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The role of microRNAs in *Anopheles* biology—an emerging research field

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Summary

In the last years, microRNAs (miRNAs) have been established as important post-transcriptional regulators of critical physiological processes in animals and plants. Here, we summarize what is known about miRNA biosynthesis, expression and function in the malaria vector mosquito *Anopheles gambiae* with a particular emphasis on the mosquito-parasite interactions. We discuss the important gaps in the current knowledge, including the potential of miRNA manipulation for future vector control strategies.

KEYWORDS

Anopheles gambiae, microRNA, *Plasmodium*, vector competence

1 | miRNAs IN INVERTEBRATES

The discovery of the first small noncoding RNA lin-4 in *Caenorhabditis elegans* in 1993 revolutionized the field of RNA biology.[1] However, the broad importance of microRNA (miRNA) regulation was only fully recognized with the discovery of the highly conserved miRNA let-7.[2] Since these first discoveries, model organisms such as *C. elegans* and *Drosophila melanogaster* paved the way for a better understanding of miRNA biosynthesis and mechanisms of action.

1.1 | A brief overview of miRNA biosynthesis

The details of miRNA biosynthesis have been covered by a series of excellent reviews[3-6]; therefore, here we will only briefly summarize the main steps of the canonical miRNA biosynthesis pathway. The majority of miRNA genes are independent transcription units located within intergenic regions; however, some miRNAs are found in intronic regions and are coregulated with the "host" gene.[7] The miRNA gene or cluster is transcribed by RNA polymerase type II as a long primary transcript called pri-miRNA that comprises one or several hairpin-loop structures. The pri-miRNA is processed by the microprocessor complex Drosha/Pasha into a ~70 nt stem-loop structure named pre-miRNA[8,9] that is exported from the nucleus to the cytoplasm by the double-stranded RNA (dsRNA)-binding receptor Exportin-5.[10] In the cytoplasm, the complex of the RNase Dicer and the dsRNA-binding protein loquacious (loqs) recognizes the dsRNA structure and cleaves off its terminal stem-loop to release a ~22 nt miRNA-miRNA* duplex.[11-13] One strand (also called arm) of the miRNA duplex is then loaded into the Argonaute (Ago) protein effector complex known as RNA-induced silencing complex (RISC). The mechanism underlying the miRNA arm selection is still not well understood. The seed sequence at the position 2-7 at the miRNA 5′-end directs RISC to its mRNA targets.[14] Binding of RISC to the target mRNA leads either to mRNA cleavage or in inhibition of its translation.[15-17]

1.2 | Worms and flies as model systems to study miRNAs

The RNase Dicer was first identified as a key enzyme in the RNA interference (RNAi) pathway in *Drosophila*.[18] In fact, the *Drosophila* genome encodes two *Dicer* genes with distinct functions. The product of *Dicer1* is essential for pre-miRNA cleavage, whereas Dicer2 is required for siRNA maturation from long dsRNAs.[19] Both Dicers interact with specific isoforms of the protein loquacious that are generated by alternative splicing. Dicer1 interacts with the two isoforms loqs-PA and loqs-PB to produce mature miRNAs, whereas a complex of Dicer2 and loqs-PD generates endo- and exo-siRNAs. Therefore, loqs isoforms and *Dicer* genes are specific for the maturation of a certain subset of short noncoding RNAs.[20] Furthermore, similar dichotomy is observed for Ago proteins; Ago1 primarily uploads miRNAs, whereas Ago2 forms the effector complex with siRNAs.[21]

Disruption of the miRNA biosynthesis pathway causes early lethality in *Drosophila* and mice, highlighting the important role of miRNAs in development.[22-24] The role of miRNAs in development has been extensively studied in *Drosophila*, which undergoes major metamorphic changes during its life cycle. Larvae transit through four instar stages before developing into pupae. The precise timing of these transitions or moults is regulated by rapid biosynthesis of the insect steroid hormone ecdysone.[25] Functional analyses of individual miRNAs exposed a tight link between hormonal regulation and miRNA signalling. For instance, miR-bantam (bantam) regulates tissue growth and patterning by repressing ecdysone release from hormone-producing cells.[26,27] The miRNA, miR-965 modulates expression levels of the ecdysone receptor, thereby decreasing tissue sensitivity to the hormone and controlling exact timing of morphogenesis.[28] On the other hand, repression of miR-965 by ecdysone during pupariation allows proliferation and migration of histoblasts (cells that form the adult abdominal epithelium during morphogenesis).[28] These are just two of many examples that demonstrate the crucial role of miRNAs in hormonal regulation during development.

