Asian Journal of Biotechnology and Genetic Engineering

1(1): 28-43, 2018; Article no.AJBGE.40646

Application of Genetically Modified Mosquitoes (*Anopheles* Species) in the Control of Malaria Transmission

Okoro, Onyekwere Joseph[1], Nnamonu, Emmanuel Ikechukwu[2], Ezewudo, Bede Izuchukwu[1*] and Okoye, Ikem Chris[1]

[1]Department of Zoology and Environmental Biology, University of Nigeria, Nsukka, Enugu State, Nigeria.
[2]Department of Biology, Federal College of Education, Eha-Amufu, Enugu State, Nigeria.

Authors' contributions

This review article was written in collaboration between all authors. Author OOJ designed the outline of the manuscript, managed the literature searches and wrote the first draft of the manuscript. Author NEI modified and improved the quality of the first draft of the manuscript. Author EBI proof-read, conducted further literature searches and wrote the final manuscript. Author OIC supervised the writing of the manuscript. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/AJBGE/2018/40646
Editor(s):
(1) Tsygankova Victoria Anatolyivna, Professor, Department for Chemistry of Bioactive Nitrogen-Containing Heterocyclic Compounds, Institute of Bioorganic Chemistry and Petrochemistry of National Academy of Sciences of Ukraine, Ukraine.
Reviewers:
(1) E. Mennan Yildirim, Adnan Menderes University, Turkey.
(2) Molobe Ikenna Daniel, Nigeria.
(3) Esraa Ashraf Ahmed ElHawary, Ain Shams University, Egypt.
(4) Marylene de Brito Arduino, Brazil.
Complete Peer review History: http://www.sciencedomain.org/review-history/24480

Review Article

Received 13th February 2018
Accepted 16th April 2018
Published 7th May 2018

ABSTRACT

Mosquito species of the *Anopheles gambiae* complex represent the major vectors of human malaria and they pose an enormous burden on global health and economies. Every year 300–500 million people are infected by malaria and over a million people die as consequence of *Plasmodium* parasite infections. Disease endemic countries often do not have the economic resources and the logistics to sustain control efforts like the massive and prolonged use of insecticides, the use of Long Lasting Insecticide Treated Nets (LLITN), Indoor Residual Sprays (IRS), Larviciding (abortion of metamorphosis) and adequate environmental sanitation. New control strategies that have

**Corresponding author: E-mail: ezewudobede@gmail.com;*

sustainable effects are desperately needed. This article, therefore, considered the unprecedented effort aimed at generating new molecular tools and a comprehensive knowledge of biology and the genetics of *Anopheline* mosquitoes which has culminated in the sequencing of the *A. gambiae* genome and development of gene transfer technology for a series of vectors species. The article also looked into the molecular advances that have been made to express genes that can block the transmission of *Plasmodium* in model systems or express traits facilitating the implementation of sterile insect techniques for vector control.

Keywords: Mosquito; malaria; control; genetics; sequencing.

1. INTRODUCTION

Mosquito-borne diseases are still a major human and animal health problem in many countries. Malaria is caused by a bite of female *Anopheles* mosquito infected with a protozoan parasite of the genus *Plasmodium* and is endemic in 106 countries and responsible for about 225 million clinical cases and 781,000 deaths annually [1,2]. According to the latest survey, as released in November 2017, the survey shows that in 2016, there were about 219 million cases of malaria recorded in 91 countries, usurping that of 2015 with an increase of 5 million cases and an estimated 445,000 malaria-related deaths in 2016, a similar number (446,000) to 2015 [3]. Malaria mortality rates have fallen by 47% globally since 2000 and by 54% in the WHO African Region [4]. Over a century ago, Ronald Ross was the first to establish the role of mosquitoes to malaria transmission and control, a discovery for which he was honoured and recognized worldwide as the second Nobel Prize winner in Physiology and Medicine in 1902 [5]. Further studies by Batista Grassi and other scientists revealed that only mosquitoes of genius *Anopheles*, and not others such as *Culex* or *Aedes* genera, have the capacity of transmitting malaria to humans. With hundreds of species of *Anopheles* mosquitoes, medical entomology has stated that only a few *Anopheles* species are important carriers of human malaria [6]. Not all individual mosquitoes or populations are equally competent as vectors, even within those *Anopheles* species that of medical importance in human malaria.

In Africa, the *Anopheles gambiae* is the major vector of *Plasmodium falciparium* and also, one of the most recognized malaria vectors in the world [7]. It depends solely on human blood, with its larvae developing temporarily from bodies of water produced by anthropogenic activities (e.g., irrigation of farmlands or flooded human or domestic animal footprints), and adults inhabiting primarily in human surroundings.

The World Health Organization (WHO) through their malaria eradication campaign recorded a great success in eradicating malaria from Europe and noticeably decreased its prevalence in many other parts of the world, mainly through enlightenment programs that involved mosquito control using antimalarial drugs like chloroquine during the 1950s and early 1960s. The most part of the Sub-Saharan Africa was not among the beneficiary from the malaria eradication campaign program, but the evenly distribution and availability of chloroquine and other cheap antimalarial drugs undoubtedly helped to control malaria mortality and morbidity. Surprisingly, malaria in Africa is again on the increase due to the advent of malaria parasites that are resistant to chloroquine and mosquitoes that have developed resistance to the insecticides used in controlling malaria transmission. In addition, control programs based on insecticide-treated bed nets, widely advocated by WHO and are under serious threat by the development of insecticide resistance in *A. gambiae* and other carriers of *Plasmodium* causing malaria.

The knowledge of mosquito-pathogen relation-ships and mosquito molecular biology has made it possible to produce mosquito strains that are incapable of transmitting various parasites or viruses. Transgenic strains of mosquitoes have been developed and evaluations of these to replace or suppress wild vector populations, reduce transmission and deliver public health gains are an imminent prospect.

1.1 Life History of Anopheles Mosquito

Mosquitoes grouped into 41 genera are estimated to have about 3,500 species. Females of the genus *Anopheles* is the only mosquito that transmit human malaria with approximately 430 *Anopheles* species, only small group of these species (30-40) transmit malaria (i.e., are "*Plasmodium* carriers") in nature. Female *Anopheles* mosquitoes feed on blood meals in order to carry out egg production, thereby

creating a bond between the human and the mosquito in the parasite life development. The successful completion of the life cycle (from the "gametocyte" to the "sporozoite" stages) of the *Plasmodium* in the mosquito depends on some key factors. The most key factors are ambient temperature and humidity (higher temperatures facilitate the parasite growth in the body of the mosquito) and also depends on the *Anopheles* to survive in the body of the mosquito after adapting to new environmental constraints pose by the host to allow the parasite to complete its development (either "multiple fission of spores" or "outside" developmental cycle) lasting for a period of 10 to 18 days. The presence of the parasites in mosquito host does not show any remarkable symptom which is not the same when the parasites infect the human host.

