Journal of Medical Entomology, 60(2), 2023, 282–293
https://doi.org/10.1093/jme/tjac182
Advance Access Publication Date: 7 December 2022
Research

ENTOMOLOGICAL SOCIETY OF AMERICA
SHARING INSECT SCIENCE GLOBALLY

OXFORD

Molecular Biology/Genomics

Identification of MicroRNAs in the West Nile Virus Vector *Culex tarsalis* (Diptera: Culicidae)

Sultan Asad [1] Ahmed M. Mehdi [2] Sujit Pujhari [1,3] Claudia Rückert [4,5] Gregory D. Ebel [5] and Jason L. Rasgon[1,6,]

[1]Department of Entomology, The Center for Infectious Disease Dynamics, and the Huck Institutes of the Life Sciences, The Pennsylvania State University, University Park, PA, USA, [2]The University of Queensland, Brisbane, Australia Diamantina Institute, Faculty of Medicine, The University of Queensland, Translational Research Institute, Brisbane, QLD, Australia, [3]Department of Pharmacology Physiology and Neuroscience, University of South Carolina School of Medicine, Columbia, SC, USA, [4]Department of Biochemistry and Molecular Biology, University of Nevada Reno, Reno, NV, 89557, USA, [5]Department of Microbiology, Immunology and Pathology, Center for Vector-borne Infectious Diseases, College of Veterinary Medicine and Biomedical Sciences, Colorado State University, Fort Collins, CO, 80523, USA, and [6]Corresponding author, e-mail: jlr54@psu.edu

Subject Editor: David Severson

Received 9 May 2022; Editorial decision 7 November 2022.

Abstract

MicroRNAs (miRNAs) are a group of small noncoding RNAs that regulate gene expression during important biological processes including development and pathogen defense in most living organisms. Presently, no miRNAs have been identified in the mosquito *Culex tarsalis* (Diptera: Culicidae), one of the most important vectors of West Nile virus (WNV) in North America. We used small RNA sequencing data and in vitro and in vivo experiments to identify and validate a repertoire of miRNAs in *Cx. tarsalis* mosquitoes. Using bioinformatic approaches we analyzed small RNA sequences from the *Cx. tarsalis* CT embryonic cell line to discover orthologs for 86 miRNAs. Consistent with other mosquitoes such as *Aedes albopictus* and *Culex quinquefasciatus*, miR-184 was found to be the most abundant miRNA in *Cx. tarsalis*. We also identified 20 novel miRNAs from the recently sequenced *Cx. tarsalis* genome, for a total of 106 miRNAs identified in this study. The presence of selected miRNAs was biologically validated in both the CT cell line and in adult *Cx. tarsalis* mosquitoes using RT–qPCR and sequencing. These results will open new avenues of research into the role of miRNAs in *Cx. tarsalis* biology, including development, metabolism, immunity, and pathogen infection.

Key words: *Culex tarsalis*, microRNAs, blood feeding

Background

West Nile virus (WNV) is one of the most prevalent mosquito-borne illnesses in the continental United States and 1 in 150 infected people develops a very severe neuroinvasive disease such as West Nile encephalitis or meningitis (McDonald et al. 2021, Petersen et al. 2013, Nash et al. 2001). It is primarily transmitted by mosquitoes in the genus *Culex*, mainly *Cx. pipiens*, *Cx. quinquefasciatus*, and *Cx. tarsalis* (Hayes 1989, Turell et al. 2005). While much progress has been made in other mosquito vectors, very little molecular research has focused on *Cx. tarsalis*, leaving us with limited tools for targeting these mosquitoes or their vectorial capacity for specific pathogens. Due to lack of vaccines or specific treatments, there is need for novel approaches to prevent WNV spread (Ulbert 2019).

MicroRNAs (miRNAs) are a potential new tool for curbing vector-borne diseases because they can indirectly or directly inhibit pathogens (Zhang et al. 2013, Slonchak et al. 2015), and because they contribute to the control of important biological events. miRNAs are small (19-25 nt) genetically encoded noncoding RNAs that regulate post transcriptional gene expression (Bartel and Chen 2004, Bartel 2009) by binding imperfectly to mRNAs either at the 3′-untranslated regions (3′-UTR), 5′-untranslated regions (5′-UTR), or coding regions. Though the 2–8 nucleotides at the 5′ end of the miRNA ('seed region') must perfectly complement the target transcript, the rest of the miRNA sequence can have mismatches and bulges (Bartel 2009, Rigoutsos 2009, Schnall-Levin et al. 2010). miRNAs are transcribed inside the nucleus into a primary miRNA transcript which is processed into a pre-miRNA by

Drosha and Pasha. The pre-miRNA is then exported to the cytoplasm with the help of exportin proteins. Once in cytoplasm, the pre-miRNA is processed by Dicer I into the ~22 nucleotide mature miRNA. The mature miRNA is loaded into the microRNA induced silencing complex (miRISC) where it becomes single-stranded. The single-stranded miRNA–miRISC complex then targets specific mRNAs based on sequence similarity, leading to targeted mRNA degradation or modulation of protein expression (Flynt et al. 2010, Asgari 2018). miRNAs are critical for a variety of cellular processes including development (Flynt et al. 2010), immunity (Momen-Heravi and Bala 2018), and pathogen response (Miesen et al. 2016, Saldana et al. 2017). This makes them prime candidates to investigate to better understand host–pathogen interactions in mosquito vectors of medically important pathogens.

More than 3,000 mosquito species are present worldwide, but only a handful are represented in miRbase—the primary database of all known miRNAs across the animal kingdom (Kozomara et al. 2019). This lack of miRNA annotation limits research progress that may help combat mosquito vectors and the pathogens they spread.

Cx. tarsalis is a neglected yet important vector of many medically important viruses such as WNV (Goddard et al. 2002, Venkatesan et al. 2009, Goldberg et al. 2010), West Equine Encephalitis (WEEV) (Barnett 1956), St. Louis Encephalitis (SLEV) (Hammon and Reeves 1943), and Cache Valley virus (CVV) (Ayers et al. 2018) in North America. Despite many studies focusing on the ecology, feeding behavior, and vector competence of this mosquito, there is little information regarding miRNAs in *Cx. tarsalis* (Venkatesan and Rasgon 2010, Dodson et al. 2018, Gorris et al. 2021). Here we have mined and analyzed publicly available data (including a dataset of small RNAs as well as the *Cx. tarsalis* genome) to identify 106 miRNAs that are present in *Cx. tarsalis* mosquitoes. The presence of 10 randomly chosen miRNAs was confirmed both in vitro and in vivo in adult mosquitoes.

Results and Discussion

Data Used in This Study

We accessed publicly available small RNA data generated from the *Cx. tarsalis* CT cell line (Ruckert et al. 2019) and passed it through our in-house pipeline (Fig. 1) to identify miRNAs in *Cx. tarsalis*. Briefly, sequence read archive (SRA) files (Supp Table S1 [online only]) were directly downloaded into CLC Genomics Workbench. All reads were trimmed by removing the RA3 adapter (TGGAATTCTCGGGTGCCAAGGG) sequences from the 3′ end of raw sequencing reads. Any reads with low quality scores or that did not have an adapter sequence were excluded. After removal of the RA3 adapter sequences we saw a cluster of RNA sequences 21–24 nucleotides in length with a peak at 22 nucleotides, which is the most abundant size of previously identified mosquito miRNAs (Fig. 2A). To further examine the profile of the trimmed RNA reads, we used the web-based server sRNAtoolbox (Aparicio-Puerta et al. 2019) to determine that approximately 70.5 percent of the reads were mappable to miRbase miRNAs (Fig. 2B).

Discovery of miRNAs and Their Potential Targets in *Cx. tarsalis*

miRNAs play a pivotal role in insect development (Alvarez-Garcia and Miska 2005, Zhang et al. 2009), reproduction (Song et al. 2018, Zhang et al. 2019), metabolism (Ling et al. 2017), and longevity (Ma et al. 2017), insecticide resistance (Li et al. 2015, Etebari et al. 2018), immunity, and host–pathogen interactions (Saldana et al. 2017, Wong et al. 2020), and have been shown to govern processes relevant to public health in various mosquito species (Slonchak et al. 2014, Yan et al. 2014, Dubey et al. 2019). We first identified *Cx. tarsalis* miRNAs by mapping our sequences to previously identified miRNAs from other mosquito species (i.e., we searched for putative orthologs). Using CLC Genomics Workbench 20, small RNA reads from 3 biological replicates of the *Cx. tarsalis* CT cell line were mapped onto mature miRNAs of both *Ae. aegypti* and *Cx. quinquefasciatus* using default parameters. We considered miRNAs to be true hits only if they were present in at least two out of the three biological replicates. Our analysis found 60 and 84 mature miRNAs in *Cx. tarsalis* using *Cx. quinquefasciatus* (Fig. 3A) and *Ae. aegypti* (Fig. 3B) mature miRNAs as reference, respectively, with an overlap of 58 miRNAs (Fig. 3C; Supp Table S2 [online only])

