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Caspar-like Gene Depletion Reduces *Leishmania* Infection in Sand Fly Host *Lutzomyia longipalpis*[*][S]

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Background: Caspar is a negative regulator of the inducible IMD immune signaling pathway.
Results: Depletion of Caspar expression led to a reduction in *Leishmania* populations in sand fly gut.
Conclusion: Disrupting expression of a single sand fly immune gene disrupts *Leishmania* development.
Significance: This study suggests that IMD-activated effectors have the potential to disrupt *Leishmania* population development and abort infections in sand fly vector.

Female phlebotomine sand flies *Lutzomyia longipalpis* naturally harbor populations of the medically important *Leishmania infantum* (syn. *Leishmania chagasi*) parasite in the gut, but the extent to which the parasite interacts with the immune system of the insect vector is unknown. To investigate the sand fly immune response and its interaction with the *Leishmania* parasite, we identified a homologue for *caspar*, a negative regulator of immune deficiency signaling pathway. We found that feeding antibiotics to adult female *L. longipalpis* resulted in an up-regulation of *caspar* expression relative to controls. *caspar* was differentially expressed when females were fed on Gram-negative and Gram-positive bacterial species. *caspar* expression was significantly down-regulated in females between 3 and 6 days after a blood feed containing *Leishmania mexicana* amastigotes. RNA interference was used to deplete *caspar* expression in female *L. longipalpis*, which were subsequently fed with *Leishmania* in a blood meal. Sand fly gut populations of both *L. mexicana* and *L. infantum* were significantly reduced in *caspar*-depleted females. The prevalence of *L. infantum* infection in the females fell from 85 to 45%. Our results provide the first insight into the operation of immune homeostasis in phlebotomine sand flies during the growth of bacterial and *Leishmania* populations in the digestive tract. We have demonstrated that the activation of the sand fly immune system, via depletion of a single gene, can lead to the abortion of *Leishmania* development and the disruption of transmission by the phlebotomine sand fly.

The phlebotomine sand fly *Lutzomyia longipalpis* is the most important vector of American visceral leishmaniasis in Central and South America. *Leishmania infantum* is the parasite naturally transmitted during the bite of this sand fly, although in the lab this sand fly is permissive; it allows the growth and transmission of several other *Leishmania* species including *Leishmania mexicana*. Gut microbial homeostasis is of key importance in phlebotomine sand fly transmission of *Leishmania* parasites. In contrast to the malaria parasite *Plasmodium*, the medically important *Leishmania* parasite resides in the gut of the insect during its entire time in the insect vector, and thousands of parasites are often observed packed inside the gut with little apparent pathogenic effect on the host. We do not know whether the insect tolerates the *Leishmania* parasite in the gut or whether here is immune suppression and presume that the parasite is able to resist some or all of the antibiotic activities of the sand fly.

Dipteran insects, as exemplified by *Drosophila*, possess an innate immunity thought to rely on two main types of effectors that act synergistically to restrict microbial growth; antimicrobial peptides and reactive oxygen species (1). Production of these antimicrobials is carefully regulated to allow for growth of commensal microbes and also limit nonspecific damage to the gut epithelia by reactive oxygen species (2). Antimicrobial peptide production is governed by the signaling immune pathways Toll, immune deficiency (IMD)[3] with JAK-STAT having an additional immune function. Epithelial immune effector induction is thought to be under the control of IMD and not Toll, probably because of the direct contact with MAMP receptors in epithelial cells (3). Repression of the NF-κB antimicrobial peptide system is needed to prevent the loss of potentially beneficial bacteria, and the insect innate immune pathways are partly governed by negative regulators. The *caspar* gene was first described from *Drosophila* mutants with hyperactivated immune responses. The *Caspar* gene encodes a protein homologue of the human Fas-associating factor 1, which is associated

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[3] The abbreviations used are: IMD, immune deficiency; contig, group of overlapping clones; ds, double-stranded; MAMP, microbiol associated molecular patterns; JAK-STAT, janus kinase signal transducer and activator of transcription; TNF/NF, Tumor Necrosis Factor/Nuclear Factor; FBS, fetal bovine serum; GFP, green fluorescent protein.

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with elements of the TNF/NF-κB pathway and which negatively controls the IMD pathway (4).

Both phlebotomine sand flies and mosquitoes are from the order Diptera, in common with *Drosophila*, and belong to the same suborder Nematocera. Studies on Anopheline mosquito-*Plasmodium* interactions showed that Toll and IMD pathways respond to parasite infections, and these responses are specific to different *Plasmodium* species (5–7). Moreover, RNAi-mediated knockdown of the Caspar gene is capable of reducing *Plasmodium falciparum* survival in the anopheline insects (8). However, depletion of Caspar in *Aedes aegypti* did not affect infection by the nematode *Brugia malayi* that cause lymphatic filariasis (9).

The phlebotomine sand fly immune response is poorly studied, and a detailed description of the relevant pathways awaits the publication of the sand fly genome. In *Phlebotomus papatasi*, an anti-Gram-negative activity was detected in the insect hemolymph, as well as lysozyme-like activity against Gram-positive bacteria and phenoloxidase activity (10). In *L. longipalpis*, it was shown that bacterial challenge can induce antibacterial peptides in the insect hemolymph (11). Additionally, cultured cells generated a nonspecific response to virus-like particles (12). Only one study has examined the sand fly immune response toward *Leishmania* parasites in detail. Defensin was induced in *Phlebotomus duboscqi* by bacterial and *Leishmania* challenge, with antiparasitic activity against the promastigote forms of *Leishmania major* (13).

In this study, we identify and describe Caspar, a putative negative immune regulator in *L. longipalpis*. We used the RNAi approach developed in our laboratories (12, 14–16) to evaluate the role of Caspar in controlling both the naturally transmitted parasite *L. infantum* and an additional species *L. mexicana* that is successfully transmitted by this insect in the laboratory.

EXPERIMENTAL PROCEDURES

Ethics Statement—All procedures involving animals were approved by the ethical review committee of the University of Liverpool and performed in accordance with United Kingdom government (Home Office-approved Project License PPL40/2958) and European Community regulations.

