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Reactive Oxygen Species-mediated Immunity against *Leishmania mexicana* and *Serratia marcescens* in the Phlebotomine Sand Fly *Lutzomyia longipalpis*[*] [S]

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Background: Reactive oxygen species are part of the sand fly innate immune system.
Results: ROS production in the gut increases in response to a bacterial pathogen but not to *Leishmania.*
Conclusion: Sand flies tolerate the presence of *Leishmania* by differential response of the ROS system.
Significance: The successful use of sand flies as vehicles for *Leishmania* transmission relies partially on the parasite circumventing the ROS immune response.

Phlebotomine sand flies are the vectors of medically important *Leishmania*. The *Leishmania* protozoa reside in the sand fly gut, but the nature of the immune response to the presence of *Leishmania* is unknown. Reactive oxygen species (ROS) are a major component of insect innate immune pathways regulating gut-microbe homeostasis. Here we show that the concentration of ROS increased in sand fly midguts after they fed on the insect pathogen *Serratia marcescens* but not after feeding on the *Leishmania* that uses the sand fly as a vector. Moreover, the *Leishmania* is sensitive to ROS either by oral administration of ROS to the infected fly or by silencing a gene that expresses a sand fly ROS-scavenging enzyme. Finally, the treatment of sand flies with an exogenous ROS scavenger (uric acid) altered the gut microbial homeostasis, led to an increased commensal gut microbiota, and reduced insect survival after oral infection with *S. marcescens*. Our study demonstrates a differential response of the sand fly ROS system to gut microbiota, an insect pathogen, and the *Leishmania* that utilize the sand fly as a vehicle for transmission between mammalian hosts.

Phlebotomine sand flies are the insect vectors responsible for the transmission of leishmaniases worldwide (1). This group of diseases is caused by parasites of the genus *Leishmania* and threatens ~350 million people in 88 countries around the world (2). Moreover, phlebotomines are also able to transmit other diseases caused by bacteria and viruses (3–5). *Lutzomyia longipalpis* (Lutz and Neiva, 1912) is the main vector species that transmits American visceral leishmaniasis in Brazil (6), American visceral leishmaniasis being one of the most important and dangerous forms of this collection of diseases (7, 8). Although its distribution is restricted to the New World (9, 10), this species has proven to be an excellent model organism given its permissiveness to different species of *Leishmania* (11), wide distribution in urban environments (12), and extensive research regarding laboratory rearing (8, 13).

In recent years, evidence from other dipteran species has highlighted the role of reactive oxygen species (ROS)[2] in insect immunity by regulating potential insect pathogens and determining the profile of the commensal gut microbiota (14–16). ROS are oxygen-derived radical species formed during cell respiration, mainly derived from mitochondrial electron transport. This group includes superoxide anion ($O_2^{\cdot-}$), the hydroxyl radical ($OH^{\cdot}$), and hydrogen peroxide (H_2O_2). Although H_2O_2 does not have unpaired electrons, it is usually considered as a ROS because it can be easily transformed into the highly reactive $OH^{\cdot}$ via a Fenton-like reaction (17, 18). Moreover, H_2O_2 can react with hypochlorous acid, superoxide anion, nitric oxide ($NO^{\cdot}$), and peroxynitrite, generating singlet molecular oxygen, a strong oxidant (19). ROS production in excess has deleterious effects in the cell, damaging lipids, proteins, and DNA (20). Eukaryotic cells are able to regulate ROS levels through the production of antioxidant enzymes. $O_2^{\cdot-}$ is produced by a NADPH oxidase and transformed to H_2O_2 by superoxide dismutase, whereas H_2O_2 is reduced to H_2O by catalase (17).

In *Drosophila melanogaster* ROS are actively produced in the midgut at a basal level in the presence of commensal microbiota (15, 21) and highly generated upon bacterial oral challenge (22). In *Anopheles gambiae*, ROS modulate immunity against bacteria and *Plasmodium* (14, 23). Studies done with *A. gambiae* showed that *Plasmodium* refractory strains were in a constant oxidative stress state exacerbated by blood-feeding and contributed to a higher *Plasmodium* melanization rate in comparison with strains susceptible to the malaria parasite (23). Superoxide anions are secreted into the midgut lumen of the adult *Aedes aegypti* mosquito, and blood meal ingestion decreased ROS levels via blood heme-activated protein kinase C (24). The

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[S] This article contains supplemental Table 1 and Figs. 1–4.
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[2] The abbreviations used are: ROS, reactive oxygen species; UA, uric acid feed; ABF, after blood-feeding; Contig, group of overlapping clones.

complexity of the role of ROS in gut microbe homeostasis was further underlined by the suggestion that gut bacteria-derived ROS may kill *Plasmodium* in the anopheline mosquito (25).

Our previous work has shown that ROS scavenging by means of antioxidant supplementation decreased survival of adult *L. longipalpis* and led to activation of the phenoloxidase cascade, which was proposed to be due to bacterial proliferation (26). The purpose of the present study was to investigate the ROS activities in the gut of *L. longipalpis* after feeding *Leishmania mexicana* and *Serratia marcescens*, a bacterial insect pathogen also found in wild sand fly populations (27). We manipulated ROS balance in infected flies to analyze its effect during vector-microbe homeostasis. The results suggest that *Leishmania* infections do not elicit ROS production within the *L. longipalpis* midgut, whereas *Serratia* infections increase ROS generation inside the sand fly gut.

EXPERIMENTAL PROCEDURES

Sand Fly Rearing—All experiments were performed using insectary-reared *L. longipalpis* from a colony first started with individuals caught in Jacobina, Brazil. Insects were kept under standard laboratory conditions (28). Sand flies were fed with 70% w/v sucrose solution in cotton wool (unless stated differently in experiments), kept under a photoperiod of 8 h of light/16 h of darkness, a temperature of 27 °C (±2), and a relative humidity of >80% inside the rearing cages. The females in the colony were fed on rabbit blood via a Hemotek membrane feeder (Discovery Workshops) at 37 °C. All procedures involving animals were performed in accordance with the United Kingdom government (Home Office) and European Commission regulations.

