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Transgenesis as a Tool to Reduce Parasite -Vector Interaction: a Review on the Progress for the Use of Genetically Manipulated *Anopheles* Mosquitoes to Control Malaria

Rania Ali El Hadi Mohamed[a]*, Wafa Abdullah Al-Megrin[b]

[a,b]*Biology Department, Faculty of Science, Princess Nourah Bint Abdull Rahman University, Riyadh 11491 Saudi Arabia*

[a]*Email: raniaelhadi@yahoo.com*

[b]*Email: parasitologist.2013@gmail.com*

Abstract

Malaria kills millions of people every year, imposing major economic and social burdens. Despite many efforts the classical control interventions which focus mainly on vector management and treatment of affected individuals with drugs. These interventions have proven inadequate to stop the transmission of *Plasmodium* parasites, subsequently the spread of malaria by *Anopheles* mosquitoes. The progressive numbers of insecticide-resistant insects and drug-resistant parasites have led to the search for a novel arsenal of strategies for inhibiting *Plasmodium* infection of mosquitoes. This work reviews current knowledge on genetic manipulation in mosquitoes that holds promise for development of transgenic mosquito refractory to malaria parasites transmission.

Keywords: Transgenic mosquitoes; vector competence; *Anopheles*; *Plasmodium*; symbiotic bacteria

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* Corresponding author.
E-mail address: raniaelhadi@yahoo.com.

1. Introduction

Worldwide a variety of vector borne diseases are transmitted to humans by mosquitoes such as malaria which is transmitted by anopheline mosquitoes [1]. Nearly half of the world population is at risk of contracting malaria and over one million people, mostly African children under the age of die, die of the disease every year [2]. The burden of malaria in developing countries (with deficit of qualified and motivated human resources, lack of technological expertise and limited financial resources) represents a major international challenge [3].

Various prevention and treatment strategies are being used to reduce malaria burden such as intermittent prophylaxis for pregnant women and children, insecticide-treated nets, indoor residual spraying of insecticides and anti-malarial combination therapies. Despite this progress made for the control of malaria there are limitations associated with these proven control strategies [4]. Consequently, this brought consideration of developing of new tools to eliminate arthropod-borne infectious pathogens or to block their transmission such as genetically modified mosquitoes (GMM) [5].

These studies performed by different scientific investigators, worldwide, included investigation of trans-genes capable to block infection in the host or parasite propagation inside the vector, searches for advanced approaches to avoid insecticides resistance by arthropods, search for tools to avoid drug resistance by the parasite. In this scenario researchers have been working on construction of transgenic mosquitoes refractory to malaria parasite. Research on transgenic mosquitoes to control malaria and genetically manipulated *plasmodium falciparum* to avoid parasite resistance to drugs will be the subjects of this review.

2. Principles of genetically modified (GM) mosquitoes: How they can be produced?

When a mosquito takes an infectious blood meal, the ingested gametocytes differentiate into male and female gametes that then mate to generate zygotes. Still in the midgut, zygotes differentiate into motile ookinetes [6]. At 24 hours, the motile ookinete invades the midgut epithelium and differentiates into an oocyst. About 2 weeks later, the oocyst ruptures, releasing thousands of sporozoites into the mosquito body cavity. At this stage, the parasites migrate to the salivary glands from where they can be transmitted to another host during a subsequent blood feed. Oocyst and sporozoite populations are severely compromised by mosquito-mounted immune responses, but the escape of a small proportion of parasites is sufficient for transmission to persist.

Recent technical advances in vector biology made possible a new strategy to combat malaria: genetically modifying the mosquito to reduce its vectorial competence. However, one crucial unresolved aspect of this approach is how to introduce effector transgenes, whose products interfere with parasite development in the mosquito, into wild mosquito populations in the field.

