Reactive Oxygen Species Modulate *Anopheles gambiae* Immunity against Bacteria and *Plasmodium*

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The involvement of reactive oxygen species (ROS) in mosquito immunity against bacteria and *Plasmodium* was investigated in the malaria vector *Anopheles gambiae*. Strains of *An. gambiae* with higher systemic levels of ROS survive a bacterial challenge better, whereas reduction of ROS by dietary administration of antioxidants significantly decreases survival, indicating that ROS are required to mount effective antibacterial responses. Expression of several ROS detoxification enzymes increases in the midgut and fat body after a blood meal. Furthermore, expression of several of these enzymes increases after a blood meal, indicating that ROS are required to mount effective antibacterial responses. Expression of several ROS detoxification enzymes increases in the midgut and fat body after a blood meal. Further reduction of catalase expression by double-stranded RNA-mediated gene silencing promoted parasite clearance by a lytic mechanism and was expected to lead to higher local levels of hydrogen peroxide. Reduction of catalase expression by double-stranded RNA-mediated gene silencing promoted parasite clearance by a lytic mechanism and reduced infection significantly. High mosquito mortality is often observed after *P. berghei* infection. Death appears to result in part from excess production of ROS, as mortality can be decreased by oral administration of uric acid, a strong antioxidant. We conclude that ROS modulate *An. gambiae* immunity and that the mosquito response to *P. berghei* involves a local reduction of detoxification of hydrogen peroxide in the midgut that contributes to limit *Plasmodium* infection through a lytic mechanism.

The *Anopheles gambiae* mosquito is the most important vector of human malaria in Africa, a disease caused by *Plasmodium* parasites that affects more than 500 million people every year and causes more than one million deaths (1). The mosquito is infected upon ingestion of *Plasmodium* gametocytes that transform to gametes. Their fertilization takes place in the lumen of the midgut and gives rise to motile ookinetes that traverse midgut epithelial cells and develop into oocysts. Oocyst formation is a bottleneck in the parasite life cycle, as most parasites die either during midgut invasion or as they come in contact with components of the mosquito immune system present in the hemolymph (2, 3). Several mosquito factors that inhibit or promote *Plasmodium* infection have been recently characterized (4–6) including reactive oxygen species (ROS)² (7, 8).

ROS are generated by byproducts of mitochondrial respiration or as part of the immune response to pathogens (9, 10). In vertebrates, activated macrophages undergo a respiratory burst in which superoxide anion (O₂·⁻) is produced by a NADPH oxidase and transformed to H₂O₂ by superoxide dismutase (SOD). Subsequently, myeloperoxidase uses H₂O₂ as a substrate and produces hypochlorous acid (HOClO), a highly bactericidal compound. Insect hemocytes (functionally similar to vertebrate neutrophils and macrophages) are also capable of generating a respiratory burst (11–15); however, the precise mechanism by which ROS are generated in these cells is not well understood.

ROS are potentially toxic to the host, so it is important that they be generated transiently and kept well localized. Host cells are protected from oxidative damage by enzymes that detoxify ROS such as SOD that detoxifies O₂·⁻ and catalase, glutathione peroxidase (Gpx), and thioredoxin peroxidase that detoxify H₂O₂.

We have previously shown that differences in systemic levels of ROS in the mosquito influence melanotic encapsulation responses against *Plasmodium* (7). A mosquito strain genetically selected to be highly susceptible (S) to *Plasmodium* has lower levels of hemolymph H₂O₂ than the unsselected G3 strain. Furthermore, a strain that has been selected to melanize *Plasmodium* and is refractory (R) to infection has significantly higher levels (2–3 times) than the parental G3 and the S strains (7). Dietary supplementation of R females with antioxidants decreases hemolymph levels of H₂O₂ and inhibits parasite melanization (7). In this study we explore how differences in ROS levels affect mosquito survival after a bacterial challenge.

Ookinetes develop and midgut invasion take place in the complex environment of a blood meal undergoing digestion.

