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Aedes aegypti D7 long salivary proteins modulate blood feeding and parasite infection

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ABSTRACT Mosquito saliva facilitates blood meal acquisition through pharmacologically active compounds that prevent host hemostasis and immune responses. Here, we generated two knockout (KO) mosquito lines by CRISPR/Cas9 to functionally characterize D7L1 and D7L2, two abundantly expressed salivary proteins from the yellow fever mosquito vector *Aedes aegypti*. The D7s bind and scavenge biogenic amines and eicosanoids involved in hemostasis at the bite site. The absence of D7 proteins in the salivary glands of KO mosquitoes was confirmed by mass spectrometry, enzyme-linked immunosorbent assay, and fluorescence microscopy of the salivary glands with specific antibodies. D7-KO mosquitoes had longer probing times than parental wildtypes. The differences in probing time were abolished when mutant mice resistant to inflammatory insults were used. These results confirmed the role of D7 proteins as leukotriene scavengers *in vivo*. We also investigated the role of D7 salivary proteins in *Plasmodium gallinaceum* infection and transmission. Both KO lines had significantly fewer oocysts per midgut. We hypothesize that the absence of D7 proteins in the midgut of KO mosquitoes might be responsible for creating a harsh environment for the parasite. The information generated by this work highlights the biological functionality of salivary gene products in blood feeding and pathogen infection.

IMPORTANCE During blood feeding, mosquitoes inject saliva into the host skin, preventing hemostasis and inflammatory responses. D7 proteins are among the most abundant components of the saliva of blood-feeding arthropods. *Aedes aegypti*, the vector of yellow fever and dengue, expresses two D7 long-form salivary proteins: D7L1 and D7L2. These proteins bind and counteract hemostatic agonists such as biogenic amines and leukotrienes. D7L1 and D7L2 knockout mosquitoes showed prolonged probing times and carried significantly less *Plasmodium gallinaceum* oocysts per midgut than wild-type mosquitoes. We hypothesize that reingested D7s play a vital role in the midgut microenvironment with important consequences for pathogen infection and transmission.

KEYWORDS gene editing, arthropod, hematophagy, vascular biology, *Plasmodium*

Vector-borne diseases account for more than 17% of all infectious diseases and cause more than 700,000 deaths annually (1). *Aedes aegypti* mosquitoes transmit dengue, Zika, and chikungunya viruses as well as parasites such as filaria or *Plasmodium gallinaceum* (2, 3). Mosquito probing, or the intradermal search for blood, is a required event for vector-borne pathogen transmission (4–6). During probing, mosquito saliva is transported from the salivary glands and injected into the host skin. It alters the skin microenvironment, preventing hemostasis and inflammatory responses and promoting pathogen transmission (7–10).

Editor Bruno Lemaitre, Ecole Polytechnique Federale de Lausanne, Lausanne, Switzerland

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The authors declare no conflict of interest.

See the funding table on p. 14.

Received 25 August 2023
Accepted 27 September 2023
Published 1 November 2023

Mosquito saliva consists of a complex array of secreted salivary proteins and other components. D7 proteins are among the most abundant proteins in the saliva of blood-feeding arthropods, including mosquitoes, sand flies, and black flies (11). Expression is female-specific and is almost exclusive to the salivary glands of the adult stages, specifically, the distal lateral lobes of glands (12, 13). Proteins involved in blood feeding are stored in the medial and distal lateral lobes, whereas proteins related to sugar metabolism localize to the proximal portion of the lateral lobes (14). There are two subfamilies of D7 proteins in mosquitoes: the long-forms of 27–30 kDa, which show two independent binding domains, and the short-forms that range from 15 to 20 kDa and have a single binding domain (12, 15–20). *Aedes aegypti* has two D7 long-forms (D7L1 and D7L2) and four short-forms, whose functions are not known (11, 21). D7L1 and D7L2 exhibit a multifunctional mechanism of ligand binding: the N-terminal domain binds cysteinyl leukotrienes, while the C-terminal domain shows high affinity to biogenic amines such as norepinephrine, epinephrine, serotonin, and histamine (17, 18). The binding capabilities of the AeD7 long-forms suggest that they scavenge biogenic amines and eicosanoids at the bite site, inducing vasodilation and inhibiting platelet aggregation.

Gene editing techniques are valuable tools for studying gene function and have been used to characterize the importance of arthropod salivary gland genes and their coded proteins (22, 23). In this work, we evaluated the importance of the two salivary D7 long-forms in mosquito blood feeding and pathogen transmission using gene edited-knockout (KO) mosquitoes. D7L1 and D7L2-KO mosquitoes showed prolonged probing times. We also demonstrated that D7 long-forms are important for pathogen infection because KO mosquitoes had fewer *Plasmodium gallinaceum* parasites than wild-type (WT) mosquitoes. During blood feeding, salivary proteins are injected into the host skin, and some of them are reingested along with blood (24). We hypothesize that the reingested D7s play a vital role in the midgut microenvironment, which has important consequences for pathogen infection in the mosquito. This work demonstrates the role of salivary D7 long-form proteins in mosquito blood feeding and pathogen infection.

RESULTS

D7L1 and *D7L2* knockout mosquitoes do not express D7L1 and D7L2 proteins

To investigate the biological function of the D7 long-forms by loss-of-function studies, we generated two lines of homozygous KO mosquitoes (*D7L1*-KO and *D7L2*-KO) by utilizing the non-homologous end-joining pathway of DNA repair to generate the disruption (25). *Aedes aegypti* embryos (n = 950) were injected with Cas9 mRNA and a mixture of synthetic guide RNAs specifically designed to target exons 2 and 3 of either the AAEL006424 gene (*D7L1*) or the AAEL006417 gene (*D7L2*) (Fig. S1), G_0 individuals (n = 188) were backcrossed to WT of the opposite sex, and G_1 individuals were screened for mutations by high-resolution melting analysis (HRMA) and confirmed by Sanger sequencing (Fig. S2A through C). For *D7L1*-KO, 17 and 3 nt deletions were detected after the Cas9 cleavage points of sgRNA6424-1 and sgRNA6424-2, respectively. For *D7L2*-KO, a 13 nt insertion and a 6 nt deletion were detected at the Cas9 cleavage points of sgRNA6417-1 and sgRNA6417-3, respectively (Fig. S2B and C). To remove the potential off-target effects and restore colony genetic diversity, heterozygous individuals for each mutation were crossed with WT mosquitoes, and heterozygous progeny were selected for four additional generations before generating each stable homozygous line. Both $D7L1^{(-/-)}$ and $D7L2^{(-/-)}$ contained frameshift mutations and premature termination codons; thus, they were expected to be loss-of-function (Fig. S2D). A double-heterozygous mosquito line was generated by crossing $D7L1^{(-/-)}$ virgin females with $D7L2^{(-/-)}$ males and $D7L2^{(-/-)}$ virgin females with $D7L1^{(-/-)}$ males. After the crosses, each set of females was blood fed, and their offspring were combined and denominated double heterozygous. These mosquitoes have one intact allele for each D7 long-form gene [$D7L1^{(-/+)}$ $D7L2^{(-/+)}$].

