

RESEARCH ARTICLE

# Catalase protects *Aedes aegypti* from oxidative stress and increases midgut infection prevalence of Dengue but not Zika

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## Abstract

### Background

Digestion of blood in the midgut of *Aedes aegypti* results in the release of pro-oxidant molecules that can be toxic to the mosquito. We hypothesized that after a blood meal, the antioxidant capacity of the midgut is increased to protect cells against oxidative stress. Concomitantly, pathogens present in the blood ingested by mosquitoes, such as the arboviruses Dengue and Zika, also have to overcome the same oxidative challenge, and the antioxidant program induced by the insect is likely to influence infection status of the mosquito and its vectorial competence.

### Methodology/Principal findings

We found that blood-induced catalase mRNA and activity in the midgut peaked 24 h after feeding and returned to basal levels after the completion of digestion. RNAi-mediated silencing of catalase (AAEL013407-RB) reduced enzyme activity in the midgut epithelia, increased H<sub>2</sub>O<sub>2</sub> leakage and decreased fecundity and lifespan when mosquitoes were fed H<sub>2</sub>O<sub>2</sub>. When infected with Dengue 4 and Zika virus, catalase-silenced mosquitoes showed no alteration in infection intensity (number of plaque forming units/midgut) 7 days after the infectious meal. However, catalase knockdown reduced Dengue 4, but not Zika, infection prevalence (percent of infected midguts).

### Conclusion/Significance

Here, we showed that blood ingestion triggers an antioxidant response in the midgut through the induction of catalase. This protection facilitates the establishment of Dengue virus in the

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midgut. Importantly, this mechanism appears to be specific for Dengue because catalase silencing did not change Zika virus prevalence. In summary, our data suggest that redox balance in the midgut modulates mosquito vectorial competence to arboviral infections.

### Author summary

Mosquitoes ingest large amounts of blood, a rich and abundant source of energy to sustain egg production. Blood digestion offers challenges to the insect, like managing high concentrations of heme and iron, pro-oxidant and potentially toxic molecules derived from hemoglobin. Mosquitoes and other blood-feeding arthropods have evolved adaptations to overcome this problem, such as the activation of catalase, an antioxidant enzyme that protect tissues against toxic free radicals. Mosquitoes act as important vectors of human diseases because during a blood meal they might also ingest microorganism circulating in our blood, such as dengue and zika virus. The adaptive antioxidant program that protects mosquito tissues against the oxidative challenge imposed by a blood meal might also influences the ability of virus to establish infection and disseminate from the midgut to the salivary glands. We show here that catalase differentially influences the number of infected midguts after mosquitoes were challenged with blood contaminated with virus, being beneficial to Dengue-4 but neutral do Zika, suggesting that redox metabolism may have distinct roles on mosquito vector competence towards different arbovirus.

### Introduction

Arthropod-borne viral (arboviral) diseases, such as Dengue, Chikungunya, and especially Zika, have recently occupied a central spot in the global discussions concerning infectious diseases due to the rapid spread of cases worldwide and the associated increase in syndromes such as microcephaly and Guillain–Barré, prompting the World Health Organization to declare Zika a public health emergency [1]. Over two million people live in areas where Zika has been reported, highlighting the risk of major epidemics, especially in Central and South Americas, as well as Southeast Asia [2]. Concerning Dengue, more accurate epidemiological data are available, and the number of annual infections could be as high as half a billion [3]. Currently, there is no vaccine to prevent new infections and no effective treatment options for sick individuals. Strategies targeting *Aedes aegypti*, the mosquito vector, such as utilizing the anti-viral effects of Wolbachia [4,5] and the sterile insect technique [6,7], are attractive possibilities under implementation. To stop mosquito spread of arboviral diseases, we need to further elucidate the molecular interactions between the virus and the vector. This knowledge will help to explain, for example, the observed differences in susceptibility to viral infections of mosquito strains/populations [8,9].

Reactive oxygen species (ROS) have emerged as central molecules in a wide array of pathological as well as physiological processes, including signaling, immunity, cell proliferation and differentiation [10]. The biological actions of ROS are based on their ability to donate or receive electrons from biomolecules, triggering a diverse set of events associated with the normal function of cells. However, under oxidative stress, elevated levels of ROS may disrupt redox signaling pathways, leading to a non-homeostatic state commonly associated with disease [11]. Therefore, the correct balance between ROS-generating systems (such as mitochondria, endoplasmic reticulum or NADPH oxidases) and ROS-detoxifying reactions (including

antioxidant enzymes such as catalase, which detoxifies H<sub>2</sub>O<sub>2</sub> into water and oxygen) is critical for maintaining homeostasis in virtually all studied organisms.