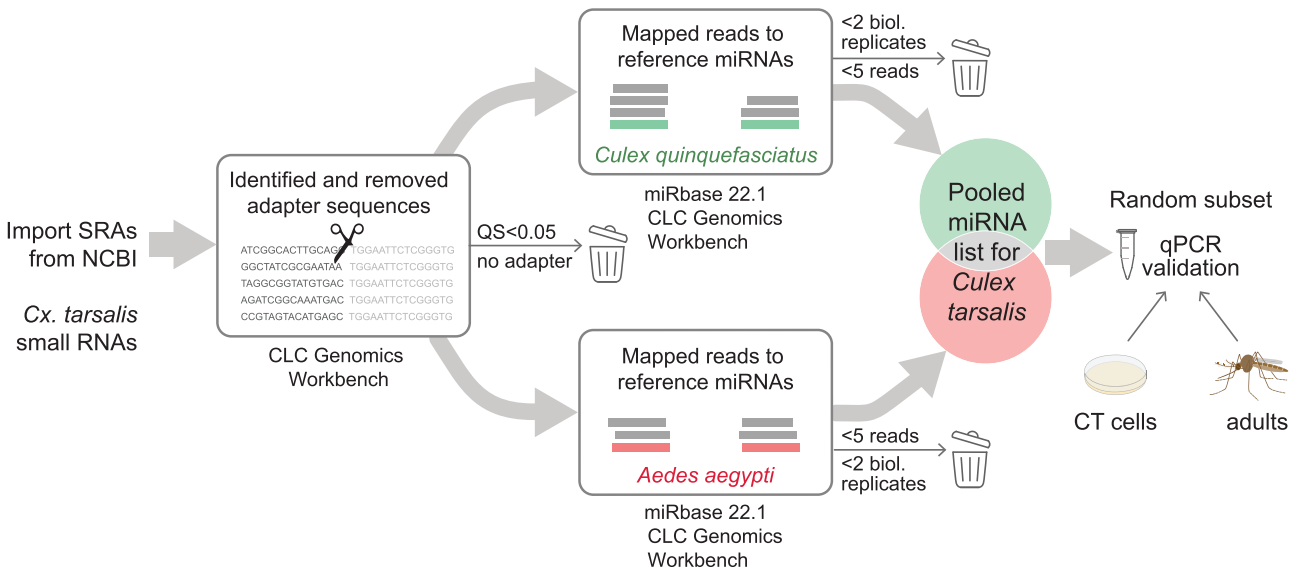

Fig. 1. Schematic diagram of miRNA discovery pipeline. We used an integrated approach combining bioinformatics and wet laboratory experiments to identify and validate *Cx. tarsalis* miRNAs. Briefly, small RNA sequences from *Cx. tarsalis* were aligned to the annotated miRNAs of both *Cx. quinquefasciatus* and *Ae. aegypti* to identify putative orthologs. A random subset of these was validated by qRT–PCR. We also used genomic data to identify novel miRNAs in *Cx. tarsalis* (not pictured). See Methods for full details. QS, quality score. SRA, sequence read archive.

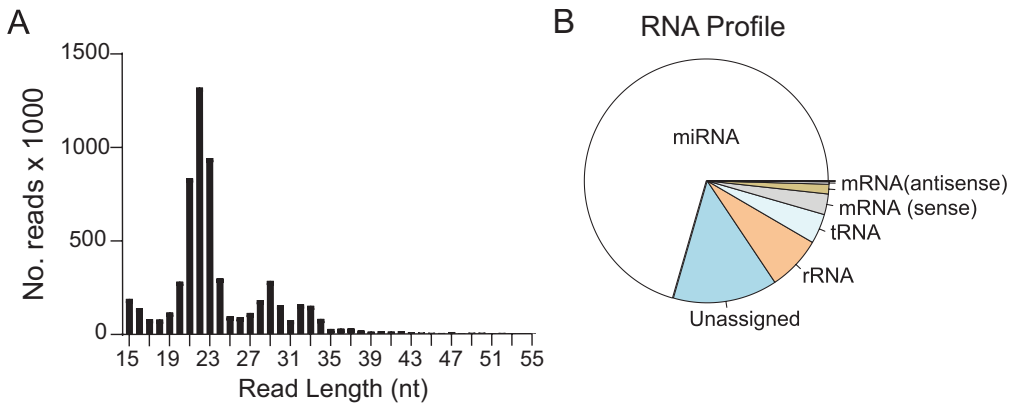

Fig. 2. Small RNA sequencing data. (A) Read length distribution after adapter removal. (B) RNA profile of analyzed small RNA reads. Approximately 70.5% of reads mapped to known miRNAs in miRbase.

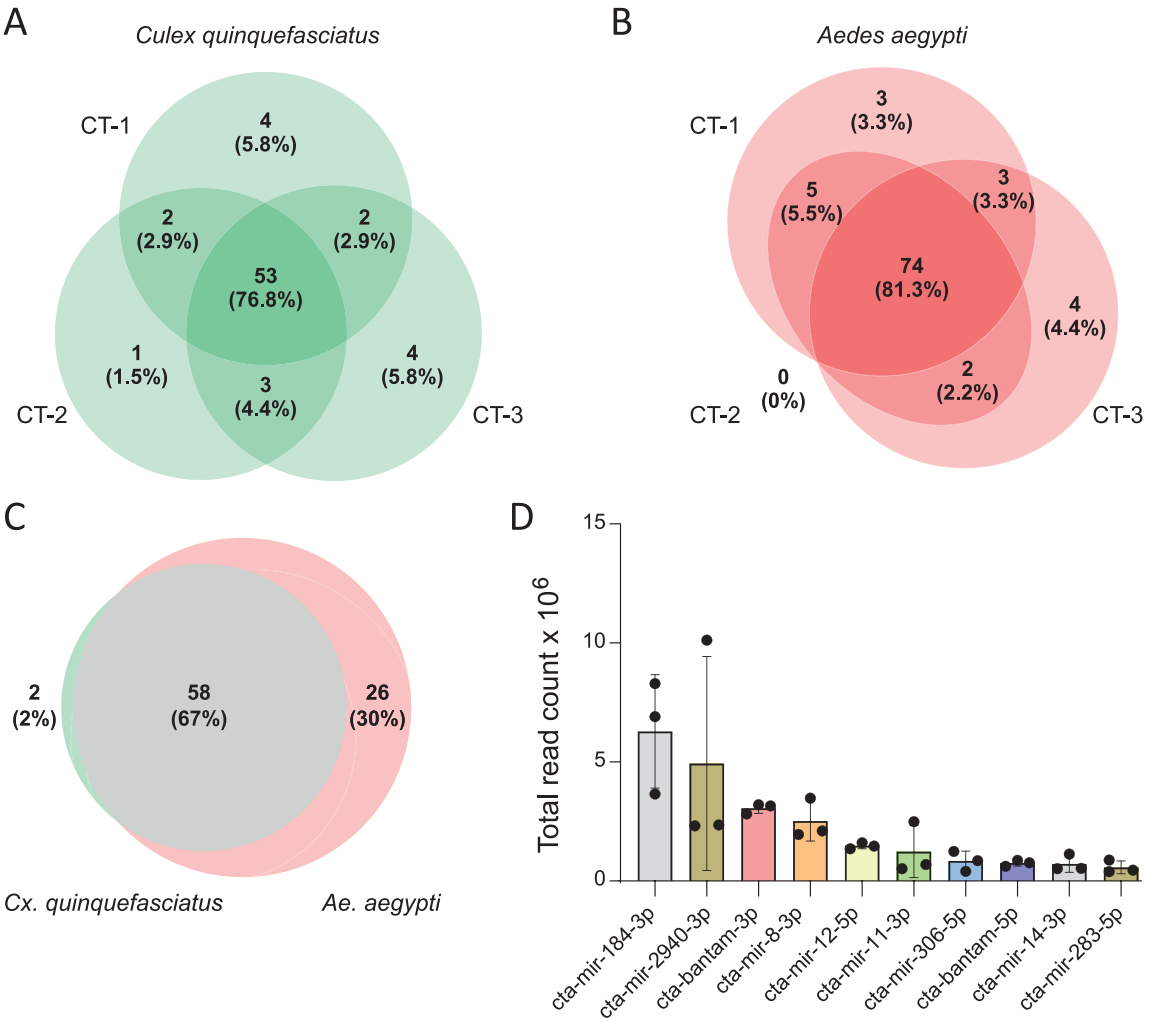

Fig. 3. Identification of *Cx. tarsalis* miRNAs. (A–B) Quantitative Venn diagrams of miRNAs identified by homology with reference sequences from *Cx. quinquefasciatus* (A) and *Aedes aegypti* (B). (C) Overlap of miRNAs identified using the *Cx. quinquefasciatus* and *Ae. aegypti* annotations. (D) The ten most abundant miRNAs in *Cx. tarsalis*. Each bar represents the expression value (in read counts) of an individual miRNA found in the SRA reads, while error bars represent the ± standard deviation and dots show individual biological replicates.

(Bardou et al. 2014). In total, we identified 86 high-confidence miRNAs in *Cx. tarsalis* mosquitoes via orthology (Table 1) with miRNA cta-miR184-3p showing the highest number of read counts (Fig. 3D). The conservation of these miRNAs over evolutionary time suggests they have functional roles (Tarver et al. 2013). To further investigate each miRNA's potential to target messenger RNAs, we used the RNAhybrid online server (Rehmsmeier et al. 2004). We found each miRNA has the potential to target multiple genes

Table 1. 86 *Culex tarsalis* miRNAs identified by homology to *Ae. aegypti* and/or *Cx. quinquefasciatus*, as indicated by Y (yes, ortholog found) or N (no ortholog found)

