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INVITED REVIEW

The *Anopheles gambiae* transcriptome – a turning point for malaria control

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Abstract

Mosquitoes are important vectors of several pathogens and thereby contribute to the spread of diseases, with social, economic and public health impacts. Amongst the approximately 450 species of *Anopheles*, about 60 are recognized as vectors of human malaria, the most important parasitic disease. In Africa, *Anopheles gambiae* is the main malaria vector mosquito. Current malaria control strategies are largely focused on drugs and vector control measures such as insecticides and bed-nets. Improvement of current, and the development of new, mosquito-targeted malaria control methods rely on a better understanding of mosquito vector biology. An organism's transcriptome is a reflection of its physiological state and transcriptomic analyses of different conditions that are relevant to mosquito vector competence can therefore yield important information. Transcriptomic analyses have contributed significant information on processes such as blood-feeding parasite–vector interaction, insecticide resistance, and tissue- and stage-specific gene regulation, thereby facilitating the path towards the development of new malaria control methods. Here, we discuss the main applications of transcriptomic analyses in *An. gambiae* that have led to a better understanding of mosquito vector competence.

Keywords: transcriptomics, *Anopheles gambiae*, malaria, vector, *Plasmodium* spp.

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Introduction

Anopheles mosquitoes represent a global health problem largely concentrated in the developing world. There are more than 450 *Anopheles* species and at least 60 are recognized as vectors of human malaria (Manguin, 2008). Only female mosquitoes are able to transmit the *Plasmodium* parasite through blood feeding (Cohuet *et al.*, 2010). Parasites undergo a complex developmental cycle within the mosquito, involving several developmental changes transversal of the midgut and salivary glands (SG) from where the sporozoites can be transmitted to a new host (Blandin *et al.*, 2004). Upon ingestion of gametocytes, the parasites differentiate into male and female gametes, which escape from the host red blood cell (RBC) membrane and fuse to form zygotes. The zygotes develop into motile ookinetes, within the blood bolus, that traverse the midgut epithelium and develop into oocysts on the basal side, producing thousands of sporozoites that are released into the haemocoel and migrate to the SG (Akinosoglou *et al.*, 2015).

Anopheles gambiae is considered the most efficient malaria vector in the world and is also the best studied (Coetzee, 2004). Its high vector capacity is the result of several factors, such as being a relatively long-lived species (Olayemi & Ande, 2009) with a short larval developmental period and its ability to adapt to the man-made environment, being considered highly anthropophilic (Magesa *et al.*, 1991; Bockarie *et al.*, 1993; Costantini *et al.*, 1996; Awolola *et al.*, 2002).

The genome sequence of the malaria mosquito *An. gambiae* was first released in 2002 (Holt *et al.*, 2002), offering new possibilities for research and discoveries

