

CHAPTER 7.5.

POTENTIAL IMPACT OF INTEGRATING THE STERILE INSECT TECHNIQUE INTO THE FIGHT AGAINST DISEASE-TRANSMITTING MOSQUITOES

R. S. LEES¹, D. O. CARVALHO² AND J. BOUYER^{2,3}

¹*Liverpool School of Tropical Medicine, Liverpool, United Kingdom
Rosemary.Lees@lstm.ac.uk*

²*Joint FAO/IAEA Division of Nuclear Techniques in Food and Agriculture,
International Atomic Energy Agency, A-1400 Vienna, Austria*

³*CIRAD, UMR ASTRE, F-34398, Montpellier, France*

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SUMMARY

More than three thousand million people live with the risk of malaria. Due to the widespread resistance of mosquitoes to insecticides and of parasites to chemotherapies, previous gains made in disease reduction are being reversed. In addition to this perennial threat, there is now a rapid invasion of *Aedes* mosquitoes across the globe and the associated spread of the arboviruses (arthropod-borne viruses) they carry. One half of the world's population is now at risk of dengue, and chikungunya (having emerged from Africa) is an increasing public-health problem in Asia and the Americas. The economic and social costs of these diseases is so great that, in some areas, they have slowed the development of nations. Current vector-control methods are inadequate (especially against container-breeding species) because they are losing their effectiveness, the global burden of mosquito-borne diseases is increasing, and no specific drugs or effective global vaccines are available to treat or prevent the diseases. Therefore, there is a need for additional suppression methods to be applied as part of Integrated Vector Management (IVM). Since the early 2000s, there has been a renewed interest in applying the sterile insect technique (SIT) against mosquito vectors of disease. The explosive outbreaks of the Zika virus (and associated birth defects) across the tropics increased the urgency. The recent availability of technology to rear and release the sterilized males of many mosquito species on a large scale has increased the expectation that the SIT could help reduce the suffering caused by mosquito-borne diseases. Much progress has been made in developing the SIT technology for mosquitoes, based on historic SIT efforts and the experiences gained in the successful large-scale application of the technique against agricultural pest species. The SIT is a suitable technology for suppressing mosquitoes because: (1) they can be mass-reared in a laboratory, (2) natural sexual dimorphism in many species aids sex separation, and (3) females become refractory after mating. There has been a perception that mosquitoes are more vulnerable than many pest species to damage during handling, sterilization, and release. However, technological and methodological improvements can cope with this lower robustness, and indeed take advantage of their smaller size and weight. Nevertheless, the need for perfect sex separation for male-only release to preclude any biting and disease transmission by released females, remains a technical bottleneck to scaling the SIT beyond small-scale pilot trials. As a remedy for this, genetic sexing strains are being developed. However, until they are available, combining the SIT with cytoplasmic incompatibility conferred by *Wolbachia* infection (incompatible insect technique (IIT)) has been proposed as an advantageous strategy. The advantage of including the IIT is that *Wolbachia* infection may prevent potential disease transmission by any released females, whereas sterilization guarantees that such females cannot reproduce, avoiding the loss of the cytoplasmic incompatibility due to *Wolbachia* establishment in the target population. Another advantage of simultaneous IIT use is that it enables the radiation dose to be minimized. Other challenges remain, particularly in release technology and quality control. Nevertheless, in recent years, pilot trials have been conducted or have been initiated, e.g. China, Germany, Greece, Italy, Mauritius, Mexico, Singapore, and Thailand, achieving encouraging results in suppressing adult populations of *Aedes* species. Area-wide releases, focused on urban and suburban settings, appear particularly promising in terms of sustainable and cost-effective IVM of *Aedes* vectors (eventually provided commercially by the private sector) because they can protect many people concentrated in relatively small areas. In the case of *Anopheles* vectors, the SIT may become a useful complementary tool, especially against outdoor-biting species which are not well-controlled by mosquito nets.

1. INTRODUCTION

Mosquito-transmitted dengue is now the world's most common mosquito-borne viral disease; over the last 50 years the incidence has grown more than 30-fold (Bhatt et al. 2013). Dengue viruses are estimated to infect about 400 million people per year, and over half of the world's population is at risk of the disease. Moreover, chikungunya virus emerged from Africa in the mid-2000s, and spread across Asia and the Americas in 2013 (Mayer et al. 2017). Due to the increasing spread of invasive mosquito species (Kraemer et al. 2015), these arboviruses were already becoming a major international public-health concern, even before Zika virus outbreaks occurred in the South Pacific in 2013 and in the Americas in 2015 (Mayer et al. 2017). Epidemics of the Zika virus in the Americas were associated with cases of microcephaly and other congenital

abnormalities. On 1 February 2016 the World Health Organization (WHO) declared a public-health emergency of international concern. To date there are no drugs or effective global vaccines available to treat or prevent Zika; this situation led to calls for an urgent response against its *Aedes* vectors. Even though one of the best vaccines ever developed exists for yellow fever (another arbovirus), it is re-emerging in some countries (including Angola, Brazil, China, Democratic Republic of Congo, and Kenya (Zwizwai 2017)).

Subsequent to a major expansion of its geographical range, *Aedes aegypti* (L.) is the most important vector of arboviruses worldwide, particularly in tropical and subtropical urban settings (Mayer et al. 2017). *Aedes albopictus* (Skuse) is also a major public-health concern because it is a very good vector of several arboviruses including dengue, chikungunya, and Zika (Mitchell 1995). The epidemiological risks associated with *Ae. albopictus* are increasingly great in Europe, where significant disease outbreaks are now occurring (Gossner et al. 2018). While arboviruses are increasing in impact and notoriety, 3.2 thousand million people remain at risk of malaria, transmitted by Anopheline mosquitoes; in 2016 alone, an estimated 216 million new cases of malaria and 445 000 deaths occurred (WHO 2017b).

1.1. Burden of Disease

Seven of the major vector-borne diseases (malaria, lymphatic filariasis, leishmaniasis, dengue, Japanese encephalitis, yellow fever, and Chagas' disease) share a largely overlapping pattern of global distribution; many parts of the world are at risk of up to six of these (Fig. 1) (Golding et al. 2015). Controlling the mosquito vectors would simultaneously combat several co-localized diseases, improving the benefit/cost ratio and sustainability of control efforts, particularly Integrated Vector Management (IVM) programmes, which could include a sterile insect technique (SIT) component. Alongside the human suffering (morbidity and mortality) caused directly by mosquito-borne diseases, they also exert a high financial burden -- direct costs of treatments and prevention activities, and indirect costs through loss of human productivity and subsequent impact on a region's economy. The concentration of malaria cases in Africa means that malaria in particular constitutes a major obstacle to sustainable development and poverty eradication on the continent.

The cost of treating cases of disease can vary greatly depending on the economic and social setting. The direct and indirect costs may fall on the state, on the patients and their families, or on both. In Ghana, for example, the treatment cost for each malaria episode varies from USD 5.70 to 48.73, depending on the severity of disease; the household is responsible for 55% of the treatment cost. Indirect costs, such as a funeral, can in some African countries be equivalent to one year's total household income (Sicuri et al. 2013). The Philippines carries the fourth-highest burden of dengue cases in South-East Asia; an analysis by Edillo et al. (2015) estimated that over 840 000 clinically diagnosed cases led to direct medical costs of USD 345 million (USD 3.26 per capita). Thirty-five percent of cases were treated as outpatients, representing 10% of indirect costs, compared with the 65% of patients who were hospitalized and constituted 90% of direct costs.