ROS metabolism influences critical parameters of insect physiology, including fecundity [12,13], immune response [14,15] and vector competence in the interaction between *Anopheles* and *Plasmodium* [16–22]. In hematophagous arthropods, such as the *Aedes aegypti* mosquito, blood digestion in the midgut releases heme, a pro-oxidant molecule. Cells subjected to high concentrations of heme, such as gut epithelial cells after a blood meal, must maintain redox balance to avoid oxidative stress. Blood-sucking organisms have evolved a series of adaptations against the deleterious effects of excess heme [23–29]. One such mechanism is the activation of antioxidant enzymes, such as catalase, which reduces H<sub>2</sub>O<sub>2</sub> and prevents its contact with heme/iron, a reaction known to generate highly toxic ROS [30,31]. An interesting possibility concerning hematophagous insect vectors is that the antioxidant protection induced upon blood feeding could also defend human pathogens being carried by the mosquitoes, such as arboviruses, from blood-induced oxidative challenge.

Our results demonstrated that a blood meal up-regulated catalase mRNA and activity in the midgut epithelium and that silencing of catalase through RNAi reduced mosquito fecundity and resistance to hydrogen peroxide feeding. Catalase silencing had no effect on the intensity of infection with Dengue or Zika viruses. Interestingly, it reduced the prevalence of Dengue infection, but had no effect on the prevalence of Zika-infected females. This indicates that the redox environment of the midgut can alter mosquito susceptibility to infection with some flaviviruses.

## Materials and methods

### 2.1 –Ethics statement

All animal care and experimental protocols were conducted in accordance with the guidelines of the Committee for Evaluation of Animal Use for Research of the Universidade Federal do Rio de Janeiro (CEUA-UFRJ). The protocols were approved under the registry CEUA-UFRJ 155/13. Dedicated technicians at the animal facility at the Institute of Medical Biochemistry (UFRJ) carried out all aspects related to rabbit husbandry under strict guidelines to ensure careful and consistent handling of the animals.

### 2.2 –Mosquitoes

Two– 10-day-old *Aedes aegypti* females (red-eye strain) were used in all the assays and were maintained in 12-h light-dark periods at 28°C and 80% relative humidity. Females were fed *ad libitum* with cotton pads soaked in a 5% sucrose solution or allowed to feed on rabbit blood.

### 2.3 –Catalase activity

Mosquitoes were cold-anesthetized and dissected in 50% ethanol. The midgut epithelia was separated from the blood bolus and collected in PBS (10 mM sodium phosphate buffer and 150 mM NaCl, pH 7.4) supplemented with a protease inhibitor cocktail (50 µg/mL SBTI, 1mM benzamidine, 1mM PMSF). Samples were mechanically homogenized with a pestle and stored at -80°C until use. Catalase activity was measured following H<sub>2</sub>O<sub>2</sub> absorbance (240 nm for 1 min) according to the protocol described by Aebi [32] in the presence of mosquito homogenates. The protein concentration was determined according to Lowry [33]. For *in vivo* inhibition experiments, mosquitoes were fed blood supplemented with different doses of 3-amino-1,2,4-triazole (AT), a catalase inhibitor. For *in vitro* inhibition, AT was incubated for 30 minutes at 4°C with tissue homogenates before measurements of enzymatic activity.

## 2.4 –RNA extraction and quantitative PCR (qPCR)

Total RNA (pools of 5–10 midgut epithelia) was extracted with TRIzol reagent (Invitrogen) according to the protocol suggested by the manufacturer and treated with DNase I. cDNA was synthesized using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) according to standard protocols. The sequence CAT1A –AAEL013407 –RB (Transcript ID from VectorBase) was used to design the catalase primers. The primer sequences used for qPCR experiments were CAATGAACTGCACCGACAAC (forward) and AGCCTCATCCA GAACACGAC (reverse). The sequence AAEL003396-RA (Transcript ID from VectorBase) was used as a housekeeping gene and corresponded to the ribosomal gene rp49 [34]. The corresponding primers for the housekeeping gene were GCTATGACAAGCTTGCCCCCA (forward) and TCATCAGCACCTCCAGCT (reverse). qPCR was performed using the SYBR Green PCR Master Mix (Applied Biosystems). Relative gene expression was calculated using the method described by Livak and Schmittgen [35].