Sand Fly Infections—For parasite infections, *L. mexicana* MNYC/BZ/62/M379 promastigotes were kindly donated by Prof. P. Bates and kept at 26 °C in M199 medium supplemented with 25 μg/ml gentamicin sulfate (Sigma), 1× Eagle's Basal Medium vitamins (Invitrogen), and 20% fetal calf serum (PAA Laboratories). Promastigotes were subpassaged into fresh medium when cultures reached late log phase. Axenic amastigotes were obtained from promastigotes as described previously with some modifications (29). Briefly, promastigotes were centrifuged at 2000 rpm for 10 min, resuspended in Grace's medium supplemented with 25 μg/ml gentamicin sulfate (Sigma), 1× Eagle's Basal Medium vitamins (Invitrogen), and 20% fetal calf serum (PAA Laboratories) at pH 5.5, and incubated at 32 °C until fully transformed amastigotes were present in the flask. Axenic amastigotes were maintained and subpassaged in supplemented Grace's medium at 32 °C. For sand fly infections, amastigotes were resuspended in 1 ml of rabbit blood (2×10^6 parasites/ml) and fed to the insects through a chick skin membrane via a Hemotek feeder at 37 °C. Insects were kept under standard laboratory conditions until required for experimental work. For bacterial infections, *S. marcescens* (NCIMB 1377) was inoculated on LB agar plates and incubated at 26 °C for 20 h. Bacterial suspensions were prepared by transferring a colony of *S. marcescens* into 5 ml of LB broth and incubating overnight at 37 °C under shaking. The suspension was centrifuged at 13,500 rpm for 5 min, resuspended in PBS, and diluted to a concentration of 5.7×10^6 cfu/ml. Bacteria were then diluted in heat-inactivated blood to a final concentration of 1.14×10^4 cfu/ml and offered to sand flies via a Hemotek feeder as explained above.

RNA Extraction and Gene Relative Expression by RT-PCR—Individual sand flies were homogenized in 50 μl of TRI Reagent® (Ambion, Austin, TX) and kept at −80 °C until needed. RNA was extracted following the manufacturer's protocol. Total RNA was quantified using a NanoDrop® (NanoDrop Technologies, Wilmington, DE) and normalized to 10 ng/μl. RT-PCR was performed with a SuperScript® III one-step RT-PCR system with a Platinum® *Taq* DNA polymerase kit (Invitrogen) executing 25 cycles and following the manufacturer's protocol (primers listed in supplemental Table 1). Relative expression was normalized using a housekeeping gene (GenBank™ AM088777, 60 S ribosomal protein L3). RT-PCR products were analyzed by 1.5% w/v agarose/ethidium bromide gel electrophoresis, and changes in gene expression were determined by densitometric measurement of bands using the GeneSnap/GeneTools software (Syngene).

ROS Regulatory Gene Sequence Analysis—Putative gene sequences of *L. longipalpis* catalase, Cu/Zn superoxide dismutase, and peroxiredoxin and oxidative resistance protein 1 (OXR1) were obtained from a cDNA library constructed from sand fly whole bodies (AM105518, AM095907, AM102380, and AM097733, respectively) (30). Putative gene sequences of *L. longipalpis* catalase, superoxide dismutase, and peroxiredoxin were obtained from a midgut-specific expressed sequence tag library (ABV60342, ABV60343, and ABV60347, respectively) (31). BLAST was used to compare midgut catalase, superoxide dismutase, and peroxiredoxin and whole body OXR1 sequences with the National Center for Biotechnology Information database (32). Conserved residues in those protein families were retrieved from the Conserved Domains Database (CDD) (33). Multiple alignment, phylogenetic analysis, and Neighbor-joining cladograms were performed with MEGA package (34).

H_2O_2 Profile—Sand flies were infected with either *Leishmania* or *Serratia* after 3 days after emergence. Insects were dissected at 24, 48, 72, and 96 h after infection. The control group was blood-fed, noninfected flies. H_2O_2 was also measured in sugar-fed flies, 1 day before infection/blood-feeding. At each time point, two pools of four midguts were homogenized in 60 μl of PBS per pool containing 2 mg/ml of the catalase inhibitor 3-amino-triazole. Samples were flash-frozen in liquid N_2 and kept at −80 °C. Prior to assay, samples were thawed and centrifuged at 16,000 rpm, 5 min at 4 °C. Additional replicates were performed at 48 h after infection using fresh samples ($n = 6$ pools of four midguts) to validate the use of frozen samples for hydrogen peroxide assays. Five μl of the supernatant were assayed for H_2O_2 using the Amplex Red® hydrogen peroxide/peroxidase assay kit (Invitrogen) following the standard protocol as recommended by the manufacturer. All assays were carried out in triplicate. The experiment was performed twice.

In Vivo Detection of ROS—Sand flies were infected with either *Leishmania* or *Serratia* after 3 days after emergence and dissected at 1, 24, 48, 72 h, and 7 days after infection. The control group was blood-fed, noninfected flies. ROS production was also measured in sugar-fed flies, 1 day before infection/

blood-feeding. At each time point, five midguts were dissected for *in vivo* detection of ROS as described previously (35). Briefly, midguts were dissected in L-15 (Leibovitz) medium (Sigma) and incubated with 30 μmol of dihydroethidium in L-15 medium for 5 min in a dark chamber on a mini orbital shaker (70 rpm) at room temperature. After 3×5-min washes under the same conditions to remove dihydroethidium, individual midguts were transferred to 10-well slides. ROS production was monitored via an inverted fluorescence microscope using a U-MWG fluorescence cube (excitation, 530–560 nm; emission, 590 nm). Images were captured using a $\times 10$ objective and analyzed by ImageJ. A semiquantitative approach was used to compare fluorescence between samples. Based on tiff image files saved using the NIS-Elements BR 3.00 imaging software (Nikon), the sand fly midguts were delimited using the paintbrush tool of the ImageJ program, and mean intensity was measured inside the midgut, minimizing interference from background fluorescence. Mean intensity values were then used to compare fluorescence between samples after incubation.

dsRNA-mediated Catalase Knockdown—Sense and antisense catalase-specific primers (supplemental Table 1) flanked by the T7 promoter site amplified by PCR a 1472-bp product obtained from a normalized whole body *L. longipalpis* cDNA library (30) that was used as template for double-stranded RNA synthesis. Transcription reactions and column purification with the MEGAscript RNAi kit (Ambion®) followed the manufacturer's protocol. dsRNA purity was assessed by 1.5% w/v agarose/ethidium bromide gel electrophoresis, and dsRNA was quantitated using a NanoDrop ND-1000 spectrophotometer (LabTech). dsRNA was eluted with nuclease-free water at 65 °C, concentrated to 4.5 μg/μl with a Martin Christ® RVC 2–25 rotational vacuum concentrator, and stored at −80 °C. Enhanced green fluorescent protein (eGFP) dsRNA was produced from a 653-bp amplicon of the pEGFP-N1 expression plasmid (Clontech) and used as a "mock-injected" control. RNA interference (RNAi) was achieved by dsRNA injections as described previously (36). After injections, sand flies were transferred to cages, kept under standard rearing conditions, and infected with *Leishmania* 72 h after injections. Insects were dissected 94 h after infection, 15 midguts were homogenized individually in 50 μl of PBS, and parasites were counted using a hemocytometer. Three whole sand flies were reserved for individual RNA extraction and knockdown evaluation by RT-PCR. Relative expression data were shown as mean ± S.E. from three biological replicates.

