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Evaluation of the Function of a Type I Peritrophic Matrix as a Physical Barrier for Midgut Epithelium Invasion by Mosquito-Borne Pathogens in *Aedes aegypti*

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ABSTRACT

In addition to modulating blood meal digestion and protecting the midgut epithelial cells from mechanical and chemical damage, a biological function attributed to the mosquito type I peritrophic matrix (PM) is preventing or reducing pathogen invasion, especially from *Plasmodium* spp. Previously, we demonstrated that chitin is an essential component of the PM and is synthesized *de novo* in response to blood feeding in *Aedes aegypti*. Therefore, knocking down chitin synthase expression by RNA interference severely disrupts formation of the PM. Utilizing this artificial manipulation, we determined that the absence of the PM has no effect on the development of *Brugia pahangi* or on the dissemination of dengue virus. However, infectivity of *Plasmodium gallinaceum* is lower, as measured by oocyst intensity, when the PM is absent. Our findings also suggest that the PM seems to localize proteolytic enzymes along the periphery of the blood bolus during the first 24 hours after blood feeding. Finally, the absence of the PM does not affect reproductive fitness, as measured by the number and viability of eggs oviposited. Key Word: Mosquito(es)

INTRODUCTION

ADULT MOSQUITOES FORM a type I peritrophic matrix (PM) in the midgut following blood feeding. The PM is a baglike structure composed of chitin, protein, and glycoproteins in a proteoglycan matrix (Moskalyk et al. 1996), which underlies the epithelial cells of the midgut (Shao et al. 2001). Three major functions have been attributed to the mosquito PM, including (1) preventing or reducing pathogen invasion, (2) modulating blood meal digestion, and (3) protecting the epithelial cells from mechanical and chemical damage (Abraham and Jacobs-Lorena 2004; Lehane 1997; Wang and Granados 2001). In *Aedes aegypti*, which transmits various mosquito-borne pathogens including *Plasmodium gallinaceum* (an avian malaria), *Brugia pahangi* (a filarial worm), and arboviruses such as dengue virus, PM becomes evident at the light or electron microscopy level 4 to 8 hours after blood feeding, attains mature thickness and texture by 12 hours, then forms crescentric layers by 24 hours (Perrone and Spielman 1988). Because the time line of PM maturation coincides with the development of *Plasmodium* ookinetes and their invasion and penetration of midgut epithelial cells (Han et al. 2000), it has been postulated that the PM functions as a physical barrier against *Plasmodium* infection (Abraham and Jacobs-Lorena 2004; Shahabuddin et al. 1993). In contrast,

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other mosquito-born pathogens, such as arboviruses and filarial worms, may penetrate or infect the midgut epithelial cells prior to PM formation. The potential for the PM to be a physical barrier to infection or to raise the infection threshold for these pathogens has long been discussed (Chamberlain and Sudia 1961; Hardy et al. 1983; Orihel 1975; Stohler 1961), and although it has been concluded that the PM probably does not represent a physical barrier for these pathogenic infections (Christensen and Sutherland 1984; Higgs 2004; Houk et al. 1979; Perrone and Spielman 1988), this has not been directly evaluated.

Whether the PM is a physical barrier for *Plasmodium* has been examined by artificially manipulating the thickness of the PM. When latex particles in a Ringer's solution are fed to *Ae. aegypti* and followed by a blood meal 24 hours later (double-feeding regimen), a thickened PM is formed and a significant reduction in *P. gallinaceum* oocysts results (Billingsley and Rudin 1992). On the other hand, this double-feeding regimen prevented PM formation in *Anopheles stephensi* but did not influence the infectivity of this mosquito to *P. berghei* (Billingsley and Rudin 1992). These studies concluded that the PM may act as a partial but not absolute barrier for *Plasmodium* infection. In another experiment, feeding *Ae. aegypti* with a blood meal containing a chitinase inhibitor, which results in a greatly thickened PM, had a negative impact on *P. gallinaceum* infection of the midgut epithelial cells (Shahabuddin et al. 1993). However, in the same study, *Ae. aegypti* exposed to a blood meal containing exogenous chitinase, which disrupted PM formation, showed no significant difference in *P. gallinaceum* prevalence as compared with control mosquitoes (Shahabuddin et al. 1993).

Because the PM is positioned between the ingested blood bolus and the midgut epithelium cells, it has been suggested that the PM acts as a semipermeable membrane, modulating the passage of molecules between the ecto- and endo-PM compartments and preventing the microvilli from being clogged by luminal contents (Borovdsy 1986; Lehane 1997; Van Handel and Romoser 1987). Using the double-feeding regimen, Billingsley and Rudin (1992) demonstrated that neither the presence of a thickened PM nor the absence of a PM had any effect on the ability of the midgut to digest a blood meal (Billingsley and Rudin 1992). In contrast, preventing PM formation by feeding a blood meal containing anti-PM antibodies limited the rate of blood meal digestion in *Ae. aegypti* (Villalon et al. 2003). It was suggested that this treatment slowed down the diffusion of hydrolytic enzymes across the PM, but the absence of the PM did not affect the rate of egg laying.

