First International Symposium on Ranaviruses • Joint Meeting of Ichthyologists and Herpetologists •

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Ranaviruses: An Emerging Threat to Ectothermic Vertebrates

Organizer: Matthew Gray[1]; Phone: 865-974-2740; Email: mgray11@utk.edu

Co-organizers: Jesse Brunner[2], Debra Miller[1,3], Jason Hoverman[4], and Andrew Storfer[2]

Affiliations: [1]Center for Wildlife Health, University of Tennessee, Knoxville, TN; [2]School of Biological Sciences, Washington State University, Pullman, WA. [3]Department of Pathobiology, College of Veterinary Medicine, University of Tennessee, Knoxville, TN; [4]Department of Ecology and Evolutionary Biology, University of Colorado, Boulder, CO.

Foreword**:** There is increasing awareness from the scientific community that emerging infectious diseases pose a significant threat to global biodiversity. Emerging diseases have been documented in various organisms including fish and herpetofauna. In particular, a group of viruses in the genus *Ranavirus* (Family Iridoviridae) that cause disease in amphibians, reptiles and fish appear to be an emerging threat for many populations. While ranaviruses were historically overlooked as significant pathogens of ectothermic vertebrates, increasing reports of mass mortality events over the past two decades have raised conservation concern. Ranavirus-associated die-offs in larval and adult amphibians have been documented in the Americas, Europe, and Asia. Death rates often exceed 90% during an outbreak. Ranavirus infections also have been reported in wild and cultured fish populations worldwide. While research on reptiles has been slower to accumulate, recent evidence suggests that ranaviruses are capable of causing morbidity and mortality in free-ranging populations. Together, these widespread die-offs have sparked a diversity of research programs addressing the ecology and evolution of ranavirus-host interactions, potential reservoirs and transmission dynamics, molecular techniques for identifying and characterizing ranaviruses, immunological and histopathological responses to infection, hypothesized causes for emergence, and potential conservation strategies to control emergence. The **purpose** of the *First International Symposium on Ranaviruses* is to bring together scientists from around the world to learn and share information about the impact of this pathogen on ectothermic vertebrates. In total, 24 scientists from 9 countries are participating with expertise in herpetology, ichthyology, ecology, veterinary medicine, immunology, genetics, and molecular biology. An important **outcome** of this symposium will be developing new research collaborations and identifying strategies aimed at reducing or preventing the emergence of ranaviruses.

Ranavirus impacts on wild *Ambystoma opacum* (Marbled Salamander) larvae. Locality: Gourley Pond, Great Smoky Mountains National Park. Photo Credit: Matt Niemiller.

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Keynote Address – Ranaviruses: Past present and Future

G. Chinchar[1], D. Whitley[1], E. Yu[1], G. Chen[2], and J. Robert[2]

[1]University of Mississippi Medical Center, Jackson, MS (email: vchinchar@umc.edu)
[2]University of Rochester School of Medicine, Rochester, NY

Since their discovery by Granoff and his colleagues in the mid-1960s, ranaviruses (RV) have been variously viewed as possible oncogenic viruses; representatives of a heretofore unknown family (*Iridoviridae*) of large DNA viruses; models of viral transcription, genome replication, virus-mediated host-shutoff, and the transcriptional role of DNA methylation; and agents of emerging disease among cold-blooded vertebrates. Although the broad outlines of RV replication were elucidated in the 1970s–1980s through work with frog virus 3 (FV3), the roles of most viral proteins remain unknown. Early attempts at teasing out viral gene function through temperature-sensitive mutants, provided some information on viral nucleic acid metabolism, but for the most part were limited in their usefulness. Recent studies using knock down (KD) mediated by antisense morpholino oligonucleotides (asMO) and siRNA and knock out (KO) following homologous recombination to replace the targeted gene with a double-selective marker (the puromyocin-resistance gene fused to the gene for green fluorescent protein) have provided the opportunity to systematically monitor essential and non-essential replicative and virulence gene function. In addition, the application of molecular tools to ecological studies has provided a facile way for field biologists to identify potential pathogens, quantify infections, trace the evolution of ecologically important viral species, and begin to understand the role of RVs in animal infections. Current RV research is focused on understanding the genetic and molecular basis for virulence; transmission, persistence, and disease manifestation in nature; intrinsic and extrinsic factors that contribute to outbreaks; determinants of geographic range, host range, and host shifts; and the role of the host immune system in protection from disease. Progress will only come through a concerted effort of molecular and field virologists, ecologists, and immunologists working together to resolve these questions.

Biosketch: Dr. Victor Chinchar received his B.S. from the University of Notre Dame in 1972 and a PhD from Indiana University (Bloomington, IN) in 1978. As a graduate student, he worked on projects involving non-human picornaviruses and somatic cell hybridization. From 1978–1981 he was a post-doctoral fellow at St. Jude Childrens' Research Hospital (Memphis, TN) where he examined transcriptional events in paramyxovirus- and rhabdovirus-infected cells. From 1981–1984 he worked as a Research Associate/Assistant Member on two projects: the generation and characterization of temperature-sensitive and drug-resistant mutants, and the development and characterization of monoclonal antibodies targeted to FV3 proteins. In August, 1984 he joined the Department of Microbiology at the University of Mississippi Medical Center (UMMC) as an Assistant Professor, and is currently a Professor within the department and the Associate Dean of the School of Graduate Studies. His laboratory is interested in FV3 replication and is attempting to identify FV3 genes involved in viral replication and immune evasion using antisense morpholino oligonucleotides and siRNAs to knock down viral gene expression and determine function by changes in phenotype. In addition, he collaborates with members of the UMMC Comparative Immunology group in studies related to anti-viral defense mechanisms in catfish.

Victor Chinchar (Ph.D.)

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Inhibition of iridovirus protein synthesis and virus replication by antisense morpholino oligonucleotides targeted to the major capsid protein, the 18 kDa immediate-early protein, and a viral homolog of RNA polymerase II

Robert Sample [a], Locke Bryan [a], Scott Long [a,1], Sai Majji [a], Glenn Hoskins [b], Allan Sinning [b], Jake Olivier [c], V. Gregory Chinchar [a,*]

[a] Department of Microbiology, University of Mississippi Medical Center, Jackson, MS 39216, USA
[b] Department of Anatomy, University of Mississippi Medical Center, Jackson, MS 39216, USA
[c] Department of Preventive Medicine, University of Mississippi Medical Center, Jackson, MS 39216, USA

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Abstract

Frog virus 3 (FV3) is a large DNA virus that encodes ~100 proteins. Although the general features of FV3 replication are known, the specific roles that most viral proteins play in the virus life cycle have not yet been elucidated. To address the question of viral gene function, antisense morpholino oligonucleotides (asMOs) were used to transiently knock-down expression of specific viral genes and thus infer their role in virus replication. We designed asMOs directed against the major capsid protein (MCP), an 18 kDa immediate-early protein (18K) that was thought to be a viral regulatory protein, and the viral homologue of the largest subunit of RNA polymerase II (vPol-IIα). All three asMOs successfully inhibited translation of the targeted protein, and two of the three asMOs resulted in marked phenotypic changes. Knock-down of the MCP resulted in a marked reduction in viral titer without a corresponding drop in the synthesis of other late viral proteins. Transmission electron microscopy (TEM) showed that in cells treated with the anti-MCP MO assembly sites were devoid of viral particles and contained numerous aberrant structures. In contrast, inhibition of 18K synthesis did not block virion formation, suggesting that the 18K protein was not essential for replication of FV3 in fathead minnow (FHM) cells. Finally, consistent with the view that late viral gene expression is catalyzed by a virus-encoded or virus-modified Pol-II-like protein, knock-down of vPol-IIα triggered a global decline in late gene expression and virus yields without affecting the synthesis of early viral genes. Collectively, these results demonstrate the utility of using asMOs to elucidate the function of FV3 proteins.

Keywords: Frog virus 3; Ranavirus; Antisense morpholino oligonucleotides

Introduction

Members of the family *Iridoviridae* comprise a diverse group of large, double-stranded DNA-containing viruses that include two genera infecting invertebrates (*Iridovirus* and *Chloriridovirus*) and three genera targeted to cold-blooded vertebrates (*Ranavirus*, *Lymphocystivirus*, and *Megalocytivirus*) (Chinchar et al., 2005). FV3, the type species of the genus *Ranavirus*, is the best characterized member of this family, and its study has elucidated the basic features of iridovirus replication (Willis et al., 1985; Chinchar, 2002; Williams et al., 2005). The complete genomic sequences of eleven iridoviruses have been determined including those for FV3 and four additional members of the genus *Ranavirus*: tiger frog virus (TFV), Singapore grouper iridovirus (SGIV), grouper iridovirus (GIV), and *Ambystoma tigrinum* virus (ATV) (He et al., 2002; Jancovich et al., 2003; Tan et al., 2004; Song et al., 2004; Tsai et al., 2005). Sequence

* Corresponding author. Fax: +1 601 984 1708.
E-mail address: vchinchar@microbio.umsmed.edu (V.G. Chinchar).
[1] Current address: Department of Biochemistry, St. Jude Children's Research Hospital, Memphis, TN 38101, USA.

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Family *Iridoviridae*: Poor Viral Relations No Longer

V.G. Chinchar(✉), A. Hyatt, T. Miyazaki, T. Williams

Contents

Abstract Members of the family *Iridoviridae* infect a diverse array of invertebrate and cold-blooded vertebrate hosts and are currently viewed as emerging pathogens of fish and amphibians. Iridovirid replication is unique and involves both nuclear and cytoplasmic compartments, a circularly permuted, terminally redundant genome that, in the case of vertebrate iridoviruses, is also highly methylated, and the efficient shutoff of host macromolecular synthesis. Although initially neglected largely due to the perceived lack of health, environmental, and economic concerns, members of the genus *Ranavirus*, and the newly recognized genus *Megalocytivirus*, are rapidly attracting

V.G. Chinchar
Department of Microbiology, University of Mississippi Medical Center, Jackson, MS USA
vchinchar@microbio.umsmed.edu

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Are Ranaviruses Capable of Contributing to Amphibian Species Declines?

M. Gray

Center for Wildlife Health, Department of Forestry, Wildlife and Fisheries, University of Tennessee, Knoxville, TN 37996, USA; (email: mgray11@utk.edu)

Ranaviruses have caused mass mortality in wild and captive amphibians on 5 continents, affecting at least 12 families (8 Anura, 4 Urodela). Despite widespread die-offs, the prevailing thought remains that ranaviruses are incapable of contributing to amphibian species declines. Epidemiological theory specifies that local extirpation of a host by a pathogen is possible under three conditions: (1) frequency dependent transmission, (2) broad host range with asymptomatic carriers, or (3) existence of an environmental reservoir. For ranaviruses, it is possible that all three conditions are met. Condition (1): Frequency dependent transmission can occur through direct contact between breeding adults and among larvae especially for species that exhibit schooling behavior. Condition (2): Laboratory and field studies confirm that ranaviruses infect multiple amphibian species, with susceptibility differing among species. Further, sublethal infections are possible in clinically normal individuals. Condition (3): Interclass transmission of ranaviruses occurs among fish, reptiles and amphibians, thus providing multiple possible vertebrate reservoirs for viral persistence. Virions also have been cultured from water and dry surfaces for >90 days, indicating that survival outside the host may be significant. Two long-term studies provide evidence that local extirpation and amphibian species declines caused by ranaviruses are possible. Future research directions should include expanding controlled studies on the: (1) susceptibility of amphibian species to various ranavirus isolates, (2) occurrence of interclass transmission among relevant ectothermic vertebrate species, and (3) environmental persistence of ranavirus virions. Long-term population monitoring and pathogen surveillance also is needed at sites with reoccurring die-offs.

Biosketch: Dr. Matthew Gray is an associate professor of wildlife ecology in the Department of Forestry, Wildlife and Fisheries at the University of Tennessee (UT), and a member of the UT Center for Wildlife Health. His research with ranaviruses has focused on broad scale surveillance in the eastern United States and controlled experiments that assess relative susceptibility of North American species to ranavirus and possible mechanisms for emergence. To date, we his lab has completed standardized testing on the susceptibility to ranavirus infection and disease for 25 amphibian species (18 anurans, 7 urodeles), with the intent of completing 40 species by August 2012. They have also have tested whether susceptibility differs among developmental stages (egg, hatchling, larva, metamorph) for 7 anuran species. Other experiments have focused on testing whether the natural stress of exposure to an insect predator increases susceptibility, the effects of nitrogenous waste on ranavirus emergence, and the efficacy of disinfectants at inactivating ranavirus. Current experiments are investigating the role of community composition impacting the likelihood of an outbreak, occurrence of interclass transmission among amphibians, reptiles and fish, and environmental persistence of ranavirus virions.

Matthew Gray (Ph.D.)

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Frog virus 3 prevalence in tadpole populations inhabiting cattle-access and non-access wetlands in Tennessee, USA

Matthew J. Gray[1,*], Debra L. Miller[2], A. Chandler Schmutzer[1], Charles A. Baldwin[2]

[1]Center for Wildlife Health, Department of Forestry, Wildlife and Fisheries, University of Tennessee, 274 Ellington Plant Sciences Building, Knoxville, Tennessee 37996-4563, USA

[2]Veterinary Diagnostic and Investigational Laboratory, University of Georgia, 43 Brighton Road, Tifton, Georgia 31793-1389, USA

ABSTRACT: Ranaviruses have been associated with most of the reported larval anuran die-offs in the United States. It is hypothesized that anthropogenically induced stress may increase pathogen prevalence in amphibian populations by compromising immunity. Cattle use of wetlands may stress resident tadpole populations by reducing water quality. We isolated a *Ranavirus* from green frog *Rana clamitans* (n = 80) and American bullfrog *R. catesbeiana* (n = 104) tadpoles collected at 5 cattle-access and 3 non-access wetlands on the Cumberland Plateau, Tennessee, USA. Sequencing confirmed *Frog virus 3* (FV3); therefore, we compared its prevalence between tadpole populations inhabiting cattle-access and non-access wetlands, and among 3 seasons (winter, summer, and autumn) in 2005. We found FV3 in both tadpole species and cattle land-use types; however, prevalence of FV3 was greater in green frog tadpoles residing in cattle-access wetlands compared to those in non-access wetlands. No difference in FV3 prevalence was detected between cattle land uses for American bullfrog tadpoles. A seasonal trend in FV3 prevalence also existed, with prevalence greater in autumn and winter than in summer for both species. In addition, we found that FV3 prevalence decreased significantly as Gosner stage increased in American bullfrog tadpoles. No trend was detected between FV3 prevalence and developmental stage for green frog tadpoles. Our results suggest that cattle use of wetlands may increase prevalence of FV3 in *Rana* tadpoles, although this effect may depend on species, season, and tadpole developmental stage.

KEY WORDS: Amphibian declines · Anthropogenic stressor · Emerging pathogen · *Frog virus 3* · Cattle · Water quality

INTRODUCTION

Ranaviruses are responsible for some of the recent declines in amphibian populations (Carey et al. 2003, Greer et al. 2005), and are considered to be an emerging pathogen (Daszak et al. 1999). In the United States, ranaviruses have been implicated as the etiologic agent in the majority of reported amphibian die-offs (Green et al. 2002). Anuran die-offs known to be caused by *Ranavirus* disease have occurred in at least 15 states of the United States and 11 species (Converse & Green 2005). Ranaviruses exist worldwide and at all elevations (Daszak et al. 1999, Converse & Green 2005). Hence, this group of viruses is potentially a global threat to amphibian populations, especially if their prevalence is increased by anthropogenic stressors (Daszak et al. 2001, Carey et al. 2003).