Obviously, the first studies investigated the most prominent examples of miRNA function. Broader characterization of miRNA functions in *Drosophila* and *C. elegans* led to very divergent observations.[29-31] In *Drosophila*, 80% of miRNA knockouts (KOs) displayed clear phenotypes in survival, fertility, lifespan or behaviour, whereas the majority of miRNA KOs in *C. elegans* did not produce gross detectable phenotypes.[29-31] The concept integrating these differences has been proposed by Hornstein and Shomron[32], who suggested that some miRNAs may act to buffer stochastic perturbations and thus confer robustness to complex systems. It is possible that the complex *Drosophila* lifestyle and unstable culturing conditions offered a better background for manifestation of the miRNA KO phenotypes than the more controlled *C. elegans* model. Several experimental and/or modelling approaches corroborated the hypothesis that miRNAs serve as a buffer.[28,33-36] As already indicated, miR-965 generates a threshold for ecdysone signalling, thus decreasing sensitivity towards variations in ecdysone levels.[28] Furthermore, miR-7 contributes to successful development of the *Drosophila* sensory organs in the context of large temperature fluctuations[34] and let-7 in *C. elegans* confers robustness to cell fate decision during pathogen infections.[36]

To date, model organisms greatly contributed to our current understanding of the mechanism and function of miRNAs and established important tools and concepts to interrogate roles of individual miRNAs in nonmodel organisms.

2 | miRNA EXPRESSION IN *A. GAMBIAE*

2.1 | miRNA identification and annotation

The availability of the *A. gambiae* genome offered an excellent opportunity for computational homology searches of miRNA genes using *Drosophila* mature miRNAs as reference.[37] This approach discovered 91 genomic loci encoding potential miRNAs of which 41 were identical to their *Drosophila* orthologs. Further searches of miRBase-annotated pre-miRNAs identified six additional miRNAs.[38] The major drawback of the homology-based analysis is that by definition, it detects only broadly conserved miRNA genes. This limitation was partially overcome by a conservative *ab initio* prediction coupled with homology searches of 16 *Anopheles* genomes.[39] This study validated 58 previously identified miRNAs, discovered five new genes and revealed 21 miRNAs common to all species within the *Anopheles* complex.

Unbiased approaches by RNA sequencing (RNAseq) of small RNA libraries and direct cloning dramatically increased the number of miRNAs identified in *A. gambiae*.[40-42] They validated most of the computationally predicted miRNAs and discovered 103 new miRNA genes. In addition, RNAseq of *A. gambiae* females offered the first quantitative estimation of miRNA expression levels and identified bantam, miR-8, miR-10, miR-184, miR-263 and miR-281 as the most abundant and highly expressed miRNAs.[40] Studies in late larval stages confirmed high levels of expression of these miRNA and revealed stage-specific enrichment of miR-11, miR-276 and miR-306.[41] Conservation of miR-8 and miR-10 in all *Anopheles* species indicates a conserved role of these abundantly expressed miRNAs in mosquito biology.[39] Interestingly, sequences of the 20 most abundant miRNAs make up for 96% of all RNAseq reads, highlighting a dramatic difference in expression levels of miRNAs.[41] In addition to canonical miRNA genes, Castellano et al.[41] identified the first two mirtrons in *A. gambiae*. Mirtrons are miRNAs that originate from short introns with potential to form hairpins and are generated by a noncanonical miRNA biosynthesis pathway. Splicing and debranching of such introns generates hairpin-loop structures similar to pri-miRNAs. These molecules are exported from the nucleus to the cytoplasm for further Dicer processing.[43,44] The functional roles of *A. gambiae* mirtrons remain to be characterized. As of today, a total of 168 potential miRNAs have been identified.

2.2 | miRNA expression during development

The overall development of *A. gambiae* is similar to that of *Drosophila* with the exception of the aquatic nature of mosquito larval and pupal stages. Mosquito larvae undergo four instar stages before developing into pupae (Figure 1). It is likely that similar to *Drosophila*, these transitions are regulated by the steroid hormone ecdysone.[45,46] The miRNA expression in developmental stages of *A. gambiae* has been largely understudied. Only one report investigated miRNA expression patterns in late larval stages, whereas three studies examined immature stages in other *Anopheles* species *A. stephensi* and *A. anthropophagus*.[41,47-49] In contrast to *A. gambiae*, gender-biased miR-2, miR-7 and miR-1175 in *A. stephensi* are enriched in male larvae.[41,49] Furthermore, high levels of let-7 expression in late larval and pupal stages in *A. gambiae* and *A. stephensi* are reminiscent of let-7 expression pattern during *Drosophila* development,[41,48-51] suggesting a conserved role of this miRNA in *Anopheles* development. However, further functional analyses are needed to address its function.

