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Article

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

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[3]. Silencing Lrch4 attenuates cytokine induction by LPS and multiple other TLR ligands and dampens the in vivo innate immune response [3].

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].

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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.

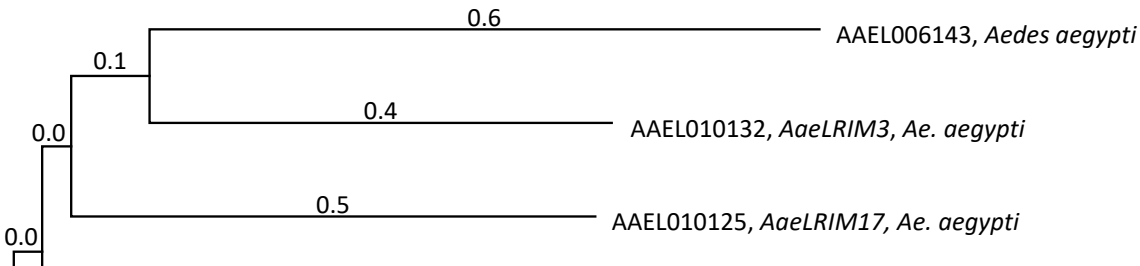

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Figure 1. The evolutionary history was inferred using the Neighbor-Joining method [45]. The optimal tree with the sum of branch length = 4.948 is shown. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary 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**).

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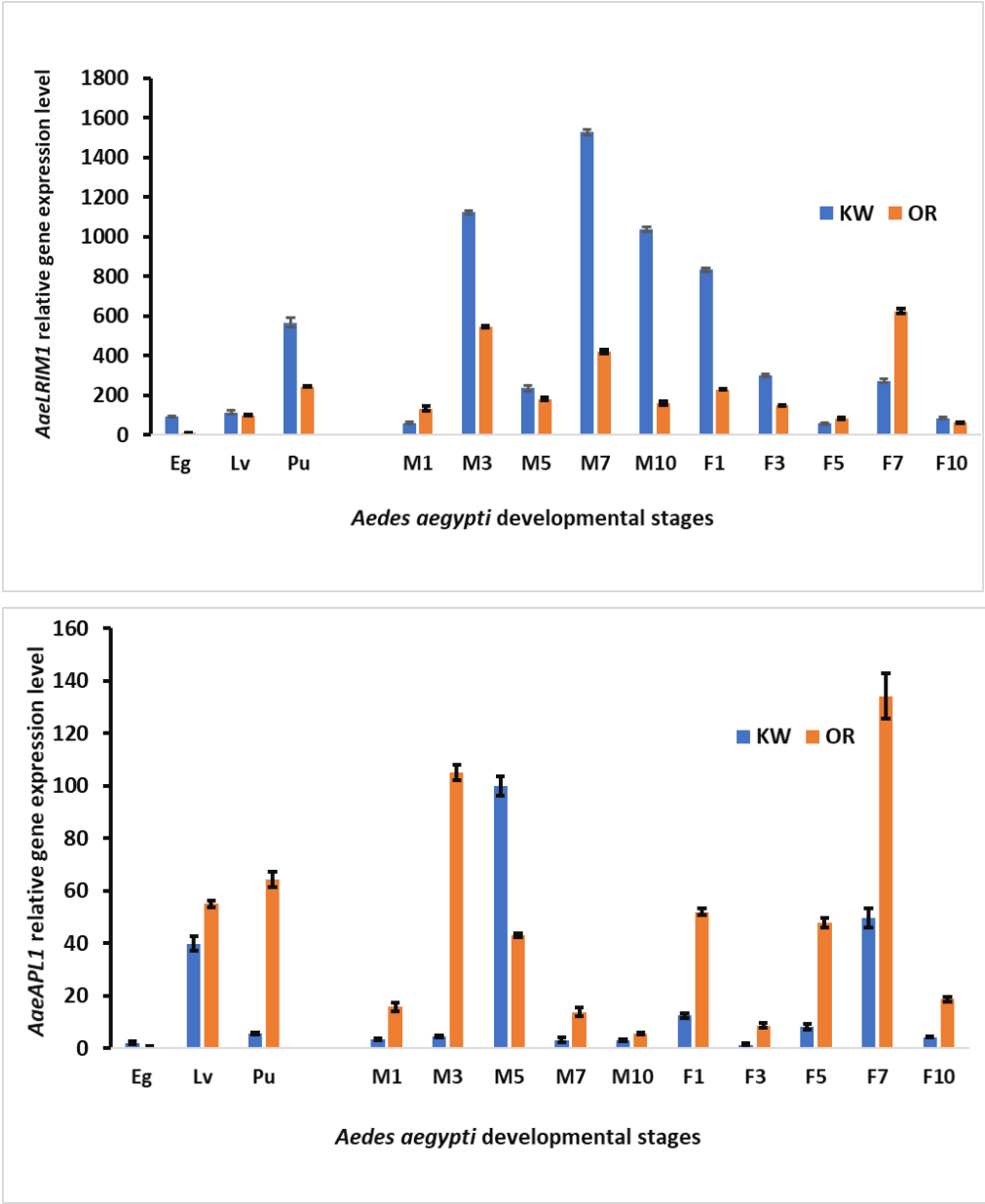