Ranaviruses are most lethal to amphibian larvae (Gantress et al. 2003), with mortality rates often >90% (Converse & Green 2005). *Ranavirus* transmission has been documented via water bath (Brunner et al. 2004, Pearman et al. 2004, Harp & Petranka 2006), cannibal-

*Email: mgray11@utk.edu

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REVIEW

Ecology and pathology of amphibian ranaviruses

Matthew J. Gray[1,*], Debra L. Miller[1,2], Jason T. Hoverman[1]

[1]274 Ellington Plant Sciences Building, Center for Wildlife Health, Department of Forestry Wildlife and Fisheries, Institute of Agriculture, University of Tennessee, Knoxville, Tennessee 37996-4563, USA

[2]Veterinary Diagnostic and Investigational Laboratory, College of Veterinary Medicine, University of Georgia, 43 Brighton Road, Tifton, Georgia 31793, USA

ABSTRACT: Mass mortality of amphibians has occurred globally since at least the early 1990s from viral pathogens that are members of the genus *Ranavirus*, family *Iridoviridae*. The pathogen infects multiple amphibian hosts, larval and adult cohorts, and may persist in herpetofaunal and osteichthyan reservoirs. Environmental persistence of ranavirus virions outside a host may be several weeks or longer in aquatic systems. Transmission occurs by indirect and direct routes, and includes exposure to contaminated water or soil, casual or direct contact with infected individuals, and ingestion of infected tissue during predation, cannibalism, or necrophagy. Some gross lesions include swelling of the limbs or body, erythema, swollen friable livers, and hemorrhage. Susceptible amphibians usually die from chronic cell death in multiple organs, which can occur within a few days following infection or may take several weeks. Amphibian species differ in their susceptibility to ranaviruses, which may be related to their co-evolutionary history with the pathogen. The occurrence of recent widespread amphibian population die-offs from ranaviruses may be an interaction of suppressed and naïve host immunity, anthropogenic stressors, and novel strain introduction. This review summarizes the ecological research on amphibian ranaviruses, discusses possible drivers of emergence and conservation strategies, and presents ideas for future research directions. We also discuss common pathological signs of ranaviral disease, methods for diagnostic evaluation, and ranavirus surveillance methods. Inasmuch as ranaviral disease is listed as a notifiable disease by the World Organization for Animal Health and is a threat to amphibian survival, we recommend that biosecurity precautions are implemented by nations to reduce the likelihood of transporting ranavirus virions among populations. Biosecurity precautions include disinfecting footwear and equipment that comes in contact with surface water inhabited by amphibians and testing commercially shipped amphibians for the pathogen. We also encourage natural resource organizations to establish routine surveillance programs for ranaviruses in wild amphibian populations.

KEY WORDS: *Ambystoma tigrinum* virus · Anuran · Bohle iridovirus · Urodela · Emerging infectious disease · Frog virus 3 · Iridovirus · Salamander

INTRODUCTION

Amphibian populations are declining globally (Houlahan et al. 2000, Stuart et al. 2004). While there are a number of factors that have contributed to these declines, emerging infectious diseases have been linked to single- and multiple-population die-offs (Collins & Storfer 2003, Daszak et al. 2003, Wake & Vredenburg 2008). *Batrachochytrium dendrobatidis* and several viral types within the genus *Ranavirus* have been associated with most of the reported amphibian mass mortality events (Berger et al. 1998, Green et al. 2002, Carey et al. 2003a). Ranavirus-associated mortality has been reported on 5 continents, at all lati-

*Email: mgray11@utk.edu

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Amphibian Ranavirus Transmission and Persistence

J. Brunner

School of Biological Sciences, Washington State University, P.O. Box 644236, Pullman, WA
(email: JesseBrunner@wsu.edu)

While amphibian ranaviruses are transmissible via several routes—contaminated water, fomites, casual contact, and ingestion via cannibalism and necrophagy—most transmission seems to require close contact. This suggests that transmission is density-dependent. This is true at low densities, according to mesocosm experiments, but at ecologically relevant densities, transmission is essentially a frequency-dependent process. This, in concert with the potential for continued transmission from dead animals, suggests that ranavirus epidemics can extirpate their host populations. How, then, does ranavirus persist to cause recurrent epidemics in the larval segment of amphibians populations? Evidence of ranavirus being able to persist in the environment is mixed, but it is clear that many environments are inhospitable. Similarly, while ranaviruses have a wide host range, there are no demonstrated instances of long-term persistence in a reservoir host species, although this likely reflects a lack of research. In at least some environments there are no alternate hosts. In these places it appears that ranavirus persists in the form of occasional chronic, transmissible infections of their primary host. Just how common this phenomenon is is not known. There is a clear need for more research on 1) the relative importance of transmission from environmental sources, infected carcasses, and live hosts, and 2) transmission between the various members of a pond community. There is also a need to quantify ranavirus persistence in the environment and on fomites, which will help elucidate the risks of repeated epidemics and the translocation of these virulent pathogens.

Biosketch: Dr. Jesse Brunner is an assistant professor in the School of Biological Sciences at Washington State University in Pullman, Washington having just moved from SUNY College of Environmental Sciences and Forestry in Syracuse, New York, where he had a similar position. Dr. Brunner earned his PhD from Arizona State University in 2004, where he worked on questions related to persistence, virulence, and transmission of the *Ambystoma tigrinum* virus. He then worked at the Cary Institute of Ecosystem studies on the community ecology of Lyme disease and other tick-borne diseases. The hallmark of his lab is the approach we take: we combine theoretical models with manipulative experiments, observational studies, and statistical modeling to tease apart the mechanisms that underpin the ecology and evolution of infectious diseases.

Jesse Brunner (Ph.D.)

Evolutionary Ecology Research, 2009, **11**: 1169–1188

Testing assumptions of the trade-off theory of the evolution of parasite virulence

Jesse L. Brunner[1] and James P. Collins[2]

[1]Department of Environmental and Forest Biology,
SUNY College of Environmental Science and Forestry, Syracuse, New York
and [2]School of Life Sciences, Arizona State University, Tempe, Arizona, USA

ABSTRACT

Background: Parasite replication is essential for transmission, but is thought to have inevitable virulent effects. The trade-off theory of parasite virulence asserts that parasites balance virulence (the increased death rate of infected hosts), which shortens the infectious period and thus reduces transmission opportunities, against transmissibility (the probability of transmission given a contact) to maximize overall transmission.

Questions: To what extent are virulence and transmissibility parasite traits? Are these traits correlated such that more virulent infections are more transmissible?

Methods: We infected tiger salamander (Ambystoma tigrinum) larvae with nine isolates of the Ambystoma tigrinum virus (ATV) and then exposed naive larvae to these infected larvae, measuring mortality rates in both to test the heritability of virulence. We then exposed five lineages of A. tigrinum larvae to five ATV isolates in a factorial design and measured mortality rates and virus shedding in each host–virus combination to determine the extent to which transmissibility and virulence are traits of the host and parasite, and whether they are related.

Results: Virulence is a heritable trait of virus isolates, but the variation among isolates is swamped by the much larger differences among host lineages. Transmissibility is clearly a viral trait. Within a given host lineage or across host–virus combinations there was little evidence that more virulent infections were also more transmissible. These results do not support the trade-off theory of virulence, but may reflect selection for alternative routes of ATV transmission.

Keywords: evolution of virulence, ranavirus, tiger salamander, trade-off theory, transmissibility.

INTRODUCTION

Parasite virulence, defined as the parasite-induced rate of host mortality, is often thought to be a product of parasite replication within a host. Greater replication rates in a host translate into greater virulence, but also into more transmission propagules. More virulent infections may therefore be more transmissible (i.e. more likely to be transmitted given an

Correspondence: J.L. Brunner, Department of Environmental and Forest Biology, SUNY College of Environmental Science and Forestry, Syracuse, NY 13210, USA. e-mail: jbrunner@esf.edu

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Escape from the pond: stress and developmental responses to ranavirus infection in wood frog tadpoles

Robin W. Warne[1]*, Erica J. Crespi[1] and Jesse L. Brunner[2]

[1]*Department of Biology, Vassar College, 124 Raymond Ave, Poughkeepsie, New York 12604, USA; and* [2]*Department of Environmental and Forest Biology, State University of New York College of Environmental Science and Forestry, Syracuse, New York 13210, USA*

Summary

1. Animal populations exhibit considerable variation in their susceptibility to infection by emerging diseases, yet it is poorly understood how environmental and intrinsic factors contribute to these patterns. Considering that intrinsic factors (e.g. life history stage, nutritional state) can impact immune function, knowledge of the physiological mechanisms that mediate susceptibility to infection may improve our understanding of the emergence of disease in natural populations.

2. Ranavirus outbreaks have been associated with die-offs of amphibians worldwide. While the ecological factors associated with epidemics have been widely studied, little is known about how physiological factors mediate amphibian responses to ranavirus infection.

3. The neuroendocrine hypothalamus-pituitary-interrenal axis (HPI) is a physiological system central to coordinating energy balance and development. It is known to both stimulate and inhibit immune function in vertebrates in different contexts. We hypothesized that the HPI axis would also mediate responses to ranavirus infection. We used wood frog (*Rana sylvatica*) larvae and ranavirus isolated from recent die-offs of local wood frog populations to examine the physiological responses to infection.

4. In addition to increasing odds of death with increasing doses of virus in an LD_{50} study, we saw a 1·7-fold increase in the odds of death with each increase in Gosner stage at the time of infection.

5. We then examined the HPI stress response of prometamorphic tadpoles exposed to a lethal dose of ranavirus. Infected tadpoles exhibited significantly elevated corticosterone levels, more rapid developmental changes, and a greater decrease in body weight relative to controls over 6 days after exposure.

6. Although elevated corticosterone mobilizes resources and enhances immunity, its acceleration of metamorphosis may be maladaptive in response to ranavirus infection, because it can draw energy away from expensive immune responses. These findings provide insight into how the balance of energy between development and immune function may contribute to patterns of ranavirus infection in pre-metamorphic amphibians.

Key-words: ranavirus, emerging disease, dose effects, corticosterone, stress response, development, wood frog

Introduction

Emerging diseases, such as chytridiomycosis and ranavirus infection, cause mass mortality and even extinctions in amphibian populations worldwide (Carey 2000; Lips *et al.* 2006; Gray, Miller & Hoverman 2009; Kilpatrick, Briggs & Daszak 2010). While it is known that amphibian populations vary in their susceptibility to infection (Carey, Cohen & Rollins-Smith 1999; Brunner, Richards & Collins 2005; Cotter *et al.* 2008; Lips *et al.* 2008; Garner *et al.* 2009), the underlying causes of this variation are unclear. Environmental factors such as pollution and environmental change are often invoked to explain these differences because they can directly or indirectly decrease the immune competence

*Correspondence author. E-mail: rowarne@vassar.edu

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Amphibian Susceptibilities to the Emerging Amphibian Pathogen Ranavirus

J. Hoverman

Department of Ecology and Evolutionary Biology, University of Colorado, Boulder, CO 80309-0334
(email: jason.hoverman@colorado.edu)

Ranaviruses have been implicated as a major cause of reported amphibian die-offs in the United States. One of the hypothesized factors in the recent emergence of ranaviruses in amphibian populations is novel strain introduction (i.e., pathogen pollution). While pathogen pollution has been identified as a significant concern, the role of species-susceptibility to novel versus endemic strains is just beginning to be explored. For example, when 19 larval amphibian species from 7 families were challenged with two ranavirus isolates: endemic frog virus 3 (FV3) and an isolate from an American bullfrog culture facility, susceptibilities varied markedly among species. The isolates showed little host specificity and all but one species experienced mortality or infection following exposure. Moreover, 53% of the species experienced over 50% mortality following exposure to the ranaculture isolate. Mortality post-exposure to the ranaculture isolate was on average 2.3X greater than post-exposure to FV3, the type species for ranavirus. These findings suggest that amphibian culture facilities may be sources of novel ranaviruses, and highlight the potential threat of pathogen pollution associated with the international and interstate commerce of amphibians. Currently, there is limited information on the occurrence or spread of novel ranavirus isolates in wild amphibian populations and whether pathogen pollution is a driver of disease outbreaks. There is a need for studies to characterize the species/strains of ranaviruses involved in die-off events to help guide conservation and management efforts.

Biosketch: Dr. Jason T. Hoverman is currently a post-doctoral research associate in the Department of Ecology and Evolutionary Biology at the University of Colorado, Boulder. He received a BS (2000) and PhD (2007) from the University of Pittsburgh. From 2007 to 2010, Dr. Hoverman was a post-doctoral research associate in the Department of Forestry, Wildlife and Fisheries at the University of Tennessee, Knoxville where his is now an adjunct assistant professor. He has diverse research interests that include community ecology, phenotypic plasticity, behavior, ecotoxicology, and disease ecology. This research has primarily utilized freshwater aquatic systems (e.g., ponds, wetlands) and their associated taxa (e.g., amphibians, snails, insects, pathogens). Over the past several years, his research has examined host-pathogen interactions within amphibian communities with a focus on trematodes and ranaviruses.

Jason Hoverman (Ph.D.)

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First Report of *Ranavirus* Infecting Lungless Salamanders

MATTHEW J. GRAY*
DEBRA L. MILLER**
and
JASON T. HOVERMAN
Center for Wildlife Health, Department of Forestry, Wildlife and Fisheries
University of Tennessee, 274 Ellington Plant Sciences Building
Knoxville, Tennessee 37996, USA

***Veterinary Diagnostic and Investigational Laboratory*
University of Georgia, 43 Brighton Road
Tifton, Georgia 31793-1389, USA

**Corresponding author; e-mail: mgray11@utk.edu*

Ranaviruses are a group of pathogens belonging to the genus *Ranavirus* (Family Iridoviridae) that have been linked to catastrophic die-offs of larval amphibians in North America and elsewhere (Daszak et al. 2003). They also have been identified as the etiologic agent in the mass mortality of adult Common Frogs (*Rana temporaria*) and Common Toads (*Bufo bufo*) in the United Kingdom (Cunningham et al. 1996; 2007a,b). In the United States, ranaviruses are responsible for the majority of disease-related mortality events in amphibians (Green et al. 2002; Muths et al. 2006). There is evidence that ranaviruses may be an emerging infectious disease (Storfer et al. 2007), possibly due to novel strain introduction (Picco and Collins 2008) or increased occurrence of anthropogenic stressors on the landscape (Forson and Storfer 2006; Gray et al. 2007). Recognizing the potential threat of ranaviruses to global amphibian biodiversity, the World Organization for Animal Health (OIE) recently listed this pathogen as a notifiable disease, requiring proof of *Ranavirus*-negative results before commercial shipment of amphibians (OIE 2008). The OIE identifies field surveillance as a critical component of risk assessment for ranaviruses (OIE 2008). Although surveillance for the amphibian pathogen *Batrachochytrium dendrobatidis* has become widespread (e.g., Chatfield et al. 2009; Rothermel et al. 2008), testing amphibians for *Ranavirus* occurs less frequently.

