Supporting Online Material for

Epithelial Nitration By A Peroxidase/NOX5 System Mediates Mosquito Antiplasmodial Immunity

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Materials and Methods

Mosquitoes. A. gambiae (G3 strain) mosquitoes were reared at 28°C, 80% humidity under a 12 h light/dark cycle and maintained on a 10% Karo syrup solution during adult stages.

Infections of mosquitoes with P. berghei. Infections were performed using transgenic GFP *P. berghei* (ANKA 2.34 strain) parasites (*1*) maintained by serial passage in 3-4-week-old female BALB/c mice (Charles River, Wilmington, MA) from frozen stocks. Mice infectivity was determined by parasitemia levels and *in vitro* exflagellation assays following standard procedures previously described (*2*). Female mosquitoes (5 days old) were fed when mice reached 3-6% parasitemia and 1-2 exflagellations/field. Uninfected blood-fed and *P. berghei* infected mosquitoes were kept at 21°C and 80% humidity. Infection levels were determined by dissecting midguts 7-8 days post-feeding. The number of parasites in individual mosquitoes was scored in a fluorescent microscope and the distributions between the different experimental groups were compared using the nonparametric Kolmogorov-Smirnov test. All phenotypes were confirmed in three independent experiments. All mouse experiments were conducted under the NIH protocol LMVR5, which has been approved by the NIAID Institutional Animal Care and Use Committee.

RNA Isolation, cDNA Synthesis, and q-PCR. Total RNAs were isolated from *A. gambiae* midguts using the RNeasy Mini Kit (Qiagen, Los Angeles, CA) or Trizol (Invitrogen,

Carlsbad, CA) according to the manufacturer's instructions. First-strand cDNA was synthesized using the QuantiTect Reverse Transcription Kit (Qiagen) with integrated genomic DNA removal. Gene expression was assessed by SYBR green quantitative Real-Time PCR (qPCR) (DyNAmo HS; New England Biolabs, Beverly, MA) in a Chromo4 system (Bio-Rad, Hercules, CA). PCR involved an initial denaturation at 95ºC for 15 min, 44 cycles of 10 sec at 94°C, 20 sec at 56°C, and 30 sec at 72°C. Fluorescence readings were taken at 72°C after each cycle. A final extension at 72°C for 5 min was completed before deriving a melting curve (70–95°C) to confirm the identity of the PCR product. Measurements of q-PCR were made in duplicate. Relative quantitation results were normalized using the ribosomal protein S7 gene as positive internal control (*3*). The primers used were as follows (sequences are indicated from 5′ to 3′): for S7, F-AGAACCAGCAGACCACCATC and R-GCTGCAAACTTCGGCTATTC; for HPX2, F-CCGCTTCTACAACACGATGA and R-CGACCAGATGGGCAAGTAT; for HPX7, F-ACTGTACCCTCGAAGCTGGA and R-GCGTGGGATTGAGAAAGGTA; for HPX8, F-AGTCACGTACGCATCGTCAG and R-ACATCACTCGCAGGACACAG; for DBLOX, F-GCTGAGTTCGGAAGACGAC and R-CAATTTGTGGCATGGAGTTG; for NOS, F-GCTCGAACTATCTGGCCAAC and R-CCACTCTTGCCAGAACGAAC; NOX5, F-TCATGCATCGCTACTGGAAG and R-CCAGAAAAGTCCACCTTGG; for IMPer, F-GGGTGCTGTGTGACAATACG and R-CCATCGCGTTAAATTCACCT and for TEP1, F-CAGATGGTTCGTTTGGTGTG and R-GCAATGCCGTAACATAC.