Figure 2. *AaeLRIM1* and *AaeAPL1* relative expression in the all developmental stages, from eggs (Eg), larvae (Lv), pupae (Pu), and adults *Ae. aegypti*, including male (M1, male 1-d-old; M3, male 3-d-old; M5, male 5-d-old; M7, male 7-d-old; and M10, male 10-d-old) and female (F1, female 1-d-old; F3, 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 *Aedes 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 similary, 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

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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*.

Treatment	**Pillai's Trace**	**df (numerator and denominator**	**p-value**	**Standardized Coefficients**	**Canonical**
				AaeLR1M1	***AaeAPL1***
Strain	0.99	2, 11	<0.0001	12.12	-10.76
Stage	1.99	4, 24	<0.0001	16.28	8.35
Strain x Stage	1.89	4, 24	<0.0001	11.09	-11.57

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 *Aae*LRIM1, 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*.

Treatment	**Pillai's Trace**	**df (numerator and denominator**	**p-value**	**Standardized Coefficients**	**Canonical**

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				AaeLR1M1	*AaeAPL1*
Strain	0.99	2, 39	<0.0001	47.84	-7.90
Sex	1.00	2, 39	<0.0001	49.46	-4.80
Age	1.98	8, 80	<0.0001	49.48	-3.43
Strain x Sex	1.00	2, 39	<0.0001	48.66	-1.11
Strain x Age	1.93	8, 80	<0.0001	46.59	-9.05
Sex x Age	1.99	8, 80	<0.0001	49.49	-4.55
Strain x Sex x Age	1.98	8, 80	<0.0001	49.52	-4.06

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±1.78log10 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±0.81log10 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 5.81±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*.

Strains	Initial dose in bloodmeal	Freshly fed	3 days post infection	7 days post infection	10 days post infection
Key West	6.4±0.09	4.30±0.0	3.96±0.26	4.11±1.78	6.57±0.05
Orlando	6.4±0.08	4.17±0.39	3.66±0.27	3.58±1.23	4.49±0.34

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[2a] 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*.

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

[2b] 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=GSE11). 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**). *AaeLRIM1* (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**). *AaeLRIM1* (AAEL010286-RA) was significantly upregulated 3-fold (**Table 3B**). Comparing ZIKV infected Key West *Ae. aegypti* with the Key West control at 7 dpi, 4 differentially expressed (DE) transcripts related to LRRP were significantly dysregulated (2 upregulated and 2 downregulated, **Table 3C**). *AaeLRIM1* (AAEL010286-RA, p-adj 5.0×10^{-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 2.8×10^{-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

Transcript ID	Log2FC	p-adj	Gene description
AAEL001401-RA	3.2722	2.3×10^{-27}	leucine-rich immune protein (Short)
AAEL001402-RA	3.2947	3.3×10^{-33}	leucine-rich immune protein (Short)

