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Antiviral RNAi Mechanisms to Arboviruses in Mosquitoes: microRNA Profile of *Aedes aegypti* and *Culex quinquefasciatus* from Grenada, West Indies

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Simple Summary: RNA interference (RNAi) is a biological process that can be used as a powerful tool to manipulate mosquito-transmitted viruses. As our knowledge of RNAi in the mosquito increases, so do the targets to interrupt mosquito life cycles and, therefore, their burden on human health. This study provides the miRNA profiles of two major mosquito vectors of arthropod-borne pathogens in Grenada, *Aedes aegypti* (Linnaeus, 1762) (Diptera: Culicidae) and *Culex quinquefasciatus* Say, 1823 (Diptera: Culicidae).

Abstract: Mosquito-borne arboviruses, such as dengue virus, West Nile virus, Zika virus and yellow fever virus, impose a tremendous cost on the health of populations around the world. As a result, much effort has gone into the study of the impact of these viruses on human infections. Comparatively less effort, however, has been made to study the way these viruses interact with mosquitoes themselves. As ingested arboviruses infect their midgut and subsequently other tissue, the mosquito mounts a multifaceted innate immune response. RNA interference, the central intracellular antiviral defense mechanism in mosquitoes and other invertebrates can be induced and modulated through outside triggers (small RNAs) and treatments (transgenesis or viral-vector delivery). Accordingly, modulation of this facet of the mosquito's immune system would thereby suggest a practical strategy for vector control. However, this requires a detailed understanding of mosquitoes' endogenous small RNAs and their effects on the mosquito and viral proliferation. This paper provides an up-to-date overview of the mosquito's immune system along with novel data describing miRNA profiles for *Aedes aegypti* and *Culex quinquefasiatus* in Grenada, West Indies.

Keywords: mosquito immunity; siRNA; miRNA; piRNA; RNA interference; arbovirus; signaling pathways

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1. Introduction

Due to an increasingly globalized economy, arthropod adaptation to expanding urbanization and other obstacles to efficacious mosquito control, the spread of mosquitoes has led to an ever-climbing number of arbovirus infection cases over the past several years (reviewed in [1,2]). Along with an increased amount of overall reported cases of arboviral infections, the emergence and re-emergence of mosquito-associated viruses such as dengue virus (DENV), West Nile virus (WNV), chikungunya virus (CHIKV) and Zika virus (ZIKV) [3] have also increased [4–14]. All known mosquito-borne arboviruses are RNA viruses characterized as single-stranded positive sense (genera *Flavivirus* and *Alphavirus*), single-stranded negative sense (genera *Orthobunyavirus* and *Phlebovirus*) or double-stranded RNA (genus *Seadornavirus* (formerly *Coltivirus*)) [15] (Table 1).

Table 1. Taxonomy of some important mosquito-borne arboviruses of humans.

Order	Families	Major Genera	Examples of Viruses
Bunyavirales	*Peribunyaviridae* *Phenuiviridae*	*Orthobunyavirus* *Phlebovirus*	Bunyamwera, California encephalitis, La Crosse Rift Valley fever
Unassigned	*Flaviviridae*	*Flavivirus*	Dengue, Japanese Encephalitis, St. Louis encephalitis, West Nile, Yellow fever, Zika
Unassigned	*Reoviridae*	*Seadornavirus*	Banna
Unassigned	*Togaviridae*	*Alphavirus*	Chikungunya, Eastern equine encephalitis, Mayaro, O'nyong-nyong, Sindbis, Western equine encephalitis

Arboviral infections are common causes of morbidity and mortality worldwide, but their impact on disease burden is underreported [16]. Limitations in health systems and the lack of appropriate surveillance systems in endemic areas contribute to our incomplete knowledge of arbovirus incidence and related complications [16]. Nevertheless, due to their rapid geographical spread, it is known that the viruses of the Flavivirus genus in the Flaviviridae family contribute the most to mortality on a year-to-year basis. Members of this family include DENV, ZIKV, WNV, yellow fever virus (YFV) and Japanese encephalitis virus (JEV) [17]. The mosquito *Aedes aegypti* is the main vector for the flaviviruses that cause the most mortality and morbidity: YFV, DENV and ZIKV [18]. Of the aforementioned diseases, dengue causes the greatest human disease burden, with an estimated 10,000 deaths and 100 million symptomatic infections per year across over 125 countries [19]. Though typically a disease associated with tropical climates, transmission may occur in other climates as well, particularly in urban settings where case numbers are increasing [20]. *Aedes aegypti* is also a vector for YFV, an arbovirus endemic in tropical areas of Africa and mainland Central and South America. The World Health Organization estimates there are approximately 200,000 cases of yellow fever worldwide each year, resulting in 30,000 deaths. Large epidemics of yellow fever occur when infected people introduce the virus into heavily populated areas with high mosquito density and where most people have little or no immunity due to lack of vaccination [20–22]. Another flavivirus that recently has expanded its geographic distribution is ZIKV. This viral infection is most famously associated with birth defects following the infection of pregnant women and occasionally Guillain–Barre syndrome following the resolution of the initial infection [23].