Germline transformation of *A. stephensi* was first reported in 2000 [7], and other important malaria vectors have since been transformed [8,9]. In the process of transformation, a mobile genetic element is used to insert into the mosquito genome a gene of interest that is under the control of a specific promoter. Choice of promoters and effector genes are some of the most important factors for generating mosquitoes that are refractory to *Plasmodium* infection and in limiting the adverse fitness effects exerted by transgene expression. Genetic drive

systems to integrate the transgene into wild mosquito populations are also essential for the implementation of genetically modified mosquitoes as tools for control of malaria transmission [10]. To target *Plasmodium* parasites during the developmental cycle, an effective anti-*Plasmodium* transgene must be expressed in a relevant tissue (midgut, fat body, and salivary glands) at a relevant time (time in which the parasite is exist in that tissue). The promoter used for transgene expression will determine the timing and the mosquito tissue in which the transgene will be expressed [10].

Transmission blocking vaccines have been adopted as a mechanism for malaria control [11]. These vaccines consist of antibodies that are ingested by the mosquito with the blood meal and interfere with parasite development. Proteins expressed on the surface of gametes (e.g. Pfs47/48, Pfs230) and ookinetes (e.g. Pfs25 and Pfs28) have been tested for such vaccines [12, 13]. Antibodies against these proteins bind to the parasite and presumably block ookinete invasion of the midgut epithelium. Various investigations revealed that polyclonal antibodies against mosquito midgut proteins interfere with *Plasmodium* oocyst formation have been published [14], but in no case have the relevant antigens been identified [15].

Of all the tissues that sporozoites come in contact with, they can invade only the salivary gland. When the mosquito bites another vertebrate host, transmission is completed by release of sporozoites from the salivary glands [16]. The invasion of salivary glands by sporozoites is thought to be mediated by receptor–ligand-like interactions resulting from the binding of parasite surface ligands to specific receptors on the salivary glands [17].

This is interpreted to indicate that sporozoites have some mechanism for differentiating among the multiple mosquito organs suspended in the hemocoel. Electron microscope studies of sporozoite interactions with salivary glands also lend support for a receptor–ligand model [18].

A study showed that there were species-specific recognition properties of sporozoites for salivary glands. *Plasmodium knowlesi* sporozoites could recognize and invade salivary glands from *Anopheles dirus* even when the glands were transplanted to a non-permissive host, *An. freeborni*. Conversely, these sporozoites could not infect *An. freeborni* salivary glands under any circumstances [19]. Competent parasite ligands for salivary gland recognition and invasion include the circumsporozoite protein (CSP). The CSP is the major protein on the surface of sporozoites, and may account for as much as 10% of the protein located there [20]. Some of the monoclonal antibodies made to *P. gallinaceum* CSP blocked sporozoite invasion of *Ae. aegypti* salivary glands [21].

In anophelines, midgut specific transgene expression has been achieved using the Carboxypeptidase [22], peritrophin [23], *Antryp1*, and *G12* [24] promoters, the vitellogenin promoter has been used to drive transgene expression in the mosquito fat body [25], and the *apyrase* [26] and *anopheline antiplatelet protein* [27] promoters can drive transgene expression in the salivary glands. Conditional transgene expression in *A. stephensi* midguts under the control of the *SRPN10* promoter has also been shown [28].

3. Anopheles spp control: Current situation and gene manipulation as an alternative control method

Vector control remains generally the most effective method to prevent malaria because there is no available vaccine for the disease [32, 33]. Most of the vector control strategies focus on components with insecticidal activity that would persist in the environment in which these were applied. Most, if not all, new developments in the control of anophelines presented incremental improvements of this concept [34].

Occurrence of drug-resistant parasites and insecticide-resistant mosquitoes, have been contributing to re-emergence and difficulties in controlling important arthropod-borne diseases [35]. Insecticide resistance was, and is still today, viewed as an unavoidable consequence of widespread insecticide use that can either be managed or overcome by the discovery of new compounds with desirable (i.e., long-term mosquitocidal) characteristics such as insecticide treated nets (ITNs) [36] and the renewed acceptability of DDT for vector control following the agreement to the Stockholm Convention on Persistent Organic Pollutants [37]. Both the use of ITNs and indoor residual spraying (IRS) target mosquito vectors in the domestic environment, and interest in peridomestic control strategies (e.g., larval control) is slowly reviving [38,39].

