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The Role of NF- κ B Factor REL2 in the *Aedes aegypti* Immune Response

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Abstract

Mosquitoes transmit numerous diseases that continue to be an enormous burden on public health worldwide. Transgenic mosquitoes impervious to vector-borne pathogens, in concert with vector control and drug and vaccine development, comprise an arsenal of means anticipated to defeat mosquito-spread diseases in the future. Mosquito transgenesis allows tissue-specific manipulation of their major immune pathways and enhances the ability to study mosquito-pathogen interactions. Here, we report the generation of two independent transgenic strains of *Aedes aegypti* overexpressing the NF- κ B transcriptional factor REL2, a homologue of *Drosophila* Relish, which is shown to be under the control of the vitellogenin promoter in the mosquito fat body after a blood meal. We show that this REL2 overexpression in the fat body results in transcriptional activation of Defensins A, C, and D, and Cecropins A and N, as well as translation and secretion of Defensin A protein into the hemolymph. We also demonstrate that induction of REL2 results in the increased resistance of the mosquito to tested Gram-negative and Gram-positive bacteria. Importantly, induction of transgenic REL2 leads to the significant decrease in susceptibility of *Ae. aegypti* to *Plasmodium gallinaceum* infection. Consistently, RNAi knockdown of REL2 in wild-type mosquitoes results in a delay in Defensin A and Cecropin A expression in response to infection and in increased susceptibility to both bacteria and *P. gallinaceum*. Moreover, our transgenic assays demonstrate that the N-terminus of the mosquito REL2, which includes the His/Gln-rich and Serine-rich regions, plays a role in its transactivation properties.

Keywords

Mosquito; transgenesis; antimicrobial peptides; *Plasmodium*; Relish; Rel transactivation domain

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1. Introduction

Despite attempts at intervention, malaria continues to kill more than 2 million people annually, worldwide. Lack of an effective anti-malarial vaccine, growing drug resistance of the malaria parasite, and increasing insecticide resistance of the mosquito vector all contribute to the intransigence of this problem. It is therefore necessary to find alternative ways to control mosquito-spread diseases. Transgenic mosquitoes that are resistant to the malaria parasite, vector control, and drug and vaccine development comprise an arsenal of methods anticipated to defeat malaria in the future. Transgenic modification of the mosquito vector requires profound knowledge of mosquito immunity, which can be achieved through detailed studies using model experimental systems.

The insect immune system consists of structural, cellular, and humoral components (Lemaitre and Hoffmann, 2007). The humoral innate immunity in insects is controlled by signal transduction pathways that resemble immune pathways of mammals (Hoffmann and Reichhart, 2002; Hoffmann, 2003; Lemaitre and Hoffmann, 2007). In both insects and mammals, pattern recognition receptors (PRRs) recognize pathogens and participate in the activation of signaling immune pathways. Such activation results in the nuclear translocation of NF- κ B/REL transcriptional factors. Finally, NF- κ B proteins induce an array of genes encoding immune effector molecules, including antimicrobial peptides (AMPs) (Ghosh et al., 1998; Baeuerle and Baltimore, 1996; Hoffmann and Reichhart, 2002; Hoffmann, 2003; Lemaitre and Hoffmann, 2007). Two well-characterized immune signaling pathways lead to the activation of NF- κ B factors in *Drosophila*: Toll and Immune-deficiency (Imd). The Toll pathway, responding to fungi and most Gram-positive bacteria, is similar to mammalian Toll-like receptors (TLRs) and IL-1 receptor signaling pathways. The Imd pathway responds mostly to Gram-negative bacteria and is similar to the mammalian tumor-necrosis factor (TNF) immune pathway (Hoffmann, 2003). Signaling through the Toll pathway leads to activation and nuclear translocation of NF- κ B factors Dorsal and Dif. Signaling through the Imd pathway results in cleavage and nuclear translocation of the third NF- κ B transcriptional factor, Relish (Hedengren et al., 1999; Stöven et al., 2000). *Drosophila* Relish has been shown to be a major regulator of AMPs. These NF- κ B factors induce the transcription of all 21 AMPs encoded in the *Drosophila* genome (Lemaitre and Hoffmann, 2007). Additionally, Relish synergistically cooperates with Dif in transcriptional activation of AMPs (Han and Ip, 1999; Tanji et al., 2007).

Sequencing and annotation of the *Anopheles gambiae* (Holt et al., 2002; Christophides et al., 2002) and *Aedes aegypti* (Nene et al., 2007; Waterhouse et al., 2007) genomes have revealed that mosquitoes lack the NF- κ B factor Dif. Therefore, they rely on two NF- κ B factors: Dorsal orthologue REL1 and Relish orthologue REL2. These mosquito REL factors are involved in immunity, and several studies have shown that signaling through REL1 and REL2 in the mosquito activates AMPs (predominantly Cecropins and Defensins) and anti-*Plasmodium* molecules such as CTL-1, LRIM-1, and TEP-1 (Osta et al., 2004; Meister et al, 2005; Shin et al., 2005; Frolet et al, 2006; Xi et al., 2008).

Ae. aegypti REL2 has three isoforms that are products of an alternative splicing of the *Rel2* gene (Shin et al., 2002). The predominant isoform of the *Aedes* REL2 (REL2-Long) contains the His/Gln (Q/H)-rich and SRR regions, the REL-homology (RHD and IPT) domains, the inhibitor I κ B-like ankyrin domain, and the Death domain. This form is similar to *Drosophila* Relish and the mammalian p105 and p100 Rel/NF- κ B transcription factors. The second (REL2-Short) isoform is similar to REL2-Long, although it lacks the ankyrin and Death domains. The third (I κ B-type) transcript has an I κ B-like domain intact, but lacks most of the N-terminal sequence and RHD domain. *An. gambiae* *Rel2* is similar to *Ae. aegypti* *Rel2*; however, it gives rise to only two isoforms—Long (Full) and Short (Meister et al, 2005). Interestingly, two Relish

isoforms with similar features to mosquito REL2-Long (Full) and REL2-Short isoforms have also been identified in the silkworm *Bombyx mori* (Tanaka et al., 2007).

In *An. gambiae*, REL2 is involved in regulating expression of Cecropin1, Cecropin3, and Gambicin, and also the *Plasmodium* antagonist LRIM1. Moreover, Anopheles REL2 has been implicated in the mosquito defense against Gram-negative and Gram-positive bacteria, and the rodent malaria parasite *Plasmodium berghei* (Meister et al., 2005). These authors further showed that the Anopheles REL2-F (Long) and REL2-S (Short) isoforms are involved in resistance against the Gram-positive *Staphylococcus aureus* and the Gram-negative *Escherichia coli* bacteria, respectively. The specific roles of *Ae. aegypti* REL2 isoforms still remain unknown.

Mosquito transformation has become a valuable tool in deciphering anti-pathogen immunity. Several prior studies have reported successful mosquito transformations using transposable elements that allow incorporation of genes of interest into the mosquito genome under control of tissue-specific promoters (Coates et al., 1998; Jasinskiene et al., 1998; Moreira et al., 2000; Kokoza et al., 2000; Kokoza et al., 2001a; Shin et al., 2003; Ito et al., 2004; Kim et al., 2004; Bian et al., 2005; Franz et al., 2006; Smith et al., 2007). Fat-body-specific *Vitellogenin* (*Vg*) is one of the best studied promoters, allowing strong tissue specificity and blood-meal-controlled expression of the genes of interest (Kokoza et al., 2000; Kokoza et al., 2001b; Nirmala et al., 2006). Previously, we successfully created a transgenic *Ae. aegypti* mosquito that overexpressed an N-terminally truncated form of REL2-Short in the fat body after a blood meal (Shin et al., 2003). It demonstrated a Relish Immune Deficient (RIMD) phenotype, in which the expression of Defensin A and Cecropin A in response to bacterial infection was abolished, and the mosquitoes were highly susceptible to Gram-negative bacteria. Thus, the essential role of *Aedes* REL2 in AMP production and antibacterial defense was proven functionally (Shin et al., 2003).

In this study, we employed mosquito transgenesis to generate two *Ae. aegypti* independent transgenic strains overexpressing REL2 in the mosquito fat body after a blood meal (PBM) to further study properties of REL2 in the mosquito *Ae. aegypti*. We used the complete REL2-Short isoform for the transgenic construct, because it lacks the I κ B ankyrin domain and is therefore available for nuclear translocation and transcription initiation without additional cleavage. Here, we report the effect of overexpression of the REL2-Short isoform, under the *Vg* promoter PBM, on the expression of AMPs, and antimicrobial and anti-*Plasmodium* resistance. Furthermore, we evaluated the results obtained through studying the transgenic phenotype by conducting REL2 RNAi study in wild-type mosquitoes. To our knowledge, this is the first report to describe effects of transgenic overexpression of REL2 in the adult mosquito *in vivo*.

2. Materials and methods

2.1. Mosquito rearing

The *Ae. aegypti* wild-type UGAL/Rockefeller strain and two transgenic strains, REL2-A and REL2-B (REL2 gain-of-function strains), were maintained in a laboratory culture at 27°C and 85% humidity. Larvae were provided with standardized larvae diet “mosquito chow”, consisting of ground and sifted rat food, yeast, and lactalbumin in a ratio of 1:1:1 by weight (Hays and Raikhel, 1990). Adult mosquitoes were fed on 10% glucose and water. For all experiments, 4-day-old previtellogenic females fed blood on White Leghorn chickens.

