



REVIEW ARTICLE

New weapons to fight malaria transmission: A historical view

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Abstract

The stagnation of our fight against malaria in recent years, mainly due to the development of mosquito insecticide resistance, argues for the urgent development of new weapons. The dramatic evolution of molecular tools in the last few decades led to a better understanding of parasite–mosquito interactions and coalesced in the development of novel tools namely, mosquito transgenesis and paratransgenesis. Here we provide a historical view of the development of these new tools and point to some remaining challenges for their implementation in the field.

Key words: Biology, Ecology and Behavior, Disease transmission and blood feeding arthropods, Malaria transmission, Medical and Veterinary Entomology, Symbiotic relationships

The intolerable burden of malaria

The numbers are staggering. It is estimated that in 2020, 241,000,000 (241 million) malaria cases and 627,000 (more than half a million) malaria deaths occurred worldwide. And very worrisome, this represents an increase of 14 million cases and 69 thousand deaths compared to the previous year (WHO World Malaria Report, 2021). On the positive side, significant headway has been made to cut down malaria cases and deaths since the beginning of this century, mainly using insecticide-impregnated bed nets and indoor residual insecticide spraying. However, these gains are gradually being reversed with the development of mosquito insecticide resistance and changes of mosquito behavior toward outdoor biting, where the use of insecticides is not practical. In summary, the only approach currently used to combat transmission – killing the mosquito – after initial success, is losing effectiveness mainly due to development of insect resistance by the obligatory mosquito vector. There is an urgent need to develop new weapons and approaches, so we can return to saving a large number of lives.

Development of molecular tools, from the “Dark Ages” onwards

The structure of DNA was first published almost 70 years ago (Watson & Crick 1953). A practical method for DNA

sequencing was first published about 25 years later (Sanger *et al.* 1977). These events served as a basis for a flurry of important discoveries in the field of molecular biology, including gene cloning (Morrow *et al.* 1974), RNA splicing (Berget *et al.* 1977), characterization of promoters, regulation of gene expression, and advances in many other fields of knowledge. The discovery of RNA interference (RNAi) in *Caenorhabditis elegans* (Fire *et al.* 1998) and its later use to manipulate gene expression in the mosquito (Blandin *et al.* 2002), revolutionized the investigation of gene function in cells and organisms in present times.

Relevant to this discussion, the fruit fly *Drosophila melanogaster* was among the first organisms to be genetically engineered. This was accomplished by use of the *Drosophila* P transposable element to insert DNA encoding an eye color gene in its genome (Spradling & Rubin 1982). Substantial research activity in *Drosophila* developmental genetics had already started much earlier. In stark contrast, molecular investigation of insect vectors was dormant during this time. The first mosquito gene to be cloned was published in the late 1980s (James *et al.* 1989). This dormant state of vector biology is surprising, given its medical importance. This state of affairs was profoundly changed with the MacArthur Foundation’s creation in 1989 of the Vector Biology Network. This was an international consortium of leading researchers recruited from various fields who were charged to apply

modern molecular approaches to the study insect vectors of disease (Beaty *et al.* 2009). This MacArthur initiative was remarkably effective, as reflected by the dramatic increase in the number of vector-related publications in leading journals and in the number of vector grants funded by the US National Institutes of Health (Beaty *et al.* 2009).

The first mosquito genetic modification was published in 1998. It used the house fly Hermes transposable element to insert an eye color gene into *Aedes aegypti* (Jasinskiene *et al.* 1998). This was followed 2 years later, by the germline transformation of the *An. stephensi* malaria vector, using a *Drosophila* Minos transposable element (Catteruccia *et al.* 2000), followed the next year with the transformation of the main African vector *An. gambiae* (Grossman *et al.* 2001).