Leishmania Parasites—*L. mexicana* (MNYC/BZ/62/M379) amastigotes were maintained in early exponential growth by weekly transfer in Grace's insect medium containing 20% FBS, with 25 μg/ml gentamicin sulfate (Sigma), pH 5.5, and cultured at 32 °C. *L. infantum* (syn. *Leishmania chagasi*, HOM/BR/76/M4192) promastigotes were maintained in Medium 199 supplemented with 10% FBS and BME vitamins (Invitrogen) with 25 μg/ml gentamicin sulfate (17).

Insects—All of the experiments were performed using sand flies from a laboratory colony of *L. longipalpis* established from sand flies caught in Jacobina (Bahia, Brazil) using standard methods described by Modi (18). The insects were fed on 70% sucrose *ad libitum* and blood-fed on heparinized rabbit blood when needed. The insects were maintained at 27 ± 1 °C, humidity of 80–95%, and a photoperiod schedule of 12 h light/12 h dark.

Feeding Blood and Leishmania Parasites—Three-day-old female sand flies were fed through chick skin membrane on heparinized rabbit blood. Artificial infection was performed with heparinized rabbit blood containing either *L. mexicana* amastigotes or *L. infantum* early log phase promastigotes (2×10^6 parasites/ml).

Sucrose Feeding and Antibiotic Feeding—A solution of 70% sucrose or 70% sucrose supplemented with a mixture of penicillin (200 units/ml), streptomycin (0.2 mg/ml), and amphotericin B (0.5 μg/ml) (Sigma) was offered to insects on cotton wool and changed daily.

Bacterial Feeding—*Escherichia coli* (K12 RM148), *Micrococcus luteus*, *Ochrobactrum sp.* (OM1, Jacobina colony isolate), *Pantoea agglomerans* (NCIMB11392), and *Serratia marcescens* (NCIMB 1377) strains were grown on LB agar overnight. Individual colonies were transferred to liquid LB medium, grown overnight, centrifuged, and resuspended in 20% (w/v) sucrose solution to 0.2 A_{600} and offered on cotton wool as fresh preparations every day to the sand flies.

Caspar cDNA Sequence—*caspar* partial cDNA sequence was identified from NCBI dbESTs database. The complete *caspar* coding sequences was predicted from *L. longipalpis* genomic data available at the NCBI Sequence Read Archives Database. The next generation sequences from file SRX016813 (accession numbers SRR036068, SRR036069, SRR036071, SRR036073, SRR036074, SRR036076, SRR036079, and SRR036080) and SRX016812 (accession numbers SRR036070, SRR036072, SRR036075, SRR036077, SRR036078, and SRR036081) were originally generated in LS454 platform. The conversion from fastq to fasta file format was performed by Galaxy platform (19) and used as a database for a local search. The partial sequence of *caspar* was used as a query for Blast searching. The obtained reads were subsequently assembled with CAP3 program (29), and the contig sequence was resubmitted for a blastx analysis against the NCBI nr database for sequence confirmation. The complete CDS of *caspar* was manually reviewed based on alignments against expressed sequence tags retrieved from GenBank™ and splicing sites prediction with the NNSPLICE 0.9 (20) available online at Berkeley Drosophila Genome Project website. To confirm *caspar* CDS prediction specific oligonucleotides were designed onto the predicted start and stop codons, and the complete ORF of Caspar was amplified by PCR from *L. longipalpis* cDNA prepared with total RNA extracted from L3 larvae. The amplified fragment was cloned into pGEM-T Easy (Promega) and sequenced. The identity of all cDNA sequences was assigned by similarity using BLAST search, and standard ClustalW multiple alignment tool Cladogram analysis was performed using MEGA4 software with a neighbor joining method with bootstrap 10,000 (21). Conserved domains were predicted by the MyHits (22) and InterProScan (23) on-line analysis tools.

RNA Extractions and RT-PCR—Total RNA was extracted in biological triplicates from pools of three whole bodies of *L. longipalpis* larvae or adults using TRI reagent (Ambion). RT-PCR was performed using SuperScript III one-step RT-PCR Platinum *Taq* HiFi (Invitrogen) according to the manufacturer's instructions, with 10 ng of template RNA, and gene-specific primers (Caspar-F, 5′-CCAAAGAGGAGGCAAGAAAGA-3′, and Caspar-R, 5′-TTCCGCTTCAAGACGCATA-3′, using 24 cycles. Transcription of genes was normalized based on tran-

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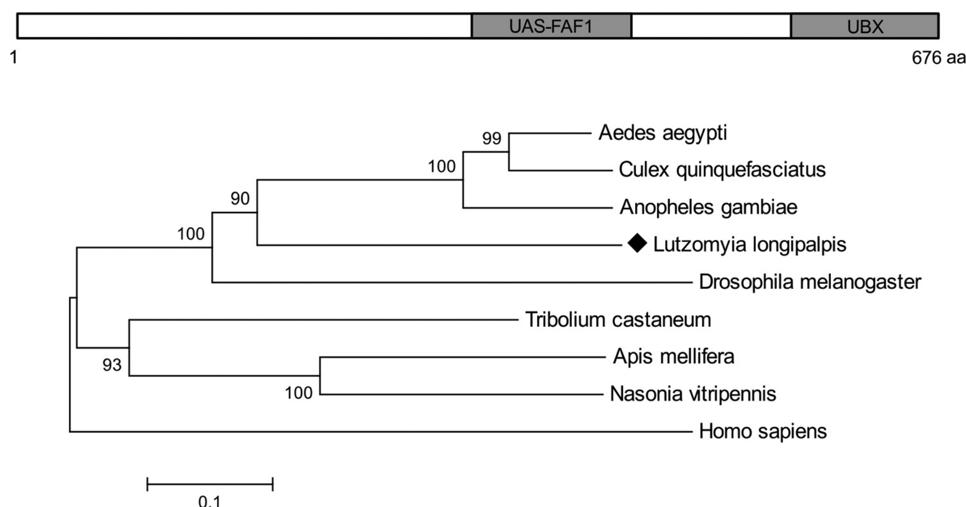

FIGURE 1. **Characterization and phylogenetic comparison of *L. longipalpis* Caspar gene sequence.** *Upper diagram*, representation of Caspar sequence possessing 676 amino acid (*aa*) residues with UAS-FAF1-like domain and a UBX ubiquitin-like domain. *Lower diagram*, molecular phylogenetic tree of the Caspar family proteins from Dipteran insects (mosquitoes *A. aegypti*, *Culex quinquefasciatus*, *A. gambiae*, and *D. melanogaster*) together with human and three other insects species.

scription of a housekeeping gene (AM088777, 60 S ribosomal protein L3). RT-PCR products were resolved by standardized 2% agarose 0.03 ng/ml ethidium bromide gels. Band intensity was determined by densitometric measurements using the software ImageJ 1.42q from standardized gel images captured in 70 ms with the GeneSnap system (Syngene, Cambridge, UK). Differential transcription of genes was determined by the ratio between target gene band intensity and the corresponding 60 S L3 products obtained from multiplex RT-PCR.

