Transcriptional profile of *Aedes aegypti* Leucine-Rich Repeat Proteins in response to Zika and Chikungunya viruses

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**Abstract:** *Aedes aegypti* (L.) is the primary vector of chikungunya, dengue, yellow fever and Zika viruses. The leucine-rich repeats (LRR)-containing domain is evolutionarily conserved in many proteins associated with innate immunity in invertebrates and vertebrates, as well as plants. We focused on the *AaeLRIM1* and *AaeAPL1* gene expressions in response to Zika virus (ZIKV) and Chikungunya virus (CHIKV) infection using a time course study, as well as the developmental expressions in the eggs, larvae, pupae, and adults. RNA-seq analysis data provided 60 leucine-rich repeat related transcriptions in *Ae. aegypti* in response to Zika virus (Accession number: GSE118858, https://www.ncbi.nlm.nih.gov/gds/?term=GSE118858). RNA-seq analysis data showed that *AaeLRIM1* (AAEL012086-RA) and *AaeAPL1* (AAEL009520-RA) were significantly upregulated 2.5 and 3-fold during infection by ZIKV 7-days post infection (dpi) of an *Ae. aegypti* Key West strain compared to an Orlando strain. The qPCR data showed that LRR-containing proteins *AaeLRIM1*, *AaeAPL1* and five paralogues were expressed 100-fold lower than other nuclear genes, such as defensin, during all developmental stages examined. Together, these data provide insights into transcription profiles of LRR proteins of *Ae. aegypti* during its development and in response to infection with emergent arboviruses.

**Keywords:** *Aedes aegypti*, Leucine-Rich Repeat Proteins, Zika virus, Chikungunya virus, immune responses, gene expression

1. Introduction

The leucine-rich repeats (LRR)-containing domain is noted to be evolutionarily conserved among many proteins correlated with innate immunity in an array of organisms including invertebrates, vertebrates, and plants [1]. Innate immunity is a conserved host response that serves as the host's first line of defense by sensing pathogen-associated molecular patterns through germline-encoded pattern recognition receptors [2]. Additionally, research generated by Aloor et al. (2018) indicates that LRR and calponin homology containing 4 (Lrch4) conform to the horseshoe-shaped structure typical of LRRs in pathogen-recognition receptors, and that the best structural match in the protein database is to the variable lymphocyte receptor of the jawless vertebrate hagfish.
Several human studies have assisted in identifying the function of Nucleotide-binding-site-(NBS)-LRR proteins which have also been utilized in plants for pathogen detection of pathogen products such as avirulence genes [4, 5]. Polymorphisms in the Nucleotide-binding oligomerization domain (NOD), LRP and Pyrin domain containing 12 (NLRP12), are associated with depression and coronary artery disease in trauma-exposed humans [6]. NLRP6, a member of the nucleotide-binding domain and LRR-containing (NLR) innate immune receptor family, participates in the progression of intestinal inflammation and enteric pathogen infections. This is important in disease pathogenesis because it responds to internal ligands that lead to the release of AMPs and mucus, thus regulating the regeneration of intestinal epithelial cells [7]. Toll-like receptor (TLRs) and NOD-like receptors, through their LRR domain, sense pathogens in mammals through pattern-recognition receptors responsible for activating antimicrobial defenses and stimulating the adaptive immune response [8].

Mutations in the genes encoding LRR-containing proteins - which impact protein folding, aggregation, oligomerization, stability, protein-ligand interactions, disulfide bond formation, and glycosylation - are associated with over sixty human diseases including high myopia, mitochondrial encephalomyopathy, Parkinson’s disease and Crohn’s disease [9, 10].

Mosquitoes lack an adaptive immune system [11] and so they are fully dependent on mounting an innate immune response to fight infection [12-15]. Research indicates that mosquito vectors and mosquito cell lines produce both humoral and cellular immune responses against invading pathogens [16, 17]. Leucine-rich repeat immune proteins (LRIMs) are a mosquito-specific family of putative innate receptors [18-20]. Two LRR proteins, LRIM1 (leucine-rich repeat immune protein 1) and APL1 (Anopheles Plasmodium-responsive leucine-rich repeat 1), have been recognized as major mosquito factors that regulate parasite infection and parasite loads [21]. In Anopheles mosquitoes, LRIM1, APL1C, and LRR-containing proteins activate complement-like defense responses against malaria by forming a disulphide-bridge that interacts with thioester-containing protein 1 (TEP1), a complement C3-like protein [22, 23]. APL1 of Anopheles gambiae is a family of variable LRR proteins required for cell-mediated protection as shown using a rodent model malaria parasite, Plasmodium berghei [24]. The An. gambiae APL1 genomic locus circumscribes three distinct genes (APL1A, APL1B and APL1C), however, only the product of APL1C acts as a P. berghei antagonist [24]. LRIM1 and APL1C both play a significant role in the anti-Plasmodium response. Accordingly, silencing of these genes results in an altered response against Plasmodium infection [20].

A genome-wide study found that variations or polymorphisms in the LRIM1 and APL1C proteins were correlated with resistance and susceptibility to Plasmodium infection [25]. Additional studies offered mechanisms for controlling Plasmodium early during the infection process by targeting the ookinete or oocyst stages of oocyst [26, 27]. Nonetheless, no detailed information has been generated for pathogen response by the LRR-containing proteins of immune factors in Aedes mosquitoes.

Aedes aegypti (L.) is a vector for transmitting emergent arboviruses including chikungunya, dengue, yellow fever and Zika viruses. Chikungunya fever is primarily transmitted to humans through mosquito vectors, Ae. aegypti and Ae. albopictus. It is a viral disease belonging to the family Togaviridae, and genus Alphavirus. Recent outbreaks of chikungunya fever occurred from 2004-2006 spanning Kenya in 2004 (Eastern/Central/Southern African, ECSA, CHIKV lineage) and the island of La Réunion in 2005-2006 (Indian Ocean CHIKV lineage). It later emerged in the New World in 2013 on St. Martin Island (Asian CHIKV lineage), eventually spreading throughout the Americas [28-31].
Over the span of the past 12 years, it is estimated that more than four million human cases of chikungunya infection have occurred worldwide [32]. Symptoms of infection include a rash, fever, headache, joint pain, and muscle pain [33] along with the chance of developing chronic musculoskeletal diseases [34]. There is currently no vaccine available for CHIKV. Accordingly, controlling the mosquito vectors is the primary method utilized to reduce the risk of disease transmission.

