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

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**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 (TGAGATTCTCGGGTGCCAAGGG) 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. 2019), 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.](Image)

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.
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.
| Name          | Sequence                          | Length | Ae. aegypti | Cx. quinquefasciatus |
|--------------|-----------------------------------|--------|-------------|----------------------|
| cta-bantam-3p| UGAGAUCAUUUGAAAGCUGA              | 21     | Y           | Y                    |
| cta-bantam-5p| CCGGUGUUCAUUUCGACGUAUCGAC        | 23     | Y           | Y                    |
| cta-let-7-5p | UGAGGUAGUGUUGUAAGUAGU            | 21     | Y           | Y                    |
| cta-miR-10-5p| ACCCGUAGAUCGGGAAUUGUU            | 22     | Y           | Y                    |
| cta-miR-100-5p| ACGCGUAGAUCGGGAAUUGUU          | 22     | Y           | Y                    |
| cta-miR-11-3p| CAUCACAGUGAGUUUUUGCG            | 22     | Y           | Y                    |
| cta-miR-11-5p| CGAGAACUCGGGCGUGACG            | 20     | Y           | Y                    |
| cta-miR-12-3p| UGAGAUAACUACGGGACUGU            | 23     | Y           | Y                    |
| cta-miR-13-3p| UAAUCAGGGAAUAAAGCAGG           | 22     | Y           | Y                    |
| cta-miR-14-3p| UCAGGUAAAUUUCUCCUCUCUAU         | 22     | Y           | Y                    |
| cta-miR-1584-3p| UGAGCGGAAGAUCGAAGG            | 22     | Y           | Y                    |
| cta-miR-1889-3p| CAGUGUACAGAUGGGGCUU            | 22     | Y           | Y                    |
| cta-miR-1889-5p| UAACUGUCAUAGUAAGACG            | 22     | Y           | Y                    |
| cta-miR-1890-3p| UGAAACUCUUGAGUUGACUGC         | 22     | Y           | Y                    |
| cta-miR-190-5p| AGAUAGUUGAUAUUGCUUGUG           | 24     | Y           | Y                    |
| cta-miR-210-3p| CUUGUCGCGUGAGCACACGUCUAU        | 22     | Y           | Y                    |
| cta-miR-232-5p| CUAAGUACUCAGCCCGGACG           | 22     | Y           | Y                    |
| cta-miR-263a-5p| AUUGGCACUGGAAAGAUCAGG         | 22     | Y           | Y                    |
| cta-miR-273-3p| UCAGGUACUCCAGAUGG             | 20     | Y           | Y                    |
| cta-miR-273-5p| UAGGAACUUCUACGCGUUCUC         | 22     | Y           | Y                    |
| cta-miR-277-3p| AGCGGAGUAGAAGAUCUU            | 19     | Y           | Y                    |
| cta-miR-277-5p| UAAACUGCUAUCGUGGACG           | 22     | Y           | Y                    |
| cta-miR-278-3p| UCGGUAGCGACUCUGGCUU          | 22     | Y           | Y                    |
| cta-miR-279-3p| UGACUAGAUCACUCAUAUA            | 22     | Y           | Y                    |
| cta-miR-281-3p| UGUCUAGGAAUUCUGGCUCU          | 21     | Y           | Y                    |
| cta-miR-281-5p| AAGAGAGCUAACGCGGACG           | 22     | Y           | Y                    |
| cta-miR-283-3p| CAUAUCAGCUGGUUAUUGCCG         | 23     | Y           | Y                    |
| cta-miR-285-3p| UAGCACCACUUCGAAACUCAG         | 22     | Y           | Y                    |
| cta-miR-2941-3p| UAGUACGGCUGAAGAUCUCAGG       | 22     | Y           | Y                    |
| cta-miR-2a-3p| UAUACACGGCAAGCUGUAAGGC        | 23     | Y           | Y                    |
| cta-miR-305-3p| CGGCAUUCUGGAGAUGAC           | 20     | Y           | Y                    |
| cta-miR-305-5p| AUUGUACUCAUCAGCGUGGCUCU        | 22     | Y           | Y                    |
| cta-miR-306-5p| UCAGGUACUGAGUACUGUCAG        | 22     | Y           | Y                    |
| cta-miR-308-3p| AAUACAGGAGAAPAUGUC            | 18     | Y           | Y                    |
| cta-miR-31-3p| UGCGAAAGAUGGCAUCUUGCGAUGUA     | 23     | Y           | Y                    |
| cta-miR-315-5p| UUUGAUUGUGUGUGCAAGG           | 22     | Y           | Y                    |
| cta-miR-317-3p| UGCAACAGCUGGUGUUAUCU          | 21     | Y           | Y                    |
| cta-miR-31-5p| UGCAACAGCUGGUGUUAUCU          | 21     | Y           | Y                    |
| cta-miR-33-5p| UGCUAGUUGAAGUUGAUCUCA         | 21     | Y           | Y                    |
| cta-miR-375-3p| UUUGUGGUUGGUGGUUGAACG         | 22     | Y           | Y                    |
| cta-miR-7-5p| UGGAACUAGUAUGAUAUUGUUGG       | 23     | Y           | Y                    |
| cta-miR-71-3p| UUCACUAAUCUGGUAAUCUGA         | 22     | Y           | Y                    |
| cta-miR-71-5p| AGAAAGACAGGGUAGUGAGA         | 22     | Y           | Y                    |
| cta-miR-79-5p| GCUGUUGGGCUCUUGUAGUAGUA        | 23     | Y           | Y                    |
| cta-miR-8-3p| UAAUACUGUACGGGAAAGAGU         | 22     | Y           | Y                    |
| cta-miR-8-5p| UAUCUAACGGGACGCAUAGC         | 22     | Y           | Y                    |
| cta-miR-87-3p| GUGGACAAAUUUCUGAGGUGU         | 22     | Y           | Y                    |
| cta-miR-92a-3p| AUUGCACUUGUGCCCGGCUC         | 19     | Y           | Y                    |
| cta-miR-957-3p| UGAAACGGGCUAAGGACGACG         | 22     | Y           | Y                    |
| cta-miR-965-3p| UAGAAGUAGGCUAAAGAGCAG         | 22     | Y           | Y                    |
| cta-miR-970-3p| UGCAUAAGACAGACGCCGCUAAU      | 21     | Y           | Y                    |
| cta-miR-980-3p| UAAGCGUCUGUAGCGG           | 19     | Y           | Y                    |
| cta-miR-988-3p| CCCCUGUGUGCAACUGACG          | 21     | Y           | Y                    |
| cta-miR-988-5p| UGUGUGCUGUUGUGCAACGGA         | 22     | Y           | Y                    |
| cta-miR-996-3p| UGCAUGAUAUCAGUUGCUGU         | 20     | Y           | Y                    |
| cta-miR-998-3p| UAGCACCACUAGAAGGACCG         | 19     | Y           | Y                    |
| cta-miR-999-3p| UGUAUACUGUAGACUGGUCU         | 22     | Y           | Y                    |
| cta-miR-9c-3p| UAAACUGUAAAGACGACG           | 22     | Y           | Y                    |
| cta-miR-9c-5p| UCUGUUGGUAAUACGCGUGA         | 22     | Y           | Y                    |
| cta-miR-2952-3p| AGAGCGUCAGCAGCGGUGGC        | 23     | N           | Y                    |
| cta-miR-2951-5p| UAGAUGCGGCAUGACUGAGGC        | 22     | N           | Y                    |
| cta-miR-11893-3p| UUCUGACUAUUACGCUACCU       | 22     | Y           | N                    |
| cta-miR-11899-3p| UAGAAGCUGUUAUAAUUGGCU        | 23     | 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 3p and 5p. 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 3p, 5p, and both 5p and 3p of pre-miRNAs. Forty-nine out of the 86 (57%) orthologous mature 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 miRNAs (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) (Nielsen 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 variation we report in the CT cell line hold true in live mosquitoes. More work is needed to determine if the patterns of variation we report in the CT cell line hold true in live mosquitoes.