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‡ The abbreviations used are: ROS, reactive oxygen species; Cat, catalase; Gpx, glutathione peroxidase; PF, post-feeding; PI, post-infection; R, refractory to *Plasmodium*; S, susceptible to *Plasmodium*; SOD, superoxide dismutase; PBS, phosphate-buffered saline; ds-, double-stranded.
Blood-meal digestion and absorption by the midgut provides the nutrients required for oogenesis (16). The fat body uses amino acids obtained from the blood meal to synthesize vitellogenin, a major storage protein that is transported into the developing oocyte. These metabolic changes are expected to increase the rate of ROS generated, and indeed, H$_2$O$_2$ levels in hemolymph increase, and midgut expression of ROS detoxifying enzymes is induced 24 h post-feeding (PF) (7, 17–21). In this study we investigate how ingestion of a Plasmodium-infected meal affects ROS production and detoxification. Our results indicate that Plasmodium infection exacerbates systemic oxidative stress after a blood meal and can result in substantial mosquito mortality. Paradoxically, at the local level midgut epithelial cells suppress expression of catalase, a major enzyme involved in H$_2$O$_2$ detoxification, in response to ookinete invasion. Reduced catalase activity is expected to increase local levels of H$_2$O$_2$. Further reducing catalase expression by dsRNA-mediated silencing decreases ookinete survival in G3 females through a lytic mechanism that does not involve melanization. We propose that, besides the well characterized strategy of increasing ROS production in response to pathogens, local reductions in ROS detoxification enzymes can also contribute to increased local ROS concentration as immune effector molecules.

**EXPERIMENTAL PROCEDURES**

**An. gambiae Strains and Malaria Parasites—**Three An. gambiae strains were used: the refractory L3–5 (R) (22, 23), the unselected susceptible G3, and the highly susceptible 4A r/r (S) (23). Mosquitoes were reared at 27 °C and 80% humidity on a 12-h light-dark cycle under standard laboratory conditions. The two Plasmodium berghei strains used, ANKA 234 and CTRP$^{-}$ (24), were maintained by serial passage in 3–4-week-old female BALB/c mice or as frozen stocks.

**Bacterial Challenge of Mosquitoes—**Three-day-old adult females were injected with a mixture of Escherichia coli and Micrococcus luteus. Bacterial cultures were grown to an optical density of 0.5 (600 nm) in LB broth, and 200 μl from each culture were mixed and centrifuged for 5 min at maximum speed. The supernatant was discarded, and the pellet was washed twice with PBS and resuspended in 125 μl of PBS. Mosquito survival was monitored daily for 8 days after intrathoracic injection of 0.138 μl of either PBS or bacterial suspension using a microinjection system and a micromanipulator (Nanoject II microinjection system; Drummond). Three groups of 30 mosquitoes were used for each treatment, and survival curves were kept at 27 °C, and 6 h after treatment, groups of 10 were washed twice with PBS and resuspended in 125 μl of PBS. Mosquito survival was monitored daily for 8 days after intrathoracic injection of 0.138 μl of either PBS or bacterial suspension using a microinjection system and a micromanipulator (Nanoject II microinjection system; Drummond). Three groups of 30 mosquitoes were used for each treatment, and survival curves were kept at 27 °C, and 6 h after treatment, groups of 10 were collected.

**H$_2$O$_2$ Injection of Mosquitoes—**Sugar-fed 4-day-old An. gambiae G3 females were injected as above with 18 nmol of H$_2$O$_2$ (in 69 nl); controls received H$_2$O injection. Mosquitoes were kept at 27 °C, and 6 h after treatment, groups of 10 were collected.

**P. berghei Infection of Mosquitoes—**Female mosquitoes (5 days old) were infected with P. berghei by feeding on anesthetized infected BALB/c mice. The infectivity of the mice was established by determining the parasitemia and by performing an exflagellation assay as described previously (25). In all the studies mice having parasitemias between 4 and 8% and 2–3 exflagellations/field under 400× magnification were used to infect mosquitoes. Blood-fed infected and control mosquitoes were kept at 21 °C and 80% humidity. P. berghei midgut infection was quantified 24 and 48 h post-infection (PI) by immunofluorescence using mouse anti Pbs21 antibody as previously described (26) or by light microscopy 11 days PI with mercurochrome staining (0.05% in water).