Gene expression analysis with RNA extracted from the salivary glands of the KO mosquito lines showed that *D7L1* transcripts were completely absent from the *D7L1*-KO

mosquitoes. *D7L2* expression was unaffected by the loss of *D7L1*. Likewise, *D7L2* was completely absent from the salivary glands of *D7L2*-KO mosquitoes, with no effect on *D7L1* (Fig. 1A). The double heterozygous strain showed close to 50% (27.0% ± 2.72% and 53.0% ± 2.47% for *D7L1* and *D7L2* genes, respectively) expression levels that corresponded to the protein expression of only one allele. Protein expression levels were generally consistent with gene expression patterns (Fig. 1B). Although no differences were observed for D7L1 transcript level in *D7L2*-KO mosquitoes compared to wild-type mosquitoes (Fig. 1A), ELISA experiments showed a significant decrease in D7L1 at the protein level (Fig. 1B). However, mass spectrometry of saliva from *D7L2*-KO mosquitoes did not show this decrease in D7L2 protein (Fig. 1C). Immunofluorescence assays with specific antibodies raised against D7L1 and D7L2 further supported the absence of D7 proteins in the salivary glands of the correspondent KO mosquitoes Fig. S3. Mass spectrometry studies with saliva from WT and KO mosquitoes confirmed the total absence of the correspondent D7 protein for each KO strain. Differential expression analysis with the number of unique mapping peptides showed a conserved pattern between the WT and the KOs, indicating that the genomic mutations caused by the gene editing approach did not alter the proteome of the saliva to a great extent (Fig. 1C). Moderate expression changes were observed in a small number of other salivary proteins, the significance of which remains to be determined. Scaling plots confirmed that replicate samples were highly similar to each other and were different from both other groups (Fig. S4). Whole proteomes from the saliva of either WT or *D7*-KO mosquitoes are detailed in Table S1.

Mosquito saliva is reingested during blood feeding (24). We demonstrated that *D7*-KO mosquitoes lack this reingested portion of D7 proteins in their midguts after blood feeding on chicken, as visualized by immunochemistry experiments (Fig. S5 A through D). Sections of WT mosquitoes stained with specific antibodies demonstrated the presence of D7L1 and D7L2 in their salivary glands and midgut (Fig. S5B). In *D7L1*-KO mosquitoes, only D7L2 is detected in the salivary glands and midgut, while *D7L2*-KO mosquitoes contain only D7L1 in both salivary glands and midgut. However, all mosquitoes harbored apyrase, a salivary protein known to be reingested during feeding (24), in the extracted midgut blood boluses (Fig. 1E). Figure S6 shows amplified images of mosquito sections stained with hematoxylin and eosin and immunostained with specific antibodies against D7 proteins. Additional experiments were performed with mosquitoes immediately after blood feeding on mice. Blood was dissected out of the midguts and used for Western blot analysis with specific antibodies against D7L1 and D7L2 proteins (Fig. 1D and F). Western blot analysis clearly showed that blood dissected from *D7L1*-KO mosquitoes lacked D7L1 protein. Similarly, D7L2 was not present in the blood dissected from *D7L2*-KO mosquitoes.

D7L1- and *D7L2*-KO mosquitoes show longer probing time on vertebrate hosts

Probing time is the interval from the initial insertion of the mosquito mouthparts in the skin until the visualization of the first traces of blood in the midgut (26). Both *D7*-KO mosquito groups showed extended probing times when fed on mouse [4.7× and 4.1×, for $D7L1^{(-/-)}$ and $D7L2^{(-/-)}$, respectively] or chicken [3.0× and 2.6×, for $D7L1^{(-/-)}$ and $D7L2^{(-/-)}$, respectively] compared to WT, but no differences were observed when blood fed using an artificial membrane feeder (Fig. 2A through C), suggesting the lack of these proteins does not affect the capacity of the mosquitoes to ingest blood when the membrane feeders are used. The probing time range and medians are shown in Table S2. Double heterozygous mosquitoes that contained one intact allele of each *D7* longform showed probing times similar to WT, suggesting each gene is haplosufficient. However, when mice (B6.129S2-Alox5tm1Fun) deficient in leukotrienes due to the lack of arachidonate 5-lipoxygenase were used, KO mosquitoes showed probing times comparable to WT mosquitoes (Fig. 2D). Our results indicate that D7L1 and D7L2 contribute to shorten

