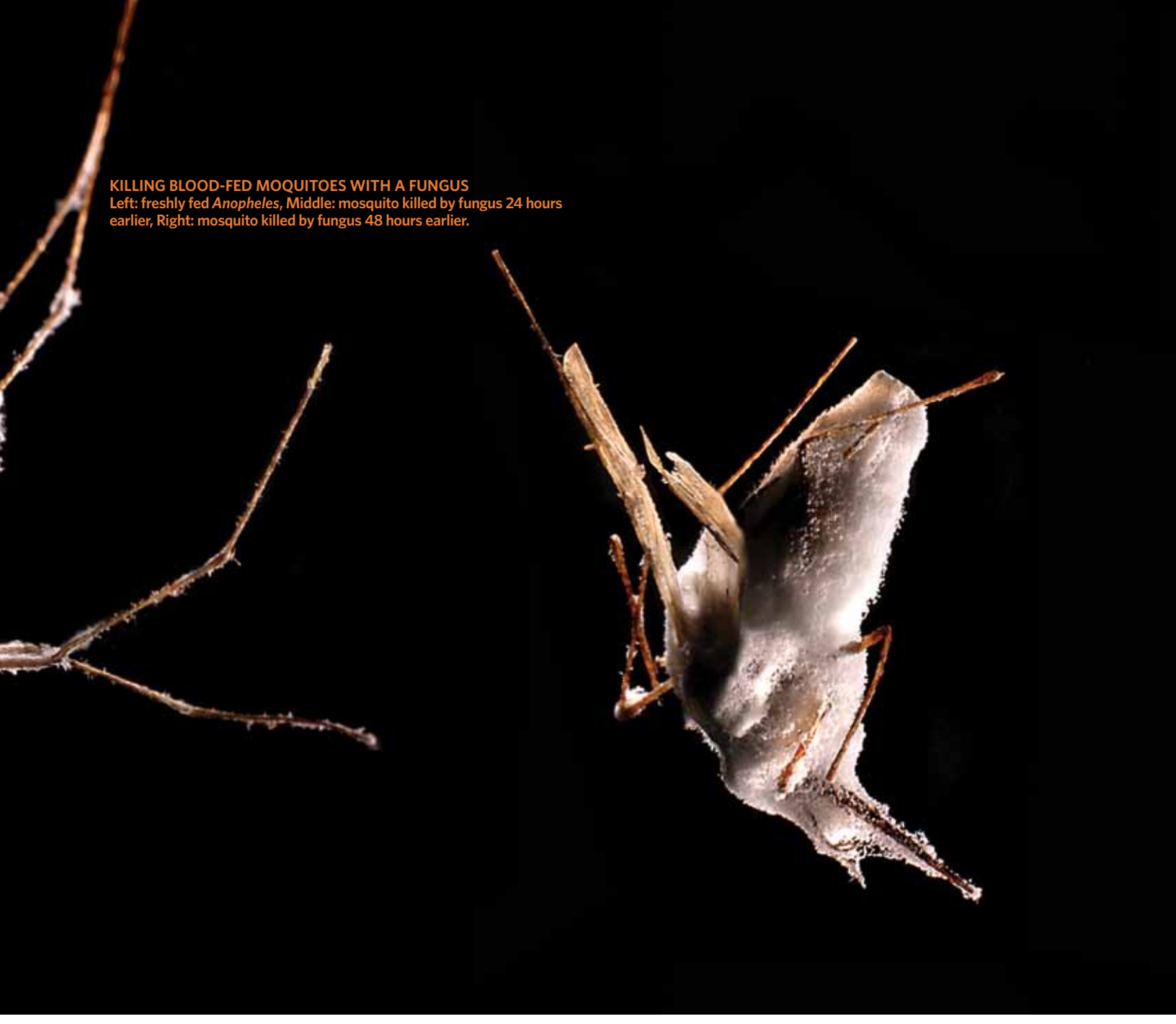
Evolution, Resisted

SCIENTISTS ARE TRYING TO DESIGN THE LAST MALARIA CONTROL AGENT THE WORLD WILL EVER NEED. **By Elie Dolgin**

Image by Hugh Sturrock

Entomologist Simon Blanford attaches a spray nozzle onto the top of a jar of white-powdered fungus immersed in a concoction of mineral oils. He leans forward into a fume hood and applies an even coating of fungal spores onto cut-up strips of disposable coffee cups taped against the back wall.