Culex species are the principal vectors for WNV and JEV [18]. Of particular concern are two closely related species; *Culex pipiens*, considered the most common mosquito in the northern regions of the U.S. (north of 39° N), and *Culex quinquefasciatus*, which is dominant south of 36° N [24,25]. Additionally, *Culex* can be found in both urban and suburban locales as well as temperate and tropical regions across the world [25–28].

Other notable diseases belong to the Alphavirus genus in the Togaviridae family: CHIKV, Sindbis virus (SINV), Semliki Forest virus (SFV) and Ross River virus (RRV), among others [29]. Unfortunately, most arboviral diseases exist without specific treatment or vaccines. Thus, control of the mosquito population and personal protection via chemical and physical repellents remains the primary mode of limiting viral transmission.

1.1. Virus Infection and Immune Responses in the Mosquito

Pathogens can enter mosquitoes through a break in the outer cuticle [30,31], but most enter the mosquito when it feeds on an infected host [32–35]. Once ingested, a virus infects mosquitoes via their midgut epithelial cells and triggers cellular and humoral components of the mosquito's innate immune system to contain the infection [36,37]. It is believed that once a virus replicates and emerges from midgut epithelial cells, the virus subsequently spreads to the hemocoel (the open circulatory system of the mosquito) [38]. Viruses thereafter spread via hemolymph circulation to other tissues, including the salivary glands, where infection and replication must occur prior to transmission to another host during hemotophagy [39]. The dissemination of viruses from the midgut to the salivary glands is not well understood [40]. The eventual transmission of the virus is therefore

reliant on the ability of the virus to travel from the midgut to the salivary glands, along with the ability of the virus to survive immunological barriers along its path.

While mosquitoes lack an adaptive immune response, they have a robust innate immune system comprising interacting aspects of both cellular and humoral defenses [32]. Most of the knowledge on insect antiviral innate immunity was elucidated from studies of the genetic model insect *Drosophila melanogaster* [41], but recently, mosquito-specific research has enhanced our understanding [42–44].

Hemocytes are the main component of the cellular arm of immunity. Hemocytes utilize cell-mediated phagocytosis, melanization, nodulation and lysis [45–48]. Conversely, the humoral response is mediated by mosquito pattern recognition receptors (PRRs) that sense conserved viral structures or pathogen-associated molecular patterns (PAMPs), the first molecular line of pathogen detection [49]. After pathogen recognition, antimicrobial peptides (AMPs), reactive oxygen species (ROS), nitrogen intermediates and components of the phenoloxidase cascade system of melanization carry out an immune response to the said pathogen [32,50,51]. These molecules are secreted into the hemolymph of the mosquito following production in the fat body, the primary site of the humoral response [18]. The transcription of genes that encode for these AMPs is dependent on several signaling cascade pathways: the Janus kinase signal transducer and activator of transcription (JAK-STAT), the Toll pathway and the immune deficiency (Imd) pathways [44]. In addition to the aforementioned, the strongest and most complex antiviral mechanism in the mosquito is the RNA interference pathway (RNAi), explicated at length below [52].

An improved understanding of the relationship between mosquito immune system and arbovirus infection is key to developing new vector-control methods. In recent years, one of the most exciting areas of research pertaining to this relationship has been the viral modulation of RNAi mechanisms. This paper will expound on RNAi mechanisms as well as the ideas behind how they may be controlled in order to make the mosquito less able to host various arboviruses.

1.2. RNA Interference Pathways

RNA interference (RNAi) is a post-transcriptional genetic mechanism that is involved in many physiological and pathological processes across all animals. Specifically, this involves several classes of small RNAs that as act as templates for proteins that identify and modulate the expression of other endogenous and foreign genetic material. Three RNAi pathways have been characterized in insects: small interfering RNAs (siRNAs), microRNAs (miRNAs) and PIWI-interacting RNAs (piRNAs) [53–57]. Small interfering RNAs compose a principal element of the antiviral response in the mosquito [18], while the contributions of piRNA and miRNA to antiviral activity are less clear.

1.2.1. siRNA

The siRNA interference pathway begins when the mosquito's cells sense exogenous dsRNA (e.g., viral genomes or replication intermediates) or endogenous dsRNA created by the host's own double-stranded transcripts [58]. An endonuclease protein called Dicer-2 cleaves long dsRNA into strands of ~21 nucleotides in length [59]. A derivative strand can now bind an argonaute protein (Ago2) where the double helix is split, and a single guide strand is selected to stay attached to Ago2. The combination of the Ago2, RNA and other associated proteins is known as the RNA-induced silencing complex (RISC). Thereby, the siRNA directs RISC to bind to complementary sequences of mRNA. Once bound, RISC completes a target-specific siRNA-mediated degradation of the mRNA [60] (see Figure 1).