dsRNA Synthesis. A 218-bp fragment of the LacZ gene was amplified using the primers F-GAGTCAGTGAGCGAGGAAGC and R-TATCCGCTCACAATTCCACA (sequences are indicated from 5′ to 3′) and cloned into the pCRII-TOPO vector (Invitrogen, Carlsbad, CA) following the manufacturer's instructions. For NOX5, a 425 bp fragment was amplified using the following primers: F-TCGTACGAATGGTCGAGTGA and R-CCAAACTGGTCGCACTTGTA and cloned into a pCRII-TOPO vector. For DBLOX, a 527 bp fragment was amplified using the primers: F-CCCGGACTGGTGGACGAGATTTC and R-TCATTAGTCCCCGCCAACGTCCAGATTC. For HPX8, a 498 bp fragment was amplified using the primers: F-TTCGGGTTGACGCGGTTGCTGTTT and R-TCATTAACGGGACGGGCGGGTTATTGCTC. For TEP-1, a fragment of 500-600 bp was amplified using the primers: F-TTTGTGGGCCTTAAAGCGCTG and R-ACCACGTAACCGCTCGGTAAG. For IMPer gene, a 462 bp fragment was amplified using the primers F-TCGGTGCTGGAAAAGGATGG and R-GTTGGCGCGAGCTAAACACG. The fragments of all genes mentioned above were cloned into pCRII-TOPO vector (Invitrogen, Carlsbad, CA). T7 promoters were incorporated into all these fragments by amplifying the cloned insert using the primers: M13FGTAAAACGACGGCCAGT and M13R-CTCGAGTAATACGACTCACTATAGGGCAGGAAACAGCTATGAC. For HPX2, total RNA was extracted from carcass of infected-fed mosquitoes 24 h post-infection and cDNA was synthesized as described previously. First, a 1280 bp fragment of the gene was amplified using external primers: F- TACGAGTGTGGCCTCTTCCT and R-TAGCAAACGGTTTCGAGGTT. This initial sequence was amplified again in order to

obtain a smaller fragment (640bp) of the gene using internal primers with T7 promoters: F-TAATACGACTCACTATAGGGACGACGACGGTGTGTACAAG and R-TAATACGACTCACTATAGGGATACTCGGCCGAATCGAAC. For HPX7, the same methodology was used to first amplify a 920 bp fragment of the gene using the primers: F-AACTGTCCTCCACGCTGTTT and R-CACCCTGGGCAAGTATTCAC; and then amplify a 520 bp sequence using the primers: F-TAATACGACTCACTATAGGGATCAAAACGAAGGCCAAATG and R-TAATACGACTCACTATAGGGCCAGCGAGATCAACTGGTTC. The PCR product was used as a template to synthesize dsRNA *in vitro* using a MEGAscript RNAi kit (Ambion, Austin, TX). dsRNA was loaded to RNA purification column supplied by the kit, eluted with water and concentrated to 3 μg/μl using a Microcon YM-100 filter (Millipore, Billerica, MA).

Cloning HPX2 into T7/CT-TOPO Vector. A 1965 bp fragment was generated using the following HPX2 specific primers: F-ATGGAAAGGCTCGCGACGCTCGATCG and R-AACGAGTCGCGCAAGATCGAGCGACG. The PCR product was cloned into T7/CT-TOPO TA vector in TOP10 F' cells (Invitrogen, San Diego, CA). Single colonies were screened for insertion of the construct in proper orientation by RT-PCR. Plasmids from positive colonies were purified by the GeneElute Plasmid Preparation Miniprep kit (Sigma Aldrich, St. Louis, MO) and sequenced to confirm that the cDNA was in frame.

Expression and Isolation of Recombinant HPX2. A construct in the correct translation frame was used to transform BL21 (DE3) pLysS cells and express recombinant HPX2 protein. Individual colonies were incubated with LB media containing ampicillin (100µg/ml) and chloramphenicol (34µg/ml) overnight. In the morning the cultures were diluted 1:20 and grown at 37°C until they reached an OD_{600} of O.5. IPTG was added to a final concentration of 1mM, and the cells were collected 3 h later by centrifugation and the pellets kept at 4°C. The protein was insoluble and present in inclusion bodies. Inclusion bodies were purified using a modified version of the Bio-Rad Profinity Ni-NTA column purification protocol. Briefly, the cells were resuspended and sonicated twice in PBS. After each sonication, the inclusion bodies were centrifuged and washed with PBT (PBS + 0.5% Triton X-100) to remove cell membranes/lipids, and again with 1X PBS to remove any residual detergent. The inclusion bodies were solubilized using denaturing conditions (20 mM Sodium Phosphate, 8 M Urea, 500 mM NaCl, pH 7.8) and incubated with the Profinity Ni-NTA column (Bio-Rad, Hercules, CA) overnight at 4°C, with circular rocking. The resin was placed in a column and the protein was purified under denaturing conditions using an imidazole step gradient (washed with 10 and 25 mM imidazole and eluted with 50, 100 and 250 mM imidazole). The fractions were dialyzed against Dulbeco's PBS (Sigma Aldrich, St. Louis, MO), subjected to SDS-PAGE. A mixture of the fractions containing pure protein and bands of the induced protein cut from Coomassie blue-stained acrylamide gels of fractions that contained other protein bands was used for the immunizations. This antigen mixture was sent to Primm Biotech (Cambridge, MA) to immunize rabbits and generate polyclonal antibodies against the HPX2 recombinant protein. The specificity of the anti-HPX2 antibody was determined by