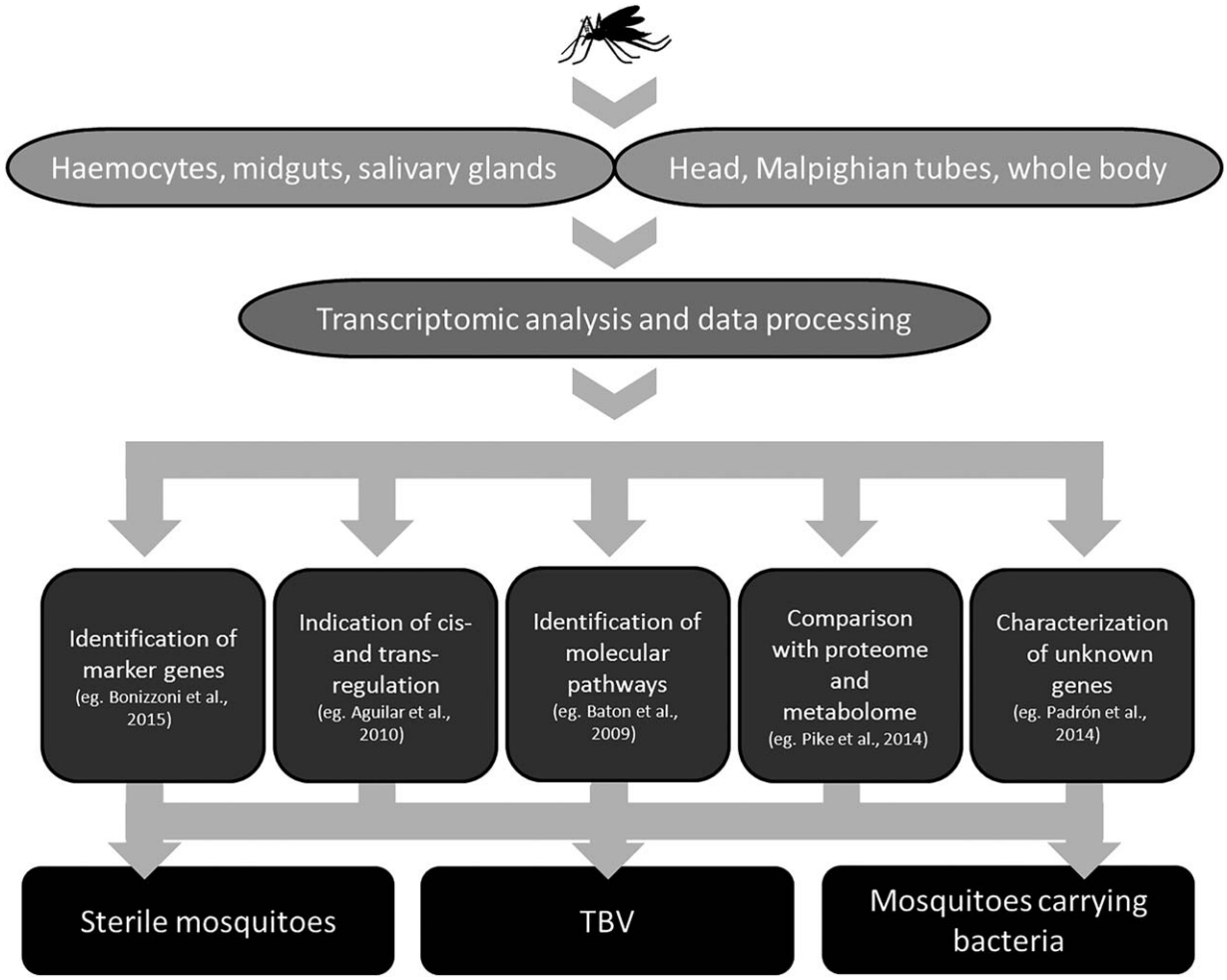

Figure 1. Schematic model of transcriptomic analyses of *Anopheles* spp. mosquito tissues (eg uninfected or infected by parasites) analysed according to condition. The results of such studies could lead to the development of control measures such as sterile mosquitoes, transmission-blocking vaccines (TBVs) and symbiont-carrying mosquitoes (Baton *et al.*, 2009; Aguilar *et al.*, 2010; Padrón *et al.*, 2014; Pike *et al.*, 2014; Bonizzoni *et al.*, 2015).

that could contribute towards the development of malaria control measures. However, the reference genome assembly is still in need of improvement (Lawniczak *et al.*, 2010) largely because of the high heterozygosity of the source DNA (Turissini *et al.*, 2014).

The development of DNA microarrays in the late 1990s initiated a brand new era for gene expression profiling, as probes and microarrays for several vectors, hosts and pathogens became commercially available, and the technology itself more accessible (Thompson *et al.*, 2001). Although widely used and responsible for significant advances, microarrays still presented some limitations such as dependence on the existing genomic sequence, signal saturation for transcripts with high abundance and high background noise owing to nonspecific hybridization (Qian *et al.*, 2014). The transcriptomic field experienced another leap forwards with the invention of high-throughput sequencing technologies, allowing high-resolution transcriptome analyses, even at the single-cell level, through RNA sequencing (RNA-Seq) (Streets *et al.*, 2014). The amplification of cDNAs followed by RNA-Seq offers a more complete analysis of the transcriptome and the possibility to discover novel and low-abundance transcripts (Tang *et al.*, 2010). The number of genomics and transcriptomics studies performed on the *An. gambiae* species complex has increased substantially over the past decade, allowing the identification and quantification of novel and rare transcripts and transcript splice-forms, even for samples with low mRNA quality and quantity. Transcriptomics analyses focused on mosquito–pathogen interactions have for example generated a wealth of data that can facilitate the development of new malaria control methods based on genetically modified mosquitoes and transmission-blocking vaccines (TBVs; Zou *et al.*, 2011).

Herein, we review the transcriptomic analyses of *An. gambiae* and discuss how these data sets have been,

and are being, used to identify new mosquito-based transmission-blocking targets. Owing to the relevance for vector competence, these studies have been focused on the characterization of mosquito response to *Plasmodium* spp. infection, blood feeding, and tissue-specific expression, as well as on mosquito insecticide resistance (Fig. 1). Other biological features relevant to vector competence, such as reproduction and life span influencing the ability of mosquito populations to transmit malaria and the impact of microbiome on parasite infection are also discussed (Blumberg *et al*., 2013; Lefevre *et al*., 2013).

Transcriptomics analysis of *An. gambiae* infected with *Plasmodium* spp.

The malaria parasite's complex life cycle in the mosquito vector comprises numerous molecular interactions that can serve as targets for the development of transmission-blocking interventions (Vlachou *et al*., 2005; Dong *et al*., 2006; Akinosoglou *et al*., 2015).

