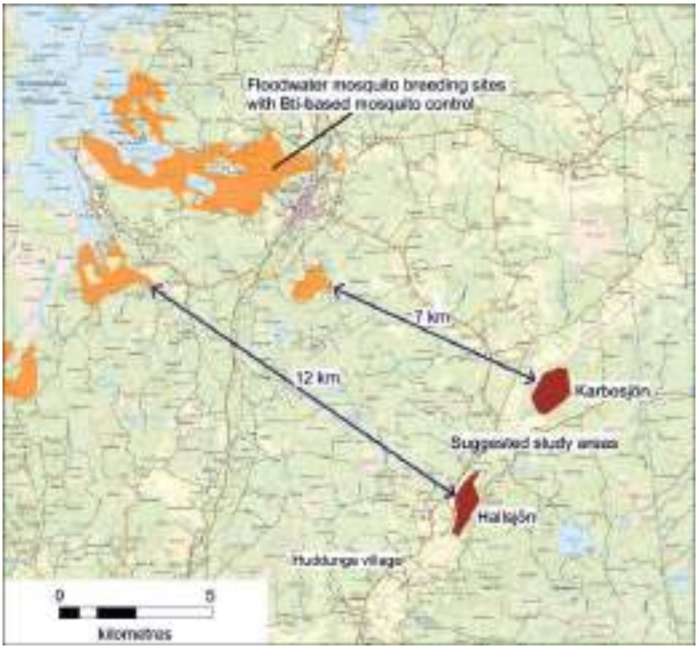

Figure 8. The two suggested areas, Hallsjön and Karbosjön for a pilot study of the SIT against the floodwater mosquito Aedes sticticus *are located northeast of Huddunge village and are isolated from breeding sites close to the River Dalälven. All breeding sites are included in routine* Bti-*based mosquito control which protects the study areas from massive reintroduction of* Ae. sticticus *females.*

The enormous mosquito nuisance problems around Lake Hallsjön were the motive to initiate mosquito control using VectoBac G spread from helicopter, as previously described. A first survey of mosquito abundance in the Hallsjön area was carried out in 2004, and in 2005 mosquito control was commenced. From 2005 onwards, the abundance of female *Ae. sticticus* is regularly monitored with CDC-trapping once every second week from May until September each year (Fig. 9).

The relative abundance of blood-seeking *Ae. sticticus* females before initiation of treatments was about 16 000 per CDC-trap and night. After several years of treatment with VectoBac G, the maximum number of female *Ae. sticticus* collected any time during summer is 11-44 per CDC-trap and night, representing a 99.97% reduction. This proves the excellent population suppression resulting from professional VectoBac G larviciding and confirms the almost total elimination of *Ae. sticticus* larvae as observed within 24 h after each treatment.