	Name	Sequence	Length	*Ae. aegypti*	*Cx. quinquefasciatus*
1	cta-bantam-3p	UGAGAUCAUUUUGAAAGCUGA	21	Y	Y
2	cta-bantam-5p	CCGGUUUUCAUUUUCGAUCUGAC	23	Y	Y
3	cta-let-7-5p	UGAGGUAGUUGGUUGUAUAGU	21	Y	Y
4	cta-miR-10-5p	ACCCUGUAGAUCCGAAUUUGUU	22	Y	Y
5	cta-miR-100-5p	AACCCGUAGAUCCGAACUUGUG	22	Y	Y
6	cta-miR-11-3p	CAUCACAGUCUGAGUUCUUGCU	22	Y	Y
7	cta-miR-11-5p	CGAGAACUCCGGCUGUGACC	20	Y	Y
8	cta-miR-12-3p	UGAGUAUUACAUCAGGUACUGGU	23	Y	Y
9	cta-miR-13-3p	UAUCACAGCCAUUUUGACGAGU	22	Y	Y
10	cta-miR-14-3p	UCAGUCUUUUUCUCUCUCCUAU	22	Y	Y
11	cta-miR-184-3p	UGGACGGAGAACUGAUAAGGGC	22	Y	Y
12	cta-miR-1889-3p	CACGUUACAGAUUGGGGUUUCC	22	Y	Y
13	cta-miR-1889-5p	UAAUCUCAAAUUGUAACAGUGG	22	Y	Y
14	cta-miR-1890-3p	UGAAAUCUUUGAUUAGGUCUGG	22	Y	Y
15	cta-miR-190-5p	AGAUAUGUUUGAUAUUCUUGGUUG	24	Y	Y
16	cta-miR-210-3p	CUUGUGCGUGUGACAACGGCUAU	23	Y	Y
17	cta-miR-252-5p	CUAAGUACUAGUGCCGCAGGAG	22	Y	Y
18	cta-miR-263a-5p	AAUGGCACUGGAAGAAUUCACGG	23	Y	Y
19	cta-miR-275-3p	UCAGGUACCUGAAGUAGCGC	20	Y	Y
20	cta-miR-276-3p	UAGGAACUUCAUACCGUGCUCU	22	Y	Y
21	cta-miR-276-5p	AGCGAGGUAUAGAGUUCCU	19	Y	Y
22	cta-miR-277-3p	UAAAUGCACUAUCUGGUACGAC	22	Y	Y
23	cta-miR-278-3p	UCGGUGGGACUUUCGUCCGUUU	22	Y	Y
24	cta-miR-279-3p	UGACUAGAUCCACACUCAUUAA	22	Y	Y
25	cta-miR-281-3p	UGUCAUGGAAUUGCUCUCUUU	21	Y	Y
26	cta-miR-281-5p	AAGAGAGCUAUCCGUCGACAGU	22	Y	Y
27	cta-miR-283-5p	CAAUAUCAGCUGGUAAUUCUGGG	23	Y	Y
28	cta-miR-285-3p	UAGCACCAUUCGAAAUCAGUAC	22	Y	Y
29	cta-miR-2941-3p	UAGUACGGCUAGAACUCCACGG	22	Y	Y
30	cta-miR-2a-3p	UAUCACAGCCAGCUUUGAAGAGC	23	Y	Y
31	cta-miR-305-3p	CGGCACAUGUUGGAGUACAC	20	Y	Y
32	cta-miR-305-5p	AUUGUACUUCAUCAGGUGCUCU	22	Y	Y
33	cta-miR-306-5p	UCAGGUACUGAGUGACUCUCAG	22	Y	Y
34	cta-miR-308-3p	AAUCACAGGAGUAUACUG	18	Y	Y
35	cta-miR-31-5p	UGGCAAGAUGUUGGCAUAGCUGA	23	Y	Y
36	cta-miR-315-5p	UUUUGAUUGUUGCUCAGAAAGC	22	Y	Y
37	cta-miR-317-3p	UGAACACAGCUGGUGGUAUCU	21	Y	Y
38	cta-miR-33-5p	GUGCAUUGUAGUUGCAUUGCA	21	Y	Y
39	cta-miR-375-3p	UUUGUUCGUUUGGCUCGAGUUA	22	Y	Y
40	cta-miR-7-5p	UGGAAGACUAGUGAUUUUGUUGU	23	Y	Y
41	cta-miR-71-3p	UCUCACUACCUUGUCUUUCAUG	22	Y	Y
42	cta-miR-71-5p	AGAAAGACAUGGGUAGUGAGAU	22	Y	Y
43	cta-miR-79-5p	GCUUUGGCGCUUUAGCUGUAUGA	23	Y	Y
44	cta-miR-8-3p	UAAUACUGUCAGGUAAAGAUGU	22	Y	Y
45	cta-miR-8-5p	CAUCUUACCGGGCAGCAUUAGA	22	Y	Y
46	cta-miR-87-3p	GUGAGCAAAUUUUCAGGUGUGU	22	Y	Y
47	cta-miR-92a-3p	AUUGCACUUGUCCCGGCCU	19	Y	Y
48	cta-miR-957-3p	UGAAACCGUCCAAAACUGAGGC	22	Y	Y
49	cta-miR-965-3p	UAAGCGUAUAGCUUUUCCCAUU	22	Y	Y
50	cta-miR-970-3p	UCAUAAGACACACGCGGCUAU	21	Y	Y
51	cta-miR-980-3p	UAGCUGCCUAGUGAAGGGC	19	Y	Y
52	cta-miR-988-3p	CCCUUGUUGCAAACCUCACGC	21	Y	Y
53	cta-miR-988-5p	UGUGUGCUUUGUGACAACGAGA	22	Y	Y
54	cta-miR-996-3p	UGACUAGAUUACAUGCUCGU	20	Y	Y
55	cta-miR-998-3p	UAGCACCAUGAGAUUCAGC	19	Y	Y
56	cta-miR-999-3p	UGUUAACUGUAAGACUGUGUCU	22	Y	Y
57	cta-miR-9c-3p	UAAAGCUUUAGUACCAGAGGUC	22	Y	Y
58	cta-miR-9c-5p	UCUUUGGUAUUCUAGCUGUAGA	22	Y	Y
59	cta-miR-2952-3p	AGAGCUCAGCACGCAGGGGUGGC	23	N	Y
60	cta-miR-2951-5p	UAGUACGGCCAUGACUGAGGGC	22	N	Y
61	cta-miR-11893-3p	UUCCUGACUUAUACGCUUACCU	22	Y	N
62	cta-miR-11899-3p	UAUGACCGAUUUAAUAUAUGGCU	23	Y	N
63	cta-miR-12-5p	UGAGUAUUACAUCAGGUACUGGU	23	Y	N

Table 1. Continued

	Name	Sequence	Length	*Ae. aegypti*	*Cx. quinquefasciatus*
64	cta-miR-13-5p	CAGUACUUAUGUUAUGCUCUCU	22	Y	N
65	cta-miR-275-5p	CGCGCUAAGCAGGAACCGAGAC	22	Y	N
66	cta-miR-277-5p	CGUGUCAGAAGUGCAUUUACA	21	Y	N
67	cta-miR-278-5p	ACGGACGAUAGUCUUCAGCGGCC	23	Y	N
68	cta-miR-282-3p	ACAUAGCCUGACAGAGGUUAGG	22	Y	N
69	cta-miR-286a-3p	UGACUAGACCGAACACUCGCGUCCU	25	Y	N
70	cta-miR-286b-3p	UGACUAGACCGAACACUCGUAUCCC	25	Y	N
71	cta-miR-2940-3p	GUCGACAGGGAGAUAAAUCACU	22	Y	N
72	cta-miR-2940-5p	UGGUUUAUCUUAUCUGUCGAGGC	23	Y	N
73	cta-miR-2944a-5p	GAAGGAACUUCUGCUGUGAUCUGA	24	Y	N
74	cta-miR-2944b-3p	UAUCACAGCAGUAGUUACCUGA	22	Y	N
75	cta-miR-2945-3p	UGACUAGAGGCAGACUCGUUUA	22	Y	N
76	cta-miR-2945-5p	AGCGGGUCCGUUUCUAGUGUCAUG	24	Y	N
77	cta-miR-2b-3p	UAUCACAGCCAGCUUUGAUGAGCU	24	Y	N
78	cta-miR-2c-3p	UAUCACAGCCAGCUUUGAUGAGC	23	Y	N
79	cta-miR-307-3p	CACAACCUCCUUGAGUGAGCGA	22	Y	N
80	cta-miR-308-5p	CGCGGUAUAUUCUUGUGGCUUG	22	Y	N
81	cta-miR-34-5p	UGGCAGUGUGGUUAGCUGGUUG	22	Y	N
82	cta-miR-79-3p	UAAAGCUAGAUUACCAAAGCAU	22	Y	N
83	cta-miR-92a-5p	CGGUACGGACAGGGGCAACAUU	22	Y	N
84	cta-miR-92b-3p	CGGUACGGACAGGGGCAACAUU	22	Y	N
85	cta-miR-92b-5p	AAUUGCACUUGUCCCGGCCUGC	22	Y	N
86	cta-miR-9b-5p	UCUUUGGUGAUUUUAGCUGUAUGC	24	Y	N

to regulate their expression (Supp Table S4 [online only]). This is consistent with previous work showing the potential for miRNAs to target multiple transcripts within an organism (Friedman et al. 2009, Bartel 2018).

The Majority of *Cx. tarsalis* miRNAs Arise From the 3′ Arm and Sequence Variation Occurs Mainly at the 3′ End of Mature miRNAs

Mature miRNAs are formed as a result of a Dicer-mediated processing of double-stranded precursor miRNA (pre-miRNA) into two arms (i.e., complementary strands) named 5p and 3p. Selective loading and retention of either 3p or 5p in the miRISC complex results in one strand becoming functionally active while the other strand is cleaved out of the complex and degraded (Asgari 2018). This process (strand selection) is influenced by the thermodynamic instability of the duplex, 5′ end starting nucleotides, and miRNA duplex length (Czech and Hannon 2011, Han et al. 2011, Lucas and Raikhel 2013). We detected mature miRNA that arose from 5p, 3p, and both 5p and 3p of pre-miRNAs. Forty-nine out of the 86 (57%) orthologous miRNAs identified in this study were derived from the 3′ arm of the pre-miRNA, indicating that there is a bias toward 3p arm processing of miRNAs in the *Cx. tarsalis* CT cell line ($P = 0.0017$, binomial test) (Fig. 4A). Further experiments are needed to verify this finding, and to determine which factors produce this bias in strand selection.