Gene expression analysis of the mosquito midgut responses to parasite infection

The mosquito midgut represents an important barrier for parasite infection, mainly because of the mosquito's immune responses (Smith *et al*., 2014). The invasion of the mosquito midgut by *Plasmodium* ookinetes promotes profound morphological changes in midgut cells, often resulting in lysis (Zieler & Dvorak, 2000; Sinden, 2002). A transcriptomic analysis revealed that more than 7% of the total mosquito transcriptome was up-regulated during the first 18–44 h after a *Plasmodium berghei*-infected blood meal. These genes were related to apoptosis, immunity, metabolism, structure and cell adhesion mechanisms. Gene silencing assays of differentially regulated genes showed that actin and microtubulin cytoskeleton proteins are crucial mediators of parasite invasion, especially at the early stages of infection (Vlachou *et al*., 2005). The presence of *Plasmodium* parasites in the midgut leads to differential expression of immune genes that mediate antiparasitic defences, mainly those that are regulated by the highly conserved nuclear factor kappa-light-chain (NF-kB) Toll and immune deficiency (Imd) signalling pathways (Dennison *et al*., 2015).

Microarray-based transcriptome analyses have also shown that *An. gambiae* responds differently to midgut invasion by different *Plasmodium* species. For example, *P. berghei* infection had a greater impact on the mosquito transcriptome, regulating hundreds of genes belonging to different functional classes, when compared to *Plasmodium falciparum*. Responses to both parasite species involved a variety of antimicrobial and other immune factors (Dong *et al*., 2006). Studies such as this suggest that the development of mosquito control strategies based on interfering with vector–parasite interactions will have to take into consideration some species-specific features.

MicroRNA (miRNA) microarray expression studies have shown that the miRNA biogenesis pathway is also involved in the response to parasite infection, and in modulating susceptibility to infection. Biryukova *et al*. (2014) compared miRNA expression levels in *An. gambiae* females after non-infected and infected blood meals, and obtained two highly expressed miRNAs, miR-317 and miR-2940, in response to *P. berghei* parasite infections. This suggests that transgenic mosquitoes expressing such *Plasmodium*-responsive miRNAs could block parasite infection and be used for malaria control (Dennison *et al*., 2015).

Transcriptomic comparison of naïve susceptible (G3 strain) and refractory (L35 strain) mosquitoes using high-throughput sequencing, with the Illumina platform, identified many novel transcripts contributing to the improvement of *An. gambiae* genome annotation and revealed new polymorphisms that could be useful for genetic studies (Padrón *et al*., 2014).

Overall, the various studies of the midgut transcriptome carried out to date have revealed processes and identified several genes that represent promising transmission blocking targets.

Gene expression analysis of mosquito haemocytes in response to parasite infection

Haemocytes are blood cells found in the mosquito haemolymph, and are known to carry out a variety of cellular defence processes. Important cellular immune responses mediated by haemocytes include phagocytosis, melanotic encapsulation and antimicrobial peptide- and complement-mediated killing (Pinto *et al*., 2009). Despite the importance of haemocytes in the mosquito's antiparasitic defences, only a few studies have explored the haemocytes transcriptome.

A genome-wide microarray-based study of *Plasmodium*- and bacteria-challenged *An. gambiae* haemocytes-like cell lines provided insights into hemocytes role in mosquito immunity, identifying numerous new immunity-related genes (Dimopoulos *et al*., 2002). A haemocyte-specific transcriptomic study identified 1485 transcripts that were induced after bacteria and/or parasite infection, most of them relating to immunity (Pinto *et al*., 2009). A study investigating the response of haemocytes to different stages of malaria parasite infection showed pathogen infection-responsive specific gene expression signatures, and co-regulation of members of the Imd and JNK pathways (Baton *et al*., 2009). These studies suggest the existence of haemocyte-specific

responses to parasites and bacteria and against the different *Plasmodium* stages.

Gene expression analysis of mosquito salivary gland responses to parasite infection

The SG of *Anopheles* spp. blood feeding by a variety of bioactive components of the saliva, thereby promoting vector reproduction and parasite transmission. The *Plasmodium* sporozoite invasion of the SG is receptor-mediated, and parasite–mosquito species specific. The identification of genes encoding putative SG receptors for sporozoites is therefore a high priority because of the potential for parasite blocking (Ghosh *et al*., 2000). Analyses of the SG transcriptome during invasion may shed light on processes involved in the interactions with the parasite. Different approaches have been used to identify transcripts that may represent malaria transmission-blocking targets.

Amongst the first transcriptomic studies on SG were those published by Arcà *et al*. (1999), Francischetti *et al*. (2002) and Lanfrancotti *et al*. (2002), which contributed to the discovery of numerous SG genes. Serial analysis of gene expression and Affimetrix-based microarray analyses showed that mosquito genes that were differentially expressed between infected and naive SG were to a significant degree related to immune responses; differential expression was also observed for genes relating to transport, and to lipid and energy metabolism (Rosinski-Chupin *et al*., 2007). A putative antihaemostatic peptide, called agaphelin, belonging to the Kazal type inhibitors, was also amongst the infection-responsive genes. Agaphelin inhibits elastase and proteinase-3, which modulate several neutrophil functions, thereby preventing arterial thrombosis without affecting homeostasis. This study show that the parasite induces mosquito immune responses and manipulates the vertebrate host for its benefit, revealing an interesting tripartite interaction amongst the parasite, vector and the vertebrate host (Waisberg *et al*., 2014).

