NEGATIVE EFFECT OF ANTIBODIES AGAINST MAXADILAN ON THE FITNESS OF THE SAND FLY VECTOR OF AMERICAN VISCERAL LEISHMANIASIS

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Abstract. Lutzomyia longipalpis expresses a salivary protein called maxadilan (MAX) that functions to dilate vertebrate blood vessels and thereby to facilitate the sand fly’s acquisition of blood. We hypothesized that antibodies specific for one of many MAX variants would inhibit vasodilatory function of that variant. *In vitro* and *in vivo* experiments showed that antibodies against a specific MAX variant decreased vasodilatory function. More specifically, antibodies against MAX blocked vasodilation of a constricted rabbit aorta. Additionally, a strain of *Lu. longipalpis*, with a nearly uniform MAX genotype, obtained a larger blood meal from naive BALB/c mice compared with mice that were either immunized with a homologous MAX genotype or sensitized to bites of flies from the same strain. Those flies taking blood from mice sensitized by sand fly bites also laid significantly fewer eggs than when they took blood from naive mice. These results have potential epidemiologic importance in light of the potential use of MAX in a vaccine or as part of a diagnostic test because they imply that a uniform MAX genotype is selected against by the vertebrate host immune response and that antigenic diversity is selected for.

INTRODUCTION

*Lutzomyia longipalpis*, the sand fly vector of *Leishmania chagasi*, is a blood-sucking arthropod that requires protein from a vertebrate blood source to mature its eggs.1 The amount of blood a female fly ingests is directly correlated with the number of eggs matured.2 Therefore, the mechanisms that have evolved to obtain blood are integrally related to individual sand fly fitness. Salivary proteins are a critically important component of the repertoire of tools of *Lu. longipalpis* specialized for obtaining blood. At the site of a bite in vertebrate skin, these proteins facilitate bleeding by blocking the hemostatic mechanisms of the vertebrate host.3 The present study focuses on maxadilan (MAX), one component of *Lu. longipalpis* saliva that is a potent vasodilator with potential epidemiologic importance.4

We recently demonstrated that MAX is a highly polymorphic peptide in natural populations of *Lu. longipalpis*5 and that its amino acid differences are recognized specifically by the vertebrate host immune system.6 The present study is predicated on the assumption that genetic drift is not the primary or sole factor responsible for genetic diversity in the MAX peptide. To investigate the potential factor(s) selecting for such diversity, we examined whether an IgG response against one MAX variant can block the function of this peptide, producing a decrease in fitness of a strain of *Lu. longipalpis* with the same MAX genotype. Fitness is defined for the purposes of the present study as a measure of the average contribution of a given genotype or allele to the subsequent generation7,8 by quantifying amount of blood taken and number of eggs laid.

Other studies have demonstrated that antibodies can either block the function of an arthropod protein or can negatively impact arthropod fitness. For example, antibody response to a single salivary protein produced a significant *in vitro* decrease in the catalytic activity of *Anopheles stephensi* apyrase.9 Antibodies against salivary can block the immunomodulatory effects of sand fly saliva.4-10 Anti-vector antibodies against a variety of vectors such as ticks and flies have been shown to reduce fecundity.11-13 However, these studies did not look at the fitness effects of a defined salivary antigen.14

In the aforementioned studies, a phenotype, such as fecundity, has been correlated with blood meal acquisition without knowing the genotype(s) influencing that phenotype on the population level. The novelty of our study is in the ability to relate the presence and absence of immune exposure to MAX, a peptide with both a fairly well-characterized phenotype and genotype, to fitness differences. Consequently, this study uniquely indicates that the vertebrate host antibody response can decrease the fitness of a strain of *Lu. longipalpis* with a homogeneous MAX genotype.

