The Rise of Ranavirus

AN EMERGING PATHOGEN THREATENS ECTOTHERMIC VERTEBRATES

By Matthew J. Gray and Debra L. Miller

Ranaviruses have been called "cold-blooded killers" (Chinchar 2002) for good reason—they are capable of causing illness and death in three ectothermic vertebrate classes (amphibians, reptiles, and fish). Experiments have also demonstrated that the virus can be passed among these groups (called interclass transmission; Bandin and Dopazo 2011), likely facilitating its persistence in aquatic systems. Ranaviruses were discovered in the 1960s (Granoff et al. 1965), yet their role in widespread die-offs of ectothermic vertebrates wasn't realized until the 1990s (Gray et al. 2009). Researchers are now racing to determine what makes ranaviruses so virulent and capable of infecting so many hosts (Lesbarrères et al. 2012).

Credit: Matt Niemiller

The larvae of marbled salamanders (above) were among several amphibian species—including spotted salamanders, wood frogs, and spring peepers—that died due to a ranavirus outbreak in Cades Cove, Great Smoky Mountain National Park.

We've been in that race for eight years after detecting ranavirus in frog communities in Tennessee farm ponds. We found that green frog (*Lithobates clamitans*) tadpoles in ponds with cattle access were 4.7 times more likely to be infected with ranavirus than those in ponds with no cattle (Gray et al. 2007). Although many factors may have contributed to this trend, we suspect that poor water quality (a stressor) and minimal vegetation (which increases contact rates among individuals) in cattle-access ponds played a role.

Since then, we've tested thousands of amphibians across Tennessee and other states and performed dozens of experiments to learn about ranavirus-host interactions. From our experience, ranavirus exists typically at low prevalence (less than 5 percent of individuals infected in a population), then emerges rapidly over a two-week period, with mortality exceeding 90 percent in multiple species. Amphibian tadpoles are most often affected, but other cold-blooded animals (such as freshwater turtles) that come in contact with the virus in water or by eating live or dead infected individuals may also succumb to the disease.

After a ranavirus outbreak, aquatic community composition and ecosystem function can change drastically as thousands of omnivorous herpetofauna die and rot at the bottom of a wetland or lake. In more than 40 years of contemporary research on pathogens affecting ectothermic vertebrates, few pathogens have been found to have as great an ability to transform aquatic ecosystems as ranaviruses.

Ominous Body of Evidence

With one in three amphibian species and over 40 percent of turtles at risk of extinction (Stuart et al. 2004, Buhlmann et al. 2009), ranavirus represents a significant threat to herpetofaunal biodiversity. An emerging pathogen is one whose distribution, prevalence in a population, or host range is increasing. Efforts to search for ranavirus in ectothermic vertebrates has increased, and there is a growing body of research that suggests this pathogen is emerging. Consider:

- Through the use of modern genetic analyses, Andrew Storfer at Washington State University found that novel ranaviruses were located in the central United States, possibly resulting from the transport of infected tiger salamanders (*Ambystoma tigrinum*) used for fishing bait (Storfer et al. 2007).

Credit: Heather Inman

Matthew J. Gray, Ph.D., CWB, is an Associate Professor of Wildlife Ecology in the Center for Wildlife Health in the Department of Forestry, Wildlife, and Fisheries at the University of Tennessee.

Credit: Heather Inman

Debra L. Miller, D.V.M, Ph.D., CWB, is a Professor of Veterinary Pathology in a split position between the Center for Wildlife Health in the Department of Forestry, Wildlife, and Fisheries and the College of Veterinary Medicine Department of Biomedical and Diagnostic Sciences at the University of Tennessee.

Credit: Matthew J. Gray

Credit: Debra L. Miller

At Great Smoky Mountains National Park, author Debra Miller (front, at right) and University of Tennessee researchers collect and catalogue salamander tail clips to be tested for ranavirus. Necrosis of the oral mucosa (see arrows) was among the lesions seen in ranaviral disease of red-eared sliders, semi-aquatic turtles common in the pet trade.

- Jason Hoverman at Purdue University (formally at the University of Tennessee) tracked the seasonal emergence of ranavirus in amphibian populations at 40 breeding sites in Tennessee, and documented a die-off involving several hundred green frog and American bullfrog (*L. catesbeianus*) larvae at one of these sites (Hoverman et al. 2012). During this study, about one-third of the sites were classified as having abnormally high prevalence of ranavirus.
- Numerous cases of amphibian die-offs caused by ranaviruses have been reported in the past 15 years, with 94 percent of reported cases occurring since 1998 (Green et al. 2002, Miller et al. 2011), which may suggest increasing geographic distribution.
- Recent emergence of a Frog Virus 3 (FV3)-like ranavirus in eastern box turtle populations (*Terrapene carolina carolina*) in Maryland, West Virginia, and Kentucky could indicate that host range is increasing (Ruder et al. 2010, Seigel and Farnsworth 2012, *The Charleston Gazette* 2012).

