



Published in final edited form as:

Science. 2011 February 25; 331(6020): 1074–1077. doi:10.1126/science.1199115.

## Development of Transgenic Fungi That Kill Human Malaria Parasites in Mosquitoes

Weiguo Fang<sup>1</sup>, Joel Vega-Rodríguez<sup>2</sup>, Anil K. Ghosh<sup>2</sup>, Marcelo Jacobs-Lorena<sup>2</sup>, Angray Kang<sup>3</sup>, and Raymond J. St. Leger<sup>1,\*</sup>

<sup>1</sup>Department of Entomology, University of Maryland, 4112 Plant Sciences Building, College Park, MD 20742, USA

<sup>2</sup>Malaria Research Institute, Department of Molecular Microbiology and Immunology, Johns Hopkins School of Public Health, 615 North Wolfe Street, E5132, Baltimore, MD 21205, USA

<sup>3</sup>Antibody Technology Group, Department of Molecular and Applied Biosciences, School of Life Sciences, University of Westminster, 115 New Cavendish Street, London W1W 6UW, UK

### Abstract

*Metarhizium anisopliae* infects mosquitoes through the cuticle and proliferates in the hemolymph. To allow *M. anisopliae* to combat malaria in mosquitoes with advanced malaria infections, we produced recombinant strains expressing molecules that target sporozoites as they travel through the hemolymph to the salivary glands. Eleven days after a *Plasmodium*-infected blood meal, mosquitoes were treated with *M. anisopliae* expressing salivary gland and midgut peptide 1 (SM1), which blocks attachment of sporozoites to salivary glands; a single-chain antibody that agglutinates sporozoites; or scorpine, which is an antimicrobial toxin. These reduced sporozoite counts by 71%, 85%, and 90%, respectively. *M. anisopliae* expressing scorpine and an [SM1]<sub>8</sub>:scorpine fusion protein reduced sporozoite counts by 98%, suggesting that *Metarhizium*-mediated inhibition of *Plasmodium* development could be a powerful weapon for combating malaria.

Nearly half of the world population is at risk of contracting malaria, and over one million people, mostly African children, die of the disease every year. Efforts to control the disease are hampered by increased resistance of parasites and vectors to drugs and insecticides (1). Emergence and spread of pyrethroid-resistant mosquitoes is a particular threat, because pyrethroid-treated bed nets are the mainstay of malaria control programs and there are no immediate prospects for new chemical insecticides (2, 3). There is consequently a pressing need for practical alternatives for malaria control (1). Several field and laboratory studies

\*To whom correspondence should be addressed. stleger@umd.edu.

The GenBank accession numbers of the sequences used in the study are P56972 (scorpine) and HQ833020 (PfNPNA-1).

#### Supporting Online Material

[www.sciencemag.org/cgi/content/full/331/6020/1074/DC1](http://www.sciencemag.org/cgi/content/full/331/6020/1074/DC1)

Materials and Methods

Figs. S1 to S9

Tables S1 to S4

References

have used fungi, such as *Metarhizium anisopliae*, that are pathogenic to adult mosquitoes. Unlike bacteria and viruses, fungal pathogens infect mosquitoes through direct contact with the cuticle and so lend themselves to strategies currently used for delivery of chemical insecticides, for example, being sprayed on indoor surfaces of houses, cotton ceiling hangings, curtains, and bed nets (4, 5) or used in outdoor odor-baited traps (6). Fungal spores persist on some treated surfaces for months (5) and can be used in insecticide-resistance management or integrated vector management because fungal infections act synergistically with various insecticides [including pyrethroids and dichlorodiphenyltrichloroethane (DDT)], and fungi are equally effective against insecticide-resistant and insecticide-susceptible mosquitoes (7, 8).

Using currently available fungal strains mosquito death is slow, but it takes about 12 to 14 days for *Plasmodium falciparum*, the causative agent of malaria, to develop in the mosquito from ingested gametocytes to infectious sporozoites. With use of a rodent malaria model, it was found that fungal biopesticides reduced transmission potential by 98% as long as mosquitoes became infected with the fungus soon after ingesting *Plasmodium* (9). A high probability of early infection is important to the success of fungal biopesticides, and the high coverage this requires may be hard to achieve in the field because of issues such as user resistance (10–12). *Metarhizium* can be engineered to kill insects faster (13), but a slow speed of kill that enables mosquitoes to achieve part of their lifetime reproductive output will reduce selection pressure for resistance to the biopesticide and translate into additional decades of effective product use (5, 10, 14). It would be highly desirable to obtain fungal strains that greatly reduce mosquito infectiousness, because this could improve disease control without increasing the spread of resistance (5). To achieve this effect, we engineered *M. anisopliae* to deliver molecules that selectively block parasite development within the vector.

