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Mini-review: Genetic enhancements to the sterile insect technique to control mosquito populations

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Abstract. The Sterile Insect Technique (SIT) uses the mass release of sterile insects as a highly effective area-wide, environmentally safe method of pest control. Various uses of genetics to enhance the sterile insect technique for mosquitoes have been proposed since the early 1950's. Using induced mutations, chromosomal rearrangements, breeding and selection researchers were able to develop traits such as sex-specific insecticide resistance and hybrid sterility. Unfortunately, selection of such traits is very laborious and can take decades to achieve. In addition this process is usually associated with severe reductions in fitness. Although several studies and control programs developed techniques to rear mosquitoes in large numbers, efficiently sort males, sterilize, distribute, and achieve localized control no large scale control of mosquitoes using SIT is currently being performed. The advent of modern biotechnology has made available a wide variety of tools to manipulate and express genes within mosquitoes on shorter time scales and with a wider range of accessible phenotypes than is possible through classical genetics. This mini review looks at a recent advance in mosquito control that promises to control *Aedes aegypti* and has the potential to be applied to many other mosquito species.

Keywords: RIDL, transgenic, sterile insect technique, SIT, mosquito, mass rearing

INTRODUCTION

The mosquitoes (Diptera: Culicidae) have been intensely studied since the end of the 1800´s when their participation in the transmission of several pathogens to humans and other vertebrates had been discovered for the first time. In 1877, Manson discovered that the disease known as elephantiasis was caused by a filarial worm and transmitted by mosquitoes of *Culex* genus, which was the first demonstration that mosquitoes transmit diseases (Chernin, 1977). From this moment on, the role of other mosquitoes as vectors of agents causing diseases, such as malaria, dengue and yellow fever, was proven (Taipe-Lagos and Natal, 2003).

In the 1950's and 60's the official programs of vector control in many countries used chemical strategies such as DDT and Paris Green combined with breeding site removal and personal protection such as bednets. This successfully eradicated malaria in southern Europe and Taiwan and reduced its morbidity in India from about 75 million to about 100,000 a year (Marchi and Munstermann, 1987; Liang, 1991). DDT was also used against *Anopheles gambiae* in the 1930's, when this species was eradicated from Northeastern Brazil (Parmakelis *et al.* 2008). In Brazil, the fight against Aedes aegypti was also initially a success with the mosquito being eradicated in 1956. This remarkable achievement was due to rigorously enforced removal of all potential breeding sites and intensive insecticide spraying. The subsequent increase in epidemics of Dengue from the 1980's to today have been caused by the re-infestation of *Aedes aegypti* and the relaxing of control measures. However, the application of the same control strategies applied in the 1950's was unsuccessful (Axtell and Arends, 1990). This was mainly due to less rigorous enforcement, inappropriate application of insecticides and the development of insecticide resistance. The use of chemical strategies in vector control around the World is problematic and largely ineffective with issues such as environmental contamination, effects on non-target organisms and the selection of resistance hampering its effectiveness (Dorta *et al.*, 1993).

Many developing countries, such as Brazil, have had large urbanization projects but without proper implementation of sanitation services. This recent expansion has increased the release of untreated effluents into the environment subsequently increasing the availability of breeding sites for a variety of mosquito species. The mosquito *Culex quinquefas-*

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ciatus, whose immature stages are able to survive in polluted water, has particularly benefitted and is rapidly increasing in numbers and distribution (Taipe-Lagos and Natal, 2003).

Axtell and Arends back in 1979 recommended the use of synthetic insecticides only in emergency situations and advocated biological control and management of the environment (i.e. breeding site removal) as a sustainable and environmentally friendly method of insect control. As the understanding of the ecological consequences of insecticide use grew, the search for alternatives became more urgent. Campaigns against Dengue at the end of the 20th century have prioritized educating people about the disease and environmentally friendly methods of control, with the use of chemicals advocated only during epidemic periods. Several natural methods of control have been developed recently, including larvicides such as those from the Bacillus genus and have been used for the biological control of vectors in many programs around the World (Cuba, 2005).