RNA-Seq has also been used for the identification of *Plasmodium* infection-regulated SG genes. A total of 2588 differentially expressed genes was found in the SG of the *An. gambiae* M form (*Anopheles colluzzii*) upon infection. RNA interference (RNAi)-mediated silencing of one gene, encoding a transmembrane glucose transporter, resulted in a lesser number of sporozoites in the SG, suggesting a potential important role of this gene during *Plasmodium* infection (Pinheiro-Silva *et al*., 2015).

Other transcriptomics studies on *An. gambiae*

Most of the mosquito salivary gland, midgut and haemocyte transcriptomic studies reported above were focused on the *An. gambiae* immune response to *Plasmodium* infection. However, transcriptome analyses relating to blood feeding, insecticide resistance, tissue and sex specificity, and the mosquito microbiota have also contributed towards a better understanding of vector competence.

Blood feeding

Adult mosquitoes feed on plant nectars to assure their survival but females need a supplementary blood meal for egg production. Blood feeding is essential for both vector propagation and parasite transmission, involving several biochemical and physiological processes. A number of transcriptomic studies have focused on mosquito blood feeding, and have significantly contributed towards our understanding on vector biology relevant for the development of biology and discovered of relevance for the development of malaria control strategies.

The ability of mosquitoes to feed efficiently depends on various factors and products, some of which are components of the saliva. The SG express about 38% of the total *An. gambiae* transcriptome, and the abundance of many salivary gland transcripts changes rapidly upon the blood meal, suggesting implications in this process (Marinotti *et al*., 2006, 2005; Das *et al*., 2010).

By combining cDNA library screening and the signal sequence trap technique, Arcà *et al*. (1999) identified 15 cDNA fragments expressed specifically in the SG of the malaria vector *An. gambiae* and some fragments showed high similarity with the *D7* and *apyrase* genes previously described in *Aedes aegypti* SG (Arcà *et al*., 1999; Lombardo *et al*., 2000). Genes encoding anti coagulants and glandins were also described by Lanfrancotti *et al*. (2002). Using western blots and real-time PCR (RT-PCR), two *An. gambiae* SG genes, *SG4* and *SG5*, were further associated with mosquito feeding behaviour and recognized as important immunogens (King *et al*., 2011).

Comparisons of transcriptome profiles of blood-fed and unfed *An. gambiae* whole-body and antennal females revealed that both are influenced by blood feeding. Mosquito response to blood feeding is discussed in several studies (Holt *et al*., 2002; Ribeiro, 2003; Vaninni *et al*., 2012). Furthermore, the feeding process is associated with odorant sensitivity changes that may coincide with the shift from host-seeking to oviposition behaviours (Rinker *et al*., 2013).

A deeper understanding of the mechanisms associated with the responses of mosquitoes to feeding and infection will help to identify vector-specific molecules that can be manipulated and antiparasite genes that can be introduced as foreign genes to block *Plasmodium* progress and disease transmission.

Insecticide resistance

In the face of increasingly widespread resistance to antimalarial drugs, the use of insecticides has become the