Table 1. Continued

| Name          | Sequence                          | Length | Ae. aegypti | Cx. quinquefasciatus |
|---------------|-----------------------------------|--------|-------------|----------------------|
| 64 cta-miR-13-5p | CAGUACUUAUGUUAAUGCUUCUCU          | 22     | Y           | N                    |
| 65 cta-miR-275-5p | CGGCGUAAAGCAGAGGCAGAGGC           | 22     | Y           | N                    |
| 66 cta-miR-277-5p | CGUGUCAAGAGCAGCAGAUCUUAC         | 21     | Y           | N                    |
| 67 cta-miR-278-5p | ACCGACGAAUAGCAGAGCAGG             | 23     | Y           | N                    |
| 68 cta-miR-282-3p | ACAUAGCCUGACAGACGGG             | 22     | Y           | N                    |
| 69 cta-miR-286a-3p | UGACUAGACCGAGAAGCAGG             | 25     | Y           | N                    |
| 70 cta-miR-286b-3p | UGACUAGACCGAGAAGCAGG             | 25     | Y           | N                    |
| 71 cta-miR-2940-3p | GCCGACGAAUAGCAGAGCAGG             | 22     | Y           | N                    |
| 72 cta-miR-2940-5p | GCCGACGAAUAGCAGAGCAGG             | 23     | Y           | N                    |
| 73 cta-miR-2944a-5p | GCCGACGAAUAGCAGAGCAGG             | 24     | Y           | N                    |
| 74 cta-miR-2944b-3p | GCCGACGAAUAGCAGAGCAGG             | 22     | Y           | N                    |
| 75 cta-miR-2945-3p | GCCGACGAAUAGCAGAGCAGG             | 22     | Y           | N                    |
| 76 cta-miR-2945-5p | GCCGACGAAUAGCAGAGCAGG             | 22     | Y           | N                    |
| 77 cta-miR-2a-3p | GCCGACGAAUAGCAGAGCAGG             | 23     | Y           | N                    |
| 78 cta-miR-307-3p | GCCGACGAAUAGCAGAGCAGG             | 22     | Y           | N                    |
| 79 cta-miR-308-5p | GCCGACGAAUAGCAGAGCAGG             | 22     | Y           | N                    |
| 80 cta-miR-34-5p | GCCGACGAAUAGCAGAGCAGG             | 22     | Y           | N                    |
| 81 cta-miR-79-3p | GCCGACGAAUAGCAGAGCAGG             | 22     | Y           | N                    |
| 82 cta-miR-92a-5p | GCCGACGAAUAGCAGAGCAGG             | 22     | Y           | N                    |
| 83 cta-miR-92b-3p | GCCGACGAAUAGCAGAGCAGG             | 22     | Y           | N                    |
| 84 cta-miR-92b-5p | GCCGACGAAUAGCAGAGCAGG             | 22     | Y           | N                    |
| 85 cta-miR-92b-5p | GCCGACGAAUAGCAGAGCAGG             | 22     | Y           | N                    |
| 86 cta-miR-9b-5p  | GCCGACGAAUAGCAGAGCAGG             | 24     | Y           | N                    |
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.
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-273 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
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% CO₂ 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 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−ΔΔCT 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.

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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.

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