The next morning, after the sopping wet strips have dried, Blanford, a senior research associate at Pennsylvania State University in State College, will return to put the cups back together. Then he'll toss in a load of young *Anopheles* mosquitoes that have just eaten a malaria-ridden blood meal, cover the cups with a mesh lining, and wait. One week later, the vast majority of the mosquitoes will die, victims of the fungus that rubbed off on their bodies from the coated cups. At least, Blanford wants it to be 1



KILLING BLOOD-FED MOQUITOES WITH A FUNGUS
Left: freshly fed *Anopheles*, Middle: mosquito killed by fungus 24 hours earlier, Right: mosquito killed by fungus 48 hours earlier.

week later, which is just short enough to prevent the transmission of malaria, but long enough to potentially circumvent the evolution of insecticide resistance—indefinitely.

Malaria kills around a million people each year, and mosquitoes have developed resistance to nearly every chemical that public health officials have thrown at them. This has rendered most existing insecticides ineffective, so new practical alternatives are critically needed. With the fungus, "we've got a product that can break resistance to insecticide now but will also work in the long run," says Andrew Read, a Penn State evolutionary biologist who is spearheading the project with his collaborator, ecological entomologist Matthew Thomas. Other scientists are trying to achieve the same feat—a new malaria treatment that also discourages resistance—using other biological control agents, such as a bacterium that shortens its host's life, and through genetic engineering. "If you design the thing right from scratch you only need one product and it should last forever," says Thomas.

But many scientists are less enthusiastic. Judging from past failures, they dismiss the Penn State researchers' plans as lofty pipe dreams. Plus, to make this goal a reality, the scientists would have to release these "biopesticides" worldwide, raising red flags about feasibility issues and potential risks.

But it's precisely the fact that this project is far-reaching and different that makes it exciting, says Thomas. "It is radical thinking," he says. "It could be life changing. I genuinely think this will work." ▶

Seven years ago, Read and Thomas, then both working in the United Kingdom, began investigating ways of using fungal species purely as a cheap, green alternative to chemical pesticides for malaria control, not as any fundamentally new approach that would halt resistance. Previously, Thomas and his wife, mycologist Nina Jenkins, were part of an international team that had developed a commercially available fungal product to target locusts and grasshoppers. "So having done all that," says Thomas, "we figured it'd be much easier to do it a second time."

One of the problems with fungal-based control agents, however, is that they don't kill insects right away. This is a sticking point with crop-eating pests, but "with malaria it doesn't matter," Thomas says, because mosquitoes take a while to become infectious.

After an *Anopheles* mosquito bites someone carrying malaria, the *Plasmodium* parasite responsible for the disease traverses the lining of the mosquito's gut and starts multiplying. Many rounds of replication follow before the *Plasmodium* progeny migrate to the mosquito's salivary glands. Only then can the mosquito infect another host. Importantly, this whole process takes between 10 to 14 days, so as long as the fungus kills the infected insects within that crucial 2-week window, it should effectively block malaria transmission.

"IT'S A REALLY OUTSIDE-THE-BOX KIND OF IDEA."

—DON GARDINER

Simon Blanford sprays his fungal concoction onto a pair of cut-up coffee cups (left), which are later taped back together and loaded with mosquitoes (right).