Previously we determined that chitin is synthesized *de novo* in the midgut of adult female *Ae. aegypti* in response to blood feeding and is an essential component for PM formation, likely serving as a framework on which peritrophic proteins assemble to form a matrix (de la Vega et al. 1998). In this study, when we use RNA interference (RNAi) to knock down *Ae. aegypti* chitin synthase (*AeCs*) expression, formation of the PM is severely disrupted (de la Vega et al. 1998). Therefore, utilizing RNAi of *AeCs* enabled us to investigate the biological function the PM might have on pathogen infection, protein digestion, and fecundity without unnatural blood feeding or pharmacologic perturbations.

MATERIALS AND METHODS

Mosquito maintenance

Mosquitoes (*Ae. aegypti*, black-eyed Liverpool strain) used in this study were reared according to described methods (Christensen and Sutherland 1984). Artificial blood meals were prepared according to Kogan (1990) with minor modification (8 mg/mL of hemoglobin, 15 mg/mL of γ-globulin, 90 mg/mL of albumin, and 2 mM of ATP (Kato et al. 2006). Mosquitoes were starved for 12 hours prior to blood feeding on a host or when blood fed through a water-jacketed membrane feeder (Kogan 1990).

Disruption of PM formation

Double-stranded RNA synthesis and injection. To produce *AeCs* (AF223577) and green florescent protein (GFP; U76561) double-stranded RNAs (dsRNAs), polymerase chain reaction (PCR) products from the corresponding cDNAs (see Table 1 for primer sequences

TABLE 1. PRIMER SEQUENCES AND RESTRICTION SITES FOR THE DOUBLE-STRANDED RNA SYNTHESIS VECTORS

cDNA template	*Forward (5′–3′)*	*RE*	*Reverse (5′–3′)*	*RE*	*Amplicon*
AeCs	tctagagctgcctgtggtcgtattc	*Xba*I	tctagagaggatcgtaccgaacatc	*Xba*I	489 bs
GFP	gaggtgaagttcgagggcga	*Nco*I*	tccatgccgagagtgatccc	*Spe*I*	371 bs

Restriction enzyme (RE) recognition sites marked by * are found in the vector, pGEM T-easy. *AeCs*, *Ae. aegypti* chitin synthase; GFP, green florescent protein.

and cloning sites) were cloned into the pGEM-T-easy vector (Promega, Madison, WI). To generate single-stranded RNA (ssRNA), plasmids were linearized by the indicated restriction enzymes (Table 1), and *in vitro* transcription was carried out from SP6 and T7 promoters using the appropriate Ampliscribe kit (Epicentre, Madison, WI). An equal amount of sense and anti-sense ssRNA was annealed to generate dsRNA, and all samples were run on a 1% agarose gel to test the integrity of synthesized dsRNA. To knock down *AeCs* expression, *AeCs* dsRNA was intrathoracically injected into individual 2-day-old female mosquitoes as described previously (Bartholomay et al. 2004). GFP dsRNA was used as an injection control.

Quantitative PCR (qPCR) for transcription analysis. Total RNA was collected from midguts of pre– and post–blood-fed (0 hour, 1 hour, 3 hours, 6 hours, 12 hours, 24 hours, 2 days, 4 days, 6 days, 8 days, 10 days, 12 days, 14 days, 16 days, and 18 days) mosquitoes using TRIzol Reagent (Invitrogen, Carlsbad, CA). Reverse transcription was carried out in a 25-μL reaction containing 2 μg of total RNA and 200 U of SuperScript II Reverse Transcriptase (Invitrogen). qPCR was performed as described previously by Kato et al. (2006) (see Table 2 for primer sequences, amplicon sizes, and annealing temperatures). RNA encoding for *Ae. aegypti* actin (*Aeact*-1; U20287) was used as a control, and all reactions were carried out in triplicate at the least. The products of qPCR reactions were sequenced to confirm their identities.

Histological preparations

Mosquito midguts were dissected into *Aedes* saline 24 hours after blood feeding, fixed with 10% formalin in phosphate-buffered saline (PBS; 20 mM KH_2PO_4, 20 mM NaH_2PO_4, 0.15 NaCl, pH 7.3) overnight, dehydrated through an ethanol-xylene series, and embedded in paraffin at 60°C. Samples were sectioned at 5 μm and stained with hematoxylin and eosin (H & E). All samples were viewed using bright field illumination on an Olympus Provis AX70 light microscope connected to a DAGE-MTI DC-330 3CCD color digital camera. Digital images were captured using Image-Pro Plus (Media Cybernetics, Silver Spring, MD) and processed using Adobe Photoshop (Adobe Systems Inc., San Jose, CA) to enhance brightness and contrast.

Effect of the PM on pathogen development/dissemination

In order to examine the effect of the absence of the PM on *P. gallinaceum, B. pahangi,* or dengue virus infectivities, mosquitoes received either no treatment, GFP dsRNA, or AeCs dsRNA injections. For *P. gallinaceum* or *B. pahangi* exposures, mosquitoes were allowed to blood feed for 30 minutes on infected White

TABLE 2. QUANTITATIVE POLYMERASE CHAIN REACTION PRIMER SEQUENCES, ANNEALING TEMPERATURES AND AMPLICON

cDNA	*Forward (5′–3′)*	*tm (°C)*	*Reverse (5′–3′)*	*tm (°C)*	*Amplicon*	*tm (°C)*
AeCs	tgagggagaatgccgtgg	59	cactgtggcttcggtcgc	60	55 bs	83
Aeact-1	ccctgaagtacccccaatgagc	59	ccatgtcatcccagttggtg	58	51 bs	81

AeCs, *Ae. aegypti* chitin synthase; Aeact-1, *Ae. aegypti* actin; tm, melting temperature.