H_2O_2 Feeding—Sand flies were kept under standard rearing conditions and allowed to feed *ad libitum* on cotton wool soaked in 5 mmol of H_2O_2 in 70% w/v sucrose solution since emergence and until the end of the experiment. Hydrogen peroxide solutions were freshly prepared from an H_2O_2 30% w/w stock solution (Sigma). Insects were infected with *Leishmania* 3 days after emergence. The control group was fed on plain 70% w/v sucrose solution. Flies were dissected 94 h after infection, 15 midguts were homogenized individually in 50 μl of PBS, and parasites were counted in a hemocytometer. Experiments were repeated twice.

Uric Acid Feed (UA), Insect Survival, and Bacteria Counts—Sand flies were kept under standard rearing conditions and allowed to feed *ad libitum* on cotton wool soaked in 10 mmol of uric acid in 7% w/v sucrose solution (pH = 8.9) since emergence and until the end of the experiment. Uric acid solution was freshly prepared every day. The control group was fed on sucrose 7% w/v, no UA (pH = 8.9). Insects were infected with *Serratia* 3 days after emergence as explained above. Control flies were fed on noninfected blood. Survival was recorded every day. Twelve flies were collected at 48 h after infection and dissected, and four pools of three midguts were homogenized in 50 μl of PBS per pool. Serial dilutions were inoculated onto LB agar plates, and colony-forming units (cfu) were counted after incubation at 26 °C for 24 h. Experiments were performed three times.

Statistical Analysis—Survival analyses were performed using the Kaplan-Meier Log Rank $\chi 2$ test. Comparisons between means of two independent groups were done with a pairwise *t* test, and multiple comparisons were done with one-way analysis of variance. For nonparametric data, multiple comparisons were done with a Kruskal-Wallis test, and pairwise comparisons were done with a Mann-Whitney test. Results are expressed as the group mean ± S.E. Significance was considered when $p < 0.05$. All data were analyzed with the SPSS Data Editor software (version 17.0, SPSS Inc.).

RESULTS

ROS Regulatory Gene Sequences from cDNA Libraries Constructed from Whole-body versus Midgut Exhibit High Similarity and Identity—Sequences corresponding to catalase, Cu/Zn superoxide dismutase, and peroxiredoxin retrieved from a sand fly whole body cDNA library showed a high level of identity and similarity against sequences obtained from a midgut-specific library (Table 1) (GenBank ABV60342, ABV60343, and ABV60347) (31), suggesting that these genes are also expressed in the midgut of *L. longipalpis*. Putative OXR1 could not be found in the midgut-specific database, and the best match to the *L. longipalpis* midgut expressed sequence tag library was a 14.5-kDa midgut protein (GenBank ABV60314, Table 1). All sequences obtained showed high levels of identity with homologous proteins from other organisms (supplemental Fig. 1, *A–E*), with conserved catalytic residues (catalase, superoxide dismutase, and peroxiredoxin) or domains (OXR1), which suggests that they are functional midgut proteins. Phylogenetic comparisons (supplemental Fig. 3) with sequences from these protein families showed a close proximity with corresponding proteins from other dipteran species, suggesting that these genes are orthologues to genes from other dipteran fly species including *Drosophila* and *Anopheles*.

Serratia and Not Leishmania Induces Changes in H_2O_2 Concentration in the Midgut—Catalase degrades toxic H_2O_2 into water and oxygen. To understand whether these changes in catalase expression have an effect on hydrogen peroxide levels, midgut-specific H_2O_2 concentration was measured in *L. mexicana-* and *S. marcescens*-infected *L. longipalpis*. Sand flies were infected, and midguts were assayed for H_2O_2 at 24, 48, 72, and 94 h ABF. Time 0 was considered as H_2O_2 concentration of non-blood-fed flies before infection. Additional replicates

TABLE 1
Putative ROS-regulatory genes

Best matched results and corresponding lowest BLASTX expected values together with putative function based on this homology of a GenBank-derived nonredundant protein database and a *Lutzomyia longipalpis* midgut cDNA library. The contig is given for each gene. A "q" following the clone identifier indicates that sequencing was from the 3′ end of the clone. The lowest BLASTX expected value (most significant similarity) together with putative function based on this homology is given. * represents best match with a putative oxidation resistance protein function.

Contig	Accession number	Best match to Non-redundant Protein Database (NRPD) excluding *L. longipalpis*	NRPD expected value	Best match to *L. longipalpis* midgut EST library	Accession number	Expected value	Identity	Similarity
							%	%
NSFM-142e04.q1k	AM105518	Putative catalase [*Phlebotomus perniciosus*]	3×10^{-11}	Putative catalase	ABV60342	1×10^{-134}	69	74
NSFM-39d09.p1k	AM095907	Putative Cu/Zn superoxide dismutase [*Phlebotomus perniciosus*]	2×10^{-73}	Putative Cu/Zn superoxide dismutase	ABV60343	6×10^{-120}	99	99
NSFM-34h03.q1k	AM102380	GI16636 [*Drosophila mojavensis*]	7×10^{-98}	Putative peroxiredoxin	ABV60347	5×10^{-159}	99	99
NSFM-22f08.p1k	AM097733	Oxidation resistance protein [*Glossina morsitans morsitans*]*	5×10^{-101}	14.5-kDa midgut protein	ABV60314	1.1	30	44

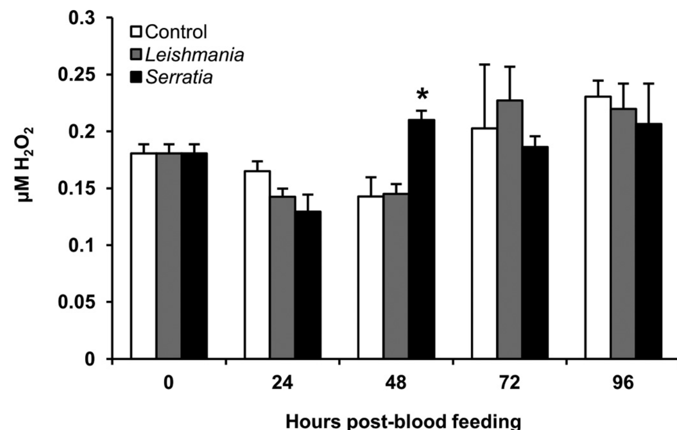

FIGURE 1. **Hydrogen peroxide concentration in the midgut of *Leishmania*- and *Serratia*-colonized sand flies.** Female flies were fed with a noninfected blood meal (*Control*), an *L. mexicana*-infected blood meal, or an *S. marcescens*-infected blood meal. Midguts of sucrose-fed sand flies were dissected 24 h before blood feeding and included in the analysis. Samples were homogenized and flash-frozen. Midguts were pooled ($n = 2$ pools of four midguts) and assayed for H_2O_2 concentration. Bar chart represents mean ± S.E. of combined samples from two independent experiments. An extra set of replicates was performed at 48 h after infection using fresh samples ($n = 6$ pools of four midguts). Data from both frozen and fresh samples were pooled for statistical analysis. *Asterisk* indicates statistical difference at $p < 0.01$ between *Serratia versus Leishmania* or control samples.