Recombinant *M. anisopliae* strains were tested for their ability to block *P. falciparum* development in *Anopheles gambiae* (Africa's principal malaria vector) (15). The 12-amino acid salivary gland and midgut peptide 1 (SM1) binds to the surface of salivary glands, thus blocking the entry of sporozoites (16). We inserted into *M. anisopliae* a synthetic gene (termed [SM1]<sub>8</sub>) that expressed eight repeats of the SM1 peptide. The scorpion (*Pandinus imperator*) antimicrobial scorpine is a hybrid between a cecropin and a defensin but is 100-fold more potent than these against *Plasmodium* (17). The single-chain antibody PfNPNA-1 is based on a recombinant human monoclonal antibody that specifically recognizes the repeat region (Asn-Pro-Asn-Ala) of the *P. falciparum* surface circumsporozoite protein and agglutinates sporozoites (18). A gene expressing the hybrid protein [SM1]<sub>8</sub>:scorpine was produced by fusing the polymerase chain reaction (PCR) product of [SM1]<sub>8</sub> to scorpine. All four genes were synthesized with the MCL1 signal peptide at the N terminus (to drive secretion) (table S1) and cloned into a plasmid downstream of the *M. anisopliae*Mcl1 promoter (PMcl1) (fig. S1). PMcl1 only expresses transgenes when the fungus encounters the mosquito hemolymph (13). The antiplasmodial genes were individually transformed into *M. anisopliae*, and we selected single-copy transformants (fig. S2) with wild-type rates of growth and sporulation (fig. S3). We also confirmed that transgenes were expressed within

20 min of the fungus contacting hemolymph and that their products interacted with salivary glands or sporozoites (fig. S4).

We inoculated adult female mosquitoes with *Metarhizium* by spraying them with spore suspensions containing  $5 \times 10^5$  spores per ml or  $5 \times 10^6$  spores per ml, because this allowed the delivery of a reproducible inoculating dose of  $7 \pm 6$  (mean  $\pm$  SEM) spores per mosquito or  $90 \pm 8$  spores per mosquito, respectively. *A. gambiae* infected by the wild-type and transgenic *Metarhizium* strains showed similar life spans (table S2 and fig. S5), indicating that the transgenic strains would not increase selection for resistance compared to the wild-type pathogen. The blood-feeding activity of mosquitoes containing *Plasmodium* was reduced by  $\sim 58\%$  within 6 days of infection with 90 spores per mosquito and by  $\sim 30\%$  within 8 days of infection with 7 spores per mosquito (transgenic or wild type) (Table 1 and table S3).

We modeled the time course of expression of antiplasmodial proteins inside *Plasmodium*-infected mosquitoes by spraying them with transformant Ma-[SM1]<sub>8</sub> (90 spores per mosquito) and measuring the binding of [SM1]<sub>8</sub> to salivary glands by immunofluorescence microscopy. [SM1]<sub>8</sub> was detected on the surface of salivary glands 2 days after fungal inoculation, and fluorescent intensity peaked at 4 days (Fig. 1). To assess the malaria control potential of *Metarhizium* strains applied to mosquitoes with advanced malaria infections, we infected mosquitoes with 90 spores per insect 11 days after feeding on *Plasmodium*-infected blood cultures and counted salivary gland sporozoites on day 17. The parental wild-type *Metarhizium* strain did not significantly reduce sporozoite density compared to control mosquitoes not infected by *Metarhizium* ( $P > 0.05$ , Mann-Whitney test). Strains expressing [SM1]<sub>8</sub>, PfNPNA-1, and scorpine reduced sporozoite counts by  $71 \pm 2.4\%$ ,  $85 \pm 3.2\%$ , and  $90 \pm 2.5\%$ , respectively (Fig. 2). The fusion [SM1]<sub>8</sub>:scorpine reduced sporozoite density to a greater extent than did [SM1]<sub>8</sub> ( $P < 0.05$ , Mann-Whitney test), but the effect was significantly less than that of scorpine alone ( $P < 0.05$ , Mann-Whitney test). However, co-inoculating mosquitoes with an equal number of spores (totaling 90 spores per mosquito) containing the scorpine and [SM1]<sub>8</sub>:scorpine genes reduced sporozoite density from  $4715 \pm 585$  to  $105 \pm 21$  ( $98 \pm 0.7\%$ ), which was significantly ( $P < 0.05$ , Mann-Whitney test) greater than the reduction achieved by scorpine alone. Presumably sporozoites surviving free scorpine in the hemolymph are being challenged by [SM1]<sub>8</sub>:scorpine binding to the surface of salivary glands. Co-infecting mosquitoes with strains expressing PfNPNA-1 and scorpine likewise achieved a  $97 \pm 0.9\%$  reduction in sporozoite counts, significantly ( $P < 0.05$ , Mann-Whitney test) greater than the reduction achieved by either scorpine or PfNPNA-1 alone.