1.2 Mosquito Vector Control Methods

Preventing or reducing malaria transmission depends entirely on control of the mosquito-carrying *Plasmodium* or altering of human-mosquito contact. Activities to control transmission should target *Anopheles* mosquito (the main vector) in the habitats of its sexually immature and adult stages in the human dwellings and immediate environment, as well as other human dwellings where human-mosquito contact occurs (e.g. schools, hospitals and workplaces). Mosquito vector control methods are any methods employed to limit or eradicate mosquito vectors in order to put to a halt their damages to health, economies, and enjoyment as it pertains to humans. Adopting Insecticide-based control measures (such as indoor spraying with insecticides, ISIs) are the best way to destroy mosquitoes that love inhabiting human living rooms. Regrettably, mosquitoes may develop resistance, as found in other insects, after a long exposure to an insecticide for several decades, an adaptive response in surviving the action of insecticide. It is well known that mosquitoes can reproduce many generations per annum, high levels of resistance can occur often. Since the discovery of insecticides in the control of malaria, scientists have studied widely and documented on the resistance of mosquitoes to insecticides. They were able to document those that have resistance to one or more insecticides to be around over 125 mosquito species. The ability of mosquitoes in developing resistance to insecticides used for indoor spraying was a major setback during the Global Malaria Eradication Campaign Program. Proper use of insecticides in eradication of mosquito can curtail the spread and development of resistance. More so, the use of insecticides in agricultural activities has often been linked to contributing in promoting mosquito resistance to insecticides.

1.3 Refractoriness as a Tool in Genetic Engineering of Mosquitoes

Some species of *Anopheles* are not really vectors of malaria, as the parasites do not complete its life cycle well (or not occur) within their body system [7]. The disparity that exists within species is also obvious. In the laboratory, scientists have been able to successfully select for strains of *A. gambiae* that are resistant/refractory to infection by malaria parasites. Those species that are refractory in nature, have an immune response that engulfs and kills the incoming foreign agents “parasites” after they have invaded the mosquito's mucosa. Researchers are investigating this response using genetic approach. They believed that a day will come when genetically modified mosquitoes that are resistant to malaria can successfully replace the conventional mosquitoes, thereby putting to an end malaria transmission.

2. METHODS OF GENETIC MANIPULATIONS

Many mosquito control approaches have failed to achieve their targets, due to the mosquito's prolific nature and genomic dynamism [8]. Adopting chemical control is now getting less attention due to potential threat to human health, killing of other organisms not targeted, insecticide refractoriness, and other ecological impacts. Other reliable approaches for mosquito control and eradication are urgently needed. Some of these approaches are:

2.1 The Sterile Insect Technique Approach (SITA)

The Sterile Insect Technique Approach (SITA) is an approach that is species-specific oriented and environmentally harmless for insect population regulation [9]. This approach relies on the mass rearing, radiation-induced sterilization, and release of a mass number of male insects into a selected and desired zone to copulate with wild-type virgin females [10]. As the mating of sterile males with the wild-type virgin females would produce no progeny, if large number of sterile males is released on the target area over a sufficient period of time, and the percentage of

multiple mating is low, the local eradication of the pest population will ensue [Fig. 1]. By decreasing or thorough eradication of the vector populations will go a long way in reducing or eradicating transmission diseases that are vector related [8]. This approach of disease control has been effectively deployed in many countries of the world [11].

The successful eradication of the New World Screw Worm, *Cochliomyia hominivorax* (the causative agent of myiasis), from the southern states of the USA, Mexico and all of the Central America was the hallmark of SITA program [12]. Presently, these areas are under surveillance from recolonization from South-American flies by introduction of a barrier in Panama that involves only infertile flies [8]. Insects are mostly sterilized with radiation, which might weaken the newly sterilized insects if the treatments are wrongly applied, thereby rendering them less fit to compete with their wild males' counterpart [13,14,15].

2.1.1 Challenges of SITA program

1. Production below desired levels due to absence of sexing strains or delay in production
2. Loss of male fitness owing to sterilization technique
3. Immigration of mated females into the target area
4. It must be stressed that different vector species need to be targeted in order to achieve the suppression of malaria parasite, rendering the application of SITA in malaria-control programs more complicated than the eradication of the screw worm.
5. Also, multiple mating of the female mosquitoes has been reported in the fields which could impair efficiency of SITA programs [16].

However, SITA could be successfully implicated in areas where there exist a simple vector-parasite relationship and where the immigration of females or other vector species is not likely to occur. These limitations may be overcome using recombinant DNA technology to engineer repressible dominant-lethal transgenes for an IIDLG strategy [13,14,15,17].

2.2 Introduction of Insects with a Dominant Lethal Gene (IIDL)

Introduction of insects with a dominant lethal/deleterious gene (IIDL) is a genetic control mechanism adopted from classical infertile insect technique (SITA) that provides a new dimension to the problems facing the control efforts [18,19,20,21,22].

This involves the introduction of genetically engineered insects by introducing a repressible "dominant lethal/deleterious" gene into the insects [23]. This gene kills the insects but could be repressed/ inactivated by treatment with an external additive known as tetracycline [24] leaving a colony to be established. When there is need for the male and female separation, tetracycline will be removed from the system, leading to the death of all females in the colony [Fig. 2].

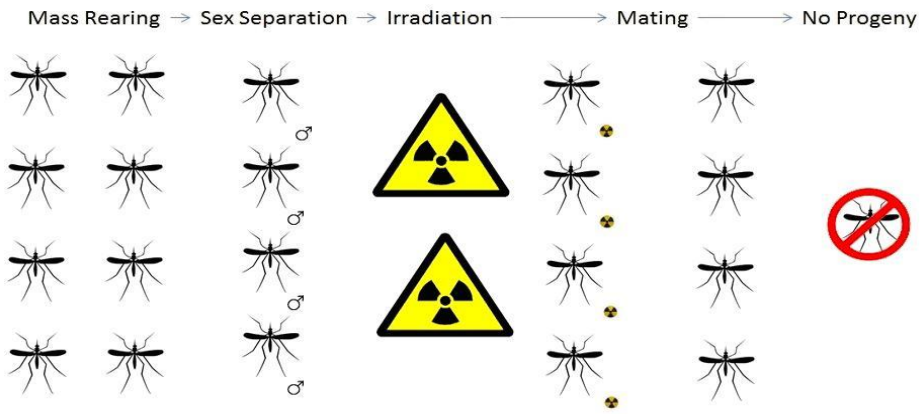

Fig. 1. Schematic expression of sterile insect technique approach (SITA): Large breeding of mosquitoes followed by careful separation of sex to ensure that only males are to be treated and made infertile by ionizing radiation and further released to copulate with wild females of the same species, resulting in no progeny/ offspring

Source [8]

The IIDL system is principled on the action of tTA (tetracycline-repressible transcriptional activator), a fusion protein that initiates sequence-specific tetracycline-repressible fusing to tRe, a tetracycline-response unit, to a true-celled transcriptional activator [8]. When tetracycline is not involved, the protein will fuse to the tRe sequence, igniting transcription from a close minimal promoter [13] [Fig. 2].

Preparing mosquitoes for release involves the deactivation of the repressor and the activation of lethal gene which will to the death of all females. During copulation with wild females, the deleterious gene of the male homozygous will produce heterozygous offsprings, leaving only males as survivors. Introduction of Insects carrying a Dominant Deleterious gene (IIDD engineering) provides another insight to many of the shortcomings of conventional SITA that have diminished its usage in mosquitoes while keeping its ecologically friendly and species-specific application [14]. Genetically modified males are homozygous for a dominant deleterious gene. Copulating with native population produces offspring that the lethal/deleterious gene are heterozygous leading to the mortality of all females and eventually, decreasing the population due to a reduction in its reproductive strength (Fig. 3.) [25,23]. Adopting IIDL means that the males will not have to be sterilized by radiation before release, making them (males) stronger when they need to compete with the wild males for mating partners. Genetic manipulation targets to achieve universal recognition by taking into consideration, the male insect's ability in locating and copulating with females of the same species [12].