The Southern Appalachian Mountain Range of North America represents a global hotspot for salamander biodiversity (Dodd 2004; Petranka 1998). In particular, lungless salamanders (Family Plethodontidae) occur in high abundance and biomass (Peterman et al. 2008; Petranka and Murray 2001) and are important components of the ecosystem (Davic and Welsh 2004). Known die-offs of Eastern Newts (*Notophthalmus viridescens*), Spotted Salamanders (*Ambystoma maculatum*), Pickerel Frogs (*Lithobates palustris*), and Wood Frogs (*L. sylvaticus*) occurred in the Southern Appalachian Mountains due to ranaviruses in 1999 and 2001 (Converse and Green 2005; Green et al. 2002). Despite these mortality events, surveillance for *Ranavirus* in Southern Appalachian amphibians has been nonexistent. Our goal was to test for the presence of *Ranavirus* in lungless salamander communities located in the Southern Appalachian Mountains. We tested for *Ranavirus* in salamander communities at three sites that differed in elevation and report prevalence by species and for each site.

Methods.—We captured adult lungless salamanders for *Ranavirus* testing on 21 April 2007 at three locations in the Great Smoky Mountains National Park, Tennessee: 1) Ash Hopper Branch (456 m elev.; 35.6836°N, 83.5375°W); 2) Chimney Tops Seeps (831 m elev.; 35.6367°N, 83.4928°W); and 3) Indian Gap Seeps (1537 m elev.; 35.61°N, 83.45°W). At all sites, we searched 1 h for salamanders in streams, seeps, and under terrestrial ground cover items (e.g., logs, rocks). We placed captured salamanders in individual 1-L plastic containers and processed up to 10 individuals per species per site. We swabbed the oral cavity and the cloaca twice each. We wore disposable gloves and changed them between animals to minimize the likelihood of cross contamination among samples. We put each swab in separate microcentrifuge tubes, placed the tubes on dry ice, and froze the swabs at -70°C within 10 h of collection. We swabbed 21, 21, and 27 salamanders at Ash Hopper Branch, Chimney Tops, and Indian Gap sites, respectively. All salamanders were released at their approximate capture location within 1 h of capture, and containers, footwear, and equipment were disinfected with 2% Nolvasan® prior to moving between sites (Bryan et al. 2009). We shipped frozen swabs overnight on dry ice to the University of Georgia Veterinary Diagnostic and Investigational Laboratory for *Ranavirus* testing.

We used conventional polymerase chain reaction (PCR) to test for the occurrence of *Ranavirus*. We extracted genomic DNA from swabs using a QIAamp DNA Mini Kit (Qiagen Inc., Valencia, California). A heminested PCR targeting a 500-bp region of the major capsid protein (MCP) gene was used following the protocol by Kattenbelt et al. (2000). The PCR products were resolved by gel electrophoresis for determination of *Ranavirus* occurrence. We randomly chose one sample per species with a distinct PCR-positive band, cut the band from the gel, and submitted the isolated product to SeqWright DNA Technology Services, Houston, Texas, for automated sequencing in the forward and reverse directions. We then performed a GenBank BLAST search (http://www.ncbi.nlm.nih.gov/Genbank.html) on the sequences to verify that positive PCR results were *Ranavirus*. Additionally, real-time quantitative PCR (qPCR) was performed following the procedure by Picco et al. (2007) on samples used for sequencing to further support our findings.

We summarized the positive test results for each species, and tallied *Ranavirus* prevalence among species for each sampling

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Anuran susceptibilities to ranaviruses: role of species identity, exposure route, and a novel virus isolate

Jason T. Hoverman[1,*], Matthew J. Gray[1], Debra L. Miller[1,2]

[1]Center for Wildlife Health, Department of Forestry, Wildlife and Fisheries, University of Tennessee, 274 Ellington Plant Sciences Building, Knoxville, Tennessee 37996-4563, USA

[2]Veterinary Diagnostic and Investigational Laboratory, College of Veterinary Medicine, University of Georgia, 43 Brighton Road, Tifton, Georgia 31793, USA

ABSTRACT: Ranaviruses are responsible for widespread amphibian die-offs, particularly with larval anurans. To understand the factors that may be contributing to the emergence of ranaviruses, we conducted 3 experiments that exposed 3 species of larval anurans to either endemic frog virus 3 (FV3) or an FV3-like isolate from a ranaculture facility. Our goals were to (1) determine the susceptibility of each species to each virus, (2) determine whether direct ingestion of virions or exposure to virions in a water bath were similarly lethal routes of transmission, and (3) quantify the effects of exposure duration on disease outcomes. We conducted our research in a controlled aquatic laboratory using a factorial combination of virus isolates, transmission routes, and exposure durations. While ranaviruses can affect many species, we found that larval anurans differ greatly in susceptibility to ranaviruses. Average mortality rates of Cope's gray tree frogs (66%) and pickerel frogs (68%) were similar but 3-fold higher than for eastern narrow-mouthed toads. Direct ingestion of the viruses increased mean infection and mortality rates by 30% and caused death about 2 times faster compared to water bath exposure. However, exposure duration did not impact mean infection or mortality rates. We also found that the ranaculture isolate increased mortality by >34% compared to FV3. Our results suggest that ranaviruses can rapidly infect and cause disease in multiple amphibian species. Given the risk associated with introducing novel ranaviruses from ranaculture facilities, we recommend that all nations adopt the protocol set forth by the World Organization for Animal Health for testing and certifying that amphibians that are commercially shipped are negative for ranavirus infection.

KEY WORDS: Emerging infectious disease · Pathogen pollution · Novel strain · Iridoviridae · Ranavirus · Frog virus 3 · Anuran

INTRODUCTION

The global emergence of infectious diseases in humans, livestock, wildlife, and plants has generated interest in understanding the factors that drive host–pathogen interactions (Daszak et al. 2000, Cleaveland et al. 2001, Dobson & Foufopoulos 2001). Disease emergence may be driven by multiple factors, including differences in species susceptibility, transmission efficiency among hosts, and whether pathogens are novel or endemic (Dobson & Foufopoulos 2001, McCallum et al. 2001, Cunningham et al. 2003). By developing a mechanistic understanding of how these factors drive disease dynamics, we can predict outbreaks and potentially manage or reduce the negative consequences associated with disease (Keesing et al. 2006).

Among the classes of vertebrates, amphibians are considered the most imperiled (Stuart et al. 2004, Wake & Vredenburg 2008). Emerging infectious diseases have been linked to amphibian declines (Daszak et al. 1999). In particular, ranaviruses are a group of amphibian pathogens that are globally distributed and have been linked to catastrophic mortality in larval

*Email: jhoverma@utk.edu

Ranavirus Publications

Gray, M. J., D. L. Miller, and J. T. Hoverman. 2009. First report of *Ranavirus* infecting lungless salamanders. Herpetological Review 40:316–319.

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Haislip, N. A., M. J. Gray, J. T. Hoverman, and D. L. Miller. 2011. Development and disease: how susceptibility to an emerging pathogen changes through anuran development. PLoS One (In press).

Ranaviruses and Amphibians: Outside the Box of Host-Parasite Relationships

D. Lesbarrères

GEARG, Department of Biology, Laurentian University, Sudbury, Canada
(email: dlesbarreres@laurentian.ca)

Pathogens are known to affect their hosts in a variety of manners and ranaviruses are no different. Traditional investigation of host-parasite relationships have focused on host life-history traits and have used the variation in those traits to assess pathogen virulence. However, the dynamic nature of adaptation and counter-adaptation between the host and the parasite may be particularly sensitive to environmental influence. Here, I will focus on the role of potential abiotic and biotic mechanisms such as temperature, larval developmental stages, and competition for resources on the prevalence and virulence of the virus. For instance, I will show that ranavirus virulence is likely density-dependent, with the effect of ranavirus infection being relatively more severe in animals held in low density. I will also present the relative susceptibility of amphibians during their different life-history stages and the potential consequences of egg infection for disease screening and experimental studies. Additionally, amphibian species differ in their susceptibility to ranaviruses and significant isolates within different strains (ATV, FV3) are numerous. An investigation of the host-pathogen genotypic interactions, in the environmental context, is needed to improve our understanding of ranavirus virulence. Ranavirus is a serious threat to amphibian populations throughout the world; therefore there is a need to investigate the environmental factors that influence its virulence so that we might begin to understand the epidemiology of ranaviral diseases and forecast disease outbreaks.

Biosketch: In the broadest sense, Dr. David Lesbarrèrres is interested in theoretical and applied questions about the evolution and ecology of amphibian species and communities. After receiving his PhD in France (2001), he joined Dr. Merilä's EGRU team in Finland for a post-doctoral position. He arrived at Laurentian University in Canada in 2004 and the past 6 years his research program has aimed at understanding amphibian population declines in human dominated landscapes. More specifically, Dr. Lesbarrèrres' focus is on two major threats: emerging infectious diseases (EIDs) and habitat fragmentation. Part of this research also investigates the fitness consequences of phenotypic and genetic variation. Both the industry and national agencies or government bodies have supported his research program. In general, his work integrates intense fieldwork coupled with molecular approaches for the analysis of population genetics and disease detection as well as laboratory experiments to estimate measures of fitness.

David Lesbarrèrres (Ph.D.)

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PLoS one

Context-Dependent Effects of Ranaviral Infection on Northern Leopard Frog Life History Traits

Pierre Echaubard[1], Kevin Little[1], Bruce Pauli[2], David Lesbarrères[1]*

1 Genetics and Ecology of Amphibians Research Group (GEARG), Department of Biology, Laurentian University, Sudbury, Canada, **2** Environment Canada, Science and Technology Branch, National Wildlife Research Centre, Carleton University, Ottawa, Canada

Abstract

Pathogens have important effects on host life-history traits, but the magnitude of these effects is often strongly context-dependent. The outcome of an interaction between a host and an infectious agent is often associated with the level of stress experienced by the host. *Ranavirus* causes disease and mortality in amphibian populations in various locations around the world, but most known cases of ranaviral infection have occurred in North America and the United Kingdom. While *Ranavirus* virulence has been investigated, the outcome of *Ranavirus* infection has seldom been related to the host environment. In a factorial experiment, we exposed Northern leopard frog (*Lithobates pipiens*, formerly *Rana pipiens*) tadpoles to different concentrations of *Ranavirus* and investigated the effect of host density on certain life-history traits, namely survival, growth rate, developmental stage and number of days from virus exposure to death. Our results suggest a prominent role of density in driving the direction of the interaction between *L. pipiens* tadpoles and *Ranavirus*. We showed that increasing animal holding density is detrimental for host fitness as mortality rate is higher, day of death earlier, development longer and growth rate significantly lower in high-density tanks. We observed a linear increase of detrimental effects when *Ranavirus* doses increased in low-density conditions, with control tadpoles having a significantly higher overall relative fitness. However, this pattern was no longer observed in high-density conditions, where the effects of increasing *Ranavirus* dose were limited. Infected and control animals fitness were consequently similar. We speculate that the host may eventually diverts the energy required for a metabolic/immune response triggered by the infection (*i.e.*, direct costs of the infection) to better cope with the increase in environmental "stress" associated with high density (*i.e.*, indirect benefits of the infection). Our results illustrate how the net fitness of organisms may be shaped by ecological context and emphasize the necessity of examining the direct/indirect costs and benefits balance to fully understand host-pathogen interactions.

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* E-mail: dlesbarreres@laurentian.ca

Introduction

Pathogens are known to affect their hosts in a variety of manners [1]. Most of the studies investigating the relationship between hosts and pathogens have focused on the direct effects that pathogens have on host life history traits, usually including measures such as body length, body weight, growth rate, or survival [2]. Traditionally, quantifying the variation in these traits following infection is used to assess pathogen virulence and host fitness effects. However, beyond the effect a pathogen can have on host-specific fitness traits, attention has also been given to the role that pathogens can play in structuring host communities and affecting population dynamics [3]. While local extinction due to pathogen exposure is rare (see [4] for an example) the extent of detrimental effects caused by a parasite may depend on biological factors such as the pathogen's mode of transmission[5], the host genotype [6], and the host condition [7,8]. Some of these features have been reported to be highly context dependent. For instance, previous studies have suggested that the degree of differential mortality suffered by infected hosts is linked to the specific host-pathogen relationship, but may also be influenced by the type and level of stress experienced by the host [9].

Relationships between pathogens, parasites and environmental disturbance have recently been addressed in human-modified systems [10], whereby pesticides or other pollutant exposure has typically been found to enhance parasite virulence [11,12] due to a reduction of the host immune function [13]. At the same time, natural environmental fluctuations can also interact with pathogen virulence. For instance, host population increase may lead to an increase in intraspecific competition for food resources (due to the reduction of per capita food availability) that may affect host traits such as body size, body weight, growth rate, and reproductive ability and in turn affect the pathogen virulence and epidemiology [14]. High density situations may also result in an increase in the contact rate between individuals that can be stressful [15], and may also boost pathogen transmission rate (*i.e.* horizontal transmission [16]) and subsequent pathogen load and virulence. For example the gray treefrog (*Hyla versicolor*) can co-occur in temporary and permanent ponds with a snail (*Pseudosuccinea columella*) that is frequently infected with the digenetic trematode *Telorchis* spp., whose free-swimming cercariae infect *H. versicolor*

Conservation Biology

Contributed Paper

Effects of Two Amphibian Pathogens on the Developmental Stability of Green Frogs

VALÉRIE ST-AMOUR,* TRENTON W. J. GARNER,† ALBRECHT I. SCHULTE-HOSTEDDE,* AND DAVID LESBARRÈRES*††

*Biology Department, Laurentian University, Sudbury, Ontario, P3E 2C6, Canada
†Institute of Zoology, Zoological Society of London, Regents Park NW1 4RY, London, United Kingdom

Abstract: *Developmental instability, measured as fluctuating asymmetry (FA), is often used as a tool to measure stress and the overall quality of organisms. Under FA, it is assumed that control of symmetry during development is costly and that under stress the trajectory of development is disturbed, resulting in asymmetric morphologies. Amphibian emergent infectious diseases (EIDs), such as* Ranavirus *and chytrid fungus, have been involved in several mortality events, which makes them stressors and allows for the study of FA. We analyzed nine populations of green frogs* (Rana clamitans) *for the presence or absence of* Ranavirus *and chytrid fungus. Individuals were measured to determine levels of FA in seven traits under the hypothesis that FA is more likely to be observed in individuals infected by the pathogens. Significantly higher levels of FA were found in individuals with* Ranavirus *compared with uninfected individuals among all populations and all traits. We did not observe FA in individuals infected with chytrid fungus for any of the traits measured. Additionally, we observed a significant association between* Ranavirus *infection and levels of FA in both males and females, which may indicate this viral disease is likely to affect both sexes during development. Altogether, our results indicate that some EIDs may have far-reaching and nonlethal effects on individual development and populations harboring such diseases and that FA can be used as a conservation tool to identify populations subject to such a stress.*

Keywords: amphibian population declines, *Batrachochytrium dendrobatidis*, chytrid fungus, developmental instability, fluctuating asymmetry, green frogs, *Rana clamitans*, *Ranavirus*

Efectos de Dos Patógenos de Anfibios sobre la Estabilidad del Desarrollo de Ranas Verdes