western blot analysis and dsRNA silencing. Unspecific antibodies to *E. coli* were removed by pre-incubating the anti-HPX2 serum with an insoluble *E. coli* acetone powder as previously described (*4*).

Gene Silencing. Female mosquitoes were injected with 69 nl of a 3-μg/μl solution of dsRNA of each gene 3 days post-emergence. Control mosquitoes were injected with dsLacZ. Between 36-48 h after injection, the mosquitoes either were fed on an uninfected (control) or a *Plasmodium*-infected mouse. All gene silencing phenotypes were confirmed in three independent experiments.

Peroxidase activity assay. Peroxidase assays using TMB (3,3′,5,5′-Tetramethylbenzidine**)** as a substrate were performed following the manufacturer's instructions (Kirkegaard & Perry Laboratories, Gaithersburg, MD). Briefly, midguts were dissected in PBS 24h post-feeding and the blood bolus content was removed. The midguts were fixed for 1 h at room temperature in 4% paraformaldehyde and 1% glutaraldehyde. Midguts were washed, homogenized and incubated for 5 min in PBS containing amino-triazole (10 mg/mL) to inhibit catalase activity. The fixed midgut fragments were incubated for 10 min in the dark at room temperature with TMB/H_2O_2 solution. The tissue was removed by centrifugation and the reaction stopped by adding 3N HCl to the supernatant. The glutaraldehyde-resistant peroxidase activity was determined by measuring the relative concentration of the end products in a spectrofluorometer plate reader (VersaMax; Molecular Devices, Sunnyvale, CA,) operating at 450 nm. For the silencing experiments, peroxidase activity was measured in midguts of control mosquitoes fed on a healthy

mouse and fed on a *Plasmodium*-infected mouse. The *Plasmodium*-induced peroxidase activity was defined as the enzymatic activity in infected midguts minus the activity in blood fed controls.

Immuno-fluorescence staining and confocal microscopy. Midguts from mosquitoes fed on uninfected and infected mice were dissected in PBS and fixed for 1 min in 4% paraformaldehyde. Midguts were opened longitudinally in PBS to remove the blood bolus and fixed for 1 h in 4% paraformaldehyde as previously described (*5*). Midgut tissues were permeabilized and blocked by incubation for 1 h in PBT solution (1% BSA, 0.1% Triton X-100 in PBS) and incubated overnight with the primary antibodies (1:300) diluted in PBT at 4 °C. Samples were washed 2-3 times with PBT and incubated for 2 h at room temperature with Alexa 555- or Alexa 488-conjugated secondary antibodies (Molecular Probes, Eugene, OR) diluted (1:500) in PBT. Tissues were washed again with PBT and mounted in Vectashield™ (Vector Laboratories, Inc., Burlingame, CT) containing 4′,6-diamidino-2-phenylindole to counter stain the nuclei. Images were obtained using a SP2 confocal microscope (Leica). The following antibodies were used: anti-HPX2 rabbit polyclonal antibody (produced as described above), anti-nitrotyrosine mouse monoclonal antibody (Calbiochem, San Diego, CA), anti-NOX5 rabbit monoclonal antibody (Sigma Aldrich, St. Louis, MO) and anti-TEP1 rabbit antibody. Anti-Pbs21 mouse monoclonal antibodies were kindly provided by Dr. Robert Sinden and anti-TEP1 rabbit polyclonal antibodies by Dr. Michael Povelones and Dr. George Christophides. For TEP1 staining experiments a PBT solution containing 1% bovine serume albumin (BSA), 0.05% Triton X-100 in PBS was used.