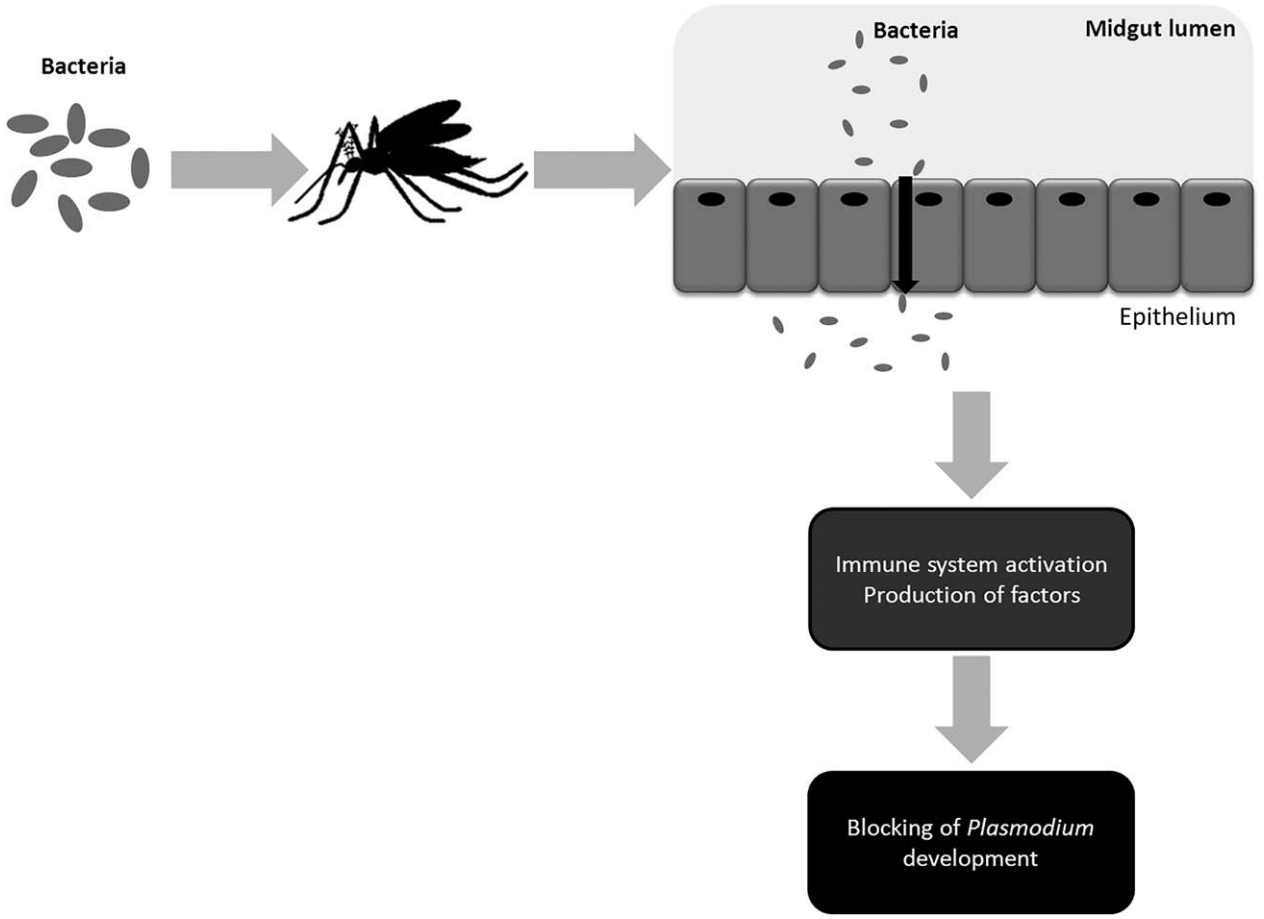

Figure 2. Schematic model of studies carried out with anti-*Plasmodium* symbiont bacteria. When ingested by mosquitoes, the bacteria traverse the midgut cells. Subsequently, by stimulating the mosquito immune system or by causing production of immune factors, the bacteria interrupt *Plasmodium* development in the midgut.

primary method of mosquito control and, consequently, malaria prevention (Bonizzoni *et al*., 2012). Chemical insecticides are successfully used in malaria control programmes, especially in areas that are particularly well suited to the propagation of mosquito vectors (Lengeler, 2004; Tanser *et al*., 2007). However, there has been an upsurge in insecticide resistance in *An. gambiae*, particularly to pyrethroids, dichlorodiphenyltrichloroethane (DDT) and carbamates, owing to the intensive use of both insecticide-treated bed-nets and indoor residual spraying (Balkew *et al*., 2010; Marcombe *et al*., 2012; WHO, 2014). Furthermore, the recurrent exposure of larvae to agricultural pollutants may select for resistance mechanisms to insecticides at the adult stage (Nkya *et al*., 2014). A number of studies on insecticide resistance have been carried out in different African countries; the data are summarized in the following paragraphs.

Both *An. gambiae* and *Anopheles arabiensis* populations in southern Benin and Nigeria were screened using an *An. gambiae* microarray detox chip for mosquitoes selected from pesticide-contaminated fields or urban pollutants (Djouaka *et al*., 2008). Data analysis allowed identification in mosquitoes from all of the collection sites of the cythocrome P450 genes (*cyp*)*6p3* and *cyp6m2* genes, known to be associated with permethrin resistance and confirmed the existence of metabolic resistance in presence or absence of the knockdown resistance (*kdr*) mutation (Djouaka *et al*., 2008).

Transcription profiling of a colonized *An. gambiae* pyrethroid-resistant strain from Odumasy, southern Ghana, indicated that the cytochrome P450 genes *cyp6z2* and *cyp6m2* are highly up-regulated in both female and male mosquitoes. The sequences of these genes differ from those found in East Africa; thus, the authors suggested that *An. gambiae* metabolic resistance may have multiple origins (Müller *et al*., 2007). Microarray analyses revealed that the cytochrome P450 genes *cyp6p3* and *cyp6z2* and a rhodopsin receptor gene are also upregulated in *An. gambiae* samples from different villages in Vallée du Kou, Burkina Faso. These