## 2.5 –RNAi silencing of catalase

A 1158-base-pair fragment of the catalase gene (AAEL013407 –RB) was amplified using cDNA from the midgut epithelia of blood-fed mosquitoes (24 h after feeding) using primers F-TTCAAGGAGTCCCAGAAGGA and R-AACCGGAATCAGAGGGA. This amplicon was subjected to a nested PCR with catalase primers that also contained a T7 binding sequence (which is necessary for RNA polymerase binding, see underlined). The primers used were TAATACGACTCACTATAGGGACTCCACTTGCTGTGCGTTT (forward) and TAATACGACTCACTATAGGGTCTCCCTTAGCAATAGCGTTG (reverse). A 773-base-pair fragment was generated, purified and used as the template for an *in vitro* transcription reaction for the synthesis of double-stranded RNA (dsRNA) for catalase (dsCat) with a MEGAscript RNAi kit (Ambion). RNAi experiments were performed via injection of 69 nL of dsCat (3 µg/µL) (or dsLacZ as an unrelated dsRNA) in the thoraxes of 2-day old female mosquitoes. Mosquitoes were used 2–3 days after the injections. A 218-bp LacZ fragment was amplified using the primers GAGTCAGTGAGCGAGGAAGC (forward) and TATCCGCTCACAATTCCA CA (reverse) and was cloned into the pCRII-TOPO vector. This plasmid was used for a subsequent PCR in which the T7 RNA polymerase promoter was also inserted, using the primer sequences GTAAAACGACGGCCAGT (M13F) and CTCGAGTAATACGACTCACTATAG GGCAGGAAACAGCTATGAC (M13R). This PCR product was used for the synthesis of the dsLacZ performed with a MEGAscript RNAi kit (Ambion).

## 2.6 –Hydrogen peroxide measurements

H<sub>2</sub>O<sub>2</sub> was measured with Amplex Red (Invitrogen) following the recommendations of the manufacturer with minor modifications. The midgut epithelia of sugar-fed mosquitoes were dissected in 2.5% BSA in PBS, the gut contents were washed out and the epithelia (pools of 5 organs) were incubated in PBS under dim light at room temperature in the presence of Amplex Red (40 µM) and 4 U horseradish peroxidase (HRP, Sigma). After a 30 min incubation, the epithelia were centrifuged, and the supernatants were collected and evaluated for fluorescence emission at 530/590 nm (Ex/Em) in a Varian Cary Eclipse Fluorescence Spectrofluorometer. The resulting values were subtracted from fluorescence readings generated by nonspecific Amplex Red oxidation by the midgut epithelia (pools of 5 organs) in the absence of HRP.

## 2.7 –Dengue 4 and Zika virus stocks

Dengue-4 was kindly provided by Dr. João Trindade Marques (UFMG–Universidade Federal de Minas Gerais, Brazil). Zika virus was obtained from Dr. Laura Helena Vega Gonzales Gil (Centro de Pesquisas Aggeu Magalhães, Fundação Oswaldo Cruz, Brazil). Viral stocks were propagated in C6/36 cells maintained in Leibovitz-15 (L-15) media (Gibco #41300–039) pH 7.4 supplemented with 5% fetal bovine serum, triptose 2.9 g/L, 10 mL of 7.5% sodium bicarbonate/L; 10 mL of 2% L-glutamine/L, 1% of non-essential amino acids (Gibco #11140050) and 1% penicillin/streptomycin at 28°C. Culture supernatants containing viral particles were harvested, centrifuged, aliquoted and stored at -80°C until use. Plaque assays (see section 2.8) were performed to determine viral titers. The Dengue-4 titer used was  $1 \times 10^7$ /mL. The Zika titer used was  $2 \times 10^7$ /mL.

## 2.8 –Mosquito infection with Dengue-4 and Zika virus

Mosquitoes were starved from sucrose (but not water) for 18–24 h and were offered a meal containing a 1:1 mix of rabbit red blood cells and L-15 media containing different amounts of Dengue-4 or Zika virus. ATP pH 7.4 at a final concentration of 1 mM was included as a phagostimulant. Viral stocks were thawed immediately before use. Mosquitoes were allowed to ingest the infectious blood through a membrane attached to an artificial feeder kept at 37°C for approximately 40 min inside a BSL-2 insectary facility. Mosquitoes were quickly cold-anesthetized, and fully engorged females were separated and housed as indicated in section 2.1 until use.