2.2. Transformation plasmid and germ-line transformation

For the mosquito germ-line transformation, we used the pBac[3×P3-EGFP, afm] transformation vector containing the eye-specific promoter (3×P3) in front of the TATA box and enhanced green fluorescent protein (EGFP), as described by Horn and Wimmer (2000). The 2.14-kb full length of the REL2-Short isoform was sub-cloned between the *Vg* promoter and SV40 polyadenylation element. This construct was introduced into the pBac[3×P3-EGFP, afm] vector at the unique *AscI* cloning site. The resulting plasmid pBac[3×P3-EGFP, afm, *Vg-REL2*] was used to inject into mosquito embryos. *Ae. aegypti* germ-line transformation was carried out as described by Kokoza et al. (2001a). Of the 1000 embryos injected, 100 live F₀ mosquitoes were recovered, and 18 female and 38 male mosquito families were organized. Then, the F₁ progeny was examined for green fluorescent glowing in the eyes. Two EGFP-expressing mosquitoes from two different families were found, crossed to wild-type mosquitoes, and gave rise to two independent transgenic mosquito lines. Therefore, the germ-line transformation resulted in establishing two independent transgenic REL2-gain-of-function mosquito lines called REL2-A and REL2-B.

2.3. Southern blot analysis

Genomic DNA was extracted from UGAL (wild type), REL2-A, and REL2-B mosquitoes using the DNeasy Tissue kit (Qiagen; Valencia, CA, USA), according to the manufacturer's instructions. For each mosquito strain, 15 µg genomic DNA was digested using *EcoRI* (Roche, Indianapolis, IN, USA) and *AscI* (NEB, Ipswich, MA, USA). DNA was precipitated, restored in 20 µl of water, and loaded on a 1% agarose gel for DNA band separation. After transfer to a nitrocellulose membrane, the DNA was hybridized with the DNA probes for right vector arm, left vector arm for *EcoRI*-restricted DNA, and the internal SV-40 polyadenylation region for *AscI*-restricted DNA.

2.4. Northern blot analysis

Total RNA was extracted from 15 female mosquitoes per time point using the Triazol method (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. A total of 5 µg RNA was used for formaldehyde gel electrophoresis followed by Northern blot and successive hybridization with *Vg*, REL2, Defensin A, and Cecropin A DNA probes. Ribosomal RNA was used as a loading control.

2.5. dsRNA production and RNAi injections

For production of dsRNA, primers against the *Ae. aegypti* REL2 RHD domain with attached T7 minimal primers were designed. They were chosen so that the PCR product would yield a 500-nucleotide-long fragment. PCR was performed using total cDNA from infected mosquitoes and a High Fidelity *Taq* Polymerase kit (Invitrogen, Carlsbad, CA, USA). The PCR product was loaded on 1x TBE gel, run for 30 min at 100 mV. The bands of expected size were cut out, and the DNA product was purified using a Qiagen gel extraction kit (Qiagen, Valencia, CA). Non-specific dsRNA against the luciferase gene from firefly (*Photinus pyralis*) was used as a control. The double-stranded RNA was produced by *in vitro* transcription with the T7 RNA polymerase using the MEGAscript T7 kit (Ambion, Austin, TX, USA). Approximately, 1 µg dsRNA in 0.5 µl of distilled water was injected into the thorax of 1-day-old CO₂-anesthetized female mosquitoes. The dsRNA-treated mosquitoes were allowed to recover for 3 days and then were used for the experiments.

2.6. Reverse transcription and PCR

To study expression dynamics of endogenous REL2 isoforms, REL1, and AMPs in the transgenic mosquitoes, total RNA was extracted from 4-day-old individual transgenic and wild-type mosquitoes—previtellogenic, 12 h and 24 h PBM using the Triazol method

(Invitrogen). The RNA from wild-type mosquitoes infected with *Enterobacter cloacae* and dissected at 24 h post-infection was used as a control. The RNA samples were treated with DNase (DNase Amplification grade, Invitrogen), and cDNAs were synthesized using Omniscript Reverse Transcriptase kit (Qiagen). cDNAs from individual mosquitoes were then tested for the presence of an infection marker, Cecropin A, after 30 cycles of PCR. Those mosquito cDNAs that showed no Cecropin A bands of an intensity comparable to the band of the control cDNA were considered to be free from infection and were pooled together. PCR with specific primers was performed under the following conditions: 35 cycles for detection of Cecropins, Dipterin, Gambicin, Holotricin, and Attacin; 30 cycles for the detection of Defensins, REL2-Long and REL2-Short isoforms, transgenic REL2, and REL1.

To evaluate efficiency of the REL2 isoform knockdowns in the mosquitoes treated with dsRNA against REL2, at 4 days after the RNAi treatment, total RNA was extracted from the fat bodies of experimental and control mosquitoes using the Triazol method (Invitrogen). RNA pooled from 12 mosquitoes was used for the experiment. The RNA samples were treated with DNase (DNase Amplification grade, Invitrogen), and cDNAs were synthesized as described above. Reverse transcription was performed with the primers specific for REL2-Long and REL2-Short isoforms as follows: REL2-Long forward primer 5'-CGTTCAACAGTCATCATGTCAACAC-3', REL2-Long reverse primer 5'-CACTTTTGCATCTCAAAGTAGTTC-3'; REL-Short forward 5'-CGTTCAACAGTCATCATGTCAACAC-3, REL2-Short reverse 5'-GATTCATCGTGTGCTACTCACTTG-3'; REL1-A forward 5'-ACGAAAGAAACGCAAGCCTA-3', REL2-A reverse 5'-ATCGTTCGTCGCTTCTCAGT-3'.

For the detection of Defensin A and Cecropin A mRNA levels in the infected mosquitoes, RT-PCR was performed with Defensin A- and Cecropin A-specific primers as follows: Defensin A forward 5'-GATCCCGAAAGGACCAACCATGC-3', Defensin A reverse 5'-ATTCCGGCAGACGCACACCTT-3'; and Cecropin A forward 5'-ATGAACTTCACGAAGTTATTT-3', Cecropin A reverse 5'-CTTTCTTAGAGCTTTAGCCCC-3'. For all experiments, the PCR products of Actin amplification were used as the PCR amplification and loading control.

2.7. Western blot analysis

Proteins were extracted from mosquitoes' whole bodies, fat bodies, and ovaries using the Triazol extraction method, according to the manufacturer's instructions (Invitrogen). For each hemolymph sample, hemolymph from 12 mosquitoes was collected by dissecting 3 female mosquitoes in 21 μ l of 1 \times phosphate buffered saline (PBS) (Cambrex, Charles City, USA) solution with 1 \times protease inhibitor (Complete Mini, Roche). Mosquitoes were dissected, and their internal organs washed in the dissection solution. A drop containing the mixture of PBS and the mosquito hemolymph was loaded on QIA shredder spin columns (Qiagen). The procedure was repeated four times, and then the hemolymph from all 12 mosquitoes was centrifuged at 3000-rcf for 1 min. The flow-through from the column was then centrifuged at 500-rcf to separate cell and serum components of the hemolymph. Next, the supernatant was carefully removed and used for Western blot analysis. For both tissue and hemolymph samples, protein concentration was measured using the Bradford method, and 5 mg protein was loaded on NuPAGE 12% Bis-Tris protein gel (Invitrogen). SDS-PAGE and protein transfer were performed using the NuPAGE Western blot system (Invitrogen). The Defensin protein was detected with Anti-DefensinA IgG (Cho et al., 1996), in 1:5000 dilutions. Immunoblots were developed using the SuperSignal West Pico detection system (Pierce, Rockford, IL, USA).

2.8. Survival experiments

To conduct survival experiments for REL2 transgenic mosquitoes, 4-day-old females were blood fed on White Leghorn chickens and then bacteria challenged at 6–10 h PBM. Bacteria *Pseudomonas aeruginosa* (ATCC 012406/10145) and *Enterobacter cloacae* were grown in Luria Bertani media (Sigma-Aldrich, St-Louis, MO, USA), and the following concentrations were used for bacterial challenge: *P. aeruginosa* OD₆₀₀ = 0.6, *E. cloacae* OD₆₀₀ = 2.8. *Staphylococcus aureus* (ATCC 133011) was grown in Nutrient Broth (Difco 23400), and a culture of *S. aureus* with OD₆₀₀ = 4.5 was used for injections. Bacterial infections were done by pricking mosquitoes in the rear part of the abdomen with an acupuncture needle (0.2 × 25mm) dipped into the bacterial culture. A group of 32 blood-fed females was used for each experiment, which was performed at least three times.

To conduct the survival experiments in the REL2 RNAi mosquitoes, the REL2 dsRNA-treated and control iLuc mosquitoes were injected with bacteria *Enterobacter cloacae* or *Enterococcus faecalis* on the 4th day-post-RNAi treatment. *E. cloacae* were grown in Luria Bertani (LB) media (Sigma-Aldrich, St-Louis, MO, USA) to OD₆₀₀ = 2.8 and then diluted 1:1000 times in the sterile LB media (Sigma). *E. faecalis* (ATCC 19433) were grown in Brain Heart Infusion (BD 237500) to OD₆₀₀ = 3.2 and then diluted 1000 times in PBS (Cambrex, East Rutherford, NJ, USA). A total of 0.5 µl of the bacteria-containing LB or PBS was injected into the mosquito thorax. Injections of 0.5 µl sterile LB media or 1× PBS (Cambrex, East Rutherford, NJ, USA) were used as controls. dsRNA-treated mosquitoes were injected on the 4th day post-RNAi treatment. Each experiment with iREL2 and iLUC mosquitoes was performed twice.