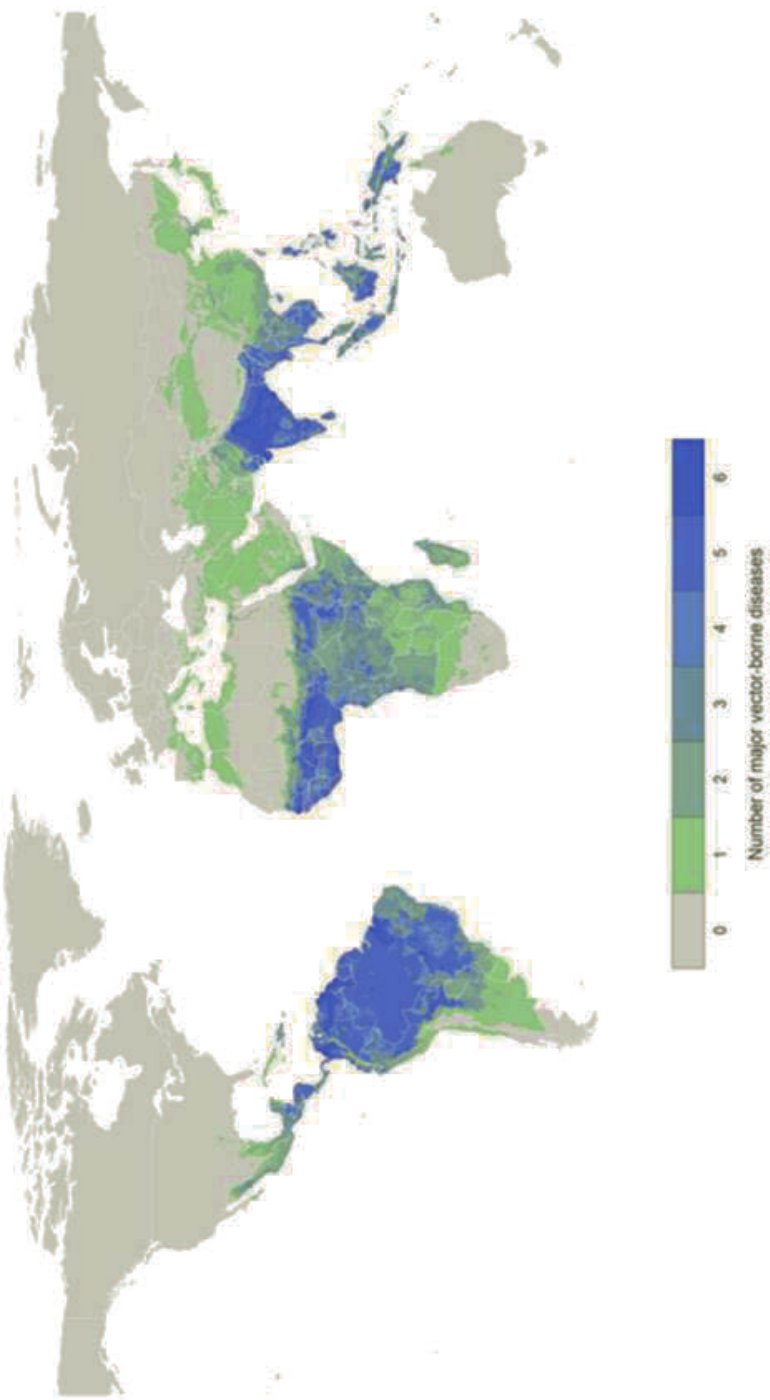


Figure 1. Combined global distribution of seven major vector-borne diseases for which integration of vector control programmes may be beneficial: malaria, lymphatic filariasis, leishmaniasis, dengue, Japanese encephalitis, yellow fever, and Chagas' disease. Colours indicate the number of vector-borne diseases that pose a risk at each 5 x 5 km grid cell. (Map from Golding et al. 2015, reproduced with permission.)

The cost of fighting dengue in 18 countries at purchasing power parity (PPP) was calculated at about USD 3.3 thousand million in 2015; using standardized PPP costs permits comparing socio-economic impacts in the different countries (Oliveira et al. 2019).

The economic burden of vector-control costs, in an attempt to reduce or prevent cases of disease, needs to be factored into estimates of the cost of disease to a country or region. To the USD 102.2 million direct cost of treating the almost 40 000 cases of dengue reported annually in Malaysia from 2007 to 2012, another USD 73.5 million (0.03% of Gross Domestic Product (GDP)) need to be added for the National Dengue Vector Control Programme, mostly for adulticidal fogging, increasing the cost by 72% (Raviwharmman Packierisamy et al. 2015).

Vector-control costs can apply even where a disease is not yet a problem or has previously been brought under control, e.g. countries (such as Mauritius) which are at risk from importation of arboviruses due to their high inflow of international visitors (Beesoon et al. 2008; Ramchurn et al. 2009) or those countries at risk of invasion by *Aedes* vectors. A balance must be made between potential disease costs and preventive vector control efforts. As the Zika virus was spreading through the Americas, the US president requested USD 1.8 billion from Congress in February 2016 to combat Zika in Costa Rica and Brazil, expenditure which was found to be justified in a study by Alfaro-Murillo et al. (2016). The judgement was based on estimated probabilities of microcephaly in babies born to infected mothers and direct medical costs, which both vary by country, and the loss of Disability-Adjusted Life Years (DALYs) per case of microcephaly (29.95 DALYs) and of Guillain-Barré syndrome (1.25 DALYs).

The wider benefits of disease reduction or eradication may be significant. A study conducted in Ghana into the economic-burden impact of malaria mentions that a 1% increase in malaria morbidity reduces economic growth by 0.41% (Asante and Asenso-Okyere 2003). Due to malaria, each business in the nation lost on average about a month's productivity per year; this corresponds to a drastic decrease in average income. A dynamic macrosimulation model to estimate the effects of eradicating malaria shows that higher economic development can be achieved in the long-term (within 30 to 50 years) from malaria eradication (Ashraf et al. 2009).

While the burden of agricultural pest insects is usually felt by individual growers and sector cooperatives, the burden of vector-borne diseases is felt by the whole population; therefore, the management of such diseases is commonly the responsibility of local organizations (including non-governmental mosquito control districts (Foley IV et al. 2021)) or supplemented by governments as part of their social policy, where it is a constitutional requirement to provide health care. Tang et al. (2004) developed a framework outlining for the USA the ten coordination and regulatory roles that government may play in health-care quality (which could be applied in any country):

- (1) purchase health care, (2) provide health care, (3) ensure access to quality care for vulnerable populations, (4) regulate health care markets, (5) support acquisition of new knowledge, (6) develop and evaluate health technologies and practices, (7) monitor health care quality, (8) inform health care decision-makers, (9) develop the health care workforce, and (10) convene stakeholders from across the health care system.

Each role proposed above would have an impact on the control of neglected diseases. In facing the continuous increase in the number of cases and disease expansion to new areas, item six highlights how critical it is to develop an effective contribution to reducing the number of transmitted cases over time -- by incorporating new strategies and control methods for vector-borne disease control (Araújo et al. 2015; Bourtzis et al. 2016).

Various countries have developed their own guidelines and protocols to deal with vector-borne diseases (based on guidelines developed by the WHO (WHO 2009, 2012)) in which they formulate evidence-based strategies and policies. For example, the Brazilian health ministry developed the National Dengue Control Plan (PNCD), operating since 2002. It comprises a great range of activities and instructions in an effort to reduce the number of dengue cases all over the country. This plan evolved after the first attempt to eradicate the mosquito in the 1970s -- aggregating and developing strategies based on different approaches including epidemiological and entomological surveillance, frequent house inspections, and insecticide application (Braga and Valle 2007). Until now, the PNCD's objectives have not been fully achieved, and the fact that dengue epidemics can still be expected every year illustrates the need for a full review and evaluation of the strategies and control methods applied. There are local problems related to achieving full implementation of the plan (a result of insufficient support from stakeholders), in addition to growing resistance to the commonly used temephos larvicide and deltamethrin adulticide (Valle et al. 2019), and the general challenge of eradicating populations of container-breeding *Aedes* vectors. Brazil alone always represents more than 95% of the number of dengue cases in Latin America (Pessanha et al. 2009; Salles et al. 2018). Until effective universal vaccines, and safe, effective, and inexpensive drugs, are developed and become available, control of the mosquito vectors of disease is likely to be the most effective method of reducing cases of disease and controlling their spread.

1.2. Need for Better Vector Control

Currently most mosquito control strategies rely primarily on the use of insecticides, but with the increasing spread and significance of resistance in vectors of malaria (Ranson and Lissenden 2016) and arboviruses (Ranson et al. 2010; Moyes et al. 2017) there is a need for sustainable tools that enhance the arsenal against key vectors, particularly in the face of public concern about the human health and environmental impact of widespread insecticide use. Although great gains have been made in reducing the burden of malaria (e.g. there were 20 million fewer malaria cases in 2017 than in 2010 (WHO 2018)), these gains have been achieved through applying artemisinin combination therapies (ACTs) and especially insecticide-treated bednets (ITNs). The 2018 World Malaria Report suggested that these gains are being reversed; no significant progress was made in reducing cases between 2015 and 2017 -- due in large part to drug and insecticide resistance becoming increasingly established and widespread (WHO 2018).

In its global vector control response 2017–2030, the WHO pointed out the urgent need for the development and integration of innovative mosquito control methods, including the SIT, particularly against *Aedes* vectors (WHO 2017a). A major

advantage of suppressing a mosquito vector population with such an integrated approach is that it can address several diseases at once; different diseases, such as arboviruses, are often transmitted by the same *Aedes* vectors, whereas other approaches, e.g. vaccination, need to be developed for each new emerging disease.

The behavioural ecology of anthropophilic mosquitoes, and in particular their use of disseminated microhabitats as oviposition sites, challenges the integrated control of these insects in many countries and climatic conditions, and prohibits a satisfactory level of population reduction (Reiter 2016). Moreover, the application of many insecticides is more and more restricted worldwide; this reduces the available vector control options, particularly in the face of spreading resistance against all but the newest classes of insecticides in vectors of both malaria (Sokhna et al. 2013) and arboviruses (Ranson et al. 2010; Grigoraki et al. 2017; Pichler et al. 2018). In addition, insecticide-treated bednets are not effective in combating *Aedes* vectors (which are active during the day).

Therefore, new techniques to control mosquitoes are under development and being evaluated in the field, including genetic control strategies targeting the reproductive capacity of disease-transmitting mosquitoes (McGraw and O'Neill 2013; Lees et al. 2015; Bourtzis et al. 2016; Flores and O'Neill 2018). Amongst these, the SIT and the incompatible insect technique (IIT) show great promise (Oliva et al. 2014).

1.3. Integration of the SIT Offers the Potential for Sustainable Mosquito Suppression

Pilot trials against mosquitoes started in about 1960 in the USA, with the goal of assessing the potential of the SIT to reduce populations of *Ae. aegypti* and *Anopheles quadrimaculatus* Say, but in both cases there was no evident population suppression in the target areas after 43–48 weeks of releases of sterile males (irradiated with gamma rays) (Morlan et al. 1962; Weidhaas et al. 1962). Many other pilot projects took place with various mosquito species in various countries using different rearing protocols, irradiation sources, and methods of sterilization (chemosterilization, translocations, inversions, and cytoplasmic incompatibility (CI)) (Benedict and Robinson 2003; Dame et al. 2009; Klassen et al., this volume). Some were able to demonstrate suppression and even elimination/eradication of the target population, e.g. elimination of *Anopheles albimanus* Wiedemann in El Salvador (Breeland et al. 1974; Dame et al. 1974).