METHODS

Production of specific IgG antibodies to MAX. A synthetic peptide (RMAX) was produced by the National Institute of Allergy and Infectious Diseases Research Technologies Branch–Peptide Synthesis and Analysis Unit (Bethesda, MD) to greater than 90% purity and was based on GenBank sequence AAA29288.1. It has been reported that MAX produced by either synthetic or recombinant technology has slightly different potencies. Moro and Lerner demonstrated that recombinant MAX is approximately 10-fold more potent than synthetic MAX.15 The reduction in potency is due to the difficulty of synthesizing a correctly folded 61-amino-acid peptide containing four cysteine residues. During synthesis there is a great possibility of incorrect intramolecular and also intermolecular disulfide bonding during oxidation.16 To minimize this problem, RMAX was put through a refolding procedure. MAX was denatured at a concentration of 10 mg/mL in denaturation buffer (6 M guanidine chloride, 0.1 M Tris-HCl, pH 8.0, 2 mM EDTA, pH 8.0–8.5, 65 mM dry dithioerythritol) for two hours at room temperature. The denatured RMAX was renatured at 10°C by diluting by 100-fold the denatured peptide into refolding buffer (0.1 M Tris-HCl, pH 8.0, 0.5 M l-arginine-HCl, 0.9 mM oxidized glutathione (GSSG), 2 mM EDTA, pH 8.0–8.5). The mixture was then incubated for 36–48 hours and dialyzed against 20 mM Tris-HCl, pH 4.0, and 100 mM urea until the conductivity was between 3 and 3.7 mMhos. The refolded peptide was purified by high-performance liquid chromatography. However, it is important to note that the refolded MAX is not a single struc-
tural species but is a mixture of species. RMAX was used as an antigen in the fitness experiments described in this report.

Six female BALB/c mice were obtained from Harlan Sprague-Dawley (Indianapolis, IN) and immunized at eight weeks of age with RMAX. Prior to each immunization, mice were sedated by an intraperitoneal injection of ketamine (60 mg/kg) and xylazine (5 mg/kg). Each mouse was injected subcutaneously with RMAX (50 μg) emulsified in Freund’s complete adjuvant (100 μL). Two weeks later, mice were injected subcutaneously with RMAX (50 μg) emulsified in Freund’s incomplete adjuvant (100 μL). Then mice were given booster immunizations with phosphate-buffered saline (PBS) and RMAX (10 μg) at two-week intervals for six weeks. Blood was collected from the mice by saphenous vein puncture one week after the last RMAX injection, and the serum fraction was used to measure antibody titers by an indirect enzyme-linked immunosorbent assay (ELISA). RMAX (5 μg/ml) was coated (50 μL/tub) on Immulon 4 microtiter plates (Dynex Technologies, Inc., Chantilly, VA) overnight at 4°C. The ELISA was carried out using standard techniques with 1:500 diluted horseradish peroxidase-conjugated goat antimouse IgG (Bio-Rad Laboratories, Hercules, CA) and ABTS (2,2'-Azinobis (3-ethylbenzothiazoline-6-sulfonic acid)-diamonium salt) substrate (Pierce, Rockford, IL). The plates were read at 405 nm in a VERSAmax™ microplate reader (Molecular Devices, Sunnyvale, CA). Mice with the highest titers of antibodies against RMAX (1:1,600–1:3,200) were used immediately in feeding experiments detailed in this report.

After immunization with RMAX and after the feeding experiments described in this report were completed, mice were given a booster immunization of RMAX (10 μg) and were killed one week later. Immediately thereafter, blood was drawn by cardiac puncture. Serum was collected and the IgG fraction was purified using an ImmunoPure® IgG (Protein A) Purification Kit (Pierce) according to the manufacturer’s instructions. The IgG fractions collected were dialyzed in Tyrode’s solution. Protein concentrations were measured using a protein assay (Bio-Rad Laboratories) and bovine serum albumin of known concentrations to make a standard curve. The protein was subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis on a 12% gel and stained with Coomassie blue.

