Fighting a fungal scourge

Researchers trying to rescue amphibians from a global fungus epidemic are finding that bacteria may be their best allies.

Amy McDermott, *Science Writer*

More than a decade ago, amphibian microbial ecologist Reid Harris watched a mother salamander as she marched in a figure-eight pattern through her clutch of soft, jellylike eggs. He knew that her strange walk, rubbing up against her brood, transferred beneficial bacteria from her skin onto the eggs to fight off fungal infection. Then something clicked. Harris wondered if she might also be showing him the solution to a scourge threatening hundreds of other amphibian species around the world.

Then, as now, amphibians were dying in droves. At least one-third of species face extinction, with at least 90 confirmed gone and another 500 in decline (1, 2). The major culprit is the skin disease chytridiomycosis, caused by the waterborne fungus *Batrachochytrium dendrobatidis*, or *Bd* for short. Under a microscope, fungal bodies anchor themselves in the translucence of frog skin and often group together to form a collection of small spheres. Chytridiomycosis kills by disrupting ion and fluid transport, eventually stopping the host's heart (3).

A particularly deadly *Bd* lineage arose in the Korean peninsula sometime in the last century, the source of a pandemic that fanned out across the world

To fight the deadliest wildlife disease on record, a fungus responsible for the decline or disappearance of hundreds of amphibian species including the Panamanian golden frog, researchers are looking to the bacteria that live on frog skin. Image credit: Flickr/Brian Gratwicke.

Eastern red-spotted newts (*Notophthalmus viridescens*) take a probiotic bath. Beneficial bacteria can protect some amphibian species from deadly fungi. Image credit: Brandon LaBumbard (photographer).

through the global amphibian trade, infecting about 700 species in six global regions over the past five decades (4). Naïve immune systems (mostly those of frogs) couldn't fight off the highly contagious and aggressive pathogen, and chytridiomycosis spread like a leveling wave, becoming the most devastating wildlife epidemic ever recorded.

The stakes were already high when Harris, now emeritus at James Madison University in Harrisonburg, VA, had his epiphany. He suspected that the salamander mother's protective antifungal bacteria could represent a line of defense against *Bd* marching right under his nose. There might be many bacteria, for that matter, already living on the backs of frogs, salamanders, newts, and toads able to fight off the fungus, he thought. If those bacterial herds could be managed, like a wildlife park, perhaps amphibian microbiomes could be manipulated to better defend against *Bd*.

Harris and colleagues set about testing a variety of amphibian skin bacteria to see which inhibited *Bd* growth (5, 6). In a landmark study published in 2009, the researchers used an antifungal bacteria isolated from salamander skin, *Janthinobacterium lividum*, known as *J. liv* for short, to protect mountain yellow-legged frogs from severe chytridiomycosis in lab experiments.

The finding kicked off an ongoing effort by Harris, who is director of disease mitigation for the Amphibian Survival Alliance in London, and others to better understand the relationship between amphibian microbiomes and *Bd*. Studies in the last 10 years show that *Bd* can change host bacterial communities, hinting at a possible link between the microbiome and disease. In parallel, researchers have also been searching far and wide for antifungal microbes such as *J. liv* that can be marshaled to protect wild animals under attack by *Bd*. They've found thousands of candidates that fight fungi in the lab, but few have been field tested. With some luck, this early work could lead to real solutions to a scourge that has victimized amphibians for years. "The idea," Harris says, "is to augment anti-*Bd* bacteria on amphibian skin."

Small Leaps

Around 1990, when researchers first realized that amphibians were dying globally, they didn't even know what was killing the animals (7). By late in the decade, histological exams of sick and dead frogs linked abnormalities in the skin to a fungus in the phylum Chytridiomycota (8). Still, many had a hard time believing a fungus alone could wreak such havoc. *Bd* was the first chytrid fungus known to parasitize vertebrates, says Joyce Longcore, a mycologist at the University of Maine in Orono, who in 1997 was the first person to culture *Bd* in the lab (9). "I knew it was a big deal," she says, "because it meant this thing we knew was killing amphibians was now in culture, and we could [do] research with it."

Researchers soon found they could clear *Bd* infections by bathing frogs in antifungal drugs (10). But deploying such treatments in the wild was unrealistic, in part, because the fungus persists in the environment, meaning treated animals would be reinfected. This need for lasting solutions for wildlife was one reason Harris looked toward bacteria. "Ideally," he says "the anti-*Bd* bacteria persists and is transmitted to other amphibians in the area, either through direct contact or indirectly through environmental transmission."

That was the dream when Harris set about his 2009 experiment on mountain yellow-legged frogs, denizens of streams in the Sierra Nevada hard-hit by *Bd*. Harris tested the efficacy of a *J. liv* bath on six captive frogs, reared in the lab from wild eggs, before exposing the animals to *Bd*. After 139 days, all the treated frogs survived whereas most of a control group that didn't get the probiotic bath had died (11).

