GRAND CHALLENGE:

Develop a Biological Strategy to Deplete or Incapacitate a Disease-Transmitting Insect Population

GOAL:

Control insects that transmit agents of disease

MODIFYING MOSQUITO POPULATION AGE STRUCTURE TO ELIMINATE DENGUE TRANSMISSION

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Scott O'Neill has spent almost half of his life studying whether an especially peculiar bacterium that resides harmlessly within many insects can help transform public health in the developing world. He may yet succeed.

As a post-doctoral researcher at the University of Illinois, and later still, while running his own lab at Yale University and then later back home again in his native Australia, O'Neill has pursued a single scientific goal: exploiting the natural characteristics of a bacterium called *Wolbachia* to eradicate dengue fever, a deadly disease transmitted to humans by mosquitoes that is spreading with increasing ferocity throughout parts of the world.

"I thought it was a pretty good idea," says 51-year old O'Neill, now the Dean of Science at Monash University in Melbourne. "Scientists don't get too many of those, so I stuck with it. Fortunately I'm pretty obsessive."

O'Neill first hatched his idea as an admittedly naive graduate student at Queensland University in Brisbane. After a professor introduced him to the *Wolbachia* bacterium, which infects insects, the novice scientist began to think if it might be possible to engineer antibodies against disease-causing pathogens into the bacterium. If he could figure out how to infect large populations of mosquitoes with the antibody-carrying bacterium, it might be possible to block the dengue virus or other pathogens before diseases like dengue fever or malaria can be transmitted from the insects to humans. While certainly clever, the notion was pretty far-fetched. *Wolbachia* was being studied by researchers at the time in *Drosophila melanogaster*, common fruit flies, and has been identified as infecting—mostly in a benign manner—about 60 percent of the world's insects, including butterflies and moths. But the bacterium didn't naturally infect the major species of dengue- transmitting mosquitoes. To bring his idea to life, O'Neill would have to figure out how to create a disease-blocking form of *Wolbachia* in the laboratory and

Left: *Wolbachia* mosquitoes are currently reared under laboratory conditions for small field trials in Australia, Vietnam and Indonesia. Scott O'Neill pictured right in the Cairns rearing facility inspects the mosquitoes before a release with Dr. Petrina Johnson.

Photo courtesy of Eliminate Dengue

then somehow get it to spread among malaria and dengue mosquitoes in the wild. As something of this nature had never been done before, making it happen would be an enormous undertaking. It could consume a single scientist's entire career.

In fact, during the effort that has consumed his career to date, O'Neill encountered numerous setbacks that left him "disillusioned and ready to give up" several times, he says. However, O'Neill also was graced with the good fortune that typically accompanies scientific success stories. And, in 2004, just two days before Christmas, O'Neill's persistence was rewarded when he was told that he had won a five-year, $6 million grant from the Bill & Melinda Gates Foundation's Grand Challenges in Global Health program to help make his scientific aspiration come true.

"I was euphoric and I was gripped by fear," the scientist recalls over coffee at Monash University. "I worried I might fail."

Instead, ten years later O'Neill is celebrated worldwide as a scientific pioneer. His labs, first at Queensland and now at Monash supported by the Gates grant, found that one *Wolbachia* strain can infect and shorten the lifespan of *Aedes aegypti*, the mosquito responsible for transmitting dengue fever to more than 50 million people a year. This reduced lifespan doesn't allow the dengue virus infection time to develop in the mosquito so that it can be transmitted from the insect's salivary glands to human blood. Moreover, the lab discovered that this and other *Wolbachia* strains block viral replication within the mosquito. In field trials in Australia beginning in 2011, mosquitoes infected with a virus-disabling *Wolbachia* were able to take over natural populations of *Ae. aegypti*. O'Neill expects that dengue rates will drop when *Wolbachia*-carrying mosquitoes fully overtake areas where the disease is now endemic, such as in Vietnam, Indonesia, Brazil and Colombia that are undertaking their own start-up *Wolbachia* projects. This expectation is supported by recent modeling results, which predict a major impact in reducing dengue transmission.[1] The Indonesia trials are being supported by the Tahija Foundation of Indonesia.

O'Neill's success so far couldn't be more timely. Despite billions of dollars annually spent on insecticides and other protective measures, cases of illness caused by dengue infections across the Americas, South-east Asia and the Western Pacific exceeded three million in 2013, up from 1.2 million five years earlier, according to the World Health Organization. The WHO reports that as many as 300 million infections occur each year, a number also sharply higher than five or 10 years ago. There is no vaccine or treatment for the virus, which typically causes flu-like symptoms—severe headache, pain behind the eyes, intense aches in muscles and joints, vomiting, swollen glands or rash. An estimated 500,000 people with severe dengue require hospitalization each year, a large proportion of whom are children. About 2.5 percent of those affected die.