Zika virus (ZIKV) was first discovered in 1947 and belongs to the family Flaviviridae, genus *Flavivirus* (CDC 2016) [35]. Spreading to the Oceania region, ZIKV caused outbreaks on Yap Island in Micronesia and French Polynesia in 2007 and 2013 respectively. In 2015, ZIKV was found to have reached Brazil spreading throughout the Americas [35, 36]. It is estimated that 1.5 million people have been infected by ZIKV in Brazil [37]. ZIKV continues to spread to new areas. Transmission in the U.S. is a major public health risk, notably for the Gulf states such as Florida and Texas where ecological conditions are favorable for the primary vector *Ae. aegypti*, as well as an increasing likelihood for virus introduction by imported cases. Manifestations of ZIKV take on different forms, the most serious of which include birth defects in humans [38] along with neurological complications that may result in Guillain-Barré syndrome, both of which are significant public health threats [39].

Mosquitoes respond to infection using an array of molecular signaling pathways and immune effector proteins. A focus on the immune system response of *Ae. aegypti* has unveiled a transcriptome analysis of genome-wide mechanisms that are implicated in defense against arbovirus infections [40-43]. No sequence-structure-function relationships of mosquito leucine-rich repeat immune proteins in *Ae. aegypti* in response to arboviruses are available, though the LRR-proteins have been compared with *An. gambiae* and *Culex quinquefasciatus* [23]. Data from *Ae. aegypti* was provided by RNA-seq analysis giving 60 leucine-rich repeat related transcriptions in response to ZIKV (Accession number: GSE118858, https://www.ncbi.nlm.nih.gov/gds/?term=GSE118858) [41]. We examined the *AaeLRIM1* and *AaeAPL1* gene expressions in response to both ZIKV and CHIKV infection using a time course study. Additionally, we investigated the developmental expressions of these genes in the eggs, larvae, pupae and adults. The current study aims to improve our understanding of the transcription profiles of *Ae. aegypti* LRR proteins during development and in response to arbovirus infection.

2. Results

2.1.1 Leucine-Rich Repeat Proteins *AaeAPL1* paralogues of *Aedes aegypti*

Evolutionary analysis of 8 paralogues of *AeaAPL1* of the *Ae. Aegypti* were conducted in MEGA7 [44] (Figure 1). The data showed that *AaeAPL1* was close related to *AaeLRIM4*. The DNA sequences producing significant alignments between *AaeAPL1* and *AaeLRIM4* was 44% identity.
Figure 1. The evolutionary history was inferred using the Neighbor-Joining method [45]. The optimal tree with the sum of branch length = 4.948 is shown. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method [46] and are in the units of the number of base substitutions per site. The analysis involved 9 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 1179 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 [44].

2.1.2 Developmental regulation of AaeLRIM1 and AaeAPL1

To understand how AaeLRIM1 and AaeAPL1 are regulated during the development of Ae. aegypti, qPCR was performed to examine relative transcription levels of AaeLRIM1 and AaeAPL1 in eggs, larvae, pupae, and male and female adults (Figure 2). In addition, we also examined 5 paralogues of AeaAPL1, i.e., AaeLRIM3, AaeLRIM4, AaeLRIM5, AaeLRIM15, AaeLRIM16, and AaeLRIM17 during developmental stages using qPCR (Figures S1A-E).
Figure 2. AaeLRIM1 and AaeAPL1 relative expression in the all developmental stages, from eggs (Eg), larvae (Lv), pupae (Pu), and adults Ae. aegypti, including male (M1, male 1-d-old; M3, male 3-d-old; M5, male 5-d-old; M7, male 7-d-old; and M10, male 10-d-old) and female (F1, female 1-d-old; F3, female 3-d-old; F5, female 5-d-old; F7, female 7-d-old; and F10, female 10-d-old) in the Key West strain (KW) and Orlando (OR) strain of Ae. aegypti. (A) AaeLRIM1; (B) AaeAPL1.

1. AaeLRIM1 and AaeAPL1 RNA Profile in Immature Stages of Ae. aegypti

MANOVA showed significant effects of mosquito strain, developmental stage, and interaction of these factors (Table 1A). For the mosquito strain effect, Key West had higher AaeLRIM1 than Orlando Ae. aegypti (Figure 2). Standardized canonical coefficients showed that AaeLRIM1 and AaeAPL1 contributed similarily, but in opposite directions (Table 1A). In contrast, Orlando had higher AaeAPL1 than Key West Ae. aegypti (Figure 2). Standardized canonical coefficients showed that AaeLRIM1 contributed twice as much as AaeAPL1 for the significant developmental stage effect (Figure 2). For the developmental stage effect, LRIM1 was significantly different between developmental stages with increases associated between each stage (Figure 2). Gene expression of AaeAPL1 was significantly higher for the larval and pupal stages compared to the egg stage. However, AaeAPL1 was lower among pupae than larvae (Figure 2). For the significant interaction, we compared less than all possible treatment groups by which developmental stage was...
compared within a given strain (e.g., Key West eggs vs. Key West larvae). Standardized canonical coefficients showed similar contribution of AaeLRIM1 and AaeAPL1 to the significant interaction, in opposite directions (Table 1A). Gene expression of AaeLRIM1 was higher for Key West than Orlando Ae. aegypti and occurred over a greater range. We observed significant increases in gene expression of AaeLRIM1 for each developmental stage for both Key West and Orlando Ae. aegypti (Figure 2). In contrast, gene expression of AaeAPL1 was higher for Orlando than Key West Ae. aegypti and occurred over a greater range. Gene expression of AaeAPL1 significantly increased for each developmental stage for both Key West and Orlando Ae. aegypti (Figure 2).