TEP1 staining and ookinete scoring. The immunofluorescence and confocal microscopy were performed using the same procedures described above for HPX2 and NOX5 staining. Pbs21 and TEP1 positive ookinetes were counted in control dsLacZ midguts or dsHPX2-silenced mosquitoes 28-30 hours post-infection with *P. berghei*. The total number of Pbs21-positive ookinetes in the LacZ contols ranged from 75 to 357 and from 85 to 792 in dsHPX2-silenced mosquitoes. The results of two independent experiments were merged and plotted as the percentage of TEP1-positive parasites on individual midguts.

Western blot. Homogenates from *A. gambiae* midguts were subjected to SDS-PAGE and/or native gel electrophoresis and blotted into nitrocellulose membranes (Invitrogen, Carlsbad, CA). The protein content of each sample was determined using BCA™ Protein Assay Kit (Thermo Scientific, Rockford, IL). The same amount of protein (30 μg/lane) from midgut homogenate samples for each experimental condition was applied in the gel. The membranes were incubated with 1 mM levamisole solution for 30 min to inhibit any internal phosphatase activity and blocked with 5% non-fat milk in TBS (10 mM Tris-HCl, 150 mM NaCl, 0.05% Tween 20, pH7.6) for 1 h at room temperature. Subsequently, the membranes were incubated over night at 4°C with the primary (anti-HPX2 or anti-NOX5) antibody (1:1,000) in blocking solution. Membranes were then washed in TBS-Tween solution and incubated with secondary alkaline phosphatase-conjugated antibody (1:5,000) (Calbiochem, San Diego, CA) diluted in blocking solution for 2 h at room temperature. Membranes were washed in TBS-Tween and TBS and developed using

Western Blue substrate for alkaline phosphatase (Promega Corporation, Madison, WI) following the manufacturer's instructions.

<u>In vitro</u> *nitration assay*. Midguts were dissected 24 h post-infection, cleaned and fixed with 4% paraformaldehyde and 0.5% glutaraldehyde for 1 h at room temperature and washed with PBS to remove the fixative. The fixed midguts were incubated with PBS buffer containing 100 µg of BSA (bovine serum albumin), 1 mM H_2O_2 and 1 mM $NaNO_2$ for 30 min at 37 °C. The midgut tissue was removed by centrifugation and the protein content of the supernatant was estimated using BCA™ Protein Assay Kit (Thermo Scientific, Rockford, IL). 3 µg of BSA in the supernatant was subjected to SDS-PAGE and western blot as described previously. Briefly, the membranes were blocked with 5% non-fat milk in TBS-Tween solution for 1 h, incubated with an anti-nitrotyrosine mouse primary antibody (1:3,000) at 4°C over night, and with a secondary alkaline phosphatase-conjugated antibody (1:5,000) for 2 h at room temperature. The membrane was developed using the Western Blue substrate for alkaline phosphatase. In parallel, commercial horseradish peroxidase (Invitrogen, Carlsbad, CA) at a final concentration of 0.3 unit/ml was used as positive control. For the negative controls, samples were pre-incubated with NaN_3 (5 mg/mL). The same amount of protein from each sample was subjected to SDS-PAGE and stained with Coomassie Blue.

<u>In vivo</u> *nitration assay*. Five midguts were dissected and fixed as described above and washed with PBS. The midguts were gently triturated and incubated in amino triazole (10 mg/mL) for 10 min. The suspension was centrifuged and the supernatant was discarded.

The pellet was incubated with levamisole 2 mM for 1 h and blocked with PBT for 1h. The washing step was repeated and the pellet was ressuspended in 50 µL of PBT. One midgut equivalent (10 µL of the 50 µL suspension) was incubated overnight with anti-nitrotyrosine mouse primary antibody diluted in PBT (1:3,000) at 4°C. Samples were washed with PBT and incubated with a secondary alkaline phosphatase-conjugated antibody (1:5,000) diluted in PBT for 2 h at room temperature. Midgut nitration was determined based on the alkaline phosphatase activity in the tissue, which is proportional to the amount of primary and secondary antibodies that bound. The relative enzymatic activity was determined using the alkaline phosphatase yellow soluble substrate (ρNPP–ρ-nitrophenylphosphate, Sigma Aldrich, St. Louis, MO) and a spectrofluorometer plate reader at 405 nm. For each experiment, the tissue homogenate from each treatment group was analyzed in quadruplicate and the fifth sample was left without primary antibody to determine the background level. The relative nitration for each experimental treatment was confirmed in at least two independent experiments. (See Tables S1 and S2).