4. Mechanism to introduce anti-Plasmodium effector transgenes into wild mosquitoes

A powerful drive mechanism is essential to spread the transgene to near establishment in the population, be tightly linked with the transgene so that separation cannot occur and have minimal impact on mosquito fitness. Potential drive mechanisms are naturally occurring “selfish” gene mechanisms with non-Mendelian inheritance [40].

Transposable elements (TEs) are mobile genetic elements that are capable of moving rapidly into populations and can be engineered to carry a transgene through a population. Today, four different transposable elements: Hermes, Mos1 (mariner), Minos, and piggyback have been widely used for germ-line transformation of numerous mosquito species including *Culex quinquefasciatus*, *Anopheles stephensi*, *Anopheles gambiae* , and *Anopheles albimanus* [41]. However, the rates of transposition for the class II transposons Hermes, Minos, Mos1, and piggybac, which have been vital for mosquito transgenesis, are not sufficient to serve as drive systems [42]. While TEs randomly integrate into a genome, HEGs use a specific DNA sequence to integrate into the chromosome through a mechanism of double-stranded DNA break repair. These enzymes are active in *A. gambiae* cells and embryos [43] and can also be engineered to carry specific DNA sequences. A breakthrough in mosquito-based genetic drive systems was recently achieved with the successful introduction of an HEG into transgenic anopheline mosquitoes [44].

In cage studies, it was shown that the genetic element could invade naive mosquito populations rapidly and may provide a novel mechanism of genetic modification of wild mosquitoes [44]. Medea, or maternal-effect-dominant embryonic arrest, causes the death of all offspring that do not inherit the Medea-bearing gene [45]. In this system, there is maternal expression of a toxin regulated by a germ line specific promoter and only zygotes expressing an antidote to the toxin will survive. As novel mosquito germline-specific promoters are discovered, such as DNA regulatory regions of the vasa gene [46], both HEGs and Medea will have tremendous potential as genetic drive systems in mosquitoes.

In order for transgenic mosquito technologies to be successfully applied, the genetically modified mosquitoes must be able to compete with wild mosquitoes. Therefore, the transgenic mosquito must be reproductively fit to ensure that the transgene will established in the population [47]. The transformation efficiency, which can be described as the percentage of fertile adults that produce transgenic progeny, does not vary substantially between the different transposable elements. In *Anopheles* mosquitoes, *piggyBac* has mainly been used for germ-line transformation. The efficiencies in *Anopheles gambiae* range from 1 to 10%, whereas in *Anopheles albimanus* and *Anopheles stephensi* mosquitoes over 10% have been observed [48, 49, 50, 51, 52]. Probably the most important factors that contribute to the efficiency of transformation are practical aspects such as the quality and concentration of the injected nucleic acid, the total insert size (piggyBac = 10–13 kb, PhiC31 ~42 kb), timing of injection (prior to pole cell formation), needle preparation, robustness of mosquito strain and ambient conditions [53]. However, some studies showed that transgenic mosquitoes are as fit as non-transgenic mosquitoes [54, 55].

5. Malaria Control using engineered symbiotic bacteria inhibit midgut of mosquito vectors

Genetic manipulations of mosquito midgut-associated bacteria (MAB) (which live in the midgut, the same mosquito compartment where the most vulnerable stages of *Plasmodium* development occur) have also been used as a tool to reduce the development of *Plasmodium* parasite inside mosquitoes. Common bacterial genera (*Enterobacter*, *Pseudomonas*, *Pantoea*, and others) have been identified [56]. The quantity of mosquito midgut bacteria increases dramatically upon blood feeding (when parasites are ingested), consequently increasing the output of the effector molecules that they are engineered to produce. Genetic modification of bacteria is much simpler and faster than genetic manipulation of mosquitoes [4].

Numerous studies revealed that Mosquitoes that have been treated with antibiotics to remove their MAB are more susceptible to *Plasmodium* infection, and reconstitution of the bacterial flora results in infections at the same level as untreated control mosquitoes [57]. When added to a parasite-laden blood meal, bacteria can interfere with parasite development [58]. Interestingly, this interference appears to be exclusive to Gram-negative (G−) bacteria but is bacterial strain dependent, suggesting some bacteria possess an anti-Plasmodium property [59]. However, no correlation between G bacteria presence and infection status was observed in field populations of *A. gambiae* and *A. funestus* from Kenya and Mali, although determination of the timing of bacterial and/or parasite acquisition by the mosquitoes was not performed [60].