In each of the data libraries used in this study, small RNA reads mapped to reference sequences from miRbase showed variability in both length and sequence. These variants are commonly known as isomiRs, and they usually arise from imperfect processing or cleavage by Drosha or Dicer (Kuchenbauer et al. 2008, Wu et al. 2009, Starega-Roslan et al. 2011), processes that primarily affect the ends of miRNAs. IsomiRs were long considered as an artifact, but recent advances in deep sequencing and computational algorithms have identified isomiRs in many species (Lee et al. 2010, Wyman et al. 2011, Amsel et al. 2017), including in *Ae. aegypti* (Etebari et al. 2015). IsomiRs have been found to act as functional miRNAs to modulate transcription and translation of target mRNAs (Cloonan et al. 2011, Llorens et al. 2013). IsomiRs are classified into three main categories: 5′ isomiRs, 3′ isomiRs, and polymorphic isomiRs, with 5′ and 3′ isomiRs subclassified according to their polymorphisms (e.g., insertion or deletion) (Neilsen et al. 2012). We used CLC Genomics Workbench 20 to describe and quantify isomiRs in *Cx. tarsalis*. We focused on insertions and deletions of nucleotides at 3′ or 5′ end of mature miRNAs and limited our discovery to a maximum of 2-nucleotide addition or deletion at either end according to default settings of CLC genome workbench. Because they are rare and may be less likely to affect miRNA function (Nielsen et al. 2012), we did not analyze polymorphic isomiRs (i.e., those arising from nucleotide substitutions) in our study. IsomiRs that were present in all three biological replicates were used for analysis (Supp Table S3 [online only]). Our results show most modifications occurred at the 3′ end of the mature miRNAs, with 2-nucleotide insertions being the most prevalent by far, followed by 1-nt insertions (Fig. 4B). Thus, our results agree with a previous study that also found the majority of isomiR sequence variations were at the 3′ end of mature miRNAs (Newman et al. 2011). 5′ variations are rare, but they typically have more functional importance because 5′ variations often alter the seed region in ways that affect regulatory ability (Tan et al. 2014). Although both cta-miR-184 and cta-miR-2940-3p showed the highest rate of isomiRs formation, there was a striking difference in the modification pattern: the former showed the majority of sequence variations at the 3′ end (Fig. 4C) while the later showed more sequence variations at the 5′ end (Fig. 4D). This suggests that cta-miR 2940-3p and its isomiRs might have an increased ability to target a wider array of transcripts due to shift in the seed region at the 5′ end. Although 5′ modifications are less frequent, they are more likely to impact function—as in the case of 5′ isomiR-9–1, which acquired the ability to downregulate the expression of two new transcripts while losing its ability to inhibit the canonical target of miR-9 (Tan et al. 2014). These results warrant further investigation into the role of isomiRs in mosquito physiology and host–pathogen interactions. More work is needed to determine if the patterns of variation we report in the CT cell line hold true in live mosquitoes.

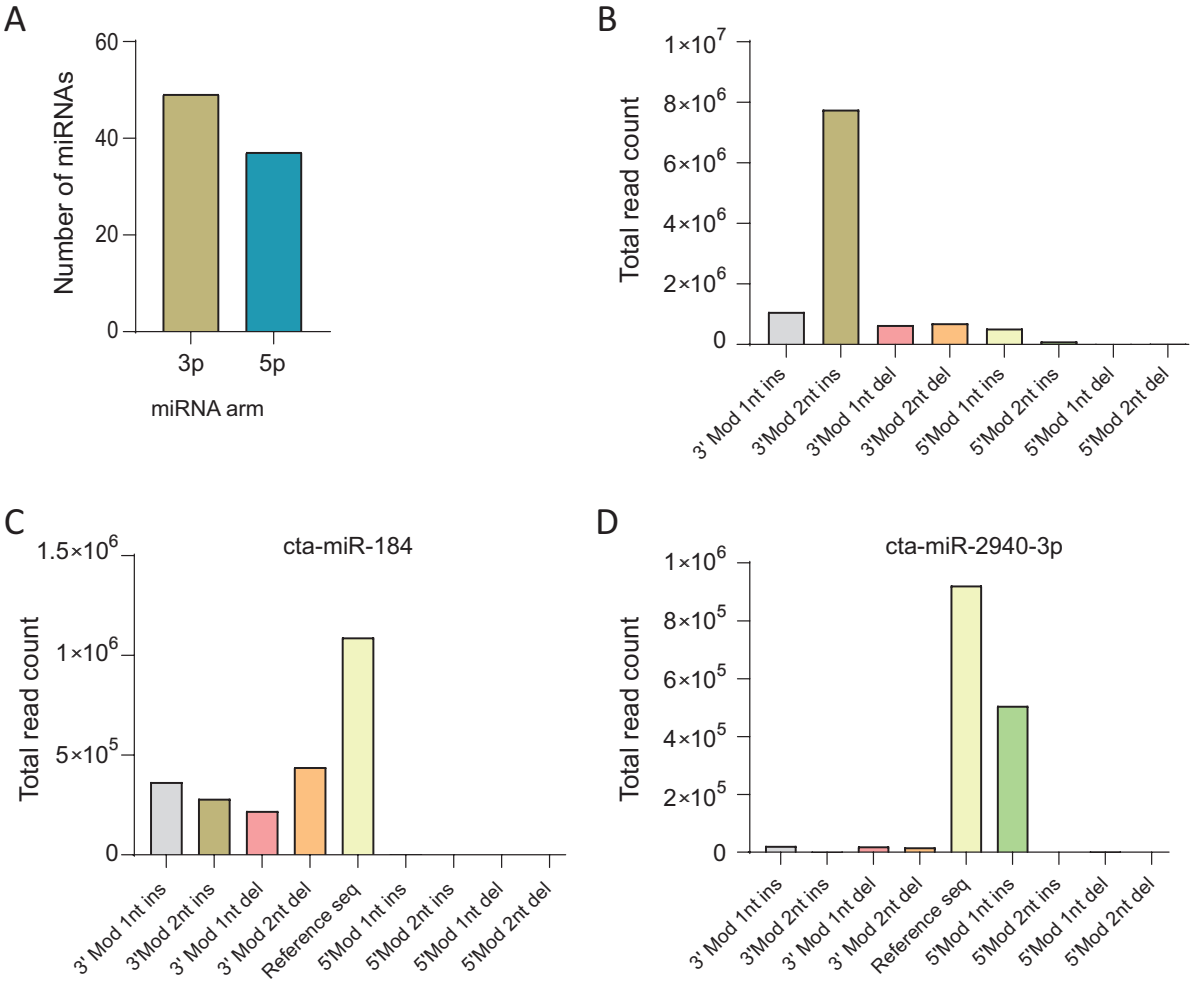

Fig. 4. *Cx. tarsalis* miRNAs arise primarily from 3p arm and most sequence variation is found at the 3′ end. (A) Number of miRNAs processed from either the 3p or 5p arm of pre-miRNAs. (B) Distribution of sequence polymorphisms of 86 mature *Cx. tarsalis* miRNA sequences. (C) Sequence polymorphisms found in cta-miR-184 are found almost exclusively at the 3′ end of the mature miRNA. (D) Sequence polymorphisms found in cta-miR-2940-3p are mainly at the 5′ end of the mature miRNA.

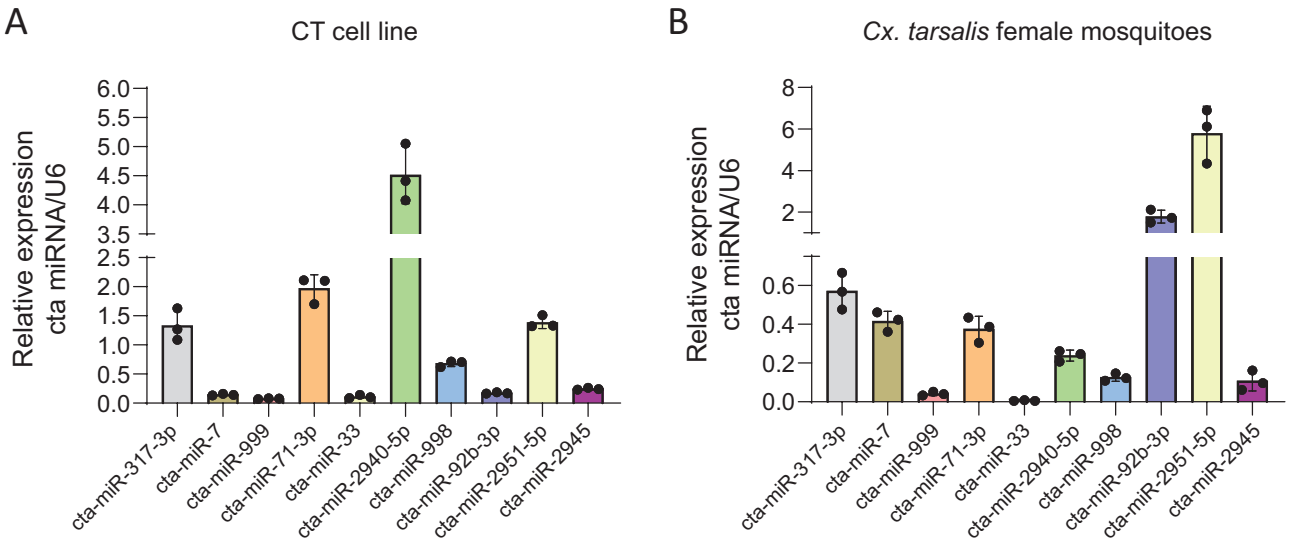

Fig. 5. Validation of *Cx. tarsalis* miRNAs. Primer extension-based confirmation of 10 randomly selected miRNAs with RT–qPCR using 3 biological replicates. U6 was used as a reference for relative quantification. Panels show relative expression of cta-miRNAs/U6 in the CT cell line (A) and in adult *Cx. tarsalis* female mosquitoes (B). Error bars represent standard deviation and dots represent individual biological replicates. Each biological replicate is made up of 5 *Cx. tarsalis* sugar-fed 7-day-old females.