**Table 1A.** MANOVA results for strain and stage effects of AaeLRIM1 and AaeAPL1 RNA profile in immature stages of Ae. aegypti.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Pillai’s Trace</th>
<th>df (numerator and denominator)</th>
<th>p-value</th>
<th>Standardized Canonical Coefficients</th>
<th>AaeLRIM1</th>
<th>AaeAPL1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Strain</td>
<td>0.99</td>
<td>2, 11</td>
<td>&lt;0.0001</td>
<td>12.12</td>
<td>-10.76</td>
<td></td>
</tr>
<tr>
<td>Stage</td>
<td>1.99</td>
<td>4, 24</td>
<td>&lt;0.0001</td>
<td>16.28</td>
<td>8.35</td>
<td></td>
</tr>
<tr>
<td>Strain x Stage</td>
<td>1.89</td>
<td>4, 24</td>
<td>&lt;0.0001</td>
<td>11.09</td>
<td>-11.57</td>
<td></td>
</tr>
</tbody>
</table>

2. AaeLRIM1 and AaeAPL1 RNA Profile in Adults (Male and Female) of Ae. aegypti

MANOVA showed significant main effects of mosquito strain, sex, and age, as well as the two-way interaction and three-way interaction (strain x sex x age). Standardized canonical coefficients showed that AaeLRIM1 contributed much more to all significant effects than AaeAPL1 (Table 1B). Because the three-way interaction was significant, we focused on pairwise comparisons of treatment groups for this effect. Specifically, we compared mosquito strains of a given sex and age (e.g., Key West, female, 1-day old vs. Orlando, female, 1-day old). For expression of AaeLRIM1, Key West was higher for 1-day 3-day, and 10-day old mosquitoes than Orlando female Ae. aegypti. In contrast, AaeLRIM1 was higher for 5-day and 7-day Orlando than Key West female Ae. aegypti. Gene expression for AaeLRIM1 was higher for all ages of Key West male Ae. aegypti except for 1-day old males. Lowest rates of expression were observed for 1-day old male Ae. aegypti for both strains and highest rates were observed for intermediate aged mosquitoes.

For gene expression of AaeAPL1, Orlando was higher for all ages than Key West female Ae. aegypti. Rates of expression were highest for 7-day old female mosquitoes and expression was lower among older females (10-day old) for both mosquito strains. For gene expression of AaeAPL1, Orlando was higher for all ages than Key West male Ae. aegypti, except 5-day old males. Rates of expression were highest for 5-day old and 3-day old male mosquitoes for Key West and Orlando, Ae. aegypti, respectively. Gene expression was low for young and old male mosquitoes (Figure 2).

**Table 1B.** MANOVA results for strain, sex and age effects of AaeLRIM1 and AaeAPL1 RNA profile in adult stages of Ae. aegypti.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Pillai’s Trace</th>
<th>df (numerator and denominator)</th>
<th>p-value</th>
<th>Standardized Canonical Coefficients</th>
<th>AaeLRIM1</th>
<th>AaeAPL1</th>
</tr>
</thead>
</table>

doi:10.20944/preprints201901.0013.v1
2.1.3 Infection in Ae. aegypti Exposed to CHIKV and ZIKV

To understand the molecular interactions of the arbovirus with permethrin resistant Ae. aegypti from Florida, qPCR was conducted to discover the gene expression in the Ae. aegypti (Key West and Orlando strains) in response to oral ingestion of ZIKV or CHIKV infected blood and ZIKV/CHIKV infection. In this study, four-day-old female Ae. aegypti adults were fed a blood meal containing 6.4 log10 pfu/mL of ZIKV (Table 2A). Fresh fed mosquitoes ingested 4.2 to 4.3 log10 pfu/mL of ZIKV. By 3 days post infection (dpi), ZIKV titer in mosquito bodies were 3.96±0.26 log10 pfu/mL and 3.66±0.27 log10 pfu/mL for the permethrin resistant and susceptible strains of Ae. aegypti, respectively. By 7 days post infection (dpi), ZIKV titer in mosquito bodies were 4.11±1.78 log10 pfu/mL and 3.58±1.23 log10 pfu/mL for the permethrin resistant and susceptible strains of Ae. aegypti, respectively. A two-tailed t-test showed no significant differences in ZIKV titer in the bodies of the two strains of Ae. aegypti (t17 = 0.77, p = 0.44). By 10 dpi, ZIKV titer in permethrin resistant strain mosquito bodies were 6.57±0.05 log10 pfu/mL, which was 100-fold higher (t4 = 8.12, p = 0.001) than the titer of the susceptible strain (4.49±0.34 log10 pfu/mL). This result demonstrated that the ZIKV replication rates were higher at this point in the infection process for the permethrin resistant strain than the susceptible strain.

Four-day-old female Ae. aegypti adults were fed a blood meal containing 8.0±0.09, 8.3±0.08 log10 pfu/mL of CHIKV (Table 2B). Fresh fed mosquitoes ingested 4.56±1.16 to 5.23±0.10 log10 pfu/mL of CHIKV. By 3 days post infection (dpi), CHIKV titer in mosquito bodies were 5.04±0.71 log10 pfu/mL and 4.7±0.81 log10 pfu/mL for the permethrin resistant and susceptible strains of Ae. aegypti, respectively. By 7 days post infection (dpi), ZIKV titer in mosquito bodies were 5.4±0.46 log10 pfu/mL and 5.8±0.48 log10 pfu/mL for the permethrin resistant and susceptible strains of Ae. aegypti, respectively. A two-tailed t-test showed no significant differences in ZIKV titer in the bodies of the two strains of Ae. aegypti. By 10 dpi, ZIKV titer in permethrin resistant strain mosquito bodies were 5.53±0.33 log10 pfu/mL, and the titer of the susceptible strain (5.20±0.49 log10 pfu/mL). This result demonstrated that the CHIKV replication rates were similar at this point in the infection process for the permethrin resistant strain and the susceptible strain.