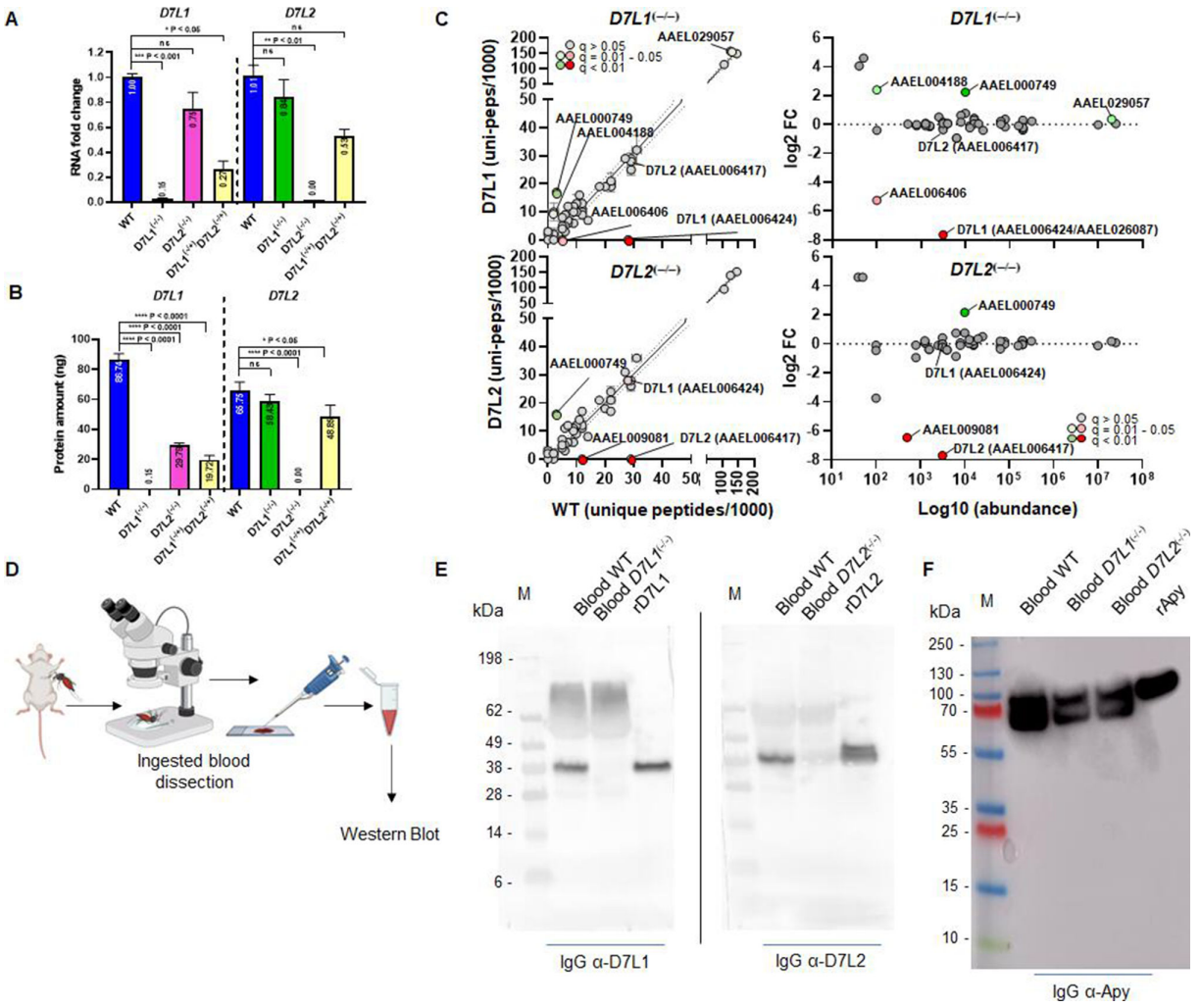

FIG 1 Absence of *D7L1* and *D7L2* at transcript and protein levels in *D7L1*-KO *and D7L2*-KO mosquitoes. (A) Gene expression analysis of AAEL006424 (*D7L1*) and AAEL006417 (*D7L2*) in the salivary glands of wild-type mosquitoes, homozygous CRISPR-generated AAEL006424 [*D7L1* $^{(-/-)}$]-KO mosquitoes, homozygous CRISPR-generated AAEL006417 [*D7L2* $^{(-/-)}$]-KO mosquitoes, and double heterozygous mosquitoes [*D7L1*$^{(-/+)}$ *D7L2*$^{(-/+)}$]. Expression levels are expressed as the fold change levels of RNA with WT as the control sample. RNA levels were analyzed by qPCR, and the *S7* gene was used as a reference gene to standardize the results. Results from two independent experiments with a total of five biological replicates are plotted. Samples were run in technical duplicates. Results are presented as the mean ± SEM. Multiple comparisons were performed by the non-parametric Kruskal-Wallis test. (B) AeD7L1 and AeD7L2 protein amount per salivary gland pair of WT, *D7L1* $^{(-/-)}$, *D7L2* $^{(-/-)}$, and double heterozygous mosquitoes [*D7L1*$^{(-/+)}$ *D7L2*$^{(-/+)}$]. Protein amounts were determined by quantitative ELISA. Serial dilutions of recombinant D7L1 and D7L2 were used to create a standard curve to infer values of protein present in the salivary glands. Results are presented as the mean ± SEM of five biological replicates from a representative of three independent experiments. Multiple comparisons were performed by one-way ANOVA (Dunnett's multiple comparison test). (C) Comparisons of mass spectrometry results of saliva from the different mosquito groups. Differential expression of peptides in the saliva of WT and *D7*-KO mosquitoes. The total number of unique peptides mapping to each gene was plotted after linear normalization (total unique peptides ranged from 845 to 957 per sample). Simple linear regression line (solid) and 95% confidence intervals (dotted) are indicated. The *Y*-axis indicates the log2 fold change (Log2FC) between WT and *D7*-KO samples for each gene product. Positive values indicate increased expression and negative values represent decreased expression in the respective *D7*-KO strain. Log10 abundance was calculated based on the formula {log10[average unique peptides (WT) + 1] × [average unique peptides (*D7L1*-KO) +1] × [average unique peptides (*D7L2*-KO) + 1]}. For all plots, differentially expressed genes based on EdgeR analysis of the raw data are indicated based on *q*-values (FDR set at 0.05). The same set of two WT samples was used for comparison with each *D7*-KO strain. *P* values are indicated as follows: ns: $P > 0.05$; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; and ****$P < 0.0001$. (**D**) Schematic representation of the experiments to determine the presence of D7 reingested proteins in D7-KO mosquito midguts. Western blot studies with blood dissected from mosquitoes fed on mice. (E and F) Detection of D7 proteins in ingested blood by Western blot with specific antibodies. Seventy-five micrograms of protein extracts from ingested blood extracted from midguts immediately after blood feeding of WT, *D7L1*$^{(-/-)}$, and *D7L2*$^{(-/-)}$ mosquitoes and 30 ng of recombinant proteins (rAeD7L1, rAeD7L2, and rApyrase as an internal control) were separated on a NuPAGE 4%–12% Bis-Tris gel and analyzed by Western blot using specific IgG antibodies (α-D7L1, α-D7L2, and α-Apy) raised in rabbits. HRP-goat anti-rabbit antibodies were used at a 1:5,000 dilution. A representative figure of two independent experiments is shown. SeeBlue Plus2 Pre-stained protein ladder and Page ruler Plus Pre-stained Protein Ladder (Thermo Fisher) were used as protein standards for panels (E) and (F), respectively.

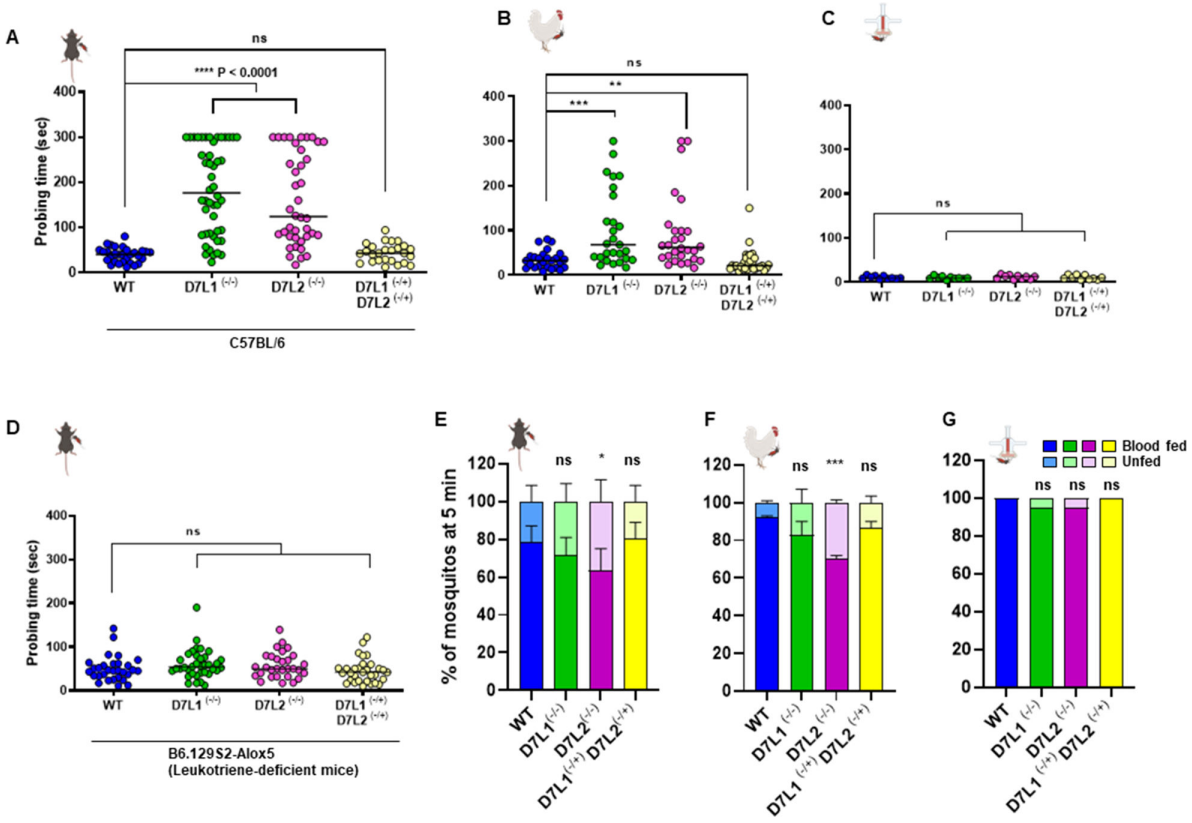

FIG 2 Probing time and blood-feeding success of WT and *D7*-KO mosquitoes. (A) Probing time of individual mosquitoes fed on C57BL/6, (B) chicken, and (C) artificial membrane feeders or (D) leukotriene-deficient mice (B6.129S2-Alox5tm1Fun). For each plot, samples were compared using either a one-way ANOVA for normally distributed samples or the non-parametric Kruskal-Wallis test for non-normally distributed samples. For multiple comparisons, WT was used as the control group. Three independent experiments carried out by two independent operators with a total of at least 30 mosquitoes were assayed per group. (E–G) Feeding success after 5 min of exposure to (E) mice, (F) chicken, and (G) artificial membrane feeder. Feeding status data are presented as mean values of two independent experiments. Each independent experiment consisted of two biological replicates of 100 mosquitoes each. Bars indicate SEMs. For multiple comparisons, WT was selected as the control group. Contingency analyses were performed by Fisher's exact tests. *P* values are indicated as follows: ns: $P > 0.05$; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; and ****$P < 0.0001$.

probing times on vertebrates through leukotriene binding, confirming the role of D7 proteins as leukotriene scavengers.