Double-stranded RNA Synthesis—Caspar DNA template was amplified by RT-PCR as described above with a second pair of gene specific primers added by T7 site at their 5′ ends (T7-CasparDS-F, 5′-CCGTAATACGACTCACTATAGGGAAC-CCAGTGGTGATTTCCTCG-3′, and T7-CasparDS-R, 5′-CCGTAATACGACTCACTATAGGGATAGCGTTTCATC-TGCATCCATC-3′). The GFP DNA template was obtained from a PCR using pBluescript (Stratagene). The PCR products of Caspar and GFP were used as templates for transcription reactions with the Megascript RNAi kit (Ambion) according to the manufacturer's instructions. The purity of the dsRNA was determined by 2% agarose gel electrophoresis and quantified using a Nanodrop ND-1000 spectrophotometer, which was subsequently concentrated to 4.5 μg/μl using a vacuum concentrator.

Double-stranded RNA Microinjections—Double-stranded RNAs were microinjected as described (16). Recently emerged (<8 h) *L. longipalpis* females were injected with 32 nl of 4.5 μg/μl Caspar or GFP (mock-injected group) dsRNA solution in RNase free water. After injections, the flies were kept in cages (>90% relative humidity) and supplied with 70% (w/v) sucrose solution *ad libitum*. The insects were subsequently infected with *L. mexicana* or *L. infantum* (2×10^6 parasites ml^{-1}) 3 days after microinjections. Additional control groups were sand flies without injections and flies mock injected (pricked) with a sterile needle.

Verification of Caspar Depletion—The depletion of the Caspar gene in sand flies injected with Caspar dsRNA, GFP dsRNAs, or uninjected was evaluated by RT-PCR using pools of three sand flies from each group collected in triplicate 3 and 4 days after microinjections.

Bacterial Quantification in Caspar-depleted Insects—Detection of bacterial DNA by quantitative PCR was performed with an Opticon 2 (Mj Research) using the QuantiTect SYBR Green PCR kit (Qiagen). Total DNA was extracted from pools of 10 whole female sand flies using the DNeasy blood and tissue kit (Qiagen), either from sugar-fed flies (20% sucrose) or from flies injected with Caspar or GFP dsRNA and collected at 3 and 4 days after the treatment. Each sample was diluted to a final concentration of 50 ng of DNA μl^{-1} and added to the quantitative PCR. The quantitative PCR was performed in triplicate in a total volume of 25 μl with 0.3 μM of each universal bacteria-specific primers as previously described (24). *Asaia* sp. DNA was amplified from bacterial lysates using universal bacterial primers 27F and 1492R (25) and used as standard for determining bacterial copy numbers.

Parasite Population in Caspar-depleted Insects—The midguts of dsRNA microinjected insects were dissected 5 days after infection and homogenized in 50 μl of 0.15 M NaCl. The number of *Leishmania* parasites present in the midgut of sand flies injected with Caspar dsRNA, mock injected, and not injected was estimated using a hemocytometer after fixation in 2% (w/v) formaldehyde in PBS.

Statistical Analysis—All of the experiments were analyzed for statistical significance using the software SPSS v17.0. The Kruskall Wallis test was used for multiple comparisons, and the Mann-Whitney test was used for pair wise comparisons. $p < 0.05$ was considered statistically significant.

RESULTS

Caspar cDNA Sequence Identification—The Caspar cDNA sequence (AM093416 *L. longipalpis* (26)) predicted translation showed a putative amino acid sequence of 676 amino acids (supplemental Fig. S1) with an estimated molecular mass of 76 kDa and pI of 5.31 containing a UAS-FAF1 like domain (ProSite

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access PS50033) (residues 375–510) and an UBX ubiquitin-like domain (ProSite access PS50033) (residues 596–673) (Fig. 1). The sand fly Caspar-like amino acid sequence shared 43 and 46% identity with *Drosophila melanogaster* and *A. gambiae* sequences, respectively. Multiple alignment of this sequence with FAS-like sequences from other organisms (supplemental Fig. S2) was used to construct a cladogram, in which the *L. longipalpis* Caspar-like sequence was shown to be closely related to the three other Culicidae (Fig. 1). A broader clade containing all of the nematoceran sequences grouped with Caspar from *Drosophila* to form a clade with all dipteran sequences. Caspar sequences from coleopterans, homopterans, and hymenopterans clustered together but were placed more distantly from all dipteran sequences.

Caspar Expression Profile—Semiquantitative RT-PCR was performed with samples obtained from developmental stages and adults of *L. longipalpis*. Expression of the genes was noted in all stages. Generally there was no clear pattern in relative transcription of Caspar in L3, L4, pupae, and newly emerged females (Fig. 2*A*). The response of the sand flies toward microbial infection of the gut was initially investigated by treating the females with a mixture of penicillin, streptomycin, and amphotericin via the sugar meal. There was a trend of an increase in Caspar when compared with non-antibiotic-treated insects that was statistically significant at 2 days post-sugar feeding (Fig. 2*B*).