## 2.9 –Plaque assays

Dengue-4 plaque assays were performed in BHK-21 cells and Zika plaque assays were performed in Vero cells maintained in DMEM (Gibco #12100–046) supplemented with sodium bicarbonate, 1% L-glutamine (200 mM, Gibco #25030081), 10% fetal bovine serum and 1% penicillin/streptomycin and seeded as monolayers (approximately 70% confluency) onto 24-well plates 12–24 h before the experiment. Seven days after the infectious meal, mosquitoes were surface-sterilized with 70% ethanol (20 seconds) and rinsed twice with sterile PBS. Midguts were dissected using clean glass slides and forceps in sterile PBS and transferred to sterile Eppendorf tubes contain 200 µl of DMEM (same as above) and 50–100 mg of sterile glass beads (Scientific Industries SI-BG05–0.5 mm diameter). Midguts were individually stored at -80°C until use. Midgut tissue was disrupted to liberate viral particles by vortexing the tubes for 10 minutes at room temperature. The samples were then centrifuged at  $10,000 \times g$  at 4°C and serially diluted in DMEM. One hundred µl of each sample was added to Vero (Zika) or BHK-21 (Dengue-4) cell culture monolayers and gently shaken for 15 minutes at room temperature, followed by an additional 45 minutes without shaking at 37°C and in a 5% CO<sub>2</sub> incubator. Subsequently, 700 µl of DMEM containing 2% FBS and 0.8% methylcellulose (Sigma #M0512. Viscosity 4,000 cP) was added to each well. Plates were incubated at 37°C and 5% CO<sub>2</sub> for five days. Samples were stained with a 1% crystal violet solution in a 1:1 (v:v) mixture of methanol/acetone for 1 h at room temperature and washed with water to remove excess dye. Then, individual plaque forming units (PFU) were visually counted.

## 2.10 –Statistical analysis

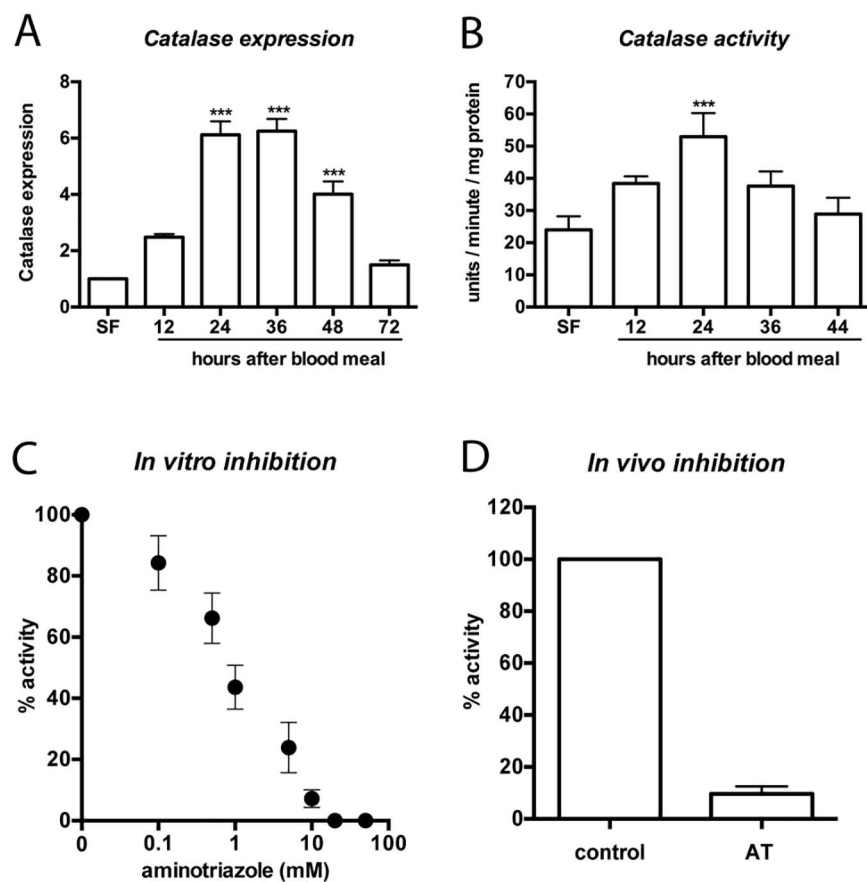
All experiments were carried out independently at least two times, and statistical analyses were performed with GraphPad Prism software. The appropriate tests are described in the figure legends.

