

Genetically Modified Insects: Science, Use, Status and Regulation

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Abstract

A major change is underway in applied genetic engineering of pest insects. The use of genetically modified (GM) insects in laboratories is widespread, well-developed and non-controversial. This review examines the technology used to produce GM insects and their potential uses. Since 2006 however, several strains and strategies have begun to be moved to field use. Insects differ from plants in several relevant aspects and regulatory frameworks available for environmental release of genetically modified organisms (GMOs) are now being adapted for GM insects, as countries make decisions regarding their research and development. The last few years have seen national approvals given for open field releases, particularly of GM mosquitoes. As a consequence of this activity, authorities are also reviewing their regulatory frameworks and requirements for the field release and deployment of GM insects. This review will also provide the current approval status of such insects including field tests and national decisions regarding field-testing, before examining those regulatory frameworks and consider whether there are unique points required for the biosafety and risk assessment of GM insects.

Keywords: Genetically modified insects, SIT, regulation, field testing, pest management.

Riassunto

Un cambiamento importante è in corso nell'ingegneria genetica applicata agli insetti parassiti. L'uso di insetti geneticamente modificati (GM) in laboratorio è molto diffuso, ben sviluppato e non controverso. Il rapporto esamina la tecnologia utilizzata per la produzione di insetti geneticamente modificati e le loro potenziali applicazioni. Dal 2006 tuttavia, diversi ceppi e strategie di utilizzo hanno cominciato a essere spostati sul campo. Gli insetti differiscono dalle piante in diversi aspetti rilevanti, e quadri normativi disponibili per il rilascio ambientale di organismi geneticamente modificati (OGM) sono ora in corso di adattamento per gli insetti, dal momento che i paesi stanno prendendo decisioni riguardo alla loro ricerca e sviluppo. Gli ultimi anni hanno visto approvazioni date a livello nazionale per i rilasci in campo aperto, in particolare di zanzare GM. Come conseguenza di questa attività, le autorità stanno anche rivedendo i loro quadri normativi e i requisiti per il rilascio di campo e la diffusione di insetti GM. Questa recensione fornirà anche lo stato attuale di approvazione degli insetti, comprese le prove di campo e le decisioni nazionali in materia, prima di esaminare i quadri normativi e considerare se ci sono dei punti unici richiesti per la valutazione della biosicurezza e il rischio derivante dagli insetti GM.

1. INTRODUCTION

Pest insects cause significant economic damage and harm to mankind. Insects transmit human, animal and plant diseases and also directly attack both plants and animals; this causes damage and losses and also impacts trade (Benedict, 2003; Deguine *et al.*, 2009; Kongsin *et al.*, 2010; Lee *et al.*, 2010; Murtola *et al.*, 2010; Pérez-Guerra *et al.*, 2010). Efforts to control insect pests have predominantly relied on the use of chemical insecticides. However this approach is under increasing pressure around the world, due to increased resistance in the pests, and lower acceptance of negative effects such as pesticide residues in food and the environment, contamination of aquatic and terrestrial environments as well as ground waters, and effects on non-target organisms. In addition, new active ingredients for insecticides are increasingly difficult to identify, and their development time and the cost of registration is increasing. This has stimulated the search for new forms of pest control. Genetics-based insect control strategies, based on the classical Sterile Insect Technique (SIT), are becoming increasingly viable. The close affinity to established SIT and biological control methods means that there is a large body of experience on which to draw when using GM insects in this way, from mass-rearing of insects, quality control, release mechanisms and field monitoring. This adds a degree of familiarity and confidence and removes some of the uncertainty around the use and implementation of novel GM insect strategies.

There are several strategies in the field when considering GM insects, which may be classified by:

- a) the propensity of the genetic trait to establish or spread, although it should be noted that these are not necessarily alternatives, as self-limiting strategies can be aimed at population replacement, or self-sustaining ones aimed at suppression (for example)
 - Self-limiting
 - Self-sustaining (including non-GM methods, such as *Wolbachia*)
- b) or by desired outcome, e.g.
 - Population suppression
 - Population replacement (conversion of the insects to a less harmful form)
 - Other, e.g. use of GM insects as flying needles for vaccination
- c) or by the method by which the heritable modification is achieved
 - Classical genetics
 - Recombinant DNA methods

- Other methods for introducing foreign or novel DNA sequence
- Paratransgenesis (the transformation of intracellular organisms associated with insects).

A brief review of each of these types will be given, but thereafter this review focuses on self-limiting strategies as these are the most advanced in terms of field use and potential deployment for pest control.

1.1. The propensity of the genetic trait to establish or spread

1.1.1. Self-limiting strategies

In self-limiting strategies, the novel trait is expected to disappear more-or-less rapidly from the environment after release. The trait may be maintained in the environment over the longer term only by periodic release of additional modified insects. The use of genetically “sterile” insects is a clear example of a self-limiting approach. Such methods are widely regarded as the least controversial and lowest risk of new genetic control methods (Pew Initiative on Food and Biotechnology, 2004). This approach builds on the operational precedents established by the successful use over 50 years of radiation-sterilised (non-GM) insects to control certain agricultural pest insects, known as Sterile Insect Technique (SIT). SIT involves inundative releases of sterile insects to mate with the target pest populations and thereby reduce their reproductive potential. Mating of released sterile males with native females leads to a decrease in the females’ reproductive potential because their offspring do not survive (Knipling, 1955; Dyck *et al.*, 2005). Ultimately, if males are released in sufficient numbers over a sufficient period, this leads to the local elimination or suppression of the pest population. SIT is species-specific and has at most indirect effects on other ‘non-target’ pest species. This ‘birth control’ strategy is therefore environmentally clean and sustainable. SIT approaches are most suited to reducing low populations to very low levels, in contrast to insecticides which are best at reducing high populations to low ones. Single-sex releases, i.e. of sterile male insects without accompanying females, are likely to be desirable in most or perhaps all cases. In some cases adult females are potentially damaging, e.g. mosquitoes and some tephritids; and more generally sterile females may reduce SIT effectiveness by ‘distracting’ sterile males from seeking and courting wild females. However if only sterile males are released a 3- to 5-fold improvement in effectiveness per released male in large-scale trials of sterile Mediterranean fruit fly (medfly, *Ceratitis capitata*) (Rendón *et al.*, 2000) was ascertained. However, for many insect species large-scale sex separation is not feasible without

genetic methods ('genetic sexing'). Consequently, several programmes, for example against New World screwworm, release both males and females, even though male-only release is considered more preferable. Insects for SIT can be sterilised chemically, by gamma irradiation or X-rays, biologically (e.g. by use of *Wolbachia*, described below), or more recently by genetics (Catteruccia *et al.*, 2009). There are several advantages to using GM insects in SIT programmes, including cost reduction through genetic sexing and the use of genetic markers, expanding the range of insects that can be used in SIT, which is presently restricted in part by the need to find a sterilising dose of radiation that is not too damaging, and by improving the mating competitiveness of released male sterile insects (Thomas *et al.*, 2000; Alphey, 2007; Alphey *et al.*, 2008; Catteruccia *et al.*, 2009; Papathanos *et al.*, 2009; Alphey *et al.*, 2010; Morrison *et al.*, 2010). Sterile male approaches are 'self-limiting' as the released males themselves will die out in the environment after their own short lifespan. A transgene or transgenes inducing sterility or lethality in this context will also disappear rapidly from the environment; repeated releases of sterile insects are required to maintain a population of sterile males in the environment in the longer term. GM insects for SIT approaches are now available and are currently being evaluated in open field release programmes of increasing scale (Alphey, 2010; Harris *et al.*, 2011; Simmons *et al.*, 2011). Programmatic use of GM insects in SIT-based plant pest control and public health vector control programmes is likely within the next few years.