Insect control strategies based on chemical and biological control have had some notable successes but in many cases control has not been sustainable in the long term. This can be attributed to many reasons, including insecticide resistance, re-invasion, environmental damage and poor control program implementation. Currently mosquitoes are increasing in distribution; *Aedes aegypti* and *Aedes albopictus* have reinvaded areas they were successfully removed from and infesting new areas at an alarming rate (Dégallier *et al.*, 1996). This has led to an increase in the annual reported incidents of dengue from 122,174 in the 70's to 884,462 from 2000-2005 (Teixeira *et al.*, 2005). Recent results for the use of DDT were not as promising in India or in Zanzibar because of the evolution in the resistance of some vectors to insecticides (Curtis and Lines 2000; Hargreaves *et al.*, 2000). In southern Africa, resistance to pyrethroids has led to the return of the use of DDT in homes even with its risks to the environment and human health, which was followed by an increase in the number of malaria cases (Hargreaves *et al.*, 2000). New methods of control are desperately needed.

A species-specific, effective and environmentally friendly technique of insect control has been around for decades and has been widely used in the control of agricultural pests (Bushland *et al.*, 1955). The Sterile Insect Technique (SIT) has been applied on several occasions to control mosquitoes but with limited success (reviewed below). With the advent of modern biotechnology it has become possible to improve the applicability, efficacy, safety and efficiency of the SIT. This mini-review looks at mosquito SIT and recent advances in molecular biology that promise to control mosquitoes with particular emphasis on a technology that is close to field application.

The sterile insect technique and its application to control mosquitoes. The idea that you could control a pest insect species by releasing large numbers of sterile adults has been around since the 1930's (Knipling, 1955; Curtis, 2006). The technique is simple in principle; rear large numbers of your chosen pest insect, sterilize and release at appropriate sites (Dyck *et al.* 2005; Alphey *et al.*, 2008). Any successful mating with the sterile insect will result in no offspring. If enough sterile insects are released the population will decline and, if given enough time, could be eliminated. Highly successful, area-wide SIT programs have been conducted against the screwworm fly *Cochliomyia hominivorax* in the USA, Mexico and Central America; also in Libya, where SIT was used in the successful control of a serious outbreak in 1989 (Lindquist *et al.*, 1992). Other targets of area-wide SIT programs include the Mediterranean fruit fly (medfly) *Ceratitis capitata* in various parts of Latin America (Hendrichs *et al.* 1995) and the codling moth in Canada (IAEA, 2001). These programmes have been conducted on a massive scale – the El Piño facility in Guatemala alone produces around 3 billion sterile male medflies per week. SIT is a proven, cost-effective and environmentally safe strategy for eradication or suppression of target populations.

Despite these successes with agricultural pests SIT has had limited success on disease vectors, the elimination of tsetse from Zanzibar being an exception (Msangi *et al.*, 2000; Vreysen *et al.*, 2000). So why has the SIT not been applied to many more insect pests and disease vectors? The main challenges are (1) being able to rear the insects in enough numbers for mass release, (2) the ability to efficiently sort males from females, (3) having an efficient method of sterilizing large numbers of insects with minimal effects on fitness, (4) an effective method to distribute the sterile male insects and (5) a quick and efficient method to identify released individuals. There are many insect species that can be reared in the laboratory and potentially in enough numbers for an SIT program, but many of these are difficult to sterilize with irradiation without severely affecting fitness (Barry *et al.* 2003; Helinski *et al.*, 2008). Mosquitoes are also an ideal choice for SIT and the early successes with agricultural pests led to a number of trials to control mosquitoes during the 1970's and 80's on several different species of mosquito including *Aedes aegypti*, *Aedes albopictus, Culex pipiens, Culex quinquefasciatus, Anophleles albimanus* and *Anopheles gambiae* (reviewed in Benedict and Robinson, 2003). Several different methods of sterilization were used for these trials on mosquitoes including irradiation, chemical, cytoplasmic incompatibility and translocations (or other chromosomal rearrangements). Although many of these trials showed a reduction in the mosquito population, very few achieved eradication in the release area or long term control (Benedict and Robinson, 2003).

