MALARIA CONTROL

Transgenic *Metarhizium* rapidly kills mosquitoes in a malaria-endemic region of Burkina Faso

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Malaria control efforts require implementation of new technologies that manage insecticide resistance. *Metarhizium pingshaense* provides an effective, mosquito-specific delivery system for potent insect-selective toxins. A semifield trial in a MosquitoSphere (a contained, near-natural environment) in Soumousso, a region of Burkina Faso where malaria is endemic, confirmed that the expression of an insect-specific toxin (Hybrid) increased fungal lethality and the likelihood that insecticide-resistant mosquitoes would be eliminated from a site. Also, as Hybrid-expressing *M. pingshaense* is effective at very low spore doses, its efficacy lasted longer than that of the unmodified *Metarhizium*. Deployment of transgenic *Metarhizium* against mosquitoes could (subject to appropriate registration) be rapid, with products that could synergistically integrate with existing chemical control strategies to avert insecticide resistance.

An estimated 2 billion people live in areas where mosquito-borne diseases are endemic. The burden is heaviest in sub-Saharan Africa, where >200 million cases of malaria are reported annually and many children succumb to the disease (*1*). Pyrethroid-impregnated bed nets or indoor residual spraying on walls and ceilings represents the mainstay of malaria vector control (*2*). However, in recent years anopheline malaria vectors in sub-Saharan Africa have gained sufficient resistance to render chemical insecticides ineffective (*3*, *4*). There is consequently a pressing need for practical alternatives for malaria control that can overcome insecticide resistance (*1*). Some *Metarhizium* spp. have strains pathogenic to adult mosquitoes and are considered to be environmentally friendly (*5*, *6*). The application of *Metarhizium* spores inside traditional houses in Tanzania reduced the number of infectious bites, but complete protection was prevented by the pathogen's low virulence (slow killing and high inoculum loads) and low persistence (*7*). To remedy these deficiencies, we engineered a strain of *Metarhizium pingshaense* (Mp-Hybrid) to express the U.S. Environmental Protection Agency (EPA)–approved calcium-activated potassium (K_{Ca}) and voltage-gated calcium (Ca_V) blocker ω/κ-hexatoxin-Hv1a (Hybrid) under the control of a hemolymph-specific promoter. In laboratory studies, Mp-Hybrid killed mosquitoes faster and at lower spore doses than wild-type (WT) fungus (*8*).

Because of the complexity of environmental conditions, we cannot be certain of the extent to which lab efficacy data can be extrapolated to a field situation. With an approach similar to the pathway proposed in the World Health Organization (WHO) *Guidance Framework for Testing of Genetically Modified Mosquitoes*, the next step before epidemiological studies is to test Mp-Hybrid outside the laboratory in conditions simulating nature (*9*). As nature is too complex to mimic indoors, a contained field trial provides the best means to assess pathogenic efficacy as measured by survivability, increased fungal lethality, and the likelihood that insecticide-resistant mosquitoes will be eliminated. In this study, we have shown that a transgene can substantially improve the performance of a malaria vector control intervention in a setting where the disease is endemic.

The trial was conducted in a contained, semifield facility (MosquitoSphere) (fig. S1) near a rural village in a region of Burkina Faso where malaria is endemic, as part of an international effort to advance the use of transgenic approaches for malaria control. Burkina Faso, with >7.9 million cases of malaria in 2017, is one of 10 high-burden countries that were not on track to meet their WHO Global Technical Strategy targets for malaria in 2017 (*1*). The sphere consisted of six compartments—four of which contained WHO experimental huts for West Africa (*10*), sugar sources (plants) for adult mosquitoes, and breeding sites (plastic sheets buried in a layer of soil to allow the collection of water)—enclosed in a greenhouse frame with walls of mosquito netting to allow exposure to ambient climate conditions and simulate a natural mosquito habitat. Insecticide-resistant *Anopheles coluzzii* mosquitoes for our experiments were collected as larvae from local breeding sites and reared to adulthood in a compartment of the sphere. The sphere was purposely built to compare the efficacy of Mp-Hybrid coexpressing green fluorescent protein (GFP) with that of Mp-RFP, a strain with WT virulence expressing red fluorescent protein (RFP), against *A. coluzzii* mosquitoes. On the basis of previous studies (*6*), we found that suspending *Metarhizium* in locally produced sesame oil and spreading the suspension on black cotton sheets (fig. S2) achieves a long-term effect in the sphere, and these sheets provide a resting area for mosquitoes that have taken blood meals from calves in the huts.