We also investigated blood-feeding success, defined as the percentage of engorged mosquitoes after a fixed amount of time that was determined in a pilot experiment where 80% of mosquitoes had a full blood meal. After 5 min of blood feeding, we still detected a significant decrease in the percentage of blood-fed mosquitoes on vertebrate hosts in the *D7L2*-KO group (Fig. 2E and F). These results indicate that D7L2 contributes to blood-feeding success at 5 min. As expected, we did not observe differences in blood-feeding success rates when mosquito groups were fed using an artificial membrane system (Fig. 2G).

D7L1- and *D7L2*-KO mosquitoes show a reduction in *Plasmodium gallinaceum* infection

Because *Ae. aegypti* is a natural vector of *P. gallinaceum*, the causative agent of avian malaria, we used it as a model of pathogen infection and transmission. Wild-type and both *D7*-KO mosquito lines were allowed to feed on *P. gallinaceum*-infected chickens (day 0). After 6 days, a subset of mosquitoes was dissected and scored for the number of oocysts in the midgut (Fig. 3A). Because *D7*-KO mosquitoes have reduced blood-feeding capabilities (Fig. 2), we let all mosquito groups feed for 15 min to prevent differences in

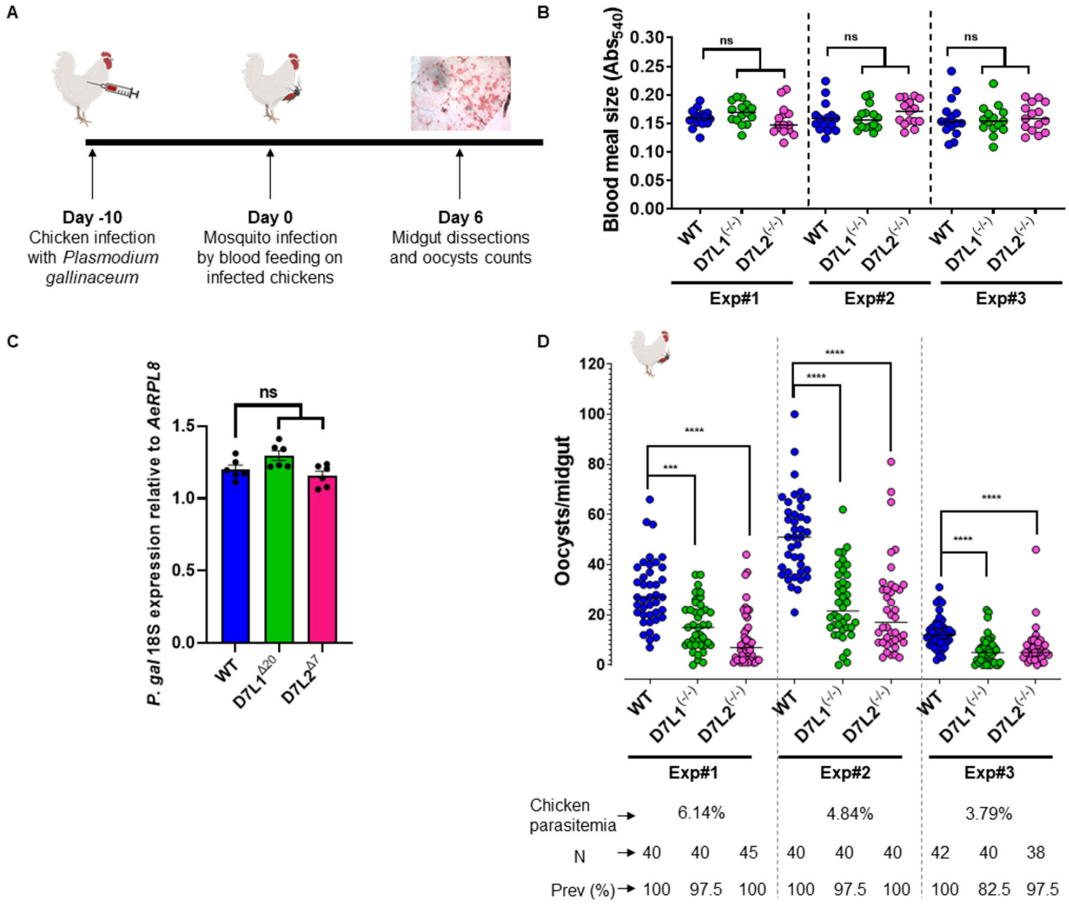

FIG 3 Mosquito infections with *Plasmodium gallinaceum* parasites. (A) Experimental plan of mosquito infections by blood feeding on infected chickens. (B) Hemoglobin content in the midguts of *P. gallinaceum*-challenged mosquitoes. Each point represents the absorbance (Abs) from a single homogenized mosquito from either WT or *D7*-KO [*D7L1*$^{(-/-)}$ and *D7L2*$^{(-/-)}$] mosquitoes. Bars indicate medians of 15 mosquitoes analyzed per experimental replicate (Exp#1, Exp#2, and Exp#3). Absorbance readings at 540 nm were determined in technical triplicates. For multiple comparisons, WT was used as the control group, and samples were compared using one-way ANOVA test. (C) *Plasmodium* 18S expression levels in the midguts from KO and WT mosquitoes at 15 min post-blood feeding on infected chicken. Results are expressed as normalized data of Ct values from *P. gallinaceum* 18S against the conserved ribosomal protein RPL8 from *Ae. aegypti*. Mean values with SEM of technical triplicates of two pools of 10 midguts per mosquito group are shown. ANOVA test resulted in no statistical difference among parasite levels at 15 min post-feeding. (D) Oocyst levels per midgut from mosquito groups fed on independent chickens. Chicken parasitemia of each experiment is shown in the figures. Dots represent the number of oocysts in individual mosquitoes, and the lines indicate median infection. Number of mosquitoes dissected (*N*); infection prevalence (prev). For multiple comparisons, WT was used as the control group, and samples were compared using the non-parametric Kruskal-Wallis test. *P* values are indicated as follows: ns: $P > 0.05$; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; and ****$P < 0.0001$.

blood meal size that could result in different parasite loads. Only fully engorged females were used for infection analysis, and a random subset was confirmed to have imbibed a similar amount of blood following an analysis of hemoglobin content in their blood meal (Fig. 3B). We also confirmed different mosquito groups had ingested similar number of parasites as demonstrated by quantitative RT-PCR (qPCR) studies with midguts from mosquitoes at 15 min post-infection (Fig. 3C).