Resumen. *La inestabilidad del desarrollo, medida como asimetría fluctuante (AF), a menudo se utiliza como una herramienta para medir estrés y la calidad integral de los organismos. Bajo AF, se asume que el control de la simetría durante el desarrollo es costoso y que bajo estrés se perturba la trayectoria del desarrollo, dando como resultado morfologías asimétricas. Las enfermedades infecciosas emergentes (EIE) en los anfibios, como* Ranavirus *y hongo chytridio, han sido involucradas en varios eventos de mortalidad, por lo que son factores estresantes y permiten el estudio de AF. Analizamos nueve poblaciones de ranas verdes* (Rana clamitans) *para la presencia o ausencia de* Ranavirus *y hongo chytridio. Los individuos fueron medidos para determinar los niveles de AF en siete atributos bajo la hipótesis de que es más probable observar la AF en individuos infectados por los patógenos. Encontramos niveles de AF significativamente mayores en individuos con* Ranavirus *en comparación con individuos no infectados entre todas las poblaciones y todos los atributos. No observamos AF en individuos infectados con el hongo chytridio para ninguna de los atributos medidos. Adicionalmente, observamos una asociación significativa entre la infección por* Ranavirus *y los niveles de AF tanto en machos como hembras, lo cual puede indicar que es probable que esta enfermedad viral afecte a ambos sexos durante el desarrollo. En conjunto, nuestros resultados indican que algunas EIE pueden tener efectos de largo alcance y no letales sobre el desarrollo de individuos y poblaciones que hospedan tales enfermedades y que la AF puede ser utilizada como una herramienta de conservación par identificar poblaciones sujetas a tal estrés.*

††*Address correspondence to D. Lesbarrères, email dlesbarreres@laurentian.ca*
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Effects of Pesticide Exposure on Susceptibility to Ranavirus in Tiger Salamanders, *Ambystoma tigrinum*

J. Kerby[1,2] and A. Storfer[2]

[1]Biology Department, University of South Dakota, Vermillion SD, (email: Jacob.Kerby@usd.edu)
[2]Biological Sciences Division, Washington State University, Pullman WA

Changing land use has forced several amphibian species to utilize wetland sites with large anthropogenic impacts. Several sites are in agricultural areas that are exposed to pesticides known to cause both direct and indirect negative effects to amphibian species. Little is understood on how pesticides alter amphibian host-pathogen dynamics, and we are in the infancy of these investigations. The few studies that have been done have revealed that pesticide exposure can increase mortality in *Ambystoma tigrinum virus* (*ATV*) exposed individuals. For example, the combined effects of the insecticide chlorpyrifos and the herbicide atrazine exhibited a monotonic effect of increased mortality with increasing pesticide concentrations. Although no synergistic effects were detected, survival was reduced from 70% in *ATV* only exposed treatments to 20% survival in the highest concentration chlorpyrifos/atrazine/*ATV* treatments. This effect was also found with another insecticide, carbaryl, when combined with *ATV* and a natural stressor of predator cue. Predator cue exposure alone produced no mortality, but survival was again dramatically reduced when combined with *ATV* and the insecticide (from 93% to 60%). These results suggest that natural stressors might play an important role in determining the effect of anthropogenic stressors on host pathogen dynamics and should be examined more closely. Further laboratory work examining other commonly used pesticides is essential, as are experiments conducted in more natural and larger mesocosms as is typical of many amphibian ecotoxicological studies. We also see a need for the long-term examination of field sites that might be influenced by both agriculture and ranavirus.

Biosketch: Dr. Jake Kerby has been an assistant professor at the University of South Dakota since 2008. He did his post-doctoral work with Andrew Storfer at Washington State University from 2006-2008 examining the interactions of contaminants on ranavirus susceptibility in amphibians. His Ph.D. work was done at UC Davis under Andy Sih where he studied the impacts of pesticides on amphibian species community interactions. Dr. Kerby is therefore an ecotoxicologist by training and focuses primarily on the direct and indirect effects of toxicants on amphibian populations. Recently, his laboratory work has focused on understanding the impacts of contaminants on disease susceptibility with both ranavirus and chytrid pathogens. Given the prominence of agriculture in Eastern South Dakota, his lab is currently examining field sites that demonstrate high pesticide runoff and examining its effect on disease prevalence in salamander and frog populations.

Jake Kerby (Ph.D.)

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ECOHEALTH

Original Contribution

Combined Effects of Atrazine and Chlorpyrifos on Susceptibility of the Tiger Salamander to *Ambystoma tigrinum* Virus

Jacob L. Kerby[1] and Andrew Storfer[2]

[1]*Biology Department, University of South Dakota, 414 E. Clark Street, Vermillion, SD 57069*
[2]*Biological Sciences, Washington State University, Pullman, WA 99164*

Abstract: Several hypotheses have been examined as potential causes of global amphibian declines, including emerging infectious diseases and environmental contaminants. Although these factors are typically studied separately, animals are generally exposed to both stressors simultaneously. We examined the effects of the herbicide atrazine and the insecticide chlorpyrifos on the susceptibility of tiger salamander larvae, *Ambystoma tigrinum*, to a viral pathogen, *Ambystoma tigrinum* virus (ATV). Environmentally relevant concentrations of atrazine (0, 20, 200 µg/L) and chlorpyrifos (0, 2, 20, 200 µg/L) were used along with ATV in a fully factorial experimental design whereby individually housed, 4-week-old larvae were exposed for 2 weeks. Atrazine alone was not lethal to larvae, and chlorpyrifos alone was lethal only at the highest concentration. When combined with ATV, chlorpyrifos increased susceptibility to viral infection and resulted in increased larval mortality. A significant interactive effect between atrazine and ATV was detected. Atrazine treatments slightly decreased survival in virus-exposed treatments, yet slightly increased survival in the virus-free treatments. These findings corroborate earlier research on the impacts of atrazine, in particular, on disease susceptibility, but exhibit greater effects (i.e., reduced survival) when younger larvae were examined. This study is the first of its kind to demonstrate decreases in amphibian survival with the combination of pesticide and a viral disease. Further examination of these multiple stressors can provide key insights into potential significance of environmental cofactors, such as pesticides, in disease dynamics.

Keywords: pesticide, disease, multiple stressors, amphibian, salamander, virus

INTRODUCTION

Amphibian declines are occurring worldwide due to a variety of anthropogenic impacts (Blaustein and Kiesecker, 2002). Whereas clear mechanisms likely underlie many of these declines (e.g., habitat loss and invasive species), other causes are far less understood and can involve subtle and complex interactions (Collins and Storfer, 2003). These "enigmatic" declines account for a large proportion of worldwide population losses and require further empirical and field-based investigation (Stuart et al., 2004). Two primary suspects for these enigmatic declines are emerging infectious diseases and contaminant exposure.

Disease is thought to play a key role in amphibian declines worldwide (Daszak et al., 2003; Stuart et al., 2004). Several diseases have been implicated in these declines:

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Correspondence to: Jacob L. Kerby, e-mail: drkerby@gmail.com

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ECOHEALTH

Original contribution

Combined Effects of Virus, Pesticide, and Predator Cue on the Larval Tiger Salamander (*Ambystoma tigrinum*)

Jacob L. Kerby,[1] Alison J. Hart,[2] and Andrew Storfer[2]

[1]*Biology Department, University of South Dakota, 414 E. Clark St, Vermillion, SD 57069*
[2]*School of Biological Sciences, Washington State University, Pullman, WA 99163*

Abstract: Emerging diseases and environmental contamination are two of the leading hypotheses for global amphibian declines. Yet few studies have examined the influence of contaminants on disease susceptibility, and even fewer have incorporated the role of natural stressors such as predation. We performed a factorial study investigating the interaction of the insecticide carbaryl, dragonfly predator cue, and the emerging pathogen *Ambystoma tigrinum* virus (ATV) on fitness correlates and disease susceptibility in tiger salamander larvae. Four week old larvae were exposed for 22 days in a 2 (0, 500 µg/l carbaryl) × 2 (control, predator cue water) × 2 (0, 1×10^4 pfu ATV) factorial designed laboratory study. Results show significant impacts to survival of larvae for both virus and predator cue treatments, as well as an interactive effect between the two, in which predator cue strongly exacerbated disease-driven mortality. There was a clear pattern of reduced survival with the addition of stressors, with those where all three stressors were present exhibiting the worst effects (a decrease in survival from 93 to 60%). On those that survived, we also detected several sub-lethal impacts in mass, SVL, and development. Predator cue and pesticide treatments significantly reduced both SVL and mass. Virus and predator treatments significantly slowed development. Stressors also exhibited opposing effects on activity. Predator cue caused a significant reduction in activity, whereas virus caused a significant increase in activity over time. These results highlight the importance of examining combined natural and introduced stressors to understand potential impacts on amphibian species. Such stressors may contribute to the emergence of ATV in particular regions, raising concerns about the influence of pesticides on disease emergence in general.

Keywords: Predator, Ranavirus, Pesticide, Multiple stressor, Tiger salamander

INTRODUCTION

Emerging infectious diseases are increasingly recognized for their crucial role in population and even community dynamics (Daszak et al. 2000). While some pathogens can play important roles in maintaining community diversity and ecosystem function (McCallum and Dobson 1995; Lafferty et al. 2006), others can threaten biodiversity by causing host extinction (Hudson and Greenman 1998; de Castro and Bolker 2005). These diseases are found in a wide variety of taxa and little is understood about how these diseases emerge and in particular which co-factors, if any, might be involved (Daszak et al. 2003). Understanding

Correspondence to: Jacob L. Kerby, e-mail: Jacob.Kerby@usd.edu

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Ranavirus Publications

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Evidence for multiple recent host species shifts among the ranaviruses (family *Iridoviridae*)

J. Jancovich[1], M. Bremont[2], J. Touchman[3], and B. Jacobs[1,3]

[1]The Biodesign Institute, Arizona State University, Tempe, AZ 85287-5401, email: james.jancovich@asu.edu
[2]Unité de Virologie et Immunologie Moléculaires, Institut National de la Recherche Agronomique, F-78352 Jouy-en-Josas, France
[3]School of Life Sciences, Arizona State University, Tempe, AZ 85287-4601

Members of the genus *Ranavirus* (family *Iridoviridae*) have been recognized as major viral pathogens of cold-blooded vertebrates. Ranaviruses (RVs) have been associated with amphibians, fish and reptiles throughout the world. At this time, the relationship between ranavirus isolates is unclear. To gain a better understanding of the relationship among ranavirus isolates and to gain insight into the evolution of the ranaviruses, we compared genomic sequences from all of the completely sequenced ranavirus isolates. Our findings suggest that the ancestral ranavirus was a fish virus and that several recent host shifts have taken place with subsequent speciation of viruses in their new hosts. The data suggesting several recent host shifts among ranavirus species increases concern that these cold blooded vertebrate pathogens may have the capacity to cross numerous poikilothermic species barriers and the potential to cause devastating disease in their new hosts. As RVs infect a wide variety of ecologically and economically important hosts, understanding RV evolution, including the importance of the unique genomic re-arrangements found among RV isolates in relation to host specificity and viral evolution, will help predict and perhaps prevent further RV epizootics. While this study does give insight into RV evolution, more genomic sequence information is needed to continue our efforts to understand the role RVs play in the environment.

Biosketch: Dr. James K. Jancovich's interest in ranaviruses began after isolating *Ambystoma tigrinum virus* in 1996. Since that time his research has focused on understanding the genomics, evolution and the host-pathogen interactions that influence ranavirus host-range and pathogenesis. Dr. Jancovich earned a Ph.D. from Arizona State University in Molecular and Cellular Biology and developed a homologous recombination system that allows us to now study ranavirus gene function by generating gene knock-out recombinant viruses. He has been a member of the Integrated Research Challenges in Environmental Biology (IRCEB) – Emerging Wildlife Diseases: Threats to Amphibian Biodiversity group since 1999 and has recently been appointed to the International Committee on Taxonomy of Viruses (ICTV), family *Iridoviridae* study group. Currently, he is a postdoctoral research associate at Arizona State University developing technology to generate more efficacious virus-based vaccines.

James Jancovich (Ph.D.)

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Evidence for Multiple Recent Host Species Shifts among the Ranaviruses (Family *Iridoviridae*)▽†

James K. Jancovich,[1] Michel Bremont,[2] Jeffrey W. Touchman,[3] and Bertram L. Jacobs[1,3]*

The Biodesign Institute, Arizona State University, Tempe, Arizona 85287-5401[1]; Unité de Virologie et Immunologie Moléculaires, Institut National de la Recherche Agronomique, F-78352 Jouy-en-Josas, France[2]; and School of Life Sciences, Arizona State University, Tempe, Arizona 85287-4601[3]

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Members of the genus *Ranavirus* (family *Iridoviridae*) have been recognized as major viral pathogens of cold-blooded vertebrates. Ranaviruses have been associated with amphibians, fish, and reptiles. At this time, the relationships between ranavirus species are still unclear. Previous studies suggested that ranaviruses from salamanders are more closely related to ranaviruses from fish than they are to ranaviruses from other amphibians, such as frogs. Therefore, to gain a better understanding of the relationships among ranavirus isolates, the genome of epizootic hematopoietic necrosis virus (EHNV), an Australian fish pathogen, was sequenced. Our findings suggest that the ancestral ranavirus was a fish virus and that several recent host shifts have taken place, with subsequent speciation of viruses in their new hosts. The data suggesting several recent host shifts among ranavirus species increase concern that these pathogens of cold-blooded vertebrates may have the capacity to cross numerous poikilothermic species barriers and the potential to cause devastating disease in their new hosts.

Iridoviruses are large, double-stranded DNA viruses that infect both vertebrate and invertebrate hosts (9, 64). The family *Iridoviridae* currently contains five genera, the *Iridovirus* and *Chloriridovirus* genera, associated with insects, the *Lymphocystivirus* and *Megalocytivirus* genera, which infect fish species, and the genus *Ranavirus*, whose members have been associated with mortality events in amphibians, fish, and reptiles (64). At this time, the type isolates for each genus in the family *Iridoviridae* have been sequenced (Table 1).

Members of the genus *Ranavirus* have been recognized as major pathogens of economically and ecologically important cold-blooded vertebrates (8, 64). For example, ranaviruses (RVs) have been isolated from amphibians in North America (6, 18, 24, 34, 35), Asia (27, 66), Australia (56), and the United Kingdom (10, 19), from fish (2, 41, 46), and from reptiles (3, 14, 30, 37, 42, 43). In fact, ranaviruses are now considered agents of emerging infectious disease (9). As interest in RVs has grown, the number of ranaviruses that have been completely sequenced has also increased. These include frog virus 3 (FV3) (58), the type virus of the genus *Ranavirus*; tiger frog virus (TFV) (27), an RV closely related to FV3 that was isolated from frogs in Asia; and *Ambystoma tigrinum* virus (ATV) (36), an RV associated with salamander mortalities in North America. In addition, two grouper iridoviruses which are also members of the genus *Ranavirus*, the grouper iridovirus (GIV) (62) and the Singapore grouper iridovirus (SGIV) (53), were recently sequenced. In addition, at the time of preparation of the manuscript, the genomic sequence of the soft-shelled turtle ranavirus (STIV) became available (29). Information obtained by comparing ranavirus genomic sequences offers insight into RV evolutionary history, identifies core groups of genes, and gives insight into the genes responsible for viral immune evasion and pathogenesis.

Previous studies have shown that RV isolates can be translocated across large distances in infected salamanders that are used as bait for sport fishing (35, 44, 51). Phylogenetic analysis was used to compare the major capsid protein (MCP) sequences from salamander RV isolates from the southern Arizona border to Canada to other RV MCP sequences (35). The data suggest that salamander RV isolates are more closely related to fish RV isolates, such as epizootic hematopoietic necrosis virus (EHNV), than to other amphibian (frog) RV isolates, such as FV3 (35). Dot plot analysis comparing the genomic sequence of ATV to those of FV3 and TFV showed two major genomic inversions (36), while the FV3 and TFV genomes showed complete colinearity. These data suggest that at some point in virus evolutionary history, an ancestral virus diverged into the salamander virus and frog virus lineages. A genomic rearrangement occurred in one of the lineages at the time of divergence or after. Subsequent host-specific evolution occurred, limiting cross transmission among isolates, in such a way that frog RVs do not cause disease during laboratory infection of salamanders and vice versa (34). There is some evidence that salamander RV isolates can be isolated from or detected in laboratory-infected frogs (52) and that a pathogen host shift is the result of the movement of these pathogens (35). Thus, the ecological and economic consequences of RVs moving in the environment include the potential of these pathogens infecting and decimating new amphibian, fish, or reptile populations. Therefore, a more complete understanding of the genetic determinants that make up RVs would help to predict future transmission events.