Multiple mechanisms could result in the inhibition of parasite infection by the presence of bacteria. It was recently identified that an *Enterobacter* bacterium isolated from wild mosquitoes in Zambia produces reactive oxygen intermediates that kill developing parasites in the midgut lumen, inhibiting *Plasmodium* prior to mosquito midgut infection [56]. Small populations of the bacterium can nearly eliminate ookinete formation in the midgut, providing proof of principle for the use of this and other bacteria to control malaria parasite transmission [56]. In general, G− bacteria show varying levels of inhibition at the early stages of parasite development, suggesting that diverse mechanisms of bacteria-mediated parasite inhibition exist [(56)]. Bacteria may play an indirect role in parasite interference through the induction of an anti-*Plasmodium* immune response in the midgut. Studies have suggested that the mosquito's anti-*Plasmodium* and antibacterial defense systems

are largely overlapping. The mosquito gut microflora has been shown to stimulate basal immune activity, which in turn is acting against the malaria parasite [57].

Disadvantage of this method is the resistant. This defect has been solved by the use of multiple effector proteins by formulating an efficient multi-effector combination. This can simply be achieved by feeding mosquitoes a mixture of GM bacteria expressing different effector genes [4]. Importantly, this approach bypasses genetic barriers of reproductively isolated mosquito populations and will hinder the spread of mosquito transgenes. Furthermore unlike mosquito transgenes, inactivation of bacterial transgenes after many generations in the field is not a problematic issue because of the easier logistics of introducing freshly transformed bacteria. Moreover, if an effector gene fails to perform as promised, introduction of alternate transgenes is relatively simple. Besides the above mentioned advantages regulations already exist regarding evaluation of bacteria to be released into the environment [4]. Each of the two methods (GM and manipulated bacteria) has their advantages and disadvantages for eventual implementation, but in the future may be combined as part of an integrated control strategy for malaria transmission [10].

6. Manipulated mosquitoes for malaria Control; advanced techniques implementation challenges

Despite the fact that several achievements have been made about *Anopheles* and *Aedes* mosquitoes there are major biotechnology challenges remaining about the improvement of the stability of a gene construct and its expression for a robust and complete interruption of pathogen transmission and the devise of safe means of spreading foreign antipathogen genes through mosquito populations in the wild. The implementation obstacles to overcome include proper risk assessment [61]. Furthermore it's essential to build the capacity (individual and community) of transfer the biotechnology in malaria-endemic countries to better address ethical, legal and social aspects of biotechnologies (e.g. transgenic mosquitoes) for promoting engagement of individuals, and communities about the development, applications, and evaluation of genetics-based methods for disease control [4].

GM mosquitoes are being developed for use in vector control related to malaria under individual institutional or national guidelines on research and biosafety in spite of the lack in directed international guidance [4]. A pioneer study conducted in Mali mapped out several crucial aspects of potential acceptance or rejection of GM mosquitoes. The study also revealed that acceptance was dependent on several conditions [61]. Recently some have advocated a total precautionary principle for genetic engineering, which would mean that no technology with more than 0% risk should ever be, attempted [62]. The UNDP/World Bank/WHO Special program for Research and Training in Tropical Diseases (TDR) has been developing the ideas of genetic control of insect vectors since a 1991 meeting on use of genetically modified (GM) mosquitoes to replace disease vectors. TDR's Steering Committee for Molecular Entomology has outlined a three-pronged effort towards developing GM mosquitoes for malaria control, with similar approaches for dengue fever and Chagas' disease [63]. First in the process for each disease is to study host–parasite interaction; second is to develop methods to transform the vector; and third is to look at population ecology and genetics and at how to replace a population of harmful vector insects with a population of non-harmful insects. Social factors need to be carefully considered [64].

