

Aedes FADD: A novel death domain-containing protein required for antibacterial immunity in the yellow fever mosquito, *Aedes aegypti*

Dawn M. Cooper^{*,1}, Ciara M. Chamberlain¹, Carl Lowenberger¹

Department of Biological Sciences, Simon Fraser University, Burnaby, B.C., Canada V5A 1S6

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ABSTRACT

Microbial infections in insects activate a series of immune responses that culminate in the production of antimicrobial peptides (AMPs). In *Drosophila*, two signaling pathways, govern the challenge-dependent expression of AMPs; the Toll and IMD pathways. While AMPs have been the subject of much research in mosquitoes, the regulation of the pathways required for AMP expression remains largely unknown. We report here the identification of *Aedes* FADD (AeFADD), a death domain protein in *Aedes aegypti*. AeFadd is expressed in all immune-competent tissues and all developmental stages examined. At the transcriptional level, AeFadd transcripts increased when challenged with *Escherichia coli* but not *Micrococcus luteus*. In both cases, we observed the induction of two AMP genes; *cecropin* and *defensin*. Loss of AeFadd function by dsRNA interference impaired the inducible expression of both AMPs, and rendered adult mosquitoes susceptible to both types of bacteria. Identifying molecules that regulate mosquito immunity may help elucidate the factors that contribute to the vectorial capacity and provide insights into general mechanisms that regulate innate immunity.

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1. Introduction

Vector-borne diseases continue to afflict a large proportion of the world's population and place a significant burden on the public health systems in many tropical and subtropical countries. Mosquitoes are among the most medically important insect vectors of human pathogens, transmitting the pathogens that cause malaria and the arboviruses that cause Dengue fever, Yellow fever and West Nile fever. Much of the current research efforts in vector biology have focused on the innate-immune responses of these insect vectors to pathogens with the hope of identifying factors that can be used as novel strategies to combat vector-borne diseases.

Insects have a potent immune response that effectively combats a broad range of pathogens, and this immunity plays a key role in mediating the interactions between pathogens and vectors. In both mammals and insects, the innate-immune system provides the first line of defense against invading pathogens. This common response between mammals and insects shares conserved pathways that

culminate in the stimulation of phagocytic activity and the production of small antimicrobial peptides (AMPs) with potent antifungal and antibacterial properties (Ferrandon et al., 2007, 2004; Hoffmann, 2003). In insects, the induction of AMPs is regulated at the level of transcription, and active peptides are expressed primarily in the insect fatbody and midgut tissues.

Drosophila has been the model organism for invertebrate immunity and the knowledge gained from these studies has been applied to many other invertebrate systems. The innate-immune response is governed by signaling pathways that trigger AMP production, phagocytosis, encapsulation and melanization. In *Drosophila*, the production of AMPs is regulated either by the Toll or IMD (immunodeficient mutant) pathways (Ferrandon et al., 2004; Hoffmann, 2003; Lemaitre and Hoffmann, 2007; Silverman and Maniatis, 2001). The Toll pathway primarily regulates the expression of two AMPs, Drosomycin and Metchnikowin, and initiates the melanization cascade and blood cell proliferation (Ferrandon et al., 2007; Lemaitre and Hoffmann, 2007). The IMD pathway regulates the induced expression of the AMPs; Cecropin, Drosocin, Defensin and Dipterican (Kaneko and Silverman, 2005; Tanji and Ip, 2005). Although some of the immune-induced genes, such as *dipterican*, are dependent on one signaling pathway only, others can be induced by both cascades suggesting interactions between the Toll and IMD pathways during septic challenge (Bangham et al., 2006).

The activation of the Toll or IMD signaling pathways depends upon the type of invading microorganism. Traditionally, the

* Corresponding author. Current address: James Hogg iCAPTURE Centre for Cardiovascular and Pulmonary Research, Providence Heart and Lung Institute at St. Paul's Hospital, University of British Columbia, Vancouver, B.C., Canada V6Z 1Y6. Tel.: +1 604 806 8346; fax: +1 604 806 8351.

E-mail addresses: dcooper@mrl.ubc.ca (D.M. Cooper), cchamberlain@mrl.ubc.ca (C.M. Chamberlain), clowenbe@sfu.ca (C. Lowenberger).

¹ Tel.: +1 778 782 3985; fax: +1 778 782 3496.

literature has indicated that infections with fungi and Gram-positive bacteria activate the Toll pathway while Gram-negative bacteria activate the IMD pathway. However, there is increasing evidence that several Gram-positive bacteria and most fungi also activate the IMD pathway (Hedengren-Olcott et al., 2004) suggesting, at least in the case of bacterial challenge, that subsets of microbial pattern recognition receptors can distinguish between different species of Gram-positive bacteria and that there may be redundancy in these signaling pathways. In general, AMP expression in response to bacterial challenge is achieved through the sensing of different forms of peptidoglycan in the cell walls of both Gram-negative and Gram-positive bacteria; with the peptidoglycan from Gram-negative bacteria differing from most Gram-positive bacteria by the replacement of lysine with *meso*-diaminopimelic acid (DAP) (Vollmer et al., 2008). In most scenarios, DAP-type peptidoglycans will stimulate the IMD pathway while the Lys-type peptidoglycans will stimulate the Toll pathway. There are, however, subsets of Gram-positive bacteria that are able to stimulate the IMD pathway through either the production of a DAP-type peptidoglycan or what appears to be a general recognition of the overall structure of the peptidoglycan and not a specific type of peptidoglycan or other cell wall components (Hedengren-Olcott et al., 2004). Together, these data suggest that grouping bacteria according to Gram stain or the type of peptidoglycan may be an over-simplification when considering the cell wall constituents that trigger immune signaling in invertebrates (Leulier et al., 2003; Hedengren-Olcott et al., 2004).