Table 2A. Zika virus titers (log10 pfu/ml) in infectious blood meals and mosquitoes for Key West and Orlando strains of Aedes aegypti.

<table>
<thead>
<tr>
<th>Strains</th>
<th>Initial dose in bloodmeal</th>
<th>Freshly fed</th>
<th>3 days post infection</th>
<th>7 days post infection</th>
<th>10 days post infection</th>
</tr>
</thead>
<tbody>
<tr>
<td>Key West</td>
<td>6.4±0.09</td>
<td>4.30±0.0</td>
<td>3.96±0.26</td>
<td>4.11±1.78</td>
<td>6.57±0.05</td>
</tr>
<tr>
<td>Orlando</td>
<td>6.4±0.08</td>
<td>4.17±0.39</td>
<td>3.66±0.27</td>
<td>3.58±1.23</td>
<td>4.49±0.34</td>
</tr>
</tbody>
</table>
Zika virus (strain PRVABC59, GenBank accession # KU501215.1) isolated from a human infected in Puerto Rico in 2015.

Table 2B. Chikungunya virus titers (log10 pfu/ml) in infectious blood meals and mosquitoes for Key West and Orlando strains of *Aedes aegypti*.

<table>
<thead>
<tr>
<th>Strains</th>
<th>Initial dose in bloodmeal</th>
<th>Freshly fed</th>
<th>3 days post infection</th>
<th>7 days post infection</th>
<th>10 days post infection</th>
</tr>
</thead>
<tbody>
<tr>
<td>Key West</td>
<td>8.0±0.09</td>
<td>5.23±0.10</td>
<td>4.7±0.81</td>
<td>5.4±0.46</td>
<td>5.20±0.49</td>
</tr>
<tr>
<td>Orlando</td>
<td>8.3±0.08</td>
<td>4.56±1.16</td>
<td>5.04±0.71</td>
<td>5.81±0.48</td>
<td>5.53±0.33</td>
</tr>
</tbody>
</table>

CHIKV (LaReunion strain LR2006-OPY1, GenBank KT449801) from a human infected on La Réunion Island in 2006 (Parola et al. 2006).

2.1.4 Leucine-Rich Repeat Proteins Changes in Transcriptome of the *Aedes Aegypti* Female Adult in Response to ZIKV Infection

To better our understanding of molecular interactions and the immune response of *Ae. aegypti* from Florida with arbovirus, we re-examined RNA-seq data to explore the changes in Leucine-Rich Repeat Proteins in the *Ae. aegypti* (Key West and Orlando strains) transcriptome in response to oral ingestion of ZIKV infected blood and ZIKV infection (Accession number: GSE118858, [https://www.ncbi.nlm.nih.gov/gds/?term=GSE118858](https://www.ncbi.nlm.nih.gov/gds/?term=GSE118858)). Data were provided by RNA-seq analysis that generated 60 leucine-rich repeat related transcriptions in the *Ae. aegypti* genome in response to Zika virus (Table S1A-S1D, Table 3A-3D). Specifically, female *Ae. aegypti* transcriptomic RNA-seq data showed that 23 genes related to Leucine-Rich Repeat Proteins (LRRP) were significantly upregulated during infection by ZIKA in 7-days post infection (dpi) *Ae. aegypti* Key West strains compared with Orlando strains. Additionally, seventeen of these genes between the two strains were upregulated more than 2-fold (p-adj ≤ 0.01; log2 fold change > ±2.0) in response to ZIKV 7 dpi (*Table 3A*). *AaelLRIM1* (AAEL012086-RA) and *AaeAPL1* (AAEL009520-RA) were significantly upregulated 2.5 and 3-fold (*Table 3A*). When comparing transcriptome profiles of two *Ae. aegypti* strains in response to the control (blood-feeding only), only three genes related to Leucine-Rich Repeat Proteins were significantly upregulated/downregulated in 7-days post infection *Ae. aegypti* in Key West strains compared with Orlando strains (*Table 3B*). *AaelLRIM1* (AAEL010286-RA, p-adj 5.0 x 10^-9, log2 fold change -3.3918) was significantly down-regulated (*Table 3C*). Analysis and comparison of mRNA expression profiles of *Ae. aegypti* Orlando strains following ZIKV infection indicated five LRRP related genes, including *AaeAPL1* (AAEL009520-RA, p-adj 2.8 x 10^-4, log2 fold change -2.2074), were significantly dysregulated (downregulated) 7-days post infection (*Table 3D*).

Table 3A. Female *Aedes aegypti* transcriptomic RNA-seq data show Leucine-Rich Repeat Proteins related genes significantly upregulated in the Zika infection in Key West strain compared with Orlando strain *Aedes aegypti* 7-days post infection

<table>
<thead>
<tr>
<th>Transcript ID</th>
<th>Log2FC</th>
<th>p-adj</th>
<th>Gene description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AAEL001401-RA</td>
<td>3.2722</td>
<td>2.3 x 10^-27</td>
<td>leucine-rich immune protein (Short)</td>
</tr>
<tr>
<td>AAEL001402-RA</td>
<td>3.2947</td>
<td>3.3 x 10^-43</td>
<td>leucine-rich immune protein (Short)</td>
</tr>
</tbody>
</table>
Table 3B. Female *Aedes aegypti* transcriptomic RNA-seq data show Leucine-Rich Repeat Proteins related genes significantly upregulated/downregulated in the Control in Key West strain compared with Orlando strain *Aedes aegypti* 7-days post injection.

<table>
<thead>
<tr>
<th>Transcript ID</th>
<th>Log2FC</th>
<th>p-adj</th>
<th>Gene description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AAEL000243-RA</td>
<td>5.2443</td>
<td>1.8 x 10^{-26}</td>
<td>leucine-rich transmembrane protein</td>
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<tr>
<td>AAEL009894-RA</td>
<td>-0.9167</td>
<td>2.9 x 10^{-4}</td>
<td>leucine-rich immune protein (Coil-less)</td>
</tr>
<tr>
<td>AAEL010286-RA</td>
<td>3.0287</td>
<td>1.5 x 10^{-5}</td>
<td>leucine-rich transmembrane protein</td>
</tr>
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</table>

Table 3C. Female *Aedes aegypti* transcriptomic RNA-seq data show Leucine-Rich Repeat Proteins related genes significantly dysregulated in the Key West strain *Aedes aegypti* 7-days post infection with ZIKV compared with Control in Key West strain.