In vitro ability of antibodies to MAX to abrogate vasodilatory function of MAX. The ability of RMAX to dilate a rabbit aortic ring preparation was compared with the ability to dilate an aortic ring pre-incubated with antibody to RMAX. Rabbit aortas were cut into 2–3 mm rings and hooked between two micro-hooks and stretched between a stable holder and an isometric transducer to 2.5 grams resting tension. The rings were then suspended in a 2–milliliter tissue-organ bath filled with Tyrode’s solution (116.4 mM NaCl, 5.4 mM KCl, 1.5 mM MgCl₂, 6.0 mM H₂O, 1.2 mM MgSO₄, 7.2 mM H₂O, 0.4 mM KH₂PO₄, 1.2 mM NaH₂PO₄·H₂O, 5.5 mM glucose, 26 mM NaHCO₃) containing 5 mM HEPES, pH 7.4. Dexamethasone (0.1 mM) was added to prevent induction of nitric oxide synthase. The solution was continually gassed with 96% O₂ and 4% CO₂. Once the resting tension had stabilized (90 minutes later), the aortic rings were contracted with phenylephrine (1 μM). After 30 minutes, when a steady contraction was achieved, anti-RMAX IgG, anti-L. chagasi IgG (purified from BALB/c mice infected with L. chagasi, a gift from L. Soong, Department of Microbiology and Immunology, University of Texas Medical Branch, Galveston, TX), or saline was added to the bath followed by RMAX 20 minutes later. The total addition volume was less than 4% of the total bath volume. An initial trial was made to find the range of doses giving inhibitions between 10% and 90% for the phenylephrine-induced contraction.

Investigations on the genetic variability of MAX from the sand fly colony used in fitness studies. To test the hypothesis that antibodies to MAX could block the function of MAX, we needed a strain of sand flies with a homogeneous MAX genotype. Therefore, it was important to determine the level of genetic and amino acid variability of MAX in the sand fly colony used in the present study. Lutzomyia longipalpis reared at the University of Texas Medical Branch (UTMB) and originating from a population of flies from Callejon, Colombia colonized for many years (Ferro C, Instituto Nacional de Salud, Bogota, Colombia) was used in all fitness experiments described. DNA was extracted individually from 18 L. longipalpis from the Callejon, Colombia colony according to a previously published protocol. The MAX gene was amplified by a polymerase chain reaction (PCR) from the DNA. The PCR products based on the genomic DNA of an individual fly were cloned using the TOPO TA Cloning system (Invitrogen, Carlsbad, CA) and INV-aF® competent Escherichia coli. Several white colonies were selected and following incubation plasmid DNA from individual clones was purified. Plasmid DNA from five positive clones from each individual insect were analyzed by single strand conformation polymorphism (SSCP) using a previously described protocol. Gels were then scanned on a digital scanner and scored by analyzing banding patterns. Separation of sample DNA was achieved primarily on the basis of differences in single-strand secondary structure. Theoretically, this technique can separate 900-basepair DNA molecules that differ by a single base pair. Results of SSCP analysis described the degree of variability on the nucleotide level in MAX. The MAX gene was amplified from plasmid DNA using BigDye™ Terminator Cycle Sequencing Ready Reaction (Applied Biosystems, Foster City, CA), cleaned on Micro Bio-Spin P-30 Tris Chromatography Column (Bio-Rad Laboratories), and sequenced on an ABI-377 automated sequencer (Applied Biosystems) according to manufacturer’s instructions. Individual DNA sequences were analyzed using DNASTAR Windows version 4.03 (DNASTAR, Inc., Madison, WI) for alignment and translation of DNA into protein sequence.