**Quantitation of Gene Expression—**Mosquito midguts and abdominal walls containing fat body were dissected on ice-cold Ashburner-PBS, placed in ice-cold RNAlater (Ambion), and stored at −70 °C until mRNA extraction. Poly(A) mRNA was isolated from groups of 10 midguts or 10 abdominal walls using Oligotex-dT beads (Qiagen) following the manufacturer's instructions. First-strand cDNA was synthesized using random hexamers and Superscript II reverse transcriptase (Invitrogen). Gene expression was assessed by SYBR green quantitative real-time PCR (DyNamo HS; New England Biolabs) in a Chromo4 system (Bio-Rad). PCR involved an initial denaturation at 95 °C for 15 min, 44 cycles of 10 s at 94 °C, 20 s at 58 °C, and 30 s 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. Quantitative real-time PCR measurements were made in duplicate. Relative quantitation results were normalized with An. gambiae ribosomal protein 57 as internal standard and analyzed by the 2$^{-ΔΔCt}$ method (27). The annotated An. gambiae genome contains four genes coding for SOD (mitochondrial Mn-SOD (ENSANGP00000020588), CuZn-SOD2 (ENSANGP00000015824), and two CuZn-SOD3 (ENSANGG00000013675) splice forms, CuZn-SOD3A (ENSANGP-00000016164) and CuZn-SOD3B (ENSANGP-00000032094)) and catalase (ENSANGP-00000021298) and Gpx (ENSANGP00000013972).

Primers used and amplified product length (bp) were the following (5’-3’): S7 (149 bp) forward (F)-AGAACCAGCA-GACCACCATC, reverse (R)-GCTGCAAATTTGGAATC- TTC; catalase (276 bp) F-GGATAAGGTGACGGCTACGA, R-GGAAAGCTACGGTACGACA, Gpx (98 bp) F-CAGGGTGCTGCTAATCCTGTA, R-CCTCTATCCGCTA TGC; Mn-SOD (136 bp) F-GGAGCACTCTTTTCCACTGC, R-GGTTGGAAGCCTCATTG; CuZn-SOD2 (171 bp) F-CACATTTCACGAGAAGGTA, R-CTGTCGCCATGAT-GAGCTT; CuZnSOD3A (310 bp) F-GGTAAGACTGCGCT- CTAGC, R-GCTCAGTCTGATCCCATGAT; CuZn-SOD3B (133 bp) F-TGCTGTAACATCGTGGCCTA, R-GCCCTCTA- ATCATCTTCTA.

**Hemolymph H$_2$O$_2$ Quantitation—**Mosquito hemolymph was collected by flushing the hemocoel. The cuticle from the last abdominal segment was torn open, 8 μl of PBS containing 2 mg/ml catalase inhibitor 3-amino-triazole (AT) were injected into the thorax, and 5 μl of flushed hemolymph was collected from the aperture in the posterior end. Hemolymph from five mosquitoes was pooled together and analyzed for H$_2$O$_2$ using the Amplex red assay fluorimetric test (Molecular Probes) following the manufacturer’s instructions.
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Catalase Activity Assay—Midguts and abdominal walls of adult An. gambiae G3 females were dissected in PBS 24 h PF, and the blood meal was carefully removed. Groups of 10 midguts or 10 abdominal walls were flash-frozen in liquid nitrogen and stored at −70 °C until assayed. Catalase activity was measured as AT-inhibitable H$_2$O$_2$ degradation with a spectrophotometric assay (Sigma, CAT100). Briefly, samples were homogenized in 50 mM potassium phosphate buffer, pH 7.0, containing 0.1% Triton X-100 and protease inhibitors (Complete, Roche Applied Science). Homogenate was centrifuged 2 min at 16 × g, and supernatant was separated for the assay. Half of each supernatant received a final concentration of 60 mM AT. The catalase assay was initiated by mixing 15 μl of supernatant with 50 mM H$_2$O$_2$ in a 100-μl total volume. The reaction was carried at room temperature for 5 min and stopped with 900 μl of 15 mM sodium azide. H$_2$O$_2$ was measured colorimetrically with 10 μl of reaction mix with 1 ml of 150 mM potassium phosphate buffer, pH 7, containing 2 mM 3,5-dichloro-2-hydroxybenzenesulfonic acid and horseradish peroxidase, and after a 15-min room temperature incubation, absorbance was read at 520 nm. Protein quantitation of midgut and abdominal wall homogenates was done with the micro BCA protein assay (Pierce).