Interest in applying the SIT against mosquitoes had waned since these initial trials in the 1960s and 1970s. Rather than technical failure, this was due largely to political instability affecting their implementation and insufficient practical governmental support (Klassen et al., this volume). Moreover, in view of the availability of new insecticides, mosquito control has relied heavily on the use of a limited number of insecticides. Recently, there has been a resurgence of interest due to: (1) increased pressure exerted by emerging arboviruses and the spread of resistant malaria, (2) the loss of current methods of control because of malaria resistance to drugs and resistance to insecticides in both *Anopheles* and *Aedes*, and (3) the availability of molecular techniques that have enabled the development of improved strains for the SIT (Bourtzis and Hendrichs 2014; Bourtzis and Tu 2018; FAO/IAEA 2018c; Lutrat et al. 2019) and made alternative versions of genetic control possible (Alphey 2014).

Releasing insects sterilized by ionizing radiation has been a very important method for the area-wide suppression, containment, and even eradication of major insect pest populations (Klassen et al., this volume). Given the experiences obtained in those programmes applying the SIT, there is reason to believe that it could also be applied -- in combination with other control methods as part of an area-wide integrated pest management (AW-IPM) approach -- to suppress disease vectors below the threshold required for disease transmission. Building on previous experiences in the 1960s and 1970s to develop the SIT for disease vectors, many parameters have been investigated to better understand, enable and optimize the application of the SIT against mosquitoes, including mass-rearing procedures, sterilization methods, transport and release methods, and trapping systems (Pepin et al. 2013; Puggioli et al. 2013; Balestrino et al. 2014a, b; Carvalho et al. 2014; Codeço et al. 2015; Lees et al. 2015; Eiras et al. 2018; Bakri et al., this volume; Dowell et al., this volume; Parker, Mamai et al., this volume; Parker, Vreysen et al., this volume; Vreysen, this volume). Part of the development of the SIT against mosquitoes has also been based on the experiences of successful programmes against other insects, e.g. those against *Ceratitis capitata* (Wiedemann) in the USA, Guatemala, Mexico, and Chile, and *Cochliomyia hominivorax* (Coquerel) in Central and North America, as well as Libya (Enkerlin, this volume; Klassen et al., this volume; Vargas-Terán et al., this volume). However, more research and testing need to be done before “mosquito SIT” reaches the same level of development as these programmes (Krafsur 1998; Vargas et al. 2008; Enkerlin et al. 2015).

Several features of mosquito biology make mosquitoes suitable targets for the SIT (Beier et al. 2014). Most of the key mosquito disease vectors can be colonized and reared in the laboratory, adapting to feeding on artificial larval diets and taking blood meals through artificial membranes. Therefore, the production of sufficient numbers to achieve an overflooding release ratio is feasible, even if reducing natural population densities using other complementary suppression techniques will often be necessary against mosquitoes, particularly the reduction of larval populations and habitats (Mangan and Bouyer, this volume).

Sexual dimorphism, particularly in *Aedes* species, can be exploited to help remove females prior to sterilization and release of males, which is an essential requirement for applying the SIT against mosquitoes -- females are the disease vectors, and releasing even small numbers that may transmit disease is problematic for the public and regulators, even if not for technical efficacy.

In comparison with fruit and tsetse flies, mosquitoes are smaller and lighter, making the challenges of handling, transport, and release more tractable (especially to take advantage of new technologies such as automated release from unmanned aerial vehicles (UAVs) (Dowell et al., this volume)). However, their small size also confers some fragility; this must be taken into account to prevent a reduction in survival or quality of released males. Finally, female mosquitoes are mostly refractory to remating -- not an absolute requirement of the SIT but beneficial in reducing the numbers for, and frequency of, release required to induce sterility in the female population (Lance and McInnis, this volume; Whitten and Mahon, this volume).

The container-breeding species, such as *Ae. aegypti* and *Ae. albopictus*, are particularly challenging to control because their life cycle relies on oviposition in

small, and often temporary, sources of water. To mitigate the risks of a whole egg batch being lost due to a small body of water drying out before the offspring can emerge and escape, and also predation or competition for space or nutrition, an Aedine female will lay her egg batch, up to 100 eggs per gonotrophic cycle, in multiple sites. Many of those sites (such as discarded drink cans or tree holes) are very small and difficult to target with chemical control, and many are difficult to remove or treat (such as water butts used to collect rain water in areas of unreliable piped-water supply or buckets in fishing communities), and may be located in urban sites which are not amenable for control, e.g. abandoned lots or balconies of apartments in high-rise buildings. This cryptic behaviour also means that females often rest in locations which are not reached by insecticide applications (Dzul-Manzanilla et al. 2017). The advantage of sterile males is that they will locate mates, which then will lay non-viable eggs, and negate the need to find and treat these hard-to-reach oviposition sites. Increasing global trade and urbanization, as well as reliance on disposable containers without sufficient waste disposal infrastructure in many areas affected by mosquito-borne disease, help explain the rapid growth in distribution of *Aedes* vectors. In this context, the reliance on the dispersal of sterile males rather than human operators is an important advantage of control efforts that integrate mosquito release over other methods; a similar advantage applies to autodissemination stations for juvenile hormones.

In the case of *Anopheles* females, these are targeted by conventional vector-control methods while they are trying to take a blood meal through an insecticide-treated bednet or resting on a wall treated with indoor residual spray (IRS), but those that remain in untreated houses or communities will be unaffected. Moreover, some exophilous species (such as *Anopheles arabiensis* Patton) that bite mainly outside will not be impacted by these methods. One major benefit of SIT application against *Anopheles* mosquitoes, in particular *An. arabiensis* which is the sole vector of malaria in most of its area of distribution, is that human operators do not need to find and treat these sites or target every mosquito, but instead male mosquitoes are released to seek out and sterilize females, whose eggs (wherever she lays them) will then be sterile.

2. DEVELOPMENT OF THE SIT TECHNOLOGY AGAINST MOSQUITOES

Increasing interest in applying the SIT, and other control methods relying on the large-scale rearing and release of insects, has led to a rapid improvement in the available technology and methodology to mass-rear, sterilize, assess the quality of, and release and conduct surveillance on, *Aedes* and *Anopheles* mosquitoes (Benedict et al. 2009a; Lees et al. 2015). A significant requirement of applying the SIT against mosquito vectors of disease is the need for male-only release, not only to maximize the efficacy of releases and the efficiency of rearing efforts, as in other insects (Franz et al., this volume; Häcker et al., this volume), but also for the public perception and regulatory challenges surrounding the release of even a small number of potentially disease-transmitting females. Pilot trials of the SIT and associated techniques have been conducted or initiated in recent years in a number of settings, against both genera of mosquitoes, and evidence of the potential for the SIT to suppress mosquito populations is being produced.

2.1. Rearing and Sterilizing Males

2.1.1. Colonization and Mass-Rearing

The need for effective colonization and rearing to maintain essential qualities in mosquitoes released for vector control have been reviewed by Benedict et al. (2009b). Colonization and establishment of a new mosquito colony is a painstaking process (FAO/IAEA 2017a, 2018b). Blood-fed females, or immature developmental stages, are collected from a field site where the species of interest is likely to dominate, and individual families are reared while morphological and/or molecular analysis is used to confirm the species. Conspecific families can then be pooled to establish a colony which is often small, and must go through a bottleneck as it becomes adapted to artificial rearing conditions, particularly feeding from an artificial membrane instead of a natural host. Once a colony is established and stabilized, laboratory-adapted mosquitoes are relatively amenable to large-scale rearing, though for a release programme to be efficient each element of the rearing process must be optimized (Parker, Mamai et al., this volume).

Key to affordable production of high-quality adults is the selection of a larval diet which provides all the nutrients needed by developing larvae to grow and establish the nutritional reserves they will require as adults for foraging and mating. Ideally, these should consist of locally available ingredients, be reliably available and of a consistent quality, and even if inexpensive yet still effective in producing high-quality adults (Khan et al. 2013 give *Anopheles stephensi* Liston as an example). A well-proven diet (consisting of tuna meal, bovine-liver powder, and vitamin mix) is effective for rearing *An. arabiensis* (Damiens et al. 2012), *Ae. albopictus* (Puggioli et al. 2013), and *Anopheles gambiae* Giles (Yahouédo et al. 2014). The addition of Brewer's yeast increases the protein content; it is particularly helpful for improving sexual dimorphism in *Aedes* rearing (Balestrino et al. 2014a). Given the cost and difficulty in obtaining bovine-liver powder, alternative diets have been validated (e.g. Bimbilé Somda et al. 2017), and other cheaper proteins, e.g. insect proteins, are also being developed (Bimbilé Somda et al. 2019).