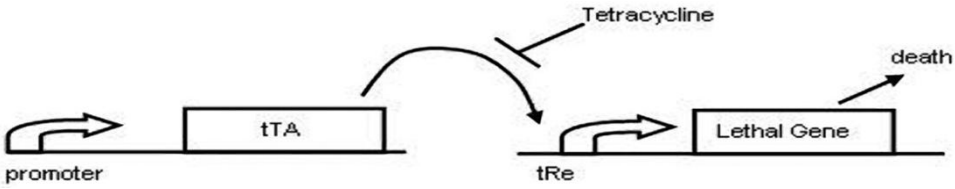

Fig. 2. Diagrammatic expression of tTA and the tetracycline-repressible system pathway. The tetracycline-repressible transcriptional activator (tTA) protein is subjected under a promoter as control. When activated, the tTA protein bonds to a specific DNA sequence, tetO, initiating expression from a nearby minimal promoter which will lead to activation of any sequence (the effector gene) subjected under the control of the minimal promoter. The synergistic effect remains that the effector gene is primarily the sequence of the promoter initiating tTA. Moreover, when there is low concentration of tetracycline, the tTA protein will not fuse with DNA, hence, the effector gene will not be expressed

Source: Modified from [13]

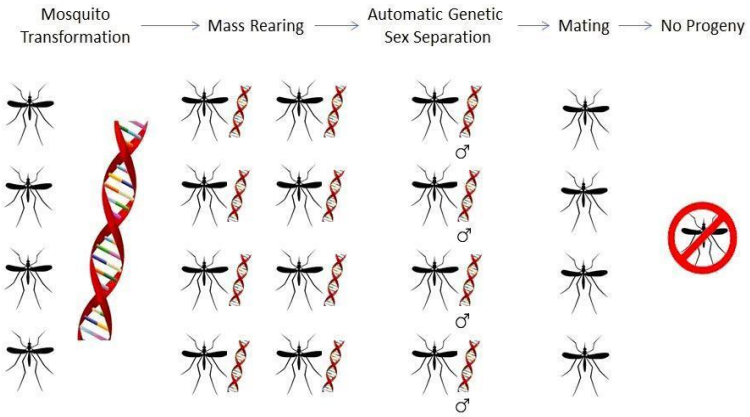

Fig. 3. Schematic expression of IIDL system: when a stable strain of genetic treated mosquitoes with female specific harmful gene is noticed, all that is required is top mass rear and eliminate the genetic repressor (tetracycline), the gene will eliminate all females leaving only males that are ready to be introduced to the wild to mate with wild females of the same species

Source [8]

Insects genetically modified to carry a female-specific deleterious (or rather incapacitating) gene could be deployed to remove females before being released or introduced [23,26,14,22]. A mechanism based on a lethal gene that acts late in development would halt the emergence of mosquitoes into adulthood, the only stage they are capable of inflicting harm, still enable them to live and involve at the larval stage, when density-dependent co-founding factors occur [19]. Simulating this mechanism indicates that fewer male mosquitoes of a late-deleterious strain need to be introduced as against those carrying an early-deleterious gene or irradiated strain to attain an equal level of control of a chosen population [23,27,10,17,22].

A female-harmful version of IIDL, with insects homozygous for one or more female-specific dominant harmful genetic make-ups, has been built in several species [22]. F1 offspring of IIDL males and wild females inherit a dominant female-specific harmful gene; the F1 females die, thereby decreasing the reproductive capacity of the wild population, but the F1 males are active and potent. This suggests a genetic sexing system encouraging male only release, either by adopting the female-harmful version of IIDL and removing the repressor from the family tree prior to release, or by introducing a bisex-harmful system with female lethality (with a separating means of repressing or initiating lethality) to allow male only release of bisex-harmful strains manipulated to kill offspring of both sexes in the wild [Fig. 3] [13].

2.3 Homing Endonuclease Genes (HEG)

Homing endonuclease genes (HEGs) are highly specific DNA endonucleases found in some viruses, bacteria and eukaryotes. They are 'selfish' genetic elements that have non-mendelian inheritance mechanisms. They spread through populations even when they provide no benefit to the host organism [28] and have been proposed to transform wild-type mosquito populations. The endonuclease promotes the movement of its encoding DNA from one allele to the other by creating a double-strand break (DSB) at a specific, long (15–40 bp) target site in an allele that lacks the HEG. Homologous DNA repair then copies the HEG to the cut chromosome in a process called 'gene conversion' [29,30].

The observation that HEGs can be engineered to cleave novel DNA sequences [31,32,33,34] offers a multitude of opportunities to utilize these elements for mosquito control. For example, HEGs could be used to disrupt genes regulating the ability of *Anopheles* mosquitoes to function as efficient vectors for *Plasmodium* parasites, or to drive recombinant refractory genes through a mosquito population, rendering them unable to transmit malaria. Alternatively, HEGs designed to target an essential mosquito gene or a gene required for female fertility could be utilized to introduce a genetic load on the population leading to population size reduction or collapse [35]. More recently, it has been suggested that a harmful selfish element subjected under the influence of a promoter which is active in individuals susceptible to *Plasmodium* infection but inactive in refractory individuals should drive alleles causing refractoriness through the population [36]. Finally, HEGs could be used to bias the sex ratio towards males, using an endonuclease that targets X-linked sequences and is expressed during male spermatogenesis from the Y chromosome [35].

2.4 *Anopheles gambiae* Epithelial Serine Protease (AgESP)

Anopheles gambiae epithelial serine protease (AgESP) is expected for *Plasmodium* parasites to successfully manipulate its way through the midgut and salivary gland epithelial barriers of mosquito. Naturally, AgESP is expressed in the submicrovillar section of mosquito midgut epithelial cells and in the basal section of the salivary glands that is of utmost important for *Plasmodium* parasites to cross these two epithelial walls. For successful completion of life cycle of the *Plasmodium* parasites in the mosquito body system, they must modify the actin cytoskeleton of mosquito epithelial cells and AgESP plays a major key role in the regulation of this process.

AgESP deactivation greatly reduces *Plasmodium berghei* and *P. falciparum* from invading the midgut thereby, preventing the transcriptional activation of gelsolin, an essential regulator of actin remodelling and a known *Plasmodium* agonist [37]. Expression of AgESP is highly initiated in midgut epithelia invaded by *Plasmodium parasites*, an indication that this protease also involves in the death of cells a response to invasion by *Plasmodium* parasites.

2.5 Altering Mosquito Sense of Smell

Vosshall's team targeted a gene called orco, which she observed that it was important for flies to be able to respond to the odors [38]. They used a genetic engineering tool called zinc-finger nucleases to specifically mutate the orco gene in *Aedes aegypti*. They injected the targeted zinc-finger nucleases into mosquito embryos, waited for them to mature, identified mutant individuals, and generated mutant strains that allowed them to study the role of orco in mosquito biology. The engineered mosquitoes showed diminished activity in neurons linked to odor-sensing.