In vivo TEP1 binding assay. The assay was performed using the same procedure described above for the *in vivo* nitration assay, but ten midguts were used for each experimental condition and anti-TEP1 rabbit serum was used as primary antibody diluted in PBT (1:1,000). *In vivo* TEP1 binding was measured in midguts of control mosquitoes fed on a healthy mouse or on a *Plasmodium*-infected mouse. The *Plasmodium*-induced TEP1 binding was defined as the difference in enzymatic activity between infected and uninfected blood-fed midguts. TEP1 binding for each experimental condition was confirmed in two independent experiments. (See Table S3).

Statistical analysis. Distributions of oocyst numbers were compared using the Kolmogorov-Smirnov test. Comparisons of mRNA expression, peroxidase activity and protein nitration levels were performed using Student's *t*-test (Prism 5.01; GraphPad Software, Inc., San Diego, CA).

References and notes

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3. K. Livak *et al*., *Methods* **25**, 4 (2001).
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6. Author Contributions: G.A.O. designed and carried out most experiments and contributed to the writing of the manuscript. J.L carried out the experiments related to HPX2 antibody production. C.B-M. contributed to the experimental design, the interpretation of results and wrote the final draft of the manuscript.

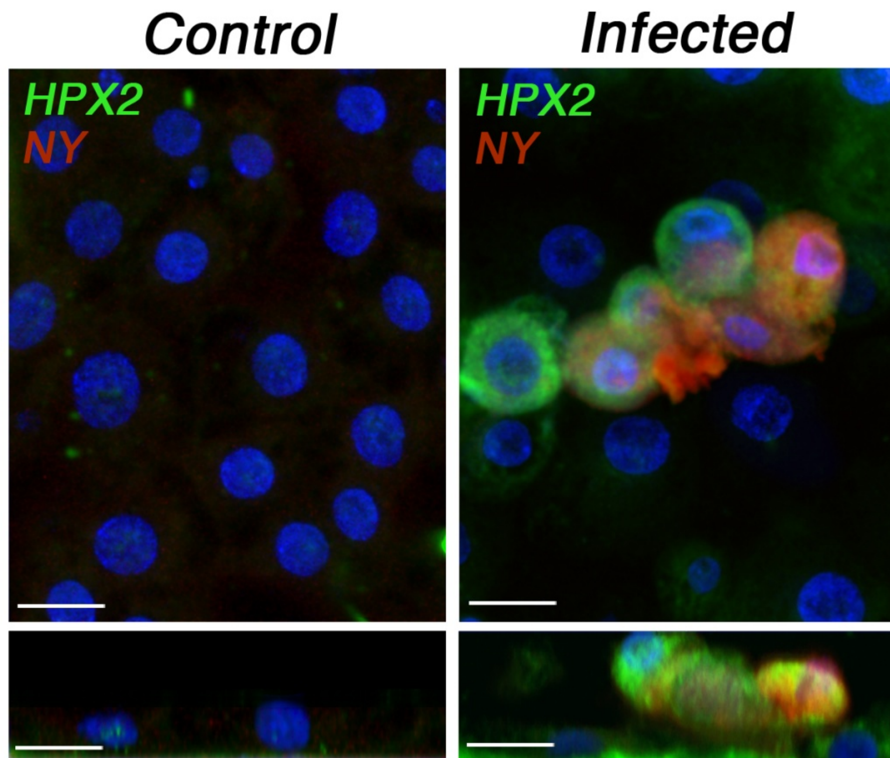

Figure S1. Immunofluorescence staining of *A. gambiae* midguts 24 hours post-feeding on a healthy (control) or a *P. berghei*-infected mouse for nitrotyrosine (red) and HPX2 (green). DAPI nuclear staining in blue (bar = 20 μm). Side views of the same images are shown in the lower panels.