During IMD signaling, DAP-type peptidoglycan recognition is mediated primarily by the membrane-bound pattern recognition receptor, PRGP-LC that interacts with the apical signaling molecule, IMD; a protein homologous to the TNFR-interacting protein, RIP (Hoffmann, 2003; Hultmark, 2003; Kaneko and Silverman, 2005; Tanji and Ip, 2005). The signaling below IMD branches to include a pathway involving the kinase dTAK1 and another pathway that includes dFADD. dTAK1 activates the IKK complex, which contains two I κ B kinases, IKK β and IKK γ (Ferrandon et al., 2004; Lemaitre and Hoffmann, 2007; Silverman and Maniatis, 2001; Silverman et al., 2000). The IKK complex directs the site-specific proteolytic cleavage and activation of Relish, which in turn activates the production of AMPs (Lu et al., 2001; Silverman et al., 2000; Stoven et al., 2003). The second signaling branch involves the adaptor protein, dFADD (*Drosophila* Fas-associated protein with Death Domain) and the apical caspase DREDD (Hu and Yang, 2000; Lu et al., 2001; Silverman et al., 2000; Stoven et al., 2003; Tanji and Ip, 2005). Both act downstream of IMD and are required for the activation of Relish, but do not act through dTAK1.

FADD molecules are best known as intracellular adaptors indispensable for FADD-mediated apoptotic signaling. FADD adaptors are death domain-containing proteins recruited to protein complexes following stimulation of death receptors, such as Fas and CD95 (Carrington et al., 2006; Cleveland and Ihle, 1995; Jeong et al., 1999). In mammals, death receptors engage ligands and stimulate the assembly of an intracellular complex containing Fas, FADD, and procaspases-8 and -10. This assembly is commonly known as DISC; death-inducing signaling complex. In this complex, procaspase-8 undergoes auto-processing, becomes activated and cleaves downstream effector caspases or the protein Bid leading to cell death (Desnoyers and Hengartner, 1997; Kaufmann and Hengartner, 2001). In *Drosophila*, this same complex includes dFADD and the caspase Dredd (Hoffmann, 2003; Kaneko and Silverman, 2005; Lu et al., 2001; Naitza and Ligoxygakis, 2004).

While most of our understanding of these pathways in insects has been based on *Drosophila* studies, much less is known about the pathways regulating immune signaling in mosquitoes. Phylogenetic studies suggest that while the genes involved in non-self recognition and intracellular signaling are highly conserved

between *Drosophila* and mosquitoes, the genes that encode effector AMPs show a greater level of diversification. The genes encoding the Toll and IMD signaling pathways appear to be principally conserved between *Drosophila* and mosquitoes, with a few important differences. Firstly, there are only two NF- κ B homologues in mosquitoes, REL1 and REL2 (homologous to *Drosophila* Dorsal and Relish, respectively). In mosquitoes, REL1 encodes two alternative transcripts that mediate the antifungal immune response (Shin et al., 2005), while REL2 encodes two (*Anopheles gambiae*) or three (*Aedes aegypti*) alternatively spliced transcripts that mediate immune responses against both Gram-positive and Gram-negative bacteria (Meister et al., 2005; Shin et al., 2003).

AMPs and NF- κ B transcription factors have been the subject of much research in mosquitoes (Bartholomay et al., 2004; Blandin et al., 2002; Lowenberger et al., 1999a,b,c; Shin et al., 2002, 2005, 2003). However, the upstream components of these immune signaling pathways have not been characterized. We previously demonstrated strong homology between two *Drosophila* caspases, DREDD and DRONC and two mosquito caspases, *Aedes* DREDD (Cooper et al., 2007a) and *Aedes* DRONC (Cooper et al., 2007b). Here we report the cloning and characterization of *Aedes* FADD (AeFADD) in the yellow fever mosquito, *A. aegypti*. AeFADD is a homologue of *Drosophila* dFADD and contains a C-terminal death domain and an N-terminal death-inducing domain. AeFADD expression is induced upon challenge with Gram-negative bacteria but not with Gram-positive bacteria. Bacterial challenge also induced the expression of two AMP genes, *cecropin* and *defensin*. The targeted knockdown of AeFADD expression, using RNAi, resulted in a reduced expression of both *cecropin* and *defensin*, and rendered adult mosquitoes susceptible to bacterial infection, indicating the essential role of AeFADD for defense against both *Escherichia coli* and *Micrococcus luteus*. The immune responses in *A. aegypti* differ from those in *Drosophila* which has significant implications to our understanding of immune responses to intracellular parasites such as viruses in insect vectors that transmit these viruses to humans.

2. Materials and methods

2.1. Insect maintenance, cDNA generation

A. aegypti (Liverpool strain) mosquitoes were reared as described previously (Lowenberger et al., 1999a,b,c). Total RNA was extracted from different developmental stages (larvae, callow pupae, black pupae, and whole adult mosquitoes) or from individual tissues (salivary glands, ovaries, midguts, and fatbody tissues). Tissues were dissected, rinsed in ice cold sterile *Aedes* saline solution (Hayes, 1953) and placed directly into Tri-reagent (Sigma, Oakville, ON, Canada). RNA extraction and purification was done as previously described (Lowenberger et al., 1999a). cDNA synthesis was done using 3 μ g of total RNA and an oligo dT primer (5'CGGGCAGTGAGCGCAACGT₍₁₂₎-3') in a 20 μ l reaction using Superscript III (Invitrogen, Carlsbad, CA, USA) at 50 °C (7). cDNA reactions were diluted to 200 μ l final volume before use in downstream applications.

2.2. Identification and sequencing of *Aedes* FADD

AeFADD was identified in a series of TBLASTN searches of the TIGR *Aedes* Gene Indices (<http://www.tigr.org/tdb/tgi/>) using *Aedes* DREDD, *Drosophila* dFADD and human FADD sequences. AeFADD was identified as an expressed sequence tag (EST) displaying significant sequence similarity to the death domain of *Drosophila*, human FADD and mouse Mort1. Primers were designed against the EST sequence and RACE PCR (Marathon cDNA amplification kit; Clontech, Mountain View, CA, USA) was used to obtain a single transcript with an open reading frame 1206 nt in length. Amplified

PCR products were cloned into pGEM T-Easy (Promega, Madison, WI, USA) and sequenced using Big Dye v3.1 chemistry (ABI Foster City, CA, USA).