RT-PCR was also performed (Fig. 3) with sand flies collected at 1, 2, 3, and 6 days after feeding on rabbit blood or a blood meal containing *L. mexicana* amastigotes (80% of sand flies were estimated to be infected after 5 days). A significant decrease in Caspar expression was recorded for infected insects compared with controls sampled 3 and 6 days after initial infection (Fig. 3). Caspar expression was subsequently examined after introducing other microorganisms into the sand fly gut by feeding live bacteria in 20% sucrose solution relative to controls fed on 20% sucrose only (Fig. 4). *E. coli* and *M. luteus* were included as standard type species. *S. marcescens* was used because of its potential as an insect pathogen that has been found in sand flies (27), and *Ochrobactrum* is found at a low level in the sand fly colony and has been found in other sand fly colonies (28). *P. agglomerans* is a commonly isolated commensal/symbiont from many species of insects including sand flies (27, 29). Expression was significantly increased in the first 24 h after feeding *E. coli* relative to the control but did not differ thereafter (Fig. 4*A*). The *M. luteus* initiated an early caspar up-regulation after 24 h followed by down-regulation after 3 and 4 days (Fig. 4*B*). Expression after feeding *Ochrobactrum sp.*, *P. agglomerans*, and *S. marcescens* showed similar overall patterns of expression (Fig. 4, *C–E*). Caspar was significantly reduced in insects fed *Ochrobactrum sp.* or *P. agglomerans* 2 and 3 days after feeding, but Caspar expression was also increased in adults fed after 5 days on *Ochrobactrum sp.* Caspar expression was significantly up-regulated 4 days after feeding with the *S. marcescens* (Fig. 4*E*).

Confirmation of Caspar Silencing—The longevity of Caspar depletion after dsRNA microinjection was investigated by sampling relative Caspar expression in uninjected, GFP dsRNA-injected, and Caspar dsRNA-injected sand flies 3 and 4 days after microinjections; the time points were selected to correspond to the time between initial sand fly infection and the time of *Leishmania* population estimation (Fig. 5). After microinjections with Caspar dsRNA, Caspar relative expression was ~3-fold in comparison with uninjected controls and ~2-fold in comparison with mock injected controls at 3 days after injection (Fig. 5; $p < 0.05$).

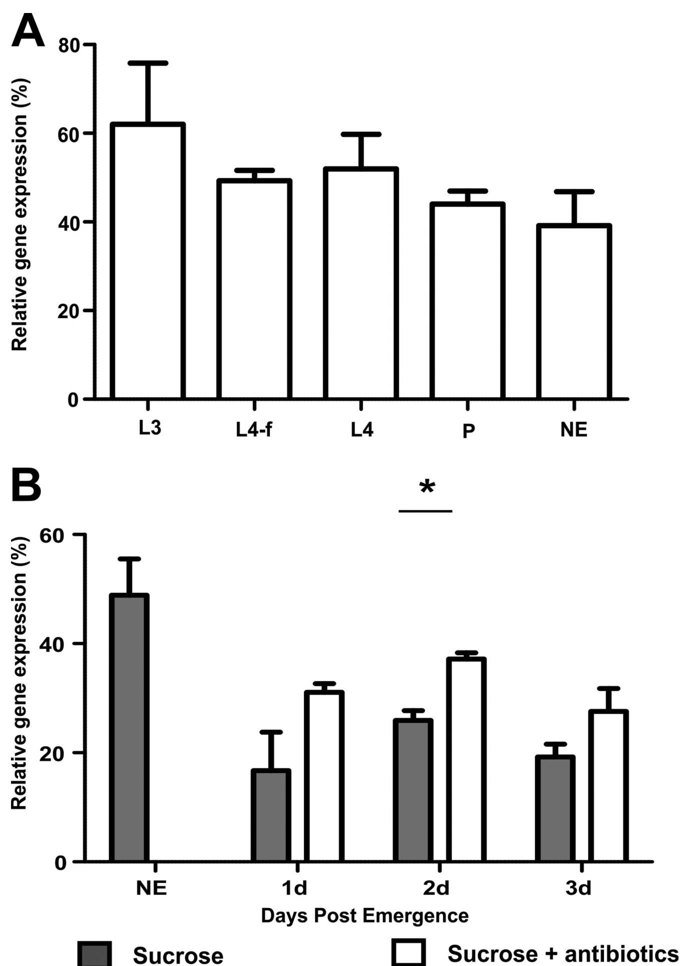

FIGURE 2. **Caspar expression.** *A*, immature developmental stages of *L. longipalpis*. Semiquantitative RT-PCR was performed on L3, L4-f (feeding), and L4 (nonfeeding) larval stages, pupae, and newly emerged females (*NE*). The *error bars* represent the standard errors for three biological replicates consisting of pools of three whole bodies. *B*, feeding antibiotics changes Caspar expression in female *L. longipalpis*. The adults were fed a solution of 70% (w/v) sucrose supplemented with antibiotics (penicillin 200 units/ml; streptomycin 0.2 mg/ml; amphotericin B 0.5 μg/ml; *open bars*) compared with controls fed 70% sucrose solution (*gray bars*) changed daily. Semiquantitative RT-PCR was performed on newly emerged females (*NE*) and females collected on days 1–3 (*1d*, *2d*, and *3d*, respectively). The *error bars* represent the standard errors for three biological replicates consisting of pools of three whole bodies per sample. *, $p < 0.05$.

Leishmania Survival in Caspar-depleted Insects—Caspar transcripts were silenced using RNAi (60% depletion after 3 days) and females fed *L. mexicana* amastigotes in a blood meal 3 days after the dsRNA microinjections. Infections in Caspar-depleted sand flies initiated with *L. mexicana* amastigotes showed a significant decrease in the size of the parasite population surviving 5 days after initial feeding in the blood meal (Fig. 6*A*, dsGFP *versus* dsCaspar $p < 0.0001$).

Two additional controls (no injections and mock injection/pricking the cuticle) were included in our RNAi experiments to

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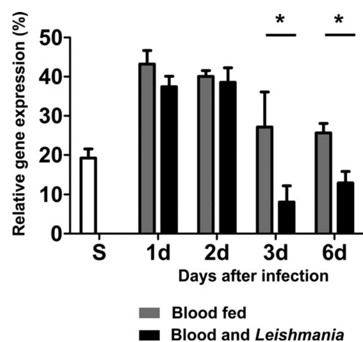

FIGURE 3. **Modulation of Caspar expression after *L. mexicana* infection of female sand flies.** Transcription of *caspar* was assessed in adults up to 6 days after feeding via a membrane on a blood meal containing *L. mexicana* amastigotes (*black bars*) compared with controls fed on blood only (*gray bars*) and sugar-fed flies (*open bars*). Semiquantitative RT-PCR was performed on females. The *error bars* represent the standard errors for three biological replicates consisting of pools of three whole bodies. *, $p < 0.05$.

account for the possibility that injections alone may affect immune gene transcription. It was interesting to note that merely pricking the cuticle was sufficient to cause a significant reduction (Fig. 6*A*; uninjected *versus* pricked $p < 0.0242$) in the population of parasites in the gut. This result suggests that general systemic immune activation prior to feeding the sand flies may influence the outcome of a gut parasite infection in these insects. The final control found no significant difference in parasite population size between pricked insects and dsGFP-injected sand flies, confirming that the double-stranded RNA did not promote nonspecific anti-leishmanial effects. Caspar-depleted sand flies possessed significantly lower numbers of parasites compared with all three controls.