We detected a significant reduction of *Plasmodium* oocyst numbers in *D7L1*- and *D7L2*-KO mosquitoes (53.5% and 66.3%, respectively) compared to the WT controls in all three biological replicates (Fig. 3D). Mosquito infections were carried out at parasitemia levels of approximately 5% (6.1%, 4.8%, and 3.8% for the first, second and third replicates, respectively). Infection experiments with higher (50.0%) and lower (2.4%) parasitemia levels resulted in a similar oocyst reduction in the *D7*-KO mosquitoes (Fig. S7). These results confirm that the reduction in oocyst numbers was independent of the chicken parasitemia.

We sought to investigate whether the reduced number of oocysts per midgut in the *D7*-KO mosquitoes resulted in a reduced transmission rate. We performed a *P. gallinaceum* transmission experiment where naive chickens were infected by the bite of five experimentally infected mosquitoes. Under these conditions, we observed a reduction in the weight gain in chickens infected by the bite of infected *D7L2*$^{(-/-)}$ mosquitoes. However, there were no differences in other clinical signs and parasitemia (Fig. S8), most probably because there were enough sporozoites to initiate an infection (27).

DISCUSSION

This article describes the role of D7L1 and D7L2 salivary proteins in mosquito blood feeding and parasite infection using knockout mosquitoes. Silencing attempts of salivary genes in mosquitoes had resulted in poor knock-down effects (22, 28), most probably due to the accumulation of transcripts in female pupae preceding overexpression in adults, which prepares the female adult for blood feeding after emergence (18). Therefore, we used CRISPR/Cas9 technologies that targeted the genes of interest in the germ cells at mosquito pre-blastoderm stages to generate mutations that were passed on to offspring. We successfully generated two independent knockout mosquito lines: *D7L1*-KO and *D7L2*-KO. Our gene expression analyses showed that *D7L1* and *D7L2* transcripts were completely absent from the correspondent KO mosquitoes. This finding is likely explained by the nonsense-mediated mRNA decay that is part of the eukaryotic mRNA quality control and regulatory process. It degrades mRNAs containing premature termination codons because of nonsense or frameshift mutations (29). At protein levels, homozygous *D7L1*- and *D7L2*-KO mosquitoes lacked the expression of the correspondent proteins, as demonstrated by ELISA, immunofluorescence, and mass spectrometry of saliva. Overall, differential expression analysis with the number of unique mapping peptides determined by mass spectrometry of saliva showed a similar pattern between WT and the KO mosquitoes. However, there were punctual genes that were up or downregulated in the KO mosquitoes. These changes could be attributed to compensatory responses for difficulty in feeding or maybe due to the presence of potential protein complexes required for stability. However, more research is needed to further clarify these observations.

For successful blood feeding, mosquitoes must find a suitable host, penetrate the host skin, locate blood vessels, and actively ingest blood until engorgement (26). A mosquito must complete the whole blood-feeding process without being noticed because impairing blood uptake has a negative impact on fecundity (30). Probing time is the first step of blood feeding and is initiated with stylet penetration and continues until the appearance of blood in the midgut (26, 30). Our experiments demonstrated that D7L1 and D7L2 play a pivotal role in the first step of blood feeding. The double heterozygous mosquitos [*D7L1*$^{(-/+)}$ *D7L2*$^{(-/+)}$] showed probing time values similar to WT mosquitoes, with no impact on blood feeding on animals suggesting that the amount of protein supplied by only one allele is enough to rescue the phenotype. This rescued phenotype by partial supplementation with one allele of each D7 also demonstrated the lack of off-target effect. We did not see an extension of probing time when mosquitoes were fed through an artificial membrane feeder, a system that lacks the vertebrate hemostatic and immune host responses. These experiments confirmed that the effects of D7L1 and D7L2 in vertebrates are essential for keeping mosquito probing time short. Similar results were observed with *Ae. aegypti* KO mosquitoes that lacked the salivary vasodilator sialokinin (23). In *Anopheles gambiae*, *D7 long form L2* (AGAP008279) weakly binds leukotrienes B4 and D4 and had a dose-dependent anticoagulant effect via the intrinsic coagulation pathway by interacting with factors XII, XIIa, and XI (20). Silencing of AGAP008279 produced a significantly lowered blood-feeding phenotype and increased probing time, supporting the hypothesis that this gene may be playing an important role in blood-feeding (31).

D7 proteins are highly expressed in the salivary glands and show a unique type of agonist inhibition called kratagonism, typically found in blood-feeding arthropods (32).

They bind biogenic amines such as serotonin, histamine, norepinephrine, epinephrine, or cysteinyl leukotrienes with high affinity (16, 17). The stoichiometry of the binding is 1:1—one molecule of salivary protein per molecule of host mediator—which explains why high concentrations of these proteins are present in the saliva (12, 15–19, 33). When mutant mice deficient for leukotriene production (B6.129S2-Alox5tm1Fun) were used in our probing time experiments, the WT phenotype was rescued for *D7*-KO mosquitoes. Our results indicate that D7L1 and D7L2 contribute to shortening probing time in vertebrates, derived from their leukotriene binding effects. This confirms the role of D7 proteins as leukotriene scavengers. Biogenic amines such as serotonin or histamine accumulate and saturate their receptors at one order of magnitude above the level required for leukotrienes (32). These results support the behavior of the double heterozygous mosquitoes, where the protein amount expressed by only one copy of each gene was sufficient to provide the observed phenotype. We also attempted to generate double homozygous mosquitoes [$D7L1^{(-/-)}$, $D7L2^{(-/-)}$] that could have provided additional important information; however, only double heterozygous were successfully generated.

Mosquito salivary proteins influence arboviral pathogenesis by altering the skin's immune environment and modulating anti-viral host immune responses (8, 34–37). On the other hand, individual saliva components, such as D7 long proteins, can have inhibitory activities against arbovirus infection (38). In addition, AeD7 long proteins interact with dengue and Zika viral particles (39, 40) and decrease viral infections *in vitro* and *in vivo* (40). Using the D7-KO mosquitoes, it would be interesting to study whether D7 proteins play a role in arbovirus infection and transmission and we aim to perform these experiments in the near future. In the case of parasitic infections transmitted by mosquitoes, *Anopheles* spp. salivary components modulate host immune responses, contributing to increased *Plasmodium* spp. infection and transmission (41–46). While human malaria is exclusively transmitted by *Anopheles* spp. mosquitoes, avian malaria can be transmitted to birds by Culicidae mosquitoes, including *Anopheles*, *Culex*, and *Aedes* spp. (3). We studied the role of *Aedes* D7 proteins in *Plasmodium gallinaceum* parasite infection and transmission using the D7-KO mosquitoes. Our parasite infection studies suggest that D7 salivary proteins may be promoting mosquito infections in the intravectorial cycle. Although there is a clear reduction of the parasitemia of the *D7*-KO mosquitoes at oocyst levels, there is not enough evidence of a reduction in sporozoite numbers in the salivary glands (data not shown). For protein supplementation experiments, we initially performed mosquito infections using an artificial membrane system where heparinized blood from an infected chicken was drawn and immediately added to artificial membrane feeders to blood feed the different mosquito groups. Then, we followed the *Plasmodium* infection in the mosquitoes and observed no differences in oocyst counts among mosquito groups. We conducted two biological replicates (Fig. S9) and, therefore, we hypothesized that the saliva containing D7 proteins (WT group) or not containing D7 proteins (KO groups) was secreted during membrane blood feeding by mosquitoes into the feeding device, mimicking what happens in the skin. However, because of a large difference in the volume of hematoma in the wound versus the volume in the artificial feeder, we believe that the salivary proteins that were demonstrated to be reingested during a blood meal are being reingested at very much lower concentrations through the artificial method due to a dilution effect and did not have any effect in the midgut's environment of the mosquitoes. Therefore, we could not use this system either for rescue experiments by mixing the parasites with recombinant proteins to blood feed the D7-KO mosquitoes or for inhibition experiments by mixing the parasites with specific antibodies against D7 proteins to blood feed the WT group. On the other hand, no effect on *Plasmodium* transmission was observed. Chickens are very sensitive to a *P. gallinaceum* infection, and transmission is known to occur even by probing infected *Aedes* mosquitoes (47). As a result, even if few oocyst numbers are present in the *D7*-KO mosquitoes, they contain enough sporozoites to initiate a transmission event (27). In addition, the longer probing time of *D7*-KO mosquitoes may

lead to a larger amount of injected sporozoites. However, this statement remains to be confirmed.