<table>
<thead>
<tr>
<th>Transcript ID</th>
<th>Log2FC</th>
<th>p-adj</th>
<th>Gene description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AAEL000243-RA</td>
<td>-6.6469</td>
<td>7.6 x 10^{-44}</td>
<td>leucine-rich transmembrane protein</td>
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<tr>
<td>AAEL003408-RA</td>
<td>2.1477</td>
<td>1.8 x 10^{-4}</td>
<td>leucine-rich transmembrane protein</td>
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<tr>
<td>AAEL009894-RA</td>
<td>0.8665</td>
<td>9.7 x 10^{-8}</td>
<td>leucine-rich immune protein (Coil-less)</td>
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<tr>
<td>AAEL010286-RA</td>
<td>-3.3918</td>
<td>5.0 x 10^{-8}</td>
<td>leucine-rich transmembrane protein</td>
</tr>
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</table>

Table 3D. Female *Aedes aegypti* transcriptomic RNA-seq data show Leucine-Rich Repeat Proteins related genes significantly dysregulated in the Orlando strain *Aedes aegypti* 7-days post infection with ZIKV compared with Control in Orlando strain.

<table>
<thead>
<tr>
<th>Transcript ID</th>
<th>Log2FC</th>
<th>p-adj</th>
<th>Gene description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AAEL009520-RA</td>
<td>-2.2074</td>
<td>2.8 x 10^{-4}</td>
<td>leucine-rich immune protein (Long)</td>
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<tr>
<td>AAEL010128-RA</td>
<td>-5.3692</td>
<td>2.8 x 10^{-6}</td>
<td>leucine-rich immune protein (Long)</td>
</tr>
</tbody>
</table>
2.1.5 AaeLRIM1 and AaeAPL1 Transcriptional Induction of ZIKV Infections in Orally Infected Ae. aegypti Females

To characterize AaeLRIM1 and AaeAPL1 expression in response to ZIKV exposure, we measured AaeLRIM1 and AaeAPL1 expressions in orally infected Ae. aegypti. MANOVA showed the significant effects of strain of Ae. aegypti, time, and their interaction (Table 4A). For the strain effect, SCCs showed that AaeLRIM1 contributed more to the significant effect than AaeAPL1 (Table 4A). Gene expression of AaeLRIM1 was significantly higher for Key West than Orlando strains. Similar contributions of gene expression of AaeAPL1 were observed for Orlando and Key West Ae. aegypti. For the significant time effect, SCCs showed that AaeLRIM1 contributed approximately 8-fold higher than AaeAPL1 (Table 4A). The highest gene expression of AaeLRIM1 was 3-hours post infection with the later time point having lower levels. For AaeAPL1, the highest levels were observed for 72, 120, and 168-hours post infection with other points being lower (Figure 3).

For the interaction, SCCs showed that AaeAPL1 contributed approximately 2-fold greater than AaeLRIM1 (Table 4A). We made less than all possible comparisons by comparing between strains and holding time constant (e.g., Key West, 3-hours vs. Orlando, 3-hours). Gene expression for AaeLRIM1 was highest for 3-hours post infection compared to other time points for both Key West and Orlando Ae. aegypti. All pairwise contrasts of treatment groups were significantly different from one another, except for 120 hours post infection (Figure 3). For the significant interaction, gene expression for AaeAPL1 was highest for Key West at 168-hours post infection and for Orlando at 72- and 120-hours post infection for Ae. aegypti. All pairwise comparisons were significantly different between each other except for 24- and 48-hours post infection between the two mosquito strains (Figure 3).

Table 4A. MANOVA results for strain and time effects of AaeLRIM1 and AaeAPL1 RNA profile in response to Zika virus infection of Ae. aegypti.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Pillai’s Trace</th>
<th>df (numerator and denominator)</th>
<th>p-value</th>
<th>Standardized Canonical Coefficients</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>AaeLRIM1</td>
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<tr>
<td>Strain</td>
<td>0.86</td>
<td>2, 31</td>
<td>&lt;0.0001</td>
<td>8.06</td>
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<tr>
<td>Time</td>
<td>1.94</td>
<td>14, 64</td>
<td>&lt;0.0001</td>
<td>8.07</td>
</tr>
<tr>
<td>Strain x Time</td>
<td>1.86</td>
<td>14, 64</td>
<td>&lt;0.0001</td>
<td>2.45</td>
</tr>
</tbody>
</table>

1 AAEEL009520-RA is the same gene as AAEEL024406.
Figure 3. AaeLRIM1 and AaeAPL1 relative expression level fold changes in Aedes aegypti female infected with ZIKV. The fold change was calculated using the $2^{\Delta\Delta C_T}$ method. \( \Delta C_T \) (Control) = \( C_T (\text{AaeLRIM1/AaeAPL1}) - C_T (\text{AeaActin}) \); \( \Delta C_T \) (infected-ZIKV) = \( C_T (\text{AaeLRIM1/AaeAPL1}) - C_T (\text{AeaActin}) \); \( \Delta\Delta C_T = \Delta C_T \) (infected-ZIKV)−\( \Delta C_T \) (Control). The 3, 12, 24, 48, 72, 120, 168, and 240 h represented gene expression post infected with ZIKV. (A) Key West strain female Ae. aegypti; (B) Orlando strain female Ae. aegypti.