The experiment was repeated using the natural host-parasite combination of *L. longipalpis-L. infantum*. In this experiment, early log phase promastigote parasite forms were used to initiate infections, and the percentage of infected sand flies was significantly reduced from 85% in dsGFP-injected controls to 45% in dsCaspar-injected (60% depletion) insects (Fig. 6*D*; $p < 0.0001$), and there was a significant reduction in parasite numbers in Caspar-depleted insects compared with the dsGFP-injected control (Fig. 6*C*; $p < 0.044$).

Promastigote initiated infections produce populations with more rapid growth kinetics compared with amastigote initiated infections; there is no lag phase in *Leishmania* population growth. Early log phase promastigotes will provide a vigorous, fast growing parasite population in the gut. This also means that the potentially sensitive transformation phase from amastigote to promastigote is not present, and sand fly host-initiated anti-leishmanial action is therefore occurring against the log phase promastigote forms and not confined to the transformation stage. The mean parasite populations present in the *L. infantum* infections were less than 25% that of the *L. mexicana* infections (Fig. 6, *A* and *C*); this was not a consequence of the use of promastigote-initiated infections, because Gossage *et al.* (17) used amastigote-initiated infections and reported more vigorous growth of *L. mexicana in vivo* compared with *L. infantum*. Therefore *L. infantum* amastigote-initiated infections may show even more significant reductions in parasite infection after Caspar depletion.

Bacterial Quantification in Caspar-depleted Insects—Total bacterial population in the female sand flies was measured 3 and 4 days after microinjections for Caspar silencing (Fig. 7). After 3 days there was no difference as measured by bacterial 16 S gene copy number. Although there was a trend toward an increase in bacterial population in Caspar-silenced sand flies after 4 days, there were no significant differences between dsCaspar-injected insects and dsGFP-injected ($n = 6$, pools of 10 insects/replicate; $p > 0.05$).

DISCUSSION

By inference from our knowledge of immune pathways in mosquitoes and *Drosophila*, we suggest that Caspar regulates the IMD signaling pathway in sand flies. Caspar depletion would then lead to IMD-related effector up-regulation leading to as yet unspecified, anti-leishmanial activities in the gut. This suggests that IMD-activated effectors have the potential to disrupt *Leishmania* population development and abort infections in the midgut lumen of the sand fly. Caspar depletion led to a fall from 85 to 45% of sand flies infected with *L. infantum*. This is the first time that depletion of a single immune gene has been shown to lead to abortion of *Leishmania* development in sand flies.

The phlebotomine sand fly has evolved to deal with a diverse microbiota throughout its life and is exposed to high titers of MAMPs. The larvae live on decomposing plant and animal material, whereas the adults feed on plant sap (30) and presumably will acquire phyllosphere-associated microbiota. Female sand flies are also telmophagic; their blood feeding is preceded by the creation of a wound and formation of a blood pool, which is contaminated with animal skin microbiota. To control the equilibrium of potentially beneficial microorganisms with harmful microorganisms acquired by sand flies, they must activate and maintain a basal immune response. From the perspective of the sand fly, the medically important *Leishmania* parasite is just one of many occupants of the gut; even in areas considered to harbor *Leishmania* epidemics, the percentage of female vectors harboring *Leishmania* may be as low as 7% (31).

Very little is known about the signaling cascades initiated after exposure to MAMPs in these sand flies, but the collection of putative immune genes described so far (26, 32–34) suggests that *Lutzomyia* is similar to *Anopheles* in the way it is regulating NF-κB activation of signaling cascades. We identified one sequence in the *L. longipalpis* cDNA libraries (26) that was similar to the *Drosophila* Caspar gene sharing higher similarity to the *A. gambiae* Caspar sequence. The sand fly amino acid sequence contains the conserved UAS and UBX domains as described in *Drosophila* (4) and in the equivalent human FAF1 (35).

Considering that Caspar is thought to be a negative regulator of the innate immune pathways, the overall profile of expression of these genes appeared unchanged throughout the juvenile developmental stages of the sand fly. The development of the gut microbiota in the larval stages is unknown, but gut microbial homeostasis will be important for an appropriate response toward pathogens and the prevention of a constant immune activation by harmless or beneficial food-associated microorganisms.

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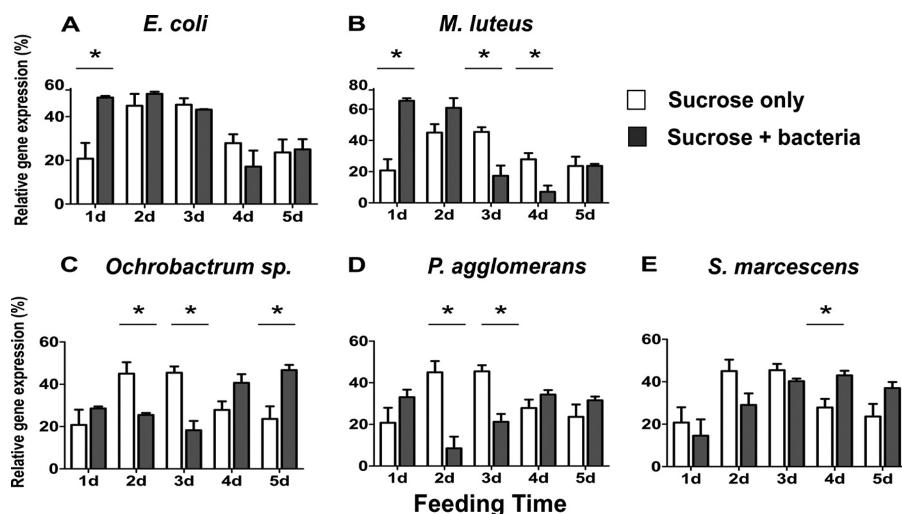

FIGURE 4. ***Per os* bacterial challenge and the expression of Caspar in female *L. longipalpis*.** *A*, *E. coli*. *B*, *M. luteus*. *C*, *Ochrobactrum sp*. *D*, *P. agglomerans*. *E*, *S. marcescens*. Transcription of *caspar* was assessed in adults fed for 5 days on 20% (w/v) sucrose supplemented with live log phase bacteria (*gray bars*) compared with controls fed sterile 20% sucrose solution (*open bars*); solution was replaced daily. Semiquantitative RT-PCR was performed on females collected on days 1–5. The *error bars* represent the standard errors for three biological replicates consisting of pools of three whole bodies. *, $p < 0.05$.