2.9. Assessment of *Plasmodium gallinaceum* oocyst and sporozoite numbers

Plasmodium gallinaceum was maintained by transmission between mosquitoes and White Leghorn chickens. In all experiments, 4- to 7-day-old transgenic mosquitoes or RNAi-treated wild-type mosquitoes on the 4th day post-RNAi treatment were infected by blood feeding on *P. gallinaceum*-infected chickens with a 9-day-old infection. At 7 days post-infection, mosquito midguts were dissected, stained with 1% mercurochrome (Sigma), and the oocyst number was calculated. To calculate the effect of REL2 overexpression on sporozoite number, mosquitoes were infected with *P. gallinaceum* (as described above) and given a second naïve blood meal at 7 days post-infection to reactivate Vg-driven REL2. After 14 days post-infection, mosquito salivary glands were dissected, and the sporozoite number was calculated using phase-contrast optics.

2.10. Statistical analyses

The statistical investigation of mosquito survival rates after bacterial infections was performed by means of Cox analysis utilizing SAS version 9.6 software (SAS Institute Inc., Cary, NC).

For the oocyst number assessment experiments, either the Student's *t*-test or Mann-Whitney-Wilcoxon test (Wilcoxon test) was used to analyze statistical differences between the wild-type and transgenic mosquitoes. The choice was made based on the results of normality test for the oocyst number distribution. For normal distributions, the Student's *t*-test was used; for those violating the normality assumption, the non-parametric Wilcoxon test was used. Both tests were performed using statistical analysis software JMP7 SAS (SAS Institute Inc., Cary, NC).

3. Results

3.1. Generation of two transgenic mosquito strains expressing REL2 in the fat body PBM

To study the role of REL2 in mosquito immunity, we have created two transgenic mosquito strains in which the full length of the REL2-Short isoform—which includes the His/Gln (Q/

H)-rich and SRR putative transactivation domain(s) as well as the REL-homology (RHD and IPT) domain—is incorporated into the mosquito genome under the control of the *Vg* promoter. A transformation vector pBac[3×P3_EGFP, afm] containing the *Vg*-REL2-Short isoform and SV40 polyadenylation region was used for mosquito germ-line transformation (Fig. 1A). The two transgenic mosquito strains, named REL2-A and REL2-B, were established as a result of the germ-line transformation and distinguished by means of green fluorescent glowing in the eyes at larval, pupal, and adult stages (not shown).

To investigate the number of transgene copies and the integrity of transgene insertion, we performed Southern blot analysis. Genomic DNA of the transgenic mosquito and wild-type strains was collected, digested with *Eco*RI and *Asc*I endonucleases, and hybridized with probes to the left arm, internal SV40 polyadenylation region, and right arm of the pBac[3×P3_EGFP, afm] transformation vector. Southern blot hybridization analysis with the probe for the internal SV40 polyadenylation region showed a single band of expected size (4.2 kb) in both transgenic strains, demonstrating the integrity of the transgene incorporation. Hybridization with the probe for the right and left vector arms yielded a single band in each transgenic strain, demonstrating a single transgene incorporation event. The bands for the right vector arm were of about the same size; however, hybridization with the probe for the left vector arm produced single bands of different sizes in two transgenic strains, validating distinct transgene incorporation regions for the two strains (Fig. 1B). Hence, Southern blot analysis confirmed generation of two distinct transgenic mosquito strains, REL2-A and REL2-B, with different sites of transgene incorporations.

3.2. Effect of the transgenic REL2 gene overexpression

To examine the effect of transgenic REL2 gene overexpression, we performed Northern blot analysis with probes for *Vg*, REL2, and AMP Defensin A. Expression of transgenic REL2 was detected as early as 2 h PBM, with the maximum at 12 h, and a decline by 48 h (Fig. 2A). At 60 and 72 h PBM, *Vg* and transgenic REL2 were no longer detectable by means of Northern analysis (data not shown). The same REL2 expression pattern was observed for the second transgenic strain (not shown). We also compared levels of the REL2-transgene expression in REL2-A and REL2-B strains at 12 and 24 h PBM. REL2-A mosquitoes appeared to have a higher degree of transgene expression than REL2-B (Fig. S1). Tissue expression of the REL2-transgene in transgenic mosquitoes was specific to the fat body and was not detected in ovaries or midgut (Fig. 2B). Thus, expression of transgenic REL2 depends on the activation of the *Vg* promoter, and occurs in a similar stage- and tissue-specific manner as the endogenous *Vg* gene.

In the transgenic mosquitoes, blood-meal-dependent expression of the transgenic REL2-Short isoform resulted in transcription of Defensin A, specifically in the mosquito fat body (Fig. 2A and B). Defensin A transcripts were detectable at 6 h PBM, reached a maximum at 12 h, and declined by 48 h. As for *Vg* and transgenic REL2, Defensin A mRNA transcripts were no longer detectable at 60 and 72 h PBM (data not shown). These dynamics of Defensin A transcripts were congruent with the expression profile of transgenic REL2, and with that of the *Vg* gene (Fig. 2A). However, in the bacteria-challenged wild-type mosquitoes, the dynamics were different: in *E. cloacae*-challenged mosquitoes, a sharp increase in Defensin A mRNA was detected at 2 h post-infection, increased over the course of infection, and remained at high levels 48 h post-infection, probably because of the persistence of the bacterial pathogen (Fig. 2A).

In order to evaluate whether transgenic overexpression of REL2-Short affects the expression of endogenous REL2 isoforms and REL1 (the main NF- κ B factor of the mosquito Toll pathway) in the fat body PBM, total RNA was extracted from blood-fed wild-type and transgenic mosquitoes at 12 and 24 h PBM, cDNA was produced as described in Materials and

Methods, and PCR was performed using primers specific to REL2 isoforms and the REL1-A isoform of REL1. No conclusive difference in the expression of REL2-Long or I κ B between wild-type and REL2 transgenics in response to a blood meal was noted. In contrast, we detected a considerable increase in the level of the REL2-Short transcript in the REL2 transgenics, likely due to the fact that REL2-Short primers recognized both endogenous REL2-S and transgenic REL2-S. The REL1A transcript appeared to be slightly enhanced in transgenic mosquitoes PBM (Fig. S2).

3.4. Effect of the transgenic REL2 gene on AMP expression

Sequencing and annotation of the *Ae. aegypti* genome revealed that it contains four different Defensins, ten different Cecropins, and also Dipterucin, Attacin, Gambicin, and Holotricin genes (Nene et al., 2007; Waterhouse et al., 2007). To study downstream targets of mosquito REL2, we designed specific primers for all AMP genes listed above and performed RT-PCR with cDNA from the transgenic and wild-type mosquitoes. We used previtellogenic and vitellogenic mosquitoes at 12 and 24 h PBM. Our data show that three of four Defensins encoded in the *Ae. aegypti* genome—Defensins A, C, and D—were expressed at the detectable levels as a result of transgenic REL2 overexpression, with a maximum expression at 12 h, and a decline at 24 h PBM. The relative levels of Defensins A, C, and D expression at 12 h PBM in transgenic mosquitoes were similar to those of bacterial-induced expression of these Defensins in control mosquitoes at 24 h post-infection (Fig. 2C).

Of ten Cecropins encoded in the *Ae. aegypti* genome, we could detect that only Cecropins A and N showed an increase in expression in response to a blood meal. Cecropin A was expressed in transgenic mosquitoes 12 h PBM. At 12 and 24 h PBM, Cecropin N was expressed at noticeably higher levels in the transgenic mosquitoes than in wild type (Fig. 2D). Dipterucin, Gambicin, Attacin, and Holotricin transcripts were increased in bacteria-challenged mosquitoes, although were not detected in response to a blood meal in either the transgenics or uninfected control wild-type mosquitoes (data not shown).

3.5. Blood-meal-activated production of Defensin A peptide in REL2 transgenic mosquitoes

Next, we studied the effect of transgenic REL2 overexpression on Defensin A protein dynamics. To analyze the tissue-specific expression of Defensin A, total bodies, fat bodies, and ovaries were collected from transgenic and wild-type female mosquitoes before blood meal and 24 h after blood feeding. Total protein was then extracted, and Western blot analysis performed using antibodies against *Ae. aegypti* Defensin A. The results showed the presence of Defensin A in transgenic female mosquitoes 24 h PBM. This AMP was detected in the transgenic mosquito fat body. No Defensin protein was detected in the mosquito ovaries, indicating tissue specificity of production of this AMP in the REL2 transgenic mosquitoes (Fig. 3A).

To examine whether Defensin was secreted into the hemolymph of transgenic mosquitoes, we collected hemolymph from previtellogenic or blood-fed transgenic females at 24, 48, and 72 h PBM, and from bacteria-challenged wild-type mosquitoes at 24, 48, and 72 h post-infection. Western blot analysis indicated the presence of Defensin peptide in the hemolymph of transgenic mosquitoes at 24 and 48 h PBM (Fig. 3B). Interestingly, the presence of the blood-meal-induced Defensin in the hemolymph of transgenic REL2 mosquitoes is relatively short compared with that due to infection. At 72 h PBM, Defensin was no longer detected in the hemolymph of transgenic mosquitoes; however, it was still abundantly present in that of bacterially challenged wild-type mosquitoes (Fig. 3B). The disappearance of Defensin A protein from the transgenic mosquito hemolymph after 48 h PBM is consistent with the dynamics of Vg, transgenic REL2, and Defensin A mRNA expression, and is most probably

due to the intrinsic properties of the *Vg* promoter. Thus, it is most likely that other AMPs activated in the fat body of REL2 transgenic mosquitoes are also secreted into the hemolymph.