EHNV was isolated in Australia from redfin perch (*Perca fluviatilis*) and rainbow trout (*Oncorhynchus mykiss*) (38, 39).

* Corresponding author. Mailing address: School of Life Sciences, Arizona State University, Tempe, AZ 85287-4601. Phone: (480) 965-4684. Fax: (480) 727-7615. E-mail: bjacobs@asu.edu.

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Innate Immune Evasion Mediated by the *Ambystoma tigrinum* Virus Eukaryotic Translation Initiation Factor 2α Homologue▽

James K. Jancovich and Bertram L. Jacobs*

Arizona State University, School of Life Sciences, and The Biodesign Institute, Center for Infectious Diseases and Vaccinology, Tempe, Arizona 85287-5401

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Ranaviruses (family *Iridoviridae*, genus *Ranavirus*) are large, double-stranded DNA (dsDNA) viruses whose replication is restricted to ectothermic vertebrates. Many highly pathogenic members of the genus *Ranavirus* encode a homologue of the eukaryotic translation initiation factor 2α (eIF2α). Data in a heterologous vaccinia virus system suggest that the *Ambystoma tigrinum* virus (ATV) eIF2α homologue (vIF2αH; open reading frame [ORF] 57R) is involved in evading the host innate immune response by degrading the interferon-inducible, dsRNA-activated protein kinase, PKR. To test this hypothesis directly, the ATV vIF2αH gene (ORF 57R) was deleted by homologous recombination, and a selectable marker was inserted in its place. The ATVΔ57R virus has a small plaque phenotype and is 8-fold more sensitive to interferon than wild-type ATV (wtATV). Infection of fish cells with the ATVΔ57R virus leads to eIF2α phosphorylation, in contrast to infection with wtATV, which actively inhibits eIF2α phosphorylation. The inability of ATVΔ57R to prevent phosphorylation of eIF2α correlates with degradation of fish PKZ, an interferon-inducible enzyme that is closely related to mammalian PKR. In addition, salamanders infected with ATVΔ57R displayed an increased time to death compared to that of wtATV-infected salamanders. Therefore, in a biologically relevant system, the ATV vIF2αH gene acts as an innate immune evasion factor, thereby enhancing virus pathogenesis.

Ranaviruses (family *Iridoviridae*, genus *Ranavirus*) are large, double-stranded DNA (dsDNA) viruses that can infect a wide variety of ectothermic vertebrates, including amphibians, reptiles, and fish (13, 70). However, the ecological and economical impacts of ranavirus infections are currently unknown, even though ranavirus infections of lower vertebrates have increased over the past few decades (13, 70). In addition, the mechanisms that enable ranaviruses to infect such a diverse group of hosts and cause, in some cases, high rates of morbidity and mortality have not been fully uncovered. While there are major epidemics associated with ranavirus infections in threatened amphibian species, commercially valuable fish, and reptiles (1–3, 8, 15, 17–19, 22, 23, 26, 28, 31, 33, 34, 40, 42–45, 52, 63, 64, 73, 75), ranaviruses are also thought to spread with fish, amphibians, and reptiles that are moved globally for bait and food and as pets (11, 15, 29, 49, 60). Because of the economical and ecological impact that these viruses have on ectothermic vertebrates, it is essential to begin to uncover the determinants of ranavirus host range and pathogenesis.

Genomic sequencing of *Ambystoma tigrinum* virus (ATV), originally isolated from tiger salamanders (*Ambystoma tigrinum stebbinsi*) in southern Arizona (28), revealed a number of genes that may enhance viral pathogenesis based on homology to other known proteins in the database (30). One gene in particular, the ATV homologue of the eukaryotic translation initiation factor 2α (vIF2αH; ATV open reading frame [ORF] 57R), has been suggested to be important for ranavirus pathogenesis (40). In addition, we have recently shown using a heterologous vaccinia virus (VACV) system that the ATV vIF2αH may play an important role in evading the host innate immune response (L. Tripuraneni, J. K. Jankovich, M. C. Heck, J. O. Langland, and B. L. Jacobs, unpublished data). By replacing the VACV innate immune evasion gene, E3L (9, 10, 16, 36, 37, 55, 61, 62), with the ATV vIF2αH gene, there is a rapid degradation of cellular PKR, an important cellular antiviral molecule that upon activation phosphorylates the eukaryotic translation initiation factor eIF2α (38, 59), thereby inhibiting protein synthesis. Therefore, we hypothesized that in a more relevant system (i.e., ATV-salamander model system), the ATV vIF2αH gene may influence viral pathogenesis by evading the host innate immune response (i.e., degrading cellular PKR). Using methods similar to those described for generating a recombinant Bohle iridovirus (47), we have generated a knockout recombinant ATV by deleting the ATV ORF 57R and then characterized this recombinant virus in cells in culture and in a salamander model. This research offers insight into a ranavirus immune evasion pathway and suggests that there is a yet-uncharacterized innate immune response in salamanders, the natural host of ATV.

MATERIALS AND METHODS

Cells and virus. Fathead minnow (FHM; ATCC CCL-42) cells were maintained in minimal essential medium (MEM) with Hank's salts (Gibco) supplemented with 5% fetal bovine serum (FBS) (HyClone) and 0.1 mM nonessential amino acids and vitamins (Invitrogen). *Epithelioma papulosum cyprini* (20) and bluegill fry (BF2; ATCC CCL-91) cells were maintained in MEM supplemented with 10% FBS and 0.1 mM nonessential amino acids and vitamins (Invitrogen). All cells were incubated at 20 to 22°C in the presence of 5% CO_2. The viruses used in this study are the *Ambystoma tigrinum* virus (wild-type ATV [wtATV]), isolated from tiger salamanders in 1996 (28), and the recombinant virus made in this study. The ATV deletion mutant lacking the vIF2αH gene (ORF 57R) was designated ATVΔ57R.

* Corresponding author. Mailing address: Arizona State University, Center for Infectious Diseases and Vaccinology, The Biodesign Institute, Box 87501, Tempe, AZ. Phone: (480) 965-4684. Fax: (480) 727-7615. E-mail: bjacobs@asu.edu.

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Host-Pathogen Coevolution: From Genes to Landscapes

A. Storfer, K. Chojnacki, and J. Eastman

School of Biological Sciences, Washington State University, Pullman, WA 99164-4236
(email: astorfer@wsu.edu)

Emerging infectious diseases threaten wildlife, livestock and humans, and are recognized as a leading scientific challenge for the 21st Century. Emerging infectious diseases are implicated in the global die-offs of amphibians, threatening many populations and species with extinction. Two critical questions arise when pathogens emerge. First – is the pathogen new or old? Second – what is the likelihood of spread? I will discuss the past 10 years of a multifaceted research program on coevolution of tiger salamanders and an emerging ranavirus throughout western North America to address these two questions. Using comparative phylogenetic methods, we show that tiger salamanders and viruses are coevolved, but human introductions of infected salamanders as fishing bait disrupts coevolutionary patterns. Due to increased densities of captive populations, increased virulence is observed in a virus strain isolated from a bait salamander population. Next, we show geographic variation in putative viral virulence genes. Using a cross-infection experimental design to test for local adaptation, we show that apparent viral adaptation is correlated with molecular evolutionary rates and particular amino acid changes in these genes. We also show that pathogen local adaptation is multi-faceted and requires estimates of infectivity, within-host growth, transmission and virulence, as opposed to the commonly used single measure of infectivity. Future studies will focus on genomic interactions of host and virus to better understand the mechanisms underlying host resistance and pathogen evasion of host defenses.

Biosketch: Dr. Andrew Storfer is an evolutionary conservation geneticist studying landscape genetics and host-pathogen coevolution in the tiger salamander-*Ambystoma tigrinum* virus system. Since graduating at the University of Kentucky with a PhD in Evolutionary Ecology in 1997, he held a Maytag Postdoctoral Fellowship at Arizona State University from 1997-1999. Dr. Storfer then continued on as an Assistant Professor at University of Florida from 1999-2001, followed by Assistant and Associate Professor in the School of Biological Sciences at Washington State University. During that time, he was awarded a Fulbright Fellowship to study at James Cook University/ University of Tasmania in 2008-2009. He was also Associate Editor of Western North American Naturalist and Diversity and Distributions. His research has been funded by: National Science Foundation, Australian Research Council, Florida Fish and Wildlife Conservation Commission, Army Corps of Engineers, Potlatch Corporation, National Park Service, Washington Department of Natural Resources; and, National Center for Ecological Analysis and Synthesis. Currently, he is Associate Director for Graduate Studies in his department, as well as serving on the Board of Governors of ASIH.

Andrew Storfer (Ph.D.)

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LETTER

Phylogenetic concordance analysis shows an emerging pathogen is novel and endemic

Andrew Storfer,[1]* Michael E. Alfaro,[1] Benjamin J. Ridenhour,[1] James K. Jancovich,[2] Stephen G. Mech,[1,3] Matthew J. Parris[4] and James P. Collins[5]

[1]*School of Biological Sciences, Washington State University, Pullman, WA 99164-4236, USA*

[2]*Molecular and Cellular Biology Program, School of Life Sciences, The BioDesign Institute, Arizona State University, Tempe, AZ 85287-4601, USA*

[3]*Biology Department, Albright College, Reading, PA 19612-5234, USA*

[4]*Department of Biology, University of Memphis, Memphis, TN 38152, USA*

[5]*School of Life Sciences, Arizona State University, Tempe, AZ 85287-4501, USA*

**Correspondence: E-mail: astorfer@wsu.edu*

Abstract

Distinguishing whether pathogens are novel or endemic is critical for controlling emerging infectious diseases, an increasing threat to wildlife and human health. To test the endemic vs. novel pathogen hypothesis, we present a unique analysis of intraspecific host-pathogen phylogenetic concordance of tiger salamanders and an emerging *Ranavirus* throughout Western North America. There is significant non-concordance of host and virus gene trees, suggesting pathogen novelty. However, non-concordance has likely resulted from virus introductions by human movement of infected salamanders. When human-associated viral introductions are excluded, host and virus gene trees are identical, strongly supporting coevolution and endemism. A laboratory experiment showed an introduced virus strain is significantly more virulent than endemic strains, likely due to artificial selection for high virulence. Thus, our analysis of intraspecific phylogenetic concordance revealed that human introduction of viruses is the mechanism underlying tree non-concordance and possibly disease emergence via artificial selection.

Keywords

Ambystoma tigrinum, *Ambystoma tigrinum* virus, amphibian declines, emerging diseases, phylogenetic concordance, virulence.

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INTRODUCTION

Emerging infectious diseases are widely recognized as a threat to public health and are increasingly appreciated as having major effects on biological communities (McCallum & Dobson 1995). In some cases, pathogens can even cause host extinctions (DeCastro & Bolker 2005). Emerging pathogens are either recently introduced to new regions or naïve hosts, or are already endemic but have increased in impact because of environmental changes or genetic changes in the host or pathogen (Daszak *et al.* 2000). Distinguishing whether emerging infectious diseases are novel or endemic is critical because each scenario necessitates different avenues for further research and possible mitigation strategies. In the case of emerging endemic pathogens, research may focus on identification and mitigation of possible environmental cofactors or assessing whether recent genetic changes increased pathogen virulence or decreased host resistance (Daszak *et al.* 2000; Jancovich *et al.* 2005; Rachowicz *et al.* 2005). In the novel pathogen case, research may focus on reasons for spread, such as: pathogen host switching, recent introductions of infected hosts to new areas, or pathogen range expansion. Efforts may then focus on identifying the source localities or species and controlling spread, such as for sudden acute respiratory syndrome (SARS; Li *et al.* 2005). However, distinguishing between the novel vs. endemic hypotheses is difficult for any pathogen and has thus rarely been accomplished because of difficulties gathering adequate data to identify the source of the pathogen, molecular information to disentangle the geographic relationships of pathogen strains, and dating the age of the pathogen (Rachowicz *et al.* 2005).

One way to determine whether pathogens are novel or endemic is to assess the extent to which they are coevolved with their host. Analyses of phylogenetic

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SHORT COMMUNICATION

Geographically variable selection in *Ambystoma tigrinum* virus (Iridoviridae) throughout the western USA

B. J. RIDENHOUR & A. T. STORFER

School of Biological Sciences, Washington State University, Pullman, WA, USA

Keywords:

Ambystoma tigrinum virus;
caspase activation and recruitment domain;
coevolution;
eIF-2α;
Iridoviridae;
phylogeny;
ranavirus;
selection;
β-OH-steroid oxidoreductase.

Abstract

We investigated spatially variable selection in *Ambystoma tigrinum* virus (ATV) which causes frequent and geographically widespread epizootics of the tiger salamander, *Ambystoma tigrinum*. To test for evidence of selection, we sequenced several coding and noncoding regions from virus strains isolated from epizootics throughout western North America. Three of the sequenced regions contained homologues for genes putatively involved in host immune evasion and virulence: eIF-2α, caspase activation and recruitment domain (CARD) and β-OH-steroid oxidoreductase. Selection analysis showed evidence of very strong purifying selection on eIF-2α, purifying selection within certain viral clades on CARD and positive selection on β-OH-steroid oxidoreductase within certain clades. Analysis using MULTIDIVTIME and Tajima's relative rate tests indicate accelerated rates of evolution within clades associated with anthropogenic movement. These clades also demonstrate greater spatial variability in selection, suggesting a lack of local adaptation (i.e. locally adapted populations should exhibit little to no selection because of absent or reduced variation in fitness once a fitness optimum is reached). Increased transfer of non-native viral strains to naïve salamander populations, in conjunction with local maladaptation as a result of local selection pressures, may explain the spread and emergence of ATV epizootics in *A. tigrinum* in western North America.

Introduction

A central question in evolutionary biology is understanding spatial patterns in the variation of species' interactions. This geographic variability in species' interactions is in part due to underlying differences in local selection pressures (Thompson, 2005; Gomulkiewicz, *et al.* 2007). Emerging infectious diseases (EIDs) – such as avian influenza, HIV and West Nile virus which are of growing human concern – may originate through changes in local selection pressures.

Emerging infectious diseases have recently been implicated as an important hypothesis for the global decline of amphibian populations (Daszak *et al.*, 2003; Stuart *et al.*, 2004; Lips *et al.*, 2006; Rachowicz *et al.*, 2006). In particular, EIDs caused by the globally distributed chytrid fungus, *Batrachochytrium dendrobatidis*, and ranaviruses are considered important causes of amphibian population epizootics and declines (Marsh *et al.*, 2002; Daszak *et al.*, 2003; Stuart *et al.*, 2004; Fox *et al.*, 2006; Lips *et al.*, 2006). By studying the evolutionary dynamics of pathogens causing amphibian EIDs, we gain a general understanding of EIDs and the conditions necessary for the emergence of pathogens from their traditional host populations.