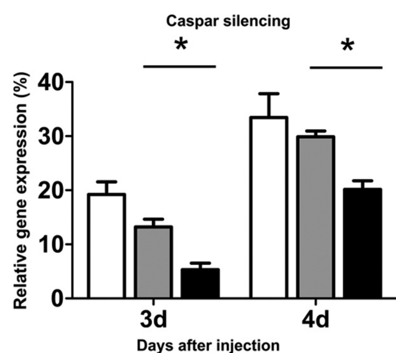

FIGURE 5. **Validation of Caspar silencing.** Caspar semiquantitative RT-PCR was performed on insects injected via the thorax with Caspar (*black bars*) or GFP dsRNA (*gray bars*) (144 ng) in sterile water. Noninjected insects (*open bars*) were also used for comparison. The samples were collected 3 and 4 days after injection. The *error bars* represent the standard errors for three biological replicates consisting of pools of three whole bodies. *, $p < 0.05$.

Feeding sucrose solution to adult sand flies during laboratory rearing is a replacement for a carbohydrate-rich plant meal. *L. longipalpis* exhibited lower Caspar transcription in sucrose-fed females when compared with sucrose with antibiotic-fed females. Bacteria are commonly associated with the gut of healthy phlebotomine sand flies in our laboratory colony,[4] and the transcription profiles confirm that all three immune signaling pathways are responding and functional in phlebotomine sand flies. Transcription of the putative negative regulator of the immune response was investigated during the 6 days following *L. mexicana* infections in comparison with a blood-fed control group. This time period covers the whole development process of the promastigote population from transformation within the blood meal during digestion through to the induction of mammalian infective metacyclic promastigotes (36).

Caspar transcription did not change during the first 2 days but was significantly down-regulated in infected insects between 3 and 6 days after *Leishmania* entered the gut; this corresponds to the period when the parasite escapes the blood meal inside the peritrophic matrix and starts to colonize the midgut lumen. Caspar may be down-regulated as a belated immune response by the sand fly host toward the large developing population of *Leishmania* parasites.

A more detailed study of Caspar expression was conducted during the 5 days after feeding on sugar meals with five different bacteria. There were significant changes in the relative expression but no clear pattern in transcription related to the different types of bacteria. This may reflect the differences in the ability of the bacteria to grow within the insect gut, as well as the type of bacteria used. There was a trend for down-regulation of Caspar in the first 3 days after feeding *S. marcescens*, but it was not significant as might be expected in response to being fed the potential pathogen. Feeding *S. marcescens* to *Drosophila* activates a local but not systemic IMD-dependent response in the midgut with Diptericin transcription (37). Another consideration is that preformed antimicrobial effectors might be present for fast release in response to sudden detection of MAMPs and that transcription is for replenishment (6).

We hypothesized that if the IMD pathway was potentially important in gut epithelial immunity, then early activation of the IMD pathway by RNAi-mediated depletion of Caspar might lead to abrogation of the *Leishmania* gut infection. Infection experiments with Caspar-depleted insects verified the hypothesis, showing significant effects on both *L. mexicana* and *L. infantum* infections. The results demonstrate the robustness of the effect of Caspar depletion on *Leishmania* infection in *L. longipalpis*. The growth of *L. mexicana*, which generally produce parasite populations more than four times larger than that of the naturally occurring *L. infantum*, was significantly impeded. The experiment also showed that merely pricking the cuticle with a sterile glass needle was sufficient to significantly reduce the promastigote population in the gut compared with uninjected controls. This control has not been included in most previous RNAi studies on insect immunity that used the injection route to administer the dsRNA. This confirms our previous finding for silencing a sand fly trypsin gene where injections

[4] M. R. V. Sant'Anna and R. J. Dillon, unpublished data.

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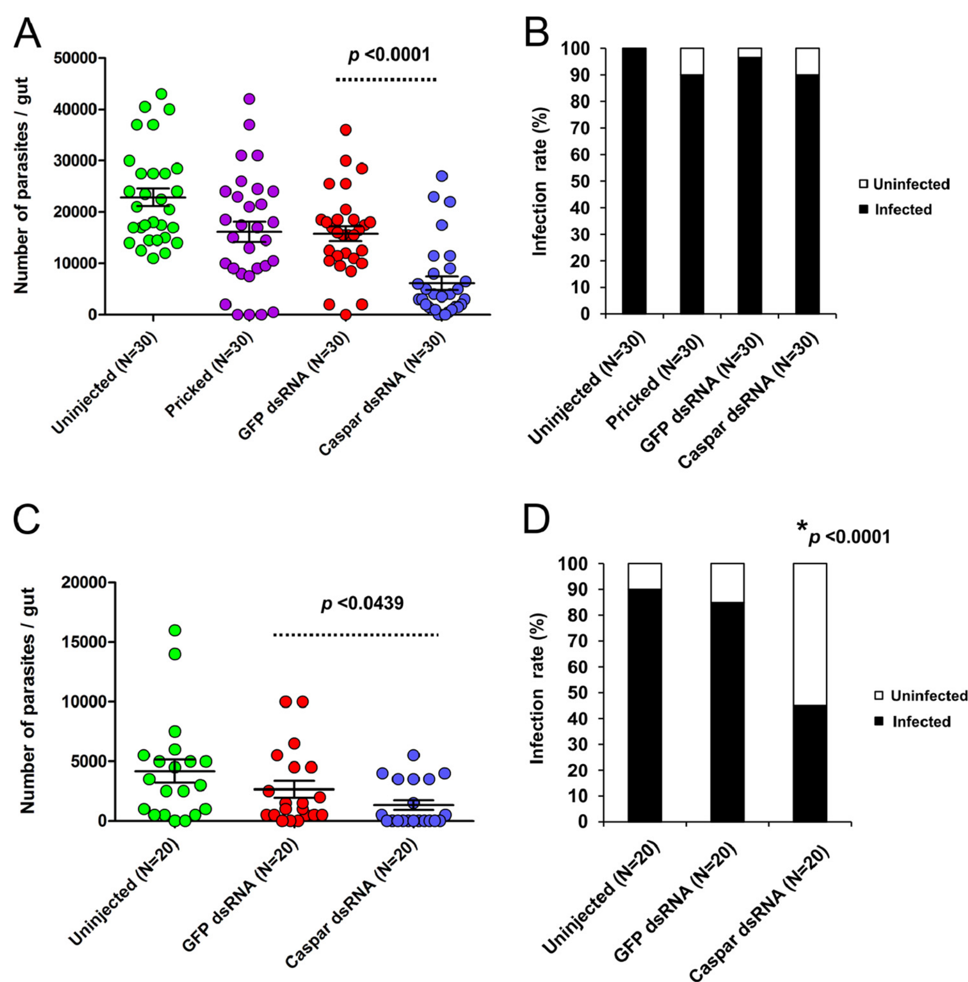