Leghorn chickens or gerbils (*Meriones unguiculatus*), respectively, 4 days after injection with dsRNA. *P. gallinaceum* infection was assayed by counting the number of oocysts from dissected midguts 7 days after blood feeding. *B. pahangi* infections were assayed by counting third-stage larvae from mosquitoes dissected 14 days after blood feeding.

For dengue virus exposure, adult female mosquitoes were fed a blood meal containing DENV-2. C6/36 cells were inoculated with stock virus and incubated at 28°C. Virus was harvested at 7 days postinfection to coincide with peak viral titer in the supernatant (data not shown). The viral supernatant was mixed with an equal volume of defibrinated sheep blood (Colorado Serum Company, Denver, CO). As a phagostimulant, ATP, at a final concentration of 2 mM, was added to the blood. Mosquitoes were fed using an isolation glove box located in a Biosafety Level 3 insectary. Infectious blood was heated to 37°C and placed in a Hemotek feeding apparatus for 1 hour (Discovery Workshops, Accrington, Lancashire, UK). Fully engorged females were separated from unfed females and counted into new cartons. We determined the titer of virus imbibed by assessing the day 0 blood meal and three mosquito samples collected immediately after feeding. At 14 days after exposure, all mosquitoes were collected for analysis. Mosquito bodies and heads were assayed for infectious virus separately to determine overall infection rate/DENV-2 titer and disseminated infection rate, respectively. Mosquito bodies were triturated in 1 mL of L-15 media (10% fetal bovine serum + 10% TPB (tryptone phosphate broth) + 100 U/mL penicillin + 100 μg/mL streptomycin + 1 μg/mL Fungizone). Each sample (100 μL) was loaded in duplicate then titrated in serial 10-fold dilutions in Vero cell culture in the first eight rows of a 96-well plate. Mosquito heads were triturated in 150 μL of L-15 medium (same as above) and titrated in serial 10-fold dilutions in Vero cells in the last four wells of the same rows as corresponding bodies. Titration plates were incubated at 37°C for 10 days, then fixed with 3:1 acetone-PBS for 10 minutes, dried, and stored at −20°C until analyzed by immunofluorescence assay. Plates were stained with a rabbit-anti-DENV hyperimmune serum at 1:100 (produced by S. Higgs in 1994) for 40 minutes at 37°C followed by a secondary antibody, anti-rabbit, immunoglobulin, biotinylated, species-species whole antibody from donkey (Amersham Biosciences, Piscataway, NJ) at 1:100 for 40 minutes at 37°C and streptavidin fluorescein (1:200) for 10 minutes at 37°C. DABCO-glycerol mounting solution was added to all plates, which were then scored using an Olympus IX70 inverted epifluorescence microscope. Body titers were

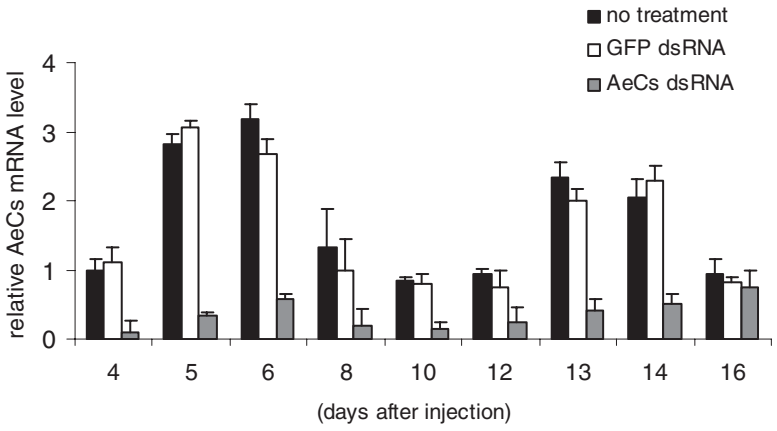

FIG. 1. Effect of *Ae. aegypti* chitin synthase (*AeCs*) dsRNA injection on relative transcript levels in *Ae. aegypti* midguts. Mosquitoes receiving no treatment (control) and mosquitoes injected with green florescent protein double-stranded RNA (dsRNA) (injection control) or *AeCs* dsRNA (chitin synthase knockdown) were blood fed twice (4 and 12 days after the injection). Total RNA was isolated from midguts at 4 to 16 days after the injection. Transcript abundance was quantified using quantitative polymerase chain reaction and calculated in comparison to the message level of the control (*Aeact*-1; U20287).