When given a choice between a human and any other animal, normal *Aedes aegypti* will reliably buzz toward the human. But the mosquitoes with orco mutations showed reduced preference for the smell of humans over guinea pigs, even in the presence of carbon dioxide, which is thought to help mosquitoes respond to human scent [38]. By disrupting this single gene, it is therefore possible to confuse the mosquito from its task of seeking humans.

2.6 Effector Genes

The term effector gene is used here for genes whose products interfere with the development of a pathogen. At least four classes of effector genes can be identified:

(1) Genes whose products interact with insect host tissues crucial for parasite development: Examples of this class are SM1, a peptide that occupies putative salivary-gland and midgut receptors for the malaria parasite [39] and phospholipase A2 (PLA2), which is a protein that acts antagonistically with the malaria ookinete invasion of the midgut [40].

(2) Genes whose products interact with the pathogen: These are genes encoding single chain monoclonal antibodies that bind to the parasite's outer surface thus blocking their development [41].

(3) Genes whose products kill the pathogen: Examples are peptides from the insect's innate immune system such as defensins and cecropins, and peptides from other sources that act as selective toxins to parasites but do not affect the host insect, such as magainins, Shiva-1, Shiva-3 and gomesin [42].

(4) Another possible strategy to reduce vector competence is by manipulation of its immune genes, for instance by using RNA interference or 'smart sprays' [43].

Another important strategic consideration is the stage of malaria parasite development to target. When a mosquito feeds on an infected blood meal, it acquires thousands of gametocytes of which only few (usually less than ten) manage to cross the midgut and form oocysts. Later, each oocyst produces thousands of sporozoites, a significant proportion of which invade the salivary gland. Because of the strong bottleneck at the level of midgut invasion, this stage of parasite development constitutes a prime target for intervention.

2.7 Paratransgenesis (Metagenomics)

Paratransgenesis, the genetic manipulation of commensals or mutualistic bacteria that alter the host's ability to transmit a pathogen, is an another way of preventing malaria infection. Bacteria can be manipulated to initiate and secrete peptides or proteins that hinder entrance of parasite or kill the parasite living in the midgut. Several bacterial endosymbioants have been identified in mosquitoes that either permanently reside within specific species/ strains or present as a predominant component of the entire microbiota of related mosquito species [44,45]. *Wolbachia* is a well-known endosymbiont bacteria of mosquitoes [46,47]. Because of their stable association and peculiar effect on the host organism (effect on age), *Wolbachia* has been described as a potential tool for suppressing vectorial ability of mosquitoes to disease transmission [48,49,50,51].

In *Anopheles stephensi*, *Asaia* bacteria were the dominant component of the whole microbiota of these mosquitoes, particularly in the female gut and in the male reproductive tract [52]. Further experimental evidences from this study also indicated that the *Asaia* bacteria are stably associated with the female guts and salivary glands, sites that are crucial for Plasmodium sp. development and transmission. In *A. gambiae* mosquitoes also, the *Asaia* bacteria are primarily localized in the midgut, salivary glands and reproductive organs [53].

Rather than genetically modifying mosquitoes, metagenomics entails genetically modifying the bacteria that inhibit the mosquito midgut.

These bacteria can be grown artificially in the laboratory and may be suitable targets for genetic simulation. Whether these bacteria are permanent or part-time inhabitants of the midgut

of adult mosquitoes remains to be investigated. For malaria to be successfully controlled, the resistant proteins or peptides exhibited by the bacteria must act on the midgut regions of the malaria parasites, stabilize their bioactivity in the regions of the midgut , and be expressed in sufficient amount. When *A. stephensi* mosquitoes were fed *Escherichia coli* that activate a binding protein of ricin and a single-chain antibody against Pbs21 also known to be a *P. berghei* ookinete surface protein, formation of oocyte was inactivated by up to 95% [54]. The use of paratransgenesis/metagenomics in malaria control will require the establishment of methods to introduce genetically engineered bacteria into wild mosquito populations.

2.8 Relevance of Metagenomics over Transgenic Mosquito in the Control of Parasite Transmission

- Bacteria live in the midgut, the same mosquito section where the highly susceptible stages of *Plasmodium* development takes place.
- The number of mosquito midgut bacteria increases dramatically with a blood meal (when parasites are ingested), correspondingly increasing the output of the effector molecules that they are engineered to produce.
- Genetic manipulation of bacteria is much simpler and faster than genetic manipulation of mosquitoes.
- Given that the use of multiple effector proteins is essential to avoid resistance, it is straightforward to formulate an efficient multi-effector combination by simply feeding mosquitoes a mixture of GM bacteria expressing different effector genes.
- Bacteria are much easier to introduce into mosquito populations than transgenes. Importantly, this approach bypasses genetic barriers of reproductively isolated mosquito populations (cryptic species) that commonly occur in areas of high malaria transmission and will hinder the spread of mosquito transgenes.
- Bacteria can be produced easily and cheaply in large quantities in disease endemic countries.
- Unlike mosquito transgenes, inactivation of bacterial transgenes after many generations in the field is not a major concern because of the easier logistics of introducing freshly transformed bacteria.

Regulations already exist regarding evaluation of bacteria to be released into the environment. A major outstanding issue is how to introduce the engineered bacteria into mosquito populations in the field.

3. ACHIEVEMENTS AND CHALLENGES OF GENETIC MANIPULATION OF MOSQUITOES

3.1 Achievements in the Genetic Transformation of Mosquito Vectors

Population replacement requires two components, a mechanism for resistance and a method to spread the gene into a population. Mechanisms of resistance (vectors unable to transmit disease pathogens) have been achieved in several mosquito species

- Transformation of *Anopheles stephensi* Patton was successfully carried out by adopting Minos transposable element and the indicator gene of the Enhanced Green Fluorescent Protein (EGFP) [55].
- Expressing a 12-amino-acid peptide (termed SM1) by *A. stephensi* that binds only to mosquito midgut and salivary-gland tissues [39], was manipulated genetically using piggyBac transposable element, the EGFP indicator gene and the artificial gene corresponding to SM1, and made unavailable to maintain the development and transmission of *Plasmodium berghei* (80% decrease in transmission) [56].
- *A. gambiae*, the most common vector of malaria transmission in Africa was remodelled using piggyBac with EG and SM1 marker was discovered to be able to bind to the midgut and salivary-gland tissues of the mosquito.
- Expression of cecropin to impair *Plasmsodium* development in *Anopheles gambiae* [42].
- A white-eyed strain of *Aedes aegypti* was remodelled (to multi-coloured eyes) in 1998, with 50% remodelling achieved with *Hermes-Cinnabar* [58], and 4% with *Mariner-Cinnabar* [58].
- A transgenic *Aedes aegypti* resistance to dengue virus (and other disease causing agents) was modified genetically [59] with a viral transducing system by adopting a double subgenomic Sindbis virus (dsSIN) containing a sequence from DEN-2 virus, to initiate resistance in *Ae. aegypti* to DEN-2 virus replication and transmission.

- An unaltered transgenic *Ae. aegypti* mosquito (*Hermes-Vg-DefA*) yielded (a blood meal induced) defensin with antibacterial activity in the adipose [60].