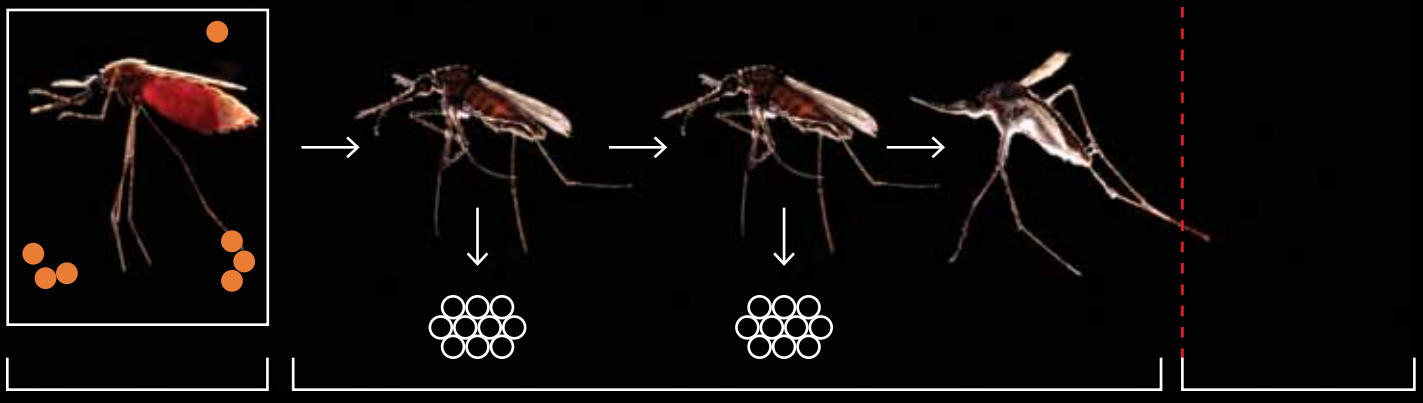
In 2005, Read and Thomas showed that this could work. Using a rodent malaria model, they found that treating ice cream tubs (they've since switched to cheaper coffee cups) with the fungal pathogen *Beauveria bassiana* killed more than 90% of mosquitoes within 14 days, and reduced the number of insects able to transmit malaria by a factor of 80.[1] At the same time, Bart Knols, a medical entomologist now at the University of Amsterdam, found that a fungal killer could be delivered in the field. His team hung cloths impregnated with *Metarhizium anisopliae* spores on the ceilings of five traditional houses in a rural Tanzanian village. After 3 weeks, Knols and his colleagues collected mosquitoes and found that 23% of *Anopheles gambiae* females—the mosquitoes that transmit most human cases of malaria—became infected and died several days earlier than uninfected controls.[2] "This could have a massive impact on the transmission of the disease," says Knols, also the managing director of K&S Consulting, a firm he cofounded that advises on infectious disease control.

A couple of years later, Read and Thomas finally realized the biggest advantage of the approach. They were preparing a review article focused on fungal pesticides for malaria control when "it dawned on us that actually maybe this [fungus] was not going to be imposing very strong selection for resistance just because of its late-life action," Read says. After the mosquito is exposed to the fungus, it lives for up to 2 weeks—enough time for two to six cycles of mosquito egg production—which means the doomed animals could comfortably reproduce, and there would be little reproductive advantage for insects that developed resistance to the fungus.

If there's hardly any selection for resistant insects, they realized, the vicious cycle of always needing to design new and better drugs might be halted—dead in the mosquitoes' tracks, as it were.

Then came the most important insight: "Wait, if that's true for the fungus it must be true for anything else" that kills only mosquitoes that have been given time to reproduce, says Read. Plus, the same approach should work for other insect-borne diseases, such as dengue fever, filariasis, and Japanese encephalitis, which also infect short-lived hosts in which the pathogen takes a while to become infectious.

How It Works



Read and Thomas's intuition stemmed from a decades-old idea that the force of natural selection acting on survival and fertility decreases with the age of an organism. Once individuals have passed their genes on to the next generation, there is little evolutionary pressure to keep them alive, so any beneficial mutations that act late in life generally confer less of an advantage than similar genetic changes operating at a young age. Thus, as long as the mosquitoes can feed and lay eggs, evolution will be largely blind to any modest differences in longevity.

After first proposing the idea in their 2007 review paper,[3] Read and Thomas then used mathematical models to show that if insecticides target older mosquitoes and if resistance to these insecticides poses a cost to individuals with that resistance, then these late-life acting agents might never be undermined by mosquito evolution.[4] Now that they had a theoretical concept, the duo could point to their earlier experimental data, which showed that it was possible to selectively kill only old, infected insects. "We proved it was practical before we had the idea, which is kind of an ass-backward way of showing it," Read says.

"It's a really outside-the-box kind of idea," says Don Gardiner, head of the Malaria Biology Laboratory at the Queensland Institute of Medical Research in Brisbane, Australia. "I would agree that you could essentially evolution-proof insecticides so that the mosquitoes don't develop resistance to them."