3.6. REL2 RNAi-mediated knockdown negatively affects Defensin A and Cecropin A expression

To validate whether the observed downstream effects of the transgenic REL2 signaling were due to the transgene function, we used RNAi to knockdown REL2-Long and REL2-Short isoforms in wild-type mosquitoes and studied the effect on Defensin A and Cecropin A expression. dsRNA against the REL2 RHD domain, which is the common region for REL2-Long and REL2-Short, was used for the knockdowns. Wild-type mosquitoes were treated with either dsRNA against RHD domain (iRHD) or control non-specific dsRNA against Luciferase gene (iLuc). On the 4th day after RNAi treatment, the isoform knockdown was confirmed using RT-PCR with REL2-Long- and REL2-Short-specific primers (Fig. 4A). RNAi-treated mosquitoes were then injected with *E. cloacae*. Total RNA was collected from both naïve, uninfected RNAi-treated mosquitoes and those infected at 1, 6, and 24 h post-bacterial challenge. Following this, RT-PCR was performed using Defensin A- and Cecropin A-specific primers. The results showed that REL2 knockdown led to more than a 6-h delay in Defensin A and Cecropin A expression in bacteria-infected mosquitoes (Fig. 4B and 4C). Thus, combined results of transgenic REL2 overexpression and REL2 RNAi treatment further suggested that this mosquito NF- κ B plays a key role in regulation of AMP expression.

3.7. Survivorship of REL2 transgenic mosquitoes to different bacterial pathogens

To study the effect of REL2 overexpression on the resistance of mosquitoes to different bacterial pathogens, we performed survival experiments for transgenics and wild-type mosquitoes using three different types of bacteria: two Gram-negative bacteria, *P. aeruginosa* and *E. cloacae*; and the Lys-type Gram-positive bacterium *S. aureus*. *P. aeruginosa* is an opportunistic human pathogen, although deadly to mosquitoes, and was shown to suppress AMP expression in infected fruit flies (Apidianakis et al., 2005). *E. cloacae* is moderately pathogenic to mosquitoes and elicits prominent induction of immune markers in response to infection (Hedengren-Olcott et al., 2004). *S. aureus* is a mild mosquito pathogen, the susceptibility to which highly increases in mosquitoes lacking Defensin or REL2 (Blandin et al., 2002; Meister et al., 2005). For the survival experiments, we used 4-day-old mosquitoes and injected them with bacteria at 6–10 h PBM. The results of our experiments indicated that transgenic REL2-A mosquitoes were more resistant to *P. aeruginosa*, *E. cloacae*, and *S. aureus* than wild-type mosquitoes (Fig. 5A, B, and C). For *P. aeruginosa*, the transgenics exhibited a 4- to 6-h delay in 50% mortality, and for *E. cloacae* and *S. aureus* the transgenics showed a 10–20% increase in mosquito survival. Similar survival profiles were observed for the REL2-B transgenic strain (data not shown). Cox analysis demonstrated a significant difference in the mortality rates between REL2-A transgenics and wild-type mosquitoes for *P. aeruginosa*, *E. cloacae*, and *S. aureus* with a *P* value < 0.05. We speculate that the increased resistance of the transgenics to bacterial pathogens is most probably due to the presence of Defensins and putatively other antimicrobial factors in the hemolymph of transgenic mosquitoes before the introduction of bacteria.

3.8. REL2 RNAi-mediated deletion results in the increased susceptibility of the mosquitoes to bacterial pathogens

Next, we investigated the effect of RNAi-mediated REL2 knockdown on the mosquito resistance to bacterial pathogens of different Gram types. We used the Gram-negative bacteria *Enterobacter cloacae* and the Gram-positive bacteria *Enterococcus faecalis*, which is a mild mosquito pathogen (Hillyer et al., 2004) that contains Lys-type peptidoglycans and activates the Toll immune pathway in *Drosophila* (Ferrandon et al., 2007). Our results demonstrated

that the knockdown of REL2 resulted in an increase in mosquito mortality to both tested bacteria (Fig. 6A and B). In iRHD-treated mosquitoes, the injections with *E. cloacae* caused 50% mosquito mortality at 24 h post-infection and, by 36 h, all iRHD-treated mosquitoes were dead. At the same time, about 40% of those treated with iLuc were still alive 5 days after bacterial challenge (Fig. 6A). The injections with *E. faecalis* resulted in 50% iRHD mosquito mortality at 72 h post-infection, and all iRHD mosquitoes were dead by day 5 post-infection; about 80% of infected iLuc mosquitoes, however, were still alive at this time point (Fig. 6B). These results are consistent with findings in the REL2 transgenic mosquitoes, where overexpression of REL2-Short had led to an increase in resistance to Gram-negative and Gram-positive bacteria. Although dsREL2 knockdown revealed only weak phenotypes with respect to Defensin A and Cecropin A expression (Fig. 4), the susceptibility to bacterial infections was strong (Fig. 6). This suggests that the decrease in Rel2 expression by iREL2 might lead to a reduced induction of other AMPs, warranting further investigation of this system in the future. Recently, a microarray-based gene expression assay in *Ae. aegypti* has revealed that 35 genes are induced as a result of Caspar depletion, a negative regulator of REL2 (Xi et al., 2008). Among genes induced by Caspar silencing were TEP13 and the AMPs Defensin E and gambicin.

3.9. Increased resistance of REL2 transgenic mosquitoes to *Plasmodium gallinaceum*

To examine the effect of overexpression of REL2 on development of the malaria parasite *P. gallinaceum* in its vector host *Ae. aegypti*, we carried out a *Plasmodium* oocyst and sporozoite number study. To address the oocyst development in the mosquito midgut, we infected REL2-A, REL2-B, and the wild-type mosquitoes with *P. gallinaceum* by allowing them to feed on *P. gallinaceum*-infected chickens. At 7 days post-infection, mosquito midguts were dissected and stained with mercurochrome, and the oocyst number was counted. The results showed that under average infection conditions REL2-A had significantly fewer oocysts than wild-type mosquitoes: most REL2-A mosquitoes had between 0 and 10 oocysts relative to the wild-type mosquitoes which had more than 40 oocysts per midgut (Student's *t*-test, $P < 0.05$) (Fig. 7A and B). Similar results demonstrating a significant reduction in oocyst number were obtained for the REL2-B mosquito strain (Wilcoxon test, $P < 0.05$) (Fig. 7C and D).

To study sporozoite development in the transgenic REL2, we first infected mosquitoes with *P. gallinaceum*. At 7 days post-infection, we gave the mosquitoes a second naïve blood meal to reactivate transgenic REL2 expression. At 14 days after infection, mosquito salivary glands were dissected, and the sporozoite number was calculated. The experiments were repeated three times, and the results demonstrated a significant decrease in sporozoite number in REL2-A mosquitoes (Student's *t*-test, $P < 0.05$) when compared with the wild-type (Fig. 7E and F). Thus, these data showed that overexpression of REL2 hinders development of *P. gallinaceum*.

3.10. REL2 is required for the resistance against *Plasmodium gallinaceum*

Finally, to study the effect of REL2 RNAi-mediated deficiency on *P. gallinaceum* development, we conducted oocyst number experiments in the REL2 RNAi-treated mosquitoes. In this trial, on the 4th day post-RNAi treatment, REL2 RNAi and control iLuc mosquitoes were infected by blood feeding on *P. gallinaceum*-infected chickens. The oocyst number was counted on the 7th day post-infectious blood meal, as described above. Our results indicated that REL2 RNAi-treated mosquitoes were significantly more susceptible to *P. gallinaceum* infection than control iLuc mosquitoes (Wilcoxon tests, $P < 0.05$) (Fig. 8A and B). Thus, combined results of REL2 overexpression and REL2 knockdowns suggest an important role for REL2 in *Ae. aegypti* anti-*Plasmodium* immunity.

4. Discussion

In this study, we used mosquito transgenesis and RNA interference to further decipher the role of mosquito REL2 in antibacterial and anti-*Plasmodium* immunity of *Ae. aegypti*. First, we generated and characterized two independent transgenic mosquito strains of *Ae. aegypti*, in which the REL2-Short isoform of NF- κ B transcriptional factor REL2 was overexpressed in the mosquito fat body under control of the *Vg* promoter. We have presented molecular and phenotypic characterization of these strains, as well as gene expression analysis, and have reported the effect of REL2 overexpression on both AMP synthesis in the adult mosquito and mosquito resistance to bacterial and *P. gallinaceum* infection. Second, we used RNAi to study the effect of REL2 knockdowns on AMP expression and resistance to bacterial and *Plasmodium* pathogens.

For both transgenic strains of *Ae. aegypti*—REL2-A and REL2-B—there was a single site for transgene incorporation, and the incorporated vector constructs were intact. Also, REL2-A and REL2-B transgenics showed distinct regions of the transgene incorporation in their genomes. Together, this knowledge allowed us to rule out the position effect of the transgenes and interpret the results of the experiments as direct effects of the transgene function. Both REL2-A and REL2-B transgenic mosquitoes demonstrated tissue-specific expression of transgenic REL2 in the fat body PBM. The pattern of the transgene expression was consistent with the dynamics of *Vg* gene expression (Kokoza et al, 2000; 2001b). Transgenic REL2 transcripts were detected at time points that corresponded to those of *Vg* transcripts, confirming blood-meal-dependent activation of the transgenic REL2.