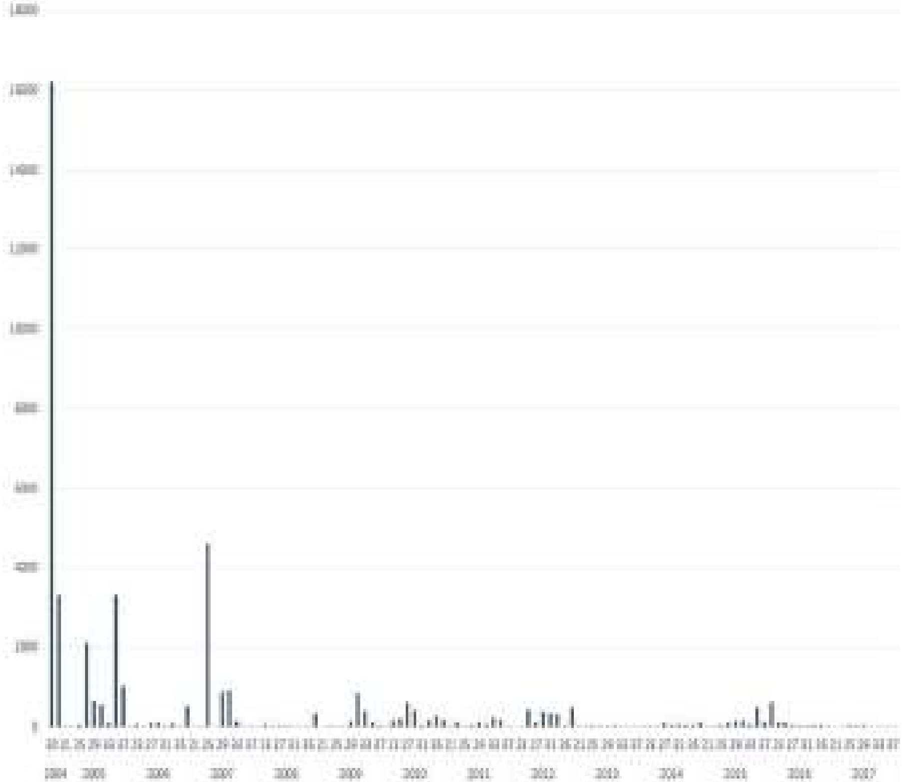

Figure 9. The abundance of blood-seeking Aedes sticticus *females at Hallsjön, central Sweden, for the years 2004 to 2017 as measured by fortnightly sampling with CDC miniature light traps baited with carbon dioxide. Mosquito control using VectoBac G commenced in 2005, inducing a very strong reduction in abundance, except for week 27 in 2007 when a mistake allowed massive mosquito emergence (data not shown).*

The size of the remaining population of surviving *Ae. sticticus* males and females after treatment is difficult to measure since there remain so few individuals and these are dispersed. However, the CDC-traps are positioned in areas with trees and bushes attracting the females produced in a much larger surrounding area of wet meadow estimated at 1-2 ha. If we use 44 females as a 10% fraction of actual numbers produced in 2 ha, the production could be supposed to be 220 females per productive ha. As males and females emerge in approximately the same abundance, this would provide us 220 males per productive ha. The total area of the two pilot areas is about 150 ha, and during a large flood it is estimated that about 75% of the area is producing *Ae. sticticus*. Thus, only 25 000 local fertile males remain in the study areas after a VectoBac G treatment. Based on the estimated abundance of local males, there will be a need for releasing approximately 250 000 sterile or incompatible males to obtain a sterile to wild male ratio of 10:1. As can be understood, these are very approximate estimates, although they probably catch the approximate general tendency.

The success of a SIT or IIT trial is crucially dependent on the release of a sufficient number of good quality sterile males to compete with the local fertile males for mating with the local females. Therefore, it might be useful to try other methods for population size estimates before the initial release of sterile or incompatible *Ae. sticticus* males in the two suggested pilot study areas.

6. PERSPECTIVES

The sterile male technique, either the SIT or the IIT, has potential to serve as an alternative solution to sole reliance on *Bti*-treatments against *Aedes sticticus,* but for an evaluation of the real potential, the suggested pilot test needs to be performed. In case of positive and encouraging results, new challenges arise.

Setting up the SIT or the IIT over the whole of the River Dalälven floodplains will require an integrated strategy with *Bti*-treatments for many years, thus the desired reduction of the control agent will not be achieved for quite some time. Sterile male release will have to start in defined subsets of the floodplains, for example in the easternmost lake system and then move westwards. There is also a risk that *Bti*-treatments will have to increase in the beginning since there are untreated areas in the current control programme. Complete area-wide coverage of breeding sites will be needed to ensure low mosquito population size and low risk for reinfestation. Thus, for approximately 10-15 years there will be a need for both large-scale *Bti*-treatments and sterile male releases. As a result of this intensive work, the use of the suppression agent may phase out completely, although it might be wise to keep the possibility of *Bti*-treatments as a backup. Release of sterile males will have to continue at a maintenance level since re-establishment of *Ae. sticticus* might occur.

In conclusion, integrating the sterile male technique into the management of a floodwater mosquito like *Ae. sticticus* means intensive work effort over many years, but the goal of an environmentally neutral mosquito control, eventually without using any suppression agent, is considered achievable. Nevertheless, this will require political decisions ensuring stakeholder commitment and the economic basis for such a project.

7. ACKNOWLEDGEMENTS

We would like to acknowledge the valuable comments and suggestions from two anonymous external reviewers, and the English language support provided by Christian Blue.

8. REFERENCES

Atyame, C. M, N. Pasteur, E. Dumas, P. Tortosa, M. Tantely, N. Pocquet, S. Licciardi, A. Bheecarry, B. Zumbo, M. Weill, and O. Duron. 2011. Cytoplasmic incompatability as a means to control *Culex pipiens quinquefasciatus* mosquito in the islands of the southwestern Indian Ocean. PLoS Neglected Tropical Diseases 5: e1440.