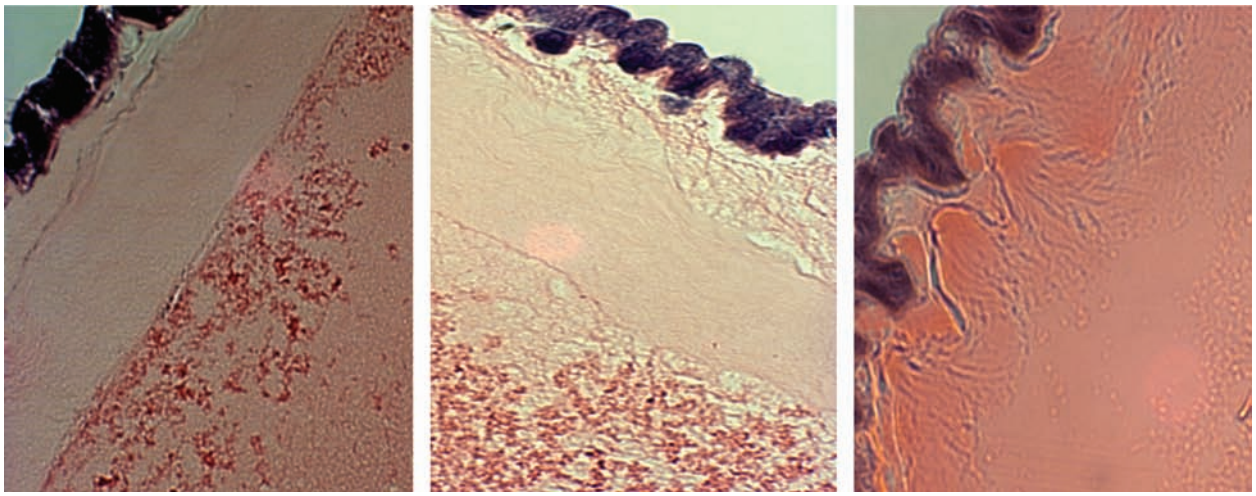

FIG. 2. Effect of *Ae. aegypti* chitin synthase (*AeCs*) double-stranded RNA (dsRNA) injection on peritrophic matrix formation in *Ae. aegypti*. Mosquitoes received either no treatment, green florescent protein (GFP) dsRNA, or *AeCs* dsRNA 4 days prior to blood feeding, and the midguts were dissected 24 hours after blood feeding. Samples were stained with hematoxylin and eosin. (**A**) Midgut from control mosquito (no treatment). (**B**) Midgut from GFP dsRNA-injected mosquito. (**C**) Midgut from *AeCs* dsRNA-injected mosquito.

calculated as infectious dose 50% endpoint titers (log10 $TCID_{50}$/mL). Infection and dissemination rates for the control and different treatment groups were compared with Fisher exact test, and body and head titers were compared by one-way analysis of variance using SPSS version 11.5 (SPSS Inc. Chicago, IL).

Effect of the PM on protein digestion

dsRNA-injected mosquitoes (*AeCs* or GFP dsRNA) were fed with an artificial blood meal containing a fluorescent-based protease detector, BODIPY FL dye-labeled proteins (Invitrogen, Carlsbad, CA), and the midguts were dissected 12 and 24 hours later. After fixing in 10% formalin, the midguts were paraffin embedded and sectioned at 5 μm. Fluorescence was visualized with an Olympus BX51 microscope (Tokyo, Japan) using a short Arc Mercury lamp (Ushio, Japan) and an GFP filter (Olympus).

Effect of the PM on longevity and fecundity

In order to examine the effect of the PM on longevity and fecundity, female mosquitoes re-

TABLE 3. EFFECT OF THE PERITROPHIC MATRIX ON *PLASMODIUM GALLINACEUM* PREVALENCE AND INTENSITY IN *AEDES AEGYPTI*

Parasitemia	*Gametocytemia*	*Treatment*	*Mean intensity*	*Prevalence (%)*
8.0%	0.8%	No treatment	2.4	87
		GFP dsRNA	25.0	85
		AeCs dsRNA	15.3*	91
7.1%	0.6%	No treatment	11.3	97
		GFP dsRNA	9.4	95
		AeCs dsRNA	8.4	95
11.2%	1.1%	No treatment	32.2	96
		GFP dsRNA	30.5	92
		AeCs dsRNA	20.9*	95
7.8%	0.5%	No treatment	9.8	92
		GFP dsRNA	10.3	88
		AeCs dsRNA	7.8	85

*One-way analysis of variance was used to determine the significance of differences among mean intensities.
AeCs, *Ae. aegypti* chitin synthase; dsRNA, double-stranded DNA; GFP, green florescent protein.

ceived either no treatment or GFP dsRNA or *AeCs* dsRNA injections and were caged together with equal numbers of male mosquitoes. Then, female mosquitoes were blood fed 4 days after dsRNA injection, caged individually, and allowed to oviposit. These same females were blood fed again 12 days after injection. The survival rate was monitored for 25 days and the number of oviposited eggs was counted and percentage of fertile eggs was calculated by hatching eggs in deoxygenated water 5 days after collection.