2.3. Tissue specific and developmental expression

We used quantitative real-time PCR (qPCR) to determine tissue specific and developmentally regulated expression of target mRNAs. All qPCR reactions were performed with a Rotor-Gene 3000 (Corbett Research, Mortlake, NSW, Australia) using the Platinum SYBR Green Supermix-UDG (Invitrogen, Carlsbad, CA, USA). We used 2.5 µl cDNA template with 12.5 µl of Platinum SYBR Green Supermix, 1 µl (10 µM) *AeFadd* sense primer (5'-AGTATCGAGCAACGTTAGAGG-3'), 1 µl (10 µM) *AeFadd* antisense primer (5'-AAGGTGCTCCAATTGCGAC-3') in 25 µl reactions under the following conditions: 50 °C for 2 min, 95 °C for 2 min, followed by 35 cycles of 95 °C for 10 s, 61.5 °C for 15 s, 72 °C for 30 s. Quantity values were generated using the 2^{-ΔΔCT} method as described previously (Livak and Schmittgen, 2001). The expression of *AeFadd* was normalized using the housekeeping gene β-actin, which was determined previously to have stable expression levels under all conditions tested. All data represent triplicate runs of cDNAs generated from at least two independent experiments. qPCR screens for *cecropin* and *defensin* were determined under the same conditions using the following primers: *cecropin*-forward 5'-ATGAAGTTCACGAAGTTATTTCTC-3' and *cecropin*-reverse 5'-CTACTTTCTTAGAGCTTTAGCCC 3' and *defensin* forward: 5'-GCGA CCTGCGATCTGCTGAG-3' and reverse 5'-TCAATTCGACAGACGCA GACC-3'.

2.4. Production of recombinant AeFADD for use in immunoprecipitation assays

Recombinant AeFADD with an N-terminal Histidine-tag and Biotin-labeled AeDREDD and AeDRONC were generated using the TNT® T7 Quick for PCR Transcription/Translation system (Promega, Madison, WI, USA). A series of PCR reactions were used to generate a Histidine-AeFADD product that contained T7 promoter sequence-spacer sequence, N₍₁₀₎-Kozak sequence-AUG-6xHis – Gene of Interest and a 3' poly-A₍₃₀₎ tail. PCR reactions were also used to generate AeDredd and AeDronc products that contained T7 promoter sequence-spacer sequence, N₍₁₀₎-Kozak sequence-AUG-Gene of Interest and a 3' poly-A₍₃₀₎ tail.

PCR products were gel purified using the Qiagen gel purification kit (Qiagen, Mississauga, ON, Canada), cloned into pGEM T-Easy (Promega, Madison, WI, USA) and sequenced using Big Dye v3.1 chemistry. *In vitro* TNT transcription and translation reactions were performed using 500 ng of PCR product as suggested by the manufacturer. AeDREDD and AeDRONC proteins were labeled with Biotin for use with the Promega Transcend Chemiluminescent Non-Radioactive Translation Detection system (Promega, Madison, WI, USA).

2.5. Interaction assays using AeFADD, AeDRONC and AeDRONC

Immunoprecipitations were used to demonstrate adaptor–caspase interactions. Twenty-five microlitres of the recombinant His-AeFADD protein were combined with 25 µl of the AeDREDD or AeDRONC proteins and allowed to mix by gentle rotation for 2 h at room temperature. The mixture was then passed over a Mag-Z zinc column (Promega, Madison, WI, USA) according to manufacturer's instructions. The bound products were washed three times with 250 µl of wash/bind buffer and eluted with 100 µl of MagZ Elution buffer. Ten microlitres of the elution was electrophoresed on a 15% SDS-polyacrylamide gel and transferred to Hybond C+–extra nitrocellulose membrane (Amersham, Piscataway, NJ, USA) in

transfer buffer (25 mM Tris, 192 g glycine, 0.01% SDS, 20% methanol, H₂O to 1 L) for 60 min using the Bio-Rad semi-dry blotting system (Bio-Rad, Hercules, CA, USA). Membranes were pre-blocked overnight at 4 °C in 5% blocking solution (5% Carnation skim milk powder in 1 × TBST (Tris-buffered saline + 0.5% Tween-20)). To detect the His-tag products the membranes were incubated in 1:5000 dilution of mouse Histidine-monoclonal antibody (Novagen, Madison, WI, USA) for 50 min with gentle agitation, washed three times in 15 ml of 1 × TBST, 5 min per wash, and then incubated with a 1:10000 dilution of goat anti-mouse antibody-HRP conjugate (Novagen, Madison, WI, USA) for 50 min with gentle agitation. The membranes were washed three times in 15 ml of 1 × TBST and once in 15 ml ddH₂O, 5 min per wash. TNT products, AeDREDD and AeDRONC were detected using the Streptavidin-HRP conjugate supplied with the Transcend Chemiluminescent Detection kit. In brief, pre-blocked membranes were incubated in 1:10000 dilution of Transcend Streptavidin-HRP (Promega, Madison, WI, USA) for 1.5 h. Membranes were then washed three times in 15 ml of 1 × TBST and twice with 15 ml of ddH₂O. Both the His-HRP and the Streptavidin-HRP conjugates were detected using the Transcend Chemiluminescent substrates according to manufacturer's instructions and exposed using Kodak Biomax light-1 film. An interaction was considered positive if the caspase was identified in the elution fraction (from the Mag-Z column) with AeFADD.