Atyame, C. M., J. Cattel, C. Lebon, O. Flores, J.-S. Dehecq, M. Weil, L. C. Gouagna, and P. Tortosa. 2015. Wolbachia-based population control strategy targetting *Culex quinquefasciatus* mosquitoes proves efficient under semi-field conditions. PLoS One 10(3): e01119288.

Bakri, A., K. Mehta, and D. R. Lance. 2021. Sterilizing insects with ionizing radiation, pp. 355–398. *In* V. A. Dyck, J. Hendrichs, and A. S. Robinson (eds.), Sterile Insect Technique – Principles and practice in Area-Wide Integrated Pest Management. Second Edition. CRC Press, Boca Raton, Florida, USA.

Becker, N. 1989. Life strategies of mosquitoes as an adaptation to their habitats. Bulletin of the Society for Vector Ecology 14: 6–25.

Becker, N. and H. W. Ludwig. 1981. Untersuchungen zur Faunistik und Ökologie der Stechmücken (Culicinae) und ihrer Pathogene im Oberrheingebiet. Mitteilungen der Deutschen Gesellschaft für allgemeine und angewandte Entomologie 2: 186–194.

Becker, N., D. Petric, M. Zgomba, C. Boase, M. Madon, C. Dahl, and A. Kaiser. 2010. Mosquitoes and their control, Second Edition. Springer Verlag, Berlin/Heidelberg, Germany. 577 pp.

Bellini, R., A. Medici, A. Puggioli, F. Balestrino, and M. Carrieri. 2013. Pilot field trials with *Aedes albopictus* irradiated sterile males in Italian urban areas. Journal of Medical Entomology 50: 317–325.

Benedict, M., and A. Robinson. 2003. The first release of transgenic mosquitoes: An argument for the Sterile Insect Technique. Trends in Parasitology 19: 349–355.

Biologisk Myggkontroll. 2019. Bekämpar översvämningsmyggor vid Nedre Dalälven.

Bourtzis, K. 2008. Wolbachia-based technologies for insect pest population control. Advances in Experimental Medicine and Biology 627: 104–113.

Bourtzis, K., R. S. Lees, J. Hendrichs, and M. J. B. Vreysen. 2016. More than one rabbit out of the hat: Radiation, transgenic and symbiont-based approaches for sustainable management of mosquito and tsetse fly populations. Acta Tropica 157: 115–130.

Brust, R. A. 1980. Dispersal behavior of adult *Aedes sticticus* and *Aedes vexans* (Diptera: Culicidae) in Manitoba. Canadian Entomologist 112: 31–42.

Caquet, T., M. Roucaute, P. Le Goff, and L. Lagadic. 2011. Effects of repeated field applications of two formulations of *Bacillus thuringiensis* var. israelensis on non-target saltmarsh invertebrates in Atlantic coastal wetlands. Ecotoxicology and Environmental Safety 74: 1122–1130.

Culbert, N. J., J. R. L. Gilles, and J. Bouyer. 2019. Investigating the impact of chilling temperature on male *Aedes aegypti* and *Aedes albopictus* survival. PLoS One 14: e0221822.

Culbert, N. J., R. S. Lees, M. J. B.Vreysen, A. C. Darby, and J. R. L. Gilles. 2017. Optimised conditions for handling and transport of male *Anopheles arabiensis*: Effects of low temperature, compaction, and ventilation on male quality. Entomologia Experimentalis et Applicata 164: 276–283.

Dame, D. A., C. F. Curtis, M. Q. Benedict, A. S. Robinson, and B. G. J. Knols. 2009. Historical applications of induced sterilisation in field populations of mosquitoes. Malaria Journal 8 (Suppl. 2): S2.

Dyck, V. A., J. Hendrichs, and A. S. Robinson (eds.). 2021. Sterile Insect Technique – Principles and practice in Area-Wide Integrated Pest Management. Second Edition. CRC Press, Boca Raton, Florida, USA. 1200 pp.

(EU) European Union. 2012. Regulation (EU) No 528/2012 of the European Parliament and of the Council of 22 May 2012 concerning the making available on the market and use of biocidal products. ISSN 1977. 2012. 677: 2985.