During the last 15 years, ranaviruses in particular have been associated with marked increases in globally distributed morbidity and mortality in fish, reptiles and amphibians (Chinchar, 2002). Based upon major capsid protein (MCP) sequence data, members of the genus *Ranavirus* in the family Iridoviridae are rapidly diverging (Marsh *et al.*, 2002). In North America, ranaviruses have been isolated from the majority of recent amphibian

Correspondence: Benjamin J. Ridenhour, Department of Biological Sciences, University of Idaho, Moscow, ID 83844-3051, USA.
Tel.: +1 208 885 4414; fax: +1 208 885 7905;
e-mail: bridenho@uidaho.edu

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Weakness of Innate Immunity Also Contributes to Susceptibility of *Xenopus* Tadpoles to FV3 Infection

J. Robert, G. Cheng, and F. De Jesús Andino

Microbiology & Immunology, University of Rochester Medical Center, Rochester, NY
(email**:** jacques_robert@urmc.rochester.edu)

Xenopus larvae have a weaker or more immature adaptive immunity than adults. We have previously shown significant differences in resistance to FV3 infection between adults and larvae. Adult frogs develop rapid innate immune responses followed by potent adaptive responses that clear FV3 within 2-3 weeks. In contrast, most tadpoles cannot clear FV3 and die within a few weeks after infection, presumably due to weaker antibody and T cell responses. However, the role of innate immunity in larval susceptibility has not been evaluated. We investigated this issue by assessing the response kinetics of several innate immune genes, and by monitoring changes in occurrence and composition of peritoneal leukocytes during FV3 infection. Using quantitative real-time PCR, we found only a modest (10-100 times lower than adult) and delayed (3 days later than adult) up-regulation of TNFα, IL-1β and IFNγ genes in leukocytes and in infected tissues, as well as a delayed induced expression of the type I IFN-inducible Myxovirus-resistance (Mx) gene. The relative fraction of macrophages and infected cells visualized by fluorescence microscopy with macrophage- and FV3-specific antibodies, increased at 3 and 6 dpi in parallel to the total number of leukocytes in the peritoneal cavity. However, unlike adults, larval macrophages appear to be resistant to FV3. This suggests that the larval innate immune effector system is distinct from the adult with a more modest and delayed anti-ranaviral response. Future plans are to characterize larval macrophages and further investigate interactions between larval innate and adaptive effector cells.

Biosketch: Dr. Jacques Robert is Associate Professor in the department of Microbiology and Immunology at the University of Rochester Medical Center, New York. His research interests are in the evolutionary and developmental aspects of tumor and viral immunity. He has worked and published extensively in the area of thymocyte differentiation, immunomodulation, and molecular evolution of immunologically relevant genes, viral immunity and phylogeny of cellular immunity. Dr. Robert's research focuses on the amphibian *Xenopus*, which he has developed as a unique versatile comparative model system to study immunity to tumors, as well as pathogenesis and immunity to ranaviruses (*Iridoviridae*) causing emerging infectious diseases in amphibians. He is principal investigator in several NIH and NSF funded awards. In addition, he is the director of an NIH-funded *Xenopus laevis* research resource for immunobiology, which is the world's most comprehensive resource specializing in the use of this species for immunological research and providing technical assistance, animals, and reagents to the scientific community.

Jacques Robert (Ph.D.)

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Innate Immune Responses and Permissiveness to Ranavirus Infection of Peritoneal Leukocytes in the Frog *Xenopus laevis*[▽]

Heidi D. Morales,[2]† Lara Abramowitz,[3] Jacqueline Gertz,[1] Jessica Sowa,[1] Ashley Vogel,[1] and Jacques Robert[1]*

Department of Microbiology and Immunology, University of Rochester Medical Center, Rochester, New York 14642[1]; Department of Neurobiology, Dana Farber Cancer Institute, Harvard Medical School, Boston, Massachusetts 02115[2]; and Department of Genetics, University of Pennsylvania Medical Center, Philadelphia, Pennsylvania[3]

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Ranaviruses such as frog virus 3 ([FV3] family *Iridoviridae*) are increasingly prevalent pathogens that infect reptiles, amphibians, and fish worldwide. Whereas studies in the frog *Xenopus laevis* have revealed the critical involvement of CD8 T-cell and antibody responses in host resistance to FV3, little is known about the role played by innate immunity to infection with this virus. We have investigated the occurrence, composition, activation status, and permissiveness to infection of peritoneal leukocytes (PLs) in *Xenopus* adults during FV3 infection by microscopy, flow cytometry, and reverse transcription-PCR. The total number of PLs and the relative fraction of activated mononucleated macrophage-like cells significantly increase as early as 1 day postinfection (dpi), followed by NK cells at 3 dpi, before the peak of the T-cell response at 6 dpi. FV3 infection also induces a rapid upregulation of proinflammatory genes including arginase 1, interleukin-1β, and tumor necrosis factor alpha. Although PLs are susceptible to FV3 infection, as evidenced by apoptotic cells, active FV3 transcription, and the detection of viral particles by electron microscopy, the infection is weaker (fewer infectious particles), more transitory, and involves a smaller fraction (less than 1%) of PLs than the kidney, the main site of infection. However, viral DNA remains detectable in PLs for at least 3 weeks postinfection, past the point of viral clearance observed in the kidneys. This suggests that although PLs are actively involved in anti-FV3 immune responses, some of these cells can be permissive and harbor quiescent, asymptomatic FV3.

Ranaviruses (RVs), of the family *Iridoviridae*, are large (165 to 169 nm) double-stranded DNA (dsDNA) icosahedral viruses that infect a wide variety of hosts, including teleosts, amphibians, and reptiles (reviewed in references 1 and 2). RVs are increasingly causing diseases and die-offs in various species of natural and captive amphibians around the world and, as such, are possibly involved in the worldwide decline of amphibian populations (4, 7, 11, 14, 15, 46, 48). Frog virus 3 (FV3), the main member and the type species of the RV genus (20), was originally isolated from the native North American leopard frog *Rana pipiens*. FV3 or FV3-like viruses are now found worldwide infecting different amphibian species, making the virus a potentially serious global threat (1, 4).

The frog *Xenopus laevis* has become an instrumental laboratory model to study immunity and pathogenesis of RVs such as FV3 and provides a realistic alternative to field studies of natural populations of endangered amphibians (reviewed in reference 32). The role of the adaptive immune response to RVs is already well established based on studies using FV3 (12, 41). However, although the critical involvement of CD8 T cells (33) and antibodies (12, 24) is now established, very little is yet known about the role of innate immune responses, especially during the early stage of infection. In addition, we have reported some evidence suggesting that macrophage-like cells in the peritoneal cavity of *Xenopus* may harbor FV3 in a fraction of animals that are otherwise asymptomatic (39). These observations laid the groundwork for the present study.

In mammals, macrophages play a key role in virus-host interactions. On the one hand, macrophages are innate immune cell effectors involved in early stages of infection by acting as phagocytic cells that engulf and digest pathogens or infected dying cells in a stimulus-dependent but non-antigen (Ag)-specific manner. In addition, macrophages recruit more phagocytic and effector cells to the area of infection by secreting chemokines such as interleukin-8 (IL-8) and proinflammatory cytokines such as IL-1β and tumor necrosis factor alpha (TNF-α) (reviewed in references 9 and 27). Macrophages are also implicated in adaptive immune responses as professional antigen presenting cells (APCs) that can process viral antigens through major histocompatibility complex class I (MHC-I) and MHC-II presentation pathways to activate CD8 and CD4 T-cell effectors, respectively. On the other hand, viruses can also remain disseminated in macrophages in a quiescent state. Indeed, several viruses (e.g., human immunodeficiency virus and herpes simplex virus 1) infect macrophages and exploit the cells' multiple functions for their own survival. Since macrophages are in constant circulation in the body, they can serve as carriers of the virus to multiple tissues (23, 44).

In the present study we have investigated the response of innate cell effectors in the peritoneal cavity during FV3 infection *in vivo*, as well as their susceptibility to FV3. Our data reveal that peritoneal leukocytes (PLs) may play a dual role in the defense against and pathogenicity of FV3 infection in amphibians.

* Corresponding author. Mailing address: Department of Microbiology and Immunology, University of Rochester Medical Center, Rochester, NY 14642. Phone: (585) 275-1722. Fax: (585) 473-9573. E-mail: Jacques_Robert@urmc.rochester.edu.
† Present address: Vesalius Research Center, Leuven B-3000, Belgium.
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Review

Emerging Ranaviral Infectious Diseases and Amphibian Decline

Jacques Robert

Department of Microbiology and Immunology, University of Rochester Medical Center, Rochester, NY 14642, USA; E-Mail: Jacques_Robert@urmc.rochester.edu; Tel.: +1-585-275-1722; Fax: +1-585-473-9573

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Abstract: Infectious diseases caused by ranaviruses (RV, family *Iridoviridae*) not only affect wild amphibian populations but also agriculture and international animal trade. Although, the prevalence of RV infections and die offs has markedly increased over the last decade, it is still unclear whether these viruses are direct causal agents of extinction or rather are the resulting (secondary) consequences of weakened health of amphibian populations leading to increased susceptibility to viral pathogens. In either case, it is important to understand the critical role of host immune defense in controlling RV infections, pathogenicity, and transmission; this is the focus of this review.

Keywords: viral immunity; *Xenopus*; Iridovirus

1. Introduction

Emerging infectious diseases (EIDs) are generally defined as diseases that are either newly recognized, novel in a population, or rapidly increasing in incidence, virulence, or geographic range [1]. While the direct impact of EIDs in human health is usually well appreciated, their threat to biodiversity is less well known. What is known, however, is that EIDs increasingly affect wildlife all over the world. In addition to playing an important ecological role, EIDs pose important problems for the conservation of endangered species, domestic and captive animals, and ultimately humans themselves [2].

With regard to amphibians, a dramatic worldwide decline of populations of multiple species was first noted about 30 years ago [3-5]. Since then, this decline has continued at an alarming rate. According to the most recent 2008 global assessment (globalamphibians.org), nearly one-third (32%),

Ranavirus Publications

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Complex Role of Macrophages in *Xenopus* Immune Defenses and Persistence of the Ranavirus FV3

J. Robert

Microbiology & Immunology, University of Rochester Medical Center, Rochester, NY
(email**:** jacques_robert@urmc.rochester.edu)

We have established *Xenopus* as a reliable model to study host immune defense in controlling infection, pathogenicity, and transmission of ranaviruses like FV3. We have shown that adult *Xenopus* resist and clear FV3 infection by developing potent anti-FV3 antibodies and efficient CD8 T cell responses that utilize macrophages as antigen-presenting cells. Despite this strong response, we have detected FV3 DNA in seemingly healthy (not deliberately infected) *Xenopus*. This observation lead us to hypothesize that FV3 is capable of establishing covert infections as seen with certain insect iridoviruses, and to investigate the possible dual role of macrophages as immune effector and permissive hosts. Accumulation of macrophages (pMc) began as early as 1 day post-infection and was correlated with an increased expression of IL-1β and TNFα pro-inflammatory cytokines. pMcs were shown to be susceptible to FV3 infection as evidenced by active FV3 transcription, and the detection of viral particles by electron microscopy and multicolor fluorescent microscopy. However, FV3 infection of pMcs resulted in the generation of fewer infectious particles, and involved a lower fraction (<1%) of pMcs than kidney tissue, the main site of infection. Notably, viral DNA remained detectable in pMcs for at least 3 weeks post-infection, past the point of viral clearance in the kidneys. These results suggest that pMcs harbor quiescent virus that may contribute to asymptomatic infection. Future plans are to characterize permissive pMc subsets using available and newly generated antibodies, follow them *in vivo* using fluorescent tracers, and identify viral immune evasion genes by reverse genetics.

Comparative Pathology of Ranavirus Infections in Wild Amphibians

D. Green

US Geological Survey, National Wildlife Health Center, Madison, WI 53711
(email: david_green@usgs.gov)

Ranavirus infections in free-living amphibians in the USA occur predominantly in larvae and metamorphs. Infections are consistently fatal to larvae; ranaviruses are rarely isolated from normal-appearing amphibians. Onset of a die-off is explosive; often hundreds or thousands of sick and dead larvae suddenly appear. Sick larvae are lethargic, swim erratically and have pinpoint or paint-brush hemorrhages in their ventral skin. Accumulation of fluids in lymphatic sacs and body cavity may be mild or severe. Internally, hemorrhages may occur in many organs and tissues of some larvae, and may be seen in muscles, heart, stomach, liver and mesonephroi. Skin ulcers may be present in some larvae and metamorphs; ulcers may be single or multiple, irregular in shape, white with red margins, and may appear on head, body or appendages. Histologically, changes are present in many organs, but especially the skin, gastro-intestinal tract, liver, pancreas, spleen and mesonephroi. Ranaviruses have tropisms for endothelium (blood vessel cells), epidermis, liver, and lympho-hematopoietic cells in the spleen, liver and renal interstitium. Vascular necrosis is detected in the lungs, sinusoids of the liver, spleen, glomeruli and submucosa of the stomach and intestine. Liver changes present as multifocal or diffuse necrosis of endothelial cells lining the sinusoids or necrosis of liver cells. Skin abnormalities begin as swelling of basal cells, thickening of the epidermis, cell necrosis and erosions or ulcers. Changes in the spleen and mesonephroi involve necrosis of glomerular capillaries, macrophages, lymphocytes and renal hematopoietic cells. Characteristic intracytoplasmic inclusion bodies are best detected in liver and skin cells.

Biosketch: Dr. David Green is a Board Certified Veterinary Pathologist and has been a Veterinary Medical Officer at the USGS National Wildlife Health Center in Madison, Wisconsin for nearly 13 years. His prior appointments include Veterinary Pathologist with the National Institutes of Health, Bethesda, Maryland (1997-1998) and Chief Veterinary Pathologist, Maryland Department of Agriculture, College Park, Maryland (1986-1997). He received a B.S. in Zoology from Oregon State University in 1971 and a DVM from Colorado State University in 1975. Dr. Green's research focuses on identifying factors responsible for wildlife disease outbreaks. His research on amphibians has focused primarily on disease surveillance, examining epidemiological patterns of amphibian disease outbreaks, and evaluating to what degree diseases pose threats for longterm amphibian conservation. His invited talks have reviewed diseases of cricket frogs (*Acris* spp) and amphibian and reptilian diseases and other mortality factors associated with urban and suburban environments.

David Green (DVM)

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Health evaluation of amphibians in and near Rocky Mountain National Park (Colorado, USA)

D. Earl GREEN* & Erin MUTHS**

* US Geological Survey, National Wildlife Health Center, 6006 Schroeder Road, Madison, Wisconsin 53711, USA

** US Geological Survey, Fort Collins Science Center, 2150 Centre Avenue, Building C, Fort Collins, Colorado 80526, USA

We conducted a health survey of amphibians in and adjacent to Rocky Mountain National Park (RMNP) to document current disease presence inside RMNP and identify disease outside RMNP with the potential to spread to the Park's amphibians. Amphibians from five sites within RMNP and seven sites within 60 km of Park boundaries were collected and examined. Necropsies (n = 238), virus isolation, bacterial and fungal cultures, and histological examinations were carried out on amphibian egg masses (outside RMNP/within RMNP: 26/22), larvae (30/42), imagos (recently metamorphosed individuals) (0/3) and adults (61/67) of five species. Marked infections by a pathogenic chytrid fungus (chyridiomycosis), *Batrachochytrium dendrobatidis*, were detected in three species (*Bufo boreas*, *Pseudacris maculata* and *Rana sylvatica*) from three of five sites within RMNP and in one of three species (*P. maculata*) from three sites outside RMNP. Of the fully metamorphosed individuals tested (*B. boreas*, *P. maculata* and *R. sylvatica*), chytridiomycosis was found in 60 % (n = 3), 46 % (n = 37) and 54 % (n = 7), respectively. Chytridiomycosis was the principal lethal pathogenic infectious disease detected in three amphibian species within or adjacent to RMNP. Higher fungi were isolated from the cloaca and skin of all five amphibian species. Watermolds (Oomycetes) were isolated from amphibian eggs or skin of all five species. No evidence of *Ranavirus* was found in cultures and histological examinations of 176 and 142 amphibians, respectively. Fifteen genera of bacteria were identified in larval and just metamorphosed amphibians, and a potentially pathogenic lungworm, *Rhabdias* sp, was identified in 61.1 % (n = 11) of *B. woodhousii* outside RMNP, but in only 2 (15.4 %) *R. sylvatica* within the Park.