2.1.6 AaeLRIM1 and AaeAPL1 Transcriptional Induction of CHIKV Infections in Orally Infected Ae. aegypti Females

To characterize AaeLRIM1 and AaeAPL1 expression in response to CHIKV exposure, we measured AaeLRIM1 and AaeAPL1 expressions in orally infected Ae. aegypti. MANOVA showed the significant effects of strain of Ae. aegypti, time, and their interaction. For all significant treatment effects, SCCs showed that AaeLRIM1 contributed approximately 2-6-fold greater than AaeAPL1 (Table #). For the strain effect, gene expression of AaeLRIM1 and AaeAPL1 was significantly higher for Key West than Orlando strains. For the time effect, gene expression of AaeLRIM1 was highest at 72-hours post infection. All time points were significantly different from one another, except 3-hours versus 120-hours post infection (Figure 4). Similarly, gene expression of AaeAPL1 was highest at 72-hours.
post infection. All time points were significantly different from one another except the following; 3-hours versus 168-hours, 24-hours versus 240-hours, and 48-hours versus 120 hours (Figure 4).

For the significant interaction, gene expression of \textit{AaeLRIM1} was highest for 24-hours and 240-hours post infection for Key West and Orlando \textit{Ae. aegypti}, respectively (Figure 4). All pairwise comparisons of treatment groups were significantly different from one another except for 48-hours and 168-hours post infection (Figure 4). The timing of the highest gene expression of \textit{AaeAPL1} was similar to observations of \textit{AaeLRIM1}, with highest levels observed for 24-hours and 240-hours post infection for Key West and Orlando \textit{Ae. aegypti}, respectively (Figure 4). All pairwise comparisons of treatment groups were significantly different from one another except for 3-hours, 120-hours, and 168-hours post infection (Figure 4).

\textbf{Table 4B.} MANOVA results for strain and time effects of \textit{AaeLRIM1} and \textit{AaeAPL1} RNA profile in response to Chikungunya virus infection of \textit{Ae. aegypti}.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Pillai's Trace</th>
<th>df (numerator and denominator)</th>
<th>p-value</th>
<th>Standardized Coefficients</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>\textit{AaeLRIM1}</td>
</tr>
<tr>
<td>Strain</td>
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<tr>
<td>Time</td>
<td>1.77</td>
<td>12, 56</td>
<td>&lt;0.0001</td>
<td>4.59</td>
</tr>
<tr>
<td>Strain x Time</td>
<td>1.79</td>
<td>12, 56</td>
<td>&lt;0.0001</td>
<td>6.20</td>
</tr>
</tbody>
</table>

![Graph showing log2 fold change over time for AaeLRIM1 and AaeAPL1 genes](image-url)
Figure 4. AaeLRIM1 and AaeAPL1 relative expression level fold changes in Aedes aegypti female infected with CHIKV. The fold change was calculated using the $2^{-\Delta\Delta CT}$ method. ΔCt (Control) = Ct (AaeLRIM1/AaeAPL1) – Ct (AeaActin); ΔCt (infected-CHIKV) = Ct (AaeLRIM1/AaeAPL1) – Ct (AeaActin); ΔΔCt = ΔCt (infected-CHIKV) – ΔCt (Control). The 3, 24, 48, 72, 120, 168, and 240 h represented gene expression post infected with CHIKV. (A) Key West strain female Ae. aegypti; (B) Orlando strain female Ae. aegypti.

3. Discussion

Identification and characterization of genes related to LRIM1 and APL1C revealed novel innate immune factors and furthered our understanding of their presumed molecular functions. Waterhouse et al. (2010) used comparative sequenced genomes: An. gambiae, Ae. aegypti, and Cx. quinquefasciatus revealed that mosquito LRIM proteins can be classified into four distinct subfamilies by a variable number of LRRs [23]. Our phylogenetic tree of paralogue showed that AaeAPL1 (or AaeLRIM2) sequence-structure-function was most closely related to AaeLRIM4.

We analyzed developmental changes in the gene expression of LRR-containing proteins in Ae. aegypti eggs, larvae, pupae, and adults. The nucleus gene LRR-containing proteins AaeLRIM1, AaeAPL1 and others five paralogues are expressed <100-fold lower than the other nuclear genes, such as defensin, during all developmental stages examined [47]. Our data show that the expression of AaeLRIM1 (AAEL012086), AaeAPL1 (AAEL009520-RA) and others five paralogues is not only regulated by development but also by the varying environmental origin (or permethrin resistant selected strain) of mosquito strains in Ae. aegypti. For both immature and adult stages, we observed higher expression of AaeLRIM1 than AaeAPL1 in Key West Ae. aegypti and higher expression of AaeAPL1 than AaeLRIM1 in Orlando Ae. aegypti. These differences in responses may be attributable to difference in insecticide resistance among the Orlando (permethrin susceptible) and Key West (permethrin resistant) strains of Ae. aegypti. However, we are unable to rule out that other differences between these two strains of Ae. aegypti (e.g., geographic origin and founder effects) may contribute to the observed differences in gene expressions. For the immature, but not adult stages, expression of AaeAPL1 and AaeLRIM1 increased with developmental stage. The strain effect for the analyses were modified by interactions with other factors, suggesting complex interactions between gene expression and immature stage, adult age, and sex. To our knowledge, this is the first exploration of AaeLRIM gene expression during Ae. aegypti development.
In *Anopheles*, two leucine-rich repeat (LRR) proteins, LRIM1 and APL1, have been shown to strongly affect *P. berghei* development in the mosquito midgut and have been identified as major mosquito factors that regulate parasite loads [18, 20-22, 48-51]. In *Ae. aegypti*, the likely LRIM1 orthologue is upregulated with other immune genes following infection with *Wolbachia* bacteria resulting in immune activation and shortened mosquito life spans [52]. However, there is no information available for revealing LRIM1 and APL1 affected by arboviruses. Our data demonstrates that gene expression of LRR-containing proteins, *AaeLRIM1* and *AaeAPL1*, not only affect regulated parasites in the *Anopheles* but are also altered by arbovirus infection in *Ae. aegypti*. Biophysical analysis of anopheles gambiae leucine-rich repeat proteins APL1A1, APL1B and APL1C can all form an extended, flexible heterodimer with LRIM1, providing a repertoire of functional innate immune complexes to protect *An. gambiae* from a diverse array of pathogens [21]. Future studies are needed to identify how proteins *AaeLRIM1* and *AaeAPL1* may influence progression of infection of arboviruses in *Ae. aegypti*. Some of the highest changes in expression of *AaeAPL1* and *AaeLRIM1* for both strains of *Ae. aegypti* occurred 24-72 hours post infection with CHIKV which approximates the time when *Ae. aegypti* acquire disseminated infections [31]. However, this response appears to be earlier for the Key West strain (approximately 24 hours), which may suggest alterations in immune responses between permethrin susceptible and resistant *Ae. aegypti* (Shin et al. un unpublished data). Changes in expression of *AaeLRIM1* and *AaeAPL1* followed a different pattern for ZIKV infected mosquitoes, suggesting gene expression changes depending on the particular arbovirus. For both strains of *Ae. aegypti*, *AaeLRIM1* tended to be highest early during infection and decline at later points. In contrast, expression of *AaeAPL1* was low early during infection and higher at later measured times.