One of the major challenges of any large scale area-wide control program of mosquitoes is to separate the males from the females. This has to be done because females bite and could potentially transmit diseases and they can distract the males from dispersing and mating with the target wild type females (Myers *et al.*, 1998). This female distraction has been demonstrated in large scale field trials using Medfly (Rendón *et al.*, 2004) and showed a three- to five-fold improvement compared to male-only release. In mosquitoes of the Genus *Culex* and *Aedes* separation of males and females can be performed based on the size dimorphism

of the pupae, male pupae are smaller than female pupae (Ansari *et al.,* 1977). This pupal size difference has been used to develop efficient sex-sorting methods and was used in India to obtain a male population with only 0.1-0.2% contamination with females (Sharma *et al.,* 1972; Ansari *et al.* 1977). New techniques of developing female-specific gene expression systems using female-specific promoters, such as the Actin-4 gene in *Aedes aegypti* (Muñoz *et al*., 2004) should enable even more efficient removal of females.

To our knowledge only two trials on mosquitoes recorded the elimination of the population within the release area, one used cytoplasmic incompatibility in *Culex quinquefasciatus* to eradicate an isolated population from the village of Okpo, near Rangoon in Myanmar (Curtis *et al*., 1982). Another used chemical sterilization of *Anopheles albimanus* with P,P-bis (1-aziridinyl)-N-methyl phosphinothioic amide to achieve 99.8% sterility in males (Breeland *et al.,* 1974; Lofgren *et al.,* 1974). Other trials that could have been a success were to be conducted on *Aedes aegypti* and *Culex fatigans* in India in the 1970's (Curtis, 2006), they had developed a mass rearing method to produce over 300,000 sterilized male *Aedes aegypti* and *Culex fatigan*s pupae per day (Singh and Razdan, 1975; Ansari *et al*., 1977) with 99.8% and 95-97% efficiency in sex sorting respectively. Methods were also developed to package, mark, transport and distribute adults (Brooks and Singh, 1975; Singh and Brooks, 1975; Singh, *et al.* 1975). Unfortunately this trial was stopped days before it was to be implemented due to a newspaper article reporting that the trial was a cover for a US bio-warfare program (Curtis, 2006). This has also unfortunately prevented any further large scale attempts to control mosquitoes using the SIT in India to date. There are currently no large scale SIT operations in operation against any mosquito species (Benedict and Robinson, 2003). However, there has been a recent attempt to develop SIT against *Aedes albopictus* in Italy (Bellini *et al.,* 2007). In this trial a method was developed to mass rear the insects to achieve a release rate of 100-1000 sterile males per hectare per week, or about 1000-10,000 males per week. They used irradiation to sterilize the mosquitoes and showed a significant decrease (about 36%) in the number of viable eggs in the release area compared to the control area, although only one treated and one control area were used and no data is given for previous numbers or population trends of mosquitoes at these sites. A larger scale trial is proposed in a more isolated urban area to avoid immigration of mated females and to develop better techniques for mass rearing and sterilization. The need for a nuclear facility near the target area increases the complexity and logistics, as well the operation costs.

These trials, taken together, indicate that SIT has considerable potential for the control of mosquitoes. Problems with mass-rearing, sex-separation, sterilization, distribution, and maintaining the competitiveness of the insect, tend to reduce the cost-effectiveness of an SIT program. For some of these key issues, especially sex-separation and sterilization, modern genetic methods could potentially provide dramatic improvements in cost-effectiveness of SIT, particularly for mosquitoes.