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AAEL001414-RA	3.3721	2.1×10^{-36}	leucine-rich immune protein (Short)
AAEL001417-RA	3.9484	2.1×10^{-5}	leucine-rich immune protein (Short)
AAEL001420-RA	3.4335	1.6×10^{-73}	leucine-rich immune protein (Short)
AAEL001649-RA	-0.2792	4.6×10^{-3}	leucine aminopeptidase
AAEL002295-RA	2.4545	1.9×10^{-44}	leucine-rich transmembrane protein
AAEL002615-RA	2.2527	4.9×10^{-12}	leucine-rich transmembrane protein
AAEL003262-RA	1.8141	3.9×10^{-5}	leucine-rich transmembrane protein
AAEL003408-RA	1.3747	5.3×10^{-6}	leucine-rich transmembrane protein
AAEL003713-RA	1.3970	7.2×10^{-4}	leucine-rich transmembrane protein
AAEL003720-RA	1.1688	5.6×10^{-5}	leucine-rich transmembrane protein
AAEL005762-RA	2.2277	2.7×10^{-5}	leucine-rich transmembrane protein
AAEL006975-RA	1.7989	8.7×10^{-5}	leucine aminopeptidase
AAEL007103-RA	3.2536	7.5×10^{-11}	leucine-rich immune protein (TM)
AAEL009520-RA[1]	3.0383	4.5×10^{-25}	leucine-rich immune protein (Long)
AAEL010125-RA	3.2598	7.0×10^{-8}	leucine-rich immune protein (Coil-less)
AAEL010128-RA	4.8149	2.4×10^{-7}	leucine-rich immune protein (Long)
AAEL010656-RA	2.7658	3.8×10^{-7}	leucine-rich immune protein (Short)
AAEL012086-RA	2.4679	1.7×10^{-12}	leucine-rich immune protein (Long)
AAEL012092-RA	2.1252	2.6×10^{-28}	leucine-rich repeat protein
AAEL012093-RA	2.2739	1.9×10^{-10}	leucine-rich transmembrane protein
AAEL012255-RA	4.2146	1.9×10^{-4}	leucine-rich immune protein (Short)

[1] AAEL009520-RA is the same gene as AAEL024406.

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.

Transcript ID	Log2FC	p-adj	Gene description
AAEL000243-RA	5.2443	1.8×10^{-26}	leucine-rich transmembrane protein
AAEL009894-RA	-0.9167	2.9×10^{-4}	leucine-rich immune protein (Coil-less)
AAEL010286-RA	3.0287	1.5×10^{-5}	leucine-rich transmembrane protein

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.

Transcript ID	Log2FC	p-adj	Gene description
AAEL000243-RA	-6.6469	7.6×10^{-44}	leucine-rich transmembrane protein
AAEL003408-RA	2.1477	1.8×10^{-4}	leucine-rich transmembrane protein
AAEL009894-RA	0.8665	9.7×10^{-8}	leucine-rich immune protein (Coil-less)
AAEL010286-RA	-3.3918	5.0×10^{-9}	leucine-rich transmembrane protein

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.

Transcript ID	Log2FC	p-adj	Gene description
AAEL009520-RA[1]	-2.2074	2.8×10^{-4}	leucine-rich immune protein (Long)
AAEL010128-RA	-5.3692	2.8×10^{-6}	leucine-rich immune protein (Long)

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AAEL001402-RA	-3.3551	6.1×10^{-8}	leucine-rich immune protein (Short)
AAEL010656-RA	-2.5623	4.4×10^{-3}	leucine-rich immune protein (Short)
AAEL012255-RA	-4.1624	3.1×10^{-5}	leucine-rich immune protein (Short)

[1] AAEL009520-RA is the same gene as AAEL024406.

2.1.5 **AaeLRIM1** *and* **AaeAPL1** *Transcriptional Induction of ZIKV Infections in Orally Infected Ae. aegypti Females*

To characterize *AaeLRIM1* and *AeaAPL1* expression in response to ZIKV exposure, we measured *AaeLRIM1* and *AeaAPL1* 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**).