We next performed a time course study and measured sporozoite prevalence (% of sporozoite-positive salivary glands) and density in mosquitoes 12 to 17 days after feeding on *Plasmodium*-infected blood and 1 to 6 days after infection with *Metarhizium*. Sporozoites began to appear on salivary glands 14 days after feeding. Co-infecting mosquitoes with *Metarhizium* strains expressing the optimal combination of scorpine and [SM1]<sub>8</sub>:scorpine reduced sporozoite density by  $>95\%$  through days 14 to 17, whereas the wild-type *Metarhizium* strain did not significantly reduce sporozoite density (Fig. 3). At day 14,  $94 \pm 2\%$  of the mosquitoes not infected with *Metarhizium* had sporozoites, compared with  $25 \pm$

1% of insects infected with transgenic strains and  $87 \pm 3\%$  of insects treated with the wild type (Table 1). When the analysis is limited only to those mosquitoes with salivary gland infections, transgenic strains reduced sporozoite density from  $1724 \pm 394$  to  $150 \pm 30$  ( $91 \pm 0.4\%$ ) at day 14, and from  $4504 \pm 324$  to  $355 \pm 50$  ( $92 \pm 1\%$ ) at day 17 (fig. S6).

The potential of *Metarhizium* strains to reduce malaria transmission was estimated by combining their impacts on mosquito mortality, blood-feeding activity, and the prevalence of sporozoites (Table 1). Infection with transgenic strains 11 days after a *Plasmodium*-infected blood meal reduced transmission by  $78 \pm 1\%$  (a total of 14 days after blood meal),  $75 \pm 2\%$  (15 days),  $77 \pm 2\%$  (16 days), and  $91 \pm 1\%$  (17 days). Infection with wild-type *Metarhizium* reduced transmission by  $16 \pm 1\%$  (14 days),  $37 \pm 2\%$  (15 days),  $54 \pm 1\%$  (16 days), and  $81 \pm 3\%$  (17 days). Thus, expression of transgenes shortened the time taken to reduce transmission, allowing *Metarhizium* to be effective even if sporogony is well advanced by the time the strain contacts the mosquito. At day 14, when the difference in impact was greatest, the transgenic strains reduced transmission ~fivefold when compared with transmission with the wild type. This is a conservative estimate because it does not take into account the 91% reduction in sporozoite density in those insects with salivary gland infections. Estimates vary as to the minimum sporozoite inoculum required to establish an infection in humans, but *A. stephensi* mosquitoes and wild African *A. gambiae* with low salivary gland infections are unlikely to transmit sporozoites (19, 20), and studies in humans have shown a clear correlation between the ability to produce a malaria infection and the intensity of salivary gland infection (21, 22).

A lower dose of spores ( $7 \pm 6$  spores per mosquito) meant that it took 7 days to reach the level of scorpine expression and the titer of hyphal bodies achieved in 3 days by ~90 spores per mosquito (fig. S7). Infecting mosquitoes with the low-spore dose 7 days after a *Plasmodium*-infected blood meal reduced prevalence (fig. S8), sporozoite density (fig. S6), and malaria transmission (table S3) to similar levels as the high dosage. By day 18, the mortality of insects infected with transgenic or wild-type strains of *Metarhizium* at low or high doses was ~90%, and 100% were dead by day 20, whereas >80% of the mosquitoes infected with *Plasmodium* alone were still alive and carrying thousands of sporozoites.