3.2 Case Studies of Successful Application of Genetically Modified Mosquitoes

The first exhibitions of GMMs happened in the Cayman Islands in 2009 and 2010 where three million engineered sterile *Aedes aegypti* mosquitoes, the primary vector of dengue fever, were introduced with the aim of lowering their population size [61,62,63,64]. Later on, in 2009–2011, genetically induced sterile *Ae. aegypti* mosquitoes were massively released in Brazil (about ten million) and also in Malaysia (about 6,000) [62,64]. The infertile GMMs were modified to have a gene that causes 96% of progeny to die before reaching adulthood [63]. Their aim was that as genetically modified sterile males mate with their female counterparts in the field, the reproductive capacity of the females will be unsuccessful, leading to low population size of the mosquito. In these case studies, introduced male GMMs were observed to be half as successful in copulating as field ones and this rate was found to be enough to reduce the population [63]. In another study that was performed in Mexico, genetically sterile strain of *A. aegypti* was determined for its capacity to promote dengue prevention efforts by involving in population reduction in a large field cage experiment. Their findings recorded a significant decrease in the target population size and still, none of the treatment populations were eliminated, possibly as a result of a fitness disadvantage associated with the genetically engineered strain [65].

3.3 Challenges of Genetic Manipulation

Despite the advantages of genetic manipulation in diseases control, genetic engineering challenges remain about the improvement of the stability of a genome and its expression for a well and complete interruption of disease transmission, improvising of best means of spreading alien anti-pathogen genes through mosquitoes in the field and the construction of safest genetic-control strategy that relies on this tool [66]. Although major achievements have been made recently, there is still need for the search for new effector molecules and promoters continue non-stop for the following two reasons. First, considering how easily parasites develop resistance to drug, it is likely that parasites will be selected that can overcome the difficulties imposed by the effector molecules. Secondly, maximum efficiency of hindering parasite development (preferably 100%) is pertinent for the genetically modified mosquito strategy to have a relevant impact on disease transmission. In addition, while many of the tools for genetic engineering of mosquitoes have been established, more studies are required in our ability to transfer this technology to the wild for the control of malaria. Others include:

3.3.1 The feasibility cost of refractoriness

To improve the likelihood of successfully introducing resistant genes into mosquitoes in the field, induced gene should impose minimal fitness load. The transgenic fitness of *A. stephensi* exhibiting the SM1 and the PLA2 induced genes was examined using different criteria, involving measurements of longevity and productivity, and use of sampling cages [67]. The SM1transgene failed to introduce a detectable fitness load, but induced genetically PLA2 mosquitoes had much decreased productivity and participated poorly with non-induced genes in cage trial studies. The reason for this minimal fitness is yet to be unraveled.

According to Catteruccia *et al*. four different genetically modified mosquito lines exhibiting fluorescent reporter proteins from an actin promoter where found to be minimally fit than the field type [57]. Reduced fitness recorded in their study could be as a result of inbreeding. Study has shown that synthesis of an alien protein in high abundance throughout an organism may likely have harmful effects on fitness [68]. As a result of this, SM1 expression was restricted to posterior midgut epithelial cells for only a few hours after a blood meal and the protein were secreted from the cells, thereby reducing fitness load. Total absence of fitness load is likely unnecessary for introducing genes into the field. Theoretical simulation indicates in the presence of appropriate drive mechanism, a gene could have an important fitness cost and still be introduced through the population [69, 70]. It is expected, since this same simulation suggests that any introduced mosquitoes would need to be approximately 100% refractory to have any role to play on malaria transmission, facilitating multiple resistant genes that may incur more fitness costs.

3.3.2 Establishing an effective drive mechanism

Two general approaches can be considered for releasing genetically modified mosquitoes in the wild: overhauling of the population or a genetic induced mechanism. Overhauling of the population, or total release, involves a significant decrease of the occupant mosquito population (for exampling, using insecticides), followed by the introduction of large numbers of resistant strain mosquitoes to occupy the deserted biological roles. This approach can be deployed as a research tool and as a field test to determine the usefulness of the genetically engineered mosquito strategy for altering malaria transmission. It should be note that, this approach cannot be deployed for large-scale control cases, because it lacks the ability to yield the required numbers of mosquitoes to achieve total overhauling on a country or continent population at wider range. Transposons also known as "jumping genes" may incur a considerable fitness cost. Transposition produces improper integration across the gene construct, some of which may alter genes and lead to alteration of genes that could be deleterious, decrease reproductive capacity or reduce fitness.

Another emphasis is that mobility of the transposons may be negatively controlled by a repressor. For example, movement of the *P* element in *Drosophila melanogaster* reduces after several generations due to an inhibitor of transposition which accumulates with time and the fly is said to accumulate the *P* (refractory/resistant) cytotype. This is valuable in feasibility studies because in cases like this, the gene(s) can be introduced or released through a population just once. In a situation where the effector gene(s) becomes altered or the parasite develops resistant to the effector gene product another gene cannot be introduced into the same population with the same transposons.

3.3.3 Mass production of transgenic mosquitoes and genetic sex determination mechanisms

Genetically induced-based methods to reduce or eliminate vector populations, such as the introduction of insects carrying a dominant deleterious, RIDD [23] show hope for some species. Instead, adopting it as a malaria control program in Africa would not be easily implemented due to incompatible subspecies leading to absence of reproduction and uncontrollable movement of mosquitoes from one village to the other. Even when implemented successfully, this method would encourage the invasion of another malaria vector to fill the vacuum left by the original vector. Therefore, replacement of field mosquitoes with genetically modified mosquitoes carrying resistant strain genes instead of population reduction or elimination approaches would be encouraged, Surprisingly, this mechanism still needs the release of large numbers of biting insects, which is ethically unacceptable due to their disturbance nature and their capability as vectors of diseases. Therefore, widespread release of transgenic mosquitoes can best be implemented using only non-biting males, promoting an easier mechanism for selection of only male mosquitoes. More still, the ability to introduce only males would provide a better hope of adopting the use of genetically modified mosquitoes acceptable to the rural communities as well as to the public.

3.3.4 Escaping resistance to the resistant/ refractory genes

Parasites occupying refractory wild mosquitoes would be difficult to select, similar to the ones under the influence of anti-malarials, and thus may lead to the development of resistant strain genes. Modifying a mosquito genetically with many resistant genes that captures different life developmental stages of parasite could reduce resistant/ refractory genes from being resistant. For instance, a genetically modified mosquito might be induced to exhibit a peptide to alter midgut and salivary gland from being infected, produced an improved encyst response to target the encysted zygote, and exhibit immune peptides to target the sporozoites. In addition, the probability of success will be greatly improved if each resistant strain is almost 100% effective as expected and if introduction of the resistant genes is enhanced with crude control methods, such as the use of insecticides in reducing wild populations before the release of engineered mosquitoes, treatment of infected individuals with drugs, and use of insecticide treated nets. The potentials of transposons or "jumping genes" may reduce as time progresses after wild release. Following the release of a new transposon into a population the transposon enjoys a period of uncontrollable activity and transmission. As a result, individuals with altered genes in the transposase or those that have established regulatory inactivation of the transposon will be chosen. Transposase silencing has garnered a lot of research most

especially in the mariner family and has been hypothesized to take place by several mechanisms, like excessive inhibition whereby an increase in transposase mechanism corresponds with reduced transposition or unorganized transposase alterations. Unorganized transposase alterations may lead to unenclosed reading frame mutations and redundant transposases that combine with active transposase for substrate termed competitive inhibition or decrease the mechanism of field-type transposase termed dominant negative complementation [71]. The activity of transposon silencing requires one to have a comprehensive knowledge of its applicability before transposons are deployed in the field.