Some papers also considered a range of ethical issues including animal rights, informed consent, community

consensus and environmental viewpoints. Researchers investigated the ethical standards for the use of GM mosquitoes for diseases control concluded that each community needs to decide its own priorities for methodology of disease policy guidance for ethical genetic engineering, and to negotiate with neighboring countries [65]. The approach to GM insects raises few intrinsic ethical issues; however, important environmental and human health concerns need to be assessed before release of any GM insects. The policy that each community adopts should be the product of open dialogue involving all sectors of society. It can be expected that this process will take years and not all communities will endorse genetic control approaches [65]. However information about the acceptance of this advanced technique in the control of malaria is limited.

7. Study limitations

All attempts at controlling the spread of diseases by transgenic mosquitoes have to subtend obstacles such as:

- Lack of risk assessment carried out on the GM insects.
- Regulations for GM insect release are also lacking, with no specific regulations existing in any country.
- Ineffective, inefficient, costly, and hazardous to varying degrees.
- Reduction or eradication of natural populations of disease vectors by introducing dominant lethal gene.

8. Recommendations

- Construct transgenic mosquito projects in malaria endemic areas in the Middle East and Africa.
- Establish a working group to develop a guidance framework document for quality standards to assess safety and efficacy and address regulatory, legal, ethical, social and cultural (ESC) issues during GMM development and testing.
- Several refractory genes will be necessary for a successful intervention both to improve the efficacy of refractoriness, and to reduce the probability that resistance to antipathogen genes will emerge in the Plasmodium population.
- Optimization of gene drive systems to deliver these refractory genes into mosquito populations.
- A broad study is required of the ecology of mosquito vectors through which the refractory genes are intended to be driven.

References

[1] Marcia and Margareth. Perspectives in the control of infectious diseases by transgenic mosquitoes in the post-genomic era – A Review. *Rio de Janeiro* 2007; 102: 425-433.

[2] Hay SI, Guerra CA, Gething PW, Patil AP, Tatem AJ, Noor AM, Kabaria CW, Manh BH, Elyazar IR, Brooker. A world malaria map: Plasmodium falciparum endemicity in 2007. *PLoS Me* 2007; 6e1000048.

[3] Sachs JD. A new global effort to control malaria. *Sciences* 2002; 298:122–124.

[4] World Health Organization. First meeting report on planning: Technical consultation on current status and

planning for future development of genetically modified mosquitoes for malaria and dengue control 2009; DOI 10.2471/TDR.10.978-924-1599238: 4-64.

[5] Bin Dajem SM, Al-Farsi HM, Al-Hashami ZS, Al-Sheikh AAH, Al-Qahtani A, and Babiker HA. Distribution of Drug Resistance Genotypes in *Plasmodium falciparum* in an Area of Limited Parasite Diversity in Saudi Arabia. *The American Society of Tropical Medicine and Hygiene* 2012; 86:782–788.

[6] Ghosh AK and Lorena MJ. *Plasmodium* sporozoite invasion of the mosquito salivary gland. *Curr Opin Microbiol* 2009; 12:394–400.

[7] Catteruccia F, Holan T, Loukeris TG. Stable germline transformation of the malaria mosquito *Anopheles stephensi*. *Nature* 2012; 405: 959–962.transformation of the malaria vector *Anopheles gambiae*, with the piggyback transposable element. *Insect Molecular Biology* 2001; 10: 597–604.

[9] Perera OP, Harrell RA, and Handler AM. Germline transformation of the South American malaria vector, *Anopheles albimanus*, with a piggyBac/EGFP transposon vector is routine and highly efficient. *Insect Molecular Biology* 2002; 11: 291–297.

[10] Cirimotich CM, April Clayton AM, and Dimopoulos G. Low- and High-Tech Approaches to Control *Plasmodium* Parasite Transmission by *Anopheles* Mosquitoes. *Journal of Tropical Medicine* 2011; doi:10.1155/2011/891342.

[11] Carter, R. Transmission blocking malaria vaccines. *Vaccine* 2001; 19: 2309- 2314.