1.1.2. Self-sustaining strategies

Self-sustaining strategies are ones in which the modification is expected to persist indefinitely in the environment, and perhaps to increase in frequency and geographic range. Such strategies at present are primarily aimed at insect vectors for human diseases (reviewed in Marshall & Taylor, 2009). Such self-sustaining strategies aim to convert or replace all insects in a population with a less harmful form, for example a form less able to transmit one or more pathogens. This might for example be achieved via a transgene which protects a mosquito from infection by *Plasmodium* species. To be effective, such a gene would have to be present and persist at a high proportion of the vector mosquitoes in a given area; unless releases are conducted on a huge scale it is likely that the frequency of the gene in the wild population will need to be increased after limited release. However, such 'refractory' genes are unlikely to be able to spread on their own (for an exception, likely confined to laboratory conditions, see Marrelli

et al. [2007] and Lambrechts *et al.* [2008]). Therefore, the assumption in the field is that such refractory genes will need to be coupled to a 'gene drive' system capable of spreading itself – and the refractory gene – despite the associated fitness cost. Maintaining the link between gene driver and refractory 'cargo' is then an additional problem (Curtis *et al.*, 2006). Finding suitable gene(s) that block amplification of the pathogen and coupling with the most appropriate drive mechanism is still in the fundamental research stage, although it is possible that the gene and the drive mechanism could be tested separately more quickly than having a fully-functional gene drive. Many biosafety and ethical issues will need to be addressed prior to open-field release of insects carrying gene-drive or self-sustaining mechanisms.

1.2. Desired outcomes

1.2.1. Population suppression

Sterile-male methods are the best known and currently most developed example of a population suppression strategy. However, a range of other strategies have been proposed. Self-limiting methods include female-killing methods, sex-ratio distortion and delayed conditional lethality (Foster *et al.*, 1988; Fryxell & Miller, 1995; Schliekelman & Gould, 2000a; Schliekelman & Gould, 2000b; Schliekelman *et al.*, 2005; Bax & Thresher, 2009). As with sterile-male methods, some of these approaches were attempted without the use of genetic engineering (e.g. Foster *et al.*, 1988; Foster *et al.*, 1991), but the use of modern genetics can make the approaches more effective, feasible and applicable to a wider range of species.

A potentially powerful genetic engineering approach involves the use of site-specific endonucleases such as homing endonuclease genes (HEGs). These can be deployed in several configurations (Burt, 2003; Deredec *et al.*, 2008), including both self-limiting and self-sustaining population suppression forms. HEGs are selfish DNA elements; by using their ability to spread through populations one can potentially drive high-fitness-cost traits such as female sterility. Modelling suggests that this can lead to global extinction of the target species (Burt, 2003; Deredec *et al.*, 2008). Proof of principle has been achieved for some of the necessary components (Windbichler *et al.*, 2011), but prototype strains have only been developed for a self-limiting sterile-male version (Windbichler *et al.*, 2008).

1.2.2. Population replacement

As discussed above, most population replacement strategies depend on the ability of the novel trait to persist and even spread in the target population and potentially beyond. Such systems are therefore generally self-sustaining. However, self-limiting approaches are also possible. These may be particularly useful in the early stages of testing of population replacement strategies as, by definition, they have lower potential to persist and spread. Inundative release may be feasible in isolated populations, especially where the released insects carry multiple copies of the transgene at different genetic loci (Rasgon, 2009). A self-limiting gene drive system has also been designed, which can give an initial increase in allele frequency after release, but still disappears in the longer term (Gould *et al.*, 2008).

1.2.3. Other outcomes

Some strategies may have intended outcomes that do not fall neatly into the above population suppression / population replacement categorisation. One example is the potential use of mosquitoes engineered to express a novel antigen in their saliva. Such 'flying needles' would potentially vaccinate suitable hosts in the release area. This was first proposed in the 1990s and revisited more recently (Crampton *et al.*, 1999; Matsuoka *et al.*, 2010; Yamamoto *et al.*, 2010). This approach is likely to have many attendant biosafety and ethical issues (e.g. control of dose, control over the number of bites received, purity of the vaccine, consent for vaccination, ability to refuse consent, etc.) and is highly unlikely to be used in the near term.

1.3. Methods by which the heritable modification is achieved

1.3.1. Paratransgenesis

The aim of paratransgenesis is to reduce vector competence by the genetic modification of symbionts living within the insect. There is a range of possibilities depending how tightly associated the microbe is with the insect. At one end of the spectrum are intracellular bacteria with no free-living form, such as *Wolbachia* species. These are transmitted vertically (mother to offspring) and are essentially non-infectious, although they can move between species on evolutionary time-scales. In many ways, inserting or modifying *Wolbachia* closely resembles inserting or modifying mitochondria; themselves thought to be remnants of once free-living bacteria. At this end of the spectrum, while the hosts are not

strictly GM insects, but contain GM microbes, in terms of risk assessment and risk management they are essentially equivalent to GM insects. As the association between microbe and insect becomes looser, the similarity between GM insects and paratransgenic systems breaks down; at the other end of the spectrum are free-living microbes that merely associate with or to some extent accumulate in insects. The majority of related research has been conducted in human disease vectors (Aksoy *et al.*, 2008; Favia *et al.*, 2008; McMeniman *et al.*, 2009; Hurwitz *et al.*, 2011), where the objective is to reduce the competence of the vector for human diseases, although there have been some agricultural applications for pests of citrus, grapevines and sugarcane (Miller *et al.*, 2006, Pittman *et al.*, 2008, Gai *et al.*, 2009). The prospects of paratransgenesis for control of insect-borne human diseases were reviewed by Coutinho-Abreu *et al.* (2010).

1.3.2. Non-GM approaches with similar outcomes

There are two non-GM strategies that use the intracellular bacteria *Wolbachia*, either in a variant of the classical SIT or as a mechanism to induce refractoriness. Brelsfoard *et al.* (2008) and Chambers *et al.* (2011) describe the use of Incompatible Insect Technique (IIT), a variant of SIT. IIT relies on embryonic lethality resulting from cytoplasmic incompatibility that is induced from the intracellular bacterium *Wolbachia pipientis* (Laven, 1967; Brelsfoard *et al.*, 2008; Alphey *et al.*, 2010). The use of *Wolbachia* to induce refractoriness to dengue virus has recently been tested in open field trials in Australia (Hoffman *et al.*, 2011; Iturbe-Ormaetxe *et al.*, 2011; O'Neill, 2011b) and is proposed to be released in Vietnam (Jeffery *et al.*, 2009). *Wolbachia* is found in many insect species, although the dengue virus vector mosquito, *Aedes aegypti*, is not normally infected with it. *Wolbachia* are transmitted maternally, like mitochondria, but manipulate the host's reproductive biology in such a way that they tend to spread through the species. Artificial infection (by micro-injection) of a species with a foreign strain of *Wolbachia* essentially adds a megabase or so of foreign DNA to its genome in a stable, heritable form, much as transgene insertion into the mitochondria would do, but with the additional ability to spread through the species. Of course this DNA occurs naturally somewhere else in the world, but then so too do most transgenes – the issue is not that they are unnatural in an absolute sense but that they did not previously occur in the new association or combination and could not have done so with any reasonable likelihood without human intervention.

In the case of the Australian trial, *Ae. aegypti* was artificially infected with specific *Wolbachia* strains isolated from a fruit fly, the presence of which in *Ae. aegypti* reduces its ability to transmit dengue. *Wolbachia*-infected mosquitoes were then released with the aim of permanent establishment of the modified form in the wild population of *Ae. aegypti* (Hoffman *et al.*, 2011; Iturbe-Ormaetxe *et al.*, 2011; O'Neill, 2011b). In the classification above, this is a clear example of a self-sustaining genetic modification aimed at population replacement. As this system was not constructed using recombinant DNA technology, such regulatory frameworks were deemed not to apply in Australia and a “work-around” was developed where the *Wolbachia*-infected mosquitoes were regulated as a veterinary chemical product (De Barro *et al.*, 2011). Although a publicly-available risk assessment was prepared for the wMelPop strain of *Wolbachia* (Murphy *et al.*, 2010), this trial used a different strain, the wMel strain, for the release. It could be considered that there were some shortcomings in the risk assessment regarding both the potential of the *Wolbachia*-infected mosquitoes to spread outside the release area, and the strain actually released not being the one assessed, but rather a more invasive one. This issue of invasiveness and spread would undoubtedly have been a focus of concern and analysis for a GM insect strategy. This highlights a degree of inconsistency which has also started to arise in the regulation of GM crops, with new methods of genome modification being developed which will not fall under the narrow definition of genetic modification in the technology and process-based EU legislation, but will have similar characteristics to many GM plants, such as herbicide tolerance (BAC, 2007; Breyer *et al.*, 2009; ACRE, 2011; Lusser *et al.*, 2011). It would clearly be more satisfactory to have a unified and consistent approach to the regulation of genetic strategies, irrespective of the precise methods used to develop the strains.