3.3.5 Sterilization

Recent advances allow several potential improvements over the methods available in early trials. All current SIT programmes use radiation to sterilize the insects. However, it has proven difficult to irradiate mosquitoes to near-complete sterility without significantly weakening them [72]. This adversely affects the ability of sterilized males to compete effectively with the wild males.

4. PRECAUTIONARY MEASURES IN THE USE OF GENETICALLY ENGINEERED MOSQUITOES FOR DISEASE CONTROL

Despite the fact that several successes have been recorded since the development and implementation of the release of genetically engineered mosquitoes for disrupting pathogen transmission mostly in *Anopheles* and *Aedes* mosquitoes, there are still many challenges pose to its implementation. These challenges range from the improvement of the stability of a gene construct and its utilization for an efficient and total disruption of pathogen transmission and the modelling of secured ways of transmitting foreign anti-pathogen genes through mosquitoes inhabiting the wild.

An assessment by Okorie et al. in Nigeria, revealed that scientists were sceptical that malaria-refractory GMMs may disperse in a random manner way beyond the points of release, which may result in transgenic mosquitoes having unpredictable effects [72]. Other serious concerns included the phobia that GMMs will cause unknown health concerns and may become refractory to fogging and insecticides. The engineering of mosquitoes such that they are no longer causing disease is risky in the context of ecological suitability and resistance as there is dearth of information about the behaviour of GMMs in the wild [64]. It is yet to be ascertained the response of GMMs in the context of behaviour, biological fitness and how genetically modified mosquitoes will mostly impact insect ecology.

Some of the challenges to overcome in implementing genetically modified mosquitoes include thorough conduct of risk assessment and management, embarking on studies that will encompass human safety and the environment, development of safest control measures principled on standardized gene-driving systems, take into consideration ethical, legal and social consequences of the introduction of GMM and public opinions. Although the introduction of GMM as disease-control approach is technically practicable, for even utilization no field release must be performed until convincing scientific evidence of humans and environmental safety and efficacy is issued and ethical, legal and social implication (ELSI) issues and general acceptance are adequately addressed.

4.1 Things to Be Considered Before GMM Can Be Deployed

4.1.1 Policy decision

Thorough safety assessment and management must be the foundation for policy decision. It requires a laid down procedure to reduce the potential risks of human and environmental consequences by expecting disastrous implications that might follow the release of GMM during investigation, by devising tracking systems for the early detection and examination of undesirable results and by deciding on intervention approaches, so that novel information can be collated and reported to avert and if needs be, correct poor health or environmental implications [68]. A well-known recommendation/requirement for scientists to endorse the introduction of GMMs in Nigeria was that there had to be proof of contingency devises available to eradicate GMMs if it becomes hazardous during the course of its introduction [74].

4.1.2 Information

Biological assessment of human and the environment safety needs to provide general knowledge about the biosafety concerns and

ensure that the information reaches the public, executives and legislatures approved by legitimate biosafety and regulatory bodies prior to any trial release should be thoroughly established [73]. Information should be made public and allowed to spread evenly in a two-way means, and informed consent should be granted from the participatory communities. Because of public health interventions, the manner for obtaining individual and group consent must be specifically stated and developed. The data should be made public to the participants so that they can gain from global expertise and reach an international agreement.

4.1.3 Environmental and health studies

Environmental and health studies for site selection should be conducted first, and based on the findings the most appropriate sites should be selected. The knowledge of the biology of mosquito should be studied to improve the knowledge of gene transfer in mosquito populations such as mating patterns, behaviour, male biology, population size and structure, the dynamism of population regulation, fitness and phenotypic implications of colonization and mass production. These will assist in identifying perfect isolated field sites and group populations in the context of genetic and ecological attributes; epidemiological qualities (transmission, disease), devise best contained semi-wild methods to enhance comprehension of the biology of (engineered) mosquitoes [75].

4.1.4 Ethical, legal and social outcomes (ELSO)

Ethical, legal and social outcomes (ELSO) of the potential utilization of GMM will also need to be considered properly, by incorporating with the scientific investigations those ELSO that are important to the utilization of GMM, and by ensuring that all parties are legally authorized have means for including their quota into the proposed control programs. The ongoing and active process of ethical examination, by a number of flora should be encouraged.

4.1.5 Communication and public awareness

There is also the need of transforming risk-assessment procedures into language(s) that can be comprehended easily by the participatory communities, and of including the end-users in the sites selection and plans for release, in clear and legally acceptable terms of informed consent, and in enhancing an understanding of the true determination of success for the programs [75]. The creation of public knowledge and trust is paramount to encourage implementation strategies that encompass the end-user communities, executives and legislatures in order to raise their awareness and instill trust about the benefits and potential hazard, to serve as an avenue to the communities to be well informed to make informed decisions about the advantages of practicing these programmes in their villages, to provide good access for communication and transmission of information, to encourage South-North research and development and create awareness in Disease-ravaging countries (DRCs) for the understanding and the proper deployment of the tool.

5. CONCLUSION

The African Malaria mosquito, *Anopheles gambiae* is probably the most dangerous of all insects [76]. This mosquito is a particularly dangerous vector as it is anthropophilic and has a long life-span. The fundamental life history for different vectorial potential depends solely on poor knowledge about the differences in mosquito functional systems, genetic make-up, and also their attitude in their environment. A good knowledge of vectorial potential may effectively enhance its manipulation for easy reduction of the burden caused by the disease. Major achievements in recent times, like the successful germ line modification and grouping of promoters, are enabling scientists to verify known refractory/ resistant genes. The isolation of additional effector genes still remains one of the areas of focus by researchers and this will be effectively possible by the presence of the *Anopheles gambiae* and *Plasmodium falciparum* genetic make-up. This knowledge can be used to genetically modify a mosquito that hinders or eliminates the *Plasmodium* during series of development in the body of the mosquito. With the likelihood of having mosquitoes that are genetically engineered, the next step is to beginning making plans on how best it would be introduced to the wild. Our interest should concentrate more on how to release the important genes into the massive number of mosquitoes in the field. Also important are an ecological survey to evaluate population structure and generational pattern. Furthermore, we must brainstorm with the ethical and political opinions involved with a mass introduction of a genetically engineered organism. Going forward,