Table 1. Summary of transcriptomic studies in *Anopheles gambiae*

Condition	Sample source	Technique	Reference
Uninfected	SG	Microarray and qPCR	Calvo *et al.* (2006)
Infected by *Plasmodium berghei*	SG	SAGE	Rosinski-Chupin *et al.* (2007)
Infected by *P. berghei*	Midgut	Microarray	Vlachou *et al.* (2005)
Infected by *P. berghei* and *Plasmodium falciparum*	Midgut	Microarray	Dong *et al.* (2006)
Infected by *P. falciparum*	Midgut	Small RNA profiling	Biryukova *et al.* (2014); Dennison *et al.* (2015)
Infected by *P. berghei*	Haemocytes	Microarray	Pinto *et al.* (2009); Baton *et al.* (2009)
Infected by *P. berghei*/bacteria	Adult mosquitoes/mosquito cell culture	Microarray	Dimopoulos *et al.* (2002)
Uninfected, insecticide resistance	Adult mosquitoes	RNA-Seq	Bonizzoni *et al.* (2012)
Uninfected, insecticide resistance	Adult mosquitoes	Microarray	Müller *et al.* (2007); Chiu *et al.* (2008); Djouaka *et al.* (2008); Mitchell *et al.* (2012); Fossog Tene *et al.* (2013); Kwiatkowska *et al.* (2013); Edi *et al.* (2014); Nkya *et al.* (2014)
Uninfected	Adult male and female mosquitoes	Microarray	Arcà *et al.* (2005); Baker *et al.* (2011); Pondeville *et al.* (2013)
Uninfected	Larvae	Microarray	Oviedo *et al.* (2009)
Uninfected	Adult male mosquitoes	Microarray	Marinotti *et al.* (2005); Gabrieli *et al.* (2014)
Uninfected	SG of female mosquitoes	Microarray	Das *et al.* (2010); Arcà *et al.* (2005)
Uninfected	Adult female mosquitoes	Microarray	Marinotti *et al.* (2006)
Uninfected	Adult female mosquitoes	RNA-Seq	Vaninni *et al.* (2012)
Uninfected	SG of female mosquitoes	SST	Arcà *et al.* (1999); Lombardo *et al.* (2000); Lanfrancotti *et al.* (2002)
Uninfected	Antennae of female mosquitoes	RNA-Seq	Rinker *et al.* (2013)
Infected by *Serratia marcescens*	Midgut of female mosquitoes	Microarray	Stathopoulos *et al.* (2014)
Infected by *Salmonella typhymurium*, *Staphylococcus aureus* and *Beauveria bassiana*	Adult female mosquitoes	Microarray	Aguilar *et al.* (2005)
Infected by *Asaia* sp.	Larvae	Microarray	Mitraka *et al.* (2013)
Infected by *Wolbachia* sp.	Mosquito cell culture	Microarray	Hughes *et al.* (2011)

qPCR, quantitative PCR; RNA-Seq, RNA sequencing; SAGE, serial analysis of gene expression; SG, salivary glands; SST, signal sequence trap technique.

findings confirm the importance of target site resistance and the presence of resistance phenotypes induced by different mechanisms (Kwiatkowska *et al.*, 2013).

The findings were similar for *An. gambiae* bendiocarb (carbamate) resistance and cross-insecticide resistance in Tiassalé, Ivory Coast. In this instance, resistance was found to be caused by target site gene mutation and duplication of P450 enzymes (Edi *et al.*, 2014). Three-dimensional docking studies performed with CYP6Z1 and CYP6Z2 against DDT revealed that, in spite of the high sequence similarity between the proteins, only CYP6Z1 can potentially metabolize DDT (Chiu *et al.*, 2008). Samples collected in Ghana, a country known to have highly DDT-resistant mosquitoes, showed that the cytochrome P450 gene *cyp6m2*, which is associated with pyrethroid resistance, was also one of the most up-regulated genes (Mitchell *et al.*, 2012).

In Yaoundé, Cameroon, malaria is transmitted by the M and S forms of *An. gambiae*, both of which are resistant to pyrethroids and DDT. Fossog Tene *et al.* (2013) used microarrays to compare expression profiles of multiple DDT-resistant samples collected from different field sites with susceptible mosquitoes. Their findings revealed that both M and S molecular forms of *An. gambiae* have a similar resistance profile. Transcripts coding for ABC transporters were up-regulated in the three populations analysed, and detoxification enzymes such as CYP6M2 and CYP6P3 showed broad substrate specificity, indicating cross-resistance.

RNA-Seq was used to study the mechanism of resistance to insecticides in *An. gambiae* mosquitoes collected in western Kenya. Bonizzoni *et al.* (2012) analysed the expression profile of deltamethrin-resistant and -susceptible mosquitoes and found that 1093 transcripts were differentially expressed, revealing a correlation with pyrethroid resistance.

The reports described above used microarrays and RNA-Seq to evaluate gene expression profiles pertaining to *Anopheles* resistance against different insecticides in several locations in Africa. The studies found that cytochrome P450 genes are associated with permethrin, benthiocarb and DDT resistance, but only CYP6Z1 could potentially metabolize DDT. The authors further suggested that these genes differ according to the collection site and that both M and S molecular forms of *An. gambiae* exhibit similar resistance profiles. The complexity of resistance