Our study shows that entomopathogenic fungi engineered to produce antimalarial peptides can block transmission by mosquitoes with advanced malaria infections. In the field, this would reduce the current need for mosquitoes to become infected with fungi soon after they pick up the malaria parasite. Furthermore, the transgenic fungi still only kill older mosquitoes, so they do not increase selection for resistance development. The level of transmission blocking achieved by the transgenic *Metarhizium* strains exceeds that reported for most genetic engineering approaches involving transgenic refractory insects (23, 24) or insect commensals (25, 26). Furthermore, these means of malaria control require the fitness of the transgenic organism to be high. In contrast, the fitness of transgenic biopesticides could be quite low (5, 10), and there is no requirement for driving mechanisms to replace field populations with engineered lines because a transgenic pathogen need not recycle. Overall, therefore, field release of a pathogen is comparatively easily handled, and, because *M. anisopliae* can infect *A. gambiae*, *A. arabiensis*, and *A. funestus*, it is an approach that

need not be specific to one or a few interbreeding populations of *Anopheles*, leaving other populations to transmit disease.

A potential problem with relying on antimalarial effects is that they might in the long run suffer from the evolution of resistant malaria parasites. However, *Metarhizium* is a tractable model for evaluating and delivering transmission blocking proteins and could be used to express multiple transgenes with different modes of action to reduce the probability of emergence of resistance to one mechanism. The diversity of antimicrobials could also offer a general approach for controlling other devastating vector-borne diseases. Scorpine inhibits dengue virus too (27), and *M. anisopliae* can infect *Aedes aegyptii* (the dengue fever vector) (13). *M. anisopliae* also infects the filariasis mosquito *Culex quinquefasciatus* (28), and different strains of *M. anisopliae* pathogenic to tsetse fly or ticks have been identified (29, 30). The pathogens these arthropods vector are likely to be susceptible to scorpine and trypanosome lytic factor (27, 31). The ability of *M. anisopliae* to express a functional singlechain antibody fragment is notable because recombinant antibodies provide a vast array of potential antiparasite and anti-arthropod effectors. These would facilitate construction of very effective, highly specific, biopesticides with minimal increased potential for negative environmental impact relative to their parental wild-type strains.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

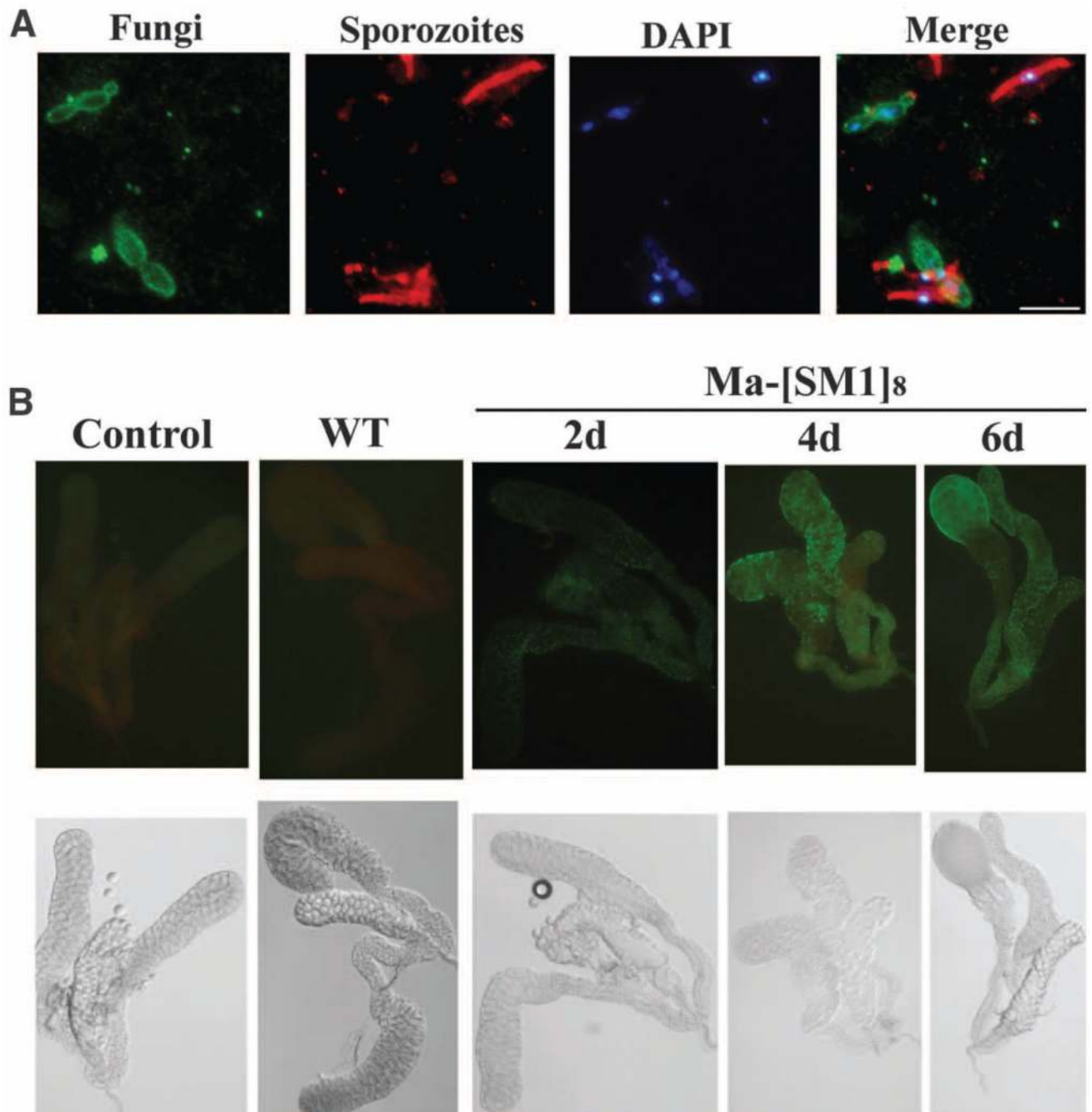
This work was supported by NIH grant 5R21A1079429-02.