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As with many scientific advances, the dengue story has its share of serendipitous events. O'Neill comes from several generations of Australian farmers and he initially pursued an agricultural degree. "I got interested in studying insects because of their role as agricultural pests and in transmitting human illnesses," he says. "Tropical diseases spread by insects was wide open for research," he says.

In 1990 O'Neill heard about a post-doctoral position at the University of Illinois that reignited his interest. At the university, O'Neill was encouraged to flesh out the idea of using the bacteria to interdict disease. *Wolbachia* is rare

among bacteria because it is passed along from infected female mosquitoes to their offspring. Moreover, as a result of a process known as cytoplasmic incompatibility, matings between male mosquitoes carrying *Wolbachia* and uninfected female mosquitoes do not produce offspring. This means that in a mix of infected and uninfected mosquitoes, offspring have a better chance of inheriting *Wolbachia*, and thus it is likely to spread and become established at high frequency in the population. In experiments in Illinois, O'Neill and colleagues were able to insert *Wolbachia* found in one type of fruit fly into the embryos of another type of fly, and then have the newly-introduced *Wolbachia* passed on to succeeding generations. In this way, they showed for the first time that it was possible to transfer the bacterium from one insect species into another. That provided evidence that it might be possible for scientists to force *Wolbachia* to spread among disease-carrying mosquitoes.

Within a few years, O'Neill was recruited to join the faculty of Yale's medical school where he began focusing on how to use the bacterium to disrupt malaria and dengue. In 1994, O'Neill and his lab were able to get a *Wolbachia* strain found in some types of mosquito to infect the *Drosophila* fruit fly and spread to offspring.[2] But, despite numerous efforts, he couldn't replicate that in a mosquito species that transmits dengue. By this time, O'Neill decided to focus solely on dengue because *Ae. aegypti* appeared simpler to work with than the mosquito strains that carry the malaria parasite.

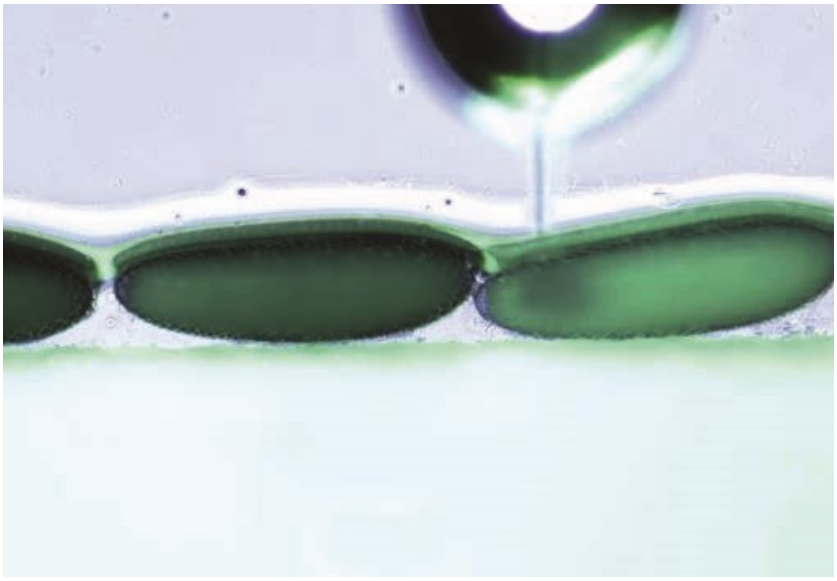

Photo courtesy of Eliminate Dengue

Scott O'Neill's lab transferred *Wolbachia* from the fruit fly to *Aedes aegypti* mosquito eggs (pictured) by microinjection. This took years, and thousands of attempts. O'Neill's lab then found that when *Wolbachia* is present in *Aedes aegypti* it stops dengue virus from growing; and if it can't grow, it can't be passed between people.

Even so, "for the longest time nothing worked," says O'Neill.

Then in 1997 a scientific paper was published by one of the world's most eminent geneticists encouraging O'Neill to press forward. Seymour Benzer at the California Institute of Technology reported the discovery of a type of *Wolbachia* he named "popcorn" that inexplicably caused widespread degeneration of tissues, including those in the brain, retina and muscle in *Drosophila*, culminating in early death of the flies.[3]

"That's it," O'Neill recalls thinking. The finding suggested there would be no need to engineer a *Wolbachia* strain to infect a mosquito with a virus-blocking antibody. Instead, O'Neill merely needed to introduce the newly-found *popcorn* variant into mosquito populations, causing infected insects to die before they lived

long enough to transmit the virus to humans. In 2001, O'Neill moved back home, taking a post at Queensland hoping the Australian government might be receptive to supporting dengue research as the disease was common in the country's northern tropical climate.