Ranavirus Symposium

From July 27-29, the Second International Symposium of Ranaviruses will be held in Knoxville, Tennessee, just before the Annual International Conference of the Wildlife Disease Association (WDA). The symposium will feature presentations and posters highlighting recent research on ranavirus. During two field trips, amphibians and turtles will be captured and sampled for ranavirus testing. To learn more, visit ranavirus.com/ranavirus/welcome.html.

To our knowledge, ranaviruses are capable of infecting amphibians from at least 14 families and over 70 individual species (Miller et al. 2011), 15 reptile species (Marschang 2011), and dozens of fish species (Whittington et al. 2010). Considering this unusually broad host range, this emerging pathogen represents a serious threat to global populations of ectothermic vertebrates.

Life-History of a Killer

Ranaviruses belong to the virus family *Iridoviridae*, and six species of *Ranavirus* are currently recognized (Chinchar et al. 2011). It is believed that ranaviruses evolved in fish and subsequently jumped to herpetofaunal hosts (Jancovich et al. 2010). The virus enters a cell by binding to it and injecting its DNA (Chinchar 2002). The cell receptor that ranavirus targets for binding is very generalized and its genetic sequence is conserved (Chinchar and Hyatt 2008), which likely contributes to its broad host range. In the laboratory, ranaviruses can infect fish, reptilian, amphibian, and mammalian cells. Because ranaviruses replicate between 12°C and 32°C (Chinchar 2002), the higher body temperature of birds and mammals precludes them from being suitable hosts (Chinchar and Hyatt 2008).

Transmission of ranavirus can occur quickly by skin-to-skin contact or exposure to the virus in water (Gray et al. 2009). Jesse Brunner of Washington State University demonstrated that ranavirus was transmitted between an infected and uninfected salamander by merely touching them together for one second (Brunner et al. 2007). Jacques Robert at the University of Rochester Medical Center detected viral transcription in the skin, intestines, and kidneys of African clawed frogs (*Xenopus laevis*) only three hours after exposure to ranavirus in water (Robert et al. 2011). He found that the most common route of entry is likely via the epithelial cells of the intestines followed by the kidney then other organs (e.g., liver, spleen), culminating with systemic infections.

The virus enters host cells and commandeers cellular processes (e.g., DNA replication, mRNA synthesis) for its own replication (Chinchar et al. 2011). Cell death can be rapid, occurring in just nine hours (Chinchar 2002), and result in significant organ necrosis and loss of function (Miller et al. 2011). In highly susceptible species such as the wood frog (*L. sylvaticus*), mortality can be as quick as three days (Hoverman et al. 2011).

It's not a pretty death. Ranaviral disease has been likened to Ebola or epizootic hemorrhagic disease for amphibians because their bodies swell and hemorrhage. Hemorrhagic lesions are a key sign in fish and can occur in reptiles. Because ranaviruses infect multiple cell types, tissue necrosis is often extensive in terminal cases. Non-lethal infections have been documented (Grayfer et al. 2012), but their role in ranavirus persistence and emergence is unclear.

In amphibians, necrosis is most prevalent in the liver, spleen, and kidney but can be found elsewhere (Miller

et al. 2011). In fish, the hematopoietic tissue is generally most severely affected. In terrestrial turtles, ranaviral lesions primarily include necrosis of the oral cavity and internal organs (usually respiratory and gastrointestinal tracts), but also may include ocular and nasal discharges. In aquatic turtles, lesions mainly include hemorrhages and ulcerations, with the latter occurring along respiratory and gastrointestinal tracts. Death is likely a consequence of organ dysfunction. Secondary infection by other pathogens also is possible.

Emergence and Its Impacts

The persistence of ranavirus in the environment is a mystery, but likely involves an interaction of high viability outside the host (Nazir et al. 2012), ability to infect multiple host species and age classes (Hoverman et al. 2011, Haislip et al. 2011), and ability to persist in some hosts as latent infections (Morales et al. 2010). Many factors that encourage ranavirus persistence exist in permanent wetlands, but die-offs are also observed in wetlands that dry annually (e.g., Harp and Petranka 2006). Jesse Brunner surmised that sublethally infected adults likely serve as carriers and shed the virus into the water when amphibians return to wetlands for breeding (Brunner et al. 2004). Other ectothermic vertebrates, such as turtles, could serve a similar role. The persistence and emergence of ranavirus in aquatic ecosystems is a cutting direction in research.