within the 48-h group were assayed using fresh, not flash-frozen, samples, and there was no significant difference between all fresh and frozen samples (analysis of variance, $p > 0.39$, $n = 6$). Only *Serratia*-infected flies exhibited a significant increase of H_2O_2 concentration at 48 h ABF when compared with the *Leishmania*-infected group ($p < 0.01$, Fig. 1). These results show that *L. mexicana* infection does not induce changes in hydrogen peroxide concentration in the midgut of *L. longipalpis* from 24 to 96 h ABF, in contrast to *S. marcescens*, which induced a significant increase in H_2O_2 concentration at 48 h after inoculation.

Serratia and Not Leishmania Induces Changes in Midgut ROS Production in Vivo—To further investigate ROS generation within *L. longipalpis* midguts, ROS levels were monitored *in vivo* in *Leishmania*- and *Serratia*-infected sand flies. *Serratia*-infected midguts showed a significant increase in ROS production at 24, 48, and 72 h ABF when compared with both *Leishmania*-infected and negative control midguts ($p < 0.02$, Fig. 2*A*). *Leishmania*-infected midguts did not show significant differences in ROS levels when compared with blood-fed negative controls (Fig. 2*A*). All *Serratia*-infected flies were dead at 7 days after infection. ROS significantly decreased at 24 and 48 h after blood feeding when compared with sugar-fed flies (Fig. 2*A*, $p < 0.05$), returning to similar values from sugar-fed flies after 72 h and 7 days after blood feeding (Fig. 2*A*). These results show that *Serratia* inoculation dramatically increases ROS production within *L. longipalpis* midgut, whereas ROS production in *Leishmania* infections did not increase in comparison with blood-fed control insects up to 7 days after infection.

Continuous H_2O_2 Feeding to Sand Flies Negatively Affects Leishmania Survival in Vivo—To analyze whether H_2O_2 has a negative effect in *Leishmania* survival inside the midgut, sand flies were allowed to feed *ad libitum* throughout the experiment on a 5-mmol H_2O_2-supplemented 70% w/v sucrose solu-

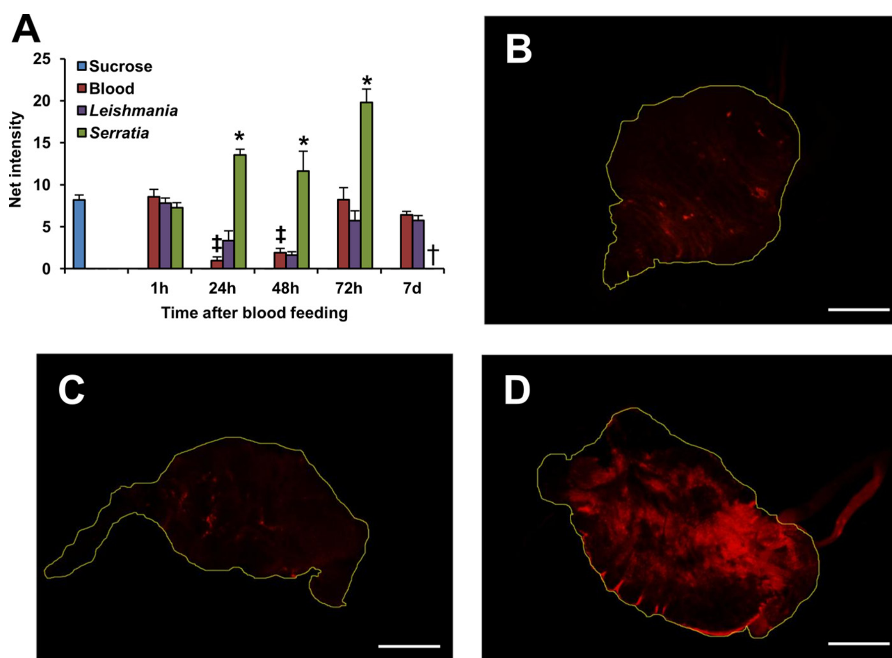

FIGURE 2. ***In vivo* detection of ROS in *Leishmania*- and *Serratia*-infected sand flies.** Female flies were fed with a noninfected blood meal, an *L. mexicana*-infected blood meal, or an *S. marcescens*-infected blood meal. Individual midguts were dihydroethidium-stained and photographed to analyze ROS production. Midguts of sucrose-fed sand flies were dissected 24 h before blood feeding and included in the analysis. *A*, bar charts represent mean values of net color intensity ± S.E. of at least five individual midguts. *Asterisk* indicates statistical difference at $p < 0.05$. *Double dagger* indicates statistical differences at $p < 0.05$ when compared with the sucrose (−24 h) group. *Dagger* represents no survivors (*Serratia* 7 days). *B–D*, selected representative images of noninfected and *Leishmania*- and *Serratia*-infected midguts, respectively. *Scale bar* represents 200 μm.

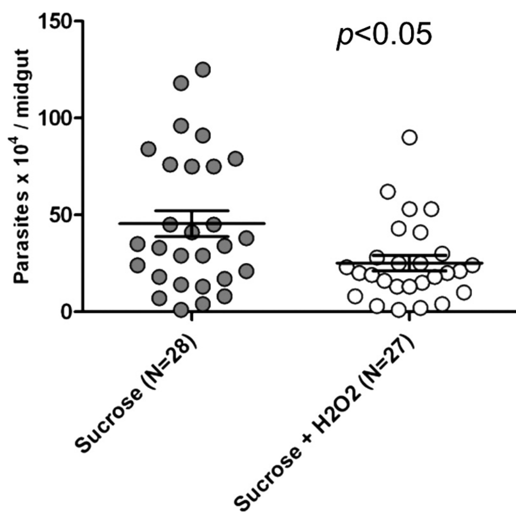

FIGURE 3. ***Leishmania* infection after continuous feeding of female *L. longipalpis* with a hydrogen peroxide-supplemented sucrose meal.** Flies were fed *ad libitum* from emergence on either 70% sucrose (*Sucrose*) or a 5-mmol H_2O_2-supplemented 70% sucrose solution (*Sucrose*+ H_2O_2) and infected 3 days after emergence. Individual midguts were dissected and sampled for *L. mexicana* at 96 h after infection. *Circles* represent the number of parasites per individual midgut. *Horizontal line* represents mean ± S.E. of combined samples from two independent experiments. Groups were compared using the Mann-Whitney *U* test.