[12] Duffy, P. E. and Kaslow, D. C. A novel malaria protein, Pfs28, and Pfs25 are genetically linked and synergistic as *falciparum* malaria transmission-blocking vaccines. *Infect. Immun* 1997; 65: 1109-1113.

[13] Healer, J., McGuinness, D., Carter, R. and Riley, E. Transmission blocking immunity to *Plasmodium falciparum* in malaria-immune individuals is associated with antibodies to the gamete surface protein Pfs230. *Parasitol* 1999; 119: 425-433.

[14] Ramasamy, M. S. and Ramasamy, R. Effect of anti-mosquito antibodies on the infectivity of the rodent malaria parasite *Plasmodium berghei* to *Anopheles farauti*. *Med. Vet. Entomol* 1990; 4, 161-166.

[15] Jacobs-Lorena, M. and Lemos, F. J. Immunological strategies for control of insect disease vectors: a critical assessment. *Parasitol Today* 1995; 11: 144-147.

[16] Riehle MA, Srinivasan P, Moreira CK and Lorena MJ. Towards genetic manipulation of wild mosquito populations to combat malaria: advances and challenges. *The Journal of Experimental Biology* 2003; 206: 3809-3816. doi:10.1242/jeb.00609.

[17] Beerntsen, B., James, A. A. and Christensen, B. Genetics of mosquito vector competence. *Microbiol. Mol. Biol. Rev* 2000; 64: 115-137.

[18] Golenda, C. F., Starkweather, W. H. and Wirtz, R. A. The distribution of circumsporozoite protein (CS) in *Anopheles stephensi* mosquitoes infected with *Plasmodium falciparum* malaria. J. *Histochem. Cytochem* 1990; 38: 475-481.

[19] Rosenberg, R. Inability of Plasmodium knowlesi sporozoites to invade *Anopheles freeborni* salivary glands. *Am. J. Trop. Med. Hyg* 1985; 34: 687-691.

[20] Nussenzweig, V. and Nussenzweig, R. S. Circumsporozoite proteins of malaria parasites. *Bull. Mem. Acad. R. Med. Belg* 1998; 144: 493-504.

[21] Warburg, A., Touray, M., Krettli, AU., Miller, LH. *Plasmodium gallinaceum*: antibodies to circumsporozoite protein prevent sporozoites from invading the salivary glands of *Aedes aegypti*. *Exp. Parasitol* 1992; 75, 303-307.

[22] Ito J, Ghosh A, Moreira LA, Wimmer EA, and Jacobs-Lorena M. Transgenic anopheline mosquitoes impaired in transmission of a malaria parasite. *Nature* 2002; 417: 6887: 452–455.

[23] Abraham EG, Donnelly-Doman M, Fujioka H, Ghosh A, Moreira L, and Jacobs-Lorena M. Driving midgut-specific expression and secretion of a foreign protein in transgenic mosquitoes with AgPer1 regulatory elements. *Insect Molecular Biology* 2005; 14:3: 271–279.

[24] Nolan T, Petris E, Muller HM, Cronin A, Catteruccia F, and Crisanti A. Analysis of two novel midgut-specific promoters driving transgene expression in *Anopheles stephensi* mosquitoes. *PLoS One* 2011; 2. DOI e16471.

[25] Chen XG, Marinotti O, Whitman L, Jasinskiene N, and James AA. The *Anopheles gambiae* vitellogenin gene (VGT2) promoter directs persistent accumulation of a reported gene product in transgenic *Anopheles stephensi* following multiple blood meals. *American Journal of Tropical Medicine and Hygiene* 2007; 76: 6: 1118–1124.

[26] Lombardo F, Lycett GJ, Lanfrancotti A, Coluzzi M, and Arc B. Analysis of apyrase 5_ upstream region validates improved *Anopheles gambiae* transformation technique," *BMC Research Notes* 2009; 2: 23-29.

[27] Yoshida S and Watanabe H. Robust salivary gland-specific transgene expression in *Anopheles stephensi* mosquito. *Insect Molecular Biology* 2006; 15: 4: 403–410.