Sensitization of mice by sand fly bite. BALB/c mice (six per group) were obtained from Harlan Sprague-Dawley and used at eight weeks of age. The exposed group were fed on by L. longipalpis females from the Callejon, Colombia colony according to a modified protocol. Briefly, prior to sand fly feeding, six mice were sedated by intraperitoneal injection of ketamine (60 mg/kg) and xylazine (5 mg/kg). During the course of two weeks, each mouse was exposed to at least 50 female sand flies twice a week. Sera were collected by saphenous vein puncture one week after the last exposure to sand fly bites and an indirect ELISA was used to measure anti-salivary gland IgG titers. The ELISA protocol is described under the subheading Production of specific IgG antibodies to MAX. The coating antigen used was two pairs of salivary glands/mL of PBS (≈2.8 μg of protein/mL) instead of RMAX. Mice with the highest titers (1:400–1:800) were used in the blood feeding experiments described in this report.
Effects of antibodies to MAX on *Lu. longipalpis* blood meal size and oviposition. A blind protocol was used to test effects of antibodies to MAX on blood meal size and oviposition of 3–4-day-old *Lu. longipalpis* from the Callejon colony, UTMB. Within each replicate of the feeding experiment, an equal number of female and male flies were placed into two cloth mesh cages (20 × 20 cm). Between replicates, the number of flies per cage ranged from 40 to 60 females and from 20 to 30 males. Females typically feed in groups and in nature males are present. We controlled for size by visual exclusion of very small or large flies. BALB/c mice, either from 20 to 30 males. Females typically feed in groups and the number of flies per cage ranged from 40 to 60 females and immunized mice or for five minutes on sand fly were allowed to feed for 10 minutes on anesthetized MAX-

...introduced into each cage. As a negative control *Phlebotomus papatasi* bites, were sedated by intraperitoneal injection of 60 mg/kg of ketamine and 5 mg/kg of xylazine and immediately introduced into each cage.

For the blood meal size experiments, Callejon colony flies were allowed to feed for 10 minutes on anesthetized MAX-immunized mice or for five minutes on sand fly–sensitized mice. As a negative control *Phlebotomus papatasi* flies were allowed to feed on MAX-immunized mice. At the end of the feeding period, the mice were removed from the fly cages, and all flies were killed by incubating the cages at -20°C for 30 minutes. The presence or absence of blood in the sand fly digestive tract was verified by light microscopy for each fly and when blood was visible the amount of hemoglobin was measured using Drabkin’s Reagent Kit (Sigma, St. Louis, MO) according to the manufacturer’s instructions. An assay was performed to correlate the volume of blood with the absorbance (540 nm). To measure the amount of blood in a sand fly, the abdomen of each fly was dissected from the rest of the body and crushed into Drabkin’s solution (250 μL), which was then placed into a well on a microtiter plate. The optical density of the resulting solution was measured at 540 nm. The volume of blood was derived from known volumes (0.2–0.6 μL) of heparinized whole mouse blood and a regression of the optical density on known volumes of blood was derived in accordance with a previous protocol.23

Given that our use of mouse hemoglobin levels as a proxy for the amount of blood in a sand fly digestive system depended largely on uniform levels of hemoglobin in our experimental group of mice, we measured hemoglobin levels in BALB/c mice. Blood was taken from each mouse by puncturing the saphenous vein. Pipette tips rinsed in heparin sulfate were used to draw up whole blood (0.4 μL) and were then rinsed into Drabkin’s solution as described earlier in this report. Two measurements were taken from each of six animals.

For the oviposition experiments, flies were allowed to feed on sensitized mice for 15 minutes. A number of sand fly parameters were controlled for: body size (by visual exclusion), prior nutritional condition, physiologic age, blood meal source and quantity, and moisture levels in the oviposition containers. The weight of each live fly was recorded using a microbalance (BP 210D; Sartorius, Goettingen, Germany) 0–15 minutes after blood acquisition because the fly begins to excrete during the blood meal, but the excretions increase in volume 15 minutes after the blood meal.24,25 Each individual female was placed into a five-dram vial oviposition container. A piece of 1 × 20-cm strip of seed germination paper (Anchor Paper, St. Paul, MN) was placed inside the vial. The portion extending out of the vial was folded along the outer side and under the outer bottom of the vial. On the fourth day after the

...blood meal, a single thickness of absorptive paper towel was completely dampened and kept in contact with the paper strip. The flies were allowed to lay eggs. A fresh piece of cotton wool soaked in 30% sucrose solution of approximately the same size was given to each fly everyday. The experiment was terminated when the fly died and the number of fully matured stage V oocytes laid and those still remaining in the fly after death were recorded.