FIGURE 6. **Caspar depletion reduces *Leishmania* populations and infection prevalence in the sand fly *L. longipalpis*.** The insects were infected with *L. mexicana* (*A* and *B*) or *L. infantum* (*C* and *D*). Females were fed via a chick skin membrane on blood containing *L. mexicana* amastigotes or *L. infantum* promastigotes. The insects were injected via the thorax with 144 ng of dsRNA in sterile water 3 days before blood feeding, and parasite populations were recorded 5 days after blood feeding. Further controls included insects without injections, insects pricked in the thorax with an empty glass microneedle, and insects injected with GFP dsRNA. The results are the products of three separate experiments (*A* and *B*) or two separate experiments (*C* and *D*). The *horizontal bars* are the standard errors of the mean. The *asterisk* in *D* denotes significant difference using chi-squared analysis between GFP dsRNA- and Caspar dsRNA-injected flies.

with the control dsRNA caused a reduction in the *Leishmania* population (15). The potential for systemic effects of a nonspecific immune activation on the development of a parasite population needs to be considered. Experiments with tsetse flies showed that injection of bacteria was sufficient to elicit a systemic immune response that resulted in a fall in trypanosome gut infection prevalence (38). However, there was no effect of sterile injection on infection prevalence in tsetse flies. Further studies on the nature of this nonspecific anti-leishmanial response are needed to establish whether this is a systemic immune-related response.

Although attempts were made to minimize bacterial populations in the sand fly gut by aseptic technique, the influence of even low levels of insect gut bacteria in the establishment of gut-colonizing *Leishmania* cannot be ignored (29, 40). The effect of gut microbiota of anopheline mosquitoes on *Plasmodium* development in the gut suggests a tripartite interplay between insect-bacteria-parasite with a combination of indirect bacterial mediated immune activation (41) and direct bacterial effects on the malaria parasite (42). Quantification of bacteria in Caspar-depleted insects suggests no difference in the low level of total bacterial populations associated with the insects at the time of feeding the *Leishmania* parasite in the blood meal. However, the possibility that Caspar-depleted insects contained a different composition of bacteria that influence *Leishmania* development cannot be ruled out.

The role of Caspar and the IMD pathway appears to be pivotal in malaria-mosquito interactions. Caspar depletion by RNAi was shown to reduce *P. falciparum* infections in all of the major malaria mosquito vectors (8). *Plasmodium sp*. infections were assessed by estimating oocyst numbers, so in mosquitoes the immune activation may be operating either on the parasite in the gut or as it traverses the midgut epithelium prior to oocyst formation on hemolymph side of the gut. This differs from the current study because the *Leishmania* parasites are not thought to penetrate through the gut epithelia, and any immune effectors must pass into the gut lumen after Caspar depletion.

The degree to which sand flies invoke an immune response against *Leishmania* infections in the natural situation is not

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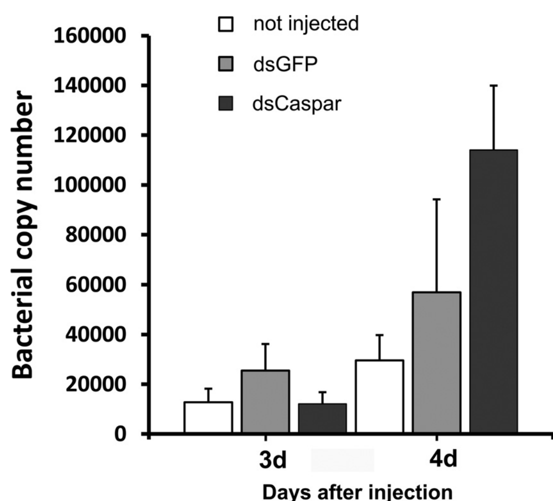

FIGURE 7. **Caspar silencing and sand fly bacterial populations.** Insects were injected with dsRNA for Caspar (*black bars*) or GFP dsRNA (*gray bars*) (144 ng), and the insects not injected (*open bars*) were used for comparison bacterial population estimated by quantitative PCR 3 and 4 days after injection. The *error bars* represent the standard errors of the mean of two separate experiments pool of 10 insects. A Mann-Whitney test was used to test for significant differences in the bacterial load ($p > 0.05$).

known, but in our experimental lab system, 80–100% infections are commonly achieved, particularly with *L. mexicana*. The metabolic costs of inducing an insect vector immune response are high (43), and this will be balanced against the potential pathogenicity of the parasite. *Leishmania* are thought to have limited effects on *L. longipalpis* longevity and fecundity (39), and this corroborates our data suggesting that there might be a limited immune response during infections.