Genetic control of mosquitoes. It was realized from the very earliest days that the advent of modern biotechnology could be used to improve SIT (Curtis, 1968; Serebrovskii, 1969). Classical biotechnology methods such as breeding and selective mutation have been used to improve SIT, such as the tsl-based genetic sexing strain of Medfly (Robinson, 2002). Many attempts to improve mosquito SIT with classical genetics have been performed using several mechanisms of control such as mutagen induced dominant lethality, sex-ratio distortion and chromosomal translocations. Each method enhances SIT by either improving mass rearing or removing the need for sterilization. Sex-ratio distortion has been developed in several mosquito species including *An. albimanus* (Seawright *et al.,* 1978), *Anopheles stephensi* (Robinson, 1986), *Anopheles arabiensis* (Lines and Curtis, 1985), *Culex quinquefasciatus* (Shetty, 1987), *Anopheles gambiae* (Curtis, 1976) and *Aedes aegypti* (Curtis *et al.,* 1976). However developments in transformation techniques (Handler and James, 2000; Handler, 2002) have led to a resurgence in the interest in using molecular tools to enhance SIT in mosquitoes (Alphey, 2007; Speranca and Capurro, 2007).
The genetic control of vectors could potentially be achieved in a variety of ways but they can generally be separated into two main methods, population replacement and population reduction. The common aspect is that the mechanism of genetic control is introduced in nature by releasing the modified organisms to mate with the wild ones (Curtis, 1968). Population reduction uses genetically sterile insects to reduce the population and population replacement aims to introduce a resistance mechanism to prevent transmission (James, 2005; Rasgon and Gould, 2005).

Population replacement requires two components, a mechanism for resistance and a method to spread the gene into a population. Mechanisms of resistance have been developed in several mosquito species, for example RNAi to reduce transmission of dengue in *Aedes aegypti* (Franz *et al.,* 2006), artificial peptides (SM1) to inhibit malaria development in *Anopheles stephensi* (Ito *et al*., 2002) and expression of cecropin to impair malaria development in *Anopheles gambiae* (Kim *et al.,* 2004). Several methods for spreading a gene into a population are currently being investigated including Wolbachia symbionts (Rasgon and Scott 2003; Sinkins and Godfray, 2004), engineered underdominance (Davis *et al.,* 2001), fitness manipulation (Hahn and Nuzhdin, 2004), multiple independently assorting loci (Schliekelman and Gould, 2000), meiotic drive systems (Mori *et al.,* 2004; Huang *et al.* 2007) and transposable elements (TEs) (Boete and Koella, 2002; O'Brochta *et al*., 2003; James, 2005).

Transgenic mosquitoes carrying a gene that acts as an effector molecule interfering with a parasite or a virus development cycle are not expected to have a fitness advantage when compared to wild mosquitoes. Therefore Mendelian inheritance alone will not be able to spread the refractory gene into a population (Vernick *et al*., 2005). To overcome this issue, a gene drive mechanism has to be developed