Statistical analysis. The Student’s *t*-test was used to determine statistically significant differences in the percent dilation of constricted rabbit aortas given different treatments. A nonparametric Kruskal-Wallis test was used to determine whether statistically significant differences existed when comparing the median blood meal size of sand flies feeding on naive mice, mice sensitized by sand fly bites, or mice immunized with RMAX. Scatter diagrams were drawn for the number of eggs laid or matured per female against the weight of the fly after taking a blood meal. Regression equations derived for two different kinds of blood meals were compared by Analysis of Variance (ANOVA) to determine whether the dependent variables (total eggs matured or eggs laid per female) and the independent variables (weight of female fed on naive mice and weight of female fed on exposed mice) produced a significant difference in regression line slopes. Such a significant difference would indicate that for the two types of blood meals, number of eggs laid/matured differs as the meal weight increases. The computer program SYSTAT version 6.0 (Systat Software, Inc., Point Richmond, CA) was used for all statistical calculations.

RESULTS

Ability of antibodies to RMAX to block vasodilation. Antibodies to RMAX were further characterized to determine whether they can neutralize the function of MAX. RMAX dilated preconstricted rabbit aortas. Vasodilation was reduced by approximately 50% when the aortas were incubated with antibodies to RMAX, compared with saline prior to the addition of RMAX (Figure 1). Other serum proteins or inorganic molecules contaminating the purified IgG fraction could explain these results. In another experiment, we controlled for this possibility by pre-incubating aortas with serum IgG purified from naive BALB/c mice (0.4 mg/mL) or from *L. chagasi*-immunized mice (0.8 mg/mL). In four independent experiments, the mean ± SE relaxation produced by RMAX alone was 22.67 ± 3.64%, while mean ± SE relaxation produced by anti-*L. chagasi* IgG, an irrelevant control, plus RMAX was 24.18 ± 2.47%. The results suggest that IgG alone does not contribute to vasoconstriction but that the interaction between anti-RMAX IgG and RMAX may inhibit the binding of RMAX to its cellular receptor pituitary adenylate cyclase activating polypeptide type I receptor (PAC-1).

Homogeneity of MAX DNA sequence from a colony population. Genetic analysis showed that the colony used in the fitness study has increased MAX amino acid homogeneity, probably due to colony inbreeding, compared with natural populations. Of 18 individual flies assayed for polymorphisms by SSCP analysis, only three distinct PCR-SSCP band phenotypes were found. Clones were made from PCR products amplified from genomic DNA obtained from each of three individual flies. DNA from six of the clones was sequenced and the predicted peptide sequences demonstrated limited
differences (Figure 2). Three of the sequences differed in amino acids and these sequences differed by a maximum of only four amino acid residues or 6.6% of the total. Some of these changes involved change from an acidic-charged residue to a non-polar residue, potentially impacting the antigenicity of the molecule. Although the differences could potentially change the chemical reactivity of the molecule, an inverse correlation was noted between the percentage of difference in amino acid residues and cross reactivity. Although clones 1a and 1e have the same amino acid sequence, the difference in bonding pattern is explained by nucleotide differences in the MAX intron. The sequence of synthetically produced RMAX matches the amino acid residues of clone 3a identically.

Effect of anti-saliva protein antibodies on blood meal size. Our findings that antibodies to RMAX may block MAX vasodilation in vitro led us to examine whether anti-saliva antibodies, present in the vertebrate host, could reduce the amount of blood taken by a fly. When individual flies were assayed for amount of hemoglobin in their blood meal, those flies biting mice pre-exposed to Lu. longipalpis bites had lesser amounts of hemoglobin than did those flies biting naive to sand flies or to any other arthropods (Figure 3A). The difference in median hemoglobin amount was statistically significant (P = 0.001). The amount of hemoglobin is a proxy for the amount of blood ingested by each fly. Based on the linear relationship between volume of blood and the optical density value representing the amount of hemoglobin, we found that flies biting naive mice took a median of 0.251 mL blood, whereas those flies biting sensitized mice took a median of 0.206 mL blood, an 18% reduction in volume.