2.6. Synthesis and microinjection of dsRNA

A 600 bp fragment of *AeFadd* was cloned into pGEM T-Easy (Promega, Madison, WI, USA) that contains SP6 and T7 promoter sites. Plasmids were linearized using *Nco*I or *Nde*I (Fermentas Burlington, ON, Canada) for 2.5 h at 37 °C. We used 5 µg of linearized plasmid to generate ssRNA products using SP6 and T7 RNA polymerase (Fermentas Burlington, ON, Canada) according to manufacturer's instructions. Transcription reactions were run for 1.5 h at 37 °C. ssRNA products were purified using the RNAaqueous 4-PCR kit (Ambion, Inc., Austin, TX, USA) as follows: 350 µl of Lysis/Binding solution and 250 µl of 64% ethanol were added to each transcription reaction, and the mixtures passed through the Ambion filter cartridges. Cartridges were washed twice with 500 µl of an 80% ethanol solution. RNA was eluted with 40 µl of preheated nuclease-free H₂O. Double-stranded RNA template was generated by mixing equal amounts of T7 and SP6 ssRNA templates and 40 units of RiboLock ribonuclease inhibitor (Fermentas Burlington, ON, Canada). The reaction was heat denatured at 65 °C for 5 min and cooled gradually to room temperature for 40 min. Product concentration was estimated using a spectrophotometer and RNA quality was visualized on a 1% agarose gel. dsRNA products were concentrated by ethanol precipitation and re-suspended in nuclease-free water to a final concentration of 3 µg/µl. A Drummond Nanoject was used to inject 184 nl (505 ng) of dsRNA into the thorax of 2-day old adult female mosquitoes. All control mosquitoes were injected with 500 ng of dsAuxin-1 RNA (*Arabidopsis*) generated as described above.

2.7. Efficiency of dsAeFadd RNA knockdown

The efficiency of *dsFadd* RNA knockdown was determined by measuring the expression levels of *AeFadd* and the AMP genes *cecropin* and *defensin* following sterile injection. Fatbody tissues were collected 24, 48, 72, and 96 h post-injection and placed directly in Tri-reagent for RNA extraction as described above. cDNA synthesis and the expression levels of *AeFadd*, and the AMP genes, *cecropin* and *defensin*, were determined using qPCR as described above.

2.8. Immune challenge and survival experiments

Immune challenges were performed by introducing *E. coli* (K12 strain) or *M. luteus* into the thorax of the mosquitoes as described previously (Lowenberger et al., 1999c). Inoculations with sterile LB medium were used as controls. Fatbody tissues were collected 24, 48, 72, and 96 h post-inoculation and placed directly in Tri-reagent for RNA extraction as described above. cDNA synthesis and the expression levels of *AeFadd*, *cecropin* and *defensin*, were determined using qPCR as described above. Survival experiments were carried out in mosquitoes injected with either dsFadd RNA or dsAuxin-1 control RNA. Groups of five mosquitoes, three per time point, were injected with 505 ng of either ds*AeFadd* RNA or control RNA and given 48 h to recover. At 48 h, mosquitoes were challenged with *E. coli* or *M. luteus* and monitored for 4 days.

3. Results

3.1. Sequence analyses

In a search for potential adaptor proteins for the previously described caspase-8 homologue *Aedes* DREDD (Cooper et al., 2007a), we identified a cDNA fragment predicted to encode a death domain-containing protein in the *A. aegypti* Gene indices

(<http://compbio.dfci.harvard.edu/tgi/tgipage.html>). Through RACE PCR we obtained a cDNA containing an open reading frame encoding a putative protein 402 aa in length, named *Aedes* FADD (AeFADD). This putative protein shares 99% identity with a putative protein named *Aedes* FADD, previously annotated by Waterhouse et al. (2007) and present in the NCBI Genbank as EAT46931. AeFADD has an overall structure similar to *Drosophila* dFADD (Hu and Yang, 2000). AeFADD shares 24% identity (62% similarity) with *Drosophila* FADD (dFADD) (Fig. 1a) and 39% identity (63% similarity) with an *A. gambiae* putative FADD protein (XP 308597). The greatest sequence similarity is found in the C-terminal death domain portion of the protein (aa 175–259) which shares 33% identity (62% similarity) with *Drosophila* and 31% identity (56% similarity) with both human FADD and mouse Mort1 (Fig. 1b). Little to no sequence similarity was found between the N-terminal region of AeFADD and any proteins in the NCBI database. However, when we used the BioinfoBank Metaserver (<http://bioinfo.pl/meta/>) to predict secondary structure, the program predicted a six α -helical bundle, similar in structure to the death-effector domain of human FADD and mouse Mort1. Because AeFADD is predicted to interact with the caspase AeDREDD, we examined the N-terminal death-effector domain of AeFADD and the N-terminal prodomain in AeDREDD for sequence homology. Limited sequence similarity was