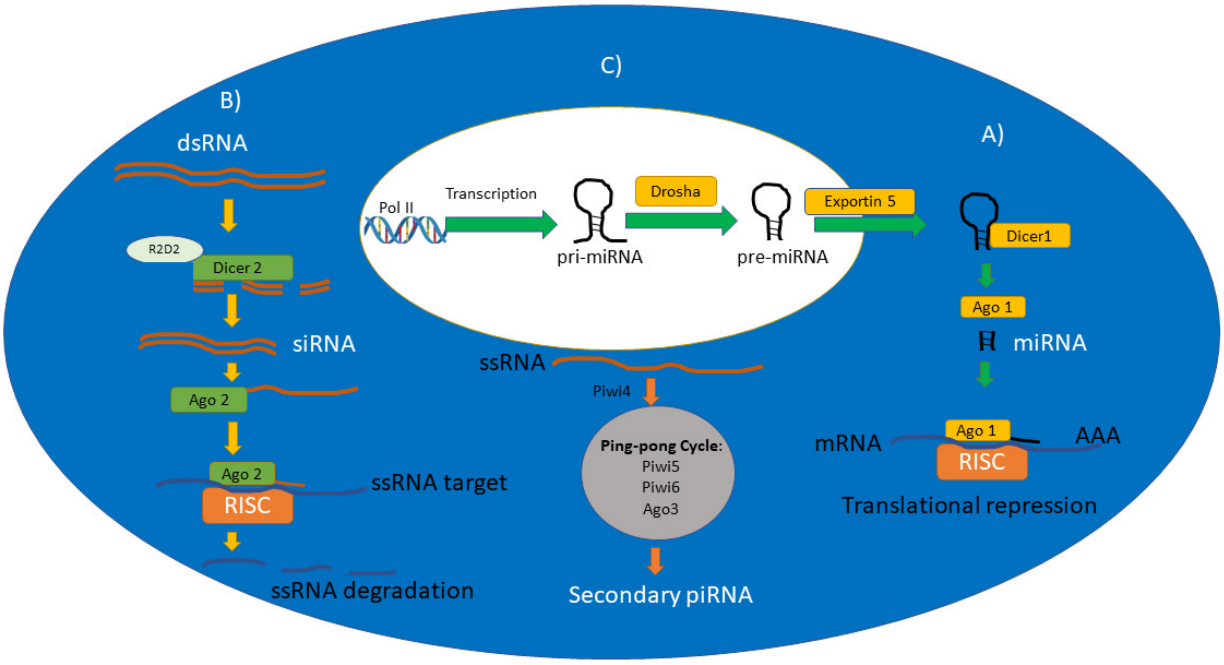

Figure 1. RNAi pathways of mosquitoes. (**A**) (**right**) miRNA pathway: miRNA transcripts are processed by Drosha to pre-miRNAs. After their transport to the cytoplasm by Exportin 5, pre-miRNAs undergo cleavage by Dicer-1 to mature miRNAs. The RISC complexes containing AGO1 regulate gene expression of mRNA targets by transcriptional repression. (**B**) (**left**) siRNA pathway: dsRNA of endogenous or exogenous origin are cleaved by Dicer-2 and its co-factor R2D2 to siRNAs. The RISC complexes containing Ago2 subsequently trigger their destruction. (**C**) (**center**) piRNA pathway: ssRNA precursors from various origins are processed to primary piRNAs by a Dicer-independent mechanism. Piwi4 activate the production of secondary piRNAs by the ping-pong cycle mechanism. The ping-pong cycle is an amplification mechanism to regulate the abundance of transcripts involving Piwi5, Piwi6 and Ago3.

Inhibition at any point in the mechanisms comprising the siRNA pathway results in increased viral replication in mosquitoes [61]. This was also shown in an experiment by Cirimotich et al. [62], wherein a recombinant Sindbis virus (SINV) was fed to *Aedes aegypti* mosquitoes with recombinant alphaviruses expressing a suppressor of RNA silencing significantly decreased the accumulation of virus-derived siRNAs. This led to large increases in virus replication and subsequent mosquito mortality [63].

Conversely, certain viruses have adapted abilities to evade or suppress RNA interference mechanisms. In the Cymbidium ringspot virus infection, the viral P19 protein binds siRNA, thereby impeding siRNA loading into the RISC [64]. Some other viruses code for proteins that directly bind to host cell RNAi machinery. For example, the cricket paralysis virus inhibits RNA silencing through direct interaction with Ago2 [65]. Turnip crinkle virus' P38 protein has been shown to mediate RNAi suppression by binding dsRNAs and siRNA duplexes [66]. The mechanism behind how viruses have evolved to be able to engage with RNAi machinery may have something to do with the high error rates associated with viral RNA-dependent RNA polymerases. These polymerases encode multifunctional proteins. Some of these proteins have functions that require RNA binding, while some may also have incidentally acquired the ability to sequester dsRNA. The latter has been found to be a common pattern associated with virus replication [67]. The best characterized viral RNAi repressors are RNA binding proteins, which can shield viral dsRNA from Dicer processing and subsequent RISC assembly. For example, the yellow fever virus (YFV) capsid protein inhibits RNA silencing by binding to mosquito dsRNAs, thereby interfering with the production of siRNA. This mechanism appears in the C proteins of other flaviviruses

such as ZIKV [68]. Other examples of RISC assembly inhibition include the B2 proteins of nodaviruses and the 1A protein of the Drosophila C virus [69].

In addition to the YFV capsid protein, research is still ongoing to determine whether other arboviruses contain genes or non-coding RNA that suppress RNAi. Research by Soldan et al. [70] and Szemiel et al. [71] on two orthobunyaviruses (La Crosse and Bunyamwera, respectively) suggests that the nonstructural gene NSs, which is typically associated with suppressing the vertebrate antiviral interferon response, may also act as a viral suppressor of RNAi.