The wet weight of Lu. longipalpis was less after feeding on BALB/c mice sensitized to Lu. longipalpis bites (0.53 mg), compared with feeding on naive mice (0.59 mg) (Figure 3B). The median weight of live Lu. longipalpis before taking a blood meal was 0.27 mg. Overall, there was a 19% decrease in the size of the blood meal due to sensitizing mice to sand fly bites.

**Figure 1.** Inhibition of the vasodilatory function of a synthetic maxadilan (MAX) peptide (RMAX) by IgG antibody to MAX. Rabbit aortic rings were suspended for 90 minutes in Tyrode's solution (0.5 mL) containing 5 mM HEPES, pH 7.4, and tensioned to 2.5 grams. Phenylephrin (1 μM) was added and allowed to stabilize for 30 minutes, then either saline (50 μL) or anti-RMAX IgG (240 μg) were added and allowed to bath the organ preparation for 20 minutes, followed by the addition of RMAX (50 ng) to both control and IgG-treated rings. The preparations were then washed and allowed to relax to obtain a zero value of contraction. Percent dilation of the aorta was recorded 10 minutes after the addition of RMAX to assess the effect of IgG. Differences between the treatment groups were found (P = 0.012) after four replicates. Error bars show the SE.

**Figure 2.** Single strand conformation polymorphism (SSCP) analysis of a polymerase chain reaction (PCR)–amplified maxadilan (MAX) gene sequence. A. Each lane represents MAX PCR-SSCP products amplified from a single fly taken from Lutzomyia longipalpis Callejon colony (University of Texas Medical Branch [UTMB]). 1, 2, and 3 represent the extent of secondary structure diversity. B, PCR-SSCP products were then amplified from six clones based on DNA from individual flies in A. C, Nucleotide sequences for PCR clones in B were obtained and protein sequence predictions were made. Amino acid residues that differ from the consensus are highlighted in bold. Only the amino acid sequences from visually distinguishable banding patterns are shown.
The next step was to determine whether antibodies against RMAX alone could reduce the size of the flies’ blood meal. When flies fed on BALB/c mice with high titers of specific antibodies against RMAX, their blood meal had a lower amount of hemoglobin than when they fed on mice completely naive to sand fly proteins (Figure 4A). The difference between the medians was statistically significant ($P = 0.038$). Specifically, flies biting naive mice took a median of 0.371 μL of blood, whereas those flies biting RMAX immunized mice took a median of 0.297 μL blood, a 20% reduction in volume. These results indicate that when *Lu longipalpis* bites an animal that has produced antibodies specific against only 1–2% of the total salivary protein content, the host response can block blood feeding.

**Figure 4.** Blood meal size of sand flies fed on mice immunized with maxadilan (MAX). A. Amount of hemoglobin in the blood meal of *Lutzomyia longipalpis* biting either mice immunized with a synthetic peptide of MAX (RMAX) (n = 57) or biting naive mice (n = 58). Individual optical density (OD) values represent hemoglobin levels in the blood meal of a single sand fly. The amount of hemoglobin was measured using Drabkins reagent. A statistically significant difference ($P = 0.038$) in hemoglobin levels was detected when comparing the group of flies biting naive mice or RMAX-immunized mice. These experiments were replicated three times using the same mouse throughout the three experiments and were conducted over a period of four days. B. Amount of hemoglobin in the blood meal of *Phlebotomus papatasi* biting either mice immunized with RMAX (n = 254) or biting naive mice (n = 260). Individual OD values represent hemoglobin levels in the blood meal of a single sand fly. The amount of hemoglobin was measured using Drabkins reagent. A statistically significant difference ($P = 0.038$) in hemoglobin levels was detected when comparing the group of flies biting naive mice or RMAX-immunized mice. These experiments were replicated three times using the same mouse throughout the three experiments and were conducted over a period of four days.