Regulatory frameworks are available for genetically modified organisms (GMOs) and are now being adapted for GM insects, as countries make decisions regarding the research and development of GM insects. The use of GM insects in laboratories is widespread, well-developed and non-controversial. Countries are now looking to their frameworks developed predominately for GM plants and adapting them for GM insects. This review will look at those frameworks and consider if there are unique points required for the biosafety and risk assessment of GM insects.

2. TECHNOLOGICAL APPROACHES

2.1. Insect transformation

The first integration of exogenous DNA into the genome of an insect, termed 'germ-line transformation', was described in 1982 (Rubin & Spradling, 1982; Spradling & Rubin, 1982). The method was based on *P* element-based transposon vectors in *Drosophila melanogaster*. That was a critical turning point both for basic and applied research. However, despite intense efforts, reproducible germ-line transformation of insects other than *Drosophila* had to wait another 13 years. There were two main reasons for this: the vector and the marker. The *P* element, and therefore *P* element-based vectors, does not seem to function outside the *Drosophilids* because of co-factor requirements, which are present only in the genera *Drosophila* (Handler et al., 1993; O'Brochta & Atkinson, 1996; Atkinson et al., 2001). Loukeris et al. (1995) used *Minos* instead of *P* to transform the Medfly; systems based on other transposons have also been developed, most notably *piggyBac*-based systems (reviewed in Handler, 2002). In respect of markers, the original transformation of *Drosophila* relied on complementation of a recessive visible mutation; initially *rosy*, but later more often *white* (Rubin & Spradling, 1982; Klemenz et al., 1987). Though the first transformations of other insects also used such complementation systems (Loukeris et al., 1995; Coates et al., 1998; Jasinskiene et al., 1998), the use of fluorescent proteins as markers has allowed transformation of wild type strains and greatly facilitated the transformation of new species. Chemical selection markers such as neomycin phosphotransferase, which confer resistance to G418, though demonstrated in *Drosophila* (Steller & Pirrotta, 1985) have been little used. Insect transgenesis has allowed for deeper understanding of the biology of insects and human disease vectors (Wimmer, 2003) and has provided an important tool for the development of new strategies to control insect pests (Alphey, 2002; Handler & Beeman, 2003; Franz & Robinson, 2011).

Genetic transformation technologies aim to delivering transgenes to the nuclei of germ cells, for a stable insect transformation. Although several methods have been proposed and tried in the past, for example electroporation and biolistics (Leopold et al., 1996, Miahle & LH, 1994), micro-injection remains by far the most widely used option for introduction of foreign DNA to the insect germ cells. The technique closely resembles that described by Rubin & Spradling (1982) but with minor modifications, to ensure embryo survival and optimum DNA delivery in different species.

A genetic vector system must also be in place to allow the integration of the transgene into the host genome. Three such systems have been described to date: transposable elements (Handler & Beeman, 2003), viruses (Olson *et al.*, 1994; Higgs *et al.*, 1995; Kamrud *et al.*, 1997) and *in vivo* recombination systems such as the FLP/FRT and Cre/loxP systems (Wimmer, 2005; Schetelig *et al.*, 2011). Virus-based expression systems permit only transient expression of a transgene. For stable integration, transposable elements have been by far the most widely used vector systems in both insect and mammalian organisms. Transposable elements are a class of selfish genetic elements which have the ability to mobilise themselves from one position in a genome to another (Kidwell & Lisch, 1997). There are three classes of transposable elements: Class I elements transpose via reverse transcription (copy themselves as RNA for transposition) and Class II elements transpose directly between DNA, removing themselves from one position into a new position entirely (without copying) (Pimpinelli *et al.*, 1995). Class III elements also exist and are referred to as miniature inverted-repeat transposable elements (MITES); they are small elements that do not encode any protein but are capable of non-replicative relocation to new insertion sites. Class II elements are used for germ-line transformation of insect species and those used belong to a sub-class of Class II elements referred to as short inverted repeat type elements. Full-length versions of these elements comprise a transposase gene flanked by short inverted repeats at each end of the element. The transposase recognises and acts on the element, especially the terminal repeats; together with host factors this is sufficient to allow transposition. Since such elements can mediate their own transposition, they are known as 'autonomous' elements. Deleted or modified versions lacking a functional transposase gene can still transpose if the transposase is provided by other means; such elements are 'non-autonomous' as they rely on an external source of the appropriate transposase. Consequently, molecular biologists can replace the transposase gene with arbitrary sequence, and still induce the resulting engineered non-autonomous transposon to 'jump' or transpose from plasmid to chromosome by supplying transposase. In practice the transposon is micro-injected into syncytial (not yet cellularised) embryos with a second 'helper' plasmid that encodes transposase but lacks the terminal sequences of the transposon and is therefore itself unable to transpose. Synthetic mRNA encoding the transposase may be used in place of the helper plasmid (Kapetanaki *et al.*, 2002). Insertions are essentially random within the genome. The transposons used for insect transformation generate short target site duplications on insertion. The target may need

to have a specific sequence to permit insertion, but these sequences are so short as to be very frequent, for example *piggyBac* inserts only in the tetranucleotide sequence TTAA. Consequently insertions are essentially randomly-located within the genome. The transposons most frequently used as vectors for non-Drosophilid insect germ-line transformation are: a) *Hermes*; b) the *mariner* element *Mos1*; c) the *TC1/mariner element Minos*; and d) *piggyBac* (Wimmer, 2003; Scolari *et al.*, 2008), of which the most commonly used is *piggyBac*.

Hermes was discovered in the housefly, *Musca domestica*, and is a member of the hAT family of transposons, most closely related to hobo (Warren *et al.*, 1994). *Minos* and *mariner* are members of the mariner/Tc family. *Minos* from *D. hydei* is closely related to Tc elements originally discovered in nematodes (Franz & Savakis, 1991). *Mariner* was discovered in *D. mauritiana* (Medhora *et al.*, 1988). The *piggyBac* transposon is part of a subclass of elements that insert exclusively in TTAA target sites and was identified as a sequence responsible for a mutation of a virus introduced into the cells of the cabbage looper moth *Trichoplusia ni* (Fraser *et al.*, 1996). Each of these elements belongs to large, divergent families of transposons which are found across a wide phylogenetic range (Robertson *et al.*, 2002; Sarkar *et al.*, 2003; Burt & Trivers, 2006).

DNA is delivered for transformation by micro-injection. The plasmid carrying the non-autonomous transposon vector and its DNA or RNA helper are co-injected into syncytial embryos (Gilbert, 2000). Since the intention is to get the DNA into the germline cells, injection aims to place the DNA in the region of the embryo where the germline precursor cells will later form, the posterior pole. Very young embryos are used, as injection must be done before the germline cells form. The time from oviposition to cellularisation ranges from 1-2 h in *D. melanogaster*, to >3 h in some Tephritids. Embryos that survive the injection process and develop to form fertile adults are termed Generation 0 or G₀. The intention is that transposition in their germline will result in germline mosaics; gametes forming from a transformed germline cell will then produce fully-transformed offspring. The G₀ insects are therefore back-crossed to an appropriate non-transgenic strain and the resulting progeny, the G₁, screened for transformed individuals. Stable transformation requires the transferral of the transgene to the offspring. The reported efficiency of most transformation systems is around 2-5% (Atkinson *et al.*, 2001). Utilisation of the above transposons has led to transformation of a number of insect species spanning

the orders Diptera, Lepidoptera, Coleoptera and Hymenoptera. Table 1 shows insect species transformed to date by transposon-mediated transgenesis.

Table 1. Summary of transposable element-mediated stable germline transformation of non-drosophilid insect species (modified from Morrison *et al.*, 2010).