there are still quite some challenges but there are reasons to be hopeful that genetic modification of mosquitoes will be successfully incorporated to our weapon in the quest to conquer malaria.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. WHO. World malaria report. WHO Global Malaria Programme; 2010. Available:http://whqlibdoc.who.int/publications/2010/9789241564106_eng. pdf Via WYSS internet
2. Cox FEG. History of the discovery of the malaria parasites and their vectors. Parasites & Vectors. 2010;3:3. DOI: 10.1186/ 1756-3305-3-5
3. WHO. World malaria report 2017. WHO Global Malaria Programme; 2017. Availabe:www.who.int/malaria/publications/world-malaria-report-2017/en/pdf
4. WHO. World malaria report 2014. Scale-up in effective malaria control dramatically reduces deaths; 2014. Availabe:www.who.int/malaria/publications/world_malaria_report_2014/en/
5. Neafsey DE, Christophides GK, Collins FH, Emrich SJ, Fontaine MC, Gelbart W, et al. The evolution of the *Anopheles* 16 genomes project. G3: Genes-Genomes-Genetics. 2013;3(7):1191-1194. DOI: 10.1534/g3.113.006247
6. Budiansky S. Creatures of our own making. Sci. 2002;298:80–86.
7. CDC. Malaria: Anopheles mosquitoes. Global health-division of parasitic diseases and malaria. 1600 Clifton road Atlanta, GA 30329-4027 USA. 800-CDC-INFO (800-234-4636), TY. 2015;888:232-6348.
8. Wilke ABB, Marrelli MT. Genetic control of mosquitoes: Population suppression strategies. Rev do Inst de Med Trop. 2012; 54(5):287–292.
9. Reiter P. Oviposition, dispersal and survival in *Aedes aegypti*: Implications for the efficacy of control strategies. Vect-Bor Zoo Dis. 2007;7:261–273.
10. Phuc HK, Andreasen MH, Burton RS, Vass C, Epton MJ, Pape G. Late-acting dominant lethal genetic systems and mosquito control. BMC Bio. 2007;5:11.
11. Pates H, Curtis CF. Mosquito behavior and vector control. Ann Rev of Entom. 2005; 50:53–70.
12. Wyss JH. Screw worm eradication in the Americas. Anna N.Y Aca Sci. 2000;916: 186–193.
13. Alphey L. Re-engineering the sterile insect technique. Insec Biochem Mole Bio. 2002; 32:1243–1247.
14. Alphey L. Engineering insects for the Sterile Insect Technique. In: Vreysen, MJB, Robinson AS, Hendrichs J. (Editors). Area-wide control of insect pests: From research to field implementation. Spr, Dordr. 2007;51-60.
15. Alphey L, Benedict M, Bellini R, Clark GG, Dame DA, Service MW. Sterile-insect methods for control of mosquito-borne diseases: An analysis. Vect Bor Zoo Dis. 2010;10:295–311.
16. Tripet F, Touré Y, Dolo G, Lanzaro GC. Frequency of multiple inseminations in field-collected *Anopheles gambiae* females revealed by DNA analysis of transfered sperm. American Journal of Trop Med Hyg. 2003;68:1–5.
17. Atkinson MP, Su Z, Alphey N, Alphey LS, Coleman PG, Wein LM. Analyzing the control of mosquito-borne diseases by a dominant lethal genetic system. Proc Nat Aca Sci. 2007;104:9540–9555.
18. Concha C, Palavesam A, Guerrero FD, Sagel A, Li F, Osborne JA, et al. A transgenic male-only strain of the New World Screwworm for an improved control program using the sterile insect technique. BMC Bio. 2016;14:72. DOI: 10.1186/s12915-016-0296-8
19. Besansky NJ, Collins FH. The mosquito genome: Organization, evolution and manipulation. Parasitol Tod. 1992;8:186–192.
20. Dorta DM, Vasukai V, Rajavel A. Evaluation of organophosphorus and synthetic pyrethroid insecticides against six vector mosquito species. Rev sau Pub. 1993;27:391–397.
21. Benedict MQ, Robinson AS. The first releases of transgenic mosquitoes: An argument for the sterile insect technique. Trends Parasitol. 2003;19:349–355.
22. Fu G, Condon KC, Epton MJ, Gong P, Jin L, Condon GC. Female-specific insect lethality engineered using alternative splicing. Nat. Biotech. 2007;25:353–357.
23. Thomas DD, Donnelly CA, Wood RJ, Alphey LS. Insect population control using

a dominant, repressible, lethal genetic system. Sci. 2000;287:2474–2476.
24. Alphey L, Nimmo D, O'connell S, Alphey N. Insect population suppression using engineered insects. In: Aksoy S. (Editor). Transgenesis and the management of vector-borne disease. Landes Biosci, Austin, Texas; 2007.
25. Heinrich JL, Scott MJ. A repressible female-specific lethal genetic system for making transgenic insect strains suitable for a sterile-release program. Proc of the Nat Acad Sci. 2000;97:8229–8232.
26. Klassen W, Curtis CF. History of the sterile insect technique. In: Dyck VA, Hendrichs J, Robinson AS (Editors). Sterile Insect Technique: Principles and practice in area-wide integrated pest management. Dordr. 2005;3–36.
27. Gong P, Epton MJ, Fu G, Scaife S, Hiscox A, Condon KC. A dominant lethal genetic system for autocidal control of the Mediterranean fruitfly. Nat Biotech. 2005; 23:453–456.
28. Stoddard BL. Homing endonuclease structure and function. Quart Rev Biophy. 2005;38:49–95.
29. Chevalier BS, Stoddard BL. Homing endonucleases: Structural and functional insight into the catalysts of intron/intein mobility. Nuc Aci Res. 2001;29:3757–3774.
30. Volna P, Jarjour J, Baxter S, Roffler SR, Monnat J. (Jr), Stoddard BL, et al. Flow cytometric analysis of DNA binding and cleavage by cell surface-displayed homing endonucleases. Nuc Aci Res. 2007;35: 2748–2758.
31. Burt A. Site-specific selfish genes as tools for the control and genetic engineering of natural populations. Proc Roy Soc B: Bio Sci. 2003;270:921–928.
32. Ashworth J, Havranek JJ, Duart CM, Sussman D, Monnat RJ (Jr), Stoddard BL. Computational redesign of endonuclease DNA binding and cleavage specificity. Nat. 2006;441:656–659.
33. Rosen LE, Morrison HA, Masri S, Brown MJ, Springstubb B, Sussman D, et al. Homing endonuclease I-CreI derivatives with novel DNA target specificities. Nuc Aci Res. 2006;34:4791–4800.
34. Smith J, Grizot S, Arnould S, Duclert A, Epinat JC, Chames P. A combinatorial approach to create artificial homing endonucleases cleaving chosen sequences. Nuc Aci Res. 2006;34:149.
35. Hahn MW, Nuzhdin SV. The fixation of malaria refractoriness in mosquitoes. Curr Bio. 2004;14:264–265.
36. Klinakis AG, Loukeris TG, Pavlopoulos A, Savakis C. Mobility assays confirm the broad host-range activity of the Minos transposable element and validate new transformation tools. Insec Mol Bio. 2000; 9:269–275.
37. Vlachou D, Schlegelmilch T, Christophides GK, Kafatos FC. Functional genomics analysis of midgut epithelial responses in *Anopheles* during *Plasmodium* invasion. Curr Bio. 2005;15:1185–1195.
38. Degennaro M, Mcbride CS, Seeholzer L, Nakagawa T, Dennis EJ, Goldman C. Orco mutant mosquitoes lose strong preference for humans and are not repelled by volatile DEET. Nat. 2013;498:487–491.
39. Ghosh AK, Ribolla PEM, Jacobs-Lorena M. Targeting *Plasmodium* ligands on mosquito salivary glands and midgut with a phage display peptide library. Proc Nat Aca Sci U.S.A. 2001;98(23):13278–13281.
40. Zieler H, Keister DB, Dvorak JA. A snake venom phospholipase A (2) blocks malaria parasite development in the mosquito midgut by inhibiting ookinete association with the midgut surface. J. Exp Bio. 2001; 204(23):4157–4167.
41. De Lara CM, Coleman J, Beerntsen BT. Virus-expressed, recombinant single-chain antibody blocks sporozoite infection of salivary glands in *Plasmodium gallinaceum*-infected *Aedes aegypti*. Americ J. Trop Med Hyg. 2000;62(4):427–433.
42. Kim W, Koo H, Richman AM, Seeley D, Vizioli J, Klocko AD, et al. Ectopic expression of a cecropin transgene in the human malaria vector mosquito *Anopheles gambiae* (*Diptera*: *Culicidae*): effects on susceptibility to *Plasmodium*. J. Med Entomol. 2004;41:447–455.
43. Christophides GK, Vlachou D, Kafatos FC. Comparative and functional genomics of the innate immune system in the malaria vector *Anopheles gambiae*. Imm Rev. 2004;198:127–148.
44. Larsson R. A rickettsia-like microorganism similar to *Wolbachia pipientis* and its occurrence in Culex mosquitoes. J. Invert Path. 2013;41:387–390.
45. Yen JH. Transovarial transmission of *Rickettsia*-like microorganisms in