Since they have an aquatic larval stage, rearing mosquitoes is more labour-intensive than other insect species targeted by the SIT. In addition, the pupal stage, lasting only 24–48 h, must be collected and transferred to a cage before adult emergence. Therefore, some level of automation and large-scale equipment for larvae and adults are required to prevent labour costs from becoming prohibitive. A tray-and-rack system (consisting of 50 trays stacked in one rack for easy filling using piped water, seeded with first-instar larvae, reared to pupation, then tilted to recover pupae for transfer to adult cages) has been validated to rear up to 200 000 *An. arabiensis* larvae (Balestrino et al. 2012) and about 900 000 *Ae. albopictus* larvae (Balestrino et al. 2014b). Accurate quantification of eggs or larvae used to seed a rearing tray is critical because density-dependent competition acts at the larval stage (affecting the speed and synchronicity of development, the size and nutritional status, and hence the performance of the resulting adults). Trays can be filled in a standardized way using a larval counter (Mamai et al. 2019). Once the trays have been tilted, *Anopheles* pupae can be separated from remaining larvae (on the basis of their different buoyancy) using a cold-water vortex system (Balestrino et al. 2011), and *Aedes* pupae are

separated using the Fay-Morlan separator (Focks 1980). Pupae are quantified volumetrically, and then the required number is transferred to adult emergence cages. In China and Singapore, ongoing programmes recover 70–80% of male pupae in only one tilting event, and sex separation is very efficient (only 0.3% female contamination). An automatic sex sorter based on a robotized Fay-Morlan separator has been developed by the Wolbaki company, with the same efficiency and a throughput of up to 150 000 pupae per hour (Xi, Z., personal communication).

Adult cages, inspired by those used for mass-rearing fruit flies, have been validated for up to 25 000 *Ae. albopictus* (Balestrino et al. 2014a; FAO/IAEA 2017b) or 15 000 *An. arabiensis* (FAO/IAEA 2017a) pupae. Adults must be blood-fed so that females can develop eggs. Adults are blood-fed using a Hemotek membrane feeder modified for mass-rearing (Damiens et al. 2013). *Anopheles* eggs are collected in water on the bottom of the oviposition cage which is then flushed out (Maïga et al. 2016); *Aedes* eggs are laid on wet filter paper which can then be removed and dried for storage (Zheng et al. 2015). Eggs can be quantified at this stage (Zheng et al. 2015; Maïga et al. 2016) before they are stored (*Aedes* only) or hatched. Mass-rearing using this system does not appear to impact male quality significantly (Soma et al. 2017) as judged in a laboratory setting. A new cheaper mass-rearing cage for *Aedes* has also been validated recently; it costs 90% less than the former IAEA reference cage and can be produced locally in any country (Maïga et al. 2019). Alternative designs for mass-rearing cages are also proving to be effective (Zhang et al. 2018).

It is essential to separate males (destined for release) from females (potential disease vectors). A perfect method for sexing mosquitoes on a mass-scale has not yet been developed (Gilles et al. 2014), although promising methods are being evaluated (section 4.1.). Nevertheless, currently *Aedes* can be separated on the basis of sexual dimorphism (differential speed of development and pupal size) (Fay and Morlan 1959; Focks 1980). In addition, the mass-removal of female *Anopheles* can be achieved by spiking blood meals with toxicants such as ivermectin (Yamada et al. 2013).

2.1.2. Irradiation

There is a perception by some that mosquitoes are more susceptible to somatic damage caused by irradiation (with the resulting reduction in performance) than other species targeted by the SIT (Bakri et al., this volume). There are reports from historic mosquito SIT projects that failures were due to poor male performance (Dame et al. 2009). Nevertheless, it is possible to select a suitable dose to induce sufficient sterility without inflicting unacceptable somatic damage, although rearing is key to ensuring a consistent high-quality output of sterile males for release (Parker, Vreysen et al., this volume). A key observation during historic mosquito SIT releases (Darrow 1968) -- that field-collected *Culex tarsalis* Coquillett males subsequently irradiated as pupae were competitive following re-release, unlike mass-reared and irradiated males -- points to the mass-rearing and handling process, and not irradiation, as the more important factor impacting male-mating success.

At the present time, sterile males for release are obtained by irradiating pupae -- they are more easily handled and less damaged by radiation than adults (Helinski et al. 2006, 2009). However, strong density-dependent efficiency related to water content and anoxia suggests a method for irradiating chilled compacted adults (that is

presently under development) will provide better results (H. Yamada, personal communication). On the other hand, pupae can be irradiated at a high density in a small volume of water, minimizing the volume to be irradiated and thereby increasing the uniformity of dose. Protocols and dose-response relationships have been established (to achieve an optimal balance between a high level of sterility and a low impact on male performance) for *Ae. albopictus* (Balestrino et al. 2010), *An. stephensi* Liston (Akram and Aslamkhan 1975), *Anopheles pharoensis* Theobald (Wakid et al. 1976), and *An. arabiensis* (Helinski et al. 2006). Even though gamma irradiators are commonly available, X-ray irradiation offers a more convenient and less costly alternative, with lower security requirements (Ndo et al. 2014; Yamada et al. 2014; Bakri et al., this volume).

2.1.3. *Quality Management and Monitoring*

Quality management and monitoring of sterile males are critical for the SIT -- to ensure good mating performance and that released males are competitive (Parker, Vreysen et al., this volume; Vreysen, this volume). Quality-control monitoring of rearing conditions is also essential to assure sexual dimorphism in *Aedes*, synchronous pupal production, consistent productivity and quality, and a reliable number of adults for release (Parker, Mamai et al., this volume; Parker, Vreysen et al., this volume). Regular measurement of important life history traits and mating competitiveness is critical in a colony reared for release. Rearing should be as standardized as possible to ensure consistent quality of material for release, and predictable synchronized development. In *Aedes* colonies it is important to maximize sexual dimorphism (key to sex-separation). Parameters such as water temperature and larval density must be optimized to consistently produce high-quality adults (Mamai et al. 2018).

A useful method for assessing if rearing conditions are optimal for a given species in a given setting was described by Valerio et al. (2016). To quantify and help minimize the impact of mass-rearing, irradiation, and handling on sterile male mosquitoes, several methods to judge the consistency, quality, and predicted performance following release have been suggested. The relative impact of different handling treatments, and combinations of different parameters, could be estimated by comparing the longevity of exposed males (Chung et al. 2018; Culbert et al. 2018b). Longevity is an important parameter of released males, also sometimes used as a proxy measure of quality.

Balestrino et al. (2017) proposed for *Aedes* pupae a system of quality control based on flight performance (an improvement on the conventional flight cylinder), and a further development has been designed (Fig. 2), to measure the flight ability of adult mosquitoes; it may be the most practical and robust of the tests for proxies of success. This parameter has been validated as a predictor of survival (a product of being able to forage for food and evade predators) and mating capacity in *Ae. aegypti* (Culbert et al. 2018a). However, it may be necessary to assess more than one quality indicator to fully understand the impact of colonization, mass-rearing, and manipulations on the overall quality of sterile males (Poda et al. 2018).

Semi-field experiments have demonstrated the ability of sterile male mosquitoes to attract and compete for mates (e.g. Howell and Knols 2009; Bellini et al. 2013a; Madakacherry et al. 2014; Yamada et al. 2014). *An. arabiensis* adults transported by

air survived and competed for matings to an acceptable degree in field cages near the intended release site in Sudan (Helinski et al. 2008). Although it is difficult to judge the effect of irradiation and other manipulations in an open field setting, this is a valuable step in evaluation. Field observations of sterile *An. arabiensis* males participating in swarms within 2 h of release (Ageep et al. 2014) is a very encouraging measure of the potential success of applying the SIT to control these mosquitoes.

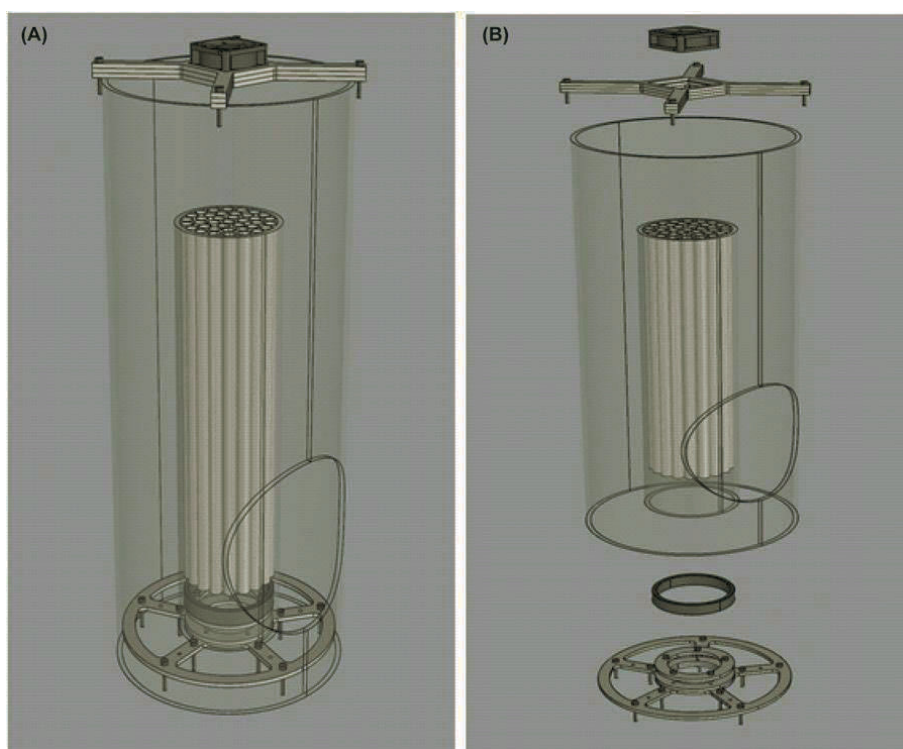


Figure 2. A: Flight test device; B: Device showing components. (Drawings from Culbert et al. 2018a, reproduced with permission.)