RESULTS

RNAi knock down of AeCs transcript

To better understand the biological role of the PM in *Ae. aegypti*, we manipulated the existence of the PM by knocking down *AeCs* expression using RNAi. In our previous study, we demonstrated that PM formation is severely disrupted when *AeCs* expression is knocked down using RNAi (Kato et al. 2006). In this study, we examined how long *AeCs* expression was compromised following injection of *AeCs* dsRNA. These data were important for studies assessing the effect of the PM on longevity and fecundity. Mosquitoes received no injection or were injected with either GFP or *AeCs* dsRNA, and all mosquitoes were blood fed at 4 and 12 days later. Following dsRNA injections and mosquito blood feeding, qPCR analysis showed that *AeCs* transcript remained at very low levels for at least 16 days after *AeCs* dsRNA injection (Fig. 1). *AeCs* showed a similar transcription profile in mosquitoes receiving no injection and those injected with GFP dsRNA. The absence of the PM was also confirmed hisologically by H & E staining after the second blood meal (Fig. 2).

Effect of the PM on Plasmodium *development*

To examine the effect of the PM on *Plasmodium* development, oocyst numbers were counted 7 days after parasite exposure in mosquitoes that received no treatment (control), GFP dsRNA (injection control), or *AeCs* dsRNA (PM lacking) injections. Mosquitoes were exposed to one of these three different treatments and then were offered a blood meal 4 days after the injection from a *P. gallinaceum*–infected chicken, and this experiment was repeated four times using four different *Plasmodium*-infected chickens. In data obtained from two of the four trials, the mean intensity (mean number of oocysts per *Plasmodium*-infected midgut) from *AeCs* dsRNA-injected mosquitoes was significantly lower than that from control mosquitoes ($df = 83$, $F = 4.043$, $p = 0.0212$; $df = 119$, $F = 21.47$, $p = 1.14 \times 10^{-8}$), and in the other two trials there was no significant difference in oocyst intensity ($df = 119$, $F = 7851$, $p = 0.4585$; $df = 83$, $F = 0.5766$, $p = 0.5640$; Table 3). No significant difference in *P. gallinaceum* infectivity was found between mosquitoes with no treatment and GFP dsRNA injection.

Effect of the PM on B. pahangi *infectivity*

To investigate the influence the absence of the PM has on *B. pahangi* infectivity, three groups of mosquitoes (no treatment, GFP dsRNA injected, or *AeCs* dsRNA injected) were exposed to *B. pahangi* infected gerbils 4 days after dsRNA injection. No significant differences were found in mean intensity of infection between the three groups of mosquitoes ($df = 83$, $F = 1.204$, $p = 0.3053$; Table 4).

Effect of PM on dengue virus dissemination

To investigate the effect of the PM on DENV infectivity and dissemination, three groups of

TABLE 4. EFFECT OF THE PERITROPHIC MATRIX ON *BRUGIA PAHANGI* INFECTIVITY IN *AEDES AEGYPTI*

Parasitemia	*Treatment*	*Prevalence (%)*	*Mean intensity*
76*	No treatment	29	6.5
	GFP dsRNA	25	8.2
	AeCs dsRNA	33	7.2

*Expressed in microfilariae/20 μL blood.

AeCs, *Ae. aegypti* chitin synthase; DNA, double-stranded DNA; GFP, green florescent protein.

TABLE 5. EFFECT OF THE PERITROPHIC MATRIX ON VIRUS TITER IN *AE. AEGYPTI* HEADS AND BODIES AT DAY 14 FOLLOWING EXPOSURE TO DENGUEVIRUS

	*Day 0 titer**		*Day 14 body titer**		*Day 14 head titer**	
Treatment	n	*Mean ± 1 SE*	n	*Mean ± 1 SE*	n	*Mean ± 1 SE*
None	2	3.52 ± 0.00	8	2.78 ± 0.46	7	1.98 ± 0.64
GFP dsRNA	2	3.24 ± 0.40	11	2.91 ± 0.43	10	1.79 ± 1.02
AeCS dsRNA	2	3.52 ± 0.00	12	3.29 ± 0.35	12	2.83 ± 0.33

*Titer as $\log_{10}$ $TCID_{50}$/mL (tissue culture infectious dose).
AeCs, *Ae. aegypti* chitin synthase; dsRNA, double-stranded DNA; GFP, green florescent protein.

mosquitoes (untreated, *AeCS* dsRNA injected, or GFP dsRNA injected) were fed a blood meal containing 5.95 $\log_{10}$ $TCID_{50}$/mL of DENV-2. Tables 5 and 6 show the results of these studies. There was no significant difference in the mean virus titer ingested by each group of mosquitoes (measured immediately after feeding; df = 2, F = 1.00, $p = 0.465$) or in mean virus titer measured in bodies and heads at day 14 between control groups (body: df = 1, F = 0.344, $p = 0.565$; head df = 1, F = 0.045, $p = 0.835$; Table 5). Whereas body and head titers between control and treatment groups were significantly different (body: df = 2, F = 4.32, $p = 0.023$; head: df = 2, F = 7.115, $p = 0.003$), no significant differences were noted in infection rates (df = 2, $\chi^2 = 0.128$, $p = 0.938$) ordissemination rates (df = 2, $\chi^2 = 1.422$, $p = 0.491$) between these groups (Table 6).