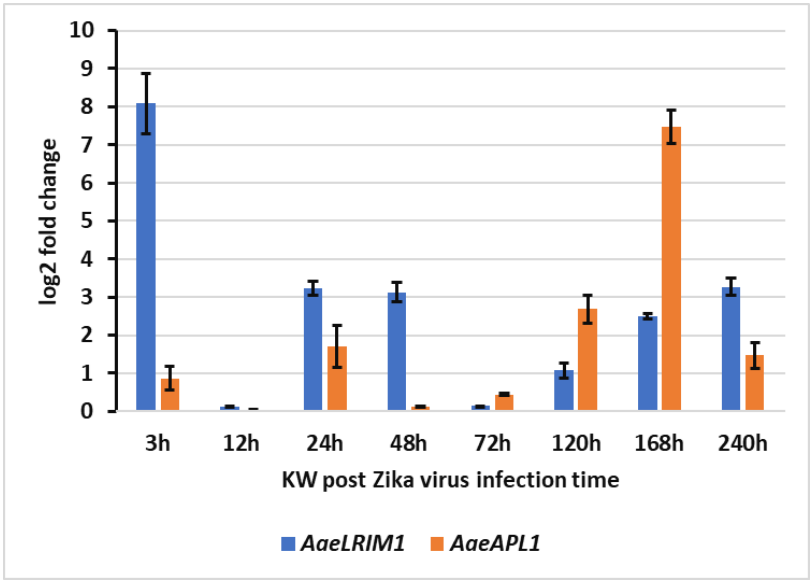
For the interaction, SCCs showed that *AaeAPL1* contributed approximately 2-fold greater than *AaeLRIM1* (**Table 4A**). We made less than all possible comparisons by comparing between strains and holding time constant (e.g., Key West, 3-hours vs. Orlando, 3-hours). Gene expression for *AaeLRIM1* was 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*.

Treatment	**Pillai's Trace**	**df (numerator and denominator**	**p-value**	**Standardized Coefficients**	**Canonical**
				AaeLR1M1	***AaeAPL1***
Strain	0.86	2, 31	<0.0001	8.06	-1.16
Time	1.94	14, 64	<0.0001	8.07	-1.01
Strain x Time	1.86	14, 64	<0.0001	2.45	5.84

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 3 January 2019 doi:10.20944/preprints201901.0013.v1



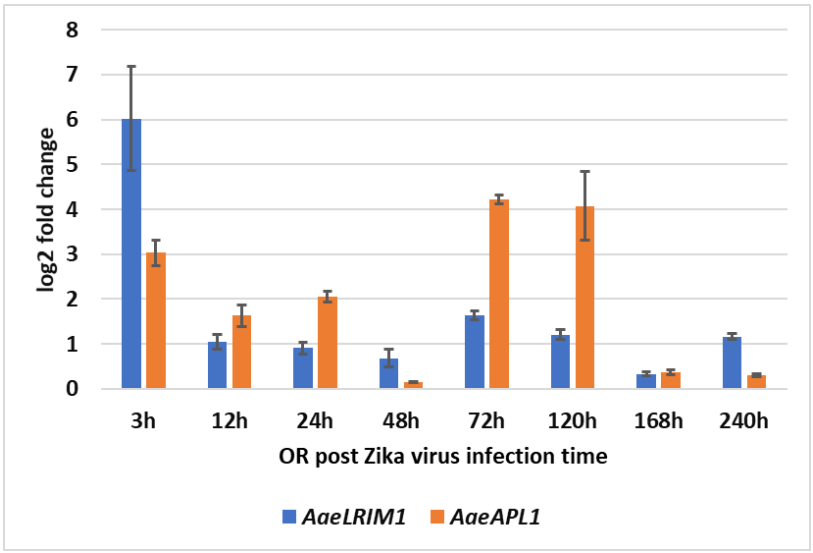


Figure 3. *AaeLRIM1* and *AaeAPL1* relative expression level fold changes in *Aedes aegypti* female infected with ZIKV. The fold change was calculated using the $2^{[-average\ \Delta\Delta CT\ ]}$ method. ΔCt (Control) = Ct (*AaeLRIM1*/*AaeAPL1*) –Ct (*AeaActin*); ΔCt (infected-ZIKV) = Ct (*AaeLRIM1*/*AaeAPL1*) – Ct (*AeaActin*); ΔΔCt =ΔCt (infected-ZIKV)−ΔCt (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