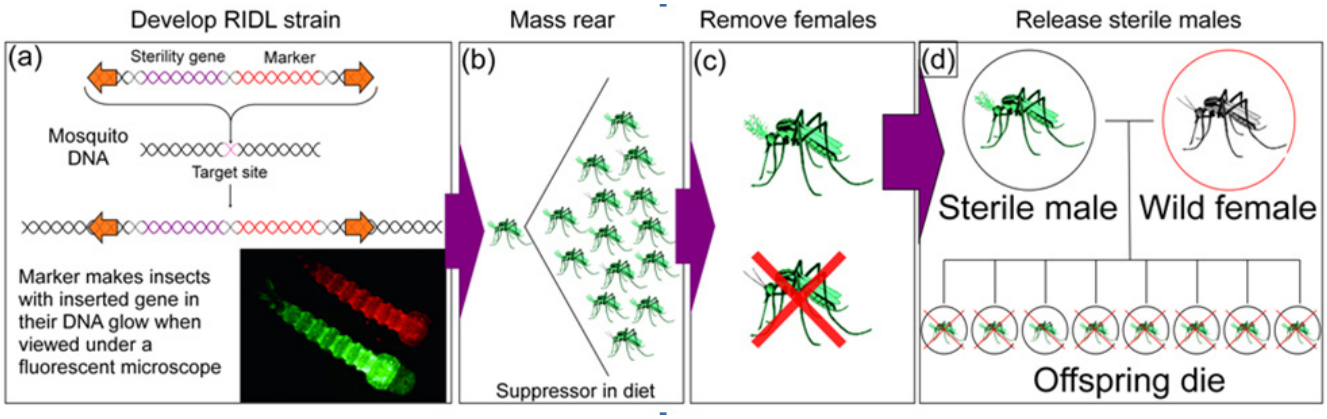

Figure 1. Population reduction using a RIDL-SIT based system. (a) The RIDL strain is developed using modern molecular biology techniques of transformation; inserting the RIDL gene system and a fluorescent marker into the insect of choice (in this case a mosquito). The inset picture in panel (a) shows green and red fluorescing Aedes aegypti larvae. Individuals expressing the fluorescent marker with a single insertion of the transgene are developed into a homozygous RIDL strain. The RIDL strain goes through rigorous testing to select a strain with a suitable phenotype (i.e. late acting or female-specific lethality, bionomic and fitness studies). Once a suitable RIDL strain is developed it can be mass reared (b) in the presence of a suppressor (a dietary supplement that suppresses the RIDL system). (c) The females are removed, either mechanically or using a female-lethal system. The males can then be released to compete with the wild type males (d) for wild type females, offspring from a successful RIDL mating will die as there is no suppressor present. Releases in large enough numbers over a sufficient time will suppress, or even eliminate, the targeted population

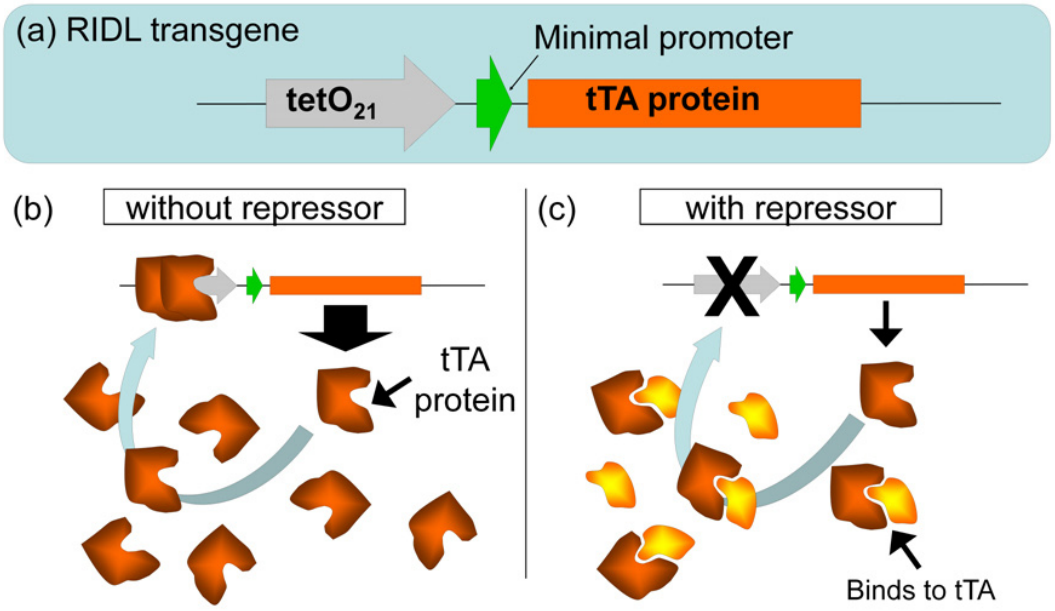

Figure 2. Schematic representing the RIDL positive feedback system in mosquitoes (adapted from (Gong et al. 2005). (a) The positive feedback system is composed of a tetO binding domain, a minimal promoter and tTA. Without the repressor (b) a small amount of tTA is produced from basal expression of the minimal promoter which then binds to the tetO binding sites. The binding of tTA enhances expression of the minimal promoter producing more tTA which in turn binds to more tetO sites. This positive feedback produces large quantities of tTA which in high enough levels are deleterious to cells. (c) The repressor is able to bind to tTA and block it from binding to tetO, preventing the positive feedback and only the basal level of tTA is produced.

taking into account the following criteria: it has to fix the effector gene at a faster rate than would be expected for Mendelian inheritance, be protected from loss of linkage between the drive mechanism and the effector gene, be resistant to mutational inactivation of effector gene, be able to evade pathogen resistance, must be efficient and robust in order to compensate for any fitness cost of the transgene and has to be safe (Sinkins and Gould, 2006).