**Figure 3.** Blood meal size and body weight post blood meal of *Lutzomyia longipalpis* fed on sensitized mice. Feeding experiments using mice sensitized to bites were repeated six times using six different mice. A. Amount of hemoglobin in the blood meal of *Lu. longipalpis* biting either mice previously exposed to *Lu. longipalpis* bites (n = 119) or biting naive mice (n = 173). Individual optical density (OD) values represent hemoglobin levels in the blood meal of a single sand fly. The amount of hemoglobin was measured using Drabkins reagent. A statistically significant difference ($P = 0.001$) in hemoglobin levels was detected between the group of flies biting naive mice or exposed mice. B. Flies fed either on naive (n = 116) mice or on mice previously exposed to sand fly bites (n = 117). Within 15 minutes after their blood meal, *Lu. longipalpis* were anesthetized at -20°C for three minutes and then weighed. There was a statistically significant difference ($P = 0.002$) in wet weight of flies biting naive or previously exposed mice. Flies were kept alive for subsequent experimentation.
For a negative control experiment, we were interested in determining whether antibodies to RMAX decrease the blood meal size of *P. papatasi* (Figure 4B). *Phlebotomus* from the Old World and *Lutzomyia* from the New World use different strategies to combat vertebrate hemostasis. These two genera diverged from their common ancestor before separation of the continents and before the major explosion of mammalian diversity. Differences in their salivary components represent the evolution of different mechanisms in solving the same problem of obtaining vertebrate blood.\(^{28}\) *Phlebotomus papatasi* has no MAX gene in its genome, but instead pharmacologic amounts of the vasodilatory and anti-platelet molecules adenosine and AMP can be found in its saliva.\(^{29}\) As was predicted, when *P. papatasi* fed on BALB/c mice immunized with RMAX, there was no detectable difference in the median hemoglobin content of their blood meal compared with when they fed on mice completely naive to sand-fly proteins (Figure 4B). These results suggest that antibodies to MAX are specifically blocking the vasodilatory function of MAX.

**Effect of anti-saliva-protein antibodies on oviposition.** Under controlled experimental conditions, *Lu. longipalpis* biting BALB/c mice previously sensitized to *Lu. longipalpis* bites laid fewer eggs (Figure 5A). Flies biting naive mice laid a median of 43 eggs or 74 eggs/mg of fly body weight, whereas those flies biting sensitized mice laid a median of 26 eggs or 49 eggs/mg of body weight. This represents a 34% reduction in the median number of eggs laid per milligram of body weight in one generation. ANOVA indicated that both sand fly blood meal weight and sensitization by sand flies produced a significant difference in the number of eggs laid (F = 14.1–40.5, degrees of freedom = 1, P = 0.0001), accounting for 26% of the variability in eggs laid. Such a reduction in eggs laid could significantly impact the size of this sand fly colony over only a few generations. Upon counting the total number of eggs matured, we found that flies biting sensitized mice matured a median of 71 eggs/mg of body weight compared with 81 eggs/mg of body weight by flies biting naive mice (Figure 5B). Again, ANOVA indicated that the variables blood meal weight and sensitization by sand flies generated a significant difference in the number of eggs matured (F = 19.7–120.0, degrees of freedom = 1, P = 0.0001), accounting for 44% of the variability in eggs matured.

**DISCUSSION**

Fitness of the sand fly *Lu. longipalpis* depends on both genetic and environmental factors and on the interrelationship between them. The set of results presented here is one of the first in any blood feeding arthropod to suggest that anti-saliva antibodies to a defined molecule may decrease blood meal size and thus may significantly influence sand fly fitness. These results lend support to our hypothesis that MAX polymorphism improves the fitness of *Lu. longipalpis*. In nature a population of *Lu. longipalpis* may repeatedly bite the same host potentially exposing the host to several generations of sand fly bites. Apparently the host response does not neutralize the function of the highly variant population of MAX molecules it is exposed to because *Lu. longipalpis* continue to obtain blood in nature (Milleron RS, unpublished data). In the present study, we suggest that differences in blood meal size and eggs laid were found because we selected a population of *Lu. longipalpis* with a highly homogeneous MAX genotype and were able to mount a strong IgG response specific for that genotype.