Effect of the PM on protein digestion, longevity, and fecundity

An artificial blood meal containing a fluorescent-based protease detector was presented to three groups of mosquitoes (no treatment, GFP dsRNA injected, or *AeCs* dsRNA injected). The midguts were dissected 12 and 24 hours after blood feeding and observed at the light microscopic level. In the midguts from control mosquitoes 12 hours post–blood feeding, a distinct layer of florescent signal was found along the periphery of the blood bolus (Fig. 3A), indicating that protease activity was highly localized to the ectoperitrophic space of the PM. Twenty-four hours post–blood feeding, protease activity was observed to be penetrating the PM and expanding to the center of the bolus (Fig. 3B). In midguts from *AeCs* dsRNA-injected mosquitoes without a PM, a more gradient florescent signal was observed (Figs. 3C and 3D). These findings suggest that the PM may delay proteolytic enzyme diffusion into the endo-PM compartment, thereby localizing the proteolytic activity to the periphery of the blood bolus during the first 24 hours after blood feeding. In the midguts from GFP dsRNA-injected mosquitoes, the same trend was observed as in the control midguts (data not shown).

To examine the effect of the absence of the PM on longevity and fecundity, we measured the survival rate, the rate and number of eggs oviposited, and egg viability (percent of larvae that hatched). The three groups of mosquitoes (no treatment, GFP dsRNA injected, or *AeCs* dsRNA injected, n = 200 per group) were caged with equal numbers of male mosquitoes for 5 days, and the females were allowed to go through two cycles of blood feeding and oviposition. The same trends were observed among the three groups of mosquitoes in the survival rate (Fig. 4), the number of eggs oviposited (Fig. 5), or the percent of larvae that hatched (Table 7), indicating that disruption of the PM does not influence longevity or fecundity of *Ae. ae-*

TABLE 6. EFFECT OF THE PERITROPHIC MATRIX ON DENGUE VIRUS DISSEMINATION MEASURED AT 14 DAYS FOLLOWING EXPOSURE OF *AE. AEGYPTI* TO A VIREMIC BLOOD MEAL

Treatments	n	*Infected*	*Disseminated*
None	18	8/18 (44%)	7/8 (88%)
GFP dsRNA	23	11/23 (48%)	10/11 (91%)
AeCS dsRNA	28	12/28 (43%)	12/12 (100%)

AeCs, *Ae. aegypti* chitin synthase; dsRNA, double-stranded RNA; GFP, green florescent protein.