Previously, we have shown that transgenic overexpression of REL2 lacking the N-terminal Q/H rich and SRR region results in an immune-deficient (RIMD) phenotype in the mosquito *Ae. aegypti* (Shin et al., 2003). This RIMD transgenic mosquito exhibited significant reduction in expression of Defensin A and Cecropin A and was highly sensitive to infection with Gram-negative bacteria. In our present work, the full-length REL2-Short isoform that includes an N-terminus with the Q/H rich and SRR region was overexpressed under the control of the *Vg* promoter in two transgenic mosquito strains. Transgenic overexpression of the complete REL2-Short isoform resulted in an enhanced expression of several AMPs and increased antimicrobial and anti-*Plasmodium* resistance. Hence, the N-terminal region that includes Q/H rich and SRR sequences plays a major role in transactivation by the REL2 NF- κ B transcription factor. Shin et al. (2002) have suggested the transactivation function of this REL2 region based on the sequence similarly with glutamine-rich transcriptional activation domains of some transcription factors, which are also associated with histidine-rich stretches. *Drosophila* Dorsal contains an N-terminal Rel homology domain (RHD), which is responsible for DNA binding and regulated nuclear import, and a C-terminal domain (CTD) that contains activation and repression motifs. Mutational experiments, however, have revealed that its N-terminal domain also plays an active role in transcriptional regulation (Jia et al., 2002). Likewise, transcriptional activation analysis has shown that both the N and C termini of RelB, a member of the Rel family of transcription factors, are required for full gene transactivation (Dobrzanski et al., 1993). More detailed functional studies are needed to decipher composition of transactivation domains in the mosquito REL2.

In the *Ae. aegypti* genome, 18 AMP genes were annotated, including four *defensin* genes: Defensins A, C, D, and E (Waterhouse et al., 2007). We studied the effect of REL2 overexpression on Defensin gene family expression. Our results showed that Defensins A, C, and D were expressed in the fat bodies of the transgenic mosquitoes in response to a blood meal. Moreover, Defensin A protein was detected in the fat body tissue and mosquito hemolymph of these transgenic mosquitoes PBM, independently of bacterial challenge. The presence of Defensin A in the hemolymph was dependent on transgenic REL2 activation, since

a decrease in transgenic REL2 expression at later time points after the blood meal resulted in Defensin A diminishing in the hemolymph. All presented observations suggest that, in *Ae. aegypti*, REL2 is a major contributor to the induction of Defensin family genes in the fat body.

Sequencing and annotation of the *Ae. aegypti* genome revealed a high diversity of the Cecropin gene family. The *Aedes* genome encodes ten *cecropin* genes, whereas *A. gambiae* possesses only four and *D. melanogaster* five (Waterhouse et al., 2007). In our study, we investigated the input of REL2 signaling on the expression of the cecropin gene family. Of ten cecropins encoded in the *Ae. aegypti* genome, only Cecropins A and N demonstrated a modest level of transcriptional activation in response to REL2 expression. Moreover, we were unable to detect expression of Attacin, Gambicin, and Holotricin in REL2 transgenic mosquitoes activated by a blood meal. This suggests that, in *Ae. aegypti*, REL2 short isoform signaling alone is not sufficient for transcriptional activation of most genes in the Cecropin gene family or of other AMPs.

There are several possible explanations for such differential AMP gene expression in response to REL2-Short overexpression. The first originates from the nature of NF- κ B factors that must form dimers for DNA binding and activation of transcription (Baeuerle and Baltimore, 1996). In *Drosophila melanogaster*, Toll and IMD pathways synergistically activate an innate immune response. Constitutive activation of Toll and PGRP-LC/IMD mimics the synergistic stimulation. In addition, RNA interference assays and promoter analyses demonstrated that cooperation of different NF- κ B-related transcription factors mediates the synergy. In *Drosophila* cell lines, Relish/Relish homodimers cause modest levels of Cecropin and Attacin expression, while Relish/Dif heterodimers enhance significantly the transcription of these AMPs (Han and Ip, 1999; Tanji et al., 2007). In our transgenic system, in which REL2 is activated by the *Vg* promoter, bypassing the entire immune response, only REL2/REL2 homodimers were expected to be available for κ B site binding in the promoters of responsive genes. Thus, it is possible that, in *Ae. aegypti*, REL2/REL2 homodimer binding to κ B sites in the promoters of Defensins A, C, and D is sufficient to initiate transcription; REL1/REL2 heterodimer formation, however, might be necessary for the transcriptional activation of other immune factors. In addition, a general requirement for JNK signaling in the expression of AMPs has recently been demonstrated by the expression of a JNK inhibitor and induction of JNK loss-of-function clones in immune responsive tissue of *Drosophila* (Delaney et al., 2006). Moreover, full activation of the *Drosophila CecropinA1* gene requires simultaneous function of NF- κ B factors and a yet-unknown factor R1 (Uvel and Engström, 2003; 2007).

REL2 transgenics exhibited increased resistance to Gram-negative and Gram-positive bacteria. Previously, the key role of REL2 in the mosquito Defensin A and Cecropin A production as well as defense against Gram-negative bacteria has been demonstrated by transgenic overexpression of a non-functional form of REL2 in *Ae. aegypti* (Shin et al., 2003). In *An. gambiae*, dsRNA knockdown of REL2 resulted in suppression of Defensin and Cecropin 1 expression and in dramatically increased susceptibility of the mosquito to Gram-negative and Gram-positive bacteria (Meister et al., 2005). In congruence with these findings, in our study, overexpression of REL2 resulted in increased resistance of *Ae. aegypti* to two Gram-negative bacteria, *P. aeruginosa* and *E. cloacae*, which are strong mosquito pathogens. In *An. gambiae*, REL2 has been implicated in the mosquito defense against the Gram-positive bacteria *S. aureus* (Meister et al., 2005). Our results indicated that overexpression of REL2 increases resistance of mosquitoes to *S. aureus*, suggesting involvement of REL2 in the defense against Gram-positive bacteria. This was further supported by the results of the survival tests in REL2 RNAi-treated mosquitoes, in which a dramatic increase in mosquito mortality was observed in response to bacterial infection by either Gram-negative or Gram-positive bacteria.

REL2-overexpressing transgenics have increased resistance to *P. gallinaceum*. On average, we observed a decrease of 90% and 50%, for REL2-A and REL2-B, respectively, in oocyst number in the midguts of the transgenics when compared with that in the wild type. Also, the transgenics exhibited a significant decrease in sporozoite number in the salivary gland when the *Plasmodium*-infected mosquitoes were given the second naïve blood meal on the 7th day post-infection to re-activate transgenic REL2 expression. The importance of REL2 for anti-*Plasmodium* immunity was further supported by the oocyst number assessment experiments in REL2 RNAi mosquitoes, in which REL2 RNAi mosquitoes had significantly higher oocyst numbers than the control RNAi insects. Likewise, RNAi depletion of REL2 in *An. gambiae* has been shown to significantly increase susceptibility of the mosquito to *P. berghei*, indicating involvement of REL2 in anti-malaria defense (Meister, et al., 2005). It is possible that the increased resistance of REL2-A and REL2-B transgenic *Ae. aegypti* mosquitoes to *Plasmodium* was due to toxicity of Defensins and Cecropins to *Plasmodium* oocysts and sporozoites. In early experiments, it was found that Defensins from dragon fly (*Aeschna cyanea*) and flesh fly (*Phormia terranova*) inhibited the development of *P. gallinaceum* oocysts and sporozoites (Shahabuddin et al., 1998). Indeed, transgenic overexpression of both Defensin A and Cecropin A in *Ae. aegypti* results in nearly complete inhibition of *Plasmodium* development and interruption of its transmission (Ahmed, Kokoza and Raikhel, unpublished results). However, we cannot exclude the possibility that increased resistance of REL2 transgenic to *P. gallinaceum* was also due to the production of other anti-*Plasmodium* factors that are controlled by REL2.

Transgenesis and RNA interference are indispensable tools, permitting in-depth study of the molecular mechanisms underlying biological processes. Our findings contribute to current knowledge of the role of NF- κ B factor REL2 in mosquito innate immunity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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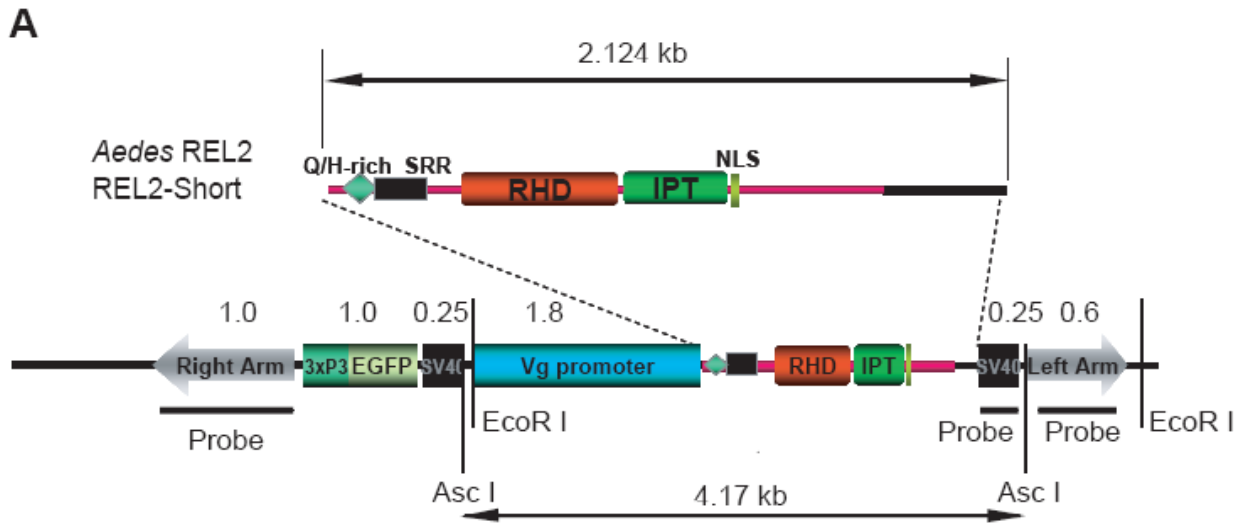
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Abbreviations