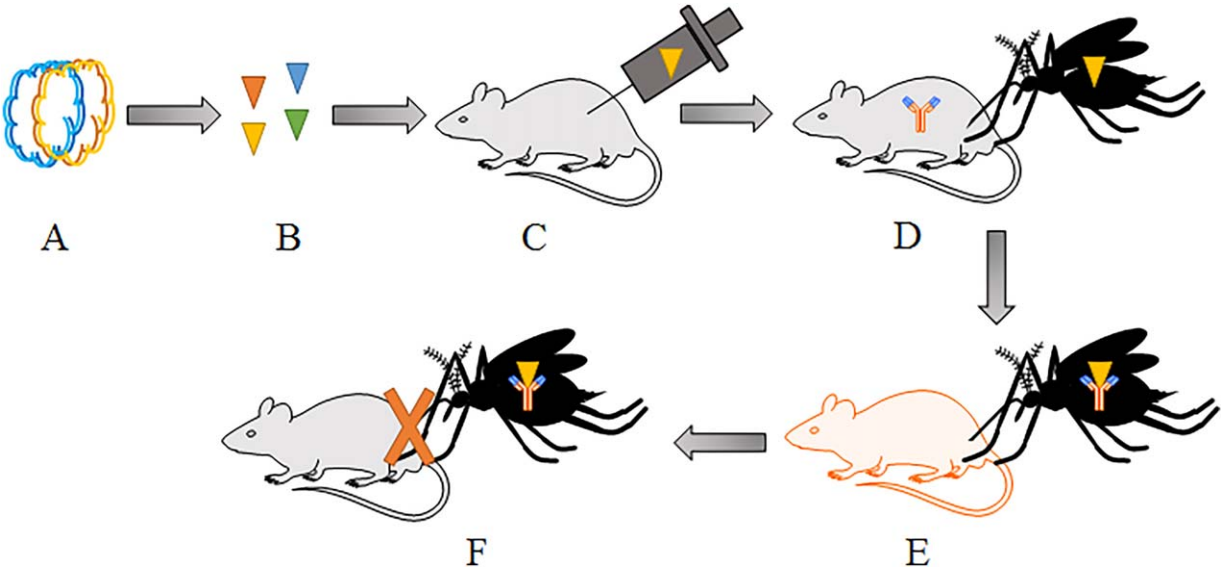

Figure 3. Model of a transmission-blocking vaccine study. (A) Transcriptomic studies reveal genes up-regulated in *Anopheles* spp. infected by *Plasmodium* parasites. (B) Up-regulated genes analysed to select candidates for immunization assays. (C) Selected candidates used as antigens for immunization assays. (D) Antigens induce the production of specific antibodies, which will be ingested during mosquito blood feeding. (E) Antibodies will then bind to target protein within the mosquito. Thus, when the mosquito subsequently takes another blood meal from a vertebrate host infected by *Plasmodium*, parasite development is interrupted. (F) Transmission to another vertebrate host is blocked. [Colour figure can be viewed at wileyonlinelibrary.com]

mechanisms must be considered when designing resistance monitoring and vector control programmes.

Tissue and sex specificity

Monitoring the transmission of *Plasmodium* parasites requires an in-depth understanding of the physiological events and interaction mechanisms that affect the mosquito vector. Mosquito control strategies based on transgenic expression of antiparasitic factors require tissue- and stage-specific promoters.

Thus, many studies have used methodologies such as RT-PCR and microarrays to identify mosquito genes and proteins with specific physiological roles that differ according to sex (Arcà *et al.*, 2005; Calvo *et al.*, 2006). In addition, a microarray-based global gene expression analysis evaluated the abundance of transcripts in the female-specific compartments of the midgut. The greater functional diversity compared to male mosquitoes was, as expected, related to the digestive process and immunity (Warr *et al.*, 2007).

Competitive two-dye hybridization on a MMC1 microarrays platform was used to conduct large-scale comparative transcriptomic analyses on samples from eight different time periods of the *An. gambiae* life cycle. The results confirmed that the same genes may show differential expression according to sex and tissue (Koutsos *et al.*, 2007).

Another transcriptional analysis yielded a profile of the whole mosquito body, including both somatic and reproductive tissues from males and females. The analysis showed that only about 30% of transcripts are present in all tissues and 54% are sexually dimorphic in at least one organ. Further, female SG have higher expression of genes associated with protein and lipid catabolism and with transport and homeostasis, whereas male gene activity is associated with both carbohydrate metabolism and transport (Baker *et al.*, 2011). The gene *cE5*, which potentially encodes an antithrombin protein that may be involved in blood feeding, was found in both male and female mosquitoes, but the corresponding protein was only found in female SG (Ronca *et al.*, 2012).

Most of the reports on SG transcriptomics concern adults, but one study compared the transcriptome of larval SG with that of whole larvae and found that genes related to the immune response are highly represented. This is to be expected, because saliva is a first-line defence against pathogens (Oviedo *et al.*, 2009).

Reducing the number of female mosquitoes in the field may have a great impact on disease control and may be achieved by interfering with mosquito reproduction. *An. gambiae* females lose the capacity to copulate after sex; a transcriptional analysis was therefore performed to analyse the morphological and behavioural changes induced by copulation. This analysis revealed that the 20-hydroxyecdysone (20E) steroid hormone, found in male *An. gambiae*, is transferred to females during sex and prevents them from mating with additional males. Thus, 20E is a regulator of female reproductive biology and may be

critical in reducing the reproductive success of *Anopheles* (Gabrieli *et al*., 2014). To identify new enzymes associated with the activity of 20E, a small-scale microarray analysis was performed to compare the expression of P450 enzymes in active and inactive steroidogenic tissues. The analysis revealed that only three genes encoding P450 enzymes, *cyp4g16*, *cyp6n1* and *cyp307a1*, are up-regulated in the active steroidogenic tissues of both male and female mosquitoes, but only *cyp307a1* is involved in steroidogenesis (Pondeville *et al*., 2013).