Population replacement is currently at the research stage of development and there are several major problems to overcome. There is currently no proven technique to drive a refractory gene into a population and there are many epidemiological and entomological risks that need to be assessed (Benedict and Robinson, 2003). Population reduction is promising from a cost effectiveness perspective as opposed to population reduction where billions of insects will need to be released, relatively few insects are needed, and indeed with an effective drive mechanism only a few insects may be required (Sinkins and Gould, 2006). However this also presents problems with containment as potentially even a few escaped insects could spread a gene through a population. One particular advantage of population reduction over population replacement is that the released individuals are sterile and do not spread any genes though a population. Inadvertent release of even millions of genetically sterile insects will only have a transient effect on the target population. There are several methods of population reduction using transgenic methods including Wolbachia (Dobson *et al.,* 2002; McMeniman *et al*., 2009) and homing endonucleases (Burt, 2003; Windbichler *et al.,* 2007). However, the use of Wolbachia and homing endonucleases are still very much at the laboratory stage of development.

A population reduction method using a strain of insects homozygous for a dominant lethal genetic system (Alphey and Andreasen, 2002; Alphey *et al.,* 2002), known as RIDL® (Release of insects carrying a dominant lethal) is now available for field use.

RIDL. RIDL technology offers a solution to many of the drawbacks of traditional SIT that have limited its application in mosquitoes while maintaining its environmentally friendly and species-specific utility. RIDL differs from conventional SIT in that the released insects are not sterilized by irradiation but are homozygous for a dominant lethal gene (Alphey, 2007). Mating with indigenous populations results in offspring that are heterozygous for the lethal gene leading to the death of all progeny and hence eventual suppression of the population due to a decrease in its reproductive capacity (Heinrich and Scott, 2000; Thomas *et al*., 2000) (Figure 1). For the purposes of mass rearing and release of the transgenic strain the dominant lethal must be repressible contingent on a permissive condition such as a food additive or an environmental variable absent in the wild but present in the rearing facility. Such a repressible RIDL system also serves to act as a failsafe for escapees since any accidental release of mosquitoes from a rearing facility are sterile without the repressor (Benedict and Robinson, 2003). Highly efficient repressible RIDL systems were first demonstrated in Drosophila models and more recently in the Mediterranean fruitfly using the tetracycline-repressible transactivator (tTA) to control expression of a toxic effector (Thomas *et al.,* 2000; Gong *et al.,* 2005). The tetracycline system depends upon the protein TetR that binds to a specific sequence of DNA (tetO) only in the absence of tetracycline. In the "tet system" TetR is fused to a eukaryotic transcriptional transactivator (tTA), which binds to tetO sequences in a tTA-responsive element driving the effector gene (Figure 2). Tetracycline is able to prevent the expression of the effector gene by binding to tTA and preventing it binding to the tetO sequences and driving the effector gene. It is also possible to make the system specific to females placing the tTA protein under the control of a female-specific promoter. The tTA protein is then expressed only in females, so the males survive with or without tetracycline but females perish in its absence.

Mathematical modeling has demonstrated the theoretical effectiveness of RIDL showing that it could prove more efficient than traditional SIT against diseases such as dengue fever (Thomas *et al*., 2000; Atkinson, 2002; Gould and Schliekelman 2004). The competitiveness of engineered mosquitoes with wild populations is controversial with some studies indicating that there is no significant fitness cost associated with the insertion of transgenes (Moreira *et al*., 2004; Marrelli *et al*., 2006) and others demonstrating a fitness effect (Catteruccia *et al.,* 2003; Irvin *et al.,* 2004). However modeling indicates that even a moderate fitness cost should not dramatically affect the critical release ratio required by RIDL particularly in comparison to the effect in competetiveness of traditional methods of sterilization (Yakob *et al*., 2008). If fitness penalties can be minimized then the effectiveness of the RIDL system can further be increased by the use of strains that are homozygous for multiple insertions of the construct; this should improve penetrance of lethality of the effector as well as acting as a backup in the event that one lethal construct is inactivated (Gong *et al.,* 2005).