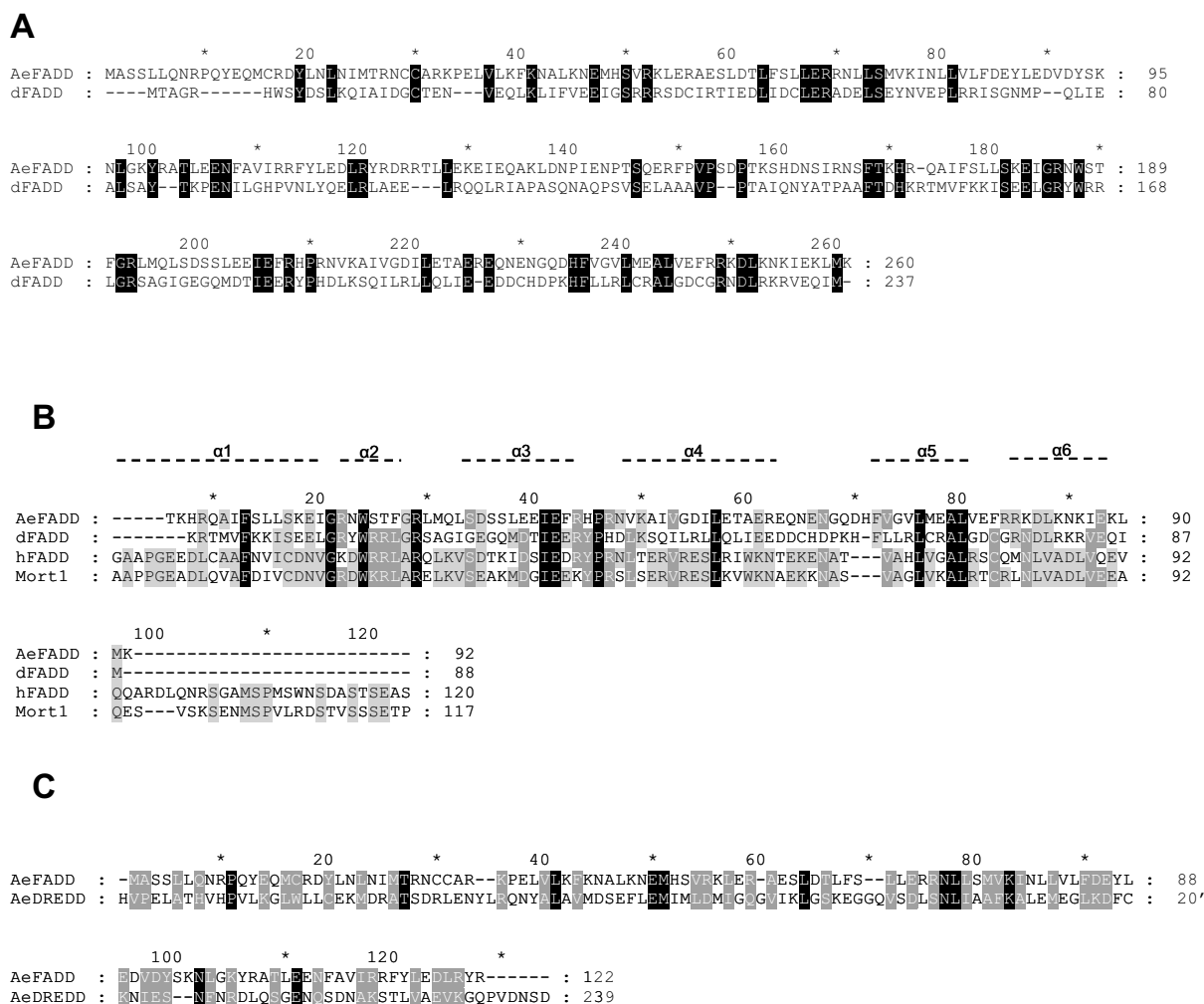


Fig. 1. Molecular characterization of *Aedes* FADD. (A) Alignment of *Aedes* FADD (AeFADD) with the closely related *Drosophila* dFADD. (B) Alignment of the C-terminal end of AeFADD with the C-terminal end of *Drosophila* dFADD, human FADD and mouse MORT1. The six bundle α -helices comprising the C-terminal death domain are marked by dashed lines. (C) Alignment of N-terminal death-inducing domain of AeFADD and the second N-terminal death-inducing domain of the caspase *Aedes* DREDD (AeDREDD). Residues highlighted in black are shared by all sequences. Those in grey highlight amino acids sharing similar properties. Alignments were constructed using Clustal X and are manually adjusted.

identified between the predicted N-terminal death-effector domain in AeFADD and a region predicted to be the second death-inducing domain in the caspase AeDREDD (10% identity and 34% similarity) (Fig. 1c). As was the case with AeFADD, we found no similarity between the N-terminal end of AeDREDD and any proteins in the NCBI database. However, an analysis of secondary structure predicted two six α -helical bundles similar in structure to the death domains of many other apoptotic proteins. These domains are similar to the death-inducing domains identified in *Drosophila* DREDD (Chen et al., 1998).

3.2. Tissue-specific and developmental expression of AeFadd

Quantitative Real-Time PCR (qPCR) revealed that *AeFadd* transcripts were detected in all developmental stages screened in this study. Transcript levels were highest in callow pupae; up to 100-fold higher than levels found in early instar larvae (Fig. 2a). *AeFadd* was found at low levels in all adult tissues with highest levels found in gut and fatbody tissues; threefold higher than levels found in salivary gland tissues (Fig. 2b).

3.3. AeFADD associates with AeDREDD

As a putative adaptor protein, AeFADD is predicted to recruit the caspase AeDREDD and *in vitro* interaction assays were used to demonstrate this interaction. The assays indicate that AeFADD interacts specifically with AeDREDD but not with the caspase AeDRONC (Fig. 3). This suggests that the interaction is mediated by the death-inducing domains present in the N-terminal ends of both proteins, as is seen with FADD-caspase associations in other systems (Cleveland and Ihle, 1995).

3.4. AeFadd, cecropin and defensin expression is up-regulated upon bacterial challenge

We examined the expression profiles of *AeFadd*, *cecropin* and *defensin* following challenge with *E. coli* and *M. luteus*. *AeFadd* transcription was up-regulated fourfold 24 h after challenge with *E. coli*, but did not change in response to challenge with *M. luteus* (Fig. 4a). Bacterial challenge resulted in the up-regulation of both *cecropin* and *defensin* (Fig. 4b). After challenge with *E. coli*, *cecropin* expression was up-regulated twofold at 8 h and 120-fold at 24 h, while *defensin* expression was up-regulated fourfold by 24 h. Challenge with *M. luteus* resulted in an eightfold up-regulation in *cecropin* expression by 24 h. In contrast, *defensin* expression was

up-regulated 80-fold at 8 h but reduced to sixfold at 24 h after challenge with *M. luteus*.

3.5. AeFadd is required for the inducible expression of cecropin and defensin

To establish a role for *AeFadd* in mosquito immune signaling, we used RNAi to knockdown *AeFadd* expression in adults. When dsRNA complementary to *AeFadd* was injected into 2-day old adult females, the mRNA levels of *AeFadd*, *cecropin*, and *defensin* were significantly reduced (Fig. 5). There was an 80–90% knockdown of *AeFadd* by day 3 post-injection that lasted for at least 5 days. The transient knockdown of *AeFadd* also resulted in a marked decrease in the expression of both *cecropin* and *defensin* by 48 h post-injection and this was maintained for 5 days. This knockdown effect was not seen in mosquitoes injected with the dsRNA control molecule obtained from *Arabidopsis thaliana* (Fig. 5).