	Species	Transposable element	Reference
Mosquitoes	Yellow fever mosquito (<i>Aedes aegypti</i>)	<i>Mariner</i>	Coates <i>et al.</i> , 1998
		<i>Hermes</i>	Jasinskiene <i>et al.</i> , 1998
		<i>piggyBac</i>	Kokoza <i>et al.</i> , 2001
	Asian tiger mosquito (<i>Aedes albopictus</i>)	<i>piggyBac</i>	Labbé <i>et al.</i> , 2010
	<i>Aedes fluviatilis</i>	<i>piggyBac</i>	Rodrigues <i>et al.</i> , 2006
	New World malaria mosquito (<i>Anopheles albimanus</i>)	<i>piggyBac</i>	Perera <i>et al.</i> , 2002
	African malaria mosquito (<i>Anopheles gambiae</i>)	<i>piggyBac</i>	Grossman <i>et al.</i> , 2001
	Indo-Pakistan malaria mosquito (<i>Anopheles stephensi</i>)	<i>Minos</i>	Catteruccia <i>et al.</i> , 2000
		<i>piggyBac</i>	Ito <i>et al.</i> , 2002; Nolan <i>et al.</i> , 2002
Southern house mosquito (<i>Culex quinquefasciatus</i>)	<i>Hermes</i>	Allen <i>et al.</i> , 2001	
Fruit flies	Mexican fruit fly (<i>Anastrepha ludens</i>)	<i>piggyBac</i>	Condon <i>et al.</i> , 2007
	Caribbean fruit fly (<i>Anastrepha suspensa</i>)	<i>piggyBac</i>	Handler & Harrell, 2001
	Oriental fruit fly (<i>Bactrocera dorsalis</i>)	<i>piggyBac</i>	Handler & McCombs, 2000
	Olive fly (<i>Bactrocera oleae</i>)	<i>Minos</i>	Koukidou <i>et al.</i> , 2006
	Queensland fruit fly (<i>Bactrocera tryoni</i>)	<i>piggyBac</i>	Raphael <i>et al.</i> , 2010
	Mediterranean fruit fly (<i>Ceratitis capitata</i>)	<i>piggyBac</i>	Handler <i>et al.</i> , 1998
		<i>Hermes</i>	Michel <i>et al.</i> , 2001
		<i>Minos</i>	Loukeris <i>et al.</i> , 1995

Other Diptera	Housefly (<i>Musca domestica</i>)	<i>piggyBac</i>	Hediger <i>et al.</i> , 2001
		<i>Mariner</i>	Yoshiyama <i>et al.</i> , 2000
	Stable fly (<i>Stomoxys calcitrans</i>)	<i>Hermes</i>	O'Brochta <i>et al.</i> , 2000
	Australian sheep blowfly (<i>Lucilia cuprina</i>)	<i>piggyBac</i>	Heinrich <i>et al.</i> , 2002
	Common green bottle fly (<i>Lucilia sericata</i>)	<i>piggyBac</i>	Concha <i>et al.</i> , 2011
	New World screwworm (<i>Cochliomyia hominivorax</i>)	<i>piggyBac</i>	Allen <i>et al.</i> , 2004
	Stalk-eyed fly (<i>Teleopsis dalmanni</i>)	<i>Minos</i>	Warren <i>et al.</i> , 2010
Wasps, bees and ants	Sawfly (<i>Athalia rosae</i>)	<i>piggyBac</i>	Sumitani <i>et al.</i> , 2003
Beetles	Harlequin ladybird (<i>Harmonia axyridis</i>)	<i>piggyBac</i>	Kuwayama <i>et al.</i> , 2006
	Red flour beetle (<i>Tribolium castaneum</i>)	<i>piggyBac</i> & <i>Hermes</i>	Berghammer <i>et al.</i> , 1999
		<i>Minos</i>	Pavlopoulos <i>et al.</i> , 2004
Butterflies and moths	Squinting bush brown butterfly (<i>Bicyclus anynana</i>)	<i>piggyBac</i> & <i>Hermes</i>	Marcus <i>et al.</i> , 2004
	Pink bollworm (<i>Pectinophora gossypiella</i>)	<i>piggyBac</i>	Peloquin <i>et al.</i> , 2000
	Silkworm (<i>Bombyx mori</i>)	<i>piggyBac</i>	Tamura <i>et al.</i> , 2000
		<i>Minos</i>	Uchino <i>et al.</i> , 2007

One area of concern for the release of transgenic insects for SIT is associated with the stability of the transgene vector and also of the expression of transgenes within it. In order to evaluate the possible risks associated with the release of transgenic insects, one needs to understand the system(s) used for the transfer of exogenous DNA into the insect's genome. As mentioned above, insect transformation is routinely mediated by transposable elements. Integrated vectors are non-autonomous transposons; that is, they require an external source of transposase for re-

mobilisation. Upon introduction into the embryo germ cells, the helper DNA plasmid or mRNA of the transposable element's transposase (see above) mediate transposition of the vector into the insect's genome, but the transposase source itself is unable to integrate. Therefore, there is no transposase source available in subsequent generations to facilitate remobilisation of the transgene.

In the absence of suitable transposase, the integrated transposon or transgene is as stable as any other gene. The issue to be addressed therefore is whether and how the transposon might be exposed to a suitable transposase, and what the consequences of such exposure might be. Transposons comprise 10% or more of the genome of *Drosophila* and other insects, however many of these copies are degenerate. Furthermore, each transposon encodes a specific transposase. Cross-mobilisation between two closely-related transposons has been detected (Sundararajan *et al.*, 1999), but in general transposases from one type of transposon are incapable of mobilising transposons of another type. If a suitable transposase is not already present in the insect's genome, the likelihood of exposure to a transposase after integration is extremely low. Were it somehow to happen, the most likely consequence – after 'no effect' – is excision and consequent loss of the transposon. Loss of part of the transposon is also possible, as is mobilisation to another site within the genome. Phylogenetic analysis shows that autonomous transposons are capable of moving from one species to another (horizontal gene transfer, HGT) over million-year time-scales (Silva & Kidwell, 2000; Robertson *et al.*, 2002; Lampe *et al.*, 2003). However, this rate would be greatly reduced for artificial non-autonomous transposons due to: (i) the need to provide exogenous transposase both to excise and to integrate; (ii) the smaller number of potential donor elements; (iii) the smaller number of potential donor individuals, and; (iv) the larger size and hence lower mobilisation rates of engineered transposons (Robertson *et al.*, 1988; Handler & Harrell, 1999; Geurts *et al.*, 2003). For any transposase-mediated event, a functional mobilisation system associated with the same or similar transposable element must be in place. Individual laboratory strains can be thoroughly tested for the existence of transposase sources, but the hypothetical risk of a transgene movement in the field remains. Previous studies have raised concern regarding the possibility of inter-genomic movement (Handler *et al.*, 2004), yet no direct evidence exists to date to support this concern for non-autonomous transposons (Lee & Langley, 2010). Even the presence of *piggyBac* elements in *B. oleae* very

closely related to the vector *piggyBac* did not lead to obvious instability of *piggyBac* elements in that species (Handler & McCombs, 2000). To reduce or eliminate even this small possibility of genetic instability, post-integration stabilisation methods have been developed (Handler *et al.*, 2004; Dafa'alla *et al.*, 2006). In the method proposed by Handler *et al.* (2004) removal of one terminus of the transgene was achieved in *D. melanogaster*; whereas Dafa'alla *et al.* (2006) removed both of the *piggyBac* transposon inverted terminal repeats in medfly, thereby rendering the transgene as stable and inert to transposase as any other gene in the insects' genome. Studies have also been conducted in both *Aedes* and *Anopheles* mosquito species on the potential re-mobilisation of *piggyBac*. It was found that in *Ae. aegypti* re-mobilisation is non-existent under a wide range of conditions (O'Brochta *et al.*, 2003; Sethuraman *et al.*, 2007), whereas in *An. stephensi*, re-mobilisation of *piggyBac* appears to occur (O'Brochta *et al.*, 2011). Consequently the potential for re-mobilisation of the transposons appears to be highly case-specific.

2.2. Uses of GM insects

There is a range of uses for GM insects, from evolutionary biology and basic laboratory research to the use of insects as factories for production of proteins, and also for pest control in both agriculture and public health areas. Some current research activities with GM insects are listed in Table 2.