Few studies have followed populations with reoccurring ranavirus die-offs, but researchers at the Zoological Society of London report ranaviruses as the likely culprit of common frog (*Rana temporaria*) declines in England (Teacher et al. 2010). Jim Petranka at the University of North Carolina in Asheville documented that recruitment of wood frogs and spotted salamanders (*Ambystoma maculatum*) was nonexistent during several years when ranavirus outbreaks occurred at the Tulula Wetland Complex in Graham County, North Carolina (Petranka et al. 2003). In the Great Smoky Mountains National Park, repeated die-offs involving multiple amphibian species have been occurring for over 10 years (Green et al. 2002, Todd-Thompson 2010), although the effects on population size are unknown. Sites with reoccurring die-offs are a conservation concern due to the possible effects on recruitment and population persistence.

Given that ranavirus infection and mortality tend to be strongly correlated ($r > 0.85$; Haislip et al. 2011, Hoverman et al. 2011, 2012), high prevalence in a population can be an indicator of emergence. Natural resource agencies should consider conducting surveillance studies to identify "infection hotspots," where ranavirus prevalence exceeds 40 percent (Hoverman et al. 2012). Wildlife Ecologist Scott Smith with the Maryland Department of Natural Resources is currently coordinating such a study among five mid-Atlantic states. After surveying 150 ponds in different physiographic regions over two years, Smith and his team hope to determine how common ranavirus is. "Anything that could lead to species loss is of grave concern," says Smith.

Upon locating ranavirus hotspots, natural resource agencies can identify mechanisms for emergence, determine population effects, and develop disease intervention strategies. Field studies should be designed in consultation with disease experts that have experience with ranaviruses. In a book chapter we wrote with David Green of the U.S. Geological Survey National Wildlife Health Center, we provide recommendations on required sample sizes to detect ranavirus given an assumed pathogen prevalence, approximate host population size, and 95 percent confidence level for detection (Green et al. 2009). For example, testing 30 individuals for ranavirus will ensure *detection* if prevalence is >10 percent. Testing 60 individuals will ensure detection if ranavirus prevalence is >5 percent. However, if the goal is to obtain a *precise, unbiased estimate* of ranavirus prevalence, the sample size should be larger. If you are willing to tolerate a 10 percent error in estimating ranavirus prevalence, at least 96 individuals should be tested; 384 individuals should be tested for a 5 percent estimation error.

Credit: Matthew J. Gray

Roberto Brenes, a Ph.D. candidate at the University of Tennessee, performs a necropsy on a black-bellied salamander as part of a long-term ranavirus surveillance study.

The reasons for ranavirus emergence at a site vary, but often are related to stressors that can be natural or anthropogenic in origin (Gray et al. 2009). A common stressor is rapid drying of a wetland, which causes

amphibian larvae to undergo metamorphosis. During metamorphosis, the immune system is endogenously suppressed (Rollins-Smith 1998), which can increase the likelihood of pathogen infection and disease. Brunner and colleagues documented that the likelihood of wood frog tadpoles dying from ranavirus increased 1.7-fold with each stage of development closer to metamorphosis (Warne et al. 2011). Agricultural pesticides and livestock usage of wetland areas also may stress hosts and increase the likelihood of ranavirus emergence (Gray et al. 2007, Kerby and Storfer 2009). A study in Arizona found that tiger salamanders were 4.3 times more likely to be infected with ranavirus in cattle-access wetlands (Greer and Collins 2008), which the authors attributed to greater contact rates because the salamanders clustered more due to less vegetation.

Credit: Katherine Edwards

Author Matthew J. Gray disinfects containers that held salamanders captured for a ranavirus surveillance study. Researchers must disinfect boots, gear, and any equipment that comes in contact with study specimens to avoid passing ranavirus among individuals and research sites.

In addition to external stressors, virus evolution may contribute to ranavirus emergence. Several studies have documented that ranaviruses isolated from captive facilities such as bullfrog farms and bait stores tend to be more virulent than ranaviruses in wild populations (Majji et al. 2006, Storfer et al. 2007, Hoverman et al. 2011). For example, 15 out of 19 amphibian species tested experienced greater mortality when exposed to a ranavirus from an American bullfrog ranaculture facility compared to a ranavirus isolated from a wild northern leopard frog (*L. pipiens*, Hoverman et al. 2011). If such captive-evolved ranaviruses are released into the wild, the effects on populations could be devastating. The emergence of ranavirus in Japan may be a case of a virulent ranavirus from American bullfrogs being released into wild populations (Une et al. 2009).