## References and Notes

1. World Health Organisation. World Malaria Report. Geneva: World Health Organisation; 2009.
2. Müller P, Donnelly MJ, Ranson H. BMC Genomics. 2007; 8:36. [PubMed: 17261191]
3. Knols BG, Bukhari T, Farenhorst M. Future Microbiol. 2010; 5:339. [PubMed: 20210542]
4. Scholte E-J, et al. Science. 2005; 308:1641. [PubMed: 15947190]
5. Thomas MB, Read AF. Nat. Rev. Microbiol. 2007; 5:377. [PubMed: 17426726]
6. Lwetoijera DW, et al. Parasites & Vectors. 2010; 3:18. [PubMed: 20233423]
7. Howard AFV, Koenraadt CJM, Farenhorst M, Knols BGJ, Takken W. Malar. J. 2010; 9:168. [PubMed: 20553597]
8. Farenhorst M, et al. PLoS ONE. 2010; 5:e12081. [PubMed: 20711409]
9. Blanford S, et al. Science. 2005; 308:1638. [PubMed: 15947189]
10. Read AF, Lynch PA, Thomas MB. PLoS Biol. 2009; 7:0001.
11. Hancock PA, Thomas MB, Godfray HCJ. Proc. Biol. Sci. 2009; 276:71. [PubMed: 18765347]
12. Hancock PA. PLoS Comput. Biol. 2009; 5:e1000525. [PubMed: 19798436]
13. Wang C, St. Leger RJ. Nat. Biotechnol. 2007; 25:1455. [PubMed: 17994009]
14. Koella JC, Lynch PA, Thomas MB, Read AF. Evol. Applications. 2009; 2:469.
15. Materials and methods are available as supporting material on *Science* Online.
16. Ghosh AK, et al. PLoS Pathog. 2009; 5:e1000265. [PubMed: 19148273]
17. Conde R, Zamudio FZ, Rodríguez MH, Possani LD. FEBS Lett. 2000; 471:165. [PubMed: 10767415]
18. Chappel JA, Rogers WO, Hoffman SL, Kang AS. Malar. J. 2004; 3:28. [PubMed: 15283866]

19. Medica DL, Sinnis P. *Infect. Immun.* 2005; 73:4363. [PubMed: 15972531]
20. Beier JC, et al. *Med. Vet. Entomol.* 1991; 5:71. [PubMed: 1768903]
21. Boyd M, Stratman-Thomas W. *Am. J. Hyg.* 1933; 17:666.
22. James SP, Nicol WD, Shute PG. *Proc. R. Soc. Med.* 1932; 29:879. [PubMed: 19990731]
23. Ito J, Ghosh AK, Moreira LA, Wimmer EA, Jacobs-Lorena M. *Nature.* 2002; 417:452. [PubMed: 12024215]
24. James AA. *J. Exp. Biol.* 2006; 206:3817. [PubMed: 14506217]
25. Rio RVM, Hu Y, Aksoy S. *Trends Microbiol.* 2004; 12:325. [PubMed: 15223060]
26. Riehle MA, Jacobs-Lorena M. *Insect Biochem. Mol. Biol.* 2005; 35:699. [PubMed: 15894187]
27. Carballar-Lejarazú R, et al. *Cell. Mol. Life Sci.* 2008; 65:3081. [PubMed: 18726072]
28. Alves SB, Alves LFA, Lopes RB, Pereira RM, Vieira SA. *J. Appl. Ent.* 2002; 126:504.
29. Maniania NK. *Biocontrol Sci. Technol.* 2002; 12:59.
30. Zhioua E, Browning M, Johnson PW, Ginsberg HS, LeBrun RA. *J. Parasitol.* 1997; 83:815. [PubMed: 9379283]
31. Thomson R, Molina-Portela P, Mott H, Carrington M, Raper J. *Proc. Natl. Acad. Sci. U.S.A.* 2009; 106:19509. [PubMed: 19858474]