[28] Lycett GJ, Kafatos FC, and Loukeris TG. Conditional expression in the malaria mosquito *Anopheles stephensi* with Tet-on and Tet-off systems. *Genetics* 2004; 167:1781– 1790.

[29] Yoshida S, Shimada Y, Kondoh D. Hemolytic C-type lectin CEL-III from sea cucumber expressed in transgenic mosquitoes impairs malaria parasite development. *PLoS Pathogens* 2007; 3: 192- 197.

[30] Meredith JM, S. Basu and Nimmo DD. Site-specific integration and expression of an anti-malarial gene in

transgenic *Anopheles gambiae* significantly reduces *Plasmodium* infections. *PLoS One* 2011; 6: 1: DOI e14587.

[31] Corby-Harris V, Drexler A, de Jong LW. Activation of Akt signaling reduces the prevalence and intensity of malaria parasite infection and lifespan in *Anopheles stephensi* mosquitoes. *PLoS Pathogens* 2010; 6: 7: DOI e1001003.

[32] Snow RW, Guerra CA, Noor AM, Myint HY, Hay SI. The global distribution of clinical episodes of *Plasmodium falciparum* malaria. *Nature* 2005; 2: 214–217 [PubMed: 15759000].

[33] Breman JG, Egan A, Keusch GT. The intolerable burden of malaria: a new look at the numbers. *Am J Trop Med Hyg* 2001; 64: 1–2 Suppl:iv–vii. [PubMed: 11425185].

[34] Hemingway J, Field L, Vontas J. An overview of insecticide resistance. *Science* 2002; 298: 96–97.

[35] Christian Epp, Raskolnikov D and Deitsch KW. A regulatable transgene expression system for cultured *Plasmodium falciparum* parasites. *Malaria journal* 2008; 7:8: doi: 10.1186/1475-2875-7-86.

[36] Hill J, Lines J, Rowland M. Insecticide-treated nets. *Adv Parasitol* 2006; 61: 77–128.

[37] Maharaj R, Mthembu DJ, Sharp BL. Impact of DDT reintroduction on malaria transmission in KwaZulu-Natal. *S Afr Med* J 2005; 95: 871–874.

[38] Killeen GF, Seyoum A, Knols BGJ. Rationalizing historical successes of malaria control in Africa in terms of mosquito resource availability management. *Am J Trop Med Hyg* 2004; 71: 87– 93.

[39] Gu W, Novak RJ. Habitat-based modeling of impacts of mosquito larval interventions on entomological inoculation rates, incidence, and prevalence of malaria. *Am J Trop Med Hyg* 2005; 73: 546–552.

[40] Sinkins SP and Gould F. Gene drive systems for insect disease vectors. *Nature Reviews Genetics* 2006; 7: 427–435.

[41] Fuchs S, Nolan T, Crisanti A. Mosquito transgenic technologies to reduce *Plasmodium* transmission. *Methods in Molecular Biology* 2013; 923: 601-622

[42] O'Brochta DA, Sethuraman N, Wilson R. Gene vector and transposable element behavior in mosquitoes. *Journal of Experimental Biology* 2003; 206: 3823–3834.

[43] Windbichler N, Papathanos PA, Catteruccia F, Ranson H, Burt A, and Crisanti. Homing endonuclease mediated gene targeting in *Anopheles gambiae* cells and embryos. *Nucleic Acids Research* 2007; 35: 5922–5933.

[44] Windbichler N, Menichelli M, Papathanos PA. A synthetic homing endonuclease-based gene drive system

in the human malaria mosquito. *Nature* 2011; 473: 212- 219.

[45] Chen CH, Huang H, Ward CM. A synthetic maternal-effect selfish genetic element drives population replacement in *Drosophila*. *Science* 2007; 316: 597–600.

[46] Papathanos PA, Windbichler N, Menichelli M, Burt A, and Crisanti A. The *vasa* regulatory region mediates germline expression and maternal transmission of proteins in the malaria mosquito *Anopheles gambiae*: a versatile tool for genetic control strategies. *BMC Molecular Biology* 2009; 10: 29-36.