## Results

### Blood feeding transiently induced catalase activity in the midgut epithelia

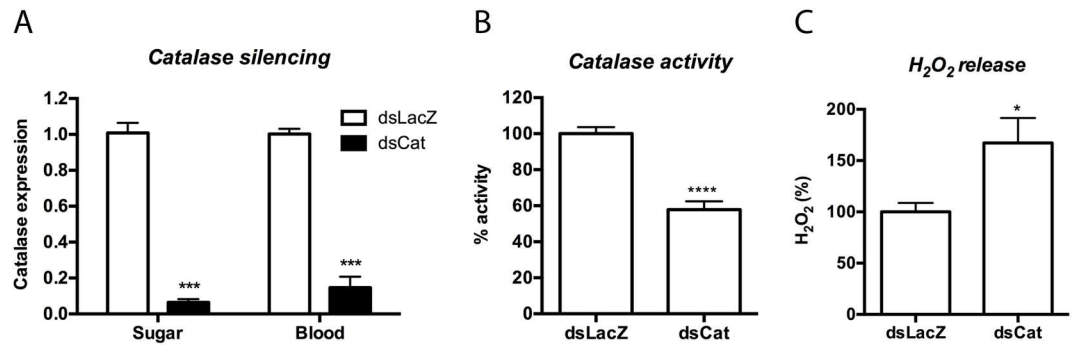
To investigate the role of catalase in the midgut of *Aedes aegypti* in response to blood feeding, we compared gene expression in the epithelia of sugar-fed (SF) and blood-fed (BF) females dissected at 12, 24, 36, 48 and 72 h after blood intake. Catalase mRNA levels increased 6-fold at 24 and 36 h after a meal and decreased to SF levels at 72 h (Fig 1A). Enzyme activity was also monitored throughout the digestion process, which spans approximately 48 h. Fig 1B shows that H<sub>2</sub>O<sub>2</sub>-removing capacity also increased in the epithelia after feeding, reaching its maximal induction at 24 h, near the peak of blood digestion [36], and returned to initial levels at 44 h.

We tested the sensitivity of catalase to 3-amino-1,2,4-triazole (AT), a well-known inhibitor [37]. Using an *in vitro* assay exposing midgut samples collected 24 h after a blood meal to different concentrations of AT, we showed that 100% of the epithelial H<sub>2</sub>O<sub>2</sub> detoxification could be abrogated with AT concentrations below 20 mM and that 50% enzyme inhibition occurred close to 1 mM. Using a similar approach, we fed females blood supplemented with 15 mM AT



**Fig 1. Catalase mRNA and activity increased in the midgut epithelia of blood-fed mosquitoes.** *Aedes aegypti* females were fed sugar (SF) or blood (BF) and dissected at the indicated time points after a blood meal. (A) Catalase mRNA expression in the epithelia was evaluated using qPCR analysis of SF and BF mosquitoes. (B) Enzymatic activity was measured as described in the Materials and Methods section. (C) Catalase inhibition by AT *in vitro*. Midgut epithelia was collected from blood-fed mosquitoes 24 h after feeding and was incubated with AT and H<sub>2</sub>O<sub>2</sub> for 30 min at 4°C; then, catalase activity was assayed. (D) Mosquitoes were fed blood supplemented with 15 mM AT and assayed for catalase activity in the epithelia \*\*\*  $p < 0.001$ . Figure 1A–B—ANOVA followed by Dunnett’s multiple comparison test.

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**Fig 2. Catalase knockdown in the midgut.** (A) Two-day-old females were injected with dsRNA against catalase (dsCat) or an unrelated control gene (dsLac). Two days after the dsRNA injection, a group of mosquitoes were fed blood, while others were fed exclusively with sugar. Twenty-four hours later, RNA was extracted for qPCR analysis. (B) Catalase activity in the epithelia was measured 24 h after a blood meal in dsRNA-treated mosquitoes. (C) Hydrogen peroxide leakage was measured in the midgut epithelia of SF mosquitoes injected with dsCat or dsLacZ. (A) \*\*\*  $p < 0.001$  (t-test). (B) \*\*\*\*  $p < 0.0001$  (t-test). (C) \*  $p = 0.0404$  (t-test).