Why did it take 16 years between the publication of *Drosophila* and mosquito transgenesis? Several factors played a role. One is the “dormancy” of vector molecular biology referred to above. Another is that after publication of the seminal work by Spradling and Rubin, a large effort was devoted by many laboratories to use the P transposable element to introduce cloned DNA into the genome of a variety of organisms and cultured cells, with no positive results, with the possible exception of one spurious event (Miller *et al.* 1987). The explanation for this failure came later, as it was determined that P element transposition activity is specific to *D. melanogaster*, whereas other elements transpose in a host-independent manner. Another limiting factor relates to the low frequency of germ line integration when injecting insect embryos. Consequently, only a small proportion of the surviving mosquitoes carry a transgene in their germ cells, necessitating a marker to identify the rare transformants. Initially *Drosophila* or *Aedes* eye color mutants were used as recipients and the transforming construct carried a gene that complements the eye mutant; the successfully transformed insects could be identified by their wild-type eye color. While this protocol was successful, it imposed a significant barrier for general use, because of the need of first isolating a host insect eye color mutant that is defective in a known gene. This restriction was overcome with the discovery of green fluorescent protein (GFP) as a cell marker (Chalfie *et al.* 1994). GFP expression obviates the need to isolate an eye color mutant, thus contributing to great progress in the generation of transgenic insects in general.

Development of novel tools to fight malaria transmission

As mentioned, the decades-old approaches to combat malaria *via* killing of the mosquitoes is gradually losing its effectiveness, in great part due to the development of vector insecticide resistance. How can we reverse this trend? The

development of new and safe insecticides that act on additional mosquito targets is being pursued, but even if successful, the risk of development of resistance once again is a concern. Clearly, the development of new approaches that can be combined with existing measures (including use of insecticides) is highly desirable.

The convergence of impressive progress made at multiple fronts in the understanding of mosquito physiology and the molecular basis for *Plasmodium*-mosquito interactions have opened the way for the development of entirely new measures. Of particular interest is the strategy of converting mosquitoes to being poor vectors of the parasite. This was the goal set at the creation of the MacArthur Vector Biology Network (Beaty *et al.* 2009), even though it sounded like science fiction at the time, as most tools to achieve this aim were not available. *Drosophila* transgenesis had long since been demonstrated (Spradling & Rubin 1982), so why not the mosquito? For this to be achieved, three main tools needed to be developed: (i) identification of a promoter that can drive expression of an anti-parasite gene in the appropriated mosquito tissue; (ii) identification of a protein that arrests parasite development in the mosquito (here forth referred to as ‘effector protein’ or simply ‘effector’); and (iii) an effective method of inserting recombinant DNA into mosquito germ cells. These tools became available in the late 1990s – early 2000s.

The most vulnerable stages of *Plasmodium* development in the mosquito occur in the midgut, where parasite numbers decrease into a strong bottleneck (Smith *et al.* 2014). The demonstration that the mosquito carboxypeptidase promoter is strongly induced in the mosquito by ingestion of blood and that the protein’s signal sequence promotes efficient secretion into the midgut lumen (Moreira *et al.* 2000) satisfied requirement (i). Since then, the carboxypeptidase has been almost universally used for driving gene expression in the midgut of transgenic mosquitoes. A constitutive gut-specific promoter – AgAper1 – derived from a peritrophic matrix gene was identified subsequently (Abraham *et al.* 2005). Moreover, a promoter from the fat body-specific vitellogenin gene was used for abundant protein secretion into the mosquito hemocoel peaking at 24 h after the blood meal (Kokoza *et al.* 2000). Later, a strong salivary gland-specific promoter was identified (Yoshida & Watanabe 2006).

An essential prerequisite for an effector [requirement (ii)] is that it efficiently and specifically inhibits parasite development and, importantly, is harmless to the mosquito and any other organism. An early effector was identified from a project that investigated the molecular basis for *Plasmodium* ookinete traversal of the mosquito midgut epithelium. A screen of a phage display library identified a peptide – SM1 – that effectively binds to the mosquito midgut epithelial cell luminal surface and importantly, strongly inhibits ookinete mosquito midgut epithelium crossing, in this way arresting parasite development (Ghosh *et al.* 2001). Another protein –

phospholipase A2 (PLA2) – that binds the mosquito midgut epithelium was also shown to interfere with ookinete crossing (Zieler *et al.* 2001). Subsequently, many other effectors have been identified, including antimicrobial peptides, venom peptides, anti-parasite antibodies, in addition to manipulation of mosquito immune genes and of regulatory RNAs.

As mentioned above, mosquito transgenesis [requirement (iii)] became a reality at about the same time as tissue-specific promoters and as the first effector proteins were identified. These findings coalesced in the genetic engineering of the first transgenic mosquito impaired in parasite transmission (Ito *et al.* 2002). This mosquito expressed the SM1 peptide from a carboxypeptidase promoter and inhibited formation of oocyst formation (the parasite form after crossing the midgut epithelium) by about 80% and drastically reduced vector competence (the mosquito's ability to transmit the parasite from an infected to a naïve mouse). A transgenic mosquito expressing PLA2 from a carboxypeptidase promoter was also shown to be significantly impaired in parasite development (Moreira *et al.* 2002). Much progress has been made since these initial discoveries in developing transgenesis as a tool to fight malaria (reviewed by Dong *et al.* 2022).