"It opens up the mind to new ways of searching for insecticides," says Joachim Kurtz, an evolutionary parasitologist at the University of Münster in Germany. Rick Paul, who studies malaria transmission at the Pasteur Institute in Paris, France, adds, "It's the first time we've ever used evolution properly at all in terms of any kind of tropical disease control."

FOR ANOTHER WAY SCIENTISTS ARE DESIGNING EVOLUTION-PROOF INSECTICIDES USING MICROSPORIDIA, GO TO THE-SCIENTIST.COM FOR AN ADDITIONAL SIDEBAR.

But many in the field remain deeply skeptical. Read and Thomas brazenly called their most recent theory paper: "How to make evolution-proof insecticides for malaria control." Many researchers felt that the title was a bit over the top, especially for a paper with no experimental data. "No entomologist should ever use the term 'evolution proof,'" says the University of California, Riverside's Brian Federici. "This type of rhetoric receives a lot of press, but mosquito gene pools are large and diverse, and so far these vectors have overcome everything humans have thrown at them."

"I'm not convinced that a late-acting insecticide would break the arms race" between mosquitoes and insecticides, agrees Martin Donnelly, who studies the evolution of insecticide resistance at the Liverpool School of Tropical Medicine. "Just because it's late-acting doesn't mean there aren't any fitness costs at earlier stages during development." Ary Hoffmann, an evolutionary biologist at the University of Melbourne, Australia, points out that the authors only considered the selective advantage of reproduction in their model, and there are other reasons why natural selection might favor long life. Read and Thomas's model "is a little bit simplistic," he says, "and the situation in nature is going to be far more unpredictable than the picture that they paint." ▶

A gust of warm, humid air blows into a cramped, walk-in insect chamber at the Johns Hopkins Malaria Research Institute in Baltimore, Md. Molecular entomologist Grant Hughes lifts the mesh cover from a tray of stagnant water swimming with mosquitoes of all life stages. With a deep breath, he sucks up all the flying adults that emerged overnight using a huge pipette resembling a turkey baster, and blows the insects into a pint-sized paper tub. He then grabs an eye-dropper, and transfers all the pupae from the tray into a small, oblong puddle of water on a plastic dish.

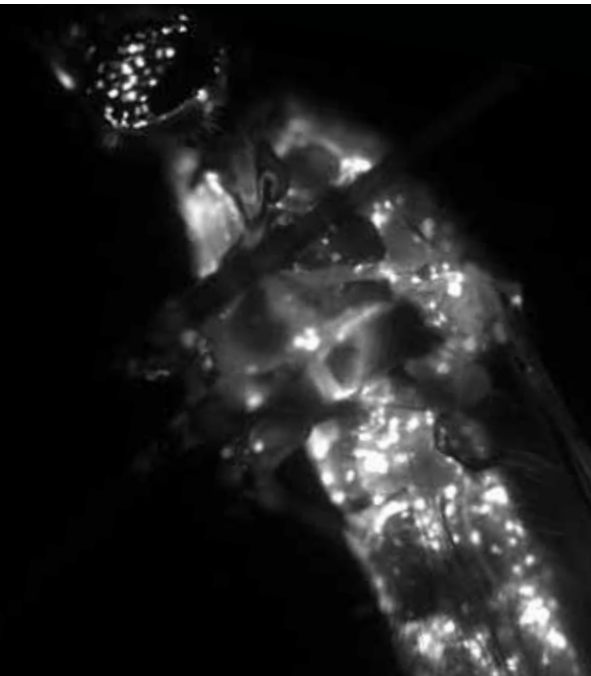
Last week, Hughes, a postdoc working with Jason Rasgon, injected female mosquitoes with *Wolbachia*, an inherited bacterium that infects more than half of all known insect species and can shorten life span, but is not known to naturally infect *Anopheles* mosquitoes, the main transmitters of malaria. Hughes mated the insects, gave them a blood meal, and let them lay eggs. Now he is collecting the offspring to check if the bacterial infection has taken hold. For the moment, the females are set aside. He'll sacrifice the males and inspect their cells' DNA for traces of the bacterium using molecular methods. "If the males are infected," says Hughes, "then I'll go and breed the females to start a population." Establishing a population of *Anopheles* mosquitoes that stably transmits *Wolbachia* bacteria from one generation to the next is one of the "holy grails of the *Wolbachia* community," says Rasgon. He has yet to get an infection to take hold, but he's making progress.