2.2. Release and Monitoring

Until now the pilot releases of sterile male mosquitoes have been small enough to permit manual release from small plastic containers. Male pupae emerge directly into these containers; they are transported to the field and opened by hand at set ground release points to release the males. This method of release has been used in all of the SIT field pilots until now, as well as in the release of 10 000 transgenic *Ae. aegypti* males per week in Juazeiro, Bahia, Brazil as part of a RIDL (Release of Insects carrying a Dominant Lethal) suppression trial (Carvalho et al. 2015; Häcker et al., this volume). However, the large-scale releases required for operational application of the SIT will necessitate more sophisticated methods of handling, transport, and release,

i.e. methods that are less labour-intensive and have the capacity to be standardized between sites. The most likely scenario is that adult males will be chilled, loaded into a release device with some level of compaction, and released either aerially or from a motorized vehicle (FAO/IAEA 2014; Chung et al. 2018). In some situations, existing infrastructure may be used, e.g. designing a release device and strategy that utilizes the coverage of large cities by bus routes, or shipping sterile mosquitoes from rearing facility to release site by commercial courier (Mains et al. 2019). Release by UAVs will probably be used to reach areas that are not reliably accessible by road, such as dense favelas or isolated rural hotspots, and importantly to increase the speed of release and uniformity of coverage while reducing costs (Benavente-Sánchez et al. 2021; Dowell et al., this volume).

It is important that release procedures do not unduly damage the males or impact their subsequent performance and survival. Laboratory studies show that mosquitoes are damaged to some degree by chilling, packing, compaction, and release into simulated field conditions (Chung et al. 2018; Culbert et al. 2018a, b). However, some degree of chilling improves survival during compaction (Chung et al. 2018), and parameters such as the chilling temperature can be optimized to minimize the impact. Also, conditions can be optimized by ensuring sufficient ventilation during chilling and transport. For example, the best chilling-temperature range for the transport of compacted *An. arabiensis* adults is 8–11°C (Culbert et al. 2017). Aedine mosquitoes appear to be more robust than Anophelines, and the time of release is likely to be more critical in the latter case.

For an SIT project to be effective, sufficient sterile males must be released in relation to the target population. In an efficient programme, males should be directed to the target area at a fine scale; this requires an understanding of the behaviour, size and spatial distribution of the population before intervention (Lees et al. 2014). Such data are also crucial in monitoring the progress, and demonstrating the success, of the project as it progresses (Vreysen, this volume). During the planning and development phase of an SIT project, it is important to trap male mosquitoes (during mark-release-recapture experiments) to make assessments about the survival of released males, distance dispersed, and level of mating competitiveness. Ongoing monitoring of the fate of released males, and the size of the target population, is necessary as an SIT programme progresses. The benefits of detailed population data are increased by making releases assisted by automated geographic information systems (GIS) (Bouyer et al., this volume). Software is available which allows releases to be tailored according to real-time surveillance data for maximum efficacy.

Since female mosquitoes are the vectors of disease, most existing traps have been designed to collect females for surveillance, and in some cases for control. It is necessary to produce male-specific tools, or to adapt the timing, location or baiting of traps to target males specifically. For *Aedes* surveillance, males and females can be collected indoors using aspirators. The BG-Sentinel trap is effective for collecting males, but most of the effective traps attract only gravid females. Thus, there is an especially urgent need for effective methods to survey Anopheline mosquitoes (Batista et al. 2019; van de Straat et al. 2019). The CDC light trap often catches few *Anopheles* mosquitoes, and other tools such as the Suna trap, sticky resting boxes, baited traps, and human-landing catches require better standardization. Swarm capture

is a powerful approach but requires great expertise, and this technique is not possible in all locations (Bimbilé Somda et al. 2018). Better understanding of the cues which attract male mosquitoes is leading to traps with improved efficiency, e.g. the Sound-GAT (Johnson et al. 2018). In Singapore, mosquito surveillance is achieved using a network of more than 30 000 GAT traps monitored weekly (N. Lee Ching, personal communication). Emerging technology for automated passive mosquito surveillance shows great promise; in theory, a network of intelligent traps could automatically identify in real time the species, sex, size, and marker status of insects entering or passing close by (FAO/IAEA 2019a).

3. PILOT TRIALS AGAINST MOSQUITO VECTORS

In this new era of interest in applying the SIT against mosquito vectors, until now the pilot trials have been on a small scale, usually focussed on isolated villages or islands. It is perhaps no coincidence that the first projects to reach pilot-scale demonstrations of the SIT are to control *Aedine* mosquitoes, given their relative amenability to mass-rearing and sex separation. These small-scale trials are sufficient to demonstrate an impact on some entomological indicators, usually the number of eggs collected per ovitrap, egg-hatch rate, and adult-catch rate. These indicators suggest success, but for the technique to be taken up by abatement districts, governments or charities for implementation on an operational scale, evidence of epidemiological impact of the SIT (reduction of disease incidence) as part of integrated management schemes will be required, involving much larger area-wide trials. Transmission of disease by mosquitoes occurs not just in the home, but also in schools and workplaces, and therefore, unless a community is very isolated with little human movement outside a given area, it will be a challenge to demonstrate disruption of transmission or case reduction. Moreover, in some circumstances, female *Aedes* appear to disperse much farther than males (up to 800 m) (Honório et al. 2003); this will make demonstration of efficacy by current randomized cluster trials very challenging. Current equipment and techniques, in particular sex separation and release methods, will need to be improved dramatically, particularly against Anophelines, before such large-scale trials are possible. Through small-scale pilot trials, many lessons are being learned, which will inform the development of larger projects, e.g. the significance of *Ae. albopictus* immigration from neighbouring vegetation into urban or semi-urban areas targeted with the SIT, and the requirement for reduction of target populations using conventional methods or IVM prior to, and in conjunction with, applying the SIT.

3.1. *Aedes albopictus* in Italy

Feasibility studies on using the SIT to target invasive *Ae. albopictus* mosquito populations were started in northern Italy in 2000 (Bellini et al. 2007). The studies included several releases of irradiated males; promising levels of induced sterility were achieved. Where release ratios were high enough, population reduction occurred (Bellini et al. 2013b).

Between 2008 and 2012, six pilot studies of sterile *Ae. albopictus* male releases were made to test the efficacy of the SIT approach to suppress mosquito populations.

The release sites were selected as being representative of urban conditions, small enough (10–17 ha) to achieve the required release ratio given the level of sterile-male output that was possible in the Bologna mosquito production unit, and well-isolated from other urban areas. The dynamics of the mosquito populations were monitored weekly in the release and control areas, using standard ovitraps. Eggs collected from the traps were counted under a stereomicroscope and hatched using standard procedures to assess fertility and, conversely, induced sterility (Bellini et al. 2007). Sterile males, released at the rate of 900–1500 males/ha/week, induced sterility levels between 15 and 70% of the background fertility of the local population. Where induced sterility reached 70%, a similar reduction was found in egg numbers collected by ovitraps. Therefore, inducing sterility levels of >80% in the native *Ae. albopictus* females for an entire season was expected to be sufficient to suppress effectively the mosquito population.

A recent meta-analysis of these trials (R. Bellini, personal communication) found that the released males demonstrated a mean competitiveness value of 0.188 (SD±0.33). This competitiveness (measured as the Capacity to Induce Sterility or CIS index) was highly variable between pilot trials despite very similar environmental conditions being experienced among the sites and similar sterile-male release methods being used in all studies. A strong temporal variability was observed, with lower values found at the beginning and at the end of the summer season (when the wild population density is usually lower and therefore the male sterile:wild ratio is higher) (Albieri et al. 2010; Carrieri et al. 2011). The strong negative correlation between the sterile:wild ratios and the competitiveness values demonstrated in this study was also observed in previous trials conducted under semi-field and field conditions using irradiated and transgenic sterile males (Harris et al. 2011; Damiens et al. 2016). In practical terms, an optimal ratio must be used in SIT operations to maximize cost-effectiveness; increasing the release ratio will not result in a proportional increase in induced sterility.

3.2. *Aedes albopictus* in Mauritius

As part of its Operational Plan for Prevention and Control of Chikungunya and Dengue, the Ministry of Health and Quality of Life in Mauritius is evaluating integrating the SIT to control *Ae. albopictus* populations; the objectives are to prevent outbreaks and the re-establishment of arboviruses in an island benefitting from large numbers of international visitors (Beesoon et al. 2008; Ramchurn et al. 2009). Several characteristics of Mauritius make it a very suitable country in which to test the feasibility of applying the SIT. Identifying potentially suitable sites for pilot-trial releases, and corresponding control areas, is straightforward due to the largely agricultural nature of the island of Mauritius. Small discrete villages exist, often located on the coast or surrounded by sugar cane fields providing geographical isolation. In some villages *Ae. albopictus* is the only Aedine mosquito present or at least the dominant species. Two suitable villages were selected for the first pilot trial: Pointe des Lascars (0.3 km², consisting of 203 houses and 800 inhabitants) and Panchvati (0.03 km², consisting of 67 houses and 270 inhabitants) (Iyaloo et al. 2014). The villages, located 1.6 km from each other, are well-matched in terms of the human

inhabitants, natural geography, infrastructure, and mosquito populations. Routine monitoring and mark-release-recapture experiments showed that the majority of ovitraps are positive for eggs throughout the year, with peaks in December-March and troughs in July-September (Iyaloo et al. 2019).