Still, by late 2003 "I was still hunting for enough money to support the development of the idea," O'Neill says.

That all changed late the next year when he won the $6 million grant from the Gates Foundation's Grand Challenges in Global Health program. "Nobody else would have entrusted us with so much money without more proof it would work."

Within the grant's first few years a graduate student, Connor McMeniman, finally was able to transfer the *Wolbachia* popcorn strain (called *w*MelPop) into a dengue mosquito, "something nobody had been able to do before," says O'Neill." McMeniman was among a crew of technicians working for hours each day using hair-thin microinjection needles under a microscope trying to get the *w*MelPop strain into just the right spot in *Ae. aegypti* embryos that would grow into adults carrying the bacterium and passing it to their offspring. Over two years, McMeniman injected 10,000 embryos. "It was a huge technical roadblock," says O'Neill. "Getting it done so early in the grant, allowed us to move quickly."

Photo courtesy of Eliminate Dengue

Residents on Tri Nguyen Island, Vietnam work with the local Eliminate Dengue project team as project collaborators to carry out weekly releases of *Wolbachia* mosquitoes during a field trial.

By 2008, the lab had found a *w*MelPop strain that persisted in offspring and cut the mosquitoes' lifespan by more than half, from 61 days to 27 days.[4] It was the first evidence that the lifespan-reducing mechanism first seen in flies could be reproduced in dengue mosquitoes. At the same time, the lab ran a parallel set of experiments in *Drosophila* that uncovered a surprising and previously unseen aspect of *Wolbachia*. Flies infected with an insect virus that normally kills them in a few days, lived twice as long when they also contained *w*MelPop. The researchers couldn't find any trace of the lethal virus in the flies containing *Wolbachia*. This suggested that *w*MelPop might actually have anti-viral properties not seen before.

In late 2008, as the Gates grant was about to expire the O'Neill lab had its "Eureka!" moment. It produced dengue mosquitoes that consistently passed

Wolbachia to their offspring, and, as an extra bonus, found the bacterium blocked dengue virus replication just as had been seen in *Drosophila* several years before.[5] "It felt like a home run," says O'Neill. "Totally unexpected."

A critical question remained: could the laboratory-infected mosquitoes spread *Wolbachia* when let loose in locales infested with native *Aedes aegypti*? They started first with a strain of *Wolbachia* (called *w*Mel) that blocks viral replication but doesn't affect mosquito lifespan, because they thought that unimpaired mosquitoes had the best chance to survive in nature. Backed with additional funding from Gates grant extensions, O'Neill obtained regulatory approvals from the Australian government's Australian Pesticides and Veterinary Medicines Authority. O'Neill's team won crucial support of communities in northern Australia to release the *Wolbachia*-modified mosquitoes. In initial tests in late 2010 in a greenhouse facility built with $2 million from the Gates Foundation, the *Wolbachia* mosquitoes invaded the small population and increased in numbers in succeeding generations.[6] The following year the O'Neill project began releasing *Wolbachia*-infected mosquitoes in selected neighborhoods in Yorkeys Knob and Gordonvale, two semi-rural towns near Cairns in Australia's northern tropical region.[7]

Even before the trials were conducted O'Neill requested CSIRO, the Commonwealth Scientific and Industrial Research Organisation, Australia's national science agency, to conduct an analysis of potential environmental and health hazards to the communities as part of the requirement of the Grand Challenges in Global Health grant that "ethical, social and cultural" concerns related to the release be addressed. Over a 10-month period in 2009, the CSIRO conducted a comprehensive study to uncover questions raised by the communities and a group of national and international scientific advisors, estimating the likelihood of 50 different types of hazards that might arise over the next 30 years. In the end, the CSIRO team found the risks to be negligible.[8]

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Armed with the CSIRO report, O'Neill also developed an innovative plan for introducing his *Wolbachia* concept to the Cairns community. He began meeting with individual residents, focus groups and local authorities. He hired a "community engagement officer" and a communications specialist. The team conducted phone surveys, canvassed residents in the two towns, published announcements of the project in local newspapers, and even ran information booths at a county fair in Gordonvale and in a large Cairns shopping mall. Eventually, O'Neill's team began meeting monthly with a "reference group" made up of residents, businesses and community leaders. In one approach that may serve as a model for releases elsewhere, the project team created a website[9] named "Eliminate Dengue" in which the project's design and monthly updates of the trials' progress were posted, along with cartoon representations of the science, data charts, and photos of the field workers releasing the insects and retrieving eggs. In addition to making the project's progress transparent to the residents near Cairns, it was designed to help enlist the support of community members as the government required property owners' permission in order to conduct releases and monitor results.