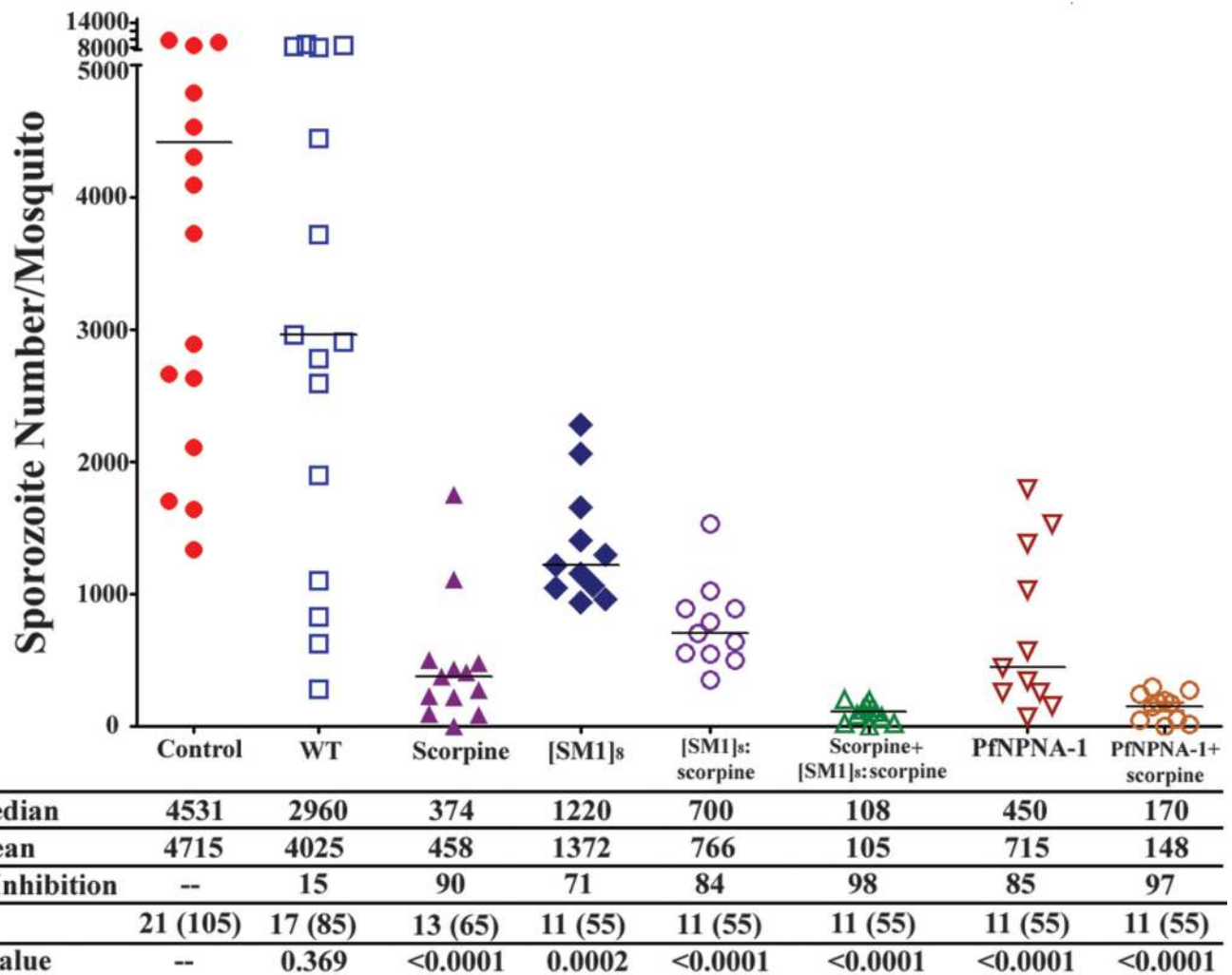




**Fig. 1.** (A) Coexistence of *Metarhizium* hyphal bodies and *P. falciparum* sporozoites in the hemolymph of infected mosquitoes. Hemolymph from mosquitoes 17 days after a *Plasmodium*-infected blood meal and 6 days after infection with *M. anisopliae* shows immunostained *M. anisopliae* (green) and *P. falciparum* (red). DNA was stained with DAPI (blue, 4',6'-diamidino-2-phenylindole). Scale bar indicates 10  $\mu$ m. (B) The timing of expression of [SM1]<sub>8</sub> by *M. anisopliae*-[SM1]<sub>8</sub> was