Table 2. Examples of GM insects, potential uses and current status

Insect species	Potential use of modified organism	Current status	Reference
Pink bollworm (<i>Pectinophora gossypiella</i>)	Improvements to the sterile insect technique	Open field and programmatic scale trials	USDA, 2008; 2009
Yellow fever/dengue mosquito (<i>Aedes aegypti</i> [Skuse])	For the control of vectors transmitting dengue	Open field trials	Phuc <i>et al.</i> , 2007; Fu <i>et al.</i> , 2010; Harris <i>et al.</i> , 2011; Hoffman <i>et al.</i> , 2011; O'Brochta <i>et al.</i> , 2011
Mediterranean fruit fly (<i>Ceratitis capitata</i>)	Protein production	Laboratory research	Markaki <i>et al.</i> , 2007

	Improvements to the sterile insect technique	Laboratory research and confined field trials	Gong <i>et al.</i> , 2005; Fu <i>et al.</i> , 2007; Schetelig <i>et al.</i> , 2009
Olive fly (<i>Bactrocera oleae</i>)	Development of the sterile insect technique	Laboratory research and contained field trials	Ant <i>et al.</i> , 2011b; Koukidou <i>et al.</i> , 2011
Cabbage looper moth (<i>Trichoplusia ni</i>)	Production of recombinant antibodies	Laboratory research	O'Connell <i>et al.</i> , 2007
	Developmental biology	Laboratory research	Tamura <i>et al.</i> , 2000; Shukla <i>et al.</i> , 2011
<i>Bombyx mori</i> and other silkworms	Protein production	Laboratory research and potential contained commercial production	Tamura <i>et al.</i> , 2000; Kato <i>et al.</i> , 2010; Wen <i>et al.</i> , 2010
Honey bee (<i>Apis mellifera</i>)	Insecticide resistance	Laboratory research	Kimura, 1997
	Comparative genomics	Laboratory research (transient expression only)	Robinson <i>et al.</i> , 2000; Evans & Weaver, 2003
<i>Aedes fluviatilis</i>	Inhibition of the malaria parasite	Laboratory research	Rodrigues <i>et al.</i> , 2006
<i>Anopheles albimanus</i>	Inhibition of malaria parasite	Laboratory research	Perera <i>et al.</i> , 2002
<i>Anopheles gambiae</i>	Inhibition of the malaria parasite	Laboratory research	Catteruccia <i>et al.</i> , 2000; Moreira <i>et al.</i> , 2002
<i>Culex quinquefasciatus</i>		Laboratory research	Allen <i>et al.</i> , 2001

*this table is not exhaustive

2.2.1. Research

Basic research accounts for the majority use of GM insects, with *D. melanogaster* having by far the major focus. Very large numbers of transgenic strains of *Drosophila* are created, maintained and shipped around the world by many laboratories. Containment levels are generally low and regulatory

requirements minimal, in part because *D. melanogaster* itself is considered to have a long history of safe use.

2.2.2. Using insects as factories

This is perhaps one of the aspects of GM insects that is most speculative or at the earliest stages of development. One application at the forefront is the use of the economically-important silkworm (*Bombyx mori*). Baculovirus-mediated transgenesis of silkworm was first described by Maeda et al. (1985) for the production of human interferon. Germ-line transformation of the silkworm has now been achieved with transposon-derived vectors, *piggyBac* (Tamura et al., 2000) and *Minos* (Uchino et al., 2007). Many pharmaceutical/veterinary recombinant proteins have now been expressed in silkworm life stages for both industrial and research purposes (Tomita et al., 2003; Pew Initiative on Food and Biotechnology, 2004b; Kato et al., 2010). Commercial protein production is now being considered. Similarly, transgenic silkworms have been used for the production of modified silk containing protein molecules to alter the characteristics of the silk, particularly trying to integrate the properties of spider silk with production capacity of the silkworm (Kato et al., 2010; Wen et al., 2010). Other applications for silkworm transgenesis include engineering resistance to disease and pathogens in the silkworms, as pathogen-induced morbidity and mortality has major economic impact in sericulture. Genetic sexing is also a desirable trait in the production of silk as male silkworms produce more silk than females (Nagaraju, 2002). Other applications in this area include the transformation of medfly to express human growth hormone (Markaki et al., 2007) and the expression of antibody fragments in whole insect larvae of *T. ni* (O'Connell et al., 2007).

There is anecdotal evidence, reported by Benedict (et al. 2010), that *Dactylopius confusus*, the cochineal beetle, is being transformed for both increased yield and altered pigment production, but results of this work are not yet publicly available. Transient expression of transgenes in honey bee (*Apis mellifera*) has also been reported (Robinson et al., 2000; Evans & Weaver, 2003).

2.2.3. Agriculture

Growth in the human population, combined with social changes, is leading to rising demand for food, which must be produced sustainably and safely.

Integrated pest management (IPM) methods are increasingly being adopted to produce crops and food in a safe and sustainable manner, especially with public demand for reduced pesticide residues in food and the growing difficulty in developing and registering new pesticides. IPM combines biological, cultural, physical and chemical tools to manage pest damage by the most economical means, and with minimal risk to health and the environment. SIT has allowed the removal of many insect pests; New World screwworm (*Cochliomyia hominivorax*) from North America and most of Central America (reviewed in Klassen & Curtis, 2005) whilst Unguja Island in Tanzania was freed of tsetse fly (*Glossina austeni*) through sterile male releases (Vreysen *et al.*, 2000). On-going releases of sterile male medfly in Guatemala, Mexico and the USA provide effective control for citrus producers in this region: the El Piño mass-rearing facility in Guatemala produces up to 3.5 billion sterile medfly males per week (Cáceres *et al.*, 2008).

Morrison *et al.* (2010) summarised these and other programmes, and discussed past and future improvements to SIT enabled by genetic technology. Since the 1990s, genetic sexing strains of medfly have been used for SIT around the world, with male-only release considered more efficient than bi-sex release (Rendón *et al.*, 2000). These strains, generated by conventional genetics, carry mutations (with rescuing wild-type alleles translocated to the male Y autosome) that allow for sex separation, and thereby male-only release, to be conducted (Franz, 2005).

Transgenic technology has not only enabled new strains of medfly to be developed, but has also permitted genetic sexing (Fu *et al.*, 2007) with a heritable marker thereby replacing the need for sterilisation by irradiation (Gong *et al.*, 2005; Fu *et al.*, 2007; Schetelig *et al.*, 2009). Further, transgenic technology can be applied to a broad range of species, with transgenic sexing strains also generated in the Mexican fruit fly (*Anastrepha ludens*; Koukidou *et al.*, 2008), the olive fly (*B. oleae*; Koukidou *et al.*, 2011) and even the dengue mosquito (*Ae. aegypti*; Fu *et al.*, 2010).

Transgenesis can provide significant advances in current SIT programmes but even more importantly, may help in implementing pest management programmes that otherwise would not have been possible. An example is the olive fruit fly, the most destructive pest of olive fruit, causing considerable crop damage in the Mediterranean region and in California.

Previous SIT attempts using irradiated mixed-sex insects achieved only limited success at suppressing populations of olive fly. The released sterile males mated with the released sterile females, instead of dispersing and seeking the wild females. It was believed at the time that sterile flies preferred to mate earlier in the day, leading to partial reproductive isolation from the wild population (Economopoulos & Zervas, 1982; Estes *et al.*, 2011). Data indicated that a genetic sexing system to allow male-only release would overcome the sexual asynchrony of the mass-reared flies. Consequently, a genetic sexing system is seen as essential for olive fly SIT. The medfly genetic sexing strains are a triumph of classical genetics; however none of the mutations and special chromosomes can be transferred to other species. For other insect pests, therefore, where none of the necessary mutations and chromosomal rearrangements are available, the use of genetic transformation approaches will be a more efficient way of developing genetic sexing strains (Franz & Robinson, 2011). The development of such strains greatly improves the prospects for the use of SIT to control field populations of olive fly and other pests for which classic SIT would be extremely challenging to use.

In SIT, sterilisation by irradiation and associated handling steps can reduce the quality - and therefore efficiency - of released insects (Holbrook & Fujimoto, 1970; Hooper & Katiyar, 1971; Mayer *et al.*, 1998; Cayol *et al.*, 1999; Lux *et al.*, 2002). An alternative means of providing the effect of sterilisation is therefore an attractive prospect. Medfly strains have been developed that, in the absence of artificial dietary supplements (in the wild, for example), produce no viable progeny (Gong *et al.*, 2005, Schetelig *et al.*, 2009). Male-sterile strains, in which sperm are damaged or killed, may also provide a means of radiation replacement that combines well with male-only release (Scolari *et al.*, 2008).