2.3 | miRNA contributions to reproduction and immunity

Adult mosquitoes transmit devastating infectious agents such as malaria parasites, dengue and zika virus. All disease-transmitting

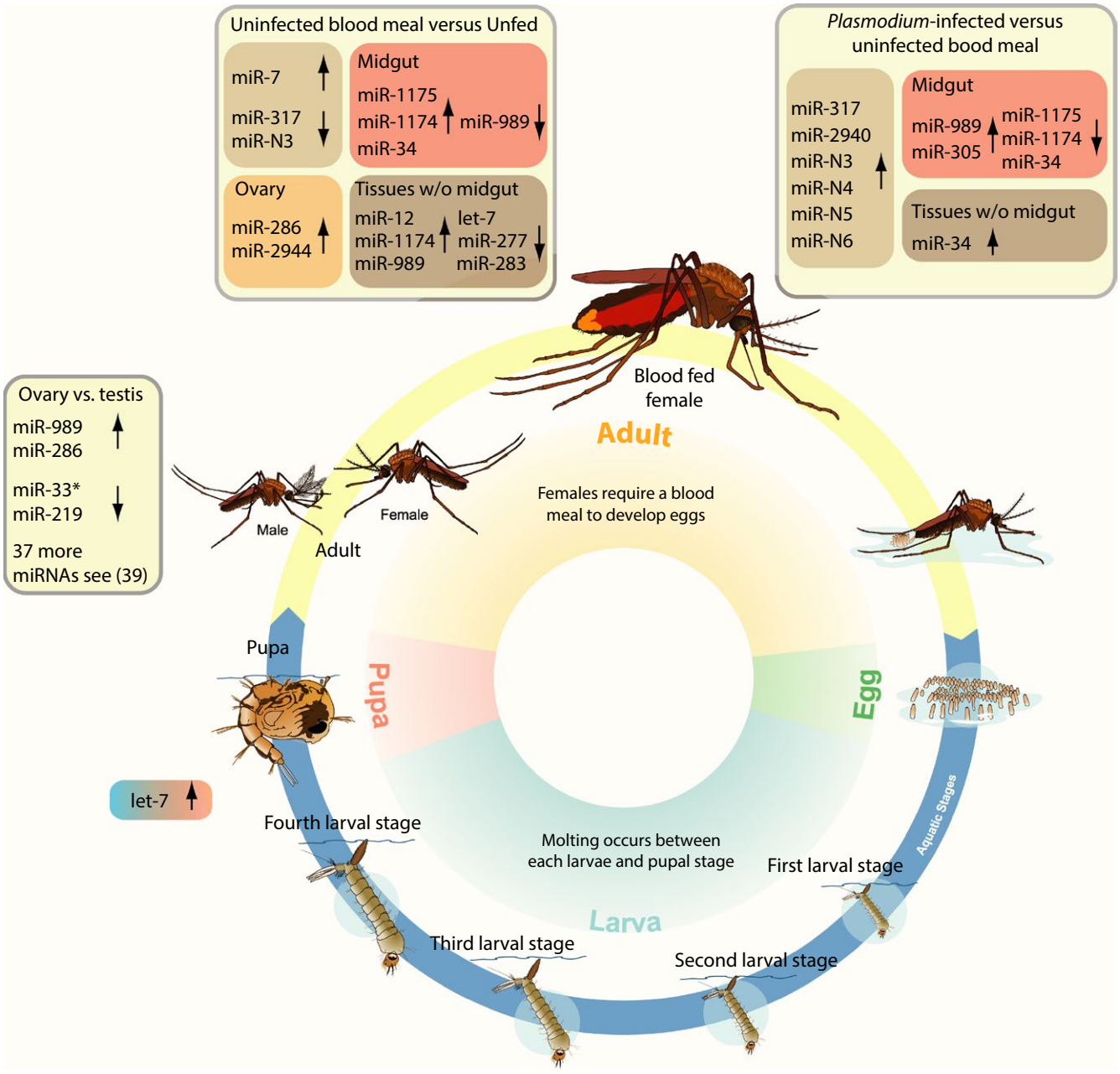

FIGURE 1 Differentially regulated miRNAs in the mosquito life cycle. Mosquito larvae hatch from eggs deposited on the water surface. The larvae undergo four instar stages before finally developing into pupae in aquatic environment (blue). Adult mosquitoes emerge from pupaes, and change their habitat from aquatic to terrestrial (yellow). To complete the life cycle, female adult mosquitoes require a blood meal to initiate egg production. The differentially regulated miRNAs of life cycle stages are depicted in the boxes. Most of the developmental stages have been completely neglected with regard to miRNA studies, whereas the reproductive organs (ovary and testis) as well as the miRNA responses to uninfected and to *Plasmodium*-infected blood feeding have been studied more extensively (adapted with permission from LadyofHats; retrieved 2016 from https://commons.wikimedia.org/wiki/File:Culex_mosquito_life_cycle_uk.svg)