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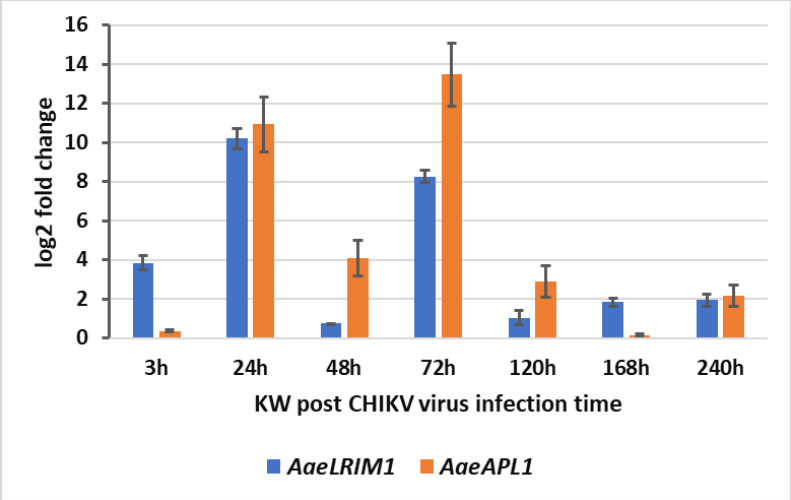
post infection. All time points were significantly different from one another except the following; 3-hours versus 168-hours, 24-hours versus 240-hours, and 48-hours versus 120 hours (Figure 4).

For the significant interaction, gene expression of *AaeLRIM1* was highest for 24-hours and 240-hours post infection for Key West and Orlando *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 *AaeAPL1* was similar to observations of *AaeLRIM1*, with highest levels observed for 24-hours and 240-hours post infection for Key West and Orlando *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).

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

Treatment	Pillai's Trace	df (numerator and denominator	p-value	Standardized Coefficients	Canonical
				AaeLR1M1	***AaeAPL1***
Strain	0.60	2, 27	<0.0001	5.90	1.23
Time	1.77	12, 56	<0.0001	4.59	2.61
Strain x Time	1.79	12, 56	<0.0001	6.20	0.79



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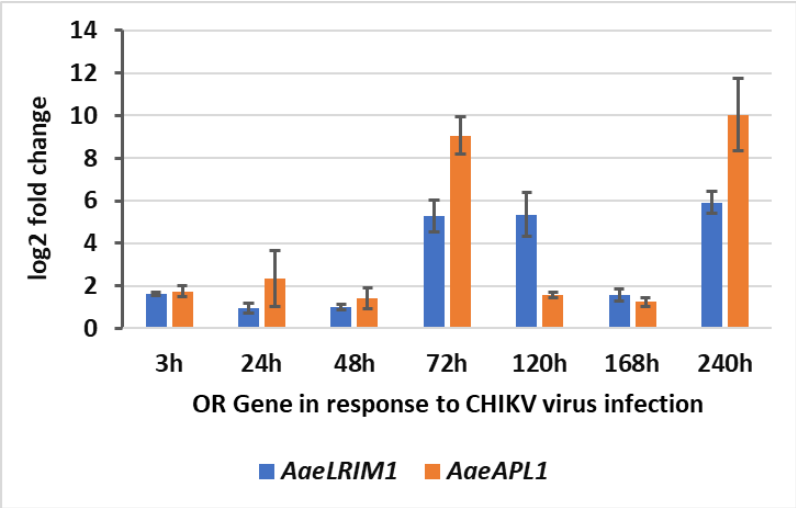

Figure 4. *AaeLRIM1* and *AaeAPL1* relative expression level fold changes in *Aedes aegypti* female infected with CHIKV. The fold change was calculated using the $2^{[-average\ \Delta\Delta CT]}$ method. ΔCt (Control) = Ct (*AaeLRIM1*/*AaeAPL1*) –Ct (*AeaActin*); ΔCt (infected-CHIKV) = Ct (*AaeLRIM1*/*AaeAPL1*) – 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.