estimated by detecting fluorescently immunostained [SM1]<sub>8</sub> binding to mosquito salivary glands. (Top row) Salivary glands with

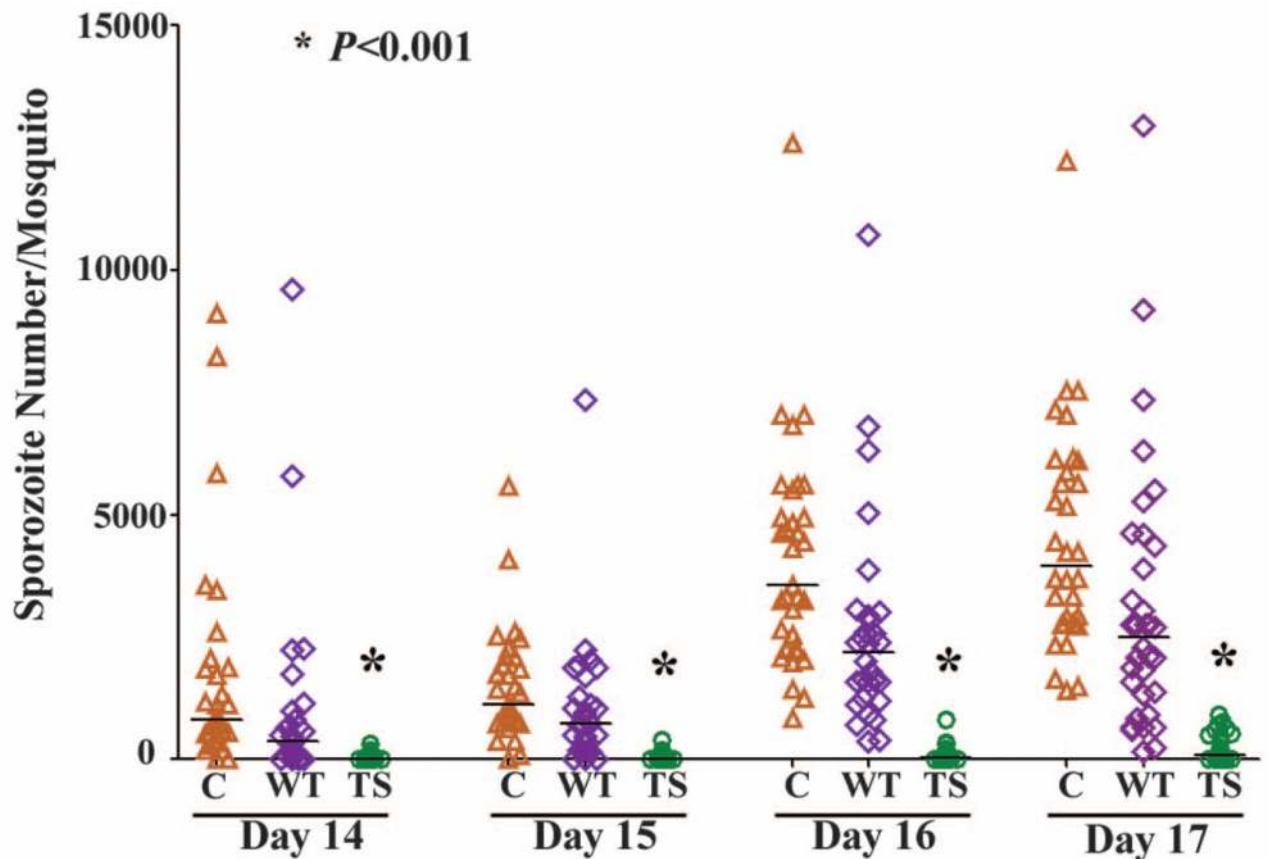
fluorescently stained [SM1] 2, 4, and 6 days postinfection. The intensity of fluorescent signal peaked at 4 days. (Bottom row) Differential interference contrast images of the salivary glands shown in the top row. Control, control salivary glands were from mosquitoes not infected with *M. anisopliae*; WT, salivary glands from mosquitoes infected with wild-type *M. anisopliae*.