Although no vaccines exist for Zika, recent efforts have found the optimal RNAi target region in the ZIKV genome [72,73]. In vitro transcription of dsRNAs from the Zika genome region spanning the NS2B-NS3-NS4A genes and subsequent evaluation of the ability of these dsRNAs to induce an effective siRNA response after injection into *Aedes aegypti* was studied. It was found that there was significant inhibition of replication of the virus in the saliva and lymph of these mosquitoes in comparison to controls [72].

1.2.2. piRNA

P-element induced Wimpy testis gene (PIWI)-interacting RNAs (piRNAs) display a broad size range (25–33 nucleotides in length) [74]. Originating from clusters in the animal genome called piRNA clusters, these clusters give rise to single-stranded RNA transcripts whose main endogenous function is to help silence transposons, thereby maintaining the structural soundness of the animal's germline [75]. Although Dicer is not involved in piRNA's mechanism, there are several ways that piRNA is post-transcriptionally processed. One method is by a ribonuclease called Zucchini (Zuc). Zuc is responsible for processing the 5′ end of piRNAs and, as such, is called Zuc-mediated processing [76]. These piRNAs are then loaded into the slicer protein, Piwi. An alternative mechanism that has been described for the processing of piRNAs is referred to as the ping-pong method, in which piRNAs are loaded into the slicer proteins, Aubergine (Aub) and Ago3. Piwi and Ago3 proteins have an estimated 10 nt of overlapping complementary bases allowing for this mechanism to take place [76,77].

The piRNA pathway has emerged as a highly important antiviral interference pathway in the cellular immune system of dipterans. Indeed, it may be as important as siRNA interference, as there is limited evidence that a piRNA pathway can be enough to mount a defense in the event of a defective siRNA-mediated pathway [78]. Though RNAi pathways are largely conserved in dipterans, some differences exist between mosquitoes and *Drosophila* [67]. The differences and similarities are important to note as much knowledge of the dipteran RNAi mechanisms is modelled from *Drosophila* [79]. *Aedes* and *Culex* mosquitoes, for example, have a larger repertoire of proteins pertinent to the piRNA pathway [67]. In *Aedes aegypti* alone, the Piwi protein family has increased to seven members (Piwi1–Piwi7) [80].

In response to RNA virus infection, piRNAs are produced. These aptly named virus-derived piRNAs (vpiRNAs) are produced mainly via the ping-pong mechanism [81]. Evidence indicates that the production of these vpiRNAs occurs in mosquito cells [67]. Associated proteins have also been demonstrated to be produced in response to infection. For example, in *Aedes aegypti* cell lines infected with SINV, Northern blotting of small RNAs of piRNA associated Ago proteins indicated a specific abundance of virus-derived piRNAs along with Ago3 [82]. It was found that antisense vpiRNAs were preferentially bound by Piwi5, while sense strands were preferentially bound by Ago3. Although a direct antiviral role for piRNA is yet to be demonstrated, there is much evidence of increased vpiRNA production in response to viral infection. For example, the presence of vpiRNAs was detected in *Aedes aegypti* and *Aedes albopictus* during CHIKV infection [83]. Morazzani et al. [83] reported that approximately 1% of the total sequenced small RNAs were derived from the virus in *Aedes aegypti*, while 1.5% of total sequenced small RNAs were of viral origin in *Aedes albopictus*. Whether silencing piRNA-associated proteins leads to increased viral replication within the mosquito is still contested. When it comes to the proteins associated

with the piRNA pathways, one cannot say for certain that targeting any specific protein will ultimately lead to increased viral replication. One case involving the knockdown of Piwi4 in *Aedes aegypti* Aag2 cells showed increased Semliki Forest virus replication [84]. On the other hand, while studies of knockdowns of Piwi5 and Ago3 in Aag2 cells predictably found a profound decline in vpiRNA expression (following Sindbus virus infection), viral replication was not affected [85]. Thus, the question of whether piRNA-clade proteins or piRNAs themselves are suitable targets for controlling viral replication persists.

There is a need for the development of a small RNA (sRNA) library as the question of what kind of RNAi is employed varies from virus to virus in different mosquitoes. For example, a study on WNV-infected *Culex* mosquitoes showed that an overwhelming majority of virus-derived sRNA read were 21 nucleotides in length and thus were siRNAs. However, there was no evidence for the role of WNV-derived piRNAs [86]. A recent study showed how increased vpiRNA presence could occur in DNA viruses so long as the mosquito is also host to the endosymbiotic bacterium, *Wolbachia pipientis* [87]. This same study confirmed increased vpiRNA production when a *Wolbachia* hosting mosquito is transinfected with *Aedes albopictus* densovirus (AalDNV-1).