Gene drives

The next challenge is how to spread the inhibitory transgenes through mosquito populations in the field. Up to the advent of CRISPR/Cas9, no tools to accomplish this goal effectively were available. However, since then, CRISPR/Cas9-based methods to spread genes through populations (commonly referred to as 'gene drives') show promising results (Nolan 2021). Two types of drives are being developed. One aims at spreading effector genes through mosquito populations to render them refractory to the parasite ('population modification') (Adolfi *et al.* 2020). Another aims at eliminating mosquito populations ('population reduction') (Hammond *et al.* 2021). Population modification has the advantage of being a more stable approach, as once implemented, little further action is required (the mosquitoes will be refractory and will transmit the refractory trait to the progeny). One challenge in this approach is to assure that relatively large 'cargo' (the DNA encoding the effector and drive genes) remains intact and functional as it is transmitted from one generation to the next. An advantage of the population reduction approach is that the technology is much advanced. A challenge is that it does nothing to modify the favorable biological niche where mosquitoes thrive, requiring continuous releases to counteract establishment of residual and migratory mosquitoes. Moreover, if a different mosquito vector species establishes in this niche, the drive loses effectiveness as mating is essential for it to work.

Paratransgenesis

As mentioned, the most vulnerable stages of *Plasmodium* development in the mosquito occurs in the midgut. After the mosquito ingests an infected blood meal, bacteria numbers increase dramatically (hundreds of folds), and they are in the blood bolus compartment side-by-side with the developing parasites. Thus, an alternative to mosquito delivery of effector proteins to the midgut parasites, is to have effectors delivered by commensal bacteria. This concept commonly referred to as paratransgenesis, was pioneered for control of *Trypanosoma cruzi* transmission, the causative agent of Chagas disease (Durvasula *et al.* 1997). Early experiments with engineered *Escherichia coli* suggested that this approach may also be used for control of malaria (Yoshida *et al.* 2001). Subsequently, a symbiotic bacterium – *Pantoea agglomerans* – that is well adapted to the mosquito gut and readily genetically manipulated was engineered to produce and secrete a variety of small effector proteins and shown to strongly inhibit *Plasmodium* development in mosquitoes (Wang *et al.* 2012). While this alternative to mosquito transgenesis was successful, it raised the important question as to how to introduce such bacteria into mosquito populations in the field. A fortuitous discovery suggested how this could be done. A bacterium – *Serratia* AS1 – was isolated from mosquito ovaries (not midgut) that is able to be transmitted vertically from female mosquitoes to their progeny and horizontally (sexually) from male to female mosquitoes during mating (Wang *et al.* 2017). Eggs were collected from a cage containing 5% female mosquitoes carrying red fluorescent *Serratia* AS1 and 5% male mosquitoes carrying green fluorescent *Serratia* AS1 and reared to adults. It was shown that 100% of the progeny carried both bacteria. Moreover, these bacteria were transmitted to at least two further generations. In a following step, *Serratia* AS1 were engineered to produce and secrete a variety of effector proteins that strongly inhibited *Plasmodium* development in the mosquito (Wang *et al.* 2017). In conclusion, *Serratia* AS1 not only can spread through mosquito populations (at least in the lab) but it also inhibits *Plasmodium* development. This is the equivalent of gene drive.

Unlike gene drive, paratransgenesis is mosquito species-insensitive, its effectors usually work with multiple *Plasmodium* species, it does not impose fitness cost to the mosquito host and producing the bacteria is low tech and inexpensive. However, many of these properties need to be verified under field conditions.