D7 salivary proteins are reingested in the mosquito midgut during blood feeding (24), and their presence may modify the midgut environment with consequences on parasite development. We hypothesize that reingested D7 proteins may be scavenging biogenic amines and eicosanoids in the ingested blood with fatal consequences for the parasite. For instance, serotonin has an effect on *Anopheles stephensi* mosquito infection with *Plasmodium* parasites (48). In the presence of physiological blood levels of serotonin, mosquitoes presented lower infection rates. Furthermore, histamine ingestion by mosquitoes enhances malaria parasite infection (49). Another hypothesis is that D7 proteins may be interacting with host receptor proteins that influence host immunity. A recent pull-down experiment showed that D7 proteins interact with human receptor proteins. In particular, the D7L1 protein (AAEL006424) directly interacted with CD86 and DC-SIGN, while the D7L2 protein (AAEL006417) interacted with CD4 and CD14 (50). However, more research is needed to understand the effect of D7 in the blood meal and parasite biology.

As a limitation of this work, we could not perform further experiments to unravel the mechanism of action of D7 proteins in oocyst reduction due to restrictions in our animal study protocols. Ideally, infecting WT mosquitoes through blood feeding on *P. gallinaceum*-infected chickens with circulating antibodies against D7 proteins would be of interest to confirm the effect of D7 in parasite mosquito infection. Studies supplementing the infectious blood meal with recombinant D7 proteins or anti-D7 antibodies could also confirm our observations. Also, further experiments are needed to evaluate whether reduced oocyst levels in the midguts have any impact on the number of sporozoites in the salivary glands.

We provide evidence supporting the conclusion that the salivary gland D7L1 and D7L2 significantly contribute to shortening probing time and ensuring blood-feeding success in *Ae. aegypti*. This work highlights the relevance of D7 proteins in blood feeding, which explains why this family of proteins has arisen and evolved from gene duplication events to permanently stay in the salivary gene repertoire of blood-feeding Nematocera. Furthermore, a lack of D7 proteins may produce a harsh environment for parasite development in the mosquito midgut when saliva is reingested during feeding. Finally, our results support transgenic approaches for studies of gain and loss of salivary functions.

MATERIALS AND METHODS

Mosquito rearing, salivary gland dissection, and saliva collection

Aedes aegypti (Liverpool strain, LVP) mosquitoes were reared in standard insectary conditions (27°C, 80% humidity with a 12-h light/dark cycle) at either the Department of Entomology and Fralin Life Science Institute, Virginia Tech, or the Laboratory of Malaria and Vector Research, NIAID, NIH. Salivary gland dissections and saliva collection were performed as previously described (23).

Generation of CRISPR/Cas9 knockout lines

Cas9 mRNA and synthetic guide RNAs (sgRNAs) targeting genes *D7L1* (AAEL006424) or *D7L2* (AAEL006417) were synthesized and injected into *Ae. aegypti* preblastoderm embryos as described previously (23, 51). Briefly, 600 ng/µL of Cas9 mRNA and 100 ng/µL of each sgRNA (Fig. S1) were injected into *Ae. aegypti* pre-blastoderm embryos as described (52). To detect editing activity, G_1 progeny was screened as adults by Phire PCR and HRMA from a piece of leg tissue (22, 23). Amplicons that differed from controls were subject to Sanger-based sequencing, and indels were identified. G_1 individuals with confirmed indels were backcrossed to the wild-type strain for four additional genera-

tions, prior to the generation of homozygous loss-of-function mutant strains through intercrossing.

Gene expression analysis by qPCR

Quantitative RT-PCR was used to determine gene expression analysis. Three pools of 10 salivary gland pairs per mosquito line {WT, homozygous *D7L1*$^{(-/-)}$, homozygous *D7L2*$^{(-/-)}$, and double heterozygous mosquitoes [*D7L1*$^{(-/+)}$*D7L2*$^{(-/+)}$]} were dissected from 1- to 2-day-old mosquitoes. Total RNA was extracted with TRIzol (Life Technologies) with Phase Lock Gel Heavy tubes (QuantaBio) following the manufacturer's instructions. One microgram of RNA was converted to cDNA using the QuantiTect Reverse Transcriptase Kit (Qiagen). The nanodrop ND-1000 spectrophotometer was used to determine all concentrations and $OD_{260}/_{280}$ ratios of nucleic acids. qPCR was carried out as previously described. Specific primers to target *D7L1* and *D7L2* genes were designed (AeD7L1_qPCR and AeD7L2_qPCR; Table S3). Briefly, in a final volume of 20 µL, the reaction mix was prepared with 2× SsoAdvanced Universal SYBR Green Supermix (Bio-Rad), 300 nM of each primer, and 100 ng of cDNA template. Two independent experiments that included five biological replicates were tested. All samples were analyzed in technical duplicates, and non-template controls were included in all qPCR experiments as negative controls. qPCR data were manually examined and analyzed by the ΔΔCt method using the CFX Maestro software version 1.1 (BioRad). ΔCt values were obtained by normalizing the data against *Ae. aegypti* 40S ribosomal protein S7 transcript as the reference gene. Samples from the salivary glands of wild-type mosquitoes were chosen as controls for the ΔΔCt values. The relative abundance of genes of interest was calculated as $2^{-\Delta\Delta Ct}$.

For *P. gallinaceum* qPCR, midguts were dissected at 15 min after mosquito feeding on chickens infected with *P. gallinaceum*. Pools of 10 midguts were added to 200 µL of TRIzol and were stored at −80°C until RNA extraction. qPCR studies were performed as described earlier with the following modifications. RNA was reverse transcribed into cDNA using the LunaScript RT Master Mix Kit (Primer Free) (NEB, E3025L) with 6 µM Random Primer Mix (NEB, S1330S) following the manufacturer's instructions in 20 µL total reaction volume. *Plasmodium* 18S ribosomal gene was used as the target gene (GeneID 39733503), while *Ae. aegypti* RPL8 transcript was used as the reference gene. Results were expressed as the *Plasmodium* levels normalized by the *Ae. aegypti* RPL8 transcript levels. Two pools of 10 midguts each were dissected from each mosquito group and technical triplicates were performed. Graphs were prepared using GraphPad Prism software version 8.02. Primer information is provided in Table S3.

Protein quantification by ELISA

AeD7L1 and AeD7L2 protein concentration per salivary gland pair of the different mosquito groups was determined by quantitative ELISA as previously described (12). Serial dilutions of recombinant D7L1 and D7L2 (17, 18) were used to create a standard curve to infer values of protein present in the salivary glands. Results are presented as the mean ± SEM of five biological replicates from a representative of three independent experiments. Multiple comparisons were performed by one-way ANOVA (Dunnett's multiple comparison test).