dsRNA-mediated Catalase Knockdown—A 496-bp DNA fragment of catalase was amplified by PCR from a full-length catalase cDNA using the primers Cat-forward (5′-GCAACAA-TACGCGCCTCTTTCA-3′) and Cat-reverse (5′-GGAATT-GACGCCAATTGC-3′) and cloned into the pCR II-TOPO vector (Invitrogen). T7 polymerase promoter sites were incorporated onto both ends of this fragment by amplification of the in vitro using the MEGAScript RNA-mediated interference kit (Ambion). dsRNA was further purified with water and concentrated to 3 μg/μl using a Microcon YM-100 filter (Millipore). Each female mosquito was injected with 69 nl (207 ng of dsRNA) at 1–2 days post-emergence using a Nanoject II microinjection system (Drummond). A 218-bp fragment of the LacZ gene was amplified using the primers LacZ-forward (5′-GAGTCGAGAGCTAGGGAACG-3′) and LacZ-reverse (5′-ATTCCGCTACACATTTCCA-3′), cloned into the pCR II-TOPO vector, and used to generate a 492-bp dsLacZ RNA using primers M13F (5′-GTAACACGACGCCATGTT-3′) and XhoI-T7-M13R (5′-GAGTCAGTGAGCGAGGAAGC-3′) and reverse 5′-CTCGAGTAATACGACTCATAAGGCGGTAGTGATGATCTCGGC-3′. The PCR product was used as template to synthesize double-stranded catalase RNA using the primers Cat-forward (5′-TACGCCCATCTTCTTCA-3′) and reverse 5′-CTCGAGTAATACGACTCATAAGGCGGTAGTGATGATCTCGGC-3′ and reverse 5′-CTCGAGTAATACGACTCATAAGGCGGTAGTGATGATCTCGGC-3′. The PCR product was used as template to synthesize double-stranded catalase RNA using the primers Cat-forward (5′-TACGCCCATCTTCTTCA-3′) and reverse 5′-CTCGAGTAATACGACTCATAAGGCGGTAGTGATGATCTCGGC-3′ and reverse 5′-CTCGAGTAATACGACTCATAAGGCGGTAGTGATGATCTCGGC-3′. The PCR product was used as template to synthesize double-stranded catalase RNA using the primers Cat-forward (5′-TACGCCCATCTTCTTCA-3′) and reverse 5′-CTCGAGTAATACGACTCATAAGGCGGTAGTGATGATCTCGGC-3′ and reverse 5′-CTCGAGTAATACGACTCATAAGGCGGTAGTGATGATCTCGGC-3′. The PCR product was used as template to synthesize double-stranded catalase RNA using the primers Cat-forward (5′-TACGCCCATCTTCTTCA-3′) and reverse 5′-CTCGAGTAATACGACTCATAAGGCGGTAGTGATGATCTCGGC-3′ and reverse 5′-CTCGAGTAATACGACTCATAAGGCGGTAGTGATGATCTCGGC-3′. The PCR product was used as template to synthesize double-stranded catalase RNA using the primers Cat-forward (5′-TACGCCCATCTTCTTCA-3′) and reverse 5′-CTCGAGTAATACGACTCATAAGGCGGTAGTGATGATCTCGGC-3′ and reverse 5′-CTCGAGTAATACGACTCATAAGGCGGTAGTGATGATCTCGGC-3′. The PCR product was used as template to synthesize double-stranded catalase RNA using the primers Cat-forward (5′-TACGCCCATCTTCTTCA-3′) and reverse 5′-CTCGAGTAATACGACTCATAAGGCGGTAGTGATGATCTCGGC-3′ and reverse 5′-CTCGAGTAATACGACTCATAAGGCGGTAGTGATGATCTCGGC-3′. The PCR product was used as template to synthesize double-stranded catalase RNA using the primers Cat-forward (5′-TACGCCCATCTTCTTCA-3′) and reverse 5′-CTCGAGTAATACGACTCATAAGGCGGTAGTGATGATCTCGGC-3′ and reverse 5′-CTCGAGTAATACGACTCATAAGGCGGTAGTGATGATCTCGGC-3′.