To allow detection of the presence of the transgene whilst rearing and in the field, these strains all carry a genetic marker expressing a fluorescent protein that can be seen using a suitable epi-fluorescence microscope. A transgenic strain of the cotton pest moth, pink bollworm (*Pectinophora gossypiella*), transformed only with a transgenic fluorescent marker (the red fluorescent protein, DsRed2), was developed to provide the existing SIT programme in south western USA with a more reliable marker of released (and irradiated) moths (Simmons *et al.*, 2011). This strain has undergone a series of cage and open-field trials to assess its suitability for SIT. In 2006-

2008, in the world's first open-field trials of a transgenic insect, the strain was radiation-sterilised and released in large numbers (over 15 million in 2008) over cotton crops in Arizona, USA, for performance assessment and programmatic demonstration.

Medfly transgenic sexing strains have undergone contained greenhouse trials to assess mating performance of males (Morrison *et al.*, 2009; Schetelig *et al.*, 2009). Similar sexing strains of olive fly were recently shown to suppress a wild-type population of olive flies in large cages (Ant *et al.*, 2011b).

2.2.4. Public health

Historically, chemical and other vector control methods (physical, environmental, biological and social/behavioural management) have helped to control some diseases (e.g. malaria and dengue) and remain the primary choice for vector control today. However, many current control approaches, including chemical controls, face increasing challenges for their use and implementation and there is a renewed demand for innovative vector control methods. In response, the genetic modification of the vector itself is a key strategy and this is thought to have considerable potential benefits for vector control, including:

- a) Specific
 - Targets a single insect pest species,
 - Chemical-free and could reduce the need for insecticides overall,
- b) Comprehensive coverage
 - Populations of mosquitoes that are inaccessible to existing control methods could be controlled using the ability of the male mosquito to find female mosquitoes,
- c) Equity
 - Will protect all individuals in the release area, irrespective of their, power, wealth, or status,
- d) Little human behavioural modification required for success
 - Some current methods require very high level levels of community participation in the control method (e.g. correct use of bed nets, emptying flowerpots, opening windows for space sprays etc.),
 - Complementary to existing integrated vector control (IVM) methods.

3. REGULATORY ASPECTS OF GM INSECTS FOR PEST CONTROL

3.1. International initiatives

Since 1991, the World Health Organization (WHO) and its Special Training

Programme in Neglected Tropical Diseases (TDR) has been taking a lead on considering the issues raised by the genetic modification of insects that are vectors of human disease (WHO/TDR, 1991) by hosting international expert consultations and other fora (Takken *et al.*, 2002; Knols *et al.*, 2006; Beatty *et al.*, 2009; WHO, 2009). In 2008, WHO/TDR funded a three-year project to examine the “Best-Practices for the use of GM mosquitoes for the control of Dengue and Malaria in disease endemic countries” with the objective of providing guidance and support to national decision-making on the use of GM mosquitoes (Mumford *et al.*, 2009; www.MosqGuide.org.uk). At the same time, it funded three three-year regional biosafety training courses on GM vectors to create networks of professionals trained for decision-making and the safe use of GM mosquitoes for vector control on a regional basis. The three regional biosafety training courses were centred at the University of Bamako, Mali; the Centre for Medical Entomology Research in Madurai, India; and PECET, University of Antioquia, Colombia. WHO/TDR is also engaged with the Foundation for National Institutes of Health (FNIH) to write a framework document for the use of GM insect vectors (WHO, 2009) that will aim to foster standardisation of procedures, comparability of results, as well as legal, ethical, social and cultural issues that should be considered in the testing of GM vectors. Although not yet published, compliance with the principles proposed in the framework document should assist with harmonised technical and ethical standards and facilitate national decision-making on the deployment of GM insect vectors as a public health tool for the control of malaria and dengue. Other biosafety, risk assessment and ethical social and cultural initiatives in the field of GM vectors and insects are underway. These were reviewed in Beech (2009b) and include activities by the United Nations Development Program in Malaysia (NRE, 2009; www.undp.org.my/), Grand Challenges in Global Health (GCGH; www.grandchallenges.org), and the Cartagena Protocol on Biosafety (CPB) Ad Hoc Technical Expert Group on Risk Assessment and Risk Management (AHTEG; http://bch.cbd.int/onlineconferences/ahteg_ra.shtml).

A phased testing approach is considered as an appropriate way to assess risk for new technologies in a wide range of fields including GM crops, chemicals and pharmaceuticals (USA EPA, 2000; Romeis *et al.*, 2008). A step-wise approach has also been taken in the evaluation of GM insects. Phases can include the following, which may or may not always be sequential, depending on facilities, experience and mutual recognition of data:

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- a) Laboratory testing in contained use conditions
 - b) Confined field testing
 - c) Open field release
 - d) Pilot operational evaluation

3.2. Contained use

Transformation of, and research with, GM insects in contained use (laboratories and quarantine facilities) is widespread and non-controversial, following established guidance for recombinant organisms (WHO, 2004a; WHO, 2004b; OGTR, 2006; Department of Biotechnology & Biotech Consortium India Ltd, 2011; NIH, 2011). Containment refers to practices that prevent unplanned or uncontrolled releases of organisms into the environment and is likely to encompass physical structures, standard operating procedures and working practices and the use of trained staff. The small sizes, high degree of mobility and in some cases long lifespan represent unique challenges in the physical containment of arthropods. Procedures are frequently species-specific and containment measures should relate to the ability of the arthropod to survive outside of the laboratory environment. Where arthropods are infected with pathogens and represent a high risk of infection or cause life-threatening diseases, they are likely to be placed in the highest biosafety category. The assignment of biosafety categories is based on risk assessment, and the containment levels are designed to manage the risk of release to the environment and protection of the public as well as to minimise exposure by laboratory workers in the facility. Risk assessment is a qualitative judgement considering the following parameters: the mobility and longevity of the arthropod, its reproductive potential, the potential for transmission of diseases in the proposed location, whether it is native or introduced in the locality, whether the arthropod is infected and what diseases it could transmit. Risk management measures can include: worker protection; physical barriers and levels of containment; operating procedures and training of staff in the rearing, handling, transport and disposal of insects; monitoring for escapees; cleaning and hygiene (NB not an exhaustive listing). There are well-accepted guidelines and publications for laboratory research with arthropods (Hoy *et al.*, 1997; Benedict *et al.*, 2003; OGTR, 2006; Benedict, 2009) from which broad risk categories can be identified:

- a) Arthropods known to be free of specific pathogens,
- b) Arthropods known to contain specific pathogens,
- c) Arthropods that may contain infectious agents or have unknown infection status,

- d) Arthropods expressing recombinant DNA (rDNA) – *risk assessment determines the containment level here, but uninfected GM arthropods that are no more dangerous than the unmodified counterpart are likely to have containment levels the same as the uninfected unmodified organism.*

In the case of arthropods expressing rDNA, the challenge is to determine the *differential in risk* between the unmodified organism and the recombinant organism. Some questions that could help determine this risk differential are:

- Does the inserted gene(s) encode a product(s) that is/are likely to alter the vector competence for the diseases it transmits?
- Does the modification increase the geographic or host range of the host organism?
- Does the modification change the reproductive potential of the host organism?
- Does the modification change the susceptibility to agents that can be used to control the host organism i.e. insecticides?
- Does the modification confer a selective advantage or disadvantage to the host organism?
- Is the modification self-limiting or self-sustaining in the environment?

Containment facilities for GM insects are likely to require official inspection and certification by national regulatory authorities, along with a case-specific risk assessment for the proposed work within the containment facility. The risk assessment may need to be submitted to, and approved by, an institutional biosafety committee prior to work taking place in the laboratory. Additional permits may be required, such as veterinary inspections of transportation packages prior to, or during shipment as insects are frequently regarded as live animals for customs inspection purposes. Additionally, the shipping of living modified organisms (LMOs) for contained use requires labelling in accordance with national and international requirements (Article 18 of the CPB). Guidance on the contained use of GM mosquito vectors has been summarised in a project, known as MosqGuide, sponsored by the WHO/TDR (WHO/TDR MosqGuide Module 2¹.)