In initial experiments, we released 100 female, insecticide-resistant *A. coluzzii* mosquitoes into each hut at dusk, allowed them to blood feed from a calf, and then collected them the next morning to monitor fungal infections (confirmed by observing fluorescent hyphae emerging from mycosed cadavers). In seven replicates, with cloths rotated between each compartment, we individually collected a total of 2402 mosquitoes and recorded their feeding status and location of capture. Overall, the majority of mosquitoes were caught on the black cloth (43.5%), with the ceiling being the second most common place for mosquitoes to be found resting (27.3%) (fig. S3). Of the mosquitoes recaptured the next morning, 93.2% were blood fed. These findings were the same for compartments containing control sheets with no fungi or sheets impregnated with Mp-RFP or Mp-Hybrid, showing that mosquitoes are not repulsed from black sheets by *Metarhizium* spores applied in this fashion. Throughout all of our experiments, none of the mosquitoes that had been collected from the compartments containing control sheets were infected with fungus. Of the *A. coluzzii* mosquitoes that were collected in the compartments with Mp-RFP (Mp-Hybrid)–impregnated sheets, 72.3% (79.5%) were infected with Mp-RFP (Mp-Hybrid) (Fig. 1): Thus, the effective coverage of our respective treatments is ~75%. A subsample of the ~25% of surviving mosquitoes was washed, and the rinse was plated on agar for counts of fluorescent colony-forming units. None were seen, which suggests that the leveling out at 20 to 25% survival is due to mosquitoes not coming into contact with the treatment over the evening of exposure, whereas infected mosquitoes typically picked up ~130 conidia from sheets (*8*).

Mp-infected *A. coluzzii* had significantly shorter life spans than uninfected mosquitoes, and within 2.5 days mosquitoes exposed to Mp-Hybrid were dying faster than those exposed to Mp-RFP. For infected mosquitoes, median lethal times for Mp-Hybrid and Mp-RFP were 5.19 ± 0.163 and 8.73 ± 0.295 days, respectively [the mean ± standard error (SE) is reported]. Fluorescent *Metarhizium* mycosis was observed on fungus-exposed cadavers, confirming mortality caused by treatment. The number of mosquitoes surviving from the uninfected control compartments never dropped below 83.7% (Fig. 1).

In the next series of experiments, we sought to establish self-sustaining *Anopheles* populations in the MosquitoSphere to determine how hypervirulence affects the generational control of mosquito populations. Initially, 1000 virgin males (sufficient males for a swarm) and 500 virgin

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females were released into each compartment; regular blood meals were provided by introducing a calf into each hut for 3 nights each week; and we daily monitored the presence of larvae, pupae, and emergent adults in each of four artificial breeding sites per compartment. Emerging adults were captured for counting in muslin netting suspended over the breeding sites and were then released into the compartmental population. The number of adults present within each compartment was also counted daily. In our first replicate, at the peak of the rainy season, adult mosquito numbers in the control compartment (containing cloth treated with sesame oil alone) increased by 921 and 1396, respectively, in the F_1 and F_2 generations, which were ~25 days apart (Fig. 2). In the hut provided with an Mp-RFP–impregnated sheet, the F_1 and F_2 populations increased by 436 and 455 adults, respectively. In huts provided with Mp-Hybrid, the F_1 and F_2 generations were 399 adults and 13 adults, respectively, indicating a collapsed population. We ran three replicates of this experiment, each lasting an average of 45 days, which is a considerable portion of the rainy season. The second two replicates had higher background attrition of mosquitoes as conditions deteriorated with the approaching dry season (Fig. 2). However, the same strong and significant trend was observed in all three replicates: that is, significant decreases in each generation of offspring in compartments with Mp-Hybrid, generational population growth in the control compartments, and stagnation of mosquito populations in the Mp-RFP compartment. The number of adults present after the F_2 generation in three replicates with Mp-Hybrid was reduced by 91.8 to 92.7% compared with those in replicates with Mp-RFP (Fig. 2).