Table 2. Novel miRNAs identified from the *Cx. tarsalis* genome and their putative target transcripts

	Name	Sequence	Length	Predicted consensus precursor sequence
1	cta-novel-1	UUAACAAUAUUUGUGUGACCUG	22	UUAACAAUAUUUGUGUGACCUGUUGUUAAUACGAUAUGAA GGUCACACGAAUAUUGCUAAGU
2	cta-novel-2	UGACUAGAGGCAGACUCGUUU	21	AGCGGGUCCGUUUCUAGUGUCAUGUGCUACGUUUGAAAGU CAUGACUAGAGGCAGACUCGUUU
3	cta-novel-3	UUUUAGACCGGUUUUGAACAC	21	UGUUCAAAACCGGUCUAAAACGUGAUUUUUCCGUUUUAGA CCGGUUUUGAACAC
4	cta-novel-4	AGAUAGGACCUUUUGAAAAAGU	22	UUUUUCAAAAGAUCCCAUCUGCUAUGGGUUUUCUAUGCAG AUAGGACCUUUUGAAAAAGU
5	cta-novel-5	AUGGCGCUGUAAAAAAGCUUUUU	23	AAAGCUUUACACAACGCCAUCUAUCAGAAAAACUUAAUGAC AAUCAGAUGGCGCUGUAAAAAAGCUUUUU
6	cta-novel-6	UAUGCUAUCUUGGGACAUGUCU	22	UAUGCUAUCUUGGGACAUGUCUGCUGUAAAAAGAUAGGAC GUGUCACAAGAUAGCACACA
7	cta-novel-7	CAUGAUAUCUUGUGACAUGUCU	22	CAUGAUAUCUUGUGACAUGUCUGCUGUAAAAAGAUAGGAC GUGUCACAAGAUAGCACA
8	cta-novel-8	AGCCGGACAGCCUCGACCUGGA	22	AGCCGGACAGCCUCGACCUGGACUACGACAUGUGGCAGGG CCAGUUCGAGUUUGUCGGGCCGGC
9	cta-novel-9	GAAGGAACUUCUGCUGUGAUCU	22	GAAGGAACUUCUGCUGUGAUCUGAGAUGUGUUCAUAUCAC AGUAGUUGUACUUUAA
10	cta-novel-10	UGACUAGACCGAACACUCGUAUC	23	GGCGAUUGUCGGCUUGGUCGCUGUCUUUACCCAAGGCACA GUUGCACUUCAGUGACUAGACCGAACACUCGUAUC
11	cta-novel-11	UAUGACCGAAUUAAUGUAUGGCU	23	CCAUAUAUGAUUUUGGUCAUAUAUUGAAUUAUGAUAUUUUA UAUGACCGAAUUAAUGUAUGGCU
12	cta-novel-12	UUCCUGACUUAUACGCUUACCCCU	24	GUAAGUAGAUAAUUCAGAAAGGCGGCAACGUUUCUUCCUG ACUUAUACGCUUACCCCU
13	cta-novel-13	UGUAACCCGUCAUGAACUGUCA	22	UGUAACCCGUCAUGAACUGUCAAAUUGGUGAAGCAGUUUG ACAGUUCUUGUUGGGUUACAUU
14	cta-novel-14	CAAACCGGAAGUUGUAAACUGU	22	AGUUCAUCAUUUCCGGUUUGAUAGAGGAAUCAAACCGGAA GUUGUAAACUGU
15	cta-novel-15	AUCCCUGCCUUCGGAUGCCUA	21	AAAGAGGUAGGGAUAACUCGCCAUCCCUGCCUUCGGAUG CCUA
16	cta-novel-16	UAUGCUAUCGUGUGACAUGUCU	22	UAUGCUAUCGUGUGACAUGUCUGCUGUAAAAAAAUAGGAC GUGUCACAAGAUAGCACUCA
17	cta-novel-17	UUUCGUUCCGGACACAAAAAUC	22	UUUCGUUCCGGACACAAAAAUCAGGAAUCGGCCUGAGGAU UUUUGUUUCCGGAAUGUCCACAGAAC
18	cta-novel-18	UCUACCGAUCGAUCUUCAUAGC	22	CGUGAAGAUCUUCCGGUGCAGACAGGCUCGUUGAGGCCAC UCAAAGUCACCAGCGUGUCUACCGAUCGAUCUUCAUAGC
19	cta-novel-19	AACAAAGCCGAUCGGAAAAGU	21	UUUUUCGUCGGCAGUGGCCAGUGUGAGAUACGCGCAACAA AGCCGAUCGGAAAAGU
20	cta-novel-20	GUAGAAUCGCGUCAAAUCGACUGA	24	AGUCGAGUGAUGCAAUUCUUUCCGCAAACAACUUUUGCCC ACUUUCGCGUAGAAUCGCGUCAAAUCGACUGA

Validation of *Cx. tarsalis* miRNAs

We randomly selected 10 miRNAs: cta-miR-317-3p, cta-miR-7, cta-miR-999, cta-miR-71-3p, cta-miR-33, cta-miR-2940-5p, cta-miR-998, cta-miR-92b-3p, cta-miR-2951-5p, and cta-miR-2945 for validation both in vitro (CT cell line) and in vivo in 7-day-old sugar-fed female *Cx. tarsalis* mosquitoes (Fig. 5A and B). Briefly, we cloned qPCR amplicons from miScript reactions into a pJET plasmid and Sanger sequenced each to confirm mature miRNA sequence (Supp Fig S1 [online only]).

Prediction and Validation of Novel miRNAs From the *Cx. tarsalis* Genome and Identification of Their Potential Target Transcripts

During the preparation of this manuscript, the *Cx. tarsalis* genome was published (Main et al. 2021), which we used to identify novel miRNAs (present in all 3 replicates) that could be mapped to the *Cx. tarsalis* genome. Our results identified 20 novel miRNAs in *Cx. tarsalis* mosquitoes (Table 2). RNAfold (rna.tbi.univie.ac.at/cgi-bin/RNAWebSuite/RNAfold.cgi) was used to determine the secondary structure of the predicted pre-miRNA sequences (Fig. 6). Primers were designed for 12 randomly selected novel miRNAs and validated through RT–qPCR in both the CT cell line and *Cx. tarsalis* mosquitoes. Our results confirm that all 12 novel selected miRNAs were expressed in CT cells (Fig. 7A), but in mosquitoes we were only able to detect 8 out of 12 (Fig. 7B), possibly reflecting physiological differences between cultured cells and live mosquitoes, or differences between embryonic (CT cell line) and adult mosquitoes. The potential target transcripts of these novel microRNAs were predicted by using the RNAhybrid online server (Rehmsmeier et al. 2004), resulting in identification of multiple RNA transcripts with putative binding sites for these miRNAs (Supp Table S4 [online only]), and increasing our confidence in these novel miRNAs.

Blood Feeding Affects miRNA Expression in *Cx. tarsalis* Mosquitoes

Blood feeding is one of the most important events in a female mosquito's life cycle. Nutrient-rich vertebrate blood is vital for egg development, has strong effects on physiology (Clements 1992,

Valzania et al. 2019), and may also expose the mosquito to blood-borne pathogens. miRNAs play a role in blood feeding in mosquitoes. In *Ae. aegypti*, miRNA-275 and miRNA-309 are upregulated upon blood feeding and are critical for blood digestion and egg development and ovary development, respectively (Bryant et al. 2010, Zhang et al. 2016). In *Anopheles stephensi*, miR-989 is downregulated 72 h post blood feeding (Mead and Tu 2008). To ask if these miRNAs are also linked to blood feeding in *Cx. tarsalis*, we examined their expression using qRT–PCR following a bloodmeal alongside non-blood fed controls. Although we did not identify miR-309 in our pipeline, we included it here because of its ovary-specific expression pattern (Zhang et al. 2016). We found a significant increase in the expression of cta-miR-275-3p at 6, 12, and 48 h post bloodmeal with the highest increase at 12 h ($P < 0.001$) validating the previous findings by Bryant et al. 2010 who found miR-275 upregulated in *Ae. aegypti* after taking a bloodmeal (Fig. 8A). Similarly, our results showed a significant increase in cta-miR-309 expression ($P < 0.0001$) at 48 h post bloodmeal, (Fig. 8B) consistent with previous reports (Zhang et al. 2016). However, in case of cta-miR-989, our results suggest that expression was modulated after intake of blood by *Cx. tarsalis*, highlighting its possible role in the reproduction of this mosquitoes species. Although there was a steady statistically significant increase in cta-miR989 expression at 6, 12, and 24 h in blood fed mosquitoes, its expression was down-regulated at 72 h. Though the decrease at 72 h post bloodmeal was not significant ($P = 0.0695$) (Fig. 8C), it showed a trend consistent with result previously reported in *An. stephensi* (Mead and Tu 2008). Our results suggest the tested miRNAs serve similar functions across diverse mosquito taxa, highlighting their biological significance in blood feeding.

Conclusions

Mosquitoes pose an increasing threat to human health, making the development of new methods for vector control an urgent public health need. Due to their role in regulating gene expression and involvement in numerous important biological events including disease states, miRNAs may help fill this gap (Hanna et al. 2019). Our study has identified a total of 106 miRNAs, including 86 already reported in other mosquitoes and 20 novel miRNAs that are present in the *Cx. tarsalis* genome. Although further studies will be required