3.6. AeFadd is required for antibacterial immunity in vivo

The survival of *AeFadd* knockdown insects was compared after challenge with *E. coli* and *M. luteus* bacteria. The *AeFadd* knockdown mosquitoes were significantly more susceptible to both *E. coli* with 40% mortality observed at 24 h, and >80% mortality by 48 h post-challenge, and *M. luteus* with 20% mortality at 24 h and >60% mortality by 48 h post-challenge than the control groups (Fig. 6). All dsRNAi-treated mosquitoes challenged with bacteria were dead 96 h post-challenge.

4. Discussion

Insects are remarkably resistant to microbial infections. In general, insects mount a multifaceted response against invading microorganisms, and use cellular mechanisms, proteolytic cascades, and the release of reactive oxygen species to eliminate invading microorganisms (Ferrandon et al., 2007, 2004; Lemaitre and Hoffmann, 2007). The best studied arm of insect immune defenses is the humoral response of which a key component is the expression of small cationic antimicrobial peptides synthesized mostly by the fatbody and secreted into the hemolymph. At least seven AMPs have been characterized in *Drosophila*. These include the antifungal peptides Drosomycin and Metchnikowin, the anti-Gram-negative peptides Attacins, Cecropins, Dipterocins and Drosocin, and the anti-Gram-positive peptide Defensin (Ferrandon et al., 2007, 2004; Lemaitre and Hoffmann, 2007).

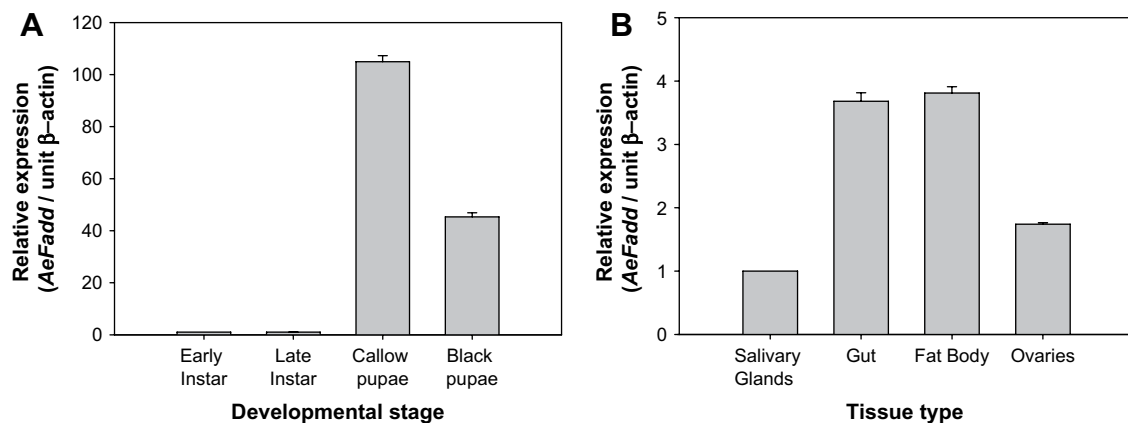


Fig. 2. Relative expression of *AeFadd* in (A) different developmental stages and (B) individual adult tissues, using Quantitative Real-time PCR. Early instar larvae and salivary gland tissues were designated as the standard and arbitrarily set to 1. The vertical axis represents the fold increase in transcript number in different developmental stages or different adult tissues relative to their given control. *AeFadd* values were normalized using the housekeeping gene β -actin.

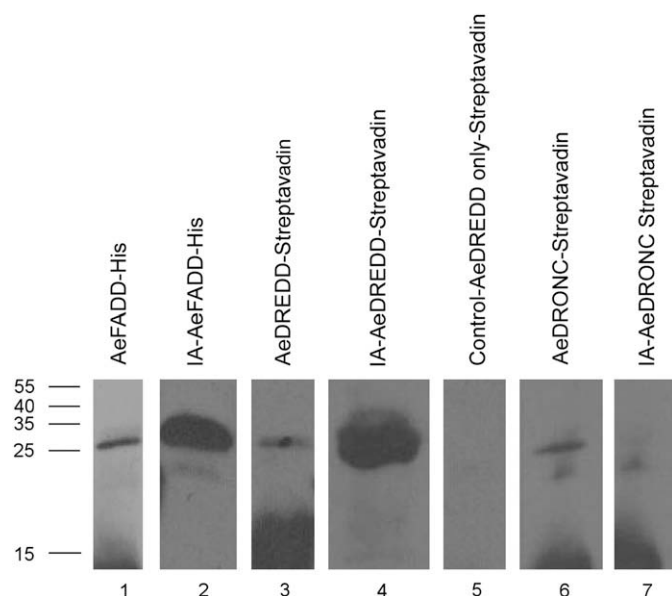


Fig. 3. *In vitro* interaction assay showing that AeFADD specifically associates with the caspase *Aedes* Dredd (AeDREDD) and not *Aedes* Dronc (AeDRONC). Interactions assays were carried out using recombinant AeFADD, AeDREDD and AeDRONC proteins produced using the Promega TNT T7 Quick for PCR Transcription/Translation and the Promega Transcend Non-radioactive translation detection system. The recombinant AeFADD contained an N-terminal His-tag, while AeDREDD and AeDRONC were labeled with Biotin. The interaction assays involved mixing equal volumes of His-AeFADD protein with either AeDREDD or AeDRONC and purified using a Mag-Z zinc column (Promega). Elution products were analyzed by SDS PAGE and western analysis using anti-His antibodies for AeFADD or a Streptavidin antibody for AeDREDD or AeDRONC. Lane 1: AeFADD TNT protein (1 μ l). Lane 2: AeFADD eluted from the column following the interaction assay (10 μ l). Lane 3: AeDREDD TNT protein (1 μ l). Lane 4: AeDREDD eluted from column following interaction assay (10 μ l). Lane 5: Control: AeDREDD eluted from column without prior interaction with AeFADD (10 μ l). Lane 6: AeDRONC TNT protein (1 μ l). Lane 7: AeDRONC eluted from column following interaction assay (10 μ l).