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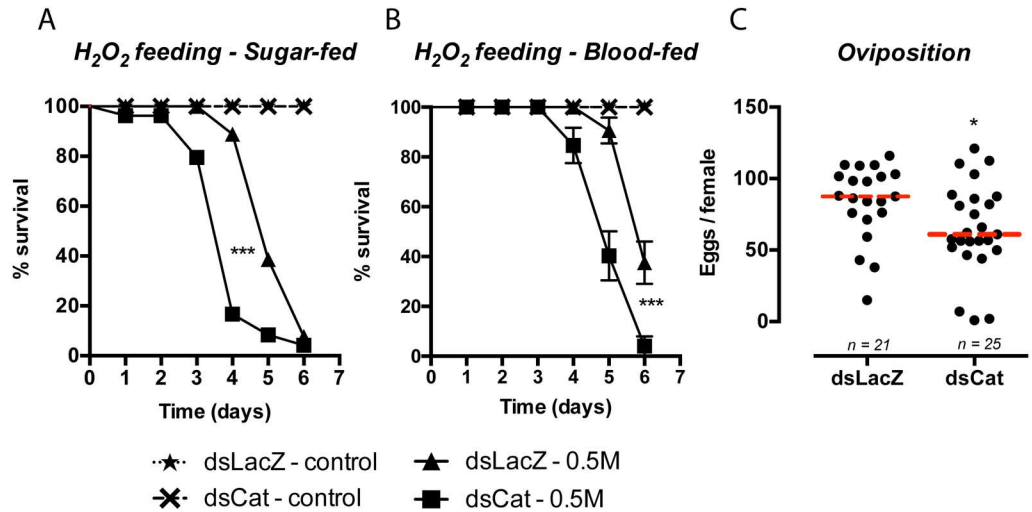
and showed that catalase activity accounted for more than 90% of H<sub>2</sub>O<sub>2</sub> removal 24 h after the meal (Fig 1D).

### RNAi silencing of catalase reduced oviposition and resistance to H<sub>2</sub>O<sub>2</sub>

We injected *Aedes aegypti* females with dsRNA against catalase and evaluated the mRNA levels in the midgut epithelia. Fig 2A shows that the catalase transcripts were reduced by 93% and 86% in SF and BF mosquitoes 24 h after feeding. Consistent with the reduced mRNA levels, we observed a decrease in catalase activity in the epithelia of blood-fed mosquitoes (Fig 2B). To determine whether catalase silencing affected midgut redox metabolism, we measured H<sub>2</sub>O<sub>2</sub> (the substrate of catalase and a diffusible ROS) released by epithelial cells. Fig 2C shows that catalase knockdown increased hydrogen peroxide levels leaked to the supernatant. Together, Fig 2A–2C confirm that the RNAi approach negatively impacted catalase activity and redox metabolism of the midgut. To address its physiological significance, we demonstrated a reduction in lifespan of both sugar-fed (SF) and blood-fed (BF) mosquitoes challenged with sucrose supplemented with H<sub>2</sub>O<sub>2</sub> (Fig 3A and 3B). The median time to death was anticipated in 1 day in the dsCatalase group, which represents 15–20% of the lifespan of mosquitoes feeding on hydrogen peroxide under the conditions tested. We also observed a small but statistically significant 22% reduction in oviposition (Fig 3C), similar to what was reported for *Anopheles gambiae* and *Lutzomyia longipalpis* [12,13].

### Catalase silencing reduces Dengue, but not Zika, midgut infection prevalence

To test the hypothesis that antioxidant protection triggered by a blood meal could influence a mosquito's infection status with different flaviviruses, we challenged catalase-silenced *Aedes aegypti* with two doses of Zika virus and measured the number of PFU per midgut (infection intensity) and the number of infected midguts (infection prevalence) seven days after administration of virus-contaminated blood. Catalase silencing did not change any of the parameters evaluated in Zika-infected females. (Fig 4A–4D). Interestingly, the highest dose offered, 10<sup>7</sup>/mL (corresponding to our maximal titer obtained from C6/36 cells supernatants), and its 100-fold dilution, (10<sup>5</sup>/mL), resulted in only a four-fold change viral loads after 7 days (10<sup>7</sup>/mL mean PFU ~ 10000; 10<sup>5</sup>/mL mean PFU ~ 3000) (Fig 4A and 4C). However, the same doses



**Fig 3. Catalase knockdown affected both resistance to H<sub>2</sub>O<sub>2</sub> and oviposition.** Catalase was silenced as described, and two days after dsRNA injection, SF (A) or BF (immediately after feeding) (B) mosquitoes were transferred to cages containing 5% sucrose supplemented with 1 M H<sub>2</sub>O<sub>2</sub> *ad libitum* (day 0). A fresh H<sub>2</sub>O<sub>2</sub> solution was provided daily. Survival was scored every 24 h. \*\*\*  $p < 0.0001$  for the comparison between dsLacZ-H<sub>2</sub>O<sub>2</sub> vs dsCat-H<sub>2</sub>O<sub>2</sub> (log-rank test). (C) Catalase-silenced mosquitoes were blood-fed and allowed to lay eggs. Each dot represents an individual mosquito. LacZ- $n = 21$ . Catalase- $n = 25$ . \*  $p = 0.37$  (t-test).