This study of the Caspar gene provides a basis to further investigate the immune signaling cascades and their role in *Leishmania* survival and development in the vector. The results suggest that *Leishmania* gut infections may invoke a "late" IMD-mediated immune response, but this is ineffective in preventing the development of a transmissible parasite population. The discovery that Caspar silencing can abort *Leishmania* transmission in the sand fly demonstrates the importance of the innate immune pathways as targets for developing new strategies to disrupt sand fly vector competence. One such scenario would be a *Leishmania* gut infection-inducible promoter that switches on the IMD pathway leading to the loss of sand fly vector competence.

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Supplemental Figure 1. Caspar nucleotide and amino acid sequence. Caspar nucleotide sequence is shown in lowercase letters and amino acid sequence in uppercase letters. Number of amino acid residues is shown on the right side of each line. Bold uppercase letters indicate UAS-FAF1 like domain (residues 375-510) and UBX ubiquitin-like domain (residues 596-673).

```
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 V  G  E  A  I  L  V  L  E  E  S  N  W  D  L  L  T  A  I  H     40
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 K  V  I  P  Q  D  D  P  P  A  E  H  P  P  P  Y  S  M  A  S     60
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 V  E  P  M  A  A  G  T  P  S  S  S  S  P  M  S  I  V  Y  N    100
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 T  K  I  R  V  R  Q  P  S  G  D  F  L  E  R  R  F  T  A  D    620
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 T  K  L  N  I  L  L  N  Y  I  T  S  V  G  L  P  V  A  E  Y    640
aaaatgttctccgggtggccaagaaaagatttgacaacgatggatacagatgaaacgcta
 K  M  F  S  G  W  P  R  K  D  L  T  T  M  D  T  D  E  T  L    660
aagaatttacgtctgtgcccacaagagacggtgatcctaaaagaacga
 K  N  L  R  L  C  P  Q  E  T  V  I  L  K  E  R                676
```
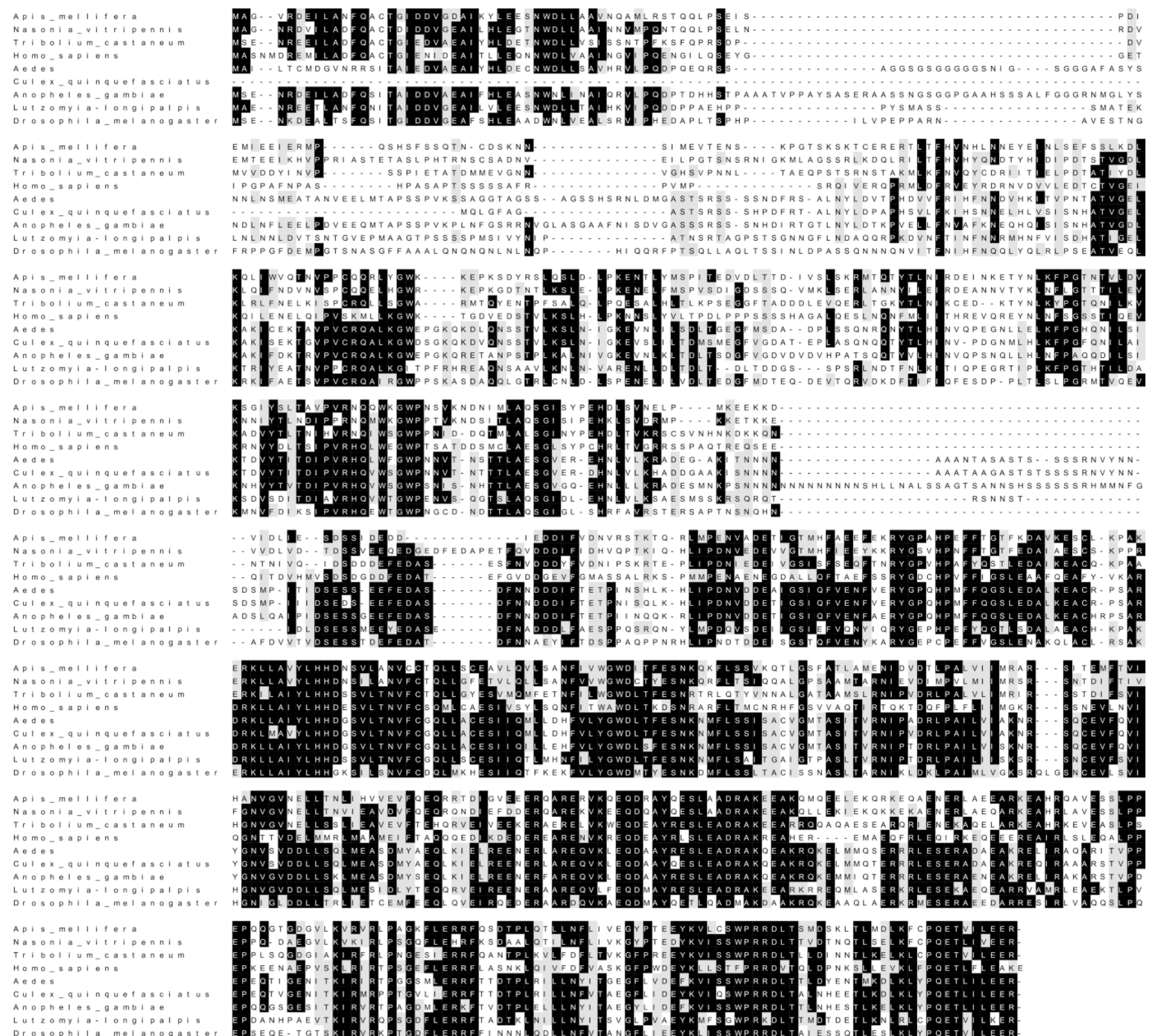

Supplemental Figure 2. Multiple alignment of Caspar-like sequences. Selected Caspar-like sequences (FAF1) from Dipteran insects *Ae.aegypti* (XP_001657027), *An. gambiae* (XP_316513), *C. quinquefasciatus* (XP_001862360), *D. melanogaster* (NP_611080), *and Lu. longipalpis* (AM093416) together with human (NP_008982) and three other insects species (XP_392750, XP_001600992, XP_975449), were aligned with ClustalW. Highly conserved amino acids are shaded in black; less conserved blocks are shaded in grey.



Caspar-like Gene Depletion Reduces *Leishmania* Infection in Sand Fly Host *Lutzomyia longipalpis*

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