Wolbachia are passed on from infected female hosts to their offspring, so if researchers can get a pathogenic strain to stick inside the mosquitoes that transmit malaria, they might provide a self-perpetuating control method. In 2003, Rasgon published a theoretical paper showing that *Wolbachia* that shorten life span should be able to spread and kill mosquitoes early enough to completely block malaria transmission.[5] Like Read and Thomas, without realizing it, Rasgon had independently devised a strategy for "evolution-proof" vector control, although he didn't frame the idea as such at the time. In fact, late-acting lethality is integral to the approach, because *Wolbachia* intimately rely on the mosquito host to survive long enough to reproduce, and pass the infection on to offspring. "It's built into the system to have that sort of dynamic," Rasgon says.

A theory is one thing; getting it to work is another. Researchers have combed through more than 30 *Anopheles* species from four different continents to find *Wolbachia* symbionts, all with negative results. This led some to speculate that it was biologically impossible for *Anopheles* to be infected with the intracellular parasite, but Rasgon has forced *Wolbachia* into cell cultures, embryos, and adult *A. gambiae* mosquitoes. In May, he reported that a life-shortening strain from fruit flies—called *popcorn*—could be successfully injected into adults, although none of these infections ever relayed to the next generation.[6] "*Wolbachia* is everywhere in that mosquito," says Rasgon. "It's in the fat body; it's in the brain; it's everywhere except the ovaries. So something is excluding it from the ovaries. Maybe this is why *Anopheles* isn't infected in nature. It's not that they can't be infected but something blocks germ-line transmission. That's sort of what we're hypothesizing now."

Rasgon has some ideas for how to get around that cellular block, but declines to comment because others are racing to solve the same problem. In February, an international consortium led by Steven Sinkins, a molecular geneticist at the University of Oxford, received a multimillion dollar European Commission grant to truncate *Anopheles*'s life span using virulent *Wolbachia*. "I'm very confident that we'll be able to get [*Wolbachia*] in [*Anopheles*]," says Sinkins. "It's just a technically difficult thing to do."

Scott O'Neill, a geneticist at the University of Queensland in Brisbane, Australia, and a member of Sinkins's consortium, has already had some success, but with a different mosquito species. Earlier this year, O'Neill reported that he could infect *Aedes aegytpi* mosquitoes—the harbingers of dengue and yellow fever viruses—with the same life-shortening *Wolbachia*, which cut the insects' life span in half.[7]



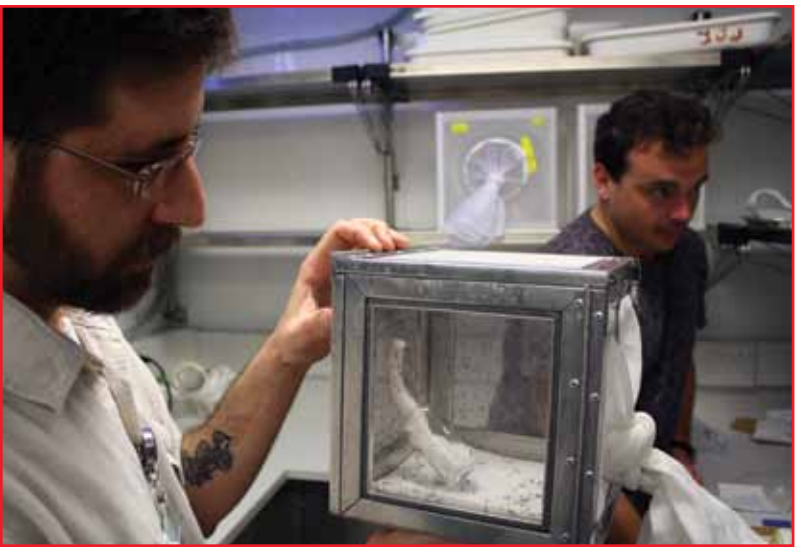
Evolution-proofing with a transgenic boost

In 2006, while trying to infect *Anopheles* mosquito cell lines with pathogenic *Wolbachia* bacteria, Johns Hopkins's Jason Rasgon had an unexpected surprise. His postdoc Xiaoxia Ren was using PCR to test for the presence of *Wolbachia* and an aberrant band kept cropping up in her negative controls. Ren brought this to the attention of her boss, but "I said, 'It's junk, don't worry about it, ignore it,'" recalls Rasgon. Ren didn't listen. "I was just curious," she says. So she sequenced the mystery PCR product anyway.