1.2.3. miRNA

MicroRNAs (miRNAs) are a class of non-coding RNA molecules that contain ~22 nucleotides and are transcribed by cellular RNA polymerase II [80]. The primary transcripts (pri-miRNAs) consist of one or several hairpin-loop structures. This pri-miRNA is then cleaved by an RNase enzyme called Drosha and the RNA binding protein, Pasha, into ~60–70 nt hairpin-shaped intermediates called pre-miRNAs. Transport is then carried out into the cytoplasm by Exportin-5, where Loquacious and Dicer-1 recognize the dsRNA structure and cleave pre-miRNAs into ~22 nt miRNA duplexes [80]. The functional diversity of miRNAs is amplified by the capacity of each miRNA locus to generate two miRNA arms from the 3p or 5p arm of the pre-miRNA, which differ in their seed sequence and target distinct sets of mRNAs [88]. One of these strands of the duplex is then recruited to the Ago1 or Ago2 protein, forming a complex known as miRISC (miRNA-containing RISC) [89]. Kobayashi et al. [90] discovered a ubiquitin ligase that they named Iru, which was found to selectively ubiquitinate empty forms of *Drosophila* Ago1. Consequently, a possible mechanism for an increased susceptibility of the mosquito to succumb to viral infection may be a depletion of Iru. It has been hypothesized that, given that Ago1 is generally more flexible and unstable when empty than in the RNA-loaded form, prolonged emptiness might make Ago more vulnerable to post-translational damage that inhibits function [91]. This would explain the need for proteins such as Iru.

The main function of miRNAs seems to be the regulation of development and physiology [53]. Processing of siRNA takes place almost entirely in the cytoplasm, while miRNA genes are transcribed into pri-miRNA by polymerase II and are processed into pre-miRNA by Drosha in the nucleus [18] (see Figure 1). This spatial difference between siRNA and miRNA processing may be responsible in part for the stronger role of siRNA in the immune response to arboviruses, which replicate in the cytoplasm [18,92]. Nevertheless, miRNAs from several arbovirus mosquito vectors have been shown to modulate host genes that control viral infection [92]. Namely, miRNA complexes specific to viral genes necessary for metabolic processes were seen in ZIKV, DENV, WNV and O'nyong-nyong virus infections [92]. Further studies are required to determine whether modulation of the miRNA pathway during arboviral infection comes as an adaptive response by the host cell made in an attempt to clear the virus or as evidence of the virus taking over the host's cellular processes [93].

In order to assess the role of miRNA in the regulation of gene expression to physiological and immune pathways, miRNA profile studies are needed to identify common and unique expression patterns of miRNA among different species of mosquitoes and under different infection conditions. Here, we present the miRNA profile of wild-caught *Aedes*

aegypti and *Culex quinquefasciatus* mosquitoes from Grenada, West Indies, and explain their potential role in immunity as determined in other studies [94,95].

2. Materials and Methods

2.1. Mosquito Collection and Processing

Three hundred *Aedes aegypti* mosquitoes and 300 *Culex quinquefasciatus* were randomly selected out of 1152 *Aedes aegypti,* and 3000 *Culex quinquefasciatus* were collected between January 2018 and December 2018 from St. George Parish as described in [96]. No specific permissions were required for this study since it was carried out on private lands. The study did not involve endangered or protected species.

2.2. Total RNA Extraction and Microarray Processing

RNA extraction was performed in batches of 30 mosquitoes at a time (10 pools) using TRIzol (ThermoFisher, Carlsbad, CA, USA). Invitrogen™ Phasemaker™ Tubes (ThermoFisher, Carlsbad, CA, USA) were used for the phase separation. RNA was DNase-treated using TURBO DNA-*free*™ (ThermoFisher, Carlsbad, CA, USA), and RNA quality was evaluated utilizing an Agilent 2100 Bioanalyzer (Agilent, Santa Clara, CA, USA) as previously described [97]. All sub-pools were pooled again for microarray processing. Samples were prepared using the FlashTag™ Biotin HSR RNA Labeling kit (ThermoFisher, Carlsbad, CA, USA) for GeneChip™ miRNA Arrays. Poly A tailing and biotin labeling were performed per manufacturer instructions. Hybridization was conducted at 48 °C and 60 rpm for 18 h. The hybridized miRNA 4.0 chips were run 2× on the Affymetrix Microarray Platform (7G scanner, hybridization oven, fluidics station). One limitation of this Microarray approach is that our results are limited to known miRNAs. All data is available in the GEO database under GSE149518. The RNA pools used for this study were previously evaluated for their virome where insect-specific viruses or animal viruses were found, but not human-associated viruses [98].

2.3. Calculation of Probe Set Statistics

Probe-level intensities were calculated using the Robust Multichip Average (RMA) algorithm, including background correction, normalization (quantile) and summarization (median polish), for each probe set and sample (2×), as is implemented in Partek Genomics Suite®, version 7.18 (2009, Partek Inc., St. Louis, MO, USA).

3. Results and Discussion

Metagenomic analysis of the samples shows the absence of arboviruses in both mosquitoes [96]. A total of 69 aae- miRNAs for *Aedes aegypti* and 47 highly-expressed cqu-miRNA for *Culex quinquefasciatus* were obtained. Table 2 show the most highly expressed miRNAs in both mosquitoes.