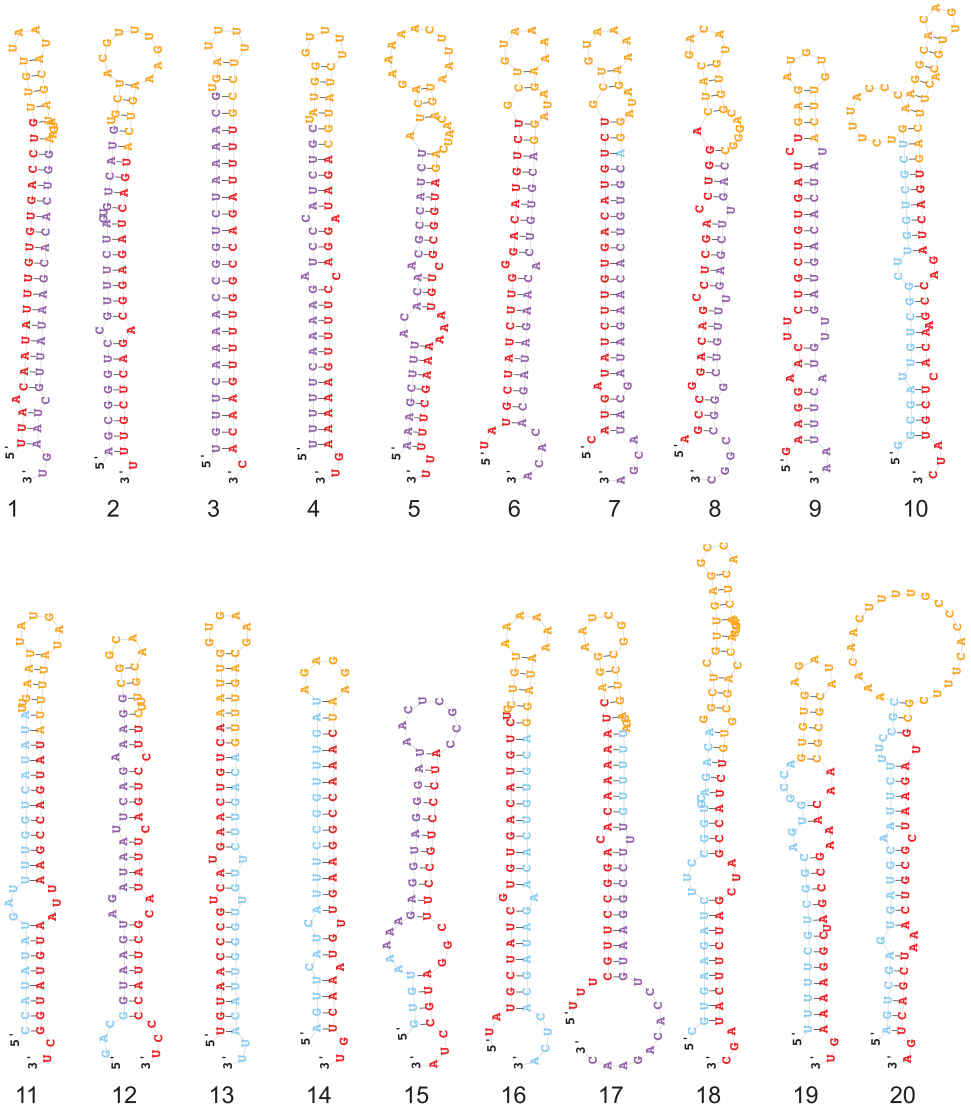

Fig. 6. Predicted secondary structures of novel miRNAs. The stem-loop structures of novel pre-miRNAs are illustrated. Mature miRNA sequences are shown in red, star sequences in purple/blue, and loop sequence in yellow. All structures were predicted using RNAfold. See Methods for full details.

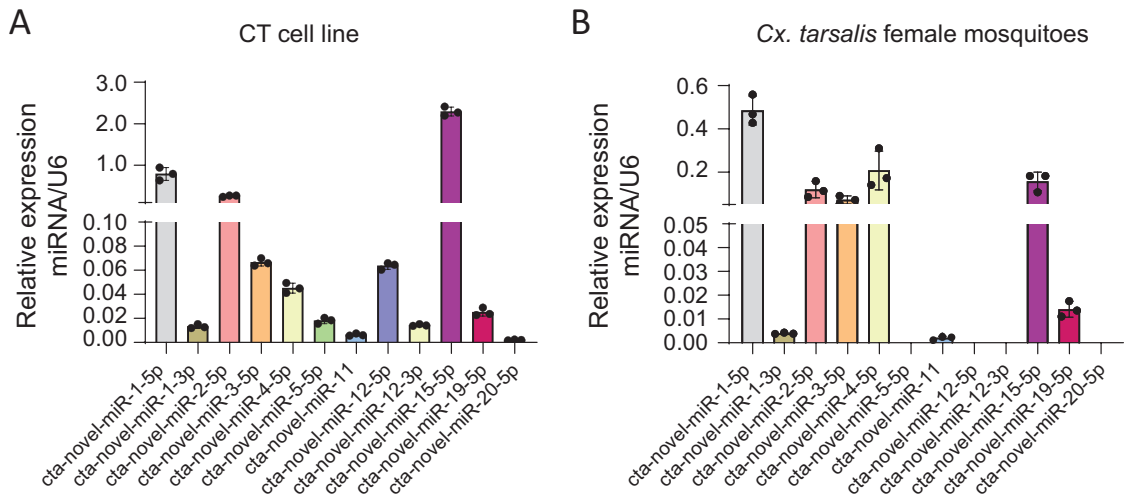

Fig. 7. Validation of novel *Cx. tarsalis* miRNAs. RT–qPCR-based amplification of novel miRNAs in CT cells (A) and *Cx. tarsalis* female mosquitoes (B). Error bars represent standard deviation and dots represent individual biological replicates. Each biological replicate is made up of 5 sugar-fed *Cx. tarsalis* 7-day-old females.

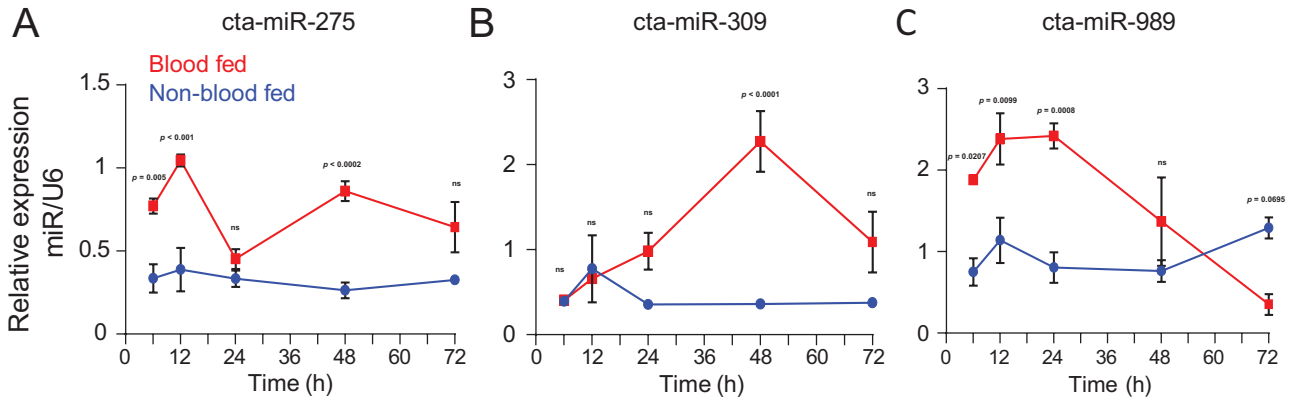

Fig. 8. Temporal expression of miRNAs in whole adult female mosquitoes following a bloodmeal. Using RT–qPCR, we measured relative expression of (A) cta-miR-275, (B) cta-miR-309, and (C) cta-miR-989. Each data point depicts the mean of three biological replicates while the error bars represent standard error of mean. Each biological replicate comprised a pool of 10 mosquitoes. Significance was evaluated by Two-Way ANOVA with Bonferroni multiple testing correction, $P < 0.05$.

to get a deeper insight into the biological context and significance of these highlighted miRNAs, these results will lay the foundation for functional studies and open new avenues for research in *Cx. tarsalis* biology and pathogen transmission.

Methods

Small RNA Libraries and Data Analysis

To identify *Cx. tarsalis* miRNAs we used a publicly available small RNA dataset from the *Cx. tarsalis* CT cell line (Ruckert et al. 2019), accessed via the National Center for Biotechnology Information Sequence Read Archive. In total, three libraries were downloaded. All libraries were generated using the TruSeq Small RNA Sample Prep Kit (Illumina) and sequenced on the Illumina HiSeq 2000 platform. The data were analyzed using CLC Genomics Workbench 20. Briefly, the sequenced library files were trimmed of adapter sequences. The sequences with low quality (quality score <0.05) and without adapters were removed. The high quality trimmed libraries were then mapped to the annotated miRNAs of *Ae. aegypti* and *Cx. quinquefasciatus* found in miRbase 22.1. Only miRNAs with a read count ≥5 were analyzed further. We used sRNAtoolbox library mode to count reads and determine expression values (read counts), using the single assignment method. We used CLC Genomics Workbench 20 to describe and quantify isomiRs. For novel miRNA prediction from the *Cx. tarsalis* genome, we used the miRDeep2 pipeline (Friedlander et al. 2012). We used RNAfold to predict secondary structures.

Target Prediction of miRNAs

RNAhybrid (Rehmsmeier et al. 2004) was used to identify the potential mRNA targets of each *Cx. tarsalis* miRNA. Briefly, each mature miRNA sequence identified in this study was allowed to hybridize to the list of all the transcripts of *Cx. tarsalis* downloaded from open science framework (https://osf.io/mdwqx/) (Main et al. 2021). The minimum free energy was set to –25 with helix constraint from 2 to 8 nucleotides of the mature miRNA without any G:U wobble.

Mosquitoes and Cell Lines

Cx. tarsalis mosquitoes (YOLO strain, BEI Resources) were reared at 26°C, 40–50% RH, and a photoperiod of 16:8 (L:D) h. This

mosquito colony is derived from wild-caught animals and has been laboratory-reared since 2003. The mosquitoes were provided with 10% sucrose solution ad libitum prior to blood feeding. The *Cx. tarsalis* cell line CT (generously provided by Dr Aaron Brault, CDC) was maintained at 28°C with 0.5% CO_2 in Schneider medium supplemented with 10% of fetal bovine serum (GIBCO) and 1% antibiotics (GIBCO). Cells were passaged once per week. For RNA extraction, cells were grown to 100% confluency in 6-well plates, and each well was considered an independent biological replicate.

Blood Feeding

Mosquitoes were starved of sucrose overnight then allowed to feed on human blood provided by anonymous donors using a membrane feeder as previously described (Brustolin et al. 2018). For each trial, 5–7-d-old female mosquitoes were separated into two cages containing ~200 individuals per cage. One cage was allowed to blood feed (hereafter the 'blood fed' group), while the other cage was provided with 10% sucrose solution instead (hereafter the 'non-blood fed' group). Thirty mosquitoes were collected from each group at each time point (6, 12, 24, 48, and 72 h post meal). Samples comprised 10 pooled mosquitoes per biological replicate and were collected in Qiazol and frozen at −80°C until RNA extraction.