3.3. Confined release

Confined release has been broadly defined by the North American Plant

¹ www.mosqguide.org.uk/Documents/Module2_Nov10.doc

Protection Organisation (NAPPO, 2007) to include not only physical confinement, such as caged releases, but also releases where the establishment and spread of the transgenic arthropods is restricted by biological, temporal, or geographic mechanisms. Confined release often forms a key step in the phased testing of transgenic arthropods and contributes to the evaluation of the strains for open release, by providing a semi-natural or larger natural environment to conduct experiments. Field cages are often temporary facilities in which research is carried out with arthropod vectors. They can be regarded as a large insectary; however the difference is that if there are GM insect vector escapees from a field cage, the vector may become established in the environment, depending on the trait that has been introduced into the mosquito. Field cages and protocols for the use of GM and SIT mosquitoes have been reviewed by Knols *et al.* (2003), Ferguson *et al.* (2008), and Helinski (2008). A semi-field system and protocol for contained trials of a self-limiting GM *Ae. aegypti* in Mexico is described by Facchinelli *et al.* (2011), and Chambers *et al.* (2011) details a semi-field cage design and results of experiments with *Wolbachia*-introgressed *Ae. polynesiensis*, the vector for lymphatic filariasis in the South Pacific. Examples of experiments that could be conducted in such environments are those that investigate possible changes in fitness and /or behaviour of the host organism (Scott *et al.*, 2006) and include mating competitiveness, and suppression of a closed population of insects. Such tests have been conducted in a large cage or semi-natural setting on a variety of genetically-sterile male insects: *Ae. aegypti* (Clark *et al.*, 2010; Wise de Valdez *et al.*, 2011), olive fly (Ant *et al.*, 2011a) and medfly (Morrison *et al.*, 2009; Schetelig *et al.*, 2009), and pink bollworm (Simmons *et al.*, 2011). The International Atomic Energy Agency (IAEA) have published guidance on routine quality control tests required for Tephritid fruit flies, and include experimental details regarding mating performance tests conducted in field cages (FAO/IAEA/USDA, 2003). Considerations for conducting confined trials of insects that contain a gene-drive mechanism have also been published (Benedict *et al.*, 2008). Protocols and procedures for the use of semi-natural environments is currently a fertile area of research and the scientific literature is developing rapidly.

3.4. Field release of GM insects

Field trials play a critical role in the evaluation of GM insects, particularly for assessing mating competitiveness, longevity and dispersal, and for determining the efficacy of the intervention in an environmental setting

typical of the insect species. Field site selection criteria are an important consideration and will be largely dependent on trial objectives and design, however there are three main considerations: scientific/technical requirements, community engagement and ethical consideration, and regulatory approval/acceptability.

3.4.1. Scientific/technical requirements

Scientific considerations have to be considered in the context of the trial objectives; however there are some general issues that should be addressed in most trials involving GM insects:

- *Presence of insect species*: Perhaps the most important parameter is that the target insect species should be present in sufficient quantities, if not abundant. If the population is too small it may not be possible to demonstrate efficacy of the intervention statistically. Baseline studies on the existing population of insects at the trial sites are essential to determine its presence and stability.
- *Field size*: The size of the trial will require a trade-off between the accuracy and validity of the study versus the resources it will require. In practice, small sites are selected that are large enough to deliver the objectives of the trial. A compact site is preferable for logistics of running the trial. Additional considerations for GM mosquitoes are the area of the site chosen versus the human population density.
- *Geographic isolation*: The site should ideally be geographically-isolated by some means (habitat, water etc.). This is principally to minimise the immigration of insects from adjacent untreated areas which could compromise the efficacy of the intervention. If geographically-isolated areas are not available, a buffer zone concept may be used around the test site. The buffer zone is treated the same as the experimental area but is not included in the sampling and monitoring processes.
- *Ecological stability*: The site should be ecologically-stable to limit the number of variables that could impact the results of the trial. In many regions, the size of the insect population fluctuates seasonally and this should be taken into account when considering the timing of the trial.
- *Presence of control sites*: Field sites should have control or comparable sites nearby, although this might not be feasible for large-scale intervention trials. In these cases, historical data on the native insect population (and also disease incidence, in the case of mosquitoes) from the local or national health authority may serve as an appropriate comparator.

Factors specific to release of sterile GM mosquitoes:

- *Human population density*: Consideration should be given to conducting a trial in a site with or without human habitation. The limitation for *Aedes* mosquitoes of conducting a trial in an uninhabited site is that there may not be any local female mosquitoes with which the released male mosquitoes may mate. Density of human inhabitation can influence the location of the trial site, as described in 'Field size' above.
- *Presence of disease*: It is the general goal of vector control interventions to ultimately prevent the spread of the vectored disease, through the reduction of the vector insect. Therefore the site should be endemic for the disease if the objective of the study is to look at transmission thresholds or demonstrate control of the disease.

3.4.2. Community engagement and ethical approval

Community engagement (CE) is a key component of acceptance and uptake of novel public health interventions. Early and effective community engagement can help to offset some of the uncertainty and corresponding need for precaution in the introduction of novel technologies (El Zahabi-Bekdash & Lavery, 2010), particularly those based on genetic modification, for which GM crops continue to be the subject of entrenched and polarised positions (Tait, 2009). The use of new drugs and vaccines delivered to individuals represents a counter position, where much effort and harmonisation of protocols for ethical consideration has taken place in the area of clinical interventions (CIOMS, 2009). However there is very little consensus, harmonisation or development of community engagement protocols for area-wide interventions in public health, such as fluoridation of water or insect-borne disease control (O'Neill, 2011a). With regard to the testing and introduction of innovative vector control strategies for use in the open field, such as genetic control of insect vectors described in this review, there has been some guidance to date (Macer, 2005; Lavery et al., 2008; Kilama, 2009; Lavery et al., 2010), but as Lavery et al. (2010) conclude

"remarkably there is no explicit body of community engagement knowledge to which researchers can turn for guidance about approaches that are most likely to be effective in different contexts..... Thus, CE practices remain as much as art as science and what makes them effective is still determined largely by a combination of intuition, experience and opinion".

However, in the absence of this guidance, community engagement and ethical approval to facilitate informed decision-making regarding trials involving GM insect vectors are an essential element in developing trust and removing uncertainty in the public health sector.

3.4.3. Regulatory approval

The first permit for the release of a GM arthropod species was in respect of a GM predatory mite in the USA in 1996 (Hoy, 2000). Since then, GM strains of the globally important cotton pest moth, pink bollworm, have been tested in both caged field and open release trials in the USA since 2002. These releases were subject to extensive regulatory review and environmental assessment. The first environmental assessment (EA) was published in the USA Federal Register in 2002, for a cage trial of transgenic pink bollworm carrying a fluorescent marker gene (USDA, 2002). A further EA was published in 2005 for an open field release of the same insect (USDA, 2006). A Final Environmental Impact Statement (EIS), in accordance with the requirements of the National Environmental Policy Act of 1969 (NEPA) has also been prepared by the US Department of Agriculture Animal and Plant Health Inspection Services (APHIS) for the use of genetically engineered (GE) fruit fly and pink bollworm in APHIS plant pest control programmes in 2008 (USDA, 2008; Rose, 2009). A Record of Decision published in the Federal Register in 2009 concluded that GE fruit fly and pink bollworm were the “*environmentally preferable alternative*” (USDA, 2009). Researchers and other commentators have also considered the use of GM insects from other contexts: legal and regulatory aspects (Pew Initiative on Food and Biotechnology, 2004; Wozniak, 2007; Donovan, 2009), safety (Benedict *et al.*, 2011; Osters & Gostin, 2011), and transformation of other insect species (see Table 1).