Antioxidant Supplementation and Mosquito Survival after P.berghei Infection—Mosquitoes received 1 mg/ml uric acid in 10% sucrose beginning 3 days post-emergence. Mosquitoes were blood-fed a control or infected mouse 5 days post-emergence. Three groups of 30 mosquitoes were used for each treatment, and survival curves were analyzed by the log rank survival test (SigmaStat).

RESULTS

ROS Levels Affect the Mosquito’s Immune Response against Bacteria—We took advantage of the genetic differences in systemic H$_2$O$_2$ levels between mosquito strains (R > G3 > S) (7) to investigate whether ROS have a general effect in the mosquito’s immune competence. Groups of 3-day-old females from each strain were challenged by injecting them with a fixed bacterial dose, and their survival followed over time. Susceptibility to bacterial infection of the three An. gambiae strains correlated directly with their systemic levels of H$_2$O$_2$ (Fig. 1A). The survival of R females, which have the highest H$_2$O$_2$ levels, was significantly higher. Conversely, survival of the S strain, which has the lowest level of H$_2$O$_2$, was significantly reduced (Fig. 1A). Furthermore, reduction of ROS in G3 mosquitoes by dietary supplementation with vitamin C or uric acid (strong antioxidants) dramatically decreased survival after bacterial challenge (Fig. 1, B and C). Vitamin C feeding also decreased the survival of mosquitoes injected with sterile PBS, but the effect was relatively mild compared with that seen in the bacteria-challenged group (Fig. 1B). Uric acid feeding did not affect mortality of uninjected mosquitoes compared with control uninjected mosquitoes (Fig. 1C). Together, these data indicate that ROS levels greatly influence the mosquito’s capacity to mount an effective immune response against bacteria.

Blood-fed Females Respond to Oxidative Stress by Increasing Systemic Expression of ROS Detoxification Enzymes—Cells protect themselves from ROS damage by inducing the expression of enzymes involved in detoxification such as SOD (superoxide anion detoxification), catalase, and Gpx (H$_2$O$_2$ detoxification). We have previously shown that the rate and magnitude of midgut induction of catalase and CuZnSOD2 expression after a blood meal is directly correlated with the ROS levels in a given An. gambiae strain. For example, induction of these two enzymes is faster and higher in the R strain, which has the highest ROS levels (7). To establish whether mRNA levels of ROS detoxification enzymes could be used as indicators of oxidative stress in different tissues, the response of An. gambiae G3 females to H$_2$O$_2$ was directly tested. Injection of H$_2$O$_2$ into adult females induces a 3- and 2-fold increase in catalase and Gpx mRNA levels, respectively (Fig. 2A). Gpx mRNA levels, respectively (Fig. 2A).
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SODs (CuZnSOD2-3) in the midgut and fat body after blood feeding (Fig. 2B) as putative indicators of oxidative stress. Blood-fed G3 females were kept at 27 °C. Catalase, CuZnSOD2, and CuZnSOD3A mRNAs are induced in both the midgut and fat body. CuZnSOD3B, however, is only induced in the midgut, whereas Gpx and MnSOD induction is limited to the fat body (Fig. 2B). In general, genes are induced earlier in the midgut where inductions are already detected by 12 h PF, whereas expression in the fat body tends to peak later (24–36 h PF) (Fig. 2B).