AMP	antimicrobial peptides
Vg	Vitellogenin
REL2	transcriptional factor REL2, homologue of <i>Drosophila</i> Relish
EGFP	enhanced green fluorescent protein
BM	blood meal
PBM	post blood meal



pBAC[3xP3-EGFP afm, Vg-REL2]

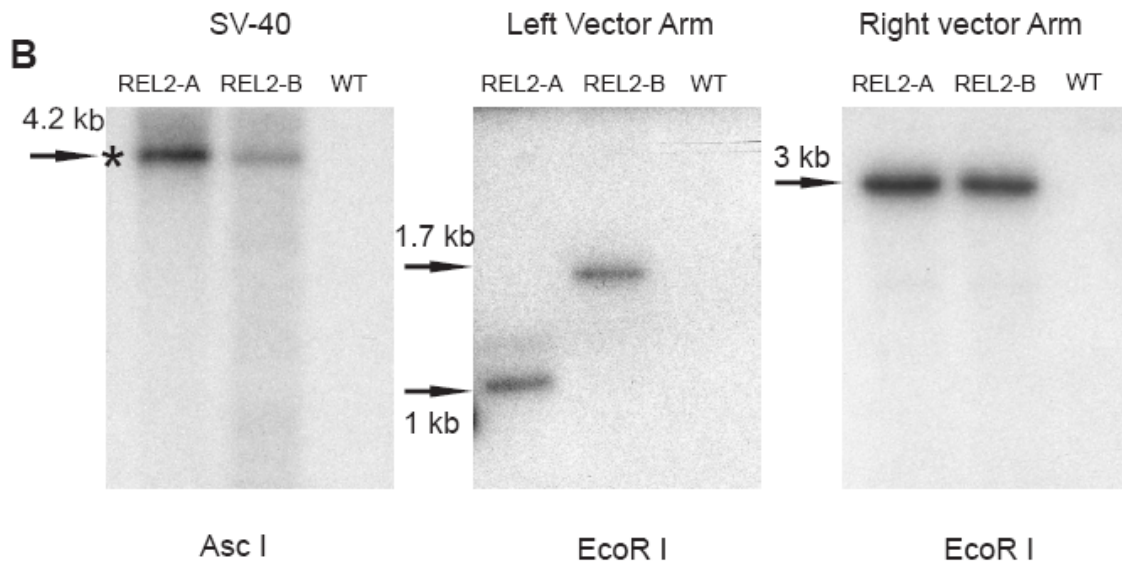
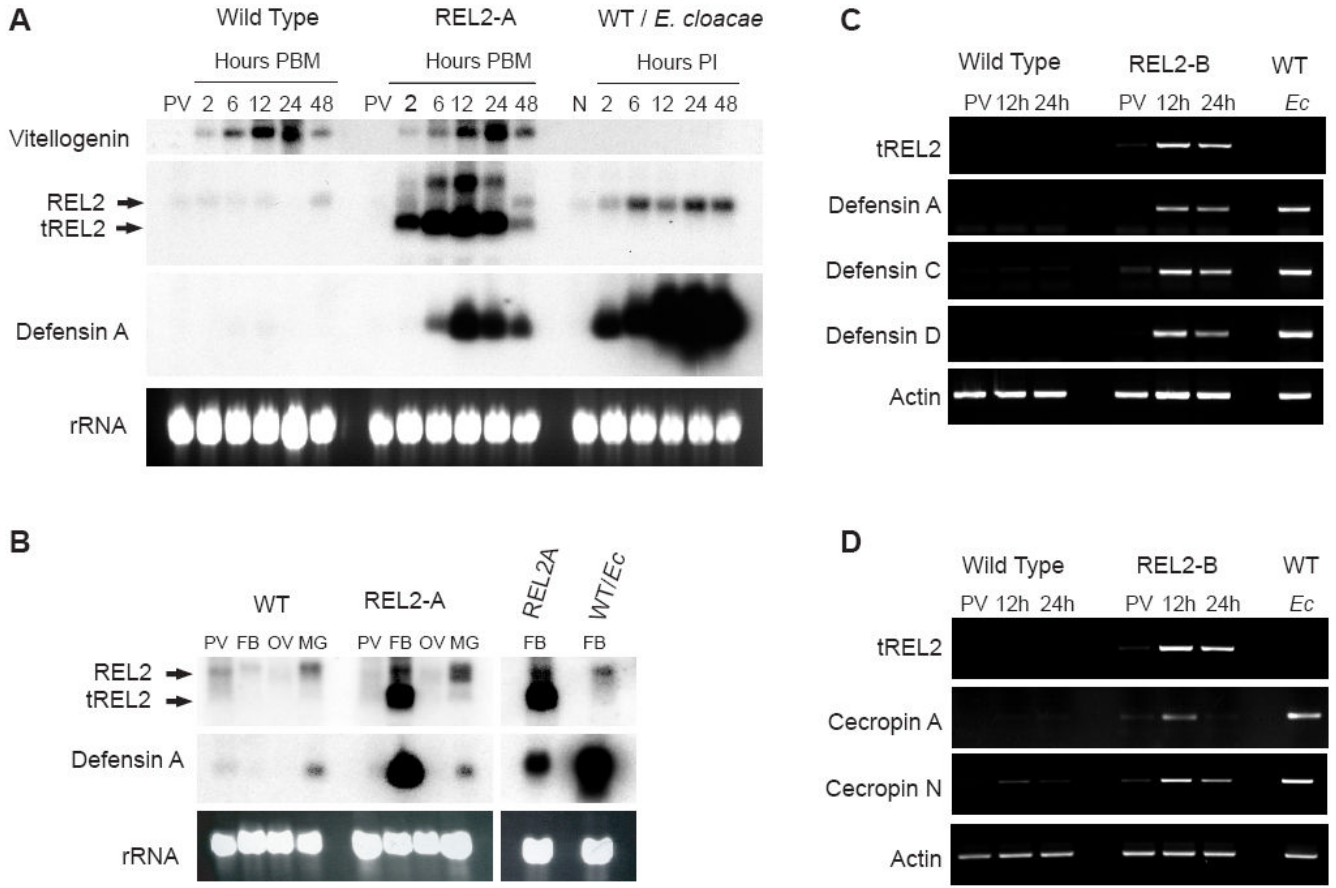


Figure 1.

Generation of two independent transgenic strains of *Aedes aegypti*—REL2-A and REL2-B (A) Schematic representation of the pBac[3xP3, EGFP afm, Vg-REL2] transformation plasmid used in germ-line transformation. The restriction sites of the restrictases used for DNA digestion are indicated; positions of the probes for right and left vector arms as well as SV40 polyadenylation region are indicated by thick black lines; double-headed arrows indicate the sizes of the REL2-Short isoform (2.124 kb) and the pBAC[3xP3-EGFP afm, Vg-REL2] construct; Q/H, glutamine/histidine-rich region, SRR, serine-rich region, RHD and IPT are parts of the REL DNA-binding domain; NLS, nuclear localization signal.

(B) Southern blot analysis of the genomic DNA extracted from REL2-A and REL2-B transgenic mosquito strains and the wild-type strain. Genomic DNA was isolated, digested using *EcoR* I and *Asc* I, and hybridized with probes specific to the internal SV40 polyadenylation region, the left and right vector arms; WT—the wild-type parental strain; asterisk indicates the band of expected size.

**Figure 2.**

Blood-meal-activated expression of transgenic REL2 and Defensin and Cecropin families of AMP in the transgenic *Ae. aegypti* mosquito strains, REL2-A and REL2-B.

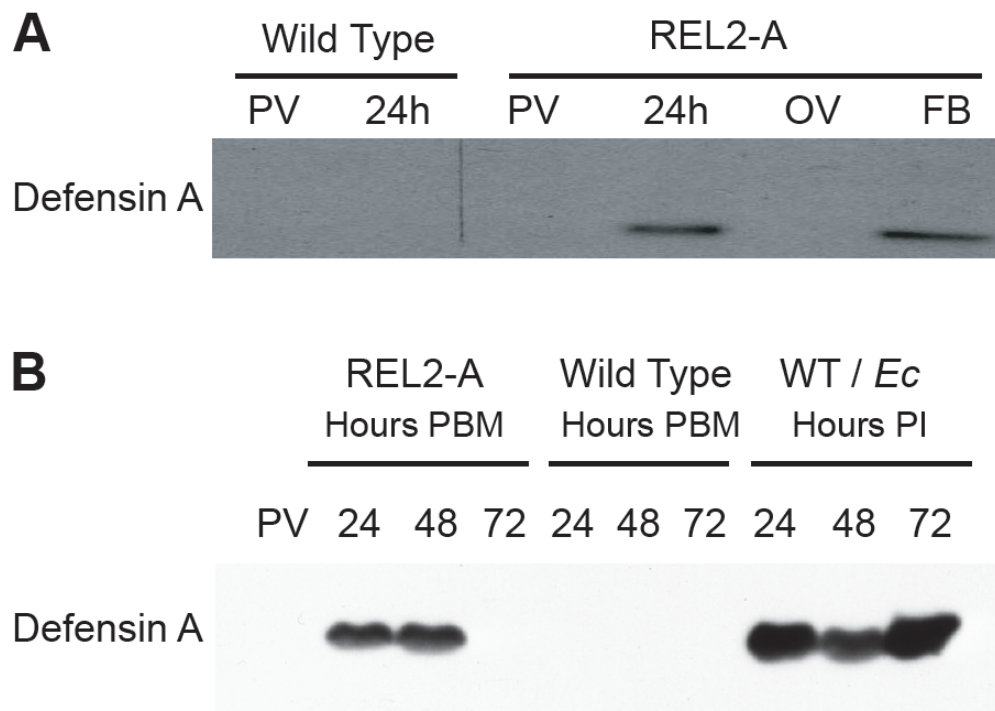
(A) Northern blot analysis: expression profiles of Vitellogenin, endogenous REL2, transgenic REL2, and Defensin A in the fat body of the REL2-A transgenic mosquitoes. Fat bodies were collected from wild-type (WT) and REL2-A mosquitoes: previtellogenic (PV) and blood-fed mosquitoes at 2, 6, 12, 24, and 48 h post blood meal (PBM). WT mosquitoes were injected with *E. cloacae*, and the fat bodies were collected from non-infected mosquitoes (n) and infected mosquitoes at 2, 6, 12, 24, and 48 h post-infection (PI). Total RNA was extracted and Northern blot analysis performed using the probes for Vg, REL2 RHD domain, and Defensin A; arrows indicate bands for endogenous REL2—REL2 and transgenic REL2—tREL2, (rRNA) ribosomal RNA—loading control.