Since 2009, there has been rapid progress in the field of innovative genetic vector control strategies and in 2010 decisions were taken by three countries to progress with open release of GM mosquitoes. The first field trial was performed in the Caribbean region, in the Cayman Islands in 2009 and subsequent trials have been conducted in 2010 on Grand Cayman, in Malaysia and Brazil. With the exception of the Cayman Islands, the releases in Malaysia and Brazil have been regulated under GMO regulations and data requirements. Typical information required by regulators includes administrative information, information on the containment strategy, information on welfare and ethical use, information on the receiving environment and release characteristics including an emergency response plan and risk assessment. These trials are variously described:

- *Grand Cayman*: Open releases of self-limiting GM *Ae. aegypti* mosquitoes were conducted in Grand Cayman, Cayman Islands in 2009 and 2010 by the Mosquito Research and Control Unit (MRCU). MRCU activities are

governed by the Mosquito Research and Control Act (2007 Revision). Importation and release of the mosquitoes was conducted under permit from the Cayman Islands Department of Agriculture, in accordance with the Draft National Conservation Act and the Animal Law (2003). A risk assessment was required which is in the public domain². Manuscripts on the results of these trials are in preparation and have been, or will be submitted, to peer-reviewed journals. Initial results have recently been published and indicated that the released GM sterile male *Ae. aegypti* successfully mated with wild females (Harris *et al.*, 2011) and that with sustained releases of the genetically-sterile males, the local *Ae. aegypti* population could be suppressed.

- *Malaysia*: The Malaysian Ministry of Natural Resources and Environment has been very proactive in capacity building regarding GMOs, and co-sponsored a workshop on the risk assessment of transgenic insects in November 2009 (NRE, 2009) as an activity towards the implementation of their primary legislation on GMOs, the Biosafety Act 2007. One of the outputs of this workshop was the publications of a hypothetical risk assessment on the open field release of a GM *Ae. aegypti* for the control of the vector that transmits dengue fever (Beech *et al.*, 2009a). Malaysia subsequently developed implementing regulations in 2010. One of the first open field releases to be evaluated under the Act was of self-limiting *Ae. aegypti* mosquitoes. In October 2010, the National Biosafety Board (NBB), acting on the positive advice of the Genetic Modification Advisory Committee (GMAC), approved an open field release of genetically-sterile self-limiting *Ae. aegypti* by the Institute for Medical Research (IMR) (GMAC, 2010). The release was conducted in an atypical environment for the insect, in an uninhabited forest in the state of Pahang in December 2010. Results have been presented at a scientific conference (Vasan, 2011) and to GMAC; a manuscript describing this work is in preparation for publication in the scientific literature. Further releases are envisaged under the same authorisation. The Malaysian Biosafety Act 2007 has been criticised as inadequate with regard to bio-ethical considerations (Hamin & Idris, 2011), specifically public information and consensus, but in this case, the Government were proactive in seeking public consultation prior to the trial in a variety of media: national newspapers, on the NRE website, and at the community level with public meetings in local languages, Mandarin and Basaha Malay (Fong, 2011).

² House of Lords Library, deposited paper 2011-0053. Available at <http://www.parliament.uk/deposits/depositedpapers/2011/DEP2011-0053.pdf>

- *Brazil*: Brazil regulates GMOs under Law 11.05 (March 2005), and has a long history of open releases of GMOs, primarily in agriculture, and consequently has a mature and well-developed regulatory framework. In December 2010 the National Biosafety Technical Commission (CTNBio) approved an open field release of self-limiting GM *Ae. aegypti* mosquitoes to suppress a wild population of *Ae. aegypti* in up to five field sites in the Bahia region¹¹.

3.4.4. Other regulatory initiatives

- *Cartagena Protocol on Biosafety (CPB)*: At the fourth Conference of the Parties, serving as the meeting of the Parties to the CPB, a number of sub-working groups were formed under the Ad Hoc Technical Expert Group (AHTEG) on Risk Assessment and Risk Management to establish a draft roadmap for risk assessment, with one sub-working group focussed on living modified mosquitoes (LMM; equivalent to GM mosquitoes). A draft guidance document on LMM was prepared by the sub-working group in 2009/2010, which was finalised at the second AHTEG meeting in April 2010 in Ljubljana, Slovenia (AHTEG, 2011). However as the document does not separate risk assessment and management of the different genetic strategies - self-limiting versus self-propagating, which have very different risk profiles and potentials - it comes across as a listing of hypothetical and occasionally scientifically-improbable risk scenarios that does not help the risk assessor evaluate potential risks from the different strategies. The CPB has also been criticised by some commentators (Angulo & Gilna, 2008; Marshall, 2010) as an inadequate vehicle for the regulation of transboundary movements of self-propagating LMM, particularly in relation to their potential to spread beyond national borders.
- *European Food Safety Authority*: EFSA has been requested by the European Commission to develop guidance documents for the risk assessment of GMOs. This has already been completed for GM plants, and a new guidance document will cover GM animals, in three sections: fish, insects and mammals/birds. To facilitate the preparation of the guidance document, EFSA recently commissioned a report on the topic of criteria for the environmental risk assessment of GM insects (Benedict *et al.*, 2010). To effectively assess environmental safety, taking into account the diversity of animal habitats, the GMO Panel is setting up three dedicated working groups of EFSA's Panels on GMOs and on Animal Health and

¹¹ Brazilian CTNBio decision on open field release of GM mosquitoes: <http://www.jusbrasil.com.br/diarios/23935599/dou-secao-1-17-12-2010-pg-48>

Welfare (AHAW). These working groups will draft specific guidance for GM fish, GM insects, and GM mammals and birds.

- As for all guidance documents, EFSA will consult Member States and relevant stakeholders during the process, early in 2012. In addition, public consultations will be held on these draft guidance documents before they are finalised and adopted by the respective Panels concerned.

4. BIOSAFETY CONSIDERATIONS

As with any new technology there could be potential human and environmental safety concerns, but as each GM insect strain is likely to have a different profile, assessments must be carried out on a case-by-case basis. The use of science-based risk assessment therefore represents a cornerstone for biosafety considerations. It is desirable however to develop a common assessment criteria framework for genetic control methods, as has been done for SIT applications of Tephritid fruit flies (FAO/IAEA, 2007), so that information is transparent and easily exchanged between countries, without negating the sovereign rights of a nation to make its own decisions. The WHO has set up a working group to develop such a framework document (WHO, 2009). Factors that could be considered in the biosafety assessment for GM insects, and specifically mosquitoes, are outlined in Table 3.

Table 3. Factors for consideration in risk assessment of GM mosquitoes

The characteristics of the recipient mosquito, including taxonomy, source, geographical distribution, mobility, longevity and reproductive potential
The characteristics of the donor organism(s), prior history of safe use, nature of pathogenicity or infectivity
The characteristics of the genetic construct, vector and mode of transformation
The genetic modification, including phenotypic expression and a description of the genetic construct, stability of phenotype and genotype, and a description of the methods that could be used to identify the GM mosquito from its non-GM counterpart
The agents that might be transmitted and whether the mosquito is or may be infected, along with the ability of the mosquitoes to transmit (one or more) pathogens
The epidemiological factors influencing transmission of disease in the proposed location
Survival, multiplication and dissemination of the GM mosquito and conditions that might affect these parameters in the receiving environment
Physical, biological, temporal or geographical parameters that limit the potential survival, multiplication and dissemination of the GM mosquito

Source: Mosqguide Module 2 (<http://www.mosqguide.org.uk/guidance.htm>)

However it is important that the outcome of any risk assessment is balanced against potential benefits derived from the expected outcomes, particularly with the introduction of GM mosquitoes that have the potential to reduce disease vector populations or convert them to a less harmful form, thereby protecting humans from disease. This approach was advocated in Morris (2011), who used a semi-quantitative approach to risk benefit analysis. The use of GM mosquitoes could require new regulatory paradigms in some jurisdictions but is essential to have proportional regulation if disease endemic countries are to benefit from the introduction of innovative technologies to address the increasing problems of insect-borne disease. Risk assessment needs to be an iterative process which adapts to new information and develops as open field releases and the deployment of GM insects becomes more commonplace.

5. CONCLUSION

The use of GM insects represents a novel and innovative tool to address the insect-borne disease of humans and crop pest losses. However despite rapid advances in the subject area, there are no widely accepted regulatory or biosafety framework that provides guidance on all aspects, although some of these are currently in development (Fontes, 2009; Benedict *et al.*, 2010; WHO, 2009). It is proposed that such a document could facilitate the standardisation of procedures and the comparability of results and conclusions, allowing robust assessments by decision-makers (WHO, 2009). However, even if such a framework document was in place, there is still a requirement for countries to develop their national guidance and policies, as well as build capacity to safely assess the risk of the increasing development and use of GM insects. It is, however, likely that the risk perception of the public and the acceptability of such risks, when balanced against potential benefits, will ultimately decide the pace of development of GM insects.

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