Plasmodium Infection Exacerbates the Oxidative Stress Induced by Blood Feeding—We tested the hypothesis that Plasmodium infection would increase systemic oxidative stress in the mosquito. This was done by comparing the mRNA expression of ROS detoxification enzymes in G3 females fed on P. berghei-infected or uninfected mice (Fig. 3, A and B). Blood-fed mosquitoes were kept at 21 °C, a temperature permissive for P. berghei development. At this lower temperature MnSOD was the only enzyme induced by 24 h PF in the fat body of females fed uninfected blood relative to sugar-fed controls (Fig. 3A). Overall, the level of induction of all genes in the fat body after an uninfected blood meal is lower when mosquitoes are kept at 21 °C than at 27 °C (see Fig. 2B). P. berghei-infected mosquitoes respond very differently, and the expression of catalase, Gpx, MnSOD, and CuZnSOD3A mRNA is significantly induced in the fat body compared with control blood-fed mosquitoes. Thus, P. berghei infection appears to exacerbate the oxidative stress induced by blood feeding. Hemolymph H2O2 levels also increase significantly 24 h after the ingestion of either an uninfected or a P. berghei-infected blood meal, but no significant difference was detected between these two groups (Table 1). Therefore, it appears that induction of ROS detoxification genes by Plasmodium infection prevents a further increase in hemolymph H2O2 levels.

**TABLE 1**

| Sample | H2O2 levels* 24 h PF (pmol/mosquito) |
|--------|-------------------------------------|
| SF     | 22.5 ± 2.2*                         |
| BF     | 44.5 ± 6.8                           |
| BI     | 54.5 ± 4.3                           |

* Results are expressed as the mean ± S.E.

**FIGURE 3.** Relative expression of ROS detoxification enzymes in An. gambiae G3 mosquitoes after P. berghei infection. A, induction of ROS detoxification enzymes mRNA in fat body of adult females fed uninfected (C) or P. berghei-infected (B) blood (21 °C). B, induction of ROS detoxification enzymes mRNA in midgut of adult females fed uninfected (C) or P. berghei-infected (B) blood (21 °C). *, significantly different (analysis of variance, p < 0.05) measurements between control and infected samples at a given time point. Bars indicate S.E.

**TABLE 2**

Effect of blood feeding on An. gambiae G3 hemolymph H2O2 levels

| Sample | H2O2 levels (pmol/mosquito) |
|--------|----------------------------|
| SF     | 22.5 ± 2.2                 |
| BF     | 44.5 ± 6.8                 |
| BI     | 54.5 ± 4.3                 |

* Results are expressed as the mean ± S.E.

**FIGURE 2.** Relative expression of ROS detoxification enzymes in response to oxidative stress in An. gambiae G3 mosquitoes. A, induction of mosquito catalase and Gpx mRNA after H2O2 injection. B, induction of ROS detoxification enzymes mRNA in midgut (●) and fat body (○) after blood feeding (27 °C). *, significantly different (analysis of variance, p < 0.05) with respect to water-injected control (A) or sugar-fed (SF) mosquitoes (B). Bars indicate S.E. C, control.
midgut invasion is prevented. Suppression of catalase induction in infected midguts (kept at 21 °C) was confirmed (Fig. 4); however, when ookinete formation was prevented by keeping mosquitoes at 27 °C, a robust induction of catalase was observed. This rules out the possibility that the lack of catalase induction in infected midguts is due to some inhibitory component(s) present in infected mouse blood. Induction of catalase mRNA in uninfected midguts is higher when mosquitoes are kept at a higher temperature (about 2.5-fold at 21 °C and more than 5-fold at 27 °C) (Fig. 4). To investigate the possibility that early zygotes or ookinetes secrete a soluble factor that suppresses catalase expression, induction of catalase mRNA in midguts infected with parasites in which the CTRP gene has been disrupted (CTRP−) was evaluated. The CTRP− mutant strain forms ookinetes that are unable to effectively invade the midgut. The induction of midgut catalase in response to CTRP− parasites was similar to that of uninfected controls, indicating that ookinete invasion is necessary for suppression of catalase expression in the midgut (Fig. 4).