mosquito species are haematophagous as they require a blood meal to initiate egg maturation (also called oogenesis; Figure 1). Within a few hours, the blood meal induces massive changes in the mosquito physiology and transcription. If infected, it also provides the environment for pathogen uptake and replication. Egg development in *A. gambiae* takes about 48 hours. During this time, blood meal digestion increases metabolic rates, digested nutrients are redistributed among the tissues and ovarian development is initiated to produce mature eggs. The onset and reset of these processes is tightly regulated in order for a mosquito to be prepared for the next blood meal.[52] On the other hand, the *Plasmodium* parasite, which is taken up with infected blood, requires 12-14 days to complete its life cycle in the mosquito. After sexual development and gamete fusion, the parasites traverse the midgut epithelium at 18-24 hours post-infection. During traversal, the majority of parasites are killed by the mosquito immune system.[53,54] Therefore, ovarian development and immune responses

to *Plasmodium* coincide in time. The surviving parasites undergo asexual replication on the basal side of the midgut epithelium during the next 12-14 days. Mature parasites migrate to and invade the salivary glands where they await injection into the vertebrate host with the next blood meal (reviewed in 55). As only female mosquitoes take a blood meal and transmit malaria as well as other diseases, miRNA functions have been mostly interrogated in female reproduction and immune responses to pathogens.

Several studies investigated changes in miRNA expression levels at early time points after blood feeding and after infection with the murine parasite *P. berghei* or the human parasite *P. falciparum*.[40,42,56] *A. gambiae* is the natural vector of *P. falciparum* in sub-Saharan Africa, nevertheless infections with the murine parasites have been helpful to investigate general mechanisms that underlie mosquito-*Plasmodium* interactions. Blood feeding induces rapid up-regulation of miR-7 and repression of miR-317 and miR-N3 as early as three hours after a blood meal.[40] In *Drosophila*, miR-7 regulates cell cycle progression of follicle cells during oogenesis by repressing the zinc finger protein tramtrack69 (Ttk69). Interestingly, Ttk69 expression is regulated by the steroid hormone ecdysone, which, in addition to larval development, plays an important role in mosquito oogenesis.[57] Further studies will be necessary to unravel the role for miR-7 during blood feeding in *A. gambiae*. Surprising results have been observed in experiments with *Plasmodium* parasites, which are believed to be recognized by signals emitted by damaged cells as a result of parasite traversal of the midgut cells.[58] Strikingly, levels of miR-317, miR-2940 and miR-N3/N4/N5/N6 were significantly up-regulated at 3 hours post-*P. berghei* infection, much earlier than the onset of midgut traversal.[40] Functions of these miRNAs in *A. gambiae* remain to be characterized. Note that accumulation of miR-92 after infection with *P. berghei* was caused by the uptake of this conserved miRNA with parasite-infected mouse blood and not by up-regulation of its expression in the mosquito.[40] Functional relevance of this finding requires further investigation.

Midgut-specific miRNA expression in *A. gambiae* was investigated by two independent approaches: direct miRNA cloning and microarray hybridization. Direct miRNA cloning from pooled midgut and midgut-depleted mosquito samples collected at 24-48 hours post-*P. berghei* infection identified miR-1174, miR-1175, miR-281 and miR-989 to be primarily expressed in the midgut.[42] Whereas midgut-specific expression of miR-1174, miR-1175 and miR-281 has been further validated by studies in other mosquito species, miR-989 was on the contrary reported to be enriched in the mosquito ovaries.[41,49,59,60] These conflicting results call for re-examination of miR-989 tissue expression patterns. Regardless of miR-989 tissue specificity, blood feeding strongly inhibited miR-989 expression in the midgut [42] and induced miR-989 in other mosquito tissues (midgut-depleted samples) or whole body samples.[41,42,49] The midgut-specific cluster of miR-1174 and miR-1175 is unique to mosquito species, and both miRNAs are up-regulated in the mosquito midgut at 24-48 hours post-blood feeding. Furthermore, expression of miR-283, let-7 and miR-277 is down-regulated by blood feeding in other tissues than midgut.[42] Although little is known about miR-277 in *Anopheles*, in *Drosophila* it controls branched-chain amino acid catabolism linked to regulation of dTOR signalling, suggesting a plausible role of miR-277 in blood digestion.[61]