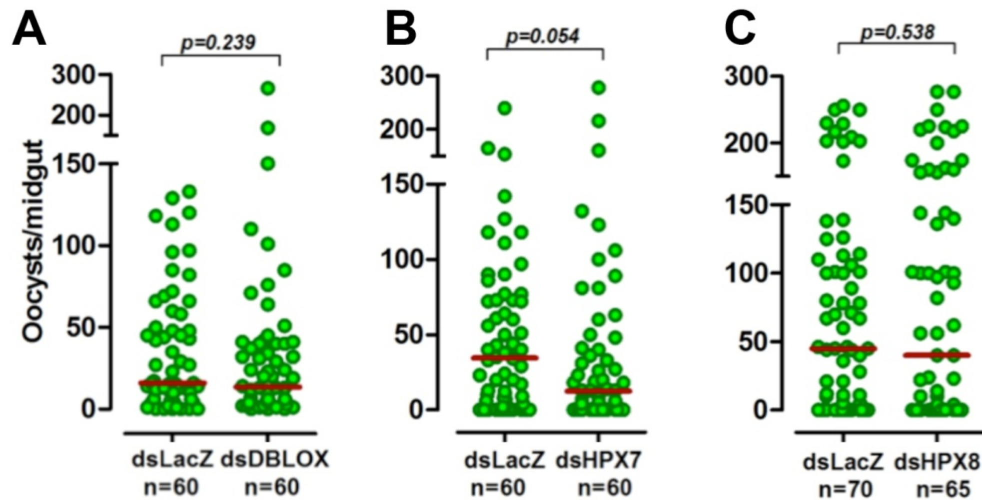

Figure S2. Effect of silencing Double peroxidase (DBLOX) (**A**), HPX7 (**B**) and HPX8 (**C**) on *P. berghei* infection 7 days post-feeding. Each circle represents the number of oocysts in an individual midgut, and the horizontal lines indicate the medians. Distributions were compared using the Kolmogorov-Smirnov test.

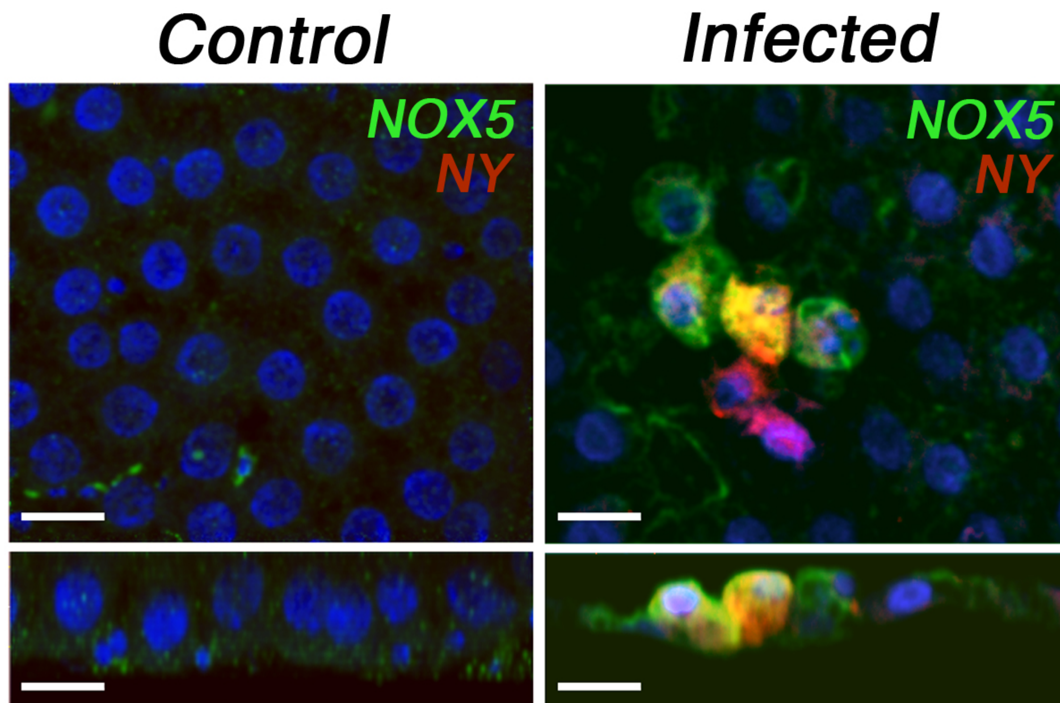

Figure S3. Immunofluorescence staining of *A. gambiae* midguts 24 hours post-feeding on a healthy (control) or a *P. berghei*-infected mouse for nitrotyrosine (red) and NOX5 (green). DAPI nuclear staining in blue (bar = 20 μm). Side views of the same images are shown in the lower panels.