"The project had gone beyond the science or managing a large laboratory," says O'Neill. "I had to develop new skills to communicate with government regulators, the people living in the communities, the media, overseeing the field sites."

THE SCIENCE:

Harnessing the anti-viral properties of *Wolbachia* to inhibit dengue transmission

Wolbachia are maternally-inherited intracellular bacteria estimated to infect over 60% of insects. To enhance transmission they manipulate the host by mechanisms including cytoplasmic incompatibility whereby uninfected females are unable to produce offspring after mating with infected males. While normally symbiotic, a virulent *Wolbachia* variant was discovered in *Drosophila melanogaster* that causes widespread tissue degeneration and halves lifespan.[3] O'Neill and colleagues reasoned that this life-shortening variant (*w*MelPop) could control transmission of vector-borne pathogens like the dengue virus, which require an extrinsic incubation period within the mosquito before becoming infectious to humans. Controlling dengue transmission by targeting mosquito longevity rather than abundance would avoid potential species eradication.

O'Neill and colleagues showed that the adapted *w*MelPop-CLA could be stably introduced into *Aedes aegypti*, the principle vector of dengue viruses, as this is not their natural host, and caused cytoplasmic incompatibility and halved lifespan also in this mosquito.[4] O'Neill and others had also discovered that some *Wolbachia* variants conferred a fitness benefit in *Drosophila* by conferring anti-viral activity. Indeed, *w*MelPop-infected *Ae. aegypti* were also largely resistant to dengue virus type 2 infection, possibly due to activation of the innate immune response or competition for host cellular resources.[5] These life-shortening and anti-viral properties together made dengue control possible in theory. However, this variant was later shown to impose various fitness costs in the host that could diminish its efficacy under certain field conditions.[14] An alternative avirulent *w*Mel variant was also tested under caged and field conditions, and also conferred resistance to dengue viral infection albeit to a lesser extent but with lower fitness costs, and could be stably established in mosquito populations.[6, 7] Modeling studies suggest that the two different *Wolbachia* variants would be best utilized to control dengue at different levels of transmission.[1]

As part of the trial, the project's field workers set small traps in people's yards where mosquitoes lay eggs. The eggs were then collected, brought back to the lab and tested for the presence of *Wolbachia*. Within weeks, 20 percent of the eggs carried the bacteria. By April, when the rainy season ended, the bacteria were present in 90 percent of the eggs. In September, after the region's dry season, 98 percent of the mosquitoes that can transmit dengue carried *Wolbachia*. This indicated to O'Neill that the virus-blocking bacterial strain was now embedded in the wild mosquito population, powerful evidence that what had been accomplished in the lab was being duplicated in a real-world environment.

The following year, the outdoor experiments were repeated in two other nearby locales. In these trials, O'Neill tested which of two bacterial strains developed in the lab would work best, the one that reduced dengue replication and was better at spreading into native mosquitoes (*w*Mel) or the one that had both life-shortening and dengue blocking properties (*w*MelPop). They found that while *w*MelPop quickly spread in the local insect population, sometime later there was a sharp decline in the number of mosquitoes carrying the strain. These results were later confirmed in Vietnam, convincing the team to move forward with *w*Mel as their best candidate to date.

By 2013, almost all mosquitoes in the Australian sites contained the bacterium, says O'Neill. In a 2014 report the Eliminate Dengue research team reported that one year after first releasing *Wolbachia* in Yorkeys Knob and Gordonvale, mosquitoes collected from that area showed strong dengue-blocking activity in the laboratory.[10] Thus far, observations of dengue activity in the Cairns regions where *Wolbachia* mosquitoes were released have been consistent with protection.[11]

As the Australian research results started coming in O'Neill began planning similar efforts in other countries. In April of 2013, the project began preliminary outdoor releases in several areas in a south central region of Vietnam, using Gates funding as well as support from the Vietnamese government. By early 2015 "close to 100 percent" of mosquitoes in a test site on Tri Nguyen Island off the south central coast of Vietnam contained *Wolbachia*.[12] The researchers began a similar field trial in two villages in Indonesia and by March 2015 about 80 percent of mosquitoes contained the bacterium.[13] Projects are also underway in Brazil and Colombia. As a result of the success to date, beginning in 2013 Eliminate Dengue received a $13 million grant from the Wellcome Trust.

While O'Neill fully expects there will be setbacks along the way, with regard to the project's success to date, O'Neill says he sometimes feels as "if it's been fated. As if we've opened a box no one looked in before and it was just waiting for us to open it."

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