Table 2. Highly expressed miRNA in both *Aedes aegypti* and *Culex quinquefasciatus*. Values under the titles *Aedes* and *Culex* represent the normalized, background-corrected fluorescence intensities for the probes on the array. Some associations of the miRNA with function and processes in mosquitoes and *Drosophila* are referenced.

miRNA (aae-, cqu-)	*Aedes*	*Culex*	Associations	Organism	Ref.
miR-1	13.46	11.98	*Wolbachia* infection	*Aedes aegypti*	[99]
miR-277-3p	12.87	12.86	Lipid metabolism	*Aedes aegypti*	[100]
miR-989	12.00	11.21	1. Blood-meal associated events 2. Plasmodium infection 3. *Wolbachia* infection 4. WNV infection	1. *Anopheles gambiae* 2. *Anopheles gambiae* 3. *Aedes aegypti* 4. *Culex quinquefasciatus*	1. [88] 2. [101] 3. [99] 4. [102]

Table 2. *Cont.*

miRNA (aae-, cqu-)	*Aedes*	*Culex*	Associations	Organism	Ref.
miR-184	11.99	11.49	1. Conserved. *Wolbachia* infection 2. CHIKV infection	1. *Aedes aegypti* 2. *Aedes aegypti/Aedes albopictus*	1. [103] 2. [104]
miR-281-5p	11.82	11.12	Midgut-specific, enhance DENV-2 replication	*Aedes albopictus*	[105]
miR-8-3p	11.54	9.67	1. Regulate production of myogenic peptide hormone 2. *Wolbachia* infection	1. *Drosophila melanogaster* 2. *Aedes aegypti*	1. [106,107] 2. [99]
miR-34-5p	11.42	12.23	*Plasmodium* infection	*Anopheles gambiae*	[101]
miR-2940-3p	11.36	11.15	*Wolbachia* infection	*Aedes aegypti*	[108]
miR-8-5p	10.79	9.52	Regulate production of myogenic peptide hormone	*Drosophila melanogaster*	[106,107]
miR-275-3p	10.68	10.44	Blood meal events	*Anopheles gambiae*	[88]
miR-100	10.35	9.86	1. CHKV infection 2. *Wolbachia* infection	1. *Aedes albopictus* 2. *Aedes aegypti*	1. [104] 2. [99]
miR-125-5p	10.21	9.29	*Wolbachia* infection	*Aedes aegypti*	[99]
miR-970	10.19	9.62	*Wolbachia* infection	*Aedes aegypti*	[109]
let-7	10.14	8.61			
miR-2941	9.82	8.47	Manipulated by *Wolbachia* during DENV-2 replication	*Aedes aegypti*	[110]
miR-317	9.57	10.32	*Wolbachia* infection	*Aedes aegypti*	[99]
miR-87	9.43	8.36			
miR-276-3p	9.33	10.23	*Wolbachia* infection	*Aedes aegypti*	[99]
miR-71-5p	9.19	8.36			
miR-2c	9.06	7.83	1. DENV infection 2. CHIKV infection	1. *Aedes aegypti* 2. *Aedes aegypti*	1. [111] 2. [104]
miR-252-5p	8.95	9.94	1. DENV infection 2. *Wolbachia* infection	1. *Aedes albopictus* 2. *Aedes aegypti*	1. [112] 2. [99]
miR-263a-5p	8.77	8.37	1. ZIKV infection 2. *Wolbachia* infection	1. *Aedes aegypti* 2. *Aedes aegypti*	1. [113] 2. [99]
miR-2940-5p	8.76	7.21	WNV infection	*Aedes aegypti* *Aedes albopictus*	[110]
miR-2b	8.75	7.34	CHIKV infection	*Aedes aegypti*	[114]
miR-2a-3p	8.59	7.32			
miR-31	8.58	8.71			
miR-11-3p	8.41	6.42			
miR-13-3p	8.26	6.37			
miR-1891	8.03	6.08	Blood meal-associated events	*Aedes albopictus*	[94,115]
miR-988-3p	7.85	6.48	Blood meal-associated events	*Anopheles gambiae*	[116]
miR-1175-5p	7.50	6.27	1. *Wolbachia* infection 2. *Plasmodium* infection	1. *Aedes aegypti* 2. *Anopheles gambiae*	1. [99] 2. [101]
miR-92b-3p	7.07	7.21	1. *Wolbachia* infection 2. WNV infection	1. *Aedes aegypti* 2. *Culex quinquefasciatus*	1. [99] 2. [102]
miR-263b-5p	7.07	7.28	1. DENV-2 infection. 2. Development	1. *Aedes albopictus* 2. *Anopheles sinensis*	1. [117] 2. [118]
miR-92a-3p	6.95	6.49	1. *Wolbachia* infection 2. Pyrethroid resistance 3. Vector–host–pathogen interaction 4. WNV infection	1. *Aedes aegypti* 2. *Culex pipiens pallens* 3. *Anopheles coluzzii* 4. *Culex quinquefasciatus*	1. [99] 2. [119] 3. [120] 4. [102]
miR-10	6.68	6.33	*Wolbachia* infection	*Aedes aegypti*	[99]

As expected, these miRNAs are largely associated with development, growth and metabolism, as suggested by other studies [94,102]. Examples of developmental miRNAs in this study that may be key in the development of control strategies are those associated with blood meal events since they lead to egg development. Of this group, miR-989, miR-275-3, miR-1891 and miR-988-3 expression levels respond to blood meal events. In *Aedes aegypti*, for example, miR-275 ensures successful blood meal digestion, fluid excretion and, consequently, egg development [121].

miRNAs in Table 2 that are most highly expressed among many species include miR-281, miR-184 and miR-989 [94]. The most frequent occurring miRNA in mosquitoes also

present in our study include miR-1, miR-8, miR-10, miR-184, mir-263, miR-275, miR-277, miR-281 and miR-317 [88,94,99,102]. Table 2 show that some of the abovementioned miRNAs are associated with arbovirus infection processes. For example, miR-281-5p, an abundant midgut-specific miRNA, was found to facilitate DENV-2 replication in *Aedes albopictus*. [105]. Additionally, miR-252-5p, which regulates the gene expression of DENV-2 E protein, may act as a cellular antiviral regulator in *Aedes albopictus* [112].