Mass spectrometry and peptide analysis

Salivary gland extracts and saliva samples were submitted for mass spectrometry at the Research and Technology Branch (NIAID, NIH). Salivary gland extracts were obtained from 10 mosquitoes from each group and two sets of saliva were collected from 50 mosquitoes each. Samples were reduced with 5 mM DTT for 40 min at 37°C, cooled to room temperature, and alkylated with 15 mM iodoacetamide for 20 min. Extracts were then hydrolyzed with 200 ng GluC endoproteinase, a serine proteinase that preferentially cleaves peptide bonds C-terminal to glutamic acid residues and results in an improved sequence coverage in mass spectrometry, for 15 h at 37°C in 40 µL reactions

buffered with 100 mM ammonium bicarbonate in the presence of 10% acetonitrile. The sample volumes were reduced under vacuum by ~50% at room temperature, samples were acidified with 0.5% trifluoroacetic acid, and desalted or concentrated on Agilent OMIX10 C18 microsolid phase extraction tips. Samples were resuspended in 10 µL of 0.1% trifluoroacetic acid, 3% acetonitrile and submitted to nano LC-mass spectrometry (Thermo Fisher Orbitrap Fusion). Two technical replicates of each sample were run.

The number of unique mapping peptides was determined, and a summary table with a single representative isoform selected for each gene was processed using EdgeR (Table S1) to determine the differential expression as previously described (22). To be included, at least three of the six samples were required to have more than three unique mapping peptides. False discovery rate (FDR) was set at 0.05.

Immunolocalization

The presence of D7L1 and D7L2 proteins in the mosquito salivary glands was visualized by immunofluorescence following the methods previously described (12, 53). Briefly, salivary glands were dissected in phosphate-buffered saline (PBS) and fixed with 4% paraformaldehyde for 30 min. After three washes with PBS, pH 7.4, samples were blocked with 1% bovine serum albumin, 0.5% Triton X-100, and PBS (blocking solution) for 1 h. Salivary glands were incubated for 16 h at 4°C with 1 µg/mL of pre-adsorbed antibodies against either D7L1 or D7L2, raised in rabbits, and diluted in blocking solution. As negative controls, salivary glands were incubated with PBS instead of primary antibody. Three 10-min washes with the blocking solution were followed by the incubation with goat anti-rabbit IgG Alexa Fluor 594 antibody (Catalog# A-11012). The excess secondary antibody was removed by another cycle of three 10-min washes. Samples were incubated with a Phalloidin Alexa Fluor 488 fluorophore (Fisher Scientific) 1:50 diluted in PBS for 20 min to stain actin in order to highlight the salivary gland wall architecture. After three additional washes, salivary glands were mounted on glass slides with Fluoroshield. Bright-field and fluorescent images were acquired in a Leica Epifluorescence Microscope, using a 20× objective with a 1× magnification. All incubations were performed in concavity slides at room temperature unless otherwise specified.

Histopathology and immunohistochemistry of mosquito samples

Mosquitoes were processed at the Infectious Disease Pathogenesis Section, Comparative Medicine Branch, NIH. Briefly, within 15 min of blood feeding, mosquitoes were washed with 80% ethanol to remove cuticle wax and were immediately fixed in 10% neutral buffered formalin for 12 h. Mosquitoes were processed with a Leica ASP6025 tissue processor (Leica Microsystems), embedded in paraffin, sectioned at 5 µm, and blocked in paraffin for histological analysis. The sections were stained with hematoxylin and eosin for routine histopathology and then evaluated by a pathologist in a blinded manner.

Formalin-fixed paraffin-embedded mosquito sections were used to perform immunohistochemical staining using two rabbit polyclonal antibodies: IgG αAeD7L1 and IgG αAeD7L2 at a dilution of 1:500. Staining was carried out on the Bond RX (Leica Biosystems) platform according to the manufacturer-supplied protocols. Briefly, 5 µm-thick sections were deparaffinized and rehydrated. Heat-induced epitope retrieval was performed using Epitope Retrieval Solution 1, pH 6.0, heated to 100°C for 20 min. The specimen was then incubated with hydrogen peroxide to quench endogenous peroxidase activity prior to applying the primary antibody. Detection with DAB chromogen was completed using the Bond Polymer Refine Detection kit (Leica Biosystems, Catalog # DS9800). Slides were finally cleared through gradient alcohol and xylene washes prior to mounting and placing coverslips.

Sections were examined by a boarded-certified veterinary pathologist using an Olympus BX51 light microscope, and photomicrographs were taken using an Olympus DP73 camera.

Western blot

Seventy-five micrograms of protein from ingested blood from WT and KO mosquitoes and 30 ng of recombinant proteins were separated on a NuPAGE 4%–12% Bis-Tris gel. Proteins were transferred to a nitrocellulose membrane (iBlot, Invitrogen) and then blocked for 1 h at 37°C with blocking buffer [5% (wt/vol) powdered nonfat milk 0.05% (vol/vol) Tween 20, Tris buffered-saline (25 mM Tris, 150 mM NaCl, pH 7.4 (TBS-T)]. Blots were incubated for 16 h at 4°C with rabbit IgG antibodies against D7L1 and D7L2 (pre-adsorbed with opposite proteins), separately. After two 5-min washes with TBS-T, blots were incubated with HRP-conjugated goat anti-rabbit IgG (Catalog # 31460, Invitrogen, dilution—1:5,000) as a secondary antibody for 1 h. Blots were washed with TBS-T five times for 5 min. Blots were developed with Femto SuperSignal West Femto Maximum Sensitivity Substrate (cat. 34094, Thermo Scientific, Rockford, IL, USA) and imaged using an Azure 300 imaging system (Azure Biosystems, Dublin, CA, USA). As an internal control for the Western blot, rabbit IgG antibodies against another salivary protein [apyrase, AAEL006347 (39)] were also tested.

Probing time

Intradermal search for blood or probing time determination was done according to Martin-Martin et al. (23). Briefly, adult female mosquitoes aged 5–10 days were deprived of sugar and water for 16 h. Mosquitoes were individually caged and offered either the back of a mouse or the breast of an immobilized chicken. The backs of 5-week-old C57BL/6 or leukotriene-deficient mice (B6.129S2-Alox5tm1Fun) were shaved the day before the experiment. Mice were anesthetized (75 mg of ketamine/kg of body weight, 10 mg of xylazine/kg of body weight, i.p.) and kept on a slide warmer during mosquito exposure. For probing time experiments in chickens, animals were immobilized, and breast feathers were removed. Probing time was also assessed on artificial membrane feeders (NDS Technologies, Inc, Vineland, NJ, USA) covered with Parafilm M (Amcor, Ann Arbor, MI, USA) filled with bovine whole blood in Acid Citrate Dextrose obtained from Lampire Biological Laboratories (Pipersville, PA, USA) supplemented with 2.4 mM ATP. Time was measured from the initial insertion of the mosquito mouthparts in the skin until visualization of the first traces of blood in the midgut. All measurements were stopped at 300 s, and mosquitoes that showed a longer probing time were recorded at 300 s. For probing time on mice and chickens, at least two independent experiments were performed until at least 26 mosquitoes per group were assayed. For probing through artificial membranes, 10 mosquitoes were tested per group. Results did not pass the D'Agostino and Pearson normality test, so one-way ANOVA Kruskal-Wallis test was used to determine statistical power for probing time on mice and chicken. For probing time through artificial membrane feeders, one-way ANOVA was used.