tion followed by *Leishmania* infections to determine the effect of chronic H_2O_2 feeding on *Leishmania* survival. Midgut homogenates of 96 h ABF sand flies fed on H_2O_2 had significantly fewer parasites when compared with negative controls fed on plain sucrose ($p < 0.05$, Fig. 3). These results show that H_2O_2 exposure *in vivo* decreases *L. mexicana* survival within the *L. longipalpis* midgut.

dsRNA-mediated Knockdown of Catalase Negatively Affects Leishmania Survival in the Midgut—Expression of four ROS regulatory genes was assessed by RT-PCR in the midgut of *L. longipalpis* infected with either *S. marcescens* or *L. mexicana*. (supplemental Fig. 4). Noninfected blood-fed flies were used as negative control. Catalase exhibited the highest variation in expression among all ROS-detoxifying gene sequences analyzed. To further understand the deleterious effects of ROS on the development of *L. mexicana* within the sand fly midgut, *L. longipalpis* catalase was knocked down by RNAi. A dsRNA-mediated catalase knockdown of >50% was achieved at 96 h after injection (Fig. 4*A*). Catalase gene depletion was detrimental to *L. mexicana* survival within *L. longipalpis* midgut at 4 days after infection (Fig. 4*B*), suggesting that changes in catalase activity within the sand fly gut had a negative outcome on *Leishmania* survival and development.

Chronic Feeding of a Potent ROS Scavenger Reduces Sand Fly Survival in Serratia-infected Flies and Increases Naturally Occurring Microbiota—To test whether ROS depletion by chronic feeding of an antioxidant would have an effect on *L. longipalpis* survival after *Serratia* inoculation, insects were fed from emergence and throughout the experiment on a uric acid-supplemented sucrose solution in cotton wool and then infected with *S. marcescens* in rabbit blood. *Serratia*-infected sand flies fed with uric acid-supplemented sucrose exhibited a significant decrease in survival when compared with *Serratia*-infected flies fed on plain sucrose solution ($p < 0.001$, Fig. 5*A*). Uric acid supplementation had no effect on survival as no significant reduction could be observed in blood-fed control flies chronically fed with uric acid in comparison with blood-fed control flies (Fig. 5*A*). To analyze whether reduction in insect

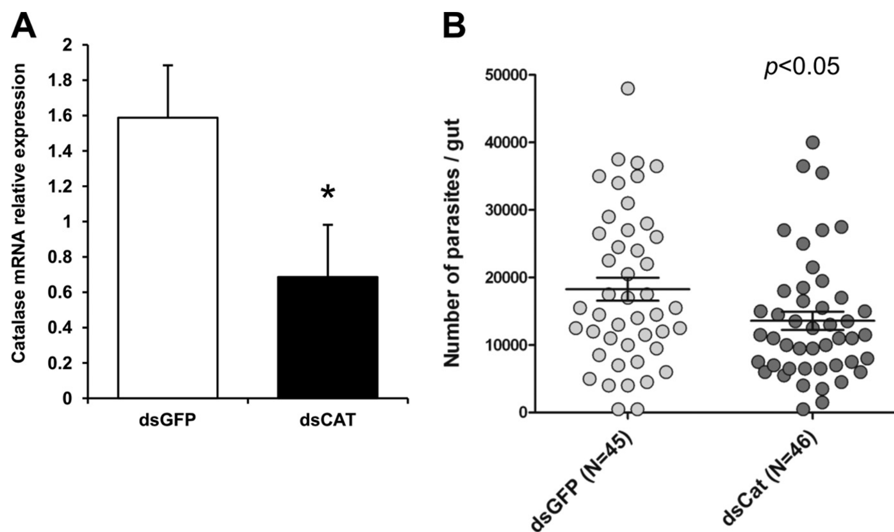

FIGURE 4. **dsRNA-mediated knockdown of catalase reduces *Leishmania* population in the midgut.** *A*, relative expression of catalase mRNA in whole fly homogenates from catalase dsRNA-injected and "mock-injected" sand flies. *B*, effect of dsRNA-mediated catalase silencing on *Leishmania* infection. Individual midguts were dissected and sampled for *L. mexicana* at 96 h after infection. *Circles* represent the number of parasites per individual midguts. *Horizontal line* represents mean ± S.E. of combined samples from three independent experiments. Groups were compared using the Mann-Whitney *U* test. *Asterisk* indicates statistical difference at $p < 0.05$.