To determine survival and dispersal of sterile males in this ecological setting, three mark-release-recapture (MRR) experiments (Itô et al., this volume; Vreysen, this volume) were performed in Pointe des Lascars (Iyaloo et al. 2019). A release rate of 6000 males/ha was applied during the winter season, and at least twice that number in the summer. When assessed in a laboratory, marking males (with a fluorescent dust) did not affect their performance (Dowell et al., this volume). After applying a dose of 40 Gy with a gamma irradiator, tests showed that: (1) dispersal was not affected by irradiation, and (2) irradiated males survived up to 12 days; on average, unirradiated males survived 4 days longer (Bakri et al., this volume).

In parallel with baseline mosquito surveillance (using ovitraps and BG-Sentinel traps), a colony of mosquitoes from the pilot villages was established in a climate-controlled insectary at the Vector Biology and Control Division. For larval diets, two animal feeds were shown to be suitable and cost-effective (USD 1/kg); they are manufactured locally (important for an island nation) (Iyaloo and Facknath 2017). Mosquitoes were reared using the tray and rack system described by Balestrino et al. (2014b), and large commercially available adult cages. Sex separation of release cohorts, done on the basis of pupal size (using graded sieves), achieved a maximum of 4% female contamination. Adult females were further removed from the release cages with an aspirator. Batches of 2000 male pupae were irradiated at 30–40 hours old, as was done for the MRR trials, and allowed to emerge into small adult cages containing a sugar meal. Adults were transported to the field in the small cages for release 3 days after emergence.

Prior to the pilot trial, staff of the Ministry of Health and Quality of Life went from house to house (in the release and control sites) inviting people to attend sensitization meetings, held in the village hall, during which the project was explained, and also a cage of male mosquitoes was used to demonstrate that they do not bite. During the trial, field officers working in the pilot sites were mobilized to talk to the public at least once per week to hear and address any concerns (D. P. Iyaloo, personal communication).

In the first mosquito SIT trial in Mauritius, IVM was applied during the first two months of the project, applying larval-source reduction, weekly *Bacillus thuringiensis israelensis* (*Bti*) applications, and biweekly pyrethroid fogging alongside sterile-male releases (Mangan and Bouyer, this volume). Each week, for 9 months, 60 000 sterile males were released, equally distributed among 10 release sites in Panchvati for the first 6 months and 20 sites for the remainder of the trial period. Ovitrap surveillance within (and in a 150-m radius around) Panchvati and within Pointe des Lascars, and biweekly 24-h collections using 8 BG-Sentinel traps in both villages, showed a significant decrease in oviposition and the adult population as a result of the IVM treatments. This was reversed in the control village when vector treatments were halted, but not in the village where sterile-male releases alone continued. Egg fertility remained stable throughout the trial period in Pointe des Lascars, but was significantly lower in Panchvati during the period of releases (except for a period in the immediate

aftermath of tropical cyclone Berguita). During the period of sterile-male releases, induced sterility reached more than 30%, the number of eggs collected per ovitrap dropped by more than 50%, and adult catch was less than one half that in the control village (D. P. Ilyaloo, personal communication). Ovitrap in the area surrounding Panchvati tended to collect more eggs than within the village, suggesting that immigration of fertile females into the treatment area was acting against the population suppression efforts.

As in the Italian SIT pilot studies, releases were more effective the lower the initial population density at the target site; low densities enabled a higher release ratio given the rearing resources available, and possibly also a greater competitive advantage for the sterile insects. Permitting sterile males to mature, and take a sugar meal before release, improved mating competitiveness. In the ecological setting of Mauritius, initiating the targeted release of sterile males in the winter season is likely to be most effective in reducing the peak summer adult population, but sustained releases over several years would be required to sustainably reduce, or even locally eliminate, populations due to the stockpile of eggs of the wild population; these eggs are laid each season and hatch when the rains arrive.

3.3. Other Pilot Trials against Mosquitoes

Many other pilot trials are ongoing or have been completed in various countries, and many more are in the planning stages (Table 1), indicating the current high level of interest and ongoing activities to develop and integrate the SIT and/or IIT, as well as transgenic approaches, against disease-transmitting mosquitoes.

4. LOOKING TO THE FUTURE

The major elements required to apply the SIT against major mosquito vectors of disease are in place. The remaining challenges, that need to be overcome before it can be applied operationally on a large scale, are just technical improvements and upscaling, particularly in terms of sex separation, methods of releasing males, and accurate monitoring of male populations.

The technology for mass-rearing mosquitoes is well advanced (section 2.1.1.; Parker, Mamai et al., this volume): mass-production of larvae in racks of large trays (Balestrino et al. 2014a), separation of larvae from pupae (Balestrino et al. 2011), and housing and feeding of adults for egg production and storage (Balestrino et al. 2014b; Maïga et al. 2017). Methodological improvements are ongoing to increase efficiency and decrease costs (directly and through automation to reduce the labour required). For example, the reuse or recycling of larval-rearing water (Mamai et al. 2017) is a significant factor in the feasibility of mass-rearing mosquitoes in water-limited environments. The costs of mass-rearing equipment are being reduced (Maïga et al. 2019) and adult and larval diets improved (Bimbilé Somda et al. 2017, 2019). Currently, two issues are still being addressed (to enable mass-deployment) -- the need for a sex-sorting system (section 4.1.) that is efficient on a large scale (particularly for Anophelines), and the development of technologies to release mosquitoes aurally using UAVs (Dowell et al., this volume).

Table 1. SIT, IIT, and transgenic pilot trials against mosquitoes

Type of approach used	Mosquito species	Location	Trial status	Reference
SIT pilots ¹	<i>Aedes aegypti</i>	Mexico	Concluded	FAO/IAEA 2018c
		Italy, Mauritius	Concluded	Bellini 2013b; D. P. Iyaloo, personal communication
	<i>Aedes albopictus</i>	La Réunion (France), Germany, Greece, Italy, Montenegro, Spain	Ongoing	TIS 2019; R. Bellini, personal communication; I. Pla Mora, personal communication
SIT/IIT pilots	<i>Aedes aegypti</i>	Thailand	Concluded	Kittayapong et al. 2018; Kittayapong 2021
		Singapore	Ongoing	FAO/IAEA 2018c
	<i>Aedes albopictus</i>	China	Concluded	Jozuka 2016; Zheng et al. 2019; Baton et al. 2021
IIT pilots for suppression – releasing only <i>Wolbachia</i> -infected males	<i>Aedes aegypti</i>	Debug-Verily in Australia and USA	Not clear	Haridy 2017; Debug-Verily 2019
		Singapore (Project <i>Wolbachia</i>)	Ongoing	Co 2019; NEA 2019a, b; Liew et al. 2021
	<i>Aedes albopictus</i>	Florida (USA)	Concluded	Mains et al. 2016, 2019
		MosquitoMate in USA	Ongoing	MosquitoMate 2019
	<i>Aedes polynesiensis</i>	Raiatea (French Polynesia)	Concluded	O'Connor et al. 2012
		Tetiaroa (French Polynesia)	Concluded	Bown 2019; Strugarek et al. 2019
IIT pilots for population replacement -- releasing also <i>Wolbachia</i> -infected females	<i>Aedes aegypti</i>	World Mosquito Programme -- Australia, Brazil, Colombia, India, Indonesia, Mexico, Pacific Islands, Sri Lanka, Vietnam	Ongoing	Flores and O'Neill 2018; WMP 2019
Transgenic mosquito pilots	<i>Aedes aegypti</i>	Oxitec -- Brazil, Cayman Islands, Panama	Concluded	Harris et al. 2011; Carvalho et al. 2015; Gorman et al. 2015
		Oxitec -- Brazil, Panama, USA	Ongoing	Oxitec 2019

¹In addition, pilot SIT projects will begin in 2019 against *Ae. aegypti* in Brazil, Cuba, Indonesia, Malaysia, the Philippines, and the USA (Florida), and against *An. arabiensis* in South Africa.

The process of handling and transporting sterile males from a mass-rearing facility to release sites could be streamlined and standardized so as to minimize the impact on male survival and performance, and to enable upscaling the release technology. For example, handling could be minimized by loading sterilized male pupae into emergence cages; after emergence the adults can be fed a sugar meal and then chilled and concentrated into transport cassettes (with the emergence cages removed) which are loaded into ground- or aerial-release vehicles.

Aerial release of mosquitoes will enable the SIT to be applied in a wider range of circumstances, including situations where access by road is difficult or the labour costs of the fine-scale release of sterile males are prohibitive. A releasing system on a UAV was tested successfully in Brazil (FAO/IAEA 2016a, 2018a). The survival of aerially released *Ae. aegypti* males was not significantly different from that of males released on the ground, suggesting that the impact of the aerial release system on insect quality was minimal. Moreover, at a sterile:wild ratio of only 3:1, the induced egg sterility reached 50% in the release area; this is very promising (J. Bouyer, unpublished data). However, further fine-tuning of the technology and approach, and the likely involvement of the private sector, will be needed to produce reliable industrial versions of the release system, and enable it to be used, eventually also commercially, in operational vector-control programmes (Dowell et al., this volume).

The priority for the operational development of the SIT against mosquitoes is to upscale field trials, demonstrate impact on mosquito populations, and ultimately prevent cases of disease. The FAO/IAEA and WHO are presently collaborating to develop common operational guidance on applying the SIT to control mosquito-borne diseases, and to offer technical guidance to countries planning to integrate the SIT into their integrated management strategies. This guidance will cover all practical aspects relating to the trial and early application of the technology, e.g. production, transport, and release of sterile males, associated quality-control parameters, clearly defined entomological and epidemiological indicators of efficacy in large-scale entomological trials, and frameworks to assess risk, safety implications, and cost-effectiveness. Guidance will follow a phase-conditional approach, and will include strategies for community and media engagement, disease surveillance under large-scale deployment, monitoring, and evaluation of success, and a description of legal frameworks for registration and regulation related to operational SIT programmes (FAO/IAEA 2019b; Bouyer et al. 2020; WHO/IAEA 2020).