**dsRNA-mediated Knockdown of Mosquito Catalase Reduces Plasmodium Infection**—To test the hypothesis that reducing catalase expression is an effective mechanism to limit *Plasmodium* infection, catalase expression was reduced systematically by dsRNA-mediated silencing in G3 *An. gambiae* females. A reduction of catalase mRNA expression of 50% was achieved 24 h PI in the whole mosquito and resulted in a decrease of 87 and 94% of catalase activity in the midgut and the fat body, respectively (Fig. 5A). Catalase knockdown led to a 39% increase in hemolymph H₂O₂ (Fig. 5A), consistent with the importance of catalase in H₂O₂ clearance. Catalase knockdown also resulted in a significant reduction in the number of parasites present in the midgut 1, 2, and 11 days PI (Fig. 5, B–D). The number of ookinetes present 1 and 2 days PI was determined by immunofluorescence; only intact parasites were counted. Reduced catalase expression resulted in extensive ookinete lysis by 2 days PI (Fig. 5C), suggesting that parasites are very labile to increased hydrogen peroxide levels during their transit through the midgut epithelium.

**Oxidative Stress Is a Major Component of Mosquito Mortality after *P. berghei* Infection**—Our data indicate that the systemic mRNA induction of several ROS detoxification enzymes is higher in *Plasmodium*-infected females (see Fig. 3A), and significant mortality is commonly observed in *P. berghei*-infected females 24–48 h PF (Fig. 6). The hypothesis that this mortality is due to increased oxidative stress in infected mosquitoes was
investigated by determining the effect of dietary supplementation with uric acid. Previous studies have shown that uric acid is an effective antioxidant in mosquitoes that can prevent loss of fecundity with aging (28) and decrease melanization of Plasmodium and implanted Sephadex beads (7). Uric acid administration significantly decreased the mortality of P. berghei-infected mosquitoes from 25 to 4% by 6 days PF (Fig. 6A), although this treatment had no significant effect on the intensity of Plasmodium infection. The median number of oocysts was 34–44 oocysts per midgut for control and uric acid-supplemented groups, respectively (Fig. 6B), indicating that reducing oxidative stress can decrease mortality even when mosquitoes are still infected with large numbers of parasites.

**DISCUSSION**

Our data indicate that genetic differences in systemic H₂O₂ levels between An. gambiae strains have broad effects in their immune response to Plasmodium (7) and other pathogens such as bacteria. The higher the systemic ROS levels in a given strain, the better the mosquitoes survive a bacterial challenge (Fig. 1A). Conversely, dietary supplementation with antioxidants (vitamin C or uric acid) can dramatically reduce the mosquito's ability to mount an effective antibacterial response (Fig. 1, B and C). Both antioxidants inhibit melanization of implanted beads and of Plasmodium parasites (7), so they may inhibit melanization of bacteria, but this is unlikely to cause mosquito mortality because blocking bacterial melanization after bacterial infection does not affect mosquito survival (29). Insect hemocytes are capable of generating a respiratory burst in response to pathogens (11–15) and also possess peroxidases that could potentially produce highly toxic microbialidal compounds (8, 30). Vitamin C administration reduces systemic levels of H₂O₂, and this could reduce the catalytic activity of peroxidases because these enzymes require H₂O₂ as a substrate.

It is interesting to note that, in contrast to their deleterious effect on immunity, the same antioxidants also have beneficial effects, as they significantly reduce age-related loss of fecundity in mosquitoes (28). These observations illustrate the importance of ROS in maintaining the balance between immunity and fecundity. In other words, although high ROS enhance immunity, this comes with a fitness cost in terms of fecundity.

Plasmodium midgut infection takes place at a time when the blood meal is undergoing digestion and nutrients are being adsorbed and transported. The rate of these physiologic responses is temperature-dependent. For example, blood meal digestion in Aedes aegypti mosquitoes is faster at higher temperatures (31). We confirmed that the mRNA expression of ROS detoxification enzymes increases after blood feeding (Fig. 2B), which is consistent with a response to the systemic accumulation of ROS such as H₂O₂ in hemolymph (Table 1). As expected, induction of these enzymes is faster and more robust at 27 °C than at 21 °C. Their expression peaks earlier in the midgut, corresponding to the time of blood-meal digestion and nutrient absorption. Induction of these genes in the fat body peaks at a time when nutrient uptake, protein synthesis, and export into the hemolymph are active in this organ.