Although mosquitoes in a compartment with Mp-Hybrid showed the lowest reproductive success, larvae appeared in breeding sites for all three replicates 1 to 2 days earlier than larvae from uninfected control mosquitoes; larvae were observed 1 day earlier in two of the three replicates with Mp-RFP than in the uninfected control (Fig. 2). Laboratory experiments reproduced this effect, with significantly ($P < 0.05$) earlier oviposition by Mp-Hybrid–infected mosquitoes (5.42 ± 0.727 days) than by untreated mosquitoes (7.02 ± 0.640 days). This effect was not significant ($P = 0.893$) for mosquitoes treated with Mp-RFP (6.88 ± 1.40 days). Dying gravid mosquitoes can sometimes bring forward their oviposition schedule, presumably to compensate for reduced chances of future reproduction (*11*). This is the first evidence that transgene expression can induce an increased terminal investment response, and it could potentially undermine transgenic control approaches by affecting the evolution of infected mosquitoes. However, Mp-Hybrid–infected mosquitoes displayed a strong decrease in both the quality and the quantity of the eggs they laid, resulting in many fewer progeny than those of Mp-RFP–treated and untreated mosquitoes (figs. S4 and S5). Only 24.7 ± 4.20% of Mp-Hybrid–infected females laid eggs, compared with 77.8 ± 3.65% of untreated mosquitoes ($P < 0.05$) (figs. S5 and S6). An average uninfected female mosquito laid 139 eggs, with 74 (53.3%) of these developing into adults, whereas an average Mp-Hybrid–infected female laid 26 eggs, with only 3 (12.9%) developing into adults (Fig. 3). Our laboratory and field data indicate that mosquitoes infected with Mp-Hybrid are laying fewer, poorer-quality eggs earlier than untreated mosquitoes.

The persistence of the fungus on treated surfaces is a key factor in determining the ultimate success of the biopesticide approach (*12*). We conducted conidial viability checks for 9 weeks of a rainy season to test the hypothesis that transgenic fungi will achieve longer-term control of mosquito populations than WT strains. Conidia on impregnated sheets gradually lost effectiveness (figs. S7 and S8). However, because Mp-Hybrid kills at much lower spore doses than the WT, sheets impregnated with Mp-Hybrid met WHO standards (>80% mortality) through week 6,

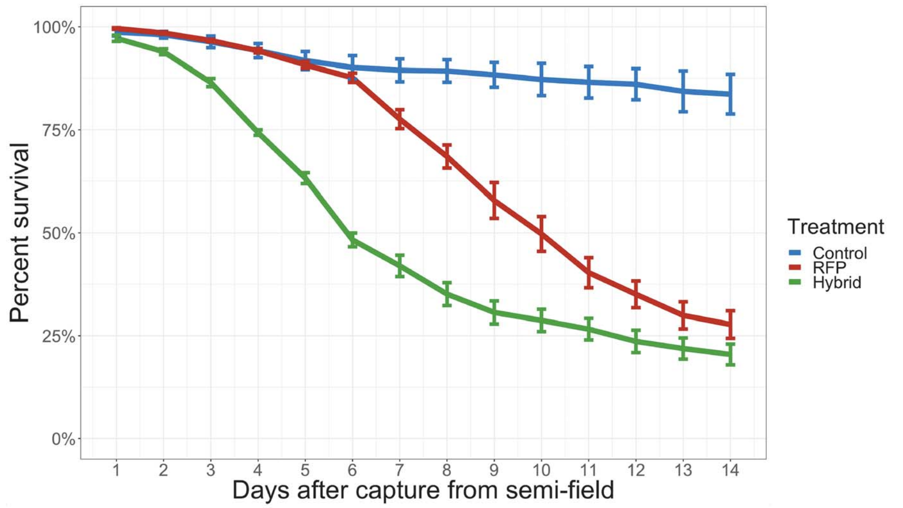

Fig. 1. Survival of mosquitoes exposed to transgenic fungi in the semifield trial. Survival curves for release-recapture experiments are shown. Mosquito survival ($n = 100$ mosquitoes per compartment) was scored after a single evening of exposure to Mp-RFP or Mp-Hybrid. Controls were exposed to neither fungus. The results are the means for seven replicates ± SE.