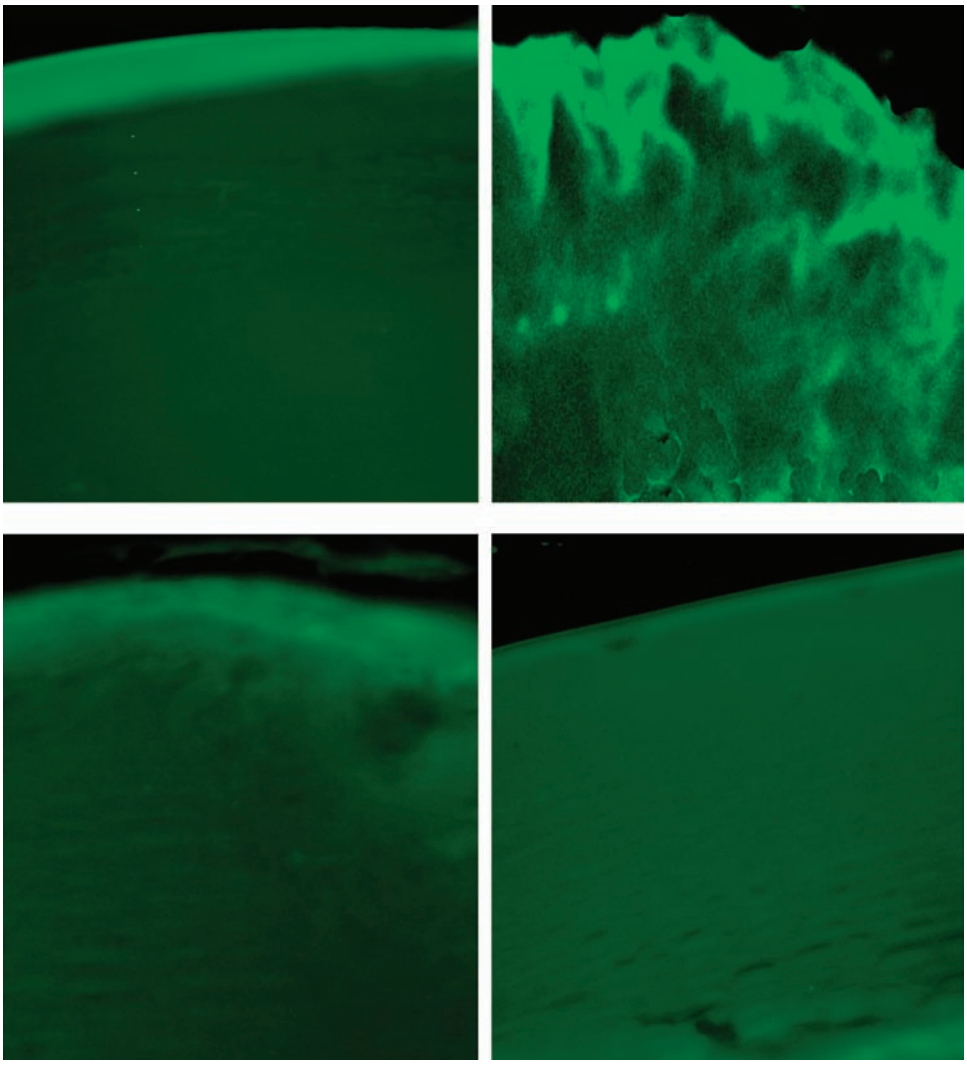

FIG. 3. Effect of *Ae. aegypti* chitin synthase (*AeCs*) double-stranded RNA (dsRNA) injection on proteolytic activity in the midgut of *Ae. aegypti*. Mosquitoes were injected with *AeCs* dsRNA and then fed 4 days later with an artificial blood meal containing a fluorescent-based protease detector. The midguts were dissected 12 and 24 hours after blood feeding, and localization of proteolytic activity in the midgut was visualized histologically. (**A**) Midgut from control mosquito 12 hours after blood feeding. (**B**) Midgut from control mosquito 24 hours after blood feeding. (**C**) Midgut from *AeCs* dsRNA-injected mosquito 12 hours after blood feeding. (**D**) Midgut from *AeCs* dsRNA-injected mosquito 24 hours after blood feeding. Dark areas in each image represent exterior of the midgut.

gypti. These results are consistent with a study in which feeding a blood meal containing anti-PM antibodies prevented PM formation but did not cause a change in the rate of egg laying by *Ae. aegypti* when compared to preimmune serum-fed mosquitoes (Villalon et al. 2003).

DISCUSSION

Mosquitoes are able to mount powerful immune defenses against invading pathogens (Beerntsen et al. 2000; Christophides et al. 2004). These defenses include physical barriers and humoral and cellular responses. Except for transovarial acquisition of some viruses, all pathogens transmitted by mosquitoes are acquired with a blood meal, and, consequently, the midgut is the first barrier encountered when establishing an infection. Within the midgut, pathogens can be exposed to abrupt changes in pH and temperature, proteolytic enzymes, and enclosure by the PM. Exiting this hostile environment as quickly as possible seems to be a viable strategy for many

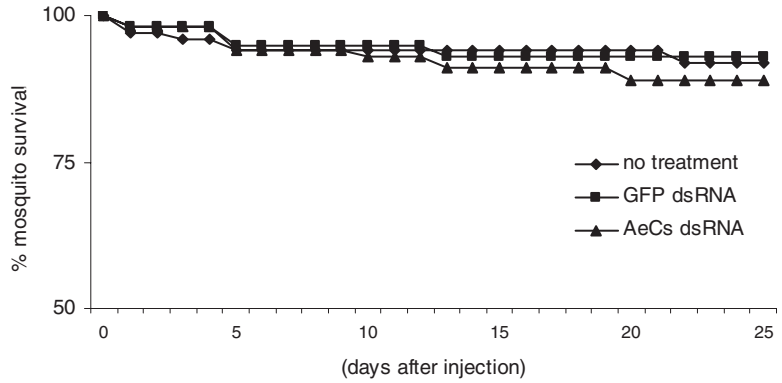

FIG. 4. Effect of *Ae. aegypti* chitin synthase (*AeCs*) double-stranded RNA (dsRNA) injection on the longevity of *Ae. aegypti*. Mosquitoes received no treatment (control), green florescent protein dsRNA (injection control), or *AeCs* dsRNA (chitin synthase knockdown) and were blood fed twice (4 and 12 days after the injection). Mosquito survival was monitored daily.

pathogens (Bartholomay et al. 2004). Microfilariae of several filarial worm species escape from the midgut by penetrating the midgut epithelial cells shortly after ingestion (usually within 90 minutes; Christensen and Sutherland 1984; Perrone and Spielman 1986), but because microfilariae fail to penetrate the midgut in certain mosquitoes, it has been speculated that the PM might serve as a limiting factor (Lehane 1997). Our data indicate that infection with a filarial worm, *B. pahangi*, does not change in the absence of the PM, and the bottleneck effect on the midgut infectivity is not due to the presence of the PM.

Due to its position between the blood and the epithelial cells, it has been suggested that the PM might be a barrier to infection of the midgut epithelial cells by arboviruses (Chamberlain and Sudia 1961; Hardy et al. 1983; Orihel 1975; Stohler 1961) or might increase the infection threshold (Hardy et al. 1983). However, the PM does not form for several hours postfeeding, whereas infectious virus may enter the midgut epithelial cells within the first hour postfeeding. Therefore, others have concluded that the PM cannot serve as a block to arbovirus infection of the mosquito midgut (Higgs 2004; Houk et al. 1979), but no definitive experiments have been conducted to demonstrate this. Our experimental data demonstrate that the infection and dissemination rates for DENV-2 are not significantly changed in the absence of the PM, being the same in untreated, GFP dsRNA-treated, and *AeCs* dsRNA-treated groups.