At first, Ren thought that Rasgon was right—it did just appear to be a garbage DNA sequence matching an unknown virus. Rasgon recalls, "She's like, 'Yeah, it was some virus,' and she turned to walk away. And I said, 'Wait, come back here. What virus?' 'Oh, some *Aedes* virus.' I said, 'OK, stop.'" They compared the phantom band's DNA to other known sequences and found that it most closely matched a densonucleosis virus, or "densovirus," that infects *Aedes* mosquitoes. But this cell line

An. gambiae adult infected with GFP-expressing densovirus

PLOS PATHOG, 4(8): E1000135, 2008.



Jason Rasgon (left) and Grant Hughes (right) are trying to infect mosquitoes with a life-shortening bacterium.

O'Neill attributes his achievement to a combination of luck and perseverance. After failing for years to introduce *Wolbachia* into the mosquitoes, O'Neill put the life-shortening *popcorn* strain into a mosquito cell culture "and just left it there," he says. A technician continued to maintain the cell line, transferring the culture medium every 4 days for 3 years before O'Neill "dusted off the project again." Remarkably, the mosquito cell line–adapted *Wolbachia* took hold when injected into *Ae. aegypti* embryos, and were stably transferred from one generation to the next. Perhaps a similar brute-force approach will be needed with *Anopheles*, says Sinkins. "We anticipate that it will take some time."

Wolbachia might not be as evolution-proof as the researchers imagine, however. Since the bacteria are vertically transmitted, Kurtz says, these "parasites" often evolve toward a more mutualistic relationship with their hosts, so eventually infection may no longer shorten life span. "This will always tend to be a problem," he says. Indeed, Hoffman showed 2 years ago that this is exactly what happened with *Wolbachia*-infected fruit flies in California over the last 20 years. And in June, he reported that some *popcorn*-infected fruit flies artificially selected for either early or late reproduction responded by living longer.[8] "When you have a bacterium that actually decreases life span then you're going to potentially get a situation where the host genome will shift to counter the effect of the bacterium," he says. "The costs of the insecticide resistance can evolve."

At Penn State, thousands of red blood cells are dancing on a microscope slide, whose image is projected onto a computer screen. Minutes ago, Read's research assistant Brian Chan withdrew a sample of blood from a malaria-infected mouse, and the drop in temperature from the rodent's warm body to room temperature has triggered the *Plasmodium* parasite to become active. Countless cells shift in shape as the malaria parasites thrash around inside like cats in a bag. Some rupture, and the eelish male gametes zip off in search of their female counterparts. The *Plasmodium* is infectious; the mouse is ready. Now, days-old mosquitoes can feast upon the malaria-ridden rodent before they are exposed to the lethal fungus. A week later, they will drop dead.

For the moment, the fungus is still in the experimental stages. But Read and Thomas hope to have enclosed field trials up and running soon. In the meantime, they are working to find chemicals that can also target just the old mosquitoes, as well as continuing to characterize different fungal isolates and their modes of action. They also want to scale up their fungal production capabilities so that the approach can readily be applied on an industrial scale. That's pretty straightforward, says Jenkins, who has adopted a production method used by commercial mushroom ▶

had never been anywhere near any *Aedes* species. Without realizing it, Ren had stumbled upon the first known densovirus naturally found in *Anopheles gambiae* (*PLoS Pathog*, 4:e1000135, 2008). The densovirus now provides another potential weapon in the much-needed arsenal against malaria. "It started off as a side project," says Ren. "But then the more we looked into it, the more interesting it became."

Densoviruses have been used as biological control agents against *Aedes* mosquitoes for decades, but never to target *Anopheles*. Part of the draw of the approach lies in its simple application: Densovirus-infected mosquito cell cultures can be ground up, sprinkled into the water where females lay their eggs, and the larvae rapidly become infected. The developing insects then continue to shed the virus into the water and pass on the parasite to their offspring, which allows both horizontal and vertical transmission. Although Rasgon's *Anopheles* densovirus does not appear to harm the mosquitoes, he is now trying to introduce genes that will release a late-acting toxin to kill the insects before they can transmit malaria—yet another technique that might circumvent mosquitoes' tendency to evolve resistance.