Prospects

To date, control of malaria transmission has relied heavily on the use of insecticides to reduce mosquito populations. As

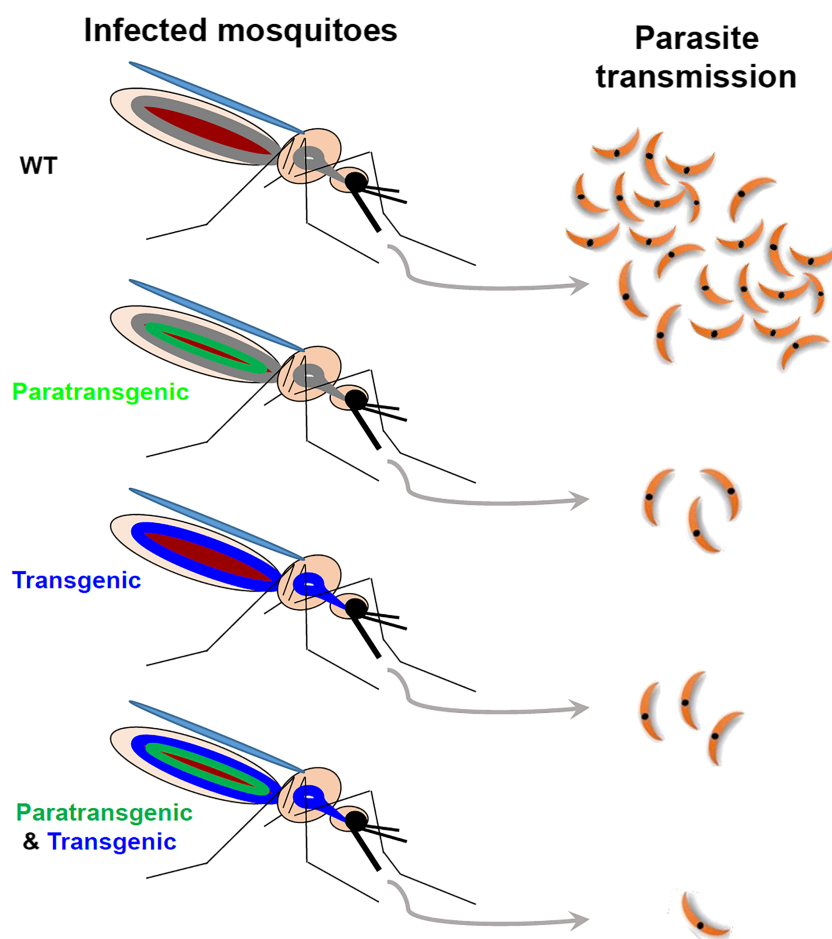


Figure 1 Prospects for implementation of genetic approaches to fight malaria. Whereas both engineering of mosquitoes (transgenesis) and engineering of mosquito symbiotic bacteria (paratransgenesis) to secrete antimalarials into the mosquito midgut have shown promising results, the combination of the two is by far the most efficient way to curtail parasite transmission. Illustrated here transgenic mosquitoes that express antimalarials in the gut and in the salivary glands. WT, wild type.

noted, the development of insecticide resistance is gradually limiting the effectiveness of this approach. Another approach, of eliminating mosquito breeding sites, is not practical in most malaria regions, as breeding sites are broadly spread and not well defined. One may then ask which of the two new strategies – transgenesis and paratransgenesis – is the most promising (Fig. 1). An important consideration is that the two are not mutually exclusive, as it is perfectly feasible to introduce inhibitory bacteria into transgenic mosquitoes. Indeed, as recently shown (Huang *et al.* 2022), the combination of transgenesis and paratransgenesis is much more effective than either individual approach.

A further important challenge relates to the regulatory, ethical, and social issues related to the introduction of genetically modified organisms in nature. Among many considerations, is that once released, it is very difficult to ‘recall’ the organisms should problems appear (Huang *et al.* 2020). While challenging, these issues are not unsurmountable, as a preliminary trial with the release of engineered mosquitoes has already been reported (Yao *et al.* 2022). As for paratransgenesis, the news is excellent. Recently, a *Serratia* has been isolated in China that not only

can spread through mosquito populations, but also naturally (without genetic engineering) strongly inhibit *Plasmodium* development in mosquitoes (Gao *et al.* 2021). Experiments are under way to verify the effectiveness of these bacteria to spread and inhibit parasite transmission under field conditions.

In conclusion, key discoveries were made in the span of a little over two decades, that bring us close to the implementation of a completely new approach to fight malaria. In this context, it is important to keep in mind that the fight of malaria is complex, and elimination of this deadly disease cannot be achieved by any single measure but must rely on the combined implementation of as many tools as possible, including insecticides, vaccines, drugs, transgenesis, and paratransgenesis.

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