[47] Chen XG, Mathur G, and James AA. Chapter 2 Gene expression studies in mosquitoes, *Advances in Geneticsm*2008; 64: 19–50.

[48] Nolan T. *piggyBac*- mediated germline transformation of the malaria mosquito *Anopheles stephensi* using the red fluorescent protein dsRED as a selectable marker. *J Biol Chem* 2002; 277:8759–8762.

[49] Perera OP. Germ-line transformation of the South American malaria vector, *Anopheles albimanus*, with a *piggyBac* /EGFP transposon vector is routine and highly efficient. *Insect Mol Biol* 2002; 11:291–297.

[50] Meredith JM. Site-specific integration and expression of an anti-malarial gene in transgenic *Anopheles gambiae* significantly reduces *Plasmodium* infections. *PLOS One* 2011; 6: DOI e14587.

[51] Windbichler N. Targeting the X chromosome during spermatogenesis induces Y chromosome transmission ratio distortion and early dominant embryo lethality in *Anopheles gambiae*. *PLoS Genet* 2008; 4: DOI e1000291.

[52] Lobo NF. High efficiency germline transformation of mosquitoes. *Nat Protoc 2006;* 1:1312–1317.

[53] Chater KF. Dispensable sequences and packaging constraints of DNA from the Streptomyces temperate phage phi C31. *Gene* 1981; 15:249–256

[54] Li C, Marrelli MT, Yan G, and Jacobs-Lorena M. Fitness of transgenic *Anopheles stephensi* mosquitoes expressing the SM1 peptide under the control of a vitellogenin promoter. *Journal of Heredity* 2008; 99: 275–282.

[55] Moreira LA, Wang J, Collins FH, and Jacobs-Lorena M. Fitness of anopheling mosquitoes expressing transgenes that inhibit *Plasmodium* development. *Genetics* 2004; 166: 1337–1341.

[56] C. M. Cirimotich CM, Dong Y, Clayton AM. Natural microbe-mediated refractoriness to *Plasmodium* infection in *Anopheles gambiae*. *Science* 2011; 332: 6031:855.

[57] Dong Y, Manfredini F, and Dimopoulos G. Implication of the mosquito midgut microbiota in the defense against malaria parasites. *PLoS Pathogens* 2009; 5: 5 DOI: e1000423.

[58] Gonzales-Ceron L, Santillan F, Rodriguez MH, Mendez D, and Hernandez-Avila JE. Bacteria in midguts of field collected *Anopheles albimanus* block *Plasmodium vivax* sporogonic development. *Journal of Medical Entomology* 2003; 40: 371–374.

[59] Pumpuni CB, Beier MS, Nataro JP, Guers LD, and Davis JR. *Plasmodium falciparum*: inhibition of sporogonic development in *Anopheles stephensi* by gram-negative bacteria. *Experimental Parasitology* 1993; 77:195–199.

[60] Straif SC, Mbogo CN, Toure AM. Midgut bacteria in *Anopheles gambiae* and *An. Funestus* (Diptera: Culicidae) from Kenya and Mali. *Journal of Medical Entomology* 1998; 35: 222–226.

[61] Touré YT, Oduola AMJ, Sommerfeld J, Morel CM. Biosafety and risk assessment in the use of genetically modified mosquiotes for disease control. In: Takken W, Scott TW, editors. Ecological Aspects for Application of Genetically Modified Mosquitoes. Wageningen: *Kluwer Academic Publishers* 2009; chapter 6: 217-222.

[62] Ho. M.W. Genetic engineering: dream or nightmare? Gateway Books, London 1998.

[63] TDR. Scientific Working Group on Insect Disease Vectors and Human Health, Geneva, WHO/HQ, 12–16 August 2002. TDR, Geneva, document TDR/SWG/VEC/03.1.

[64] Macer D. Ethical, legal and social issues of genetically modified disease vectors in public health. UNDP/World Bank/ WHO Special program for Research and Training in Tropical Diseases (TDR), Geneva 2003.

[65] Macer D. Ethical, legal and social issues of genetically modifying insect vectors for public health. *Insect Biochemistry and Molecular Biology* 2005; 35: 649–660.