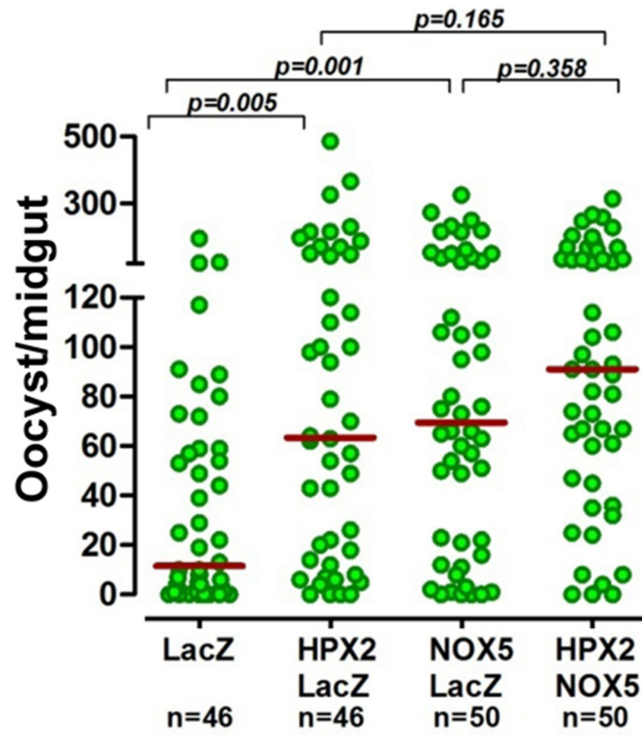

Fig. S4. Effect of double silencing NOX5 and HPX2 on *P. berghei* infection 7 days post-feeding. Each circle represents the number of parasites in an individual midgut and the medians are indicated by the horizontal lines.