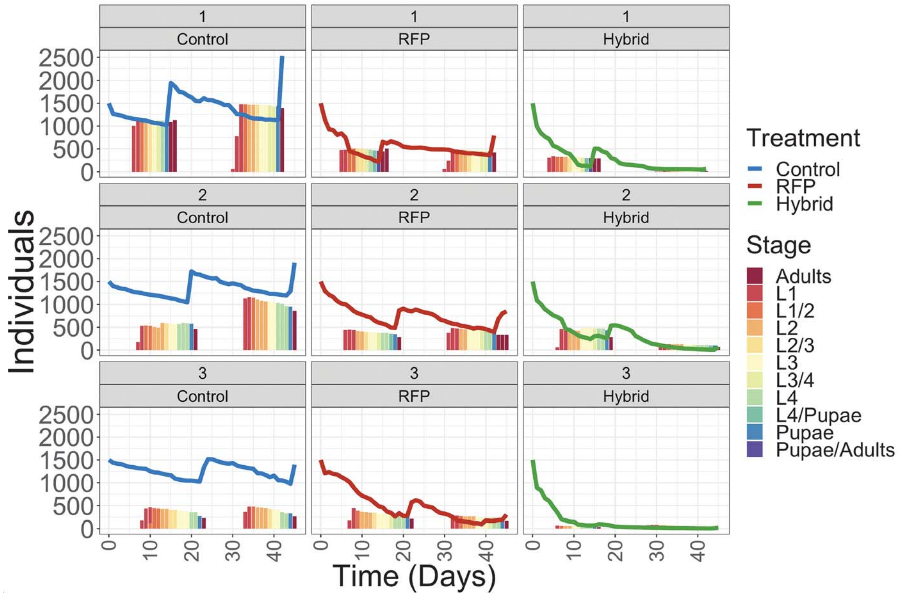

Fig. 2. Semifield-established population counts of F_1 and F_2 offspring. Graphs depict population counts of F_1 and F_2 offspring after the release of 500 female and 1000 male mosquitoes. Bars represent visual counts of the number of individuals per day in compartmental breeding sites at each developmental stage; lines represent the number of adults in the entire compartment. Treatments were monitored for two generations (45 days in duration). The results from three replicates (1, 2, and 3) are shown. L, larval instar.

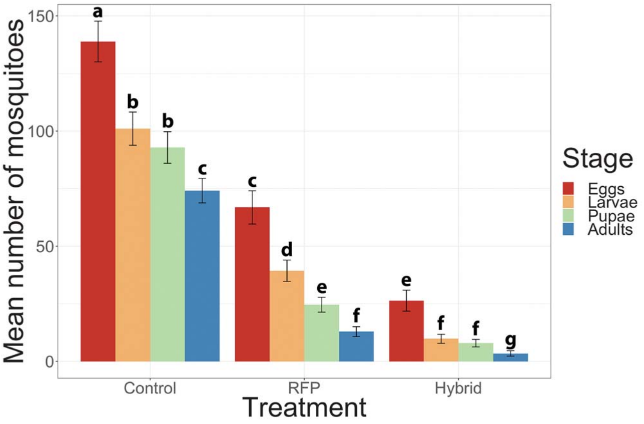

Fig. 3. Effect of fungi on fecundity. The graph shows the mean numbers of eggs, larvae, and pupae produced per individual female in laboratory experiments. The mean ± SE is reported (n = 80 females, n = 70, and n = 71 for untreated, Mp-RFP, and Mp-Hybrid mosquitoes, respectively). Letters indicate significant differences among groups ($P < 0.05$).

whereas the efficacy of the Mp-RFP no longer met that threshold after 4 weeks. From weeks 5 through 7, Mp-Hybrid killed about twice as many mosquitoes as Mp-RFP. Therefore, the long-term control potential of Mp-Hybrid is increased relative to that of the parental strain (figs. S7 and S8).

Our results show that Mp-Hybrid's increased virulence and lower inoculum load have produced a product that is more robust and easier to use than the WT fungus in a setting where malaria is endemic. This finding, together with the greatly reduced blood-feeding propensity of Mp-Hybrid–infected mosquitoes (*8*), means that Mp-Hybrid could affect malaria transmission over a broader range of lethal, prelethal, and generational conditions than unmodified *Metarhizium*. *Metarhizium* biopesticides are already registered for agricultural use in a number of African countries (*13*). The compelling urgency posed by insecticide-resistant mosquitoes has incentivized in-country regulatory agencies to engage with issues raised by transgenic *Metarhizium* and has helped to build relevant regulatory experience that may also be applied to a plethora of alternative strategies offered by transgenic entomopathogens (*14*), as well as genetically modified (GM) anophelines, when such technologies are ready for application. In contrast to GM mosquitoes, a genetically engineered *Metarhizium* strain (*15*) was approved by the EPA for use in an outdoor field trial, and transgenic microbial pest control agents have already been marketed without fanfare (*16*), providing precedents for our work. The insect neurotoxin Hybrid (traded as Versitude) was recently approved by the EPA to control lepidopteran pests and thus provides a good foundation for the current work. However, *Metarhizium* strains expressing multiple toxins are lethal at doses as low as one spore per mosquito (*8*), which will enable further improvements to efficacy once the safety of these toxins toward noninsects is confirmed.