Another mechanism of ranavirus emergence is pathogen pollution—the transport of a pathogen across large geographic distances by humans and release of it into a naïve population (Cunningham et al. 2003). It is unclear what constitutes a "large geographic distance," but it's probably related to both the dispersal distance of the host and population isolation. If host populations interact through dispersal, host immune systems co-evolve with natural changes in viral DNA. However, in isolated populations or populations separated by a geographic barrier (such as a mountain range), it is possible for viruses to evolve differently (Ridenhour and Storfer 2008). These slight changes in the virus' genome could result in enhanced virulence in a different population. Thus, if humans transport and release infected hosts over large distances, it could result in emergence of a novel ranavirus (Ridenhour and Storfer 2008). Emergence of ranavirus in some areas of the central U.S., for example, was attributed to moving tiger salamanders for the bait trade (Storfer et al. 2007, Ridenhour and Storfer 2008).

Preventing Spread, Learning More

Ranavirus can persist outside the host for greater than 30 days (Nazir et al. 2012) and be transported on objects such as sediment, boots, and nets. Recreationists and biologists can contribute to pathogen pollution if they contact contaminated water or sediment and do not disinfect footwear or equipment. Such may be the case with the spread of the amphibian chytrid fungus (*Batrachochytrium dendrobatidis*), where high occurrence has been associated with high human access (Pauza et al. 2010). We found that ranavirus prevalence in salamander communities tended to be higher at sites in the Great Smoky Mountains National Park with high access by recreationists (Gray et al. 2009b).

All biologists who work in aquatic systems or handle fish, amphibians, or reptiles should disinfect equipment and supplies that come in contact with these animals or water (Green et al. 2009). Solutions of 3 percent bleach, 0.75 percent Nolvalsan® (chlorhexidine diacetate) and 1 percent Virkon S® (potassium peroxymonosulfate) are effective at inactivating ranavirus with one-minute contact duration (Bryan et al. 2009), and can be applied easily using a pump sprayer. We recommend use of Nolvasan® because it is considered less toxic to amphibians (Hadfield and Whitaker 2005). Additionally, when handling amphibians, biologists should wear disposable vinyl or nitrile gloves rinsed with distilled water and changed before handling different animals (Chashins et al. 2008, Greer et al. 2009, Green et al. 2009).

Beyond these precautions, researchers who plan to release amphibians or other ecothermic vertebrates as part of repatriation projects should test a subset of individuals (up to 30) to verify that they are not infected with ranavirus. Tissue types that can be collected for non-lethal testing of ranavirus infection using polymerase chain reaction (PCR) include toe and tail clips; blood also can be used (Green et al. 2009, Gray et al. 2012). Tissues that are collected can be stored in 90

percent ethanol or frozen at -80°C prior to testing. If individual animals are euthanized, we recommend that infection is tested from a homogenate of the liver and kidneys, which increases detection. We found that tail clips resulted in a 20 percent false negative rate when testing for ranavirus with PCR (Gray et al. 2012). If individuals destined for translocation or repatriation test positive for ranavirus infection, we recommend they are not released. There is no current treatment (e.g., vaccine) available for ranaviral disease.

Guidance on design of surveillance studies, tests used for detecting ranavirus, laboratories that specialize in diagnosing ranaviral disease, and biosecurity precautions are available from the Global Ranavirus Consortium (GRC), a coalition of more than 30 international scientists with expertise in ranaviruses. The group's mission is to facilitate communication and collaborative research on ranaviruses among scientists, veterinarians, and field biologists. To that end, the GRC hosts a listserv (grc@listserv.utk.edu) and convenes a symposium on ranaviruses every two years, with the next scheduled for July 2013 (see box on page 52).

It's essential that wildlife biologists understand the threat of ranavirus and act quickly to address its spread. Many herpetofaunal species of great conservation concern are very susceptible to this pathogen, including the Chinese giant salamander (*Andrias davidianus*), California tiger salamander (*Ambystoma californiense*), gopher tortoise *Gopherus polyphemus*), Carolina gopher frog (*L. capito*), and dusky gopher frog (*L. sevosus*). Several freshwater and marine fish important to global markets are also highly susceptible to infection. In the U.S., for example, Thomas Waltzek of the University of Florida attributed a 2009 die-off of endangered pallid sturgeon (*Scaphirhynchus albus*) fingerlings in Missouri's Blind Pony Fish Hatchery to an FV3-like ranavirus.

Clearly, ranaviruses can impact many ectothermic vertebrate species in the wild and in captivity. We recommend that natural resource agencies and zoological facilities take a proactive role in documenting the presence of ranavirus in populations and take measures to thwart its spread. If we sit idle in the face of this lethal, emerging pathogen, our springs truly may become silent. ■

This article has been reviewed by subject-matter experts.

Go to news.wildlife.org/twp for additional resources on ranavirus.