Blood-feeding success

For each feeding experiment, three cages each containing 100 female mosquitoes were starved of sugar and water the night before the experiment. An initial experiment was performed in order to determine the time needed to get about 80% of blood-fed wild-type mosquitoes. Once that time was chosen, it was kept invariable for the other experimental groups (5 min). Mosquitoes were allowed to feed on the animals or through an artificial membrane system at 27°C in the dark for 5 min. After blood feeding, mosquitoes were scored under a stereo microscope as fed if any traces of blood were visualized in their midgut or as unfed. Two independent feeding success experiments were performed.

Plasmodium gallinaceum infection and transmission experiments

Six-week-old White Leghorn chickens (*Gallus gallus*, Charles River, Norwich, USA) were IV inoculated with washed blood containing *Plasmodium gallinaceum* gametocytes. We used the parasite strain 8A (27) passed through both chickens and mosquitoes. After

inoculation, we determined chicken parasitemia daily by checking Giemsa-stained blood smears prepared from blood droplets collected from the chicken's feet vein. The smears were fixed in absolute methanol and stained with 10% Giemsa at pH 6.4 for 10 min. Chickens with parasitemia ranging from 3% to 7% were considered acceptable for proceeding to mosquito feeding. For experiments aiming at comparing the effect of parasitemia, chicken with high (50.0%) and low (2.4%) parasitemia were used. Five-to-seven-day-old mosquitos that had been deprived of sugar for 16 h were allowed to feed to repletion on infected chickens for 15 min. Unfed or partially fed mosquitoes were removed and only fully engorged females were used in subsequent experiments. Since we had two experimental groups and one control group, we could not perform the feeding of all three groups simultaneously. For each chicken, two mosquito containers were placed simultaneously on top of the chicken and the third group was fed immediately after the first two groups were removed. To randomize any residual effect of salivary proteins on the skin, we alternated the order of the groups. Fifty mosquitoes were used per group. Blood-fed females were kept on a sugar diet until dissections. *Plasmodium* infection was evaluated at both oocyst and sporozoite levels. Midguts from *P. gallinaceum*-infected mosquitoes were dissected 6 days post-blood feeding and stained with 0.1% mercurochrome, and oocysts were counted by light microscopy. Three biological replicates were performed. To determine the difference in *P. gallinaceum* infection levels among mosquito groups, the D'Agostino-Pearson omnibus normality test was used to determine whether oocyst counts were normally distributed, and either an unpaired *t* test or Mann-Whitney test was used accordingly to assess the statistical significance of the differences between control and experimental groups. A P value < 0.05 was considered statistically significant. The percent reduction in oocysts was calculated as $100 \times \{1- [(\text{mean number of parasites in the experimental group})/(\text{mean number of parasites in the control group})]\}$.

Differences in the amount of blood ingested by each mosquito were determined by quantification of total hemoglobin using Drabkin's reagent (Sigma-Aldrich). All blood-fed mosquitoes from each group were placed in 1.5 mL tubes containing two to four zirconia-silica beads (size 2.3 mm, Research Product International) and individually homogenized in 1 mL of Drabkin's reagent for 1 min at 4,000 RPM in a Bullet Blender Storm 24 (Next Advance). Samples were cleared by centrifugation for 5 min at $13,500 \times g$ at room temperature. From the supernatants, triplicates of 200 µL were added to flat-bottom 96-well microtiter plates (Corning, Costar). Absorbance at 540 nm was determined using a VersaMax microplate reader (Molecular Devices). Results were analyzed by one-way ANOVA.

For transmission studies, mosquitoes were infected by blood feeding on a chicken that presented 12.9% parasitemia. Infected mosquitoes were maintained on sugar, and parasite infections matured in the mosquitoes. On day 20 after mosquito infection, infected mosquitoes were exposed to naive chicken for parasite transmission. Two chickens per mosquito group were used. Each chicken was exposed to five infected mosquitoes. A set of infected mosquitoes were used to determine the number of sporozoites per salivary gland. For sporozoite quantification, dissected salivary glands were collected in tubes containing 10 µL of PBS, and four pools of five salivary glands each were homogenized by pipetting 20 times with a 10 µL tip. Sporozoite numbers were determined in C-Chip disposable hemocytometers (InCyto, Thermo Fisher Scientific, Waltham, USA) by phase-contrast microscopy. Clinical signs, including weight loss, temperature, hematocrit, and chicken parasitemia were measured daily for 12 days after the mosquito challenge. For multiple comparisons, WT was used as the control group, and samples were compared using the one-way ANOVA for the sporozoite levels or the two-way ANOVA for clinical signs and parasitemia determinations.

ACKNOWLEDGMENTS

The authors thank Andre Laughinghouse, Kevin Lee, and Yonas Gebremicale for excellent mosquito rearing and technical help with *P. gallinaceum*-infected chickens, Brian Bonilla

for salivary gland dissections, and Julio Castillo for his help with the confocal microscopy. We thank Drs. Jose M. Ribeiro and Laura Amo for fruitful discussions, and Ian N. Moore for helping with immunohistochemistry experiments.

This research was supported by the Division of Intramural Research Program of the NIH/NIAID (AI001246, to E.C.) and by a subcontract from grant 1R01AI099483 (to Z.N.A.).

The study was conceptualized by E.C., Z.N.A., and I.M.-M.; methodology was designed by I.M.-M., B.B.K., A.A., A.M.-C., A.E.W., P.C.V.L., and E.C.; investigation was done by I.M.-M., B.B.K., A.A., A.M.-C, A.E.W., P.C.V.L., G.S., K.B., M.M., Z.N.A., and E.C.; the original draft of the manuscript was written by I.M.-M.; the manuscript was reviewed and edited by I.M.-M., Z.N.A., and E.C.; funding was acquired by E.C. and Z.N.A.; resources were provided by Z.N.A. and E.C.

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FUNDING

Funder	Grant(s)	Author(s)
HHS | NIH | NIAID | Division of Intramural Research, National Institute of Allergy and Infectious Diseases (DIR, NIAID)	AI001246	Ines Martin-Martin Adeline E. Williams Alvaro Molina-Cruz Paola C. Valenzuela-Leon Gaurav Shrivastava Karina Botello Mahnaz Minai Eric Calvo
HHS | NIH | National Institute of Allergy and Infectious Diseases (NIAID)	1R01AI099483	Bianca B. Kojin Azadeh Aryan Zach Adelman

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ETHICS APPROVAL

Public Health Service Animal Welfare Assurance #A4149-01 guidelines were followed according to the National Institute of Allergy and Infectious Diseases (NIAID), National Institutes of Health (NIH) Animal Office of Animal Care and Use (OACU). These studies were carried out according to the NIAID-NIH animal study protocol (ASP) approved by the NIH Office of Animal Care and Use Committee (OACUC), with approval IDs ASP-LMVR3 and ASP-LMVR102.

ADDITIONAL FILES

The following material is available online.

Supplemental Material

Supplemental figures (mBio02289-23-S0001.pdf). Fig. S1 to S9.
Supplemental legends (mBio02289-23-S0002.docx). Legends for Fig. S1 to S9 and Tables S1 to S3.
Table S1 (mBio02289-23-S0003.xlsx). Mass spectrometry results.
Table S2 (mBio02289-23-S0004.xlsx). Probing time.
Table S3 (mBio02289-23-S0005.xlsx). Primers used in this work.

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