An unexpected complexity has been observed when miRNA expression patterns were compared between *P. berghei*-infected and uninfected mosquitoes. Parasite infection attenuated expression of a number of miRNAs after a blood meal by decreasing expression levels of miR-1174, miR-1175 and miR-34 that are up-regulated by blood meal and by increasing expression levels of miR-989 that is repressed by blood meal.[42] These results suggest that *Plasmodium* infection manipulates miRNA expression programmes in the mosquito midgut. However, the significance of such manipulation and the underlying mechanisms remain largely understudied. Expression of miRNAs in the remaining tissues is not significantly modulated by *Plasmodium* infections with the exception of miR-34 whose expression levels are slightly increased by infection.[42] A microarray-based approach identified differentially regulated miRNAs in the mosquito midgut at 18 and 24 hours after *P. falciparum* infection. In this study, regulation of miRNAs by normal blood feeding was not investigated. Similar to *P. berghei* infections, expression of miR-989 was down-regulated at 18 hours post-infection, whereas at 24 hours, only miR-305 was up-regulated.[56]

Reproductive fitness directly shapes *A. gambiae* abundance and thereby critically contributes to vectorial capacity and malaria transmission. To characterize male and female reproductive programmes, miRNA repertoires of larval and adult gonads were investigated by RNAseq.[41] Tissue-specific sequencing identified 45 new miRNAs and demonstrated significant changes in expression of 41 miRNAs in the gonads of unfed females.[41] High enrichment of miR-286 and miR-2944 in the testes and ovaries of blood-fed females suggested a role for these miRNAs in gametogenesis, while high levels of miR-989 were detected in the ovaries of blood-fed females[41] (Figure 1 and Table 1).

3 | THE miRNA BIOSYNTHESIS PATHWAY IN *A. GAMBIAE*

3.1 | Conserved miRNA biosynthesis pathway

Sequencing of the *A. gambiae* genome greatly benefited the identification of the mosquito orthologs of *Drosha*, *Dicer1*, *Ago1* and *loquacious* involved in miRNA biosynthesis in *Drosophila*.[62] The crucial role of *Ago1* in mosquito miRNA biosynthesis was demonstrated by RNAseq of small RNA libraries isolated from mosquitoes before and after silencing of *Ago1* or *Ago2*.[40] Only knock-down of *Ago1* significantly reduced abundance of mature miRNAs, suggesting a conserved role of Ago1 in generation of mature miRNAs in *A. gambiae*.[40] Interesting differences in functional specificity of *loquacious* isoforms have been reported—the loqs-PA isoform that regulates exclusively miRNA biogenesis in *Drosophila*, also regulates biogenesis of endo- and exo-siRNAs in *Ae. aegypti*.[20,63] Further studies are needed to examine how these differences affect the specificity of the Dicer/loquacious complex and functional specificities of Dicer1 and Dicer2 in the mosquitoes.

TABLE 1 Summary of expression patterns of *Anopheles gambiae* miRNAs regulated by blood feeding and *Plasmodium* infection

	A. gambiae			Invertebrates		
miRNA	Regulation by blood meal	Regulation by *Plasmodium* infection	Tissue of differential expression	Function	Targets	Organism
miR-1174	Up [40,57]	Down[40]	Midgut	Sugar metabolism, blood meal digestion, egg development and survival[57]	*Serine hydroxymethyl-transferase*[57]	*Ae. aegypti*
miR-1175	Up[40,57]	Down[40]	Midgut			
miR-305	N/A	Up[54]	Midgut	*P. falciparum* development[54] Adaption to starvation via TOR and p53 pathway[60] Midgut stem cell differentiation[61] Memory formation[62]	*p53*[60]	*A. gambiae* *Drosophila*
miR-989	Down[40] Up[39,40]	Up[40,54]	Midgut Ovary	Border cell migration during oogenesis[63]		*Drosophila*
miR-7	Up[38]			Cell cycle and differentiation during oogenesis[55] Cell cycle and mass during wing growth[64] Eye morphogenesis[65] Maintenance of stem cell progenitor state[66,67]	*Tramtrack69*[55] *Cut, Senseless*[64] *iHog*[65] *Yan, Bam*[66,67]	*Drosophila*
miR-317	Down[38]	Up[38]		Morphogenesis, gonad development, larval development[68] Brain development[69] Cell cycle[70] Post-mating response[71]	*Cyclin B*[70]	*Drosophila*
miR-2940		Up[38]		Dengue virus replication[72]	*Arginine methyl-transferase*[72]	*Ae. aegypti*
let-7	Down[40]			Multiple functions; reviewed in 73	Multiple	Multiple
miR-283	Down[40]					
miR-277	Down[40]			Amino acid catabolism[59] Neurodegenerative disorder[74]	*Drep2 and Vimar*[74]	*Drosophila*
miR-34	Up[40]	Down[40] Up[40]	Midgut (midgut-depleted tissues)	Maternal miR-34 regulates neuronal development[75] Autophagy and lifespan[76] DNA damage response, cell death[77]	*Atg9*[76]	*Drosophila* *C. elegans*
miR-286	Up[39]		Ovary (and testis)			
miR-2944	Up[39]		Ovary (and testis)			
miR-12	Up[40]			*Wolbachia* proliferation[78]	*MCT1 and MCM6*[78]	*Ae. aedes*
miR-N3	Down[38]	Up[38]				
miR-N4/N5/N6		Up[38]				