P. berghei infection appears to exacerbate the oxidative stress induced by feeding, as mosquitoes respond by increasing the systemic expression of ROS detoxification enzymes (Fig. 3A). This response is effective, as no significant increase in hemolymph H₂O₂ levels was observed in infected mosquitoes (Table 1). We were surprised to find that infected midguts suppress catalase induction (Fig. 3B), because reducing catalase expression at a time of high metabolic activity would increase intracellular H₂O₂ levels. This result is consistent with previous microarray analysis of midgut gene expression that showed lower midgut catalase expression in P. berghei-infected mosquitoes (21).

The suppression of catalase mRNA is dramatic and, thus, appears to be a widespread response of midgut epithelial cells that is not limited to those relatively few cells invaded by parasites. One can envision that epithelial cells may release some “factor” in response to ookinet invasion that mediates suppression of catalase expression, even in cells that were not invaded.

Suppression of catalase expression is likely to be part of a defense response triggered by parasite invasion. Previous studies have shown that during their transit through the midgut, ookinetes trigger a series of reactions, including protein nitration, that ultimately lead to apoptosis of the invaded cell (8, 26). Cell nitration appears to be a two-step process in which NO generated by nitric-oxide synthase (32, 33) rapidly converts into nitrite. An inducible epithelial peroxidase uses nitrite and H₂O₂ as substrates and catalyzes nitration (8) in a reaction similar to what has been described in vertebrate macrophages (34). Catalase is extremely efficient, and reducing local levels of this enzyme may be necessary for H₂O₂ to be available as substrate for the peroxidase-mediated nitration reaction. ROS generated during the immune response are also potentially toxic to the host, so it is important to keep them well localized. Local generation of ROS is well documented, but we propose that reducing expression of detoxification enzymes in specific cells is also an alternative and important mechanism to mount effective immune responses while preventing self-damage.

dsRNA-mediated knockdown of catalase expression decreases midgut infection by Plasmodium (Fig. 5, B–D) and supports the hypothesis that reducing expression of this enzyme is an effective defense response against the parasite by increasing ROS levels. We have previously shown that the size

**FIGURE 6. Effect of uric acid on mosquito mortality and P. berghei infection.** A, mean mosquito survival at different days post-feeding in control and P. berghei-infected G3 An. gambiae females. B, effect of uric acid administration on P. berghei infection. C, control-fed uninfected blood; i, P. berghei infected; U.A., fed 1 mg/ml of uric acid. Bars indicate S.E.
of the ingested blood meal in mosquitoes in which catalase has been silenced is similar to that of the controls injected with dsRNA LacZ (28), indicating that the observed differences in infectivity cannot be explained by differences in blood-meal size. The reduction in infectivity after catalase silencing can already be observed 24 h Pl and appears to be due to increased ookinete lysis (Fig. 5C), as melanization responses are not observed. We have previously proposed that, to survive, *Plasmodium* ookinetes must exit the invaded midgut cell before the peroxidase-mediated nitration reactions are induced. A further reduction in catalase activity is expected to increase the activity of epithelial peroxidases and accelerate the nitration response. The proposed model predicts that under these conditions, parasites could be damaged or modified. One can envision that nitrated parasites could become “visible” to components of the immune system that mediate parasite lysis such as the thioester containing protein 1 (4).

The fact that uric acid administration can significantly reduce mortality after *P. berghei* infection (Fig. 6A) indicates that oxidative stress can be extremely high and causes lethality in infected mosquitoes. In this study uric acid was administered as a sugar solution that is stored in the crop and released slowly into the gut, and it did not affect *P. berghei* infectivity (Fig. 6B); however, direct administration of uric acid into the midgut by giving it as a sugar-free solution reduces nitration of proteins in the blood meal and increases *P. berghei* midgut infection (33). The evidence presented indicates that invasion of the mosquito midgut by *P. berghei* ookinetes leads to major changes in ROS metabolism. It is worth noting that no increase in mortality is observed when mosquitoes are infected with the human parasite *P. falciparum* (data not shown) despite the higher temperature at which infected mosquitoes are kept (27 °C instead of 21 °C), suggesting that oxidative stress is less prominent after infection with the human parasite. The mosquito oxidative response to *P. falciparum* infection is currently under investigation.

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