Studying the immune system of mosquitoes will provide insights into significant opportunities to bridge tissue damage, immune invasions mechanisms, and immune response against pathogens [11]. More importantly, unveiling newfound understanding of mosquito immunity will shed light on the fight against disease-spreading pathogens, including ZIKV and CHIKV. Understanding the mechanisms that allow pathogens to grow and replicate in mosquitoes will provide insights into the mechanisms of mosquito-pathogen interactions. Finding exact immune evasion strategies of pathogens will help produce novel strategies that are effective at controlling them.

4. Materials and Methods

**Mosquito strains and developmental stages of Aedes aegypti**

*Ae. aegypti* larvae were collected from Key West (24.55°N, 81.78°W), Florida, USA since 2011 and were initially tested for permethrin resistance, then subjected to permethrin selection for 15 generations and again assayed for resistance strain[41]. Key West strain *Ae. aegypti*, referred to as the resistant strain, was maintained at the Florida Medical Entomology Laboratory (FMEL) in Vero Beach, FL, USA. The Orlando population of *Ae. aegypti* was collected from Orlando, FL, USA and reared in the Mosquito and Fly Research Unit, Center for Medical, Agricultural and Veterinary Entomology, ARS-USDA in Gainesville, FL since 1952. The Orlando strain is recognized as a permethrin susceptible strain of *Ae. aegypti* [53]. For the experiments, mosquito eggs were hatched and reared in a rearing chamber at 27°C [47]. We sampled eggs, larvae, pupae and adults to measure developmentally regulated gene expression of Leucine-Rich Repeat Proteins in *Ae. aegypti*. We collected 100 µg of eggs, 20 larvae at 3rd instar stage, 20 pupae, 10 adult mosquitoes, 10 males and 10...
females at 1-day-old (teneral), 3-day-old, 5-day-old, 7-day-old and 10-day-old adults. The experiments were repeated three times.

**Chikungunya virus and Zika virus infection**

Four-day-old female adults were fed blood containing either CHIKV, ZIKV or blood without virus as control [41, 47]. Isolates of the Indian Ocean lineage of CHIKV (LR2006-OPY1, GenBank accession: KT449801) from Réunion and the Asian lineage of ZIKV (strain PRVABC59, GenBank accession # KU501215.1) from Puerto Rico were cultured in African green monkey (Vero) cells and used in the mosquito infection study. The detail procedures were described in the previous publication [41, 47].

Monolayers of Vero cells were inoculated with 500 μl of diluted stock virus (multiplicity of infection, 0.1) and incubated for 1 hr at 37°C and 5% CO2 atmosphere, after which 24 mL media (M199 medium supplemented with 10% fetal bovine serum, penicillin/streptomycin and mycostatin) were added to each flask and incubated for three days for CHIKV and six days for ZIKV. Freshly harvested media from infected cell cultures were combined with defibrinated bovine blood and ATP (0.005 M) and presented to mosquitoes using a membrane feeding system (Hemotek, Lancashire, United Kingdom) for one hour feeding trials. Control blood meals were prepared similarly except that monolayers of Vero cells were inoculated with media only. Samples of infected blood were taken at the time of the feedings and stored at -80°C for later determination of virus titer. Mosquitoes were fed 8.0-8.3 log10 pfu/ml of CHIKV and 6.4 log10 pfu/ml of ZIKV.

Following feeding trials, fully engorged mosquitoes were sorted using light microscopy (10X) and held in cages (h by d: 10 cm by 10 cm) maintained at a 12:12 hour light:dark photoperiod and 30°C. Mosquitoes were provided with an oviposition substrate and 10% sucrose solution on cotton pads. Cohorts of mosquitoes were killed and stored at -80°C at the following sample periods after ingesting infected blood: 3-h, 12-h (ZIKV only), 24-h, 48-h, 72-h, 120-h, 168-h, and 240-h. Mosquitoes were deprived of sucrose but not water 1-day before trials used to measure transmission on 3, 5, 7, and 10 days following ingestion of ZIKV and CHIKV infection blood.

**RNA extraction**

Samples were homogenized with a plastic pestle in 1.5 ml tubes. Total RNAs were extracted using TRIzol reagent according to the manufacturer’s instruction (Ambion, Life Technologies, Carlsbad, California 92008, USA) following the standard protocol [47]. The RNA samples were digested by DNase I (RNase-free), according to the manufacturer’s instructions (Thermo Scientific, Wilmington, Delaware USA). The purified RNA samples were quantitated by NANODROP 2000 Spectrophotometer (Thermo Scientific, Wilmington, Delaware USA).