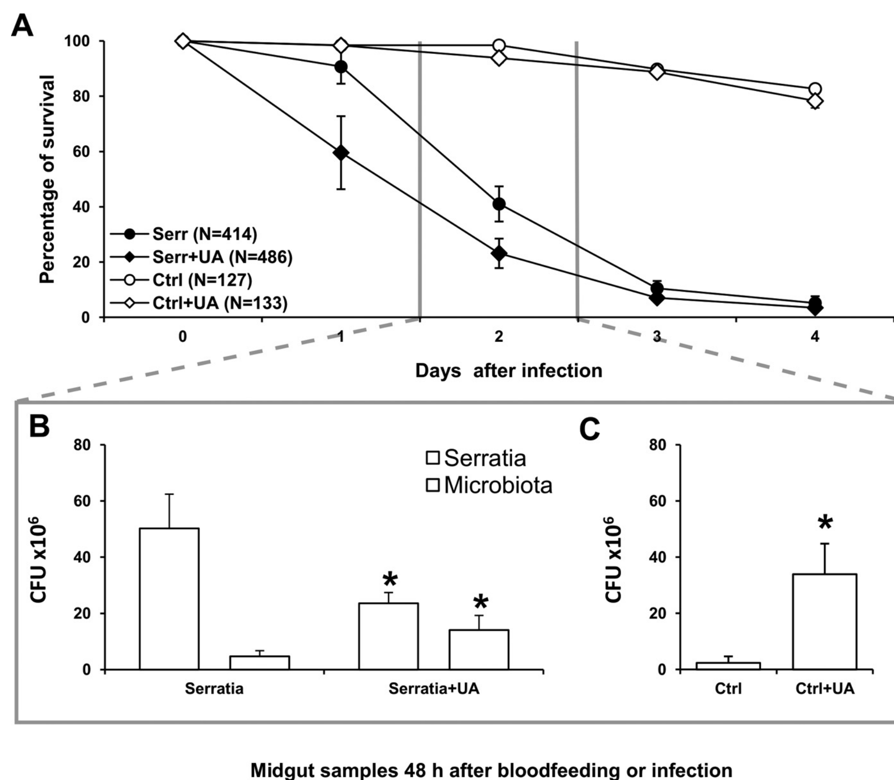

FIGURE 5. **Chronic feeding of a uric acid-supplemented sugar meal reduces survival in *Serratia*-infected flies and increases naturally occurring microbiota.** *A*, the percentage of survival of sand flies fed with: *Serratia* diluted in blood and 70% sucrose solution *ad libitum* (*Serr*); *Serratia* diluted in blood and a 10-mmol uric acid-supplemented 70% sucrose solution *ad libitum* (*Serr+UA*); noninfected blood and 70% sucrose solution *ad libitum* (*Ctrl*); and noninfected blood and a 10-mmol uric acid-supplemented 70% sucrose solution *ad libitum* (*Ctrl+UA*). *B*, effect of uric acid-supplemented sucrose feeding on *Serratia* and resident microbiota within the sand fly midgut. *C*, effect of uric acid-supplemented sucrose feeding on resident microbiota in noninoculated sand flies. Bar charts represent mean ± S.E. of combined samples from at least three independent experiments ($n = 60$). Groups were compared using the Mann-Whitney *U* test. *Asterisk* indicates statistical difference at $p < 0.05$. Survival curves were compared using the Kaplan-Meier Log Rank χ^2 test.

survival was due to an increase in *Serratia* bacterial cells within the sand fly gut, midguts of sand flies inoculated with *Serratia* with or without UA were dissected at 48 h ABF, and homogenates were diluted in PBS and inoculated onto LB agar plates. *Serratia* population in flies fed on UA-supplemented sucrose were significantly lower when compared with controls ($p \leq 0.012$, Fig. 5*B*). However, control flies (noninfected) fed on UA-supplemented sucrose exhibited a larger population of resident

microbiota ($p \leq 0.009$, Fig. 5*C*). To investigate whether naturally occurring microbial growth displayed a similar behavior in *Serratia*-infected samples, colony counts of resident microbes were performed. UA supplementation significantly increased resident microbiota numbers in *Serratia*-infected flies ($p \leq 0.037$, Fig. 5*B*).

DISCUSSION

The present study shows that ROS regulation influences the sand fly host-*Leishmania* parasite-gut microbiota interaction and that changes in midgut ROS levels by feeding uric acid (an exogenous ROS scavenger) can alter the dynamics of sand fly midgut homeostasis, favoring the growth of commensal sand fly gut bacteria.

ROS Production with Bacteria—The H_2O_2 concentration was significantly higher in *Serratia*-infected flies 2 days after blood feeding (Fig. 1). Additionally, *in vivo* detection of ROS confirmed high levels from 1 to 72 h after bacterial challenge (Fig. 2*A*). It is possible that the increase in ROS was due to epithelial cell death caused by pathogen proliferation (37). Alternatively, this increase could be part of the oxidative burst against pathogenic bacteria that has been observed in *Drosophila* (21). A similar inverse correlation between bacterial growth and ROS levels has been shown recently in *A. aegypti* (24). In a previous work, we showed that feeding sand flies with the ROS scavenger ascorbic acid was detrimental to survival (26) and suggested that mortality could be caused by bacterial infections due to ROS reduction in the midgut. In the present study, oral administration of a ROS scavenger also decreased survival in *Serratia*-infected flies continuously fed on uric acid when compared with *Serratia*-infected flies fed on sucrose solution. It is very unlikely that differences in mortality were caused by uric acid toxicity because flies fed on this antioxidant did not show any differences in survival when compared with control.

The addition of dietary uric acid increased resident gut microbiota in both *Serratia*-infected and noninfected flies but had an opposite effect on the *Serratia* population. In similar experiments performed in *Aedes* with *Enterobacter asburiae*, the addition of dietary ROS inhibitors increased both endogenous microbiota and *Enterobacter* (24). In our work, midguts were dissected 48 h after infection, when mortality reached ~80%. It is possible that survivors sampled at that time point were more resistant to infection and harbored a lower *Serratia* load. Another possibility is that the increase in the *Serratia* population was impeded by resident microbes, which were significantly higher in *Serratia*-infected flies supplemented with uric acid.

ROS Production with Leishmania—We have shown that hydrogen peroxide can kill *Leishmania in vitro* (supplemental Fig. 2) and that oral administration of H_2O_2 to infected flies is detrimental to *Leishmania* survival within the sand fly gut. RNAi-mediated gene silencing of the H_2O_2-detoxifying enzyme catalase led to lower *Leishmania* population in the gut, providing further confirmation that *Leishmania* parasites are sensitive to ROS generation. An estimated reduction of ~50% in catalase expression was achieved in catalase dsRNA-injected sand flies in comparison with mock-injected controls. Catalase knockdown sand flies exhibited a reduction in parasite numbers when compared with dsGFP-injected insects. Because only fully engorged females were selected for the experiments, it is unlikely that the size of the blood meal could account for the difference in parasite numbers. It was quite intriguing to find a lack of ROS activity in *Leishmania*-infected sand flies in comparison with *Anopheles* and *Plasmodium* (14). The fact that two different ROS (O_2^- and H_2O_2) did not exhibit induction in biochemical assays in *Leishmania*-colonized sand fly midguts raises the possibility that *Leishmania* could "evade" the oxidative burst by an unknown mechanism or avoid eliciting a ROS-based response to ensure survival within the gut. One potential scenario would be detoxification of ROS by the *Leishmania* during blood meal digestion using the antioxidant enzymes of the protozoan. Antioxidant enzymes are crucial for *Leishmania* parasites during infections inside the macrophage. It has been shown that virulence in *Leishmania* correlates with antioxidant enzyme expression in the parasite (38, 39), as well as with its resistance to hydrogen peroxide toxicity (40–42). Moreover, in an interspecies microarray performed in *L. mexicana*, one of the most up-regulated genes in promastigotes when compared with lesion amastigotes was peroxidoxin (43), which when expressed as a protein was able to break down ROS in protozoa (44–46). The hypothesis of a *Leishmania*-mediated ROS detoxification during blood meal digestion is currently being studied in our laboratory.