Classical methods of sterilization result in progeny that die early in embryogenesis. Mathematical modeling of SIT strategies has indicated that such early acting lethality can negatively affect the efficiency of any release program of *Aedes aegypti* if there is competition for resources at the larval stage (Atkinson *et al*., 2007). This is due to density-dependant stabilization of population levels by food availability, oviposition sites etc affecting the number of larvae surviving to adults. The result is that early acting lethality increases the survival rate of viable offspring by raising resource availability and can leave the adulthood population levels unchanged under certain circumstances. This effect is a substantial one: Phuc *et al*., (2007) estimated that for *Aedes aegypti* the critical release ratio for population elimination could be up to 540% greater for an early acting lethal as opposed to a late acting one. For mosquitoes competition effects are only relevant in the larval stage of development due to density and since pupae do not feed. Therefore

delaying lethality until late in development could substantially increase the effectiveness of control programs by allowing larval competition to occur. A genetically engineered late acting tetracycline repressible RIDL system was first demonstrated in the mosquito *Aedes aegypti* with the transgenic line OX513A (formerly called LA513A) (Phuc *et al*., 2007). The LA513 RIDL system resulted in death at the larval/pupal boundary with 95-97% penetrance of lethality. Despite this imperfect penetrance of lethality and the fact that the line may not be fully competitive with wild type there is no theoretical barrier to the use of the LA513 for mosquito control (Yakob *et al*., 2008).

One of the major advantages of SIT over other techniques (such as insecticides, larvicides, breeding site removal etc.) is that the males are very good at seeking out females of the same species and the technique becomes more effective as the population is reduced. Whereas these other techniques are effective at reducing large populations, they are difficult to implement on a small, dispersed population without great expense. Therefore it is desirable to have a combination of RIDL with other control methods in an integrated vector management program. Indeed any initial reduction in the mosquito population, such as larviciding, breeding site removal and insecticides will help to make technique RIDL program more efficient.

Progression to the field is the next logical step for this technology and experiments have shown encouraging results showing that OX513A males are as competitive as laboratory reared wild type males under contained semi-field conditions (Lee *et al*., 2008; Khongtak *et al*., 2009). There are safety, regulatory and public perception issues that need to be addressed before any release can be performed but these are outside the scope of this review for a recent review addressing these issues see "Macer 2003; Knols *et al.,* 2007; Macer, 2006; Beech *et al*., 2009; Vasan, 2009".

CONCLUSIONS AND FUTURE PERSPECTIVE

The SIT has been attempted for mosquito control several times over the past 60 years. However, to our knowledge there are currently no large scale mosquito control programs in operation. The recent advances in insect genetic engineering have allowed the development of sterile insects to enhance the environmentally friendly, species-specific and effective SIT and make it applicable to mosquitoes. One such method, the RIDL system, has been developed in *Aedes aegypti* which has the potential to overcome previous limitations of SIT in this species (and potentially others), providing an effective and safe method of control. RIDL technology has progressed rapidly in recent years in mosquitoes and a strain of *Aedes aegypti* is now available for field testing. This will not be the first release of a transgenic insect as this has been performed with Pink Bollworm in the USA since 2006 (Simmons, 2007; Simmons *et al.,* 2007). The mosquito RIDL is a similar system to the one developed in Pink Bollworm and this suggests that this approach is a safe and environmentally friendly method.

The RIDL technique is applicable to a wide range of mosquito vectors and recently it has also been developed in *Aedes albopictu*s, another major vector of dengue and chikungunya. These recent developments, a strain available for field testing and the possibility to develop this technique in a wide range of other mosquito species makes RIDL a powerful new tool in the mosquito control arsenal.

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