**cDNA synthesized and qPCR amplification**

cDNAs from 2 μg of total purified RNA were synthesized using a Cloned AMV First-Strand cDNA Synthesis Kit Invitrogen™ and Oligo (dT)20 primer, according to the manufacturer’s instructions (Invitrogen, Carlsbad, CA). The reaction was terminated by heat inactivation at 95°C for 5 min. The cDNA samples for qPCR from developmental stages, infected treatment, and controls were diluted by adding 80 μl ddH2O to 20 μl reaction solution [54].
The quantitative PCR (qPCR) assay for target genes *AaeLRIM1* and *AaeAPL1* and reference gene *AaeActin* in *Ae. aegypti* was achieved using a BIO-RAD C1000 Touch Thermal Cycler, CFX 96™ Real-Time System (BIO-RAD, Hercules, CA, USA). The qPCR reaction mixture with a volume of 15 µl in Multiwell Plates 96 contained 1 µl diluted cDNA, 0.5 µM primers and 1X master mix of PowerUP SYBR® Green Master Mix (Applied Biosystems, Thermo Fisher Scientific, Foster City, CA). In every qPCR run, *AaeActin* was employed as an internal control to normalize for variation in the amount of cDNA template. The qPCR primers for *AaeLRIM1* and *AaeAPL1* genes were designed from the coding region based on GenBank, Accession Number using Primer3 [http://primer3.ut.ee](http://primer3.ut.ee) (Table 3). The qPCR thermal cycling parameters were the same as previous publication [55]. Relative expression levels were calculated as follows for the developmental stages. First, *AaeLRIM1/AaeAPL1* transcript levels relative to a standard (*AaeActin*) were calculated using the formula \(ΔCT = CT(AaeLRIM/AaeAPL1) - CT(AaeActin)\). Second, an average \(ΔCT\) value for each sample was calculated. Third, relative expression levels were calculated using the equation 10,000 x 2 \(^{ΔCT}\) for the treatment and control adults. First, *AaeLRIM1* or *AaeAPL1* transcript levels relative to a standard (*AaeActin*) were calculated using the formula \(ΔCT = CT(AaeLRIM/AaeAPL1) - CT(AaeActin)\). Then, \(ΔΔCT = ΔCT\) (infected) – \(ΔCT\) (control) value for each sample was calculated. Third, relative expression levels were calculated using the equation 1x 2 \(^{ΔΔCT}\) [47, 55-58].

### Table 3. Primers from *Aedes aegypti* for qPCR reaction.

<table>
<thead>
<tr>
<th>Gene ID</th>
<th>Gene name</th>
<th>Primer name</th>
<th>Primer sequence (5′→3′)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AAEL012086</td>
<td><em>AaeLRIM1</em></td>
<td>AaeLRIM1-086-1011F</td>
<td>TGACAACCGGGTTAGGAAG</td>
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<td>TGGCCAATCATGTCTCA</td>
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<td>AAEL024406</td>
<td><em>AaeAPL1</em></td>
<td>AaeAPL1-406-115F</td>
<td>TCAACCCAGCTCAGATAC</td>
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<td>TCAGCAAGTCCACAGTGC</td>
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<td>AAEL010132</td>
<td><em>AaeLRIM3</em></td>
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<td>TGTAGCCGCAAATACACA</td>
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<td>AaeLRIM3-132-405R</td>
<td>CTGAAAGTCGTCGTTACA</td>
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<td>AAEL010128</td>
<td><em>AaeLRIM4</em></td>
<td>AaeLRIM4-128-612F</td>
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<td>AAEL007103</td>
<td>*AaeLRIM15</td>
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<tr>
<td>AAEL011197</td>
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<td>AaeActin-197-590R</td>
<td>CGTTACGTAGATCTTCC</td>
</tr>
</tbody>
</table>

1 AAEL024406 is the same gene as AAEL009520-RA

**RNA-Seq Library Sequencing, Data mining and RNA-seq analysis**

RNA-Seq library sequencing, data mining and RNA-seq analysis were featured in the previous publication [41]. Gene expression was assessed by counting the number of mapped reads for each transcript [59]. Significant up- and downregulated genes were selected using the adjusted P-value (p-adj), log2 fold-change (log2FC), or both for downstream analysis. The RNA-seq data have been deposited to NCBI (https://www.ncbi.nlm.nih.gov/gds/?term=GSE118858). RNA-seq analysis data were provided 60 leucine-rich repeat related transcriptions in the *Ae. aegypti* in response to Zika virus (Table S1A-D).
**Statistical analysis**

Multivariate analysis of variance (MANOVA) and ANOVA were used to measure developmentally regulated gene expression of *AaeLRIM1* and *AaeAPL1*. The relative contribution and relationship of *AaeLRIM1* and *AaeAPL1* to developmental treatment effects were assessed using standardized canonical coefficients (SCC) (PROC GLM, SAS 9.22). When significant effects were detected, we used univariate comparisons among treatment least-squares means for the immature stages and adult stages. Similarly, we used MANOVA to measure expression of *AaeLRIM1* or *AaeAPL1* following ingestion of CHIKV and ZIKV infected blood. We tested for all main treatment factors and interactions.

**Supplementary Materials:** Supplementary materials can be found at [www.mdpi.com/xxx/s1](http://www.mdpi.com/xxx/s1).

**Table S1**: Female *Aedes aegypti* transcriptomic RNA-seq data show Leucine-Rich Repeat Proteins 7-days post infection or post injection between Key west and Orlando strains.

**Figure S1A-E.** *AaeLRIM3, AaeLRIM4, AaeLRIM15, AaeLRIM16,* and *AaeLRIM17* relative expression in the all developmental stages.

**Figure S2A-B.** *AaeLRIM3, AaeLRIM4, AaeLRIM15, AaeLRIM16,* and *AaeLRIM17* relative expression level fold changes in *Aedes aegypti* female infected with ZIKV (Key West strain and Orlando strain).

**Figure S3A-B.** *AaeLRIM3, AaeLRIM4, AaeLRIM15, AaeLRIM16,* and *AaeLRIM17* relative expression level fold changes in *Aedes aegypti* female infected with CHIKV (Key West strain and Orlando strain).


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**Conflicts of Interest:** All authors declare no conflict of interest.

**Abbreviations**

ZIKV    Zika Virus
CHIKV  Chikungunya virus
LRR  leucine-rich repeats
LRIM1  leucine-rich repeat immune protein 1
APL1  Anopheles Plasmodium-responsive leucine-rich repeat 1

References