The role of miRNA in the mosquito's defense against arboviruses has not been well studied [122]. One of the first reports to indicate miRNA participation in antiviral mechanisms was by Slonchak et al. [110]. This study showed that the downregulation of the mosquito-specific aae-miR-2940-5p in mosquito cells acts as a potential antiviral mechanism in the mosquito host to inhibit WNV replication. The antiviral activity is a result of repressing the expression of the metalloprotease m41 FtsH gene, which is required for efficient WNV replication. High expression of miR-2940-5p and miR-2940-3p could indicate the absence of WNV in our samples which is confirmed in results previously reported by us [96]. Another study found that miR-2940-5p and miR-2940-3p were significantly downregulated upon DENV-2 infection [123]. DENV-2 was also absent in these samples as previously reported [96]. In addition, miR-2940-5p and miR-2940-3p were reported to decrease in CHIKV-infected *Aedes albopictus* [104]. Once more, the absence of CHIKV was also observed previously in the same samples [96]. Furthermore, miR-2940-5p, which is highly induced in *Wolbachia*-infected *Aedes aegypti*, was previously reported to enhance *Wolbachia* efficient maintenance and limit replication of DENV in *Aedes aegypti* [108]. These results indicate that miR-2940 downregulation may be a good indicator of arboviral infection [124].

There are a few other miRNAs in Table 2 that highlight the relevance of miRNA in the interaction of the host with *Wolbachia*, specifically miR-989, miR-2940-3p, miR-2941, miR-1175-5p and miR-92b-3p. Hussain et al. [109] described how *Wolbachia* manipulates the levels of this miRNA in *Ae. aegypti* mosquitoes in order to guarantee their persistence and survival in mosquito cells. Additionally, the upregulation of miR-2940 in *Wolbachia*-infected cells leads to downregulation of the DNA methyltransferase 2 (*AaDnmt2*) transcript levels, and this results in a reduction in the replication of DENV and an increase in *Wolbachia* replication.

A total of 18 miRNAs had a larger than two-fold difference in expression between *Aedes aegypti* and *Culex quinquefasciatus* (Table 3).

Table 3. miRNA expression and fold difference between *Aedes* and *Culex.* Fold difference calculated as *Aedes* expression/*Culex* expression. Some associations of the miRNA with function and processes in mosquitoes and *Drosophila* are referenced.

miRNA (aae-, cqu-)	*Aedes*	*Culex*	Fold Difference	Associations	Organism	Ref.
miR-1174	0.38	4.53	−11.80	1. Blood-meal associated events 2. *Plasmodium* infection	1. *Aedes aegypti*, *Anopheles gambiae* 2. *Anopheles gambiae*	1. [125] 2. [101]
miR-278-3p	4.08	0.29	13.90	1. Regulates Pyrethroid resistance 2. *Wolbachia* Infection	1. *Culex pipiens pallens* 2. *Aedes aegypti*	1. [126] 2. [99]
miR-2946	9.55	0.85	11.22	Zygote-associated	*Aedes aegypti* *Anopheles stephensi*	[127]
miR-2944b-5p	6.39	0.76	8.37	CHIKV replication	*Aedes aegypti*	[128]
miR-137	5.12	0.84	6.10			
miR-932-5p	5.03	0.90	5.60	1. Regulates Pyrethroid resistance 2. *Wolbachia* infection	1. *Culex pipiens pallen* 2. *Aedes aegypti*	1. [129] 2. [99]
miR-308-5p	6.90	1.30	5.30	1. Zika infection- 2. *Wolbachia* infection	1. *Aedes aegypti* 2. *Aedes aegypti*	1. [113] 2. [99]
miR-957	4.83	0.93	5.20	Courtship	*Drosophila melanogaster*	[130]
miR-281-3p	3.31	0.91	3.63	DENV replication	*Aedes albopictus*	[105]
miR-12-5p	5.75	1.66	3.47			

Table 3. *Cont.*

miRNA (aae-, cqu-)	*Aedes*	*Culex*	Fold Difference	Associations	Organism	Ref.
miR-306-5p	6.17	1.94	3.17	*Wolbachia* infection	*Aedes aegypti*	[99]
miR-2945-5p	2.13	0.78	2.74	DENV-2 infection	*Aedes aegypti*	[93]
miR-13-5p	2.45	0.95	2.57	Bluetongue virus infection	*Aedes albopictus*	[131]
miR-998	5.56	2.23	2.49	Conserved among mosquitoes suggesting vital function	*Anopheles. gambiae* *Aedes aegypti* *Anopheles. stephensi*	[94]
miR-1889-5p	1.98	0.80	2.49	1. DENV-2 infection 2. *Wolbachia* infection	*Aedes. albopictus,* 1. *Aedes albopictus* 2. *Aedes aegypti*	1. [117] 2. [99]
miR-305-5p	6.90	3.07	2.25	1. Aging 2. ZIKV virus infection 3. Blood-meal associated events	1. *Drosophila melanogaster* 2. *Aedes aegypti* 3. *Anopheles gambiae*	1. [132] 2. [113] 3. [88]
miR-285	8.12	3.76	2.16	Regulates Pyrethroid resistance	*Culex pipiens pallen*	[133]
miR-34-3p	4.69	2.18	2.15	1. *Wolbachia* infections 2. *Plasmodium infection*	1. *Aedes aegypti* 2. *Anopheles gambiae*	1. [99] 2. [101]
miR-71-3p	2.23	1.06	2.11	*Wolbachia* infection	*Aedes aegypti*	[99]
miR-932-3p	4.52	2.18	2.08	*Wolbachia* infection	*Aedes aegypti*	[99]
miR-9c-3p	5.27	2.56	2.06	Phagosome	*Drosophila melanogaster*	[134]