In contrast to GM mosquito approaches, Mp-Hybrid is not limited to targeting a single interbreeding population of mosquitoes (*8*). However, the narrow host range of Mp-Hybrid (tested against a panel of beneficial and pest insects) limits possible threats to nontarget insects (*8*). Furthermore, substantial off-site dispersal of Mp-Hybrid is unlikely given that its large, sticky, ultraviolet light–sensitive spores do not naturally become airborne, and application methods such as the attachment of spores to sheets in residential use sites can further reduce opportunities for movement (*17*). *Metarhizium* spp. sporulate after host death on the insect cadaver, but scavengers can intervene before sporulation occurs, preventing natural means of dispersal (*18*). Because of these factors, transgenes in entomopathogenic fungi may be much easier to contain than mosquito transgenes. Genetic containment methods developed for these fungi offer additional solutions to further allay any concerns from stakeholders (*19*).

Deployment against mosquito populations could therefore (subject to robust conference with relevant stakeholders as well as regulatory bodies) be rapid, with products integrating readily into existing control strategies and regulatory protocols. In particular, Mp-Hybrid could be applied alongside insecticides to maximize effects and to manage resistance; although unmodified biopesticides can also work synergistically with pyrethroid insecticides, these effects are observed earlier and are more pronounced in Mp-Hybrid (*20*, *21*). These synergistic effects are observed earlier and are more pronounced with Mp-Hybrid than with the WT fungus (*22*). Taken together, these results suggest that the introduction of a single transgene under the control of a native fungal promoter substantially increases both the efficacy and the applicability of fungal entomopathogens for malaria vector control, justifying further development of transgenic entomopathogenic fungi for open field application.

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ACKNOWLEDGMENTS

We recognize the indispensable efforts of our local volunteers (Z. Olivier, D. Justin, O. Moussa, S. Boureima, O. Bamory, S. Michael, O. Bema, and O. Yaya) and the support of the Soumousso community. We thank S. Touro for ensuring local administration of this project in Centre Muraz/IRSS. **Funding:** This work was supported by NIH-NIAID under award RO1-AI106998 to R.J.S. and A.D. **Author contributions:** R.S.L., A.D., B.L., E.B., and R.D. designed the research. R.S.L., A.D., B.L., and E.B. contributed to data analysis and wrote the manuscript. E.B., B.L., S.A.M., A.K.O., I.S., and E.J.G. curated and collected the data. **Competing interests:** All authors declare that they have no competing interests. **Data and materials availability:** All data are available in the main text or supplementary materials. R code to conduct this analysis is available in GitHub (*23*) and is archived at Zenodo (*24*).

SUPPLEMENTARY MATERIALS

science.sciencemag.org/content/364/6443/894/suppl/DC1
Materials and Methods
Figs. S1 to S8
Data S1 to S5

31 January 2019; accepted 7 May 2019
10.1126/science.aaw8737

Science

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Science **364** (6443), 894-897.
DOI: 10.1126/science.aaw8737

Targeting mosquitoes to target malaria

Anopheline mosquitoes transmit the malaria parasite, but the insects themselves are prone to their own infections. Lovett *et al.* engineered a specific fungal pathogen of anophelines to carry insect-selective toxins. The effectiveness of this fungus for controlling mosquitoes was trialed in near-field conditions in Burkina Faso in a setup called MosquitoSphere. Approximately 75% of wild insecticide-resistant mosquitoes released into the environment became infected with the transgenic fungus, causing population collapse within 45 days.

Science, this issue p. 894

Science (print ISSN 0036-8075; online ISSN 1095-9203) is published by the American Association for the Advancement of Science, 1200 New York Avenue NW, Washington, DC 20005.