An increasing body of sand fly sequence data (30, 31, 47, 48) allows us to infer that the sand fly gut immune response will be similar in overall organization to that of the other, more extensively studied dipterans: mosquitoes and *Drosophila*. In these insects, there is a reliance on the innate immune response mainly via the two types of effectors: ROS and antimicrobial peptides. Manipulating the sand fly ROS system revealed the potential complexity underlying immune homeostasis in the gut. The challenge for the fly is to regulate ROS production within the gut to attempt suppression of potential pathogens while allowing development of potentially beneficial microorganisms. Our results suggest that ROS are harmful to the *Leishmania* and that experimental activation of the ROS system in the sand fly results in a reduced *Leishmania* population. However, there is an apparent tolerance of the *Leishmania* by its sand fly host, allowing the development of large populations. The addition of antioxidant to the gut and subsequent effects on cohabiting bacterial species provide us with a glimpse into the "fine tuning" between ROS levels, bacterial communities, and the sand fly vector of *Leishmania*.

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REFERENCES

1. Sacks, D., and Kamhawi, S. (2001) Molecular aspects of parasite-vector and vector-host interactions in leishmaniasis. *Annu. Rev. Microbiol.* **55,** 453–483
2. World Health Organization (2010) *Control of the Leishmaniases*, WHO Technical Report Series 949, World Health Organization, Geneva, Switzerland

3. Alexander, B. (1995) A review of bartonellosis in Ecuador and Colombia. *Am. J. Trop. Med. Hyg.* **52,** 354–359
4. Comer, J. A., Tesh, R. B., Modi, G. B., Corn, J. L., and Nettles, V. F. (1990) Vesicular stomatitis virus, New Jersey serotype: replication in and transmission by *Lutzomyia shannoni* (Diptera: Psychodidae). *Am. J. Trop. Med. Hyg.* **42,** 483–490
5. Charrel, R. N., Gallian, P., Navarro-Mari, J. M., Nicoletti, L., Papa, A., Sánchez-Seco, M. P., Tenorio, A., and de Lamballerie, X. (2005) Emergence of Toscana virus in Europe. *Emerg. Infect. Dis.* **11,** 1657–1663
6. Maingon, R. D., Ward, R. D., Hamilton, J. G., Bauzer, L. G., and Peixoto, A. A. (2008) The *Lutzomyia longipalpis* species complex: does population substructure matter to *Leishmania* transmission? *Trends Parasitol.* **24,** 12–17
7. Lainson, R., and Rangel, E. (2005) *Lutzomyia longipalpis* and the eco-epidemiology of American visceral leishmaniasis, with particular reference to Brazil: a review. *Mem. Inst. Oswaldo Cruz* **100,** 811–827
8. Soares, R. P., and Turco, S. J. (2003) *Lutzomyia longipalpis* (Diptera: Psychodidae: Phlebotominae): a review. *An. Acad. Bras. Cienc.* **75,** 301–330
9. Grimaldi, G., Jr., and Tesh, R. B. (1993) Leishmaniases of the New World: current concepts and implications for future research. *Clin. Microbiol. Rev.* **6,** 230–250
10. Romero, G. A., and Boelaert, M. (2010) Control of visceral leishmaniasis in Latin America: a systematic review. *PLoS Negl. Trop. Dis.* **4,** e584
11. Myskova, J., Svobodova, M., Beverley, S. M., and Volf, P. (2007) A lipophosphoglycan-independent development of *Leishmania* in permissive sand flies. *Microbes Infect.* **9,** 317–324
12. Costa, C. (2008) Characterization and speculations on the urbanization of visceral leishmaniasis in Brazil. *Cad. Saude Publica.* **24,** 2959–2963
13. Volf, P., and Volfova, V. (2011) Establishment and maintenance of sand fly colonies. *J. Vector Ecol.* **36,** S1–S9
14. Molina-Cruz, A., DeJong, R. J., Charles, B., Gupta, L., Kumar, S., Jaramillo-Gutierrez, G., and Barillas-Mury, C. (2008) Reactive oxygen species modulate *Anopheles gambiae* immunity against bacteria and *Plasmodium*. *J. Biol. Chem.* **283,** 3217–3223
15. Ha, E. M., Oh, C. T., Ryu, J. H., Bae, Y. S., Kang, S. W., Jang, I. H., Brey, P. T., and Lee, W. J. (2005) An antioxidant system required for host protection against gut infection in *Drosophila*. *Dev. Cell* **8,** 125–132
16. Munks, R. J., Sant'Anna, M. R., Grail, W., Gibson, W., Igglesden, T., Yoshiyama, M., Lehane, S. M., and Lehane, M. J. (2005) Antioxidant gene expression in the blood-feeding fly *Glossina morsitans morsitans*. *Insect Mol. Biol.* **14,** 483–491
17. Thannickal, V. J., and Fanburg, B. L. (2000) Reactive oxygen species in cell signaling. *Am. J. Physiol. Lung Cell. Mol. Physiol.* **279,** L1005–L1028
18. Bonekamp, N. A., Völkl, A., Fahimi, H. D., and Schrader, M. (2009) Reactive oxygen species and peroxisomes: struggling for balance. *Biofactors* **35,** 346–355
19. Miyamoto, S., Ronsein, G. E., Prado, F. M., Uemi, M., Corrêa, T. C., Toma, I. N., Bertolucci, A., Oliveira, M. C., Motta, F. D., Medeiros, M. H., and Mascio, P. D. (2007) Biological hydroperoxides and singlet molecular oxygen generation. *IUBMB Life* **59,** 322–331
20. Freeman, B. A., and Crapo, J. D. (1982) Biology of disease: free radicals and tissue injury. *Lab. Invest.* **47,** 412–426
21. Ha, E. M., Oh, C. T., Bae, Y. S., and Lee, W. J. (2005) A direct role for dual oxidase in *Drosophila* gut immunity. *Science* **310,** 847–850
22. Ha, E. M., Lee, K. A., Park, S. H., Kim, S. H., Nam, H. J., Lee, H. Y., Kang, D., and Lee, W. J. (2009) Regulation of DUOX by the $G\alpha_q$-phospholipase $C\beta$-Ca^{2+} pathway in *Drosophila* gut immunity. *Dev. Cell* **16,** 386–397
23. Kumar, S., Christophides, G. K., Cantera, R., Charles, B., Han, Y. S., Meister, S., Dimopoulos, G., Kafatos, F. C., and Barillas-Mury, C. (2003) The role of reactive oxygen species on *Plasmodium* melanotic encapsulation in *Anopheles gambiae*. *Proc. Natl. Acad. Sci. U.S.A.* **100,** 14139–14144
24. Oliveira, J. H., Gonçalves, R. L., Lara, F. A., Dias, F. A., Gandara, A. C., Menna-Barreto, R. F., Edwards, M. C., Laurindo, F. R., Silva-Neto, M. A., Sorgine, M. H., and Oliveira, P. L. (2011) Blood meal-derived heme decreases ROS levels in the midgut of *Aedes aegypti* and allows proliferation of intestinal microbiota. *PLoS Pathog.* **7,** e1001320
25. Cirimotich, C. M., Dong, Y., Clayton, A. M., Sandiford, S. L., Souza-Neto, J. A., Mulenga, M., and Dimopoulos, G. (2011) Natural microbe-mediated refractoriness to *Plasmodium* infection in *Anopheles gambiae*. *Science* **332,** 855–858
26. Diaz-Albiter, H., Mitford, R., Genta, F. A., Sant'Anna, M. R., and Dillon, R. J. (2011) Reactive oxygen species scavenging by catalase is important for female *Lutzomyia longipalpis* fecundity and mortality. *PLoS ONE* **6,** e17486
27. Gouveia, C., Asensi, M. D., Zahner, V., Rangel, E. F., and Oliveira, S. M. (2008) Study on the bacterial midgut microbiota associated to different Brazilian populations of *Lutzomyia longipalpis* (Lutz & Neiva) (Diptera: Psychodidae). *Neotrop. Entomol.* **37,** 597–601
28. Modi, G. (1997) Care and maintenance of phlebotomine sand fly colonies in *The Molecular Biology of Insect Disease Vectors* (J.M. Crampton, Beard, C. B., and Louis, C. L., ed), pp. 21–30, Chapman & Hall, London
29. Bates, P. (1994) Complete developmental cycle of *Leishmania mexicana* in axenic culture. *Parasitology* **108,** 1–9
30. Dillon, R. J., Ivens, A. C., Churcher, C., Holroyd, N., Quail, M. A., Rogers, M. E., Soares, M. B., Bonaldo, M. F., Casavant, T. L., Lehane, M. J., and Bates, P. A. (2006) Analysis of ESTs from *Lutzomyia longipalpis* sand flies and their contribution toward understanding the insect-parasite relationship. *Genomics* **88,** 831–840
31. Jochim, R. C., Teixeira, C. R., Laughinghouse, A., Mu, J., Oliveira, F., Gomes, R. B., Elnaiem, D. E., and Valenzuela, J. G. (2008) The midgut transcriptome of *Lutzomyia longipalpis*: comparative analysis of cDNA libraries from sugar-fed, blood-fed, post-digested, and *Leishmania infantum chagasi*-infected sand flies. *BMC Genomics* **9,** 15
32. Altschul, S. F., Gish, W., Miller, W., Myers, E. W., and Lipman, D. J. (1990) Basic local alignment search tool. *J. Mol. Biol.* **215,** 403–410
33. Marchler-Bauer, A., Lu, S., Anderson, J. B., Chitsaz, F., Derbyshire, M. K., DeWeese-Scott, C., Fong, J. H., Geer, L. Y., Geer, R. C., and Gonzales, N. R. (2011) CDD: a Conserved Domain Database for the functional annotation of proteins. *Nucleic Acids Res.* **39,** D225–D229
34. Tamura, K., Dudley, J., Nei, M., and Kumar, S. (2007) MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Mol. Biol. Evol.* **24,** 1596–1599
35. Owusu-Ansah, E., Yavari, A., and Banerjee, U. (February 27, 2008) A protocol for *in vivo* detection of reactive oxygen species. *Nat. Protoc.* 10.1038/nprot.2008.23
36. Sant'Anna, M. R., Alexander, B., Bates, P. A., and Dillon, R. J. (2008) Gene silencing in phlebotomine sand flies: xanthine dehydrogenase knockdown by dsRNA microinjections. *Insect Biochem. Mol. Biol.* **38,** 652–660
37. Buchon, N., Broderick, N. A., Poidevin, M., Pradervand, S., and Lemaitre, B. (2009) *Drosophila* intestinal response to bacterial infection: activation of host defense and stem cell proliferation. *Cell Host Microbe* **5,** 200–211
38. Krieger, S., Schwarz, W., Ariyanayagam, M. R., Fairlamb, A. H., Krauth-Siegel, R. L., and Clayton, C. (2000) Trypanosomes lacking trypanothione reductase are avirulent and show increased sensitivity to oxidative stress. *Mol. Microbiol.* **35,** 542–552
39. Steenkamp, D. J. (2002) Trypanosomal antioxidants and emerging aspects of redox regulation in the trypanosomatids. *Antioxid. Redox Signal.* **4,** 105–121
40. Pal, S., Dolai, S., Yadav, R. K., and Adak, S. (2010) Ascorbate peroxidase from *Leishmania major* controls the virulence of infective stage of promastigotes by regulating oxidative stress. *PLoS One* **5,** e11271
41. Acestor, N., Masina, S., Ives, A., Walker, J., Saravia, N. G., and Fasel, N. (2006) Resistance to oxidative stress is associated with metastasis in mucocutaneous leishmaniasis. *J. Infect. Dis.* **194,** 1160–1167
42. Goyal, N., Roy, U., and Rastogi, A. K. (1996) Relative resistance of promastigotes of a virulent and an avirulent strain of *Leishmania donovani* to hydrogen peroxide. *Free Rad. Biol. Med.* **21,** 683–689
43. Holzer, T. R., McMaster, W. R., and Forney, J. D. (2006) Expression profiling by whole-genome interspecies microarray hybridization reveals differential gene expression in procyclic promastigotes, lesion-derived amastigotes, and axenic amastigotes in *Leishmania mexicana*. *Mol. Biochem. Parasitol.* **146,** 198–218
44. McGonigle, S., Dalton, J. P., and James, E. R. (1998) Peroxidoxins: a new antioxidant family. *Parasitol. Today* **14,** 139–145