RNA Extractions and miRNA RT–qPCR

Qiazol was used to extract total RNA from both cells and mosquitoes following the manufacturer's suggested protocol. For mosquito RNA samples, 5 sugar-fed *Cx. tarsalis* females (7-d-old) were pooled per biological replicate. RNA was quantified using a Nanodrop and 2ug of total RNA was used to synthesize miRNA cDNA using the miScript RT kit following the manufacturer's suggested protocol. cDNA was diluted 1:10 and approximately 18ng of cDNA was used to validate miRNAs identified in this study using the miScript qPCR kit according to manufacturer's suggested protocol. All qPCR reactions were performed with 3 biological replicates and 2–3 technical replicates. U6, a conserved small nuclear RNA, was used as an internal control for RT–qPCR, and we used the Qiagen qPCR data analysis Excel template to normalize expression values. The Qiagen qPCR template uses the $2^{-\Delta\Delta CT}$ CT quantitation method to compute normalized expression of means. We used two-way analysis of variance (ANOVA) with Bonferroni multiple testing correction to statistically evaluate differences in expression level between treatment groups.

Cloning and Sequencing of miRNA Amplicons

qPCR amplicons were separated using 1% agarose gel electrophoresis. Single amplicon bands were cut using a sterile surgical blade and DNA was extracted with the Zymo Gel extraction kit according to manufacturer's suggested protocol. Eluted amplicons were directly cloned into pJET1.2/Blunt vector according to the manufacturer's suggested protocol. Positive clones were sent to GENEWIZ for Sanger sequencing. The resulting sequences were analyzed with CLC Genomics Workbench 20.

Acknowledgments

We are thankful to Dr Aaron C. Brault from CDC for generously providing the CT cell line and Hillery Metz for helpful comments on the manuscript. The following reagent was obtained through BEI Resources, NIAID, NIH: *Culex tarsalis* YOLO, NR-43026.

Funding

This work was supported by NIH grants R01AI150251, R01AI128201, and R01AI116636, NSF grant 1645331, and funds from the Dorothy Foehr Huck and J. Lloyd Huck endowment to J.L.R.

Authors' Contributions

SA conducted experiments, analyzed data, and wrote the manuscript. AMM assisted with data analysis. SP assisted with data analysis. CR and GDE provided data and assisted in manuscript writing. JLR provided funding, assisted in data analysis, and wrote the manuscript.

Ethics Approval and Consent to Participate

Not applicable.

Consent for Publication

All authors have consented to publication.

Availability of Data and Material

Data are available from the NCBI-SRA depository under accession numbers SRR8936281, SRR8936288, and SRR8936289.

Supplementary Data

Supplementary data are available at *Journal of Medical Entomology* online.

References Cited

Alvarez-Garcia, I., and E. A. Miska. 2005. MicroRNA functions in animal development and human disease. *Development*. 132: 4653–4662.

Amsel, D., A. Vilcinskas, and A. Billion. 2017. Evaluation of high-throughput isomiR identification tools: illuminating the early isomiRome of Tribolium castaneum. *BMC Bioinf.* 18: 359.

Aparicio-Puerta, E., R. Lebron, A. Rueda, C. Gomez-Martin, S. Giannoukakos, D. Jaspez, J. M. Medina, A. Zubkovic, I. Jurak, B. Fromm, *et al.* 2019. sRNAbench and sRNAtoolbox 2019: intuitive fast small RNA profiling and differential expression. *Nucleic Acids Res.* 47: W530–W535.

Asgari, S. 2018. Chapter two - microRNAs as regulators of insect host–pathogen interactions and immunity, pp. 19–45. *In* G. Smagghe (ed.), *Advances in insect physiology*, vol. 55. Academic Press, San Diego, CA.

Ayers, V. B., Y. S. Huang, A. C. Lyons, S. L. Park, S. Higgs, J. I. Dunlop, A. Kohl, B. W. Alto, I. Unlu, B. J. Blitvich, *et al.* 2018. Culex tarsalis is a competent vector species for Cache Valley virus. *Parasit. Vectors*. 11: 519.

Bardou, P., J. Mariette, F. Escudie, C. Djemiel, and C. Klopp. 2014. jvenn: an interactive Venn diagram viewer. *BMC Bioinf.* 15: 293.

Barnett, H. C. 1956. The transmission of Western equine encephalitis virus by the mosquito Culex tarsalis Coq. *Am. J. Trop. Med. Hyg.* 5: 86–98.

Bartel, D. P. 2009. MicroRNAs: target recognition and regulatory functions. *Cell*. 136: 215–233.

Bartel, D. P. 2018. Metazoan microRNAs. *Cell*. 173: 20–51.

Bartel, D. P., and C. Z. Chen. 2004. Micromanagers of gene expression: the potentially widespread influence of metazoan microRNAs. *Nat. Rev. Genet.* 5: 396–400.

Brustolin, M., S. Pujhari, C. A. Henderson, and J. L. Rasgon. 2018. Anopheles mosquitoes may drive invasion and transmission of Mayaro virus across geographically diverse regions. *PLoS Negl. Trop. Dis.* 12: e0006895.

Bryant, B., W. Macdonald, and A. S. Raikhel. 2010. microRNA miR-275 is indispensable for blood digestion and egg development in the mosquito Aedes aegypti. *Proc. Natl. Acad. Sci. USA*. 107: 22391–22398.

Clements, A. N. 1992. *The biology of mosquitoes. Volume 1: development, nutrition and reproduction*, Chapman & Hall, London and New York.

Cloonan, N., S. Wani, Q. Xu, J. Gu, K. Lea, S. Heater, C. Barbacioru, A. L. Steptoe, H. C. Martin, E. Nourbakhsh, *et al*. 2011. MicroRNAs and their isomiRs function cooperatively to target common biological pathways. *Genome Biol.* 12: R126.

Czech, B., and G. J. Hannon. 2011. Small RNA sorting: matchmaking for Argonautes. *Nat. Rev. Genet*. 12: 19–31.

Dodson, B. L., S. Pujhari, and J. L. Rasgon. 2018. Vector competence of selected North American Anopheles and Culex mosquitoes for Zika virus. *PeerJ*. 6: e4324.

Dubey, S. K., J. Shrinet, and S. Sunil. 2019. Aedes aegypti microRNA, miR-2944b-5p interacts with 3'UTR of chikungunya virus and cellular target vps-13 to regulate viral replication. *PLoS Negl. Trop. Dis*. 13: e0007429.

Etebari, K., S. Osei-Amo, S. P. Blomberg, and S. Asgari. 2015. Dengue virus infection alters post-transcriptional modification of microRNAs in the mosquito vector Aedes aegypti. *Sci. Rep.* 5: 15968.

Etebari, K., M. H. Afrad, B. Tang, R. Silva, M. J. Furlong, and S. Asgari. 2018. Involvement of microRNA miR-2b-3p in regulation of metabolic resistance to insecticides in Plutella xylostella. *Insect. Mol. Biol.* 27: 478–491.

Flynt, A. S., J. C. Greimann, W. J. Chung, C. D. Lima, and E. C. Lai. 2010. MicroRNA biogenesis via splicing and exosome-mediated trimming in Drosophila. *Mol. Cell.* 38: 900–907.

Friedlander, M. R., S. D. Mackowiak, N. Li, W. Chen, and N. Rajewsky. 2012. miRDeep2 accurately identifies known and hundreds of novel microRNA genes in seven animal clades. *Nucleic Acids Res*. 40: 37–52.

Friedman, R. C., K. K. Farh, C. B. Burge, and D. P. Bartel. 2009. Most mammalian mRNAs are conserved targets of microRNAs. *Genome Res*. 19: 92–105.

Goddard, L. B., A. E. Roth, W. K. Reisen, and T. W. Scott. 2002. Vector competence of California mosquitoes for West Nile virus. *Emerg. Infect. Dis*. 8: 1385–1391.

Goldberg, T. L., T. K. Anderson, and G. L. Hamer. 2010. West Nile virus may have hitched a ride across the Western United States on Culex tarsalis mosquitoes. *Mol. Ecol.* 19: 1518–1519.

Gorris, M. E., A. W. Bartlow, S. D. Temple, D. Romero-Alvarez, D. P. Shutt, J. M. Fair, K. A. Kaufeld, S. Y. Del Valle, and C. A. Manore. 2021. Updated distribution maps of predominant Culex mosquitoes across the Americas. *Parasit. Vectors*. 14: 547.

Hammon, W. M., and W. C. Reeves. 1943. Laboratory transmission of St. Louis Encephalitis virus by three genera of mosquitoes. *J. Exp. Med*. 78: 241–253.

Han, B. W., J. H. Hung, Z. Weng, P. D. Zamore, and S. L. Ameres. 2011. The 3'-to-5' exoribonuclease Nibbler shapes the 3' ends of microRNAs bound to Drosophila Argonaute1. *Curr. Biol*. 21: 1878–1887.

Hanna, J., G. S. Hossain, and J. Kocerha. 2019. The potential for microRNA therapeutics and clinical research. *Front. Genet*. 10: 478.

Hayes, C. G. 1989. West Nile fever, pp. 59–88. *In* T. P. Monath (ed.), *The arboviruses: epidemiology and ecology*, vol. V. CRC Press, Boca Raton, Florida.

Kozomara, A., M. Birgaoanu, and S. Griffiths-Jones. 2019. miRBase: from microRNA sequences to function. *Nucleic Acids Res*. 47: D155–D162.

Kuchenbauer, F., R. D. Morin, B. Argiropoulos, O. I. Petriv, M. Griffith, M. Heuser, E. Yung, J. Piper, A. Delaney, A. L. Prabhu, *et al*. 2008. In-depth characterization of the microRNA transcriptome in a leukemia progression model. *Genome Res*. 18: 1787–1797.