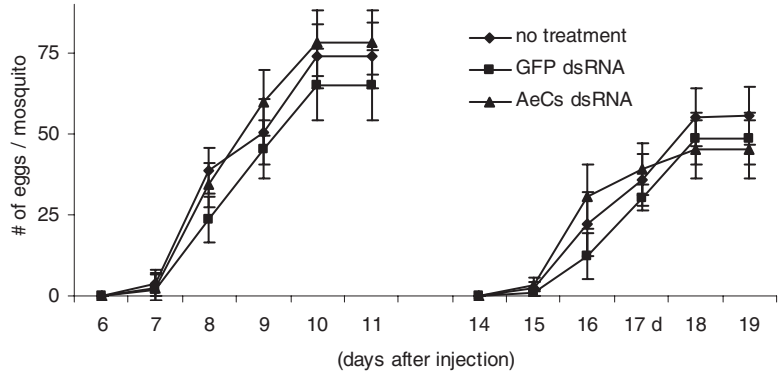

FIG. 5. Effect of *Ae. aegypti* chitin synthase (*AeCs*) double-stranded RNA (dsRNA) injection on the fecundity of *Ae. aegypti*. Mosquitoes received no treatment (control), green florescent protein dsRNA (injection control), or *AeCs* dsRNA (chitin synthase knockdown) and then were blood fed twice (4 and 12 days after the injection) and the number of eggs laid were counted daily.

Table 7. Effect of the Peritrophic Matrix on the Percentage of *Ae. aegypti* Eggs That Hatched

Treatment	*1st oviposition (%)*	*2nd oviposition (%)*
None	95.4	94.2
GFP	96.4	95.1
AeCS	97.1	96.8

AeCs, *Ae. aegypti* chitin synthase; GFP, green florescent protein.

These data therefore provide the first definitive evidence that the PM does not constitute a barrier to arbovirus infection of the *Ae. aegypti* midgut epithelium or to arbovirus dissemination to secondary tissues.

Plasmodium spp. remain in the midgut of their mosquito vectors for at least 24 hours. During this time period, it has been demonstrated that *Plasmodium* spp. use xauthurenic acid, as well as a temperature decrease of at least 5°C, as cues to initiate gametogenesis (Billker et al. 1998; Garcia et al. 1998). Following syngamy and ookinete formation, they secrete chitinase (and perhaps hydrolytic enzymes) to transverse the PM (Abraham and Jacobs-Lorena 2004; Huber et al. 1991). It is generally accepted that the PM serves as a physical barrier for invading pathogens, especially for *Plasmodium* spp. ookinete penetration of the midgut epithelial cells, but our findings suggest that *P. gallinaceum* may actually take advantage of the presence of the PM to improve midgut infection. Interestingly, similar results have been reported before, but the significance of these findings was not discussed. Shahabuddin et al (1993) disrupted PM formation by feeding an exogenous chitinase containing blood meal to *Ae. aegypti* (Shahabuddin et al. 1993). Chitinase-treated mosquitoes, lacking a PM, harbored 11.8 oocysts per midgut, whereas control mosquitoes (PBS treated) had 46.0 oocysts per midgut. The double-feeding regimen treated *An. stephensi* produced no PM, and the infectivity of *P. berghei* was lower in three out of five cases (Billingsley and Rudin 1992), but the authors of the both articles concluded that *Plasmodium* infectivity was unaffected by the presence or absence of the PM. Even though these findings are counterintuitive, there seems to be a tendency that the absence of a PM might indeed reduce *Plasmodium* infectivity.

In order to begin searching for supporting evidence for this hypothesis, we examined the effect of the absence of the PM on protein digestion, by incorporating a fluorescence-based protease detector in a blood meal. Our histological data indicate that the protease activity is localized along the periphery of the blood bolus during the first 24 hours after blood feeding. These data are supported by the fact that trypsin is also localized along the periphery of blood bolus and within the PM of the *Ae. aegypti* blood-fed midguts (Graf et al. 1986). It has been suggested that the PM limits the rate of blood meal digestion, probably by slowing down the diffusion of hydrolytic enzymes across the PM (Villalon et al. 2003). It is important to note that gametes, zygotes, and early (but not differentiated) ookinetes are sensitive to proteolytic enzymes (Gass and Yeates 1979). These parasite stages are present in the midgut during the first 24 hours after exposure to the parasite. Therefore, because the PM localizes proteolytic enzymes along the periphery of the blood bolus during the early stages of infection, the PM may function to provide a more favorable environment in which *Plasmodium* parasites can survive.

The PM occurs in the majority of insects, and the energy and resources used in the production of these structures suggest that the PM plays important biological roles (Lehane 1997). The absence of a PM in adult male mosquitoes can be viewed as strong evidence that the mosquito PM is associated with blood feeding. Our findings suggest that the PM does not protect the epithelial cells from infection/invasion by blood-borne pathogens and in fact might actually enhance the ability of *Plasmodium* to establish an infection in *Ae. aegypti*. The PM does seem to modulate blood meal digestion by delaying the diffusion of proteolytic enzymes into the blood bolus. However, we could not find any evidence to suggest that the absence of the PM negatively influences either mosquito longevity or fecundity. Therefore, even though application of these findings for other mosquito vectors, including other *Aedes*, *Anopheles*, and *Culex* spp., has not been tested, it is apparent the PM does not negatively influence the infectivity of pathogens known to be transmitted by *Ae. aegypti*. Although unlikely, it could be

argued that the PM might influence mechanisms of refractoriness in *Ae. aegypti* for pathogens that this mosquito species does not transmit, but, this is a topic for future investigations.

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