This early success with *J. liv* was so exciting that biologist Matthew Becker, then a graduate student collaborating with Harris, wondered if it would work for another, far more troubled species. Hordes of small, sunny-yellow Panamanian golden frogs once gathered along forest streams in the Cordillera Mountains that snake down Panama's narrow spine. They are a national icon—immortalized in marketplace tchotchkes of golden frogs kissing, dancing, and getting married—and a symbol of hope. But golden frogs haven't been seen in the wild since 2009. They likely succumbed to *Bd*, leaving captive populations as all that's left of the species.

Knowing there was no hope at all for reintroducing the species to the wild without protection from *Bd*, Becker repeated Harris' experiment in 2011 with 20 Panamanian goldens from the Houston Zoo. But soaking in a probiotic bath of *J. liv* before exposure to *Bd* didn't save the little yellow frogs. The bacteria multiplied on the frogs' skin over the first two weeks; then bacterial numbers started declining, and the *Bd* infection grew more severe, finally sickening and killing all the animals (12).

Such differing outcomes in two frog species, in two nearly identical experiments, crushed any expectation

that *J. liv* might be a silver bullet against *Bd*. The research community took a collective step back, says Becker, now a biologist at Liberty University in Lynchburg, VA. Biologists began to ask more basic questions about amphibian microbiomes and their relationship with the disease.

Clues on the Battlefield

More than a thousand articles have come out since 2011 exploring microbiome-related questions. Researchers now have a better sense of the variety of bacterial communities on different amphibian species, which may also help explain their differing vulnerability to *Bd* and responses to probiotic treatment (13). Scientists have also learned that *Bd* infection is associated with changes in the composition of a host's bacterial community, although it hasn't been clear if *Bd* causes those changes or merely capitalizes on them (14).

In a 2014 study led by microbial ecologist Andrea Jani and ecological modeler Cheryl Briggs, both at the University of California, Santa Barbara, field surveys showed that the relative abundances of certain bacterial species changed with the severity of *Bd* infections. To test whether *Bd* was actively changing the microbiome, Jani and Briggs experimentally infected 42 frogs with the fungus and then compared their skin microbiomes with uninfected controls over time. They found that the pathogen did, in fact, change the microbiome's composition. That doesn't necessarily imply that the microbiome protects against *Bd* or that microbiome changes make *Bd* infections worse, Jani says. Some lab research suggests the microbiome can protect frogs, Jani adds, "but we don't know that it ever does in nature."

New research, published in the past year, is starting to provide answers by examining multiple wild populations. Up in the Pyrenees Mountains, lacing the border between France and Spain, the chubby little midwife toad fell into steep decline over the last decade. But some pockets of toad populations are now bouncing back. Microbial ecologist Kieran Bates, a postdoctoral researcher at the University of Oxford and a visiting researcher at Imperial College London, wondered what set rebounding populations apart.

So Bates hiked to mountain lakes to collect and compare skin bacteria from toads in both struggling and recovering populations, looking to see if they had caught the same genotype of *Bd*. Perhaps, Bates thought, the rebounding populations had a milder, less virulent form of the fungus. But genome sequencing revealed that all the toads had the same type of *Bd*. What varied was the makeup of their commensal bacteria (15).

Bates saw consistent differences in the number and relative abundance of bacterial species between rebounding populations and declining ones. The finding "implies the microbiome is important in driving disease dynamics," Bates says. One potential explanation, which has yet to be tested, is that antifungal microbes are more abundant in recovering groups.

To get a better handle on what else might explain differences in amphibian microbiomes, Becker, and more than 40 other authors launched a major survey, the results of which were published this year (16). The researchers looked at what factors shape the assemblage of skin bacteria on each frog, toad, and salamander species across a broad range of environments around the world. One major takeaway is that the number of bacterial species inhabiting amphibian skin is inversely related to local temperatures, with greater bacterial diversity in temperate zones, and less in the tropics.

"Whether it's warm or cold, and whether it's warm or cold all the time or changing throughout the year, are the two pieces shaping what's on their skin," says coauthor Molly Bletz, a postdoctoral researcher in microbial and disease ecology at the University of Massachusetts in Boston. It's possible that different microbes colonize the skin seasonally in temperate zones, she says, whereas a few species take over year-round in the tropics.

"We didn't think it was going to be simple to begin with. But it's a lot more complex than what we first thought out, as with most things."

—Matthew Becker

Understanding what drives bacterial diversity could help researchers develop tailor-made probiotics for different species, Bletz and Becker say, ever mindful that the whole point is to fight chytridiomycosis.

Fungus Fighters

To that end, researchers are also hunting for bacterial species that carry their own antifungal defenses in the hope that they could be recruited to give the animals a better fighting chance against *Bd*. Having tested thousands of candidate bacteria, researchers have found that many do fight the disease, at least in the lab.

Doug Woodhams regularly wades into lily pad–covered ponds in search of antifungal bacteria living on wild amphibians. He hikes with a backpack loaded with Petri dishes, cotton swabs, and ice packs to collect microbes off their skin. Back in his disease ecology lab at the University of Massachusetts, Boston, Woodhams cultures the samples—searching for microbes that make chemical weapons like the striking violet pigment violacein that *J. liv* uses to inhibit *Bd* (17). The ideal, researchers say, is to find bacteria already present on an amphibian's own skin that could be amplified on the animal, sprayed around its environment, or both, to become a lasting defensive force.