A difference in the ability of MAX to dilate constricted rabbit aortas was observed. The results suggest that anti-MAX IgG has the ability to neutralize MAX vasodilatory function. However, it is important to note that these results do not necessarily describe the physiologic response at the bite site. These findings in the aorta may not be reproducible at the bite site where vasoconstriction is less important in controlling hemostasis,\(^{30}\) where smaller vessels respond differently to hemostatic proteins,\(^{31,32}\) or when the dosage of MAX is different. MAX has been shown to exhibit a biphasic dose response activity.\(^{4,33}\) For these reasons, the results obtained from our *in vitro* aorta model were confirmed *in vivo* when we observed a decrease in the fitness of sand flies biting pre-exposed BALB/c mice.

Producing a complete inhibition of sand fly blood feeding was not an expected result. The saliva of *Lu. longipalpis* contains a number of anti-hemostatic proteins that promote vertebrate blood flow by blocking vertebrate hemostasis.\(^{3,34}\) The erythema-inducing MAX opens the pre-capillary arteriolar sphincters to increase blood available to the hungry fly, while other salivary components such as apyrase and anti-clotting agents contribute to increase the capillary hemorrhage from where the fly feeds. Therefore, if the function of one of the proteins in the saliva is blocked, *Lu. longipalpis* would still be able to obtain blood from the vertebrate. In addition to salivary proteins, specialized sand fly mouthparts have evolved to penetrate the dermis where the capillaries are located and obtain blood.\(^{35–37}\) In fact, mosquitoes that are prevented from salivating are still able to obtain some blood.\(^{38,39}\)

In our experimental system, mice were anesthetized and were therefore unable to defend themselves from fly bites. The decrease detected in the blood meal size of *Lu. longipalpis* produced by pre-exposing mice to *Lu. longipalpis* salivary proteins may have been accentuated had the mice been fully alert and able to defend themselves against the sand fly. Defensive behavior results in disruption of feeding\(^{40–43}\) or even in death of the arthropod. Therefore, *Lu. longipalpis* like some mosquitoes feed most successfully on inactive, sleeping hosts and less on alert hosts.\(^{44}\) The duration of time during which a mosquito attempted to blood feed despite host defensive behavior was longest during the mosquito’s first attempt at obtaining blood and was progressively shorter thereafter.\(^{45,46}\) To mimic natural feeding conditions and partially account for the effects of host defensive behavior, we limited the amount of time that the sand flies could bite the host.

In the present study, *Lu. longipalpis* produced and laid more eggs when the blood meal source is from naive BALB/c mice rather than from mice sensitized by bites or immunized with MAX. Pre-exposing mice to sand fly bites effected *Lu. longipalpis* reproduction first by decreasing the median blood meal size and second by reducing the number of eggs produced and laid given the same amount of blood. Similar to previous results,\(^{2,47}\) these results suggest that both the amount and composition of blood affects sand fly reproductive potential and ultimately the size of subsequent generations. A practical application of these findings for people attempting to establish productive colonies of sand flies is to avoid repeated use of the same animals when feeding a genetically homogeneous colony.

Under most circumstances, an increase in fitness is defined
as a per capita increase of a genotype and theoretically, fecundity should evolve to ever-higher levels. One study of *Lu. longipalpis* found density-dependent feeding success for a field population of sand flies. Blood meal size decreased with increasing densities of female *Lu. longipalpis*; yet, increasing host abundance increased feeding success. In a natural setting, host population size is not an important limiting factor since *Lu. longipalpis* have catholic feeding preferences. Because abundance is not an absolute predictor of persistence, in the future, we are interested in carrying out these experiments over several generations.

Using MAX as a model system suggests that an arthropod salivary protein is under selection driven by the vertebrate host immune system. These findings are relevant in light of the proposed development of an anti-*Leishmania* vaccine based on MAX. Due to the epidemiologic importance of MAX, we would like to extend these results by measuring fitness differences in the laboratory in strains of *Lu. longipalpis* fixed for MAX variant X fed on mice immunized with a highly divergent variant Y. Understanding the implications of antigenic diversity for a vaccine candidate will aid in the successful design of vaccines.

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