3.2 | Regulation and function of miRNA biosynthesis pathway in response to malaria parasites

The massive physiological changes in development and after blood feeding require a coordinated network of transcriptional and post-transcriptional regulation. Interestingly, enhancer regions of genes encoding the components of the miRNA biosynthesis pathway are enriched in binding sites for the ecdysone-related transcription factor broad complex (BRC) and for the transcription factors NF-κB that regulate mosquito immune responses.[62] Sequencing of mRNAs associated with ribosomes at 22-26 hours after infection with *P. falciparum* revealed accumulation of *Dicer1*, *Dicer2* and *Drosha* transcripts,[64] suggesting that the miRNA biosynthesis pathway is responding to parasite infection. Unfortunately, a comprehensive analysis of expression patterns of the miRNA pathway components has not been performed yet.

To investigate whether miRNAs regulate *Plasmodium* development in *A. gambiae*, some components of the miRNA biosynthesis pathway have been silenced in adult mosquitoes by injection of dsRNA. *Plasmodium* development was then evaluated 10 days after infection by counting the number of parasites in the mosquito midguts.[42,56] Silencing of *Dicer1* and *Ago1* significantly increased the number of developing *P. berghei* but not *P. falciparum* parasites.[42] Note that *Ago1* silencing led to a slight decrease in the percentage of *P. falciparum*-infected mosquitoes.[56]

In summary, the role of miRNAs in *A. gambiae* responses to *P. falciparum* remains controversial. *Plasmodium* infections increase translation rates of the components of the miRNA biosynthesis pathway in midgut cells. However, inhibition of miRNA biosynthesis increases susceptibility of *A. gambiae* to *P. berghei* but does not significantly impact *P. falciparum* development. Further functional studies of individual miRNAs are needed to better understand the role of miRNAs in *A. gambiae* responses to malaria parasites.

4 | FUNCTIONAL ANALYSIS OF INDIVIDUAL miRNAs IN *A. GAMBIAE*

4.1 | Experimental analysis of miRNAs

The initial toolbox to investigate miRNA functions in mosquitoes was restricted to miRNA knockout and overexpression using transgenic approaches. The breakthrough came with the development of miRNA mimics and inhibitors such as antagomirs.[65] This innovation opened unprecedented opportunities to quickly assess miRNA functions by injection of specific inhibitors directly into mosquito adults. The antagomirs are RNA antisense oligos that (i) are reverse-complementary to the miRNA of interest, (ii) have a phosphorothionate backbone at the first two and last four nucleotides, (iii) are 2′O-methylated at all nucleotides and (iv) are coupled to cholesterol at the 3′-end.[66] Gain of function is investigated using miRNA mimics, which are small dsRNA oligos that mimic endogenous miRNAs with a characteristic two nucleotide overhang at their 3′-end.[56]