45. Levick, M. P., Tetaud, E., Fairlamb, A. H., and Blackwell, J. M. (1998) Identification and characterization of a functional peroxidoxin from *Leishmania major*. *Mol. Biochem. Parasitol.* **96,** 125–137
46. Barr, S. D., and Gedamu, L. (2001) Cloning and characterization of three differentially expressed peroxidoxin genes from *Leishmania chagasi*: evidence for an enzymatic detoxification of hydroxyl radicals. *J. Biol. Chem.* **276,** 34279–34287
47. Dostálová, A., Votýpka, J., Favreau, A. J., Barbian, K. D., Volf, P., Valenzuela, J. G., and Jochim, R. C. (2011) The midgut transcriptome of *Phlebotomus* (*Larroussius*) *perniciosus*, a vector of *Leishmania infantum*: comparison of sugar-fed and blood-fed sand flies. *BMC Genomics* **12,** 223
48. Ramalho-Ortigão, M., Jochim, R. C., Anderson, J. M., Lawyer, P. G., Pham, V. M., Kamhawi, S., and Valenzuela, J. G. (2007) Exploring the midgut transcriptome of *Phlebotomus papatasi*: comparative analysis of expression profiles of sugar-fed, blood-fed, and *Leishmania*-major-infected sand flies. *BMC Genomics* **8,** 300