**Fig. 2.**

Expression of antiplasmodials by *M. anisopliae* inhibits sporozoite invasion of salivary glands. Mosquitoes were infected with the wild-type or transgenic *Metarhizium* strains 11 days after a *Plasmodium*-infected blood meal, and 6 days later salivary glands from each treatment were dissected, pooled into groups each containing glands from five mosquitoes, and homogenized for sporozoite counting. *M. anisopliae* strains were engineered to express [SM1]<sub>8</sub>, PfNPNA-1, scorpine, and an [SM1]<sub>8</sub>:scorpine fusion protein. Mosquitoes were also co-inoculated with *M. anisopliae* strains expressing scorpine and an [SM1]<sub>8</sub>:scorpine fusion protein or with strains expressing scorpine and PfNPNA-1. *N*, the number of groups (the total number of mosquitoes is shown in parenthesis). Statistical significance was determined by Mann-Whitney test,  $\alpha = 0.05$ . The *P* values given in this figure were calculated by comparing each combination of *Plasmodium*- and *M. anisopliae*-infected mosquitoes versus control mosquitoes only infected with *Plasmodium*. The horizontal lines represent the medians.



<b>Median</b>	812	370	0	1110	730	0	3570	2183	40	3957	2500	85
<b>Mean</b>	1623	986	39	1513	1057	56	4121	2598	106	4504	3096	209
<b>% Inhibition</b>	--	39	98	--	30	96	--	37	97	--	31	95
<b>N</b>	34	32	36	31	30	33	33	30	30	32	34	34
<b>% Prevalence</b>	94	87	25	97	91	38	100	100	53	100	100	58

**Fig. 3.** Sporozoite prevalence and density in mosquitoes infected by transgenic (TS) *Metarhizium* strains expressing scorpine and [SM1]<sub>g</sub>:scorpine. Mosquitoes were each infected with ~90 *Metarhizium* spores 11 days after a *Plasmodium*-infected blood meal, and salivary glands were scored for sporozoites. Sporozoites were first detected on day 14. The absence of sporozoites determined microscopically was confirmed by PCR (fig. S9). C, control mosquitoes infected with *Plasmodium* only; WT, mosquitoes infected with *Plasmodium* and the wild-type *M. anisopliae* strain. Statistical significance was determined by Mann-Whitney test,  $\alpha = 0.05$ . The horizontal lines represent the medians.

Table 1

Estimated reductions in malaria transmission in *Metarhizium*-treated mosquitoes (estimates do not include the impact of *Metarhizium* strains on sporozoite intensity). About 90 spores per mosquito were applied to mosquitoes 11 days after they had fed on *Plasmodium*-infected blood. C indicates control mosquitoes infected with *P. falciparum* only; WT, infected with *P. falciparum* and wild-type *Metarhizium*; and TS, infected with *P. falciparum* and transgenic *Metarhizium* strains expressing scorpine and [SM1]<sub>8</sub>:scorpine. Prevalence is percent of mosquitoes with sporozoites in salivary glands. Mosquitoes able to transmit malaria were calculated by equation S.1 (10). Reduction in malaria transmission was calculated by equation S.2 (10).

Days after feeding on infected blood	14			15			16			17		
	C	WT	TS	C	WT	TS	C	WT	TS	C	WT	TS
% Mortality	0	3 ± 1.2	4 ± 0.9	0	21 ± 3	23 ± 2	3 ± 1.2	40 ± 8	40 ± 6	5 ± 1.8	60 ± 7	61 ± 5
% Prevalence	94 ± 2	87 ± 3	25 ± 1	97 ± 1	91 ± 2	38 ± 2	100	100	53 ± 3	100	100	58 ± 4
% Feeding	92 ± 5	85 ± 2	84 ± 2	90 ± 4	77 ± 5	77 ± 6	86 ± 6	64 ± 7	62 ± 5	82 ± 9	42 ± 4	41 ± 3
% Mosquitoes able to transmit malaria	86 ± 2	72 ± 2	20 ± 1	87 ± 3	55 ± 2	22 ± 2	83 ± 2	38 ± 1	19 ± 1	78 ± 1	17 ± 2	7 ± 1
% Reduction in malaria transmission	0	16 ± 1	78 ± 1	0	37 ± 2	75 ± 2	0	54 ± 1	77 ± 2	0	81 ± 3	91 ± 1