4.1. Need for Scalable and Effective Sex Separation

Although perfect sex separation may not be an absolute requirement for the efficacy of the SIT, in the case of mosquitoes (where females are the vectors of disease) the tolerance for even low-level female contamination in the release population is very low (section 2.1.1.). Options for improved sex-sorting that are currently available include using classical genetics to produce a genetic sexing strain (GSS), such as the dieldrin-sexing strain in *An. arabiensis* (Yamada et al. 2012). This system was very effective in sorting males (resistant to rearing solutions containing 2 to 4 ppm dieldrin, unlike females) but presented two drawbacks: a reduced egg fertility and thus low strain productivity, and the presence of dieldrin residues on the released males and the

associated risk of bioaccumulation in the environment (Yamada et al. 2015). Another option is to exploit the fact that only female mosquitoes feed on blood, i.e. add a toxicant to the blood meal (Yamada et al. 2013); though effective on a small scale, this may not be feasible for mass-rearing, particularly where a 100% male-release population is absolutely required.

Significant effort is being expended to develop new methods of sex separation (Papathanos et al. 2009, 2018; Bourtzis and Tu 2018). Sorting systems have been developed in *Aedes* species based on their sexual dimorphism, attempting to improve on the Fay-Morlan separator (Fay and Morlan 1959; Focks 1980) for larger-scale rearing. They enable mature pupae to be separated automatically on the basis of sex (Fig. 3) (Araújo et al. 2018; Bellini et al. 2018; Zacarés et al. 2018); at small-scale rearing up to 99% of males are recovered, and contamination by females is lower than 0.1%. An automated sieving system has been developed to separate *Ae. aegypti* pupae on the basis of smaller (male) pupae being able to pass up through openings in a submerged surface while larger (female) pupae are trapped below (Justia Patents 2017; Debug-Verily 2019). Another automatic pupae sex-separator, that can sort up to 150 000 pupae per hour with a female contamination rate below 0.3%, has been developed for *Ae. albopictus* by Wolbaki Biotech in China (Baton et al. 2021).

The size difference between *Anopheles* male and female pupae is less pronounced, and they are less robust and more easily damaged, making such sorting impossible (Mashatola et al. 2018). However, a similar system is under development to sort mature pupae of a sexing strain expressing sexual dimorphism in eye colour (K. Bourtzis, personal communication), which may be more amenable for *Anopheles* sex sorting, and the two systems may be combined to further improve the purity of the male-only release population. Nevertheless, these sexing systems are based on the sorting of pupae, making it necessary to produce female larvae; if females could be removed earlier in development, production costs could potentially be reduced by one half. Also, these methods are labour-intensive; it is very challenging to remove every female without losing a large proportion of males, further escalating the rearing costs.

Mechanical systems are unlikely ever to separate the sexes with 100% efficiency, and certainly not on a large scale; a few females will always be released. There is then a danger of “population replacement” when using only the IIT, but when the IIT and radiation-sterilization are combined, this cannot happen – any released females are sterile (and cannot transmit disease because of the *Wolbachia* infection) (section 4.2.).

A more efficient method of separation will be required before the SIT can be applied to mosquitoes on an operational scale, most likely relying on a GSS. A GSS in *An. albimanus* was produced in the 1970s for the SIT project in El Salvador but was lost after the trial was terminated (Dame et al. 2009). Producing GSSs by classical genetics is lengthy and labour-intensive because it relies on mutagenizing and screening large numbers of individual families (due to the low probability of mutagenesis producing the desired linkage event) (Lebon et al. 2018; Ndo et al. 2018). In *Aedes*, species sex is determined by a male-determining factor located on a small, non-recombining M locus on chromosome 1 and not on a heteromorphic sex chromosome (Hall et al. 2015), making it more unlikely and therefore more laborious to achieve translocation of the selectable marker to the sex-determining chromosome (Papathanos et al. 2018).

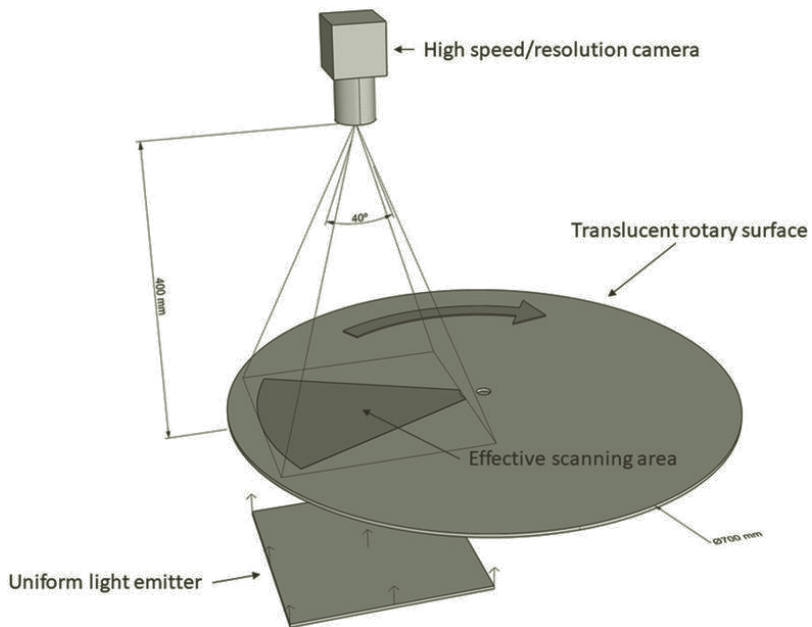


Figure 3. Upper drawing: Camera set-up for sorting male and female *Aedes* pupae based on size dimorphism. (Drawing from Zacarés et al. 2018, reproduced with permission.)
 Lower photo: Prototype laser sex-sorting machine (Bourtzis 2019).

However, a promising approach, using modern molecular techniques, is to identify *temperature-sensitive lethal* (*tsl*) genes, and to link them to sex-specific genes to enable the removal of females as first-instar larvae or even as embryos through classical genetics (as is done for *C. capitata* (Franz et al., this volume)). Modern molecular tools that can be used to produce transgenic as well as non-transgenic sexing strains have been widely reviewed (Catteruccia et al. 2009; Bernardini et al. 2018; Häcker and Schetelig 2018; Lutrat et al. 2019; Häcker et al., this volume).

An alternative approach could be to feed double-stranded RNAs (dsRNAs) to mosquito larvae (Whyard et al. 2015). By targeting both the testis genes and a female sex determination gene (*doublesex*) to induce RNA interference (RNAi), female development can be successfully inhibited (Häcker et al., this volume). To be effective, the dsRNA must be available constantly during relevant developmental stages to guarantee the silencing effect, though the cost of producing these dsRNAs may be reduced through the culture of transformed bacteria or yeast.

Flow cytometry machines (COPAS®, Union Biometrica) could be used for high-throughput separation of males from females based on the sex-specific expression of a fluorescence marker, e.g. sperm-specific expression in the testes (Marois et al. 2012). This approach is still being upscaled for mass-rearing, but relies on the use of transgenic strains, whose “open release” is limited or prohibited in some countries. Irradiating these transgenic strains to sterilize them before release would enable them to benefit from a non-GMO status in European countries, where only fertile material is considered as an organism. Under the Nagoya protocol (CBD 2014), even the release of sterile GMOs is tightly controlled, though their release is authorized in Europe (Lutrat et al. 2019). Using approaches such as CRISPR/Cas engineering, targeted mutagenesis through gene editing could be used to create GSSs which do not contain exogenous DNA and thus are not classed as transgenics, facilitating their acceptance for release (Kandul et al. 2019; Häcker et al., this volume).

4.2. Combined SIT/IIT Approach to Control *Aedes*

In parallel with the increased interest in applying the SIT against mosquito vectors of disease, there has also been much interest in developing and applying the incompatible insect technique (IIT), and exploring its potential through *Wolbachia*-based approaches to enhance the SIT (Zabalou et al. 2004; Moretti et al. 2018; Baton et al. 2021). The IIT relies on cytoplasmic incompatibility (CI) between released males (which carry a *Wolbachia* infection) and wild females (with no infection, or a different and incompatible infection), thus resulting in sterile matings. Moreover, introduced *Wolbachia* infection makes females refractory to arboviral transmission. Starting in 2011 in Australia, field trials releasing *Wolbachia*-infected *Ae. aegypti* females and males have been successful at population replacement (Hoffmann et al. 2011). However, such *self-sustaining* releases (Alphey 2014) have the intent to establish permanently the *Wolbachia* strain in the target population, a process (that unlike the SIT) is irreversible and leaves an “ecological footprint”. On the other hand, releasing only *Wolbachia*-infected males is *self-limiting*, and results in population suppression similar to the SIT, without any permanent changes in the target population (Bourtzis et

al. 2014, 2016); it is essential that only males are released, otherwise population replacement can take place.