Within the IMD pathway, a key event in AMP induction is the nuclear translocation of the NF- κ B transcription factor, Relish (Kaneko and Silverman, 2005; Silverman and Maniatis, 2001). At present, the precise sequence of events that leads to the cleavage and activation of Relish is not completely understood; however, activation of the IMD pathway triggers two distinct cascades that ultimately target Relish. Of particular interest here, bacterial sepsis

triggers the formation of the receptor–adaptor complex containing the death domain-containing protein IMD, the adaptor molecule dFADD, and the caspase Dredd (Hu and Yang, 2000; Naitza and Ligoxygakis, 2004; Silverman et al., 2000; Stoven et al., 2003). The dFADD–DREDD complex is required for Relish activation (Hu and Yang, 2000; Stoven et al., 2003) and may also be required for the second IMD cascade, which involves the recruitment of dTAK1, a homologue of the mammalian MAP3 kinase TAK1 (Hoffmann, 2003; Kaneko and Silverman, 2005; Park et al., 2004).

FADD adaptors typically contain an N-terminal death-effector domain (or death-inducing domain in *Drosophila*) and a C-terminal death domain (Carrington et al., 2006; Hu and Yang, 2000). In *Drosophila*, dFADD has been shown to interact with the caspase DREDD through N-terminal death-inducing domain interactions (Hu and Yang, 2000; Naitza et al., 2002). The N-terminal end of AeFADD and the second putative DID domain in the N-terminal region of the initiator caspase AeDREDD (Cooper et al., 2007a) reveals 10% shared identity and 34% similarity (Fig. 1c), suggesting that AeDREDD is a potential interacting partner. In this study, AeFADD interacted specifically with AeDREDD but not with the initiator caspase AeDRONC suggesting that the function of the AeFADD/AeDREDD complex may be conserved *in vivo* in mosquitoes.

AeFadd transcripts were found in all developmental stages ranging from relatively low levels of expression during early and late larval stages and significantly higher levels in callow and black pupae (Fig. 2a), and corroborates the low expression levels of BG4 in larvae previously reported (Bryant et al., 2008). The significant increase in *AeFadd* expression in callow pupae, but not in the late larval stages, suggest that the AeFADD protein may play a role in mosquito development but that it is not mediated by the same ecdysone developmental cues seen with other mosquito apoptotic genes, such as *AeDronc* (Cooper et al., 2007b). *AeFadd* transcripts were detected in greatest levels in fatbody and gut tissues (Fig. 2b), both of which have high cell turnover rates and are immune-competent tissues known to express AMPs.

Many studies examining the role of IMD signaling in mosquitoes have focused primarily on the role of the NF- κ B transcription factor, REL2 (Meister et al., 2005; Shin et al., 2003). In mosquitoes, REL2 is involved in the inducible expression of *cecropin*, *defensin*, and *gambicin*, after bacterial challenge (Bartholomay et al., 2004; Blandin et al., 2002; Kaufmann and Hengartner, 2001; Meister et al., 2005; Shin et al., 2005, 2003). While a role for REL2 has been

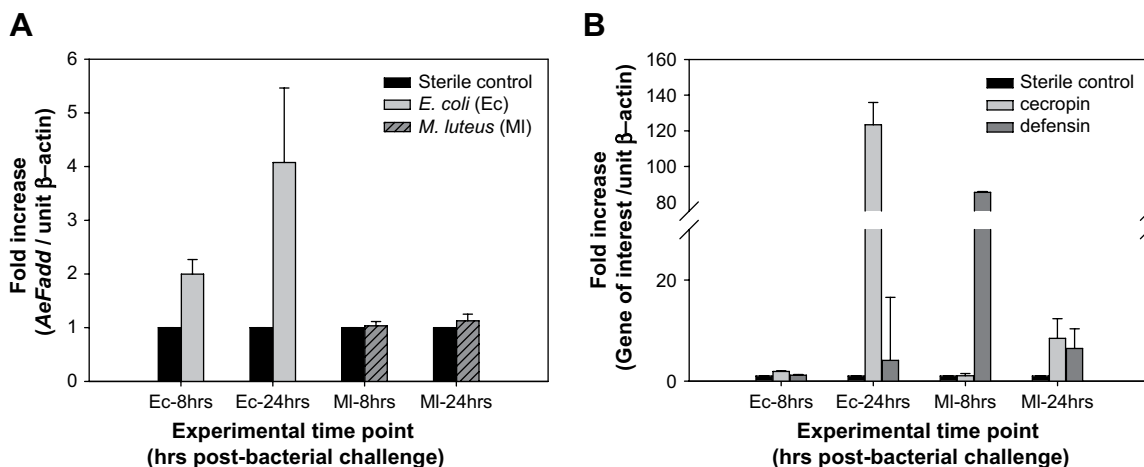


Fig. 4. Relative expression of *AeFadd* and AMPs during bacterial challenge using Quantitative Real-time PCR. (A) *AeFadd* expression in the fatbody tissues of adult mosquitoes at 8 and 24 h following *E. coli* (plain bars) or *M. luteus* (cross-hatched bars). (B) Relative expression of *cecropin* and *defensin* following challenge with *E. coli* (plain bars) or *M. luteus* (cross-hatched bars). Mosquitoes challenged with sterile LB medium were used as controls. The vertical axis represents fold increase over sterile controls. All genes were normalized using the housekeeping gene β -actin. Data represent cDNAs generated from three separate experiments. Error bars represent standard deviations.