Lee, L. W., S. Zhang, A. Etheridge, L. Ma, D. Martin, D. Galas, and K. Wang. 2010. Complexity of the microRNA repertoire revealed by next-generation sequencing. *RNA*. 16: 2170–2180.

Li, X., L. Guo, X. Zhou, X. Gao, and P. Liang. 2015. miRNAs regulated overexpression of ryanodine receptor is involved in chlorantraniliprole resistance in Plutella xylostella (L.). *Sci. Rep.* 5: 14095.

Ling, L., V. A. Kokoza, C. Zhang, E. Aksoy, and A. S. Raikhel. 2017. MicroRNA-277 targets insulin-like peptides 7 and 8 to control lipid metabolism and reproduction in Aedes aegypti mosquitoes. *Proc. Natl. Acad. Sci. USA*. 114: E8017–E8024.

Llorens, F., M. Banez-Coronel, L. Pantano, J. A. del Rio, I. Ferrer, X. Estivill, and E. Marti. 2013. A highly expressed miR-101 isomiR is a functional silencing small RNA. *BMC Genomics*. 14: 104.

Lucas, K., and A. S. Raikhel. 2013. Insect microRNAs: biogenesis, expression profiling and biological functions. *Insect Biochem. Mol. Biol*. 43: 24–38.

Ma, K. S., F. Li, Y. Liu, P. Z. Liang, X. W. Chen, and X. W. Gao. 2017. Identification of microRNAs and their response to the stress of plant allelochemicals in Aphis gossypii (Hemiptera: Aphididae). *BMC Mol. Biol.* 18: 5.

Main, B. J., M. Marcantonio, J. S. Johnston, J. L. Rasgon, C. T. Brown, and C. M. Barker. 2021. Whole-genome assembly of Culex tarsalis. *G3 (Bethesda)*. 11: jkaa063.

McDonald, E., S. Mathis, S. W. Martin, J. E. Staples, M. Fischer, and N. P. Lindsey. 2021. Surveillance for West Nile Virus Disease - United States, 2009-2018. *MMWR Surveill. Summ*. 70: 1–15.

Mead, E. A., and Z. Tu. 2008. Cloning, characterization, and expression of microRNAs from the Asian malaria mosquito, Anopheles stephensi. *BMC Genomics*. 9: 244.

Miesen, P., A. Ivens, A. H. Buck, and R. P. van Rij. 2016. Small RNA profiling in dengue virus 2-infected aedes mosquito cells reveals viral piRNAs and novel host miRNAs. *PLoS Negl. Trop. Dis*. 10: e0004452.

Momen-Heravi, F., and S. Bala. 2018. miRNA regulation of innate immunity. *J. Leukoc. Biol*. 103: 1205–1217.

Nash, D., F. Mostashari, A. Fine, J. Miller, D. O'Leary, K. Murray, A. Huang, A. Rosenberg, A. Greenberg, M. Sherman, *et al*; 1999 West Nile Outbreak Response Working Group. 2001. The outbreak of West Nile virus infection in the New York City area in 1999. *N. Engl. J. Med*. 344: 1807–1814.

Neilsen, C. T., G. J. Goodall, and C. P. Bracken. 2012. IsomiRs--the overlooked repertoire in the dynamic microRNAome. *Trends Genet*. 28: 544–549.

Newman, M. A., V. Mani, and S. M. Hammond. 2011. Deep sequencing of microRNA precursors reveals extensive 3' end modification. *RNA*. 17: 1795–1803.

Petersen, L. R., A. C. Brault, and R. S. Nasci. 2013. West Nile virus: review of the literature. *JAMA*. 310: 308–315.

Rehmsmeier, M., P. Steffen, M. Hochsmann, and R. Giegerich. 2004. Fast and effective prediction of microRNA/target duplexes. *RNA*. 10: 1507–1517.

Rigoutsos, I. 2009. New tricks for animal microRNAS: targeting of amino acid coding regions at conserved and nonconserved sites. *Cancer Res*. 69: 3245–3248.

Ruckert, C., A. N. Prasad, S. M. Garcia-Luna, A. Robison, N. D. Grubaugh, J. Weger-Lucarelli, and G. D. Ebel. 2019. Small RNA responses of Culex mosquitoes and cell lines during acute and persistent virus infection. *Insect Biochem. Mol. Biol*. 109: 13–23.

Saldana, M. A., K. Etebari, C. E. Hart, S. G. Widen, T. G. Wood, S. Thangamani, S. Asgari, and G. L. Hughes. 2017. Zika virus alters the microRNA expression profile and elicits an RNAi response in Aedes aegypti mosquitoes. *PLoS Negl. Trop. Dis*. 11: e0005760.

Schnall-Levin, M., Y. Zhao, N. Perrimon, and B. Berger. 2010. Conserved microRNA targeting in Drosophila is as widespread in coding regions as in 3'UTRs. *Proc. Natl. Acad. Sci. USA*. 107: 15751–15756.

Slonchak, A., M. Hussain, S. Torres, S. Asgari, and A. A. Khromykh. 2014. Expression of mosquito microRNA Aae-miR-2940-5p is downregulated in response to West Nile virus infection to restrict viral replication. *J. Virol*. 88: 8457–8467.

Slonchak, A., R. P. Shannon, G. Pali, and A. A. Khromykh. 2015. Human microRNA miR-532-5p exhibits antiviral activity against West Nile virus via suppression of host genes SESTD1 and TAB3 required for virus replication. *J. Virol*. 90: 2388–2402.

Song, J., W. Li, H. Zhao, L. Gao, Y. Fan, and S. Zhou. 2018. The microRNAs let-7 and miR-278 regulate insect metamorphosis and oogenesis by targeting the juvenile hormone early-response gene Kruppel-homolog. *Development*. 1: 145.

Starega-Roslan, J., E. Koscianska, P. Kozlowski, and W. J. Krzyzosiak. 2011. The role of the precursor structure in the biogenesis of microRNA. *Cell. Mol. Life Sci*. 68: 2859–2871.

Tan, G. C., E. Chan, A. Molnar, R. Sarkar, D. Alexieva, I. M. Isa, S. Robinson, S. Zhang, P. Ellis, C. F. Langford, *et al*. 2014. 5' isomiR variation is of functional and evolutionary importance. *Nucleic Acids Res*. 42: 9424–9435.

Tarver, J. E., E. A. Sperling, A. Nailor, A. M. Heimberg, J. M. Robinson, B. L. King, D. Pisani, P. C. J. Donoghue, and K. J. Peterson. 2013. miRNAs: small genes with big potential in metazoan phylogenetics. *Mol. Biol. Evol.* 30: 2369–2382.

Turell, M. J., D. J. Dohm, M. R. Sardelis, M. L. Oguinn, T. G. Andreadis, and J. A. Blow. 2005. An update on the potential of North American mosquitoes (Diptera: Culicidae) to transmit West Nile virus. *J. Med. Entomol.* 42: 57–62.

Ulbert, S. 2019. West Nile virus vaccines - current situation and future directions. *Hum. Vaccin. Immunother.* 15: 2337–2342.

Valzania, L., M. T. Mattee, M. R. Strand, and M. R. Brown. 2019. Blood feeding activates the vitellogenic stage of oogenesis in the mosquito Aedes aegypti through inhibition of glycogen synthase kinase 3 by the insulin and TOR pathways. *Dev. Biol.* 454: 85–95.

Venkatesan, M., and J. L. Rasgon. 2010. Population genetic data suggest a role for mosquito-mediated dispersal of West Nile virus across the western United States. *Mol. Ecol.* 19: 1573–1584.

Venkatesan, M., K. W. Broman, M. Sellers, and J. L. Rasgon. 2009. An initial linkage map of the West Nile virus vector Culex tarsalis. *Insect. Mol. Biol.* 18: 453–463.

Wong, R. R., N. Abd-Aziz, S. Affendi, and C. L. Poh. 2020. Role of microRNAs in antiviral responses to dengue infection. *J. Biomed. Sci.* 27: 4.

Wu, H., C. Ye, D. Ramirez, and N. Manjunath. 2009. Alternative processing of primary microRNA transcripts by Drosha generates 5' end variation of mature microRNA. *PLoS One.* 4: e7566.

Wyman, S. K., E. C. Knouf, R. K. Parkin, B. R. Fritz, D. W. Lin, L. M. Dennis, M. A. Krouse, P. J. Webster, and M. Tewari. 2011. Post-transcriptional generation of miRNA variants by multiple nucleotidyl transferases contributes to miRNA transcriptome complexity. *Genome Res.* 21: 1450–1461.

Yan, H., Y. Zhou, Y. Liu, Y. Deng, and X. Chen. 2014. miR-252 of the Asian tiger mosquito Aedes albopictus regulates dengue virus replication by suppressing the expression of the dengue virus envelope protein. *J. Med. Virol.* 86: 1428–1436.

Zhang, G., M. Hussain, S. L. O'Neill, and S. Asgari. 2013. Wolbachia uses a host microRNA to regulate transcripts of a methyltransferase, contributing to dengue virus inhibition in Aedes aegypti. *Proc. Natl. Acad. Sci. USA.* 110: 10276–10281.

Zhang, Q., W. Dou, D. Pan, E. H. Chen, J. Z. Niu, G. Smagghe, and J. J. Wang. 2019. Genome-wide analysis of microRNAs in relation to pupariation in oriental fruit fly. *Front. Physiol.* 10: 301.

Zhang, Y., X. Zhou, X. Ge, J. Jiang, M. Li, S. Jia, X. Yang, Y. Kan, X. Miao, G. Zhao, *et al*. 2009. Insect-specific microRNA involved in the development of the silkworm Bombyx mori. *PLoS One.* 4: e4677.

Zhang, Y., B. Zhao, S. Roy, T. T. Saha, V. A. Kokoza, M. Li, and A. S. Raikhel. 2016. microRNA-309 targets the Homeobox gene SIX4 and controls ovarian development in the mosquito Aedes aegypti. *Proc. Natl. Acad. Sci. USA.* 113: E4828–E4836.