As discussed above, studies of miRNAs in *A. gambiae* so far have focused exclusively on miRNA expression patterns in reproductive organs or during normal and infectious blood meals. The first functionally examined miRNA in *A. gambiae* was the midgut-specific miR-1174 whose expression is induced by blood feeding.[42,59] Inhibition of miR-1174 by antagomirs produced striking phenotypes in *A. gambiae* and *Ae. aegypti*. Mosquitoes showed defects in sugar metabolism, blood intake, egg development and had a shortened lifespan.[59] Computational target prediction and qPCR validation identified the gene encoding serine hydroxymethyltransferase (SHMT) as a potential miR-1174 target. This was confirmed in experiments with simultaneous knock-down of *SHMT* and of miR-1174 that rescued mosquito defects in sugar metabolism, blood intake and egg development. As *Plasmodium* infection represses miR-1174 expression,[42] it will be of interest to examine whether miR-1174 repression after infectious blood meal impacts *Plasmodium* development. More information with regard to regulation of parasite development is available for miR-305 and miR-989, whose expression is up-regulated by *P. falciparum* infection in the midgut.[56] Injection of miR-305 mimics negatively impacts *P. falciparum* development, whereas miR-305 inhibition causes a reverse phenotype and leads to increased parasites loads.[56] Of note, significant phenotypic variation has been reported between experiments involving miR-305. Such variability may result either from some experimental problems, or could be indicative of miR-305 buffering function detectable only under certain conditions, as described for *C. elegans*.[36] Knock-down of miR-305 reduces total bacterial loads in the midgut as measured by counting colony-forming units of plated midgut extracts.[56] However, changes in the composition of the microbiota induced by miR-305 inhibition were not further investigated. Therefore, whether miR-305 affects selected bacterial classes or exerts a more general effect on the microbial community remains unknown. The potential role of miR-305 in immune regulation is especially interesting in the light of the computational studies that predicted such well-known immune genes as the transmembrane PGN recognition protein LC (*PGRP-LC*) and the *Anopheles Plasmodium*-responsive leucine-rich repeat protein 1 (*APL1C*) to be potential targets of miR-305.[56] Functional analysis of miR-989 did not reveal any detectable phenotypes in infections with *P. falciparum*.[56] Instead, RNA-end (PARE) sequencing of ovaries identified ten potential miR-989 mRNA targets in *A. stephensi*, of which the majority encode unknown proteins with potential roles in protein binding and/or in proteolysis.[49] Further studies should address roles of miR-989 and of its target genes in *A. gambiae* oogenesis.

4.2 | Computational target prediction

Identification of miRNA targets in the cell of interest is an important step for its functional characterization. This is usually achieved by computational and/or experimental approaches. Computational target prediction based on genome-wide screening for canonical target sites represents a time- and cost-efficient tool. The canonical miRNA binding site is located in the 3′UTR of the mRNA and shows partial or contiguous base pairing to the miRNA seed sequence. However, miRNAs can also bind to noncanonical binding sites and efficiently repress target mRNAs.[67] As the exact definition of miRNA binding sites and the molecular mechanisms of successful target repression are still

incompletely understood, the algorithms that predict miRNA targets generate high number of false-positives. Poor annotations of 3′UTRs and of gene functions in nonmodel organisms further complicate identification of miRNA binding sites and their target genes. Among the web-based prediction tools available for model organisms, two feature *A. gambiae*: (i) EMBL-EBI platform microcosm targets using the miRANDA algorithm (www.ebi.ac.uk), and (ii) InsecTar database, which is an integrated database that combines miRANDA, RNAhybrid and MicroTar algorithms (www.insectar.sanbi.ac.za).[68-70] Furthermore, a number of open source algorithms can be used for analysis of custom sets of 3′UTR data. These web tools are complemented by such programs as RNAhybrid[69] and MicroInspector,[71] which look for miRNA binding sites in a specific mRNA of interest. Although these tools might be helpful to predict miRNA targets, knowledge of the miRNA phenotype following injection of miRNA inhibitors or mimics, combined with mRNA analysis, would be useful to narrow down the list of potential targets and exclude false-positives.

5 | GAPS OF KNOWLEDGE AND PERSPECTIVES

In the field of *A. gambiae*, miRNA research is still very young. Most information about the function of miRNAs is inferred from the model organisms such as *Drosophila* and *C. elegans*, but also from the more studied *Ae.aegypti*. It is becoming increasingly clear that the roles of even highly conserved miRNAs differ between organisms according to their ecological and physiological particularities. For example, habitat choices of immature stages differ significantly between aquatic mosquitoes and fruit flies. Haemophagic requirements of mosquito females also distinguish them from their *Drosophila* counterparts. Therefore, functions of miRNAs involved in development and reproduction are likely to vary between *Drosophila* and *A. gambiae*. In contrast, *Ae.aegypti* shares many aspects of *Anopheles* lifestyle and may represent a better model. In the following sections, we discuss major gaps in our knowledge of miRNAs in mosquitoes and identify promising lines of research.

5.1 | Tissue- and cell-specific miRNA expression patterns in *A. gambiae*

Currently, tissue-specific patterns of *Anopheles* miRNAs remain very incomplete. The majority of studies investigated miRNA expression in the tissues that are important for *Plasmodium* development (midgut) and for mosquito reproduction (gonads). However to advance our understanding of miRNA functions in *Anopheles*, global temporal tissue- and cell-specific miRNA expression profiles are desperately needed. Especially, mosquito blood cells (also called haemocytes), which play a key role in mosquito immune responses, have not been investigated. In mammals, miRNAs modulate outcomes of infection and inflammation by regulating blood cell differentiation and activation of immune cells (summarized in 72,73 and Entwistle & Wilson, this issue). It will be of interest to examine whether immune functions of blood cells in insects are regulated by evolutionary conserved mechanisms.