Bruce Hay, a geneticist at the California Institute of Technology in Pasadena, is also using genetic engineering to create what could provide another "evolution-proof process," he says. He created a synthetic selfish genetic element called *Medea* that could be used to drive a life-shortening gene through mosquito populations (*Science*, 316:597–600, 2007). "You'd be converting the population through genetic means to a live fast–die young life history," Hay says. "The logic behind all these approaches is exactly the same."

Many researchers are hesitant to use any genetic modification for fear of a backlash from the general public, but if it's a promising approach, it's worth pursuing, Rasgon says. "The feedback we've gotten has been generally supportive." ■

farmers. She grows the fungus in liquid culture, transfers spores to a cereal such as rice or barley, and then isolates a pure powder that can be stored indefinitely.

The Penn State researchers have also had discussions with an industrial partner, but declined to go into specifics, citing a confidential disclosure agreement. "We're pleased to be taken seriously," says Read. Many critics, however, continue to dismiss the approach. Some critics worry that if we select for mosquitoes to die earlier, then the *Plasmodium* parasite in turn will evolve to develop quicker. "We don't lose too much sleep over [this criticism] because there's already very strong natural selection for more rapidly developing malaria," says Read. As it is, most mosquitoes don't survive long enough in nature to transmit malaria. So if malaria could develop faster it would already have a huge advantage right now.

IMAGINE TRYING TO CONVINCE PEOPLE THAT THE BEST WAY TO COMBAT MALARIA IS TO ACTUALLY LET THE YOUNG, NONTRANSMITTING MOSQUITOES BREED INSTEAD OF TRYING TO KILL ALL INSECTS AS QUICKLY AS POSSIBLE.

A bigger problem might be public acceptance. Imagine trying to convince people that the best way to combat malaria is to actually let the young, nontransmitting mosquitoes breed instead of trying to kill all insects as quickly as possible. That message runs counter to conventional wisdom, not to mention World Health Organization guidelines that encourage people to avoid (via bednets) and kill (via insecticide sprays) whenever possible.

Janet Hemingway, director of the Liverpool School of Tropical Medicine, says it will be "extraordinarily difficult" to sell the late-acting control strategy in endemic malarial regions. "Trying to tell an uneducated villager in rural Tanzania or elsewhere that [letting more mosquitoes survive] doesn't matter is really not going to wash, I suspect." Thomas Scott, director of the University of California Mosquito Research Laboratory in Davis, agrees. "Are people really going to buy a product that they don't think is protecting them?"

Read and Thomas brush off this pessimistic attitude, saying that education and proper messaging are not insurmountable barriers. "What's the plan B?" asks Thomas. "What do we do next? At the moment there is no plan B other than searching for another example that 5 years down the line will fail again. We can't just do the same again. That's why we still have malaria."

This kind of response is typical "hand waving from people who are lab scientists," says Hemingway, who also heads the Innovative Vector Control Consortium, a product development partnership funded by the Bill and Melinda Gates Foundation. "That's where people who never work in the field really underestimate what it takes. They ought to do a couple months in the field and see the practicalities."

O'Neill is doing just that. In 2005, he, Hoffmann, and others received a 5-year, $6.7 million Gates Foundation Grand Challenges in Global Health Initiative grant to test the feasibility of releasing *Wolbachia*-infected *Aedes* mosquitoes in the wild. In addition to monitoring outdoor enclosures, O'Neill is working with anthropologists to establish focus groups with dengue-affected people in three countries to gain a better understanding of community attitudes toward the control tactic. "Public acceptability seems very high to the approach," he says.

Knols also received positive feedback with his fungus. "When we did the trial in Tanzania, the local population was crazy about this whole thing," he says. "They loved it. They wanted more of the fungus." And since the fungus still relies on treated bednets and indoor residual spraying—the cornerstones of existing control protocols—implementing the approach only requires a change in attitude, not a change of action. "In terms of what people actually get to see, there's not that much difference in these control methods," Knols says.

Read admits that his argument is "a hard sell," but what's the alternative? "An unsustainable approach now is going to do a lot of damage in the long run. That's the lesson of history," he says. "We can be smarter this time." ■

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