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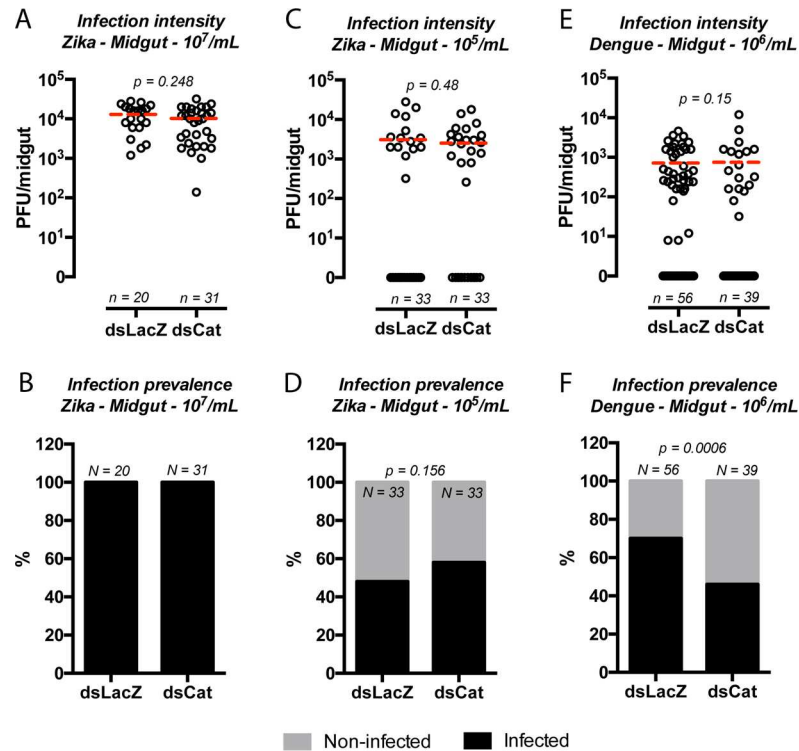
resulted in a change in infection prevalence ( $10^7$ /mL Zika viral particles produced 100% prevalence in both dsLacZ and dsCat while  $10^5$ /mL viral particles produced ~50% prevalence in both dsLacZ and dsCat) (Fig 4B and 4D). When we challenged dsCatalase-treated mosquitoes with the maximal Dengue-4 infectious dose ( $5 \times 10^6$ /mL), there was no alteration in the median infection intensity (Fig 4E). However, we observed a significant reduction in infection prevalence (70% of dsLacZ mosquitoes were infected vs 46% of dsCatalase;  $p = 0.0006 \pm$  chi-square, demonstrating that a reduction in epithelial H<sub>2</sub>O<sub>2</sub>-removing capacity through catalase knockdown reduced the ability of Dengue-4, but not Zika virus, to infect the midgut of *Aedes aegypti*.

## Discussion

Overall, we showed that a blood meal induced antioxidant protection in the midgut of *Aedes aegypti* and that RNAi-mediated knockdown of catalase resulted in reduced oviposition and lifespan when mosquitoes were challenged with H<sub>2</sub>O<sub>2</sub> and decreased midgut virus prevalence after infection with Dengue-4, but not Zika.

The levels of pro-oxidant molecules, including hydrogen peroxide, in a given tissue must be carefully monitored to maintain normal cellular functions, with deviations from the optimal concentration being potentially harmful for the organism. The increase in H<sub>2</sub>O<sub>2</sub> levels may be particularly deleterious to hematophagous arthropods because blood digestion releases heme, a pro-oxidant molecule, which may interact with cellular ROS, leading to oxidative stress [30,38], decreased reproductive output [12,13] and possibly death of the insect [39,40]. We recently demonstrated that after a blood meal, *Aedes aegypti* inhibits the metabolic generation of ROS [24]. In that report, we showed that after blood intake, heme triggers a protein kinase C-dependent mechanism that inhibits dual oxidase (Duox) activity (a source of ROS in the midgut), maintaining low ROS production during blood digestion compared with SF levels. The pattern of antioxidant enzyme expression observed in most organisms shows that they typically respond to increased levels of ROS, such as catalase in *Rhodnius prolixus* [29]. It is