Initial transcriptional studies on *A. gambiae* miRNAs focused on the early time points after *Plasmodium* infections revealing modest changes in expression levels of a few miRNAs. Numerous studies in mammals highlight crucial roles of miRNAs during chronic or long-term infections.[74-76] Therefore, future studies should investigate the role of miRNAs in the mosquito adaptation to infection. This is especially important in regard to trophic parasite dependence on mosquito nutrients.[77-79]

5.2 | Tools for functional miRNA studies

The *in vivo* injection of antagomirs and miRNA mimics is based on an invasive approach prone to side effects that are not well characterized yet. Furthermore, injection does not allow for manipulation of miRNA expression in a tissue-specific manner. The CRISPR-Cas9 technology accelerates gene editing and provides an unprecedented tool for generation of miRNA loss-of-function and mutant mosquito lines.[80] Furthermore, spatiotemporal inhibition or overexpression can be achieved by transgenesis-mediated temporal tissue-specific expression of miRNA-sponges or miRNAs.[81,82] The Raikhel laboratory has successfully applied this approach to *Ae. aegypti* for fat body-, ovary- and midgut-specific miRNA inhibition at ~24 hours post-blood meal.[66,80,83] RNA sponges contain complementary binding sites to a miRNA of interest and function as competitive inhibitors when expressed in the cells as transgenes. Introduction of a noncomplementary bulge at the site of Ago1 cleavage protects sponge RNA from destruction while allowing it to capture the target miRNA.[81] The spatiotemporal expression of sponges is achieved by guiding their expression with inducible and tissue-specific promoters. However, availability of inducible or tissue-specific promoters is another limiting factor in mosquito research.

5.3 | Post-transcriptional regulation of miRNAs

Post-transcriptional regulation of miRNAs is a new topic in miRNA research (reviewed in 84). miRNAs showing variations in exact sequence as compared to the miRBase annotations are called isomiRs. The 5′-end of miRNAs defines the target specificity and is subject to little variation. In *A. gambiae*, 5% of the sequenced miRNAs show 5′ modifications that lead to a shift in the seed sequence.[40] Whether and how these changes affect miRNA function remains to be investigated. In contrast to the conserved 5′-end, the 3′-end of miRNAs are very heterogeneous.[85] Similar to many organisms, template-independent nucleotide additions at the 3′-ends of miRNAs, such as uridylation- and adenylation, are also frequent in *A. gambiae*[85]. Functional significance of these modifications is currently under investigation. Initial evidence suggests that uridylation targets miRNA for degradation, whereas adenylation increases its stability or activity.[85-88] Therefore, 3′ modifications may critically affect miRNA regulation and function. Whereas no significant changes in 3′ and 5′ modifications have been reported after *A. gambiae* blood feeding, post-transcriptional regulation of miRNAs during mosquito development remains to be investigated.

5.4 | Potential application in vector control

Most of the research on miRNAs in mosquitoes has focused predominantly on aspects relevant to vectorial capacity such as reproduction and vector competence, which can be potentially exploited for vector control applications.[56,59,89] However, all these studies have been performed in laboratory conditions. As discussed in this review, the miRNA phenotypes may vary greatly in field mosquitoes exposed to variable environmental conditions; therefore, future studies should address miRNA function in field mosquitoes. The CRISPR-Cas9 system has revolutionized gene drive approaches offering an efficient system to spread a desired gene into natural mosquito populations.[90] This system could be potentially harnessed to modulate or totally abort expression of miRNAs of interest. As the majority of the characterized miRNAs regulate mosquito reproduction, such genetic drives can be used for species eradication.[59,80,89]

6 | CONCLUSIONS

Although the first miRNA was identified 23 years ago, the miRNA research in *A. gambiae* is still in its infancy with only three of 168 miRNAs functionally characterized. RNAseq studies crucially contributed to the identification of miRNA repertoire and to quantification of miRNA expression levels.[40,41] Nevertheless, *A. gambiae* miRNAs remain understudied with regard to their expression patterns, sites of expression and function. Further characterization of miRNA expression at the tissue or cellular level may reveal new or differentially regulated miRNAs that have not been detected at the level of the whole organism. Changes in miRNA expression are induced by both blood feeding and parasite infection. As blood feeding induced changes coincide with parasite development, they most likely also shape vector competence. These changes, at least in part, are regulated by the steroid hormone ecdysone. As miRNAs tightly regulate hormonal signalling during *Drosophila* development, it will be of interest to examine the role of mosquito miRNAs in ecdysone regulation during development and reproduction. More functional analyses of miRNAs using gene-editing technologies are awaited to reveal new fascinating paradigms in vector-parasite interactions and in mosquito biology in general.

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CONFLICT OF INTEREST

None for both authors.

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