mosquitoes. Ann N.Y Aca Sci. 2013;266: 152–161.
46. Sinkins SP. *Wolbachia* and cytoplasmic incompatibility in mosquitoes. Insec Bioch Mol Bio. 2004;34:723–729.
47. Sinkins SP, Walker T, Lynd AR, Steven AR, Makepeace BL. *Wolbachia* variability and host effects on crossing type in *Culex* mosquitoes. Nat. 2005;436:257–260.
48. Mcmeniman CJ, Lane RV, Cass BN, Fong AW, Sidhu M. Stable introduction of a life-shortening *Wolbachia* infection into the mosquito *Aedes aegypti*. Sci. 2009; 323(5910):141–144.
49. Rasgon JL. Dengue fever: Mosquitoes attacked from within. Nat. 2011;476:407–408,450–453.
50. Christodoulou M. Biological vector control of mosquito-borne diseases. Lanc Infec Dis. 2011;11:84–85.
51. Townson H. *Wolbachia* as a potential tool for suppressing filarial transmission. Ann Trop Med Parasitol. 2013;96(2):117–127.
52. Favia G, Ricci I, Damiani C, Raddadi N, Crotti E. Bacteria of the genus Asaia stably associate with *Anopheles stephensi*, an Asian malarial mosquito vector. Proc Nat Aca Sci. 2007;104(21):9047–9051.
53. Damiani C, Ricci I, Crotti E, Rossi P, Rizzi A. Mosquito-bacteria symbiosis: The case of *Anopheles gambiae* and *Asaia*. Micro Eco. 2012;60:644–654.
54. Yoshida S, Ioka D, Matsuoka H, Endo H, Ishii A. Bacteria expressing single-chain immunotoxin inhibit malaria parasite development in mosquitoes. Mol Biochem Parasitol. 2001;113:89–96.
55. Catteruccia F, Nolan T, Loukeris TG, Blass C, Savakis C, Kafatos FC. Stable germline transformation of the malaria mosquito *Anopheles stephensi*. Nat. 2000; 405(6789):959–962.
56. Itoh J, Gosh A, Moreira LA, Wimmer EA, Jacobs-Lorena M. *Transgenic anopheline* mosquitoes impaired in transmission of a malaria parasite. Nat. 2002;417:452-455. DOI: 10.1038/ 417452a
57. Jasinskiene N, Coates CJ, Benedict MQ. Stable transformation of the yellow fever mosquito, *Aedes aegypti* with the Hermes element from the housefly. Proc Nat Aca Sci U.S.A. 1998;95(7):3743–3747.
58. Coates CJ, Jasinskiene N, Miyashiro L. Mariner transposition and transformation of the yellow fever mosquito, *Aedes aegypti*. Proc Nat Aca Sci U.S.A. 1998;95(7):3748–3751.
59. Olson KE, Higgs S, Gaines PJ. Genetically engineered resistance to dengue-2 virus transmission in mosquitoes. Sci. 1996; 272(5263):884–886.
60. Kokoza V, Ahmed A, Cho WL. Engineering blood meal-activated systemic immunity in the yellow fever mosquito, *Aedes aegypti*. Proc Nat Aca Sci U.S.A. 2000;97(16): 9144–9149.
61. Beech CJ, Koukidou M, Morrison NI, Alphey L. Genetically modified insects: Science, use, status and regulation. Col Biosaf Rev. 2012;6:66–124.
62. Beisel U, Boëte C. The flying public health tool: genetically modified mosquitoes and malaria control. Sci Cult. 2013;22:38–60.
63. Facchinelli L, Valerio L, Ramsey JM, Gould F, Walsh RK, Bond G, et al. Field cage studies and progressive evaluation of genetically-engineered mosquitoes. PLoS Neg Trop Dis. 2013;7:e2001.
64. Resnik DB. Ethical issues in field trials of genetically modified disease-resistant mosquitoes. Dev World Bioet. 2014;14:37–46.
65. Moreira LA, Wang J, Collins FH, Jacobs-Lorena, M. Fitness of anopheline mosquitoes expressing transgenes that inhibit *Plasmodium* development. Gene. 2004;166(3):1337–1341.
66. Touré YT, Oduola AMJ, Sommerfeld J, Morel CM. Biosafety and risk assessment in the use of genetically modified mosquitoes for disease control, Chapter 16. Special Programme for Research and Training in Tropical Diseases (TDR), World Health Organization, 20 Avenue Appia, CH- 1211 Geneva, Switzerland. First updated on 25/6/2003 and modified on 27/4/2010.
67. Liu HS, Jan MS, Chou CK, Chen PH, Ke NJ. Is green fluorescent protein toxic to the living cells? Biochem Biophy Res Com. 1999;260:712–717.
68. Ribeiro JM, Kidwell MG. Transposable elements as population drive mechanisms: Specification of critical parameter values. J. Med Entomol. 1994;31:10–16.
69. Boete C, Koella JC. Evolutionary ideas about genetically manipulated mosquitoes and malaria control. Trends Parasitol. 2003;19(1):32–38.
70. Hartl DL, Lohe AR, Lozovskaya ER. Regulation of the transposable element mariner. Gene. 1997a;100:177–184.
71. Andreasen MH, Curtis CF. Optimal life stage for radiation sterilization of

Anopheles for sterile insect releases. Med Vet Entomol. 2005;19:238–244.

72. Okorie PN, Mckenzie FE, Ademowo OG, Bockarie M, Kelly-Hope L. Nigeria *Anopheles* vector database: An overview of 100 years' research. Plos One. 2011; 6(12).
73. Scott TW, Takken W, Knols BG. The ecology of genetically modified mosquitoes. Sci. 2002;298(5591):117–119.
74. Wheelis M, Spielman A, Regal P. Manual for assessing ecological and human health effects of genetically engineered organisms. The Edmonds Institute, Washington, USA; 1998. Availabe:http://www.edmonds-institute.org/manual.html
75. Macer D. Ethical, legal and social implications (ELSI) for the use of genetically modified disease vectors. Presentation at 6th World Congress of Bioethics, Brasilia, Brazil, UNDP/WORLD; 2002.
76. Curtis CF, Myamba J, Wilkes TJ. Comparison of different insecticides and fabrics for anti-mosquito bednets and curtains. Med Vet Entomol. 1996;10(1):1–11.

Peer-review history:
The peer review history for this paper can be accessed here:
http://www.sciencedomain.org/review-history/24480