INTRODUCTION

Boreal toads (*Bufo boreas*) currently exist as remnant populations in Rocky Mountain National Park (RMNP) (a roughly rectangular 107,625 hectares park in northern Colorado, USA; elevation range: 2,440 to 4,345 m; latitude and longitude at approximate center of park: 40°40'N, 105°60'W) where their historic range was once more extensive (CORN et al., 1997). Recent precipitous declines in two of three populations of boreal toads within RMNP

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[Communication]

A New Ranavirus Isolated from *Pseudacris clarkii* Tadpoles in Playa Wetlands in the Southern High Plains, Texas

SHANNON M. TORRENCE*[1]
Department of Natural Resources, Texas Tech University, Lubbock, Texas 79409, USA

D. EARL GREEN, CATHERINE J. BENSON, AND HON S. IP
U.S. Geological Survey, National Wildlife Health Laboratory, 6006 Schroeder Road, Madison, Wisconsin 53711, USA

LOREN M. SMITH AND SCOTT T. MCMURRY
Department of Zoology, Oklahoma State University, Stillwater, Oklahoma 74078, USA

Abstract.—Mass die-offs of amphibian populations pose a challenging problem for conservation biologists. Ranaviruses often cause systemic infections in amphibians and, in North America, are especially virulent and lethal to larvae and metamorphs. In this paper we describe a novel ranavirus isolate as well as the first recorded occurrence of ranavirus in the southern High Plains of Texas and in associated populations of the spotted chorus frog *Pseudacris clarkii.* The breeding sites were playas, that is, wetlands that fill via isolated thunderstorms that can occur infrequently; thus, not every playa has water or breeding amphibians annually. We did not detect ranavirus in sympatric anurans, but other reports document ranaviruses in *Pseudacris* spp. elsewhere. The occurrence of multiple isolates of ranavirus in a number of *Pseudacris* species suggests that this genus of frogs is highly susceptible to ranaviruses and may experience exceptionally high mortality rates from infection. Thus, the virus may contribute to substantial seasonal population declines and low seasonal recruitment, with negative impacts on populations of breeding adults in successive years.

Mass die-offs of amphibian populations pose a challenging problem for conservation biologists, and emerging infectious diseases are suspected of contributing to many of these declines (Daszak et al. 1999; Collins and Storfer 2003; Semlitsch 2003). One such disease agent is ranavirus (family Iridoviridae; Hyatt et al. 2000). Ranaviruses often cause systemic infections in amphibians and are consistently lethal in multiple amphibian genera (Green et al. 2002). Reports document ranavirus-associated mortality events in caudate and anuran populations in North America, Europe, and Australia (Cullen and Owens 2002; Docherty et al. 2003; Jancovich et al. 2003; Gray et al. 2007; Balseiro et al. 2009). Larval stages are particularly susceptible (Jancovich et al. 1997; Hyatt et al. 1998; Bollinger et al. 1999; Green et al. 2002; Collins et al. 2003), and several host, pathogen, and environmental factors may influence mortality rates (Daszak et al. 1999; Brunner et al. 2005). The variation in morbidity and mortality rates among and within species also suggests either prior exposure to the pathogen or a degree of innate resistance (Daszak et al. 1999; Pearman et al. 2004).

The 25,000 playa wetlands throughout the southern High Plains (SHP) are the primary breeding habitat of amphibians of this region (Smith 2003). These wetlands fill as a result of isolated thunderstorms and become dry when the periods between rain events are long; thus, not every playa has water or breeding amphibians every year (Smith 2003). A die-off in a naïve population that has unpredictable breeding opportunities could be detrimental to the persistence of the population, especially during periods of extended drought or where habitat loss is substantial. Here, we report the first documented case of ranavirus disease in the spotted chorus frog *Pseudacris clarkii*. This is also the first documented case of this disease in the playa wetlands of the SHP and in Texas.

Methods

In conjunction with a food web study, we sampled 12 playa wetlands in Swisher and Briscoe counties, Texas, from 4 June to 12 July, 2005 (Figure 1). For that study, we designated two hydroperiod treatments; "long-hydroperiod" playas contained water for at least 3 months before rain events in late May (i.e., the playas were already wet during the May thunderstorms), and "short-hydroperiod" playas were dry until the late-May rain events. Within each of these hydroperiod treatments, three playas were in cultivated landscapes and

* Corresponding author: froggirltx@hotmail.com
[1] Present address: Tonto Basin Ranger District, Tonto National Forest, 28079 North Arizona Highway 188, Roosevelt, Arizona 85545, USA.

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Pathological Changes Observed in European Amphibians with Ranaviral Diseases

A. Balseiro

SERIDA, Servicio Regional de Investigación y Desarrollo Agroalimentario, Centro de Biotecnología Animal. 33394 Deva-Gijón, Asturias, Spain, (email: abalseiro@serida.org)

Ranaviruses have been implicated as a cause of mass amphibian deaths worldwide. Since the 1990s the number of reported ranaviral disease outbreaks has increased greatly. In Europe, ranaviruses have caused outbreaks of high mortality in the United Kingdom, Croatia, Spain, Denmark and, recently, The Netherlands. Typically, affected animals die of systemic hemorrhagic disease. The hemorrhages are noticeable in larval amphibians, but adult animals are often found dead with no external abnormalities. In addition to systemic hemorrhagic disease, there is another disease syndrome reported in Britain that is characterized by skin ulcerations, necrosis of the digits, and no obvious internal lesions. Histologically, acute necrosis occurs throughout most organ systems of infected animals showing systemic hemorrhagic disease. Lymphoid and haematopoietic necrosis can be also observed. Round, intracytoplasmic, basophilic inclusions, consistent with ranaviral inclusions are present in epithelial cells of the skin, renal tubules and gastrointestinal tract, endothelial cells of the glomeruli, hepatocytes, cells within the spleen and exocrine glandular cells of the pancreas, and are generally associated with varying degrees of necrosis. Pyknotic cell nuclei containing condensed chromatin are also observed. Various immunohistochemistry techniques have been performed to demonstrate the distribution of the virus. Our understanding of ranavirus pathology remains in its infancy; histological examination of infected animals will be important to understanding how the virus affects various species. Ultimately, it is important to remain vigilant and establish surveillance programs to detect new outbreaks of ranaviral disease so that we can better understand the epidemiology of this pathogen and its impact on amphibian biodiversity in Europe.

Biosketch: Dr. Ana Balseiro has been employed at the Animal Biotechnology Research Center (SERIDA, Asturias, Northern Spain) since 2000. She finished her Veterinary Science studies in the University of León (Spain) in 1999 and received her Ph.D. from the same University in 2004. She has also been a member of the Spanish Society for Veterinary Pathology (SEAPV) since 2000. She has over thirteen years of experience in animal disease diagnostics. She joined SERIDA as Veterinary Pathologist for the Animal Health Area; a job which involved carrying out research projects and diagnostic pathology on domestic and wild animal species. Since 2001, she has been part of a team of researchers working on passive and active programs of wildlife disease surveillance, with particular reference to infectious diseases, dealing with emerging disease outbreaks, such as ranaviral, throughout the course of her work. She has obtained support from regional and national research institutions, being the leader of some of these projects.

Ana Balseiro (DVM, Ph.D.)

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DISEASES OF AQUATIC ORGANISMS
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Pathology, isolation and molecular characterisation of a ranavirus from the common midwife toad *Alytes obstetricans* on the Iberian Peninsula

Ana Balseiro[1,*], Kevin P. Dalton[2], Ana del Cerro[1], Isabel Marquez[1], Andrew A. Cunningham[3], Francisco Parra[2], José M. Prieto[1], R. Casais[1]

[1]SERIDA, Servicio Regional de Investigación y Desarrollo Agroalimentario, Laboratorio de Sanidad Animal, 33299 Jove, Gijón, Spain

[2]Departamento de Bioquímica y Biología Molecular, Instituto Universitario de Biotecnología de Asturias, Universidad de Oviedo, 33006 Oviedo, Spain

[3]Institute of Zoology, Regent's Park, London NW1 4RY, UK

ABSTRACT: We describe the pathology, isolation and characterisation of a virus responsible for an outbreak of a systemic haemorrhagic disease causing high mortality in tadpoles of the common midwife toad *Alytes obstetricans* in the 'Picos de Europa' National Park in northern Spain. The virus, provisionally designated as the common midwife toad virus (CMTV), was isolated from homogenates of visceral tissue from diseased toad tadpoles following inoculation on epithelioma papulosum cyprini (EPC) cells. Molecular characterisation of the virus, including sequence analysis of the DNA polymerase and major capsid protein genes, showed that the isolated virus was a ranavirus with marked sequence identity to other members of the genus *Ranavirus*. A rabbit antiserum raised against purified virions was prepared and used to definitively demonstrate systemic distribution of the virus in diseased tadpoles, indicating that the isolated virus was the primary pathogen.

KEY WORDS: Common midwife toad · *Alytes obstetricans* · *Ranavirus* · Pathology · Virology · Immunohistochemistry

INTRODUCTION

Iridoviruses encompass 5 genera: *Iridovirus*, *Chloriridovirus*, *Ranavirus*, *Megalocytivirus* and *Lymphocystivirus* (Chinchar et al. 2005). Of these, the genus *Ranavirus* contains pathogens of fish, amphibians, and reptiles (Langdon et al. 1986, Hyatt et al. 2002, Cunningham et al. 2008). Frog virus 3 (FV3) is the type species of the genus *Ranavirus* and the best-characterised member of the family *Iridoviridae* (Van Regenmortel et al. 2000). FV3 and closely related ranaviruses (e.g. RUK in the UK; Hyatt et al. 2000) have been implicated as a cause of mass amphibian deaths worldwide (Hyatt et al. 2000). In addition to FV3, other important pathogenic ranaviruses are the tiger frog virus (TFV) (Weng et al. 2002) and *Ambystoma tigrinum stebbensi* virus (ATV) (Jancovich et al. 1997, Bollinger et al. 1999). Ranaviruses have been identified as the cause of explosive disease outbreaks, with high mortality rates due to systemic disease in frogs in the US (Majji et al. 2006), Australia (Speare & Smith 1992), Croatia (Fijan et al. 1991) and the UK (Cunningham et al. 1996, 2007), and in salamanders in the US (Jancovich et al. 1997, Docherty et al. 2003) and Canada (Bollinger et al. 1999). In most disease outbreaks, death is due to systemic haemorrhaging and tissue necrosis. However, infection with RUK in the UK may present 2 main disease syndromes: a peracute disease characterised by systemic haemorrhages and a chronic disease characterised by skin ulceration with no internal gross lesions (Cunningham et al. 1996, 2007). In both presentations, however, systemic infection with ranavirus can be demonstrated (Cunningham et al. 2008).

*Email: abalseiro@serida.org

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Short Communication

Outbreak of common midwife toad virus in alpine newts (*Mesotriton alpestris cyreni*) and common midwife toads (*Alytes obstetricans*) in Northern Spain: A comparative pathological study of an emerging ranavirus

Ana Balseiro [a,*], Kevin P. Dalton [b], Ana del Cerro [a], Isabel Márquez [a], Francisco Parra [b], José M. Prieto [a], R. Casais [a]

[a] SERIDA, Servicio Regional de Investigación y Desarrollo Agroalimentario, Laboratorio de Sanidad Animal, 33299 Jove, Gijón, Spain
[b] Departamento de Bioquímica y Biología Molecular, Instituto Universitario de Biotecnología de Asturias, Universidad de Oviedo, 33006 Oviedo, Spain

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ABSTRACT

This report describes the isolation and characterisation of the common midwife toad virus (CMTV) from juvenile alpine newts (*Mesotriton alpestris cyreni*) and common midwife toad (CMT) tadpoles (*Alytes obstetricans*) in the Picos de Europa National Park in Northern Spain in August 2008. A comparative pathological and immunohistochemical study was carried out using anti-CMTV polyclonal serum. In the kidneys, glomeruli had the most severe histological lesions in CMT tadpoles, while both glomeruli and renal tubular epithelial cells exhibited foci of necrosis in juvenile alpine newts. Viral antigens were detected by immunohistochemical labelling mainly in the kidneys of CMT tadpoles and in ganglia of juvenile alpine newts. This is the first report of ranavirus infection in the alpine newt, the second known species to be affected by CMTV in the past 2 years.

In recent years, chytridiomycosis and ranavirus infections have caused outbreaks of high mortality in amphibians and appear to be linked to amphibian population declines (Daszak et al., 1999). The number of reported ranavirus outbreaks has increased greatly since the 1990s, with recurring epidemics occurring in native amphibian populations in the United Kingdom (Cunningham et al., 1996; Teacher et al., 2009).

The common midwife toad virus (CMTV) is a ranavirus originally isolated from common midwife toad (CMT) tadpoles (*Alytes obstetricans*) from the Picos de Europa National Park in Northern Spain in September 2007 (Balseiro et al., 2009). This was the first description of a ranavirus disease in Spain. No further cases of the disease were detected until August 2008, when high mortality was observed in CMT tadpoles and juvenile alpine newts (*Mesotriton alpestris cyreni*) in a pond approximately 1 km from the permanent water trough where the first outbreak occurred. In this study we report the isolation of CMTV from juvenile alpine newts, along with CMT tadpoles at the same location, and perform a comparative pathological and immunohistochemical study. No further cases of CMTV infection were detected in the permanent water trough, which had been disinfected after the outbreak in 2007.

Macroscopically, CMT tadpoles exhibited systemic haemorrhages. Juvenile alpine newts had haemorrhages on the ventral surface (Fig. 1), but not in internal organs. Systemic haemorrhages are common in ranavirus infections (Fox et al., 2006; Cunningham et al., 2008). However, in the United Kingdom, ranavirus infection can present with cutaneous ulceration but no internal gross lesions (Cunningham et al., 1996, 2008). Microscopic lesions in CMT tadpoles and juvenile alpine newts in the present outbreak were similar to those described for the systemic haemorrhagic form of ranavirus disease in CMT tadpoles previously (Balseiro et al., 2009).

Three whole CMT tadpoles and three juvenile alpine newts were fixed in 10% neutral buffered formalin immediately after death, dehydrated in graded ethanol solutions, embedded in paraffin wax, sectioned at 4 μm thickness and stained with haematoxylin and eosin (H&E). On histopathological examination, intracytoplasmic inclusion bodies (Fig. 2) were associated with small foci of necrosis in the skin, liver, kidney, pancreas and gastrointestinal tract. Pycnotic cell nuclei were also observed in these organs. Vesicles and focal thickening were observed in the epidermis in both species. In the kidneys, glomeruli were the structures most affected in CMT tadpoles, while both glomeruli and tubular epithelial cells exhibited foci of necrosis (Fig. 2C) in juvenile alpine newts. Another ranavirus, FV3, exhibits trophism for the kidney, specifically for the proximal renal tubular epithelium (Robert et al., 2005). Necrosis of neuroepithelial tissue, previously described in salamanders (Docherty et al., 2003), was not found in infected CMT tadpoles or juvenile alpine newts.