Symbiont infection

Analyses of the responses of mosquitoes to parasite infection and their relationships with symbiotic microorganisms may yield important information about host defence tools and antimicrobial immune pathways that could contribute to new disease control strategies. Below, we describe a number of studies related to the presence of bacteria and the *Anopheles* immune response.

Oduol *et al*. (2000) performed a gene expression screen of the entire transcriptome of *An. gambiae*. After immune stimulation, a protein related to the protease inhibitor α-2-macroglobulin responded strongly to infection with malarial parasites but exhibited slight to no response to bacteria, whereas other genes displayed the opposite response. In addition, Aguilar *et al*. (2005) used an Affymetrix GeneChip to evaluate the response of the *An. gambiae* transcriptome to inoculation with inactivated *Salmonella typhymurium*, *Staphylococcus aureus* and *Beauveria bassiana*. Transcriptional responses to the three microbial elicitors showed a high degree of specificity and included some transcripts that had not been previously known to be associated with immunity in *An. gambiae*.

The mosquito endosymbiont *Wolbachia pipientis* affects immune regulation and inhibits the dissemination and/or development of insect pathogens. Infection of an *An. gambiae* cell line with *Wolbachia* sp. revealed a pronounced effect on the expression of malaria-related immune genes and a significant decrease in the intensity of *Plasmodium* infection (Kambris *et al*., 2010; Hughes *et al*., 2011). These findings should be taken into account when studies to manipulate vector reproduction are performed.

Gram-negative bacteria from the genus *Asaia* have been found to be stably associated with *An. gambiae* larvae and adults. A microarray analysis revealed that *Asaia* play a crucial role during mosquito larval stages, with the genes involved in cuticle formation most greatly affected (Mitraka *et al*., 2013). *Asaia* can be introduced into mosquitoes and maintained for at least one generation, which suggests that these bacteria could be used for paratransgenic vector control.

A transcriptomic and reverse-genetic analysis was used to compare the response of septic and aseptic mosquitoes to *P. falciparum* infection and revealed that the immune response against bacteria is independent of the response against *P. falciparum*. Moreover, the study revealed a variety of putative immune genes that are regulated upon *P. falciparum* infection in the absence of midgut microbiota (Blumberg *et al*., 2013). In another study, single nucleotide polymorphism genotyping and expression profile techniques combined with RNAi-mediated gene silencing and 454 pyrosequencing found that 38 *An. gambiae* genes are associated with *Serratia marcescens* infection (Stathopoulos *et al*., 2014). The results suggest that the mosquito response to bacterial infection includes both an epithelial and an immune component. Moreover, they show that mosquito gut bacteria influence the outcome of infection with *Plasmodium*, suggesting that natural genetic variation in immune-related genes can define the gut bacterial population with high specificity.

All these findings related to parasite, mosquito vector, and midgut symbiont interactions may allow the identification of valuable new targets in the development of new methods to regulate malaria transmission (Fig. 2). For example, naturally occurring bacteria could be used to manipulate vector reproduction or as agents of paratransgenesis by being genetically modified to express antipathogen effector molecules.

Conclusions and future directions

In this review, our objective was to describe how advances in high-throughput sequencing have contributed to the information available in online databases, revealing information difficult to obtain (Table 1).

Combining and comparing the results of different approaches has yielded plentiful evidence regarding mosquito immunity and resistance mechanisms. Moreover, it has led to the identification of critical targets that may be of use in the development of TBVs and mosquito biological control strategies. We also discussed advances in designing mosquito vectors that are incapable of transmitting the malaria parasite, such as sterile mosquitoes and mosquitoes that carry symbionts.

Despite all of the efforts undertaken over the last 20 years, a protective vaccine against malaria remains elusive. However, several vaccine candidates are presently under study. RTS, S/AS01 (recombinant protein-based malaria vaccine formulated with the adjuvant AS01, also known as Mosquirix) was recently approved by the European Medicine Agency. It shows only partial protection against *P. falciparum* and therefore must be used in combination with other control measures (Agnandji *et al*., 2014). New strategies to reduce malaria transmission are needed to maintain the achievements obtained thus far. TBVs and drugs targeting both mosquitoes and

parasites may prevent human-to-mosquito transmission by targeting the sexual stages of the parasite. Vaccines are unlikely to completely control malaria, but they could fill the gap in existing interventions if used in association with other measures to eradicate malaria (Fig. 3; Williams *et al.*, 2013).

A critical point in the development of vaccines is the identification of new targets. Advances in transcriptome profiling will lead to a deeper understanding of transcriptomic dynamics and, consequently, to the discovery of novel genes, proteins and pathways associated with vector-stage parasite development. Transcriptomic, proteomic and systems biology tools are thus fundamental to the improvement and development of new strategies to control the spread of malaria and to develop more effective vaccines.

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