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 3 January 2019 doi:10.20944/preprints201901.0013.v1

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. unpublish 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 27ºC [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

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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% CO_2 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 95ºC for 5 min. The cDNA samples for qPCR from developmental stages, infected treatment, and controls were diluted by adding 80 µl ddH_2O to 20 µl reaction solution [54].

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 3 January 2019 doi:10.20944/preprints201901.0013.v1

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 PCR primers for *AaeLRPIM,* and *AaeAPL1* genes were designed from the coding region based on GenBank, Accession Number using Primer3 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, *AaeLRIM*/*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 $2^{[-\text{average } \Delta CT]}$ [47, 55]. Relative expression levels were calculated as follows for the treatment and control adults. First, *AaeLRIM1* or *AaeAPL1* transcript levels relative to a standard (*AaeActin*) were calculated using the formula ΔCT = CT (*AaeLRIM1*/*AaeAPL1*) – CT (*AaeActin*). Then, $\Delta\Delta$CT= ΔCT (infected) – ΔCT (control) value for each sample was calculated. Third, relative expression levels were calculated using the equation 1x $2^{[-\text{average } \Delta\Delta CT]}$ [47, 55-58].

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

Gene ID	Gene name	Primer name	Primer sequence (5′---3′)
AAEL012086	*AaeLRIM1*	AaeLRIM1-086-1011F	TGACAACCGGGTTAAGGAAG
		AaeLRIM1-086-1198R	TGGCCAAATCATTGTTCTCA
AAEL024406	*AaeAPL1*	AaeAPL1-406-115F	TCAACCCAGCCTCCAGATAC
		AaeAPL1-406-275R	TCAGCAGTTTCACCACTTGC
AAEL010132	*AaeLRIM3*	AaeLRIM3-132-166F	TGTAGCCCGCAATAATCACA
		AaeLRIM3-132-405R	CTGAAGTGCTCCGTTGAACA
AAEL010128	*AaeLRIM4*	AaeLRIM4-128-612F	TGTAGCCCGCAATAATCACA
		AaeLRIM4-128-830R	GCCAGATTAAGCTCCACGAG
AAEL007103	*AaeLRIM15*	AaeLRIM15-103-1522F	ATGGTATTGCGTGGAGGAAG
		AaeLRIM15-103-1676R	ATCCTATCAACCGCCCTTCT
AAEL008658	*AaeLRIM16*	AaeLRIM16-658-299F	ACACCTTCGAGAAAGCGAAA
		AaeLRIM16-658-541R	TCAACATGGGCAAATGAGAA
AAEL010125	*AaeLRIM17*	AaeLRIM17-125-555F	GCAGTACAATTCGCTGACCA
		AaeLRIM17-125-718R	CCTTAAGCCGATTGAAGCTG
AAEL011197	*AaeActin*	AaeActin-197-152F	AGGACTCGTACGTCGGTGAC
		AaeActin-197-590R	CGTTCAGTCAGGATCTTC

[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**).

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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 developmental stages (Tukey-Kramer method). Separate analyses were performed 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.

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).

Author Contributions: Conceptualization, L.Z., and B.A.; Methodology, L.Z., and B.A.; Software, B.A. and L.Z.; Validation, L.Z., and B.A.; Formal Analysis, L.Z., and B.A.; Investigation, L.Z., B.A. and DS; Resources, L.Z., B.A. and DS; Data Curation, L.Z.; Writing-Original Draft Preparation, L.Z.; Writing-Review & Editing, L.Z., B.A. and DS; Visualization, L.Z., and B.A; Supervision, L.Z.; Project Administration, L.Z.; Funding Acquisition, L.Z. and B.A..

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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

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