Until effective genetic sexing strains become available for large-scale SIT application, combining the SIT with the IIT provides important benefits for an operational scale (Arunachalam and Curtis 1985; Bourtzis and Robinson 2006; Brelsfoard et al. 2009). A strain of *Ae. albopictus*, carrying three strains of *Wolbachia* (*wAlbA*, *wAlbB*, and *wPiP*), has been shown to express strong cytoplasmic incompatibility and good mating competitiveness in semi-field tests after irradiation at 28 Gy (a fully sterilizing dose for females) (Zhang et al. 2016). Thus, due to the sterility given to males by cytoplasmic incompatibility, a lower irradiation dose can be used to sterilize females, minimizing the impact on male performance (Zhang et al. 2015a, b). At the same time, including the IIT precludes potential disease transmission by any inadvertently released females (which make up at least 1% of the release population in upscaled SIT releases using current sexing systems). On the other hand, simultaneous sterilization guarantees that such inadvertently released females cannot reproduce; this will prevent *Wolbachia* from becoming established in the target population (resulting in the loss of cytoplasmic incompatibility and creating resistance to the IIT approach) (Lees et al. 2015; Bourtzis et al. 2016; NEA 2018). However, in Europe, this approach is at present limited by the absence of regulation.

An early pilot trial of the combined IIT and SIT approach against *Ae. aegypti*, conducted in a village in Chachoengsao Province, eastern Thailand, showed a significant reduction in hatch rate, and a lower total adult catch, during 6 months of weekly releases compared with the control area (Kittayapong et al. 2018; Kittayapong 2021). Released males were infected with *Wolbachia* collected from a local *Ae. albopictus* strain, irradiated as pupae with 70 Gy using gamma rays, and confirmed to be sterile by crossing a sample of each release cohort with non-irradiated *Wolbachia*-infected females to score fertility. In a small-cage laboratory test, released males were shown to be equally competitive to non-irradiated *Wolbachia*-infected males. A total of 10 000–25 000 one-day-old males was released (100–200 per household per week) for 6 months from delivery containers in which pupae had emerged and been provided with a sugar feed. Local support was garnered in the pilot site through strong community engagement and public awareness activities, even involving householders in the releases (Dyck, Regidor Fernández et al., this volume). Adult abundance was monitored monthly using sticky traps, and collections were made with portable vacuum aspirators. Ovitrap were collected weekly to monitor hatch rate (Vreysen, this volume).

4.3. *New Opportunities in the Integrated Management of Mosquito-Borne Diseases Offered by the SIT*

The SIT technology is ready to be applied in pilot trials and small-scale population suppression programmes for integrated mosquito control (FAO/IAEA 2015; Lees et al. 2015). In the efforts to control malaria, the SIT may, under specific conditions (particularly against exophilic species such as *An. arabiensis* where conventional control by insecticide-treated nets and indoor residual sprays is less effective), become a powerful adjunct to other technologies. This would be in accordance with the World

Health Organization's Roll Back Malaria strategy (Nabarro 1999), and more recently the WHO's Global Vector Control Response 2017–2030 (WHO 2017a), which promote integrated vector management rather than reliance on any single approach to control malaria. Given the advanced state of development of the SIT against *Ae. aegypti*, and in response to the widespread epidemic of the Zika disease in 2015–2016, FAO/IAEA proposed the technology as part of an integrated Zika management strategy (FAO/IAEA 2016b).

Advances in molecular biology and biotechnology have provided several potential genetic methods to manage mosquito populations, offering different opportunities and challenges relative to the SIT (Catteruccia et al. 2009; Alphey 2014; Bouyer and Marois 2018; Flores and O'Neill 2018; Häcker et al., this volume). Also, it has been proposed that the SIT can be boosted by treating the males with biocides, like juvenile hormone analogues or specific biopesticides like densovirus, before release (Bouyer and Lefrançois 2014; Bouyer et al. 2016). During mating, or even mating attempts, sterile males could transmit these biocides to females, which would in turn transfer them to oviposition sites. As an example, treating sterile male *Ae. albopictus* with pyriproxyfen may enable the number of males released into a given area to be reduced by more than ten-fold, and achieve a more reliable and sustainable impact on dengue transmission (Pleydell and Bouyer 2019). The principle has been tested with success on a very small scale against *Ae. aegypti* in Kentucky (Mains et al. 2015), and further trials are planned in Spain and France.

The fact that the SIT is self-limiting, unlike population replacement based on *Wolbachia* or gene drive of transgenic traits, is one of its advantages. However, this is also a drawback -- releases must be sustained over time, involving permanent costs to prevent disease transmission in a given area. Other advantages of the SIT are that it is species-specific, and has no regulatory requirements (unlike most other proposed genetic control methods that require approval for releases) (Reeves et al. 2012; Hendrichs and Robinson, this volume). Also, it has a positive public perception and no restrictions on intellectual property rights -- each country is able to use its own local mosquito strains to apply the technology.

Finally, random mutations, chromosome breakages, and gross gonad damage caused by radiation eliminates the risk of resistance development -- unlike insecticides and potentially other genetic control methods (Alphey et al. 2011; Eckermann et al. 2014; Handler 2016; The Economist 2017; Häcker et al., this volume; Hendrichs and Robinson, this volume; Whitten and Mahon, this volume). Contrary to a wrong perception that this sterilization process is necessarily associated with a loss of competitiveness of the sterile males, field trials of alternative technologies, especially RIDL, demonstrated that competitiveness of transgenic males can be much lower than what has been observed with irradiated males (Benedict and Robinson 2003; Facchinelli et al. 2013). This is related primarily to the fact that in mosquitoes the SIT is based on local strains that are well-adapted to local environments, whereas transgenic strains have often been colonized for decades.

In other insects, e.g. tsetse flies, it has been demonstrated that a loss of competitiveness is usually the result of a combination of factors such as mass-rearing, handling, chilling, and transport rather than irradiation itself (Diallo et al. 2018). This will affect equally all genetic control methods based on the mass-release of males.

Optimizing the rearing conditions is critical to minimize the impact of laboratory adaptation and other manipulations on adult male quality (Bargielowski et al. 2011).

An understanding of the reasons for a negative correlation between competitiveness and the sterile:wild male ratio (seen in the SIT trials in Italy) will be instrumental for planning future mosquito control programmes that have an SIT component. One of the major hypotheses for this phenomenon is that a proportion of the female population might be protected from sterile males regardless of the release ratio because they are located in cryptic habitats, and it is difficult for the released sterile males to access these. This illustrates the importance of released sterile males matching the behaviour of wild males by, for example, aggregating in the same micro-habitats (Vreysen et al. 2011). Also, there probably is an optimum ratio of sterile to wild males beyond which no increase in the ratio results in a higher induced sterility. It is important to identify this optimal ratio because accurate estimates of sterile-male release densities would maximize the cost-efficiency of SIT projects.

Another reason might be immigration of fertile females into the target area from neighbouring sites where sterile males are not released (the females have a much higher dispersal capacity than males) (Honório et al. 2003). Therefore, a tailored distribution of sterile males in the target area and surroundings is needed so as to achieve an adequate overflooding ratio even where wild populations are at high densities, especially when releasing into urban settings characterized by many favourable cryptic micro-habitats. Hot spots with a high mosquito population density should be identified and targeted appropriately, although this is challenging with existing monitoring tools and is not currently achievable on a large scale.

The SIT is not a stand-alone technology, and needs to be applied in combination with other pest control methods as part of an area-wide integrated pest management (AW-IPM) approach (Hendrichs, Vreysen et al., this volume; Klassen and Vreysen, this volume; Mangan and Bouyer, this volume). In particular, population reduction achieved through public participation in larval-site reduction will usually be a prerequisite, and will require that there is a strong stakeholder engagement in all projects (Dyck, Regidor Fernández et al., this volume).

5. CONCLUSIONS

At present, many SIT pilot trials against mosquitoes are ongoing or in preparation (Table 1). These field trials will create important knowledge needed for future upscaling of the technology. The most illustrative recent example is the SIT/IIT pilot trial in China that successfully suppressed, and nearly eliminated, two field populations of *Ae. albopictus* over a two-year period. Millions of sterile males were released, with prior pupal irradiation. Community support for the SIT/IIT approach strongly increased following mosquito releases, as nuisance-biting decreased. This successful field trial has fully demonstrated the feasibility of area-wide application of SIT/IIT for mosquito vector control (Zheng et al. 2019).

Area-wide releases, focused on urban/suburban settings and touristic sites, appear particularly promising in terms of sustainable and cost-effective IVM with an SIT component (eventually provided commercially by the private sector), as they can protect many people concentrated in relatively small areas.

The potential impact of the successful control of mosquito vectors, through the combined use of the SIT and other control methods, can be estimated based on previous successful programmes. When the Panama Canal was constructed, mosquito control during a period of 5 y had a significant economic and social impact on the region – eliminating yellow fever and drastically reducing malaria transmission, enabling the Panama Canal to be completed, and promoting the development of the entire area (RES 2014). In Brazil in the 1930s and 1940s, similar benefits were seen when a broad range of strategies was used to eradicate the invasive *An. gambiae* (Hendrichs, Enkerlin et al., this volume). In each of these cases, the community saw these programmes as drastic, due to, for example, the use of military force to achieve the goals, but the resulting impact on morbidity and development overcame the negative reactions (Severo 1956; Ockenhouse et al. 2005; Griffing et al. 2015).

Between 1990 and 2010, the global burden from neglected tropical diseases declined by 27%; however, this reduction occurred mostly in upper-middle-income countries. If the latest WHO targets are met between 2010 and 2020, a 55% reduction in the global burden in each country would be achieved, with an even greater reduction in low- and lower-middle-income countries (Stolk et al. 2016). It is expected that including the SIT among methods used to control mosquitoes will contribute to achieving the WHO goal, and result in increased human well-being, reduced deaths from vector-borne diseases, and increased development in deeply affected areas.

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