**Fig 4. Catalase silencing impacted Dengue but not Zika midgut infection prevalence.** (A) Females were fed blood contaminated with 10<sup>7</sup> PFU/mL of Zika virus, and 7 days after feeding the number of PFU was determined in the midgut. (B) The percentage of infected midguts (infection prevalence) was scored from the same set of data as in A. (C) Mosquitoes were fed a lower dose of Zika-infected blood (10<sup>5</sup>/mL), and PFU/midgut was determined 7 days post-infection. (D) Infection prevalence of mosquitoes from C. (E) Mosquitoes were fed blood contaminated with 10<sup>6</sup> PFU/mL of Dengue 4 virus, and PFU/midgut was counted 7 DPI. (F) Infection prevalence was determined from the same group of mosquitoes. Mann-Whitney *U*-tests were used for infection intensity (A, C, E), and chi-square tests were performed to determine the significance of infection prevalence analysis (B, D, F). Statistical values and number of replicates are depicted in the corresponding figures.

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peculiar, then, that *Aedes aegypti* increases catalase expression and activity after a blood meal, especially when ROS levels were reduced compared with sugar-fed mosquitoes. We hypothesize that hematophagous mosquitoes evolved a redundant protection strategy to prevent oxidative stress following blood intake, which may explain the simultaneous decrease in ROS production and increase in antioxidant capacity. This is a major departure from most studies on ROS metabolism, where antioxidant enzymes are regulated by previous oxidant stress.

Redox metabolism has been implicated in the response of *Anopheles* mosquitoes to plasmodium infection [20,41]. While in *Anopheles gambiae* catalase knockdown reduced *Plasmodium berghei* oocyst counts, supposedly through augmented concentration of toxic H<sub>2</sub>O<sub>2</sub>, it increased oocysts of the human malaria *P. vivax* in its natural vector, *Anopheles aquasalis* [22], revealing a complex and species-specific role of catalase in the gut of mosquitoes during malaria infection. Regarding the interaction of ROS and microorganisms, high levels of free radicals are believed to be detrimental. However, an emerging concept posits that similar to the role of catalase in *P. vivax*-*An. aquasalis*, other parasites, such as *Trypanosoma cruzi*, thrive under host oxidative stress [42–45]. In the case of *Aedes aegypti*, altering redox homeostasis in the gut through catalase silencing reduced Dengue prevalence in the midgut (Fig 4F),

suggesting that ROS could antagonize infection by this specific arbovirus. However, catalase RNAi did not alter the percentage of infected midguts in females challenged with Zika (Fig 4B and 4D), indicating a differential sensitivity of flaviviruses to ROS produced by *Aedes aegypti*.

Our understanding of mosquito immunity to dengue virus has predominantly focused on classical immune genes and the roles of the Toll, Stat and RNAi pathways have been firmly established [46–49]. Additionally, genes that conventionally are not considered (or labeled) immune genes, such as lipid and redox metabolism, are also known to influence mosquito-arbovirus interactions and should not be overlooked [8,27,50]. Little is known about the molecular aspects of the interaction between *Aedes aegypti* and Zika, and antiviral mechanisms described for other flaviviruses will likely be involved [51]. In agreement with our result using Dengue-4, it was recently shown for Dengue-2 that RNAi-silencing of the ROS-producing enzymes Duox and NoxM, as well as treatment of mosquitoes with the antioxidant vitamin C, enhanced viral infection in *Aedes aegypti* [52]. The data presented here suggested that catalase silencing altered the so-called midgut infection barrier (MIB) for Dengue-4. MIB is a concept that refers to mechanisms involved in the inhibition of the initial contacts between virions and the intestinal epithelial cells, preventing the establishment of the infection [53,54]. These mechanisms may involve physical barriers, such as the peritrophic matrix, or physiological and immunological mechanisms, such as the midgut microbiota and/or the RNAi pathway, to name a few [55]. Here, catalase silencing was shown to reduce Dengue-4 prevalence possibly through an alteration in the midgut threshold of infection. If the virus is able to pass this bottleneck, then it establishes a successful cycle of replication, which was seen by the similar infection intensities of the catalase-silenced and control groups of insects. Importantly, this mechanism did not alter Zika prevalence in the midgut, indicating significant differences between how these two flaviviruses establish infections in the mosquito gut.

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