(B) Northern blot analysis: fat-body-specific expression of transgenic REL2 and Defensin A in REL2-A mosquitoes. Total bodies were collected from previtellogenic (PV) and blood-fed wild-type (WT) and REL2-A mosquitoes at 24 h post blood meal (PBM). Mosquito fat bodies (FB), ovaries (OV), and midguts (MG) were collected from blood-fed mosquitoes at 24 h PBM. Total RNA was extracted, and Northern analysis was performed with the probes for REL2 RHD domain and Defensin A; FB from WT mosquitoes injected with *E. cloacae* (*Ec*) at 24 h post-infection were used as a control of Defensin A expression; REL2—endogenous REL2, tREL2—transgenic REL2.

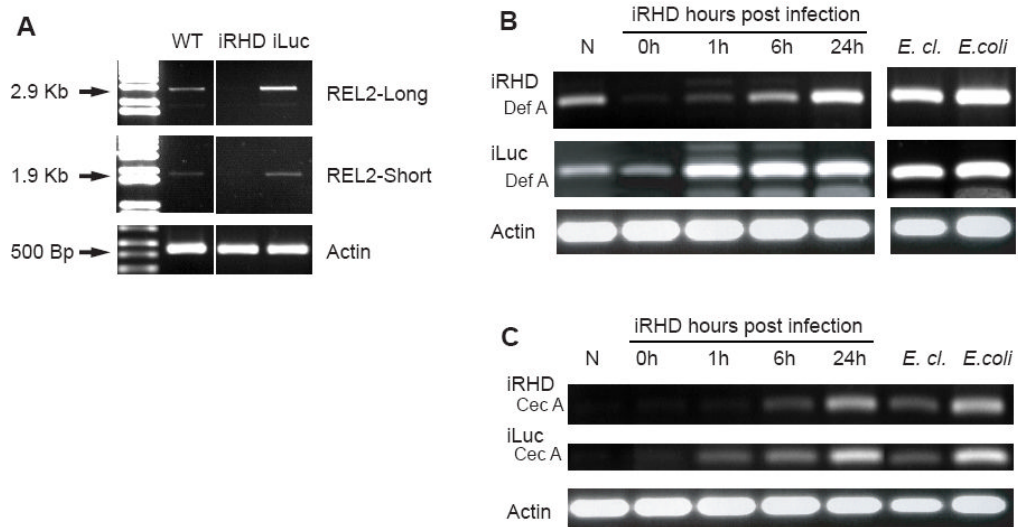
(C) Blood-meal-activated expression of transgenic REL2 and Defensins A, C, and D; and

(D) blood-meal-activated expression of transgenic REL2 and Cecropin A and N in the transgenic REL2-B strain (RT-PCR analysis). Fat bodies were collected from previtellogenic

(PV) and blood-fed mosquitoes at 12 and 24 h post blood meal; fat bodies collected from wild-type (WT) mosquitoes injected with *E. cloacae* (*Ec*) at 24 h post-infection were used as a control. Total RNA was extracted and treated with DNase I, and cDNA was synthesized and used as a template for RT-PCR with primers specific for transgenic REL2, Defensin A, C, D, and also Cecropin A and N.

**Figure 3.**

Blood-meal-activated Defensin A protein in the fat bodies (FB) and hemolymph of REL2 transgenic mosquitoes. **(A)** Blood-meal-activated Defensin A in the FB. Total bodies of previtellogenic (PV) and blood-fed mosquitoes at 24 h post blood meal (PBM), as well as FB and ovaries (OV) of blood-fed REL2-A were used for protein extraction. A 10- μ g aliquot of total protein was used per lane for SDS-PAGE, and Western blot analysis was performed using the anti-Defensin A antibodies. **(B)** Blood-meal-activated secretion of Defensin A peptide in the hemolymph of transgenic mosquitoes. Wild-type and REL2-A mosquitoes were blood fed, and control PV wild-type mosquitoes were injected with *E. cloacae*; hemolymph was collected as described in Materials and Methods at 24, 48, and 72 h PBM and at the same time points post-infection (PI). Protein (10 μ g) was loaded per lane for SDS-PAGE, and Western analysis was performed using the antibodies against Defensin A. WT/*Ec*—wild-type mosquitoes injected with *E. cloacae*.

**Figure 4.**

RNAi knockdowns of REL2-Long and REL2-Short result in the delayed expression of AMPs Defensin A and Cecropin A in wild-type, *E. cloacae*-infected mosquitoes. **(A)** Expression of REL2-Long and REL2-Short in the REL2 dsRNA-treated mosquitoes. **(B and C)** Delayed expression of Defensin A **(B)** and Cecropin A **(C)** in response to the bacterial challenge in RNAi-treated mosquitoes. iRHD—mosquitoes treated with dsRNA against REL2 RHD domain; iLuc—mosquitoes treated with dsRNA against luciferase gene. N—naïve, non-treated wild-type mosquitoes (neither RNAi nor bacteria treatments); *Ecl* and *E. coli*—wild-type mosquitoes not treated with dsRNA, at 24 h post-bacterial infection: *Ecl*—*E. cloacae* injections *E. coli*—*E. coli* injections. Actin amplification represents the loading control for both iRHD and iLuc mosquitoes, where equal amplification of actin was observed in all experiments.

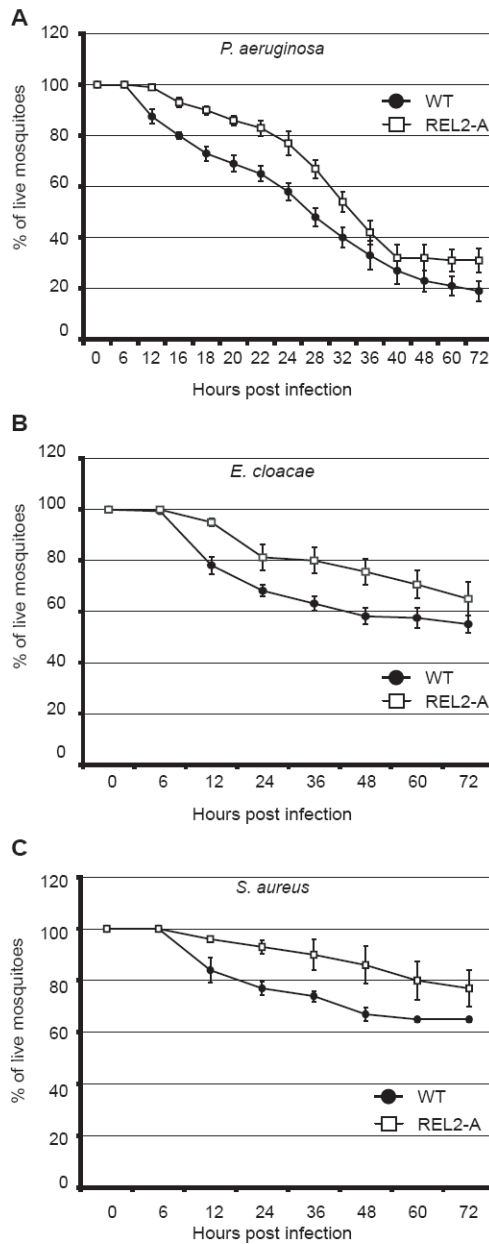


Figure 5.

Increase in survival of REL2 transgenics to Gram-negative and Gram-positive bacteria Wild-type and transgenic mosquitoes were blood fed and injected with bacteria between 6 and 12 h post blood meal. Survival was monitored by counting the number of live mosquitoes.