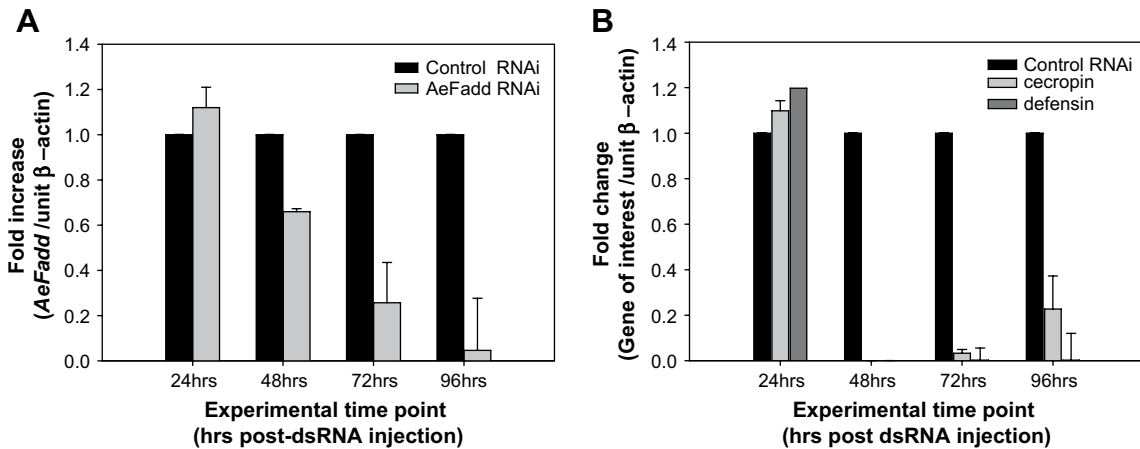


Fig. 5. Efficiency of RNAi-mediated knockdown of *AeFadd*. (A) Targeted knockdown of *AeFadd* expression in the fatbody tissues of adult mosquitoes produced a transient knockdown that lasted at least 5 days. (B) *AeFadd* function is required for the expression of both *cecropin* and *defensin* in response to sterile injection. RNAi effectiveness was determined using qPCR to quantify transcript levels. Adult mosquitoes injected with a plant dsRNA product (*Arabidopsis auxin-1*) are the controls. All experimental samples are expressed as fold increase relative to the dsRNA controls. All genes were normalized using the housekeeping gene β -actin. Data represent cDNAs generated from two separate experimental trials. Error bars represent standard deviations.

established in AMP expression, the role of upstream signaling molecules, such as *AeFADD* is unknown. In this study, *AeFadd* expression was up-regulated fourfold in response to challenge with *E. coli* but did not change in response to *M. luteus* (Fig. 4a), and as expected, challenge with both bacteria induced the expression of *cecropin* and *defensin* (Fig. 4b). Knocking down *AeFadd* expression using RNAi resulted in a significantly reduced (>80%) expression of both *cecropin* and *defensin* (Fig. 5b) indicating that *AeFadd* is required to induce AMP expression. These knockdown mosquitoes also were unable to express either *cecropin* or *defensin* in response to bacterial challenge and died within 72 h. In contrast, insects treated with the control dsRNA construct expressed normal levels of *cecropin* and *defensin* and survived the immune challenge (Fig. 6). Adult knockdown mosquitoes were more sensitive to *E. coli*, with most mortality observed within 24 h of injection but all knockdown mosquitoes died within 4 days of injection. These data suggest that *AeFadd* is required for REL2 processing and AMP expression, and for successful immune responses against both *E. coli* and *M. luteus* bacteria.

Our findings support previous studies that have established an IMD-specific REL2 function in mosquitoes. Shin et al. (2003)

established that transgenic *A. aegypti* over-expressing a dominant negative form of one REL2 splice variant (C8) was sensitive to the Gram-negative but not to the Gram-positive bacteria tested. Similarly, Meister et al. (2005) established that the targeted knockdown of both *A. gambiae* REL2 splice variants, REL2-S and REL2-F, resulted in increased sensitivity to both the Gram-negative and Gram-positive bacteria tested. The *Aedes* REL2 C8 variant is homologous to the *Anopheles* REL2-S variant, and both are required for anti-Gram-negative immunity while the *Anopheles* REL2-F variant is required for anti-Gram-positive immunity. The targeted knockdown of a more apical signaling molecule in the IMD pathway, such as *AeFADD*, should abolish REL2 processing outright, rendering mosquitoes susceptible to infections by both Gram-negative and Gram-positive bacteria as we have demonstrated here.

Our understanding of the innate-immune signaling pathways that are activated during immune challenge has increased considerably in recent years. A key advantage of studying innate immunity in mosquitoes is that we can study an integrated system in a whole organism in the absence of adaptive immune responses. Most studies of vector-parasite interactions have evaluated the immune response to parasites that freely traverse the hemocoel of the vector as they move towards the salivary glands or mouthparts for transmission. Less well studied are the responses to intracellular parasites against which AMPs and phagocytosis may not be major factors. The responses to clear these infections may be mediated by other immune factors and pathways that lead to responses such as apoptosis. It is becoming apparent that certain molecules and pathways may play roles in different but complimentary immune responses (i.e. apoptosis and IMD signaling). Teasing apart these interactions are paramount to our overall understanding of immune responses in mosquitoes. This will allow us to study in detail the direct and indirect interactions between vectors and the different pathogens they transmit, and what factors determine the compatibility between a vector and a specific pathogen, all of which may provide valuable insights into the evolution of innate immunity in general.

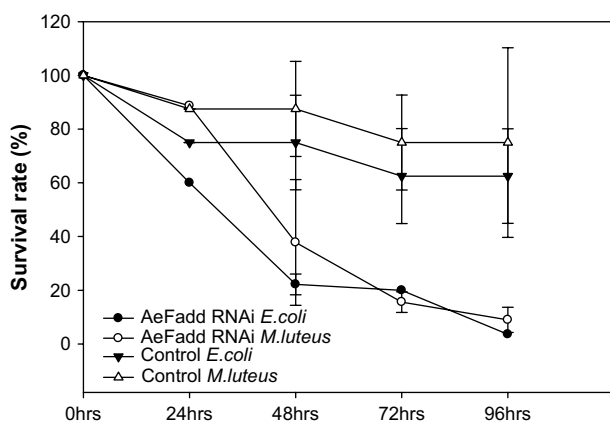


Fig. 6. *AeFadd* is required for antibacterial immunity in *Aedes aegypti*. Survival of adult mosquitoes following challenge with *E. coli* or *M. luteus*. Loss of *AeFadd* function rendered mosquitoes susceptible to Gram-negative infection (closed circles) and Gram-positive infection (open circles). Control mosquitoes (injected with dsAuxin-1RNA) were able to survive infection (closed and open triangles). Three groups of five were challenged at each time point and the survival assay was repeated twice. Error bars represent standard deviation.

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