Woodhams estimates that he and other researchers have collected and tested about 7,400 candidate bacteria in the last decade and found almost 2,000 that have anti-*Bd* properties in culture. He keeps a digital database tracking them all. But of the thousands of candidates identified so far, only about

a dozen have been tested on living captive frogs and even fewer on wild populations. Cost and red tape, such as the permitting needed to do a test in the wild, are the main bottlenecks, he says.

One of the earliest field tests was cut short by weather but did suggest the bacterial approach holds promise. In 2010, biologist Vance Vredenburg of San Francisco State University in CA, a coauthor of Harris' 2009 study, and his team caught and tagged 100 wild yellow-legged frogs in the high-elevation lakes of Dusy Basin in the Sierras, where *Bd* was a newcomer at the time. The researchers soaked 80 frogs in a *J. liv* bath, leaving the other 20 as untreated controls, then released them all. After year one, untreated frogs had more severe *Bd* infections. After two years, the researchers couldn't find any untreated frogs, suggesting they had succumbed. But 39% of the treated group had survived, which was "pretty good proof of concept," Harris says. The pond froze the following winter, killing all the remaining frogs, so the team couldn't find out how long the protection would have lasted. Still, Harris believes that the two-year field trial demonstrated the feasibility of a probiotic approach, at least for this species.

Undaunted, Woodhams hopes to begin a new field trial in western Massachusetts in the next year or two, testing antifungal bacteria on eastern red-spotted newts, an East Coast salamander species. In this case, it's not *Bd* that's threatening, but rather another chytrid fungus called *Bsal* (*Batrachochytrium salamandrivorans*). First turning up in the Netherlands in 2008, it's devastated local fire salamander populations there (18, 19). *Bsal* causes chytridiomycosis predominantly in newts and salamanders. Although it hasn't yet shown up in North America, models predict that if it does, native amphibians are in grave danger (20). Woodhams wants to be ready when it arrives.

He's now swabbing these newts, looking for one (or several) potential probiotics that can inhibit both *Bd* and *Bsal* in the lab. In the field trials, he'll catch and tag newts at several ponds, give a subset the antifungal bacteria, and spray the same probiotic around the lip of ponds. He'll continue monitoring the *Bd* load on the newts and other amphibians at the ponds, recapturing test subjects every few months. Although the animals don't get particularly sick from *Bd*, seeing how a probiotic fights that fungus, which is already a part of the wild environment, could indicate its potential to fight the much-worse *Bsal*, Woodhams says.

Keeping Perspective

When *J. liv* protected yellow-legged frogs but not Panamanian goldens, it confirmed that a single probiotic would not suffice to battle *Bd* or similar fungal epidemics. The host's own microbiome makeup will likely play a key role in determining which populations are most vulnerable to begin with and which weapons will defend best in each case.

It's now clearer, for example, why the same probiotic didn't work for yellow-legged frogs and golden frogs. *J. liv* naturally occurs in the Sierras, but in unpublished sequencing data, Becker found *J. liv* at extremely low levels on golden frogs, suggesting it is not a natural part of their microbiome. In fact, he says, the frogs may have a mechanism to fight off the bacteria, which prevents it from protecting the animals against *Bd*.

That's one reason why researchers are confident that the safest candidate probiotics are probably those already growing on local amphibian skin. That way, the microbes are also not being introduced to novel habitats. Even so, the main concern when releasing bacteria into the wild is that they will have unexpected consequences for other pond-dwellers, such as fish, water bugs, or dragonfly larvae.

If and when researchers do find a probiotic that excels in lab and field trials, the next step is testing it in the wild. That could mean catching and soaking amphibians, as Vredenburg has done and as Woodhams plans to do, as well as inoculating the environment by spraying around pond edges, a method that Woodhams intends to carry out. Most researchers agree that antifungal microbes alone are unlikely to solve the larger chytrid crisis but believe the probiotics do have great potential as one tool to fight *Bd* and now *Bsal*.

Even if probiotics don't provide a cure-all, the research may also point to less direct forms of treatment. In an article published last spring (21), Woodhams applied four antifungal bacteria to yellow-legged frogs and then exposed them to *Bd*. Although none of the bacteria protected the frogs, their presence did appear to dial up the frogs' own immune defenses, inducing greater secretion of antimicrobial peptides from their skin. Perhaps, Woodhams says, there'll be a way to induce frog skin to upregulate its own defenses using bacteria or maybe even friendly fungi to get the frog immune system to defend itself from pathogenic fungi.

Despite such signs of progress, probing and dissecting the microbiome and searching for ways to fight the *Bd* scourge with bacteria remain daunting challenges with remedies far from guaranteed. "We didn't think it was going to be simple to begin with," Becker says. "But it's a lot more complex than what we first thought out, as with most things."

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