(A) Decreased susceptibility of REL2-A transgenics to *E. cloacae* infection. Combined results of five independent experiments; total number of mosquitoes used was 155; error bars represent standard errors; P values < 0.05 (Cox analysis)

(B) Decreased susceptibility of REL2-A transgenics to *P. aeruginosa* infection. Combined results of six independent experiments; total number of mosquitoes used was 192; error bars represent standard errors; P values < 0.05 (Cox analysis)

(C) Decreased susceptibility of REL2-A transgenics to *S. aureus* infection. Combined results of three independent experiments; total number of mosquitoes used was 96; error bars represent standard errors; *P* values < 0.05 (Cox analysis)

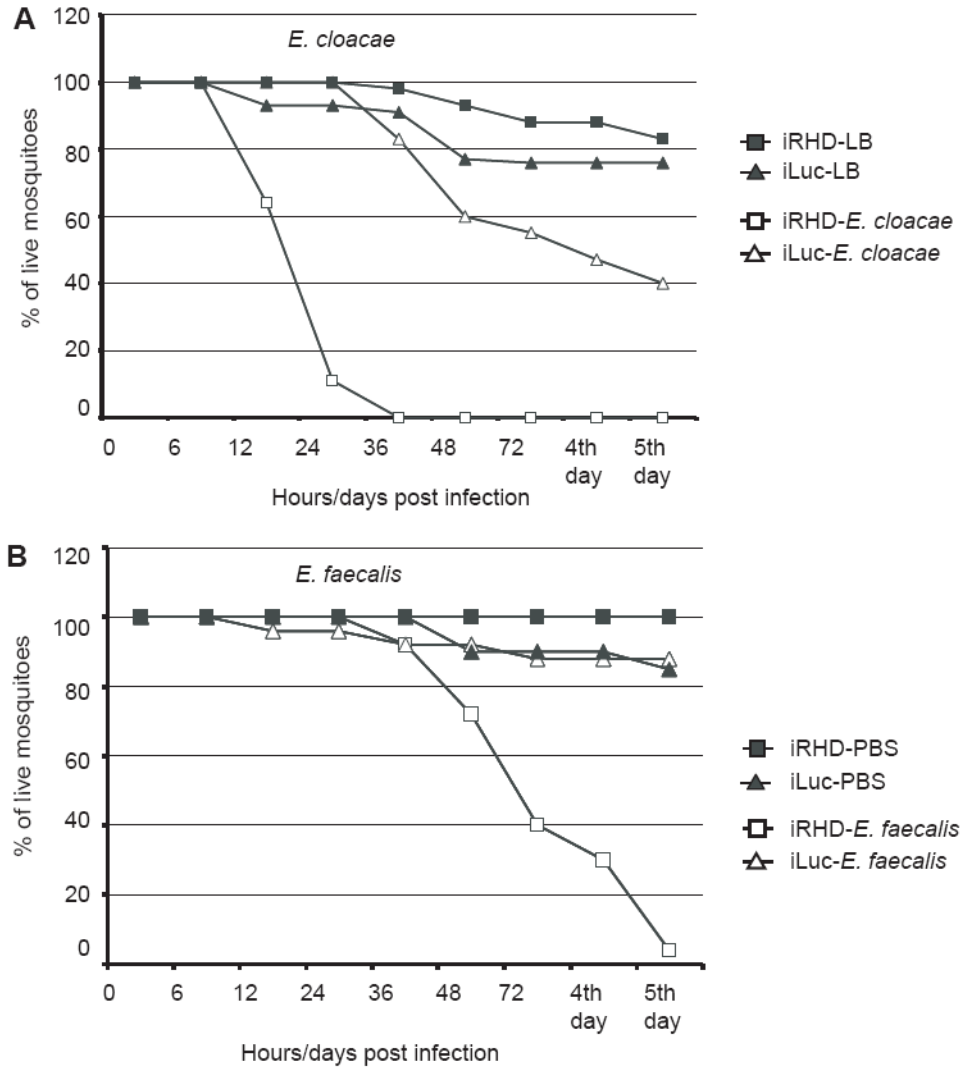
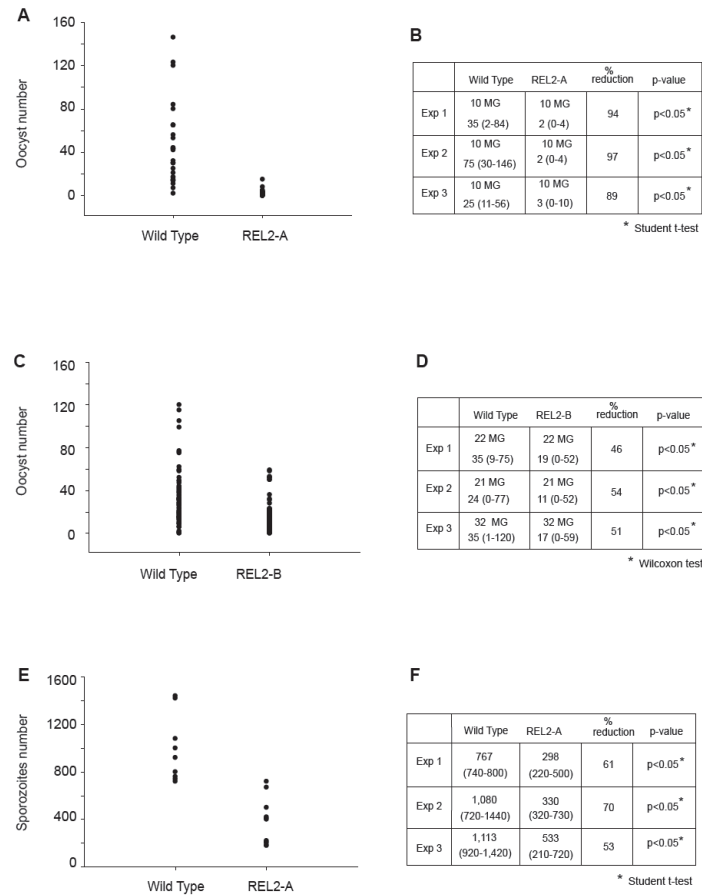


Figure 6. REL2 RNAi-mediated knockdown results in the increased susceptibility to Gram-negative and Gram-positive bacteria. REL2 sdRNA-treated mosquitoes were injected with bacteria on the 4th day after RNAi treatment. Survival was monitored by counting the number of live mosquitoes. **(A)** Increased susceptibility of iRHD-treated mosquitoes to *E. cloacae* infection. Combined results of two independent experiments. **(B)** Increased susceptibility of iRHD mosquitoes to *E. faecalis* infection; combined results of two independent experiments. iRHD—mosquitoes treated with dsRNA against REL2 RHD domain; iLuc—mosquitoes treated with non-specific dsRNA against luciferase gene; LB—mosquitoes injected with sterile LB media.

**Figure 7.**

Increased resistance of REL2 transgenic *Ae. aegypti* to *P. gallinaceum* infection. **(A)** and **(C)** Decreased number of *P. gallinaceum* oocysts in the midguts of REL2-A and REL2-B mosquitoes, respectively. **(B)** and **(D)** Numeric representation of the graphs in **(A)** and **(C)**, respectively; average oocyst number for REL2-A, REL2-B, and wild-type mosquitoes represents the average range of number of oocysts per experiment (shown in parentheses); number of midguts used per experiment is indicated; (MG) midguts. **(E)** and **(F)** Sporozoite number was significantly reduced in REL2-A mosquitoes: **(C)** *P. gallinaceum* sporozoite number in the REL2-A mosquitoes; **(D)** numeric representation of the graph in **(C)**; the table reflects average number of sporozoites and the range is indicated in parentheses.

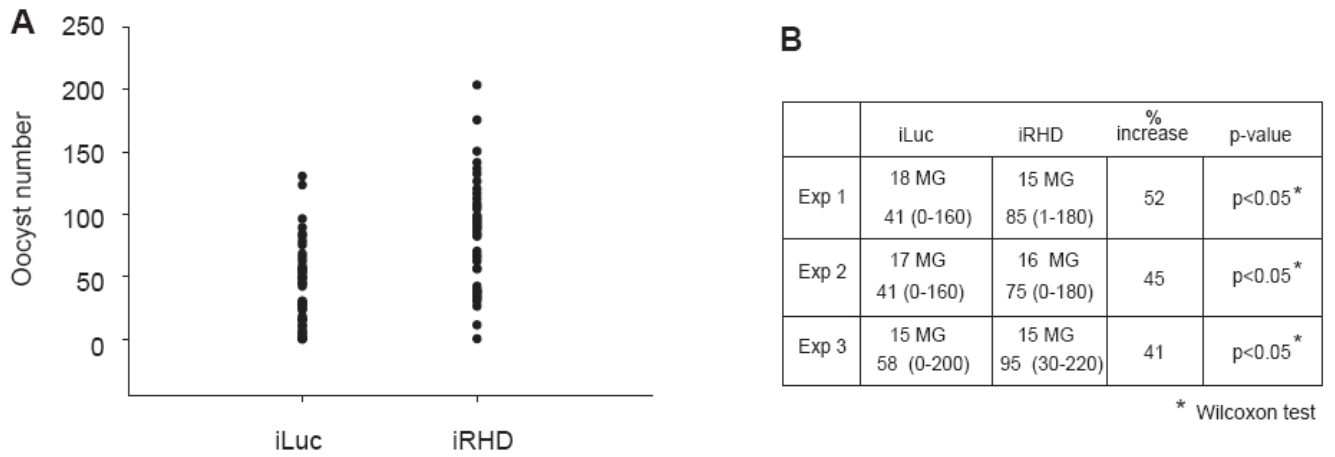


Figure 8. REL2 RNAi-mediated knockdown results in the increased susceptibility of *Ae. aegypti* to *P. gallinaceum* infection. **(A)** Increased number of *P. gallinaceum* oocysts in the midguts of iRHD-treated mosquitoes versus control iLuc mosquitoes ($P < 0.05$ Wilcoxon test). **(B)** Numeric representation of the graph in (A), average oocyst number for REL2 RNAi (iREL2), and control Luciferase RNAi (iLuc) mosquitoes represents the average range of number of oocysts per experiment (shown in parentheses). Number of midguts used per experiment is indicated; (MG) midguts.