Of the miRNAs listed in Table 3, miR-1174 is the only miRNA expressed in higher amounts in *Culex quinquefasciatus* compared to *Aedes aegypti*. miR-1174 has been found significantly upregulate post-blood meal and is specific to the female mosquito midgut in *Aedes aegypti* and *Anopheles gambiae*, suggesting a role in blood-meal-associated events. Studies have found that miR-1174 targets serine hydroxymethyltransferase, and its inhibition disrupts sugar absorption, fluid excretion, blood intake in the gut and, consequently, egg maturation and survival [125].

Table 3 also include some differentially expressed miRNA associated with arbovirus infection including miR-2944b-5p, miR-308-5p, miR-281-3p, miR-2945-5p, miR-1889-5p, miR-305-5p and miR-34-3p. For example, miR-2944b-5p affects CHIKV replication. Loss-of-function studies of miR-2944b-5p using antagomirs, both in vitro and in vivo, reveal an increase in CHIKV viral replication [128].

Among the differentially expressed miRNAs in these two common mosquitoes in Grenada, some are associated with insecticide resistance, including miR-278-3p, miR-932-5p and miR-285. For example, the conserved miR-278-3p and a target gene it modulates (*CYP6AG11*) have been critical for pyrethroid resistance in *Culex pipiens pallens* [126]. Overexpression of miR-278-3p through microinjection also led to a significant reduction in the survival rate of the mosquito. Future research by this research group intends to look for markers of resistance to pyrethroids and other insecticides in future studies.

miRNAs found in this study that have been previously reported to be associated with *Wolbachia* infection include miR-278-3p, miR-932-5p, miR-308-5p, miR-306-5p, miR-1889-5p, miR-34-3p, miR-71-3p and 932-3p (Table 3). Whether this differential expression is a signature of present *Wolbachia* infection and a potential mechanism of *Wolbachia* maintenance in the mosquito needs to be explored.

An interesting observation arrives from the miRNAs that are shared between those associated with pyrethroid resistance and those involved in *Wolbachia* infection. For example, low levels of miR-278-3p and miR-932-5p were found in pyrethroid-resistant mosquitoes in some studies. At the same time, low levels of the same miRNAs are found in mosquitoes with no *Wolbachia* infection compared to those infected with *Wolbachia* [126,129]. Here, we observed that in the mosquito population that naturally exhibits *Wolbachia* infection in Grenada (*Culex quinquefasciatus*) [98], the levels of miR-278-3p are 13.0 times higher than that of the *Aedes aegypti* population, which is not infected with *Wolbachia* in Grenada [98]. Similarly, miR-935-5p expression in *Culex* is 5.6 times higher than in *Aedes*.

The role of miRNAs in host-pathogen interactions, regardless of the pathogen, is clear from all the studies cited; however, the targets of many of them in mosquitoes need to be determined.

4. Conclusions

From simple mosquito nets and sprays to more complex genetically modified mosquitoes, effective strategies for vector control are paramount in preventing vector-borne diseases. Transgenic introduction of antiviral RNAs into mosquito genomes has already proven successful in engineering resistance to arboviruses [95,135,136], and yet questions still remain that preclude widespread release of these mosquitoes. What are the ecological implications? Are the costs of implementing such control measures prohibitive? Additionally, we know that RNAi is a main component in the mosquito immune system. There are studies demonstrating that RNAi pathways can effectively modulate the viral load in some vectors. The delivery of exogenous small RNAs to wild mosquitoes poses challenges and unknown off-target effects, and hence, the modulation of the mosquito's endogenous RNAi by other means may give us a more controlled and cost-effective solution to vector control. Many more studies are required to establish the basis of such a solution. We first need to determine profiles of small RNAs in local wild populations, determine the function of some of these molecules and finally create or modify existing transgenic methods to be able to manipulate their expression. For example, arboviruses establish persistent infections and trigger RNAi responses in mosquitoes, but their use for silencing vectors is not practical since they cause disease in vertebrate hosts [53]. Perhaps the bevy of mosquito-specific viruses offers viable alternatives for virus-mediated transgenesis. Additionally, symbionts such as *Wolbachia* have demonstrated the capacity to manipulate RNAi in some mosquitoes [94,99,124]. Here, we summarized how RNAi pathways in the immune systems of mosquitoes work and established some potential targets for vector control in *Aedes aegypti* and *Culex quinquefasciatus* by describing the miRNA profiles of these mosquitoes collected in Grenada, West Indies.

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