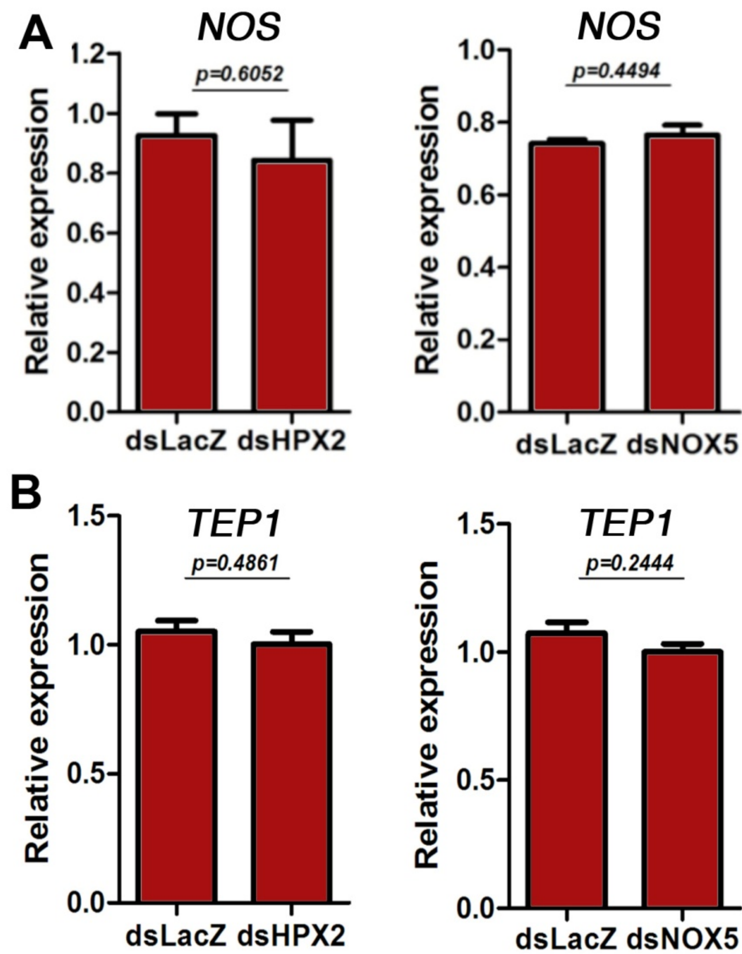

Fig. S5. Effect of HPX2 or NOX5 silencing on (**A**) NOS mRNA levels 24 h post-infection with *P. berghei* and (**B**) TEP1 mRNA levels in sugar-fed mosquitoes 2 days after dsRNA injection (mean ± SEM).

Table S1: Effect of *Plasmodium* infection and HPX2 or NOX5 silencing on *in vivo* midgut protein nitration.

Control versus Infected

	Control* (Mean±SEM)	Infected* (Mean±SEM)	P value
Experiment 1	0.186 ± 0.016	0.332 ± 0.035	0.0003
Experiment 2	0.326 ± 0.110	0.873 ± 0.315	0.0469
Experiment 3	0.223 ± 0.065	0.743 ± 0.205	0.0138

HPX2 silencing

	dsLacZ* (Mean±SEM)	dsHPX2* (Mean±SEM)	P value
Experiment 1	0.420 ± 0.068	0.250 ± 0.023	0.0150
Experiment 2	0.627 ± 0.183	0.287 ± 0.043	0.0038

NOX5 silencing

	dsLacZ* (Mean±SEM)	dsNOX5* (Mean±SEM)	P value
Experiment 1	0.529 ± 0.026	0.266 ± 0.093	0.0095
Experiment 2	0.727 ± 0.193	0.155 ± 0.023	0.0071

* Absorbance units per midgut corresponded to ρ-NPP substrate consumption by alkaline phosphatase conjugated to the secondary antibody bound to the anti-nitrotyrosine primary antibody.

Table S2: Effect of silencing IMPer or co-silencing IMPer and NOX5, HPX2 or TEP1 on *in vivo* protein nitration.

Silenced Genes	Exp 1* (Mean±SEM)	Exp 2* (Mean±SEM)
dsLacZ	0.217 ± 0.052 (a)	0.204 ± 0.019 (a)
dsIMPer + dsLacZ	0.370 ± 0.017 (b)	0.328 ± 0.023 (b)
dsIMPer + dsHPX2	0.201 ± 0.023 (a)	0.206 ± 0.039 (a)
dsIMPer + dsNOX5	0.207 ± 0.058 (a)	0.199 ± 0.048 (a)
dsIMPer + dsTEP1	0.319 ± 0.027 (b)	0.291 ± 0.050 (b)

* Significant differences between treatments in a given experiment are indicated by different letters in parentheses ($p < 0.01$). Absorbance units per midgut corresponded to ρ-NPP substrate consumption by alkaline phosphatase conjugated to the secondary antibody bound to the anti-nitrotyrosine primary antibody.

Table S3: Effect of HPX2 silencing on *in vivo* midgut TEP1 binding.

	Infected midguts	Control midguts	*Plasmodium*-induced binding*
Experiment 1			
dsLacZ (Mean±SEM)	0.539 ± 0.058	0.113 ± 0.057	0.426 ± 0.057
dsHPX2 (Mean±SEM)	0.308 ± 0.068	0.124 ± 0.029	0.186 ± 0.068
P value	0.0002	0.6200	0.0001
Experiment 2			
dsLacZ (Mean±SEM)	0.562 ± 0.067	0.176 ± 0.114	0.391 ± 0.067
dsHPX2 (Mean±SEM)	0.255 ± 0.050	0.145 ± 0.040	0.110 ± 0.050
P value	0.0001	0.5032	0.0001

* Defined as the difference in TEP1 binding between *Plasmodium*-infected and control blood-fed uninfected midguts, 28-30 hours post-feeding. (n=10 for each experimental condition). Absorbance units per midgut corresponded to ρ-NPP substrate consumption by alkaline phosphatase conjugated to the secondary antibody bound to the anti-TEP1 primary antibody.