Samples of kidneys from CMT tadpoles and juvenile alpine newts were fixed in 2.5% glutaraldehyde, embedded in resin and ultrathin sections were stained with uranyl acetate and lead citrate

* Corresponding author. Tel.: +34 985 308470; fax: +34 985 327811.
E-mail address: abalseiro@serida.org (A. Balseiro).

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Ranavirus Publications

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Comparative Pathology of Ranaviral Disease among Amphibians, Reptiles, and Fish

D. Miller

Center for Wildlife Health, Department of Forestry, Wildlife and Fisheries, University of Tennessee, Knoxville, TN 37996
(email: dmille42@utk.edu)

Pathogens within the family *Iridoviridae* have been identified as etiologic agents in amphibian, reptile and fish morbidity and mortality events throughout the world. In many cases, the pathogens of concern belong to the genus *Ranavirus*. In amphibians, gross lesions associated with ranaviruses can include swelling, erythema, ulceration and hemorrhage. Microscopic lesions in amphibians include renal tubular, hepatocellular, and splenic necrosis. Although larval amphibians are most often affected, ranaviral disease has been reported in adults of some species, especially in captivity and in wild populations in Europe. In adult amphibians, hemorrhages and cutaneous ulcerations are most often reported. In reptiles, juveniles and adults can be affected. Lesions in reptiles are frequently reported in the digestive tract, but also can include erythema and ulcerations in the skin, nasal cavities and, in chelonians, the shell. In fish, erythema and hemorrhage can be seen grossly, and necrosis of the hematopoietic tissue and occasionally other organs can be seen microscopically. In all cases, intracytoplasmic inclusion bodies are seen variably. We are just beginning to understand the cells and organs that ranaviruses target, which may differ among viral types and host species. Future research directives should include the use of advanced molecular techniques, such as *in situ* hybridization and immunohistochemistry to elucidate the pathogenesis of ranaviruses among species. We need to further explore the possibility of vertical transmission in hosts and investigate the likelihood of interclass disease transmission. Finally, vaccine development is an important research need for control of ranaviral disease in captive populations.

Biosketch: Dr. Debra Miller is an associate professor of wildlife pathology with a split appointment between the Center for Wildlife Health (Department of Forestry, Wildlife and Fisheries) and the Department of Pathobiology (College of Veterinary Medicine) at the University of Tennessee. She has studied amphibian, reptile, and fish diseases for over 11 years, and for the past 6 years, has been investigating ranavirus outbreaks and prevalence in both free-ranging and captive animals, with a concentration on amphibians. She has a special interest in documenting histopathological changes associated with ranaviral diseases. She collaborates with Matt Gray on broad scale surveillance in the eastern United States and controlled experimental studies to assess relative susceptibility of various North American amphibians. Current and future investigations include, characterizing histopathological changes associated with ranavirus infection, exploring environmental persistence of ranavirus virions; expanding susceptibility testing to other amphibians, reptiles, and fish; exploring the likelihood of interclass transmission among amphibians, reptiles and fish; investigating the role of community composition in ranaviral disease outbreaks; investigating the impact of ranaviruses and Bd on hellbender populations in Arkansas and Tennessee; and exploring treatment and control measures for ranviral disease. Other areas of research include investigating environmental and toxicological factors that affect nest-success in leatherback sea turtles and documenting histopathological changes in marine and Arctic mammals.

Debra Miller (DVM, Ph.D.)

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CONCURRENT INFECTION WITH RANAVIRUS, *BATRACHOCHYTRIUM DENDROBATIDIS*, AND *AEROMONAS* IN A CAPTIVE ANURAN COLONY

Debra L. Miller, D.V.M., Ph.D., Sreekumari Rajeev, B.V.Sc., Ph.D., Dipl. A.C.V.M., Milagros Brookins, D.V.M., Jeff Cook, Lisa Whittington, B.S., H.T., and Charles A. Baldwin, D.V.M., Ph.D., Dipl. A.C.V.M.

Abstract: Four species (*Dendrobates auratus*, *Phyllobates terribilis*, *Pyxicephalus adspersus*, and *Rhacophorus dennysi*) of captive anurans with a clinical history of lethargy and inappetence were found dead and were submitted for necropsy. Gross lesions included irregular patches of sloughed skin and rare dermal ulcerations. Histologic findings included epidermal proliferation that was most pronounced on the digits and that included intracytoplasmic chytrid organisms. Bacteria were often associated with the epidermal lesions. Intracytoplasmic inclusion bodies were observed in hepatocytes. Real-time polymerase chain reaction yielded positive results for both Ranavirus and *Batrachochytrium dendrobatidis* (Bd). Bacterial culture of internal organs yielded *Aeromonas hydrophila*. This is the first report of concurrent infections in anurans by Ranavirus and Bd and *A. hydrophila*.

Key words: *Aeromonas hydrophila*, anuran, *Batrachochytrium dendrobatidis*, concurrent infection, Ranavirus.

INTRODUCTION

In both free-ranging and captive amphibians, infections with Ranavirus and *Batrachochytrium dendrobatidis* (Bd), individually, have been responsible for morbidity and mortality. These diseases have resulted in population declines and, in the case of Bd, loss of species.[5] Furthermore, the bacterium *Aeromonas hydrophila* is an amphibian pathogen and may contribute to morbidity and mortality in both ranaviral and Bd infections.[8] Ranavirus is reported to be responsible for most anuran mortality, events in the United States,[3,9] and Bd has been responsible for amphibian declines in many parts of the world, including Australia and Panama.[1] Most often Ranavirus and Bd are documented singly or along with opportunistic *A. hydrophila* infections. However, reports of concurrent infection by all three pathogens are lacking. Herein, a case of concurrent infection by Ranavirus, Bd, and *A. hydrophila* in four species of captive anurans is described.

CASE REPORT

Multiple species of anurans in a captive facility displayed varying degrees of clinical signs, including lethargy, inappetence, and difficulty moving. Approximately 20% of the collection was affected, and six individuals who died were submitted to the University of Georgia Veterinary Diagnostic and Investigational Laboratory (VDIL) for necropsy. Included in the submission were four poison dart frogs (three *Dendrobates auratus* and one *Phyllobates terribilis*), an African bullfrog (*Pyxicephalus adspersus*), and a Chinese gliding frog (*Rhacophorus dennysi*). Based on fat and muscle stores, the frogs were in fair body condition. These frogs had a few random areas of sloughing skin on the legs, back, ventrum, or head (Fig. 1A); generalized erythema; and a few random, 2–10-mm, irregular foci of ulceration (Fig. 1B), some of which were proliferative (1 mm raised; Fig. 1B, inset). The kidneys were mildly friable, and in the African bullfrog, they were markedly congested (Fig. 1C). The livers were moderately swollen and pale, with distended gall bladders (Fig. 1D). No other significant gross changes were noted. A section of each organ (brain, heart, lung, skin, digits, skeletal muscle, spleen, kidney, liver, stomach, intestines, pancreas) was collected for virus isolation, real-time polymerase chain reaction (qPCR) for *Ranavirus* and Bd, and bacterial culture. An identical set of tissues was fixed in 10% phosphate-buffered formalin, embedded in paraffin, cut at 4-μm sections, and stained with hematoxylin and eosin (H&E) and examined microscopically. Ranavirus qPCR was performed using the protocol of Pallister et al.,[11] adapted by Picco et al.[12] Chytrid qPCR was performed using the method described by Boyle et al.[2] Bacterial and fungal cultures were performed on skin tissue using standard operating protocols at the VDIL. Briefly, the samples were inoculated onto a trypticase soy

From The University of Georgia, College of Veterinary Medicine, Veterinary Diagnostic and Investigational Laboratory, Tifton, Georgia 31793, USA (Miller, Rajeev, Brookins, Whittington, Baldwin); and Reptiland, Allenwood, Pennsylvania 17810, USA (Cook). Correspondence should be directed to Dr. Miller (millerdl@uga.edu).

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DISEASES OF AQUATIC ORGANISMS
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Efficacy of select disinfectants at inactivating *Ranavirus*

Laura K. Bryan[1], Charles A. Baldwin[2], Matthew J. Gray[3], Debra L. Miller[2, 3,*]

[1]University of Georgia, College of Veterinary Medicine, Athens, Georgia 30602, USA
[2]Veterinary Diagnostic and Investigational Laboratory, University of Georgia, 43 Brighton Road, Tifton, Georgia 31793-1389, USA
[3]Center for Wildlife Health, Department of Forestry, Wildlife and Fisheries, University of Tennessee, 274 Ellington Plant Sciences Building, Knoxville, Tennessee 37996-4563, USA

ABSTRACT: *Ranavirus* can cause disease in reptiles and amphibians. Because survival time outside of a host remains uncertain, equipment must be disinfected to prevent transmission of ranaviruses. However, disinfectant efficacy against amphibian ranaviruses has not been investigated for chlorhexidine (Nolvasan®), sodium hypochlorite (bleach), or potassium compounds. Our goal was to determine the efficacy of Nolvasan® (0.25, 0.75 and 2.0%), bleach (0.2, 1.0, 3.0 and 5.0%), and Virkon S® (1.0%) at inactivating *Ranavirus* at 1 and 5 min contact durations. Potassium permanganate ($KMnO_4$) (2.0 and 5.0 ppm) was also tested with a 60 min contact time. Nolvasan® at 0.75 and 2.0% and bleach at 3.0 and 5.0% concentration were effective for both contact durations. Virkon S® was effective for both durations, but $KMnO_4$ was not effective at either concentration. Concentrations of Nolvasan®, bleach and Virkon S® that are at least 0.75, 3.0 and 1.0%, respectively, are effective at inactivating *Ranavirus* after 1 min exposure time.

KEY WORDS: *Ranavirus* · Amphibians · Disinfection · Chlorhexidine · Pathogen pollution · Potassium peroxymonosulfate · Potassium permanganate · Sodium hypochlorite

INTRODUCTION

The genus *Ranavirus* encompasses several related double-stranded DNA icosahedral viruses of the family *Iridoviridae*. Ranaviruses cause disease in fish, reptiles and amphibians (Chinchar 2002, Converse & Green 2005, Robert et al. 2007). Ranaviruses have been implicated in large-scale die-offs of amphibians in Europe, Asia, Australia, and the Americas (Converse & Green 2005, Picco et al. 2007). In the United States, ranaviruses have been associated with significant amphibian losses in 15 states (Converse & Green 2005). These mortality events have generated global concern for the welfare of amphibians. In May 2008, the World Organization for Animal Health (OIE) classified *Ranavirus* as a notifiable pathogen (OIE 2008), imposing guidelines for the importation of amphibians across international borders. There are no treatments or vaccinations currently available for ranaviruses (Robert et al. 2007).

Captive ranaculture and zoological facilities also have experienced large-scale morbidity and mortalities due to *Ranavirus* (Miller et al. 2007, D. L. Miller unpubl.). The United States imports an average of 14.7 million wild-caught amphibians a year and exports 2 million amphibians annually to markets in Europe and Asia (Schlaepfer et al. 2005). Most exported animals are held at several locations before reaching their final destination, and imported animals are dispersed widely throughout the United States (Schlaepfer et al. 2005). Commercial trade of amphibians for pets, research, bait and consumption has the potential to spread ranaviruses to naïve environments and new hosts (Picco et al. 2007). Recent phylogenic analyses found similarities between *Ranavirus* strains associated with mortality events and those isolated from infected amphibians used for food and bait, suggesting that environmental spread of ranaviruses may be linked to human recreation and

*Corresponding author. Email: millerdl@uga.edu

Ranavirus Publications

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Amphibian Ranaviruses in Canada – Historical, Current, and Future Research Directions

D. Schock

Keyano College, 8115 Franklin Avenue, Fort McMurray, Alberta, Canada, T9H 2H7
(email): danna.schock@keyano.ca

Ranaviruses have been documented in Canadian amphibian populations from British Columbia to Prince Edward Island, and as far north as Norman Wells in the Northwest Territories. On-going research in Canada falls under four broad categories: 1) field studies that examine host range and geographic range, usually as part of studies that also investigate the prevalence of chytrid fungus infections in provincially or federally listed amphibian species; 2) field + lab studies that seek to identify ecological and environmental correlates with disease outbreaks; 3) validating and improving non-lethal diagnostic tests; and 4) viral biology including annotating viral genomes and identifying mediators of gene expression. To date, most amphibian ranaviruses documented in Canada have been isolated from ranid frogs and appear to be FV3-like. A smaller number of studies have focused on ranaviruses from tiger salamanders (Ambystomatidae) in Alberta, Saskatchewan and Manitoba, and thus far, all isolates have been identified as Ambystoma tigrinum virus (ATV). Comparatively little work has examined other families of salamanders or anurans. This is an important research need given the multi-host nature of ranaviruses. Studies that are needed for management and conservation purposes include long-term (10+ yr) studies that can address the effects of ranaviruses on long-term host population stability and persistence, the effects of ranaviruses on amphibian communities (not just populations of focal species), and studies that identify immunological and ecological correlates with disease outbreaks and effects of sublethal infections.

Biosketch: Dr. Danna Schock is a faculty member at Keyano College, which is situated in northern Alberta in the heart of Canada's oils sands. She teaches biology and environmental science. Her graduate degrees and postdoctoral projects were in ecology, especially evolutionary ecology and wildlife disease ecology. One facet of her current research examines correlations between proximity to oil sands mining activities and infectious disease dynamics (ranaviruses and *Batrachochytrium dendrobatidis*) in amphibian populations. A second facet of her research involves testing reclamation strategies that seek to use specially constructed wetlands to age and detoxify materials associated with oil sands mining. Ultimately the use of these wetlands will only be possible if they can support self-sustaining populations of endemic flora and fauna, including amphibians. Using wood frogs and a local strain of the ranavirus FV3, Dr. Schock is testing the immuno-competence of amphibians reared in water from these constructed research wetlands. She is currently collaborating with academic and government ecotoxicologists, pathologists, scientists with expertise in boreal forest ecology, and reclamation scientists in private industry.

Danna Schock (Ph.D.)

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ECOHEALTH

Original Contribution

Mortality Rates Differ Among Amphibian Populations Exposed to Three Strains of a Lethal Ranavirus

Danna M. Schock,[1,3] Trent K. Bollinger,[2] and James P. Collins[1]

[1]*School of Life Sciences, Arizona State University, Tempe, AZ 85287-4501*
[2]*Canadian Cooperative Wildlife Health Centre, Department of Veterinary Pathology, Western College of Veterinary Medicine, University of Saskatchewan, Saskatoon, SK S7N 5B4, Canada*
[3]*Faculty of Veterinary Medicine, Department of Ecosystem and Public Health, University of Calgary, Calgary, AB T2N 4N1, Canada*

Abstract: Infectious diseases are a growing threat to biodiversity, in many cases because of synergistic effects with habitat loss, environmental contamination, and climate change. Emergence of pathogens as new threats to host populations can also arise when novel combinations of hosts and pathogens are unintentionally brought together, for example, via commercial trade or wildlife relocations and reintroductions. Chytrid fungus (*Batrachochytrium dendrobatidis*) and amphibian ranaviruses (family Iridoviridae) are pathogens implicated in global amphibian declines. The emergence of disease associated with these pathogens appears to be at least partly related to recent translocations over large geographic distances. We experimentally examined the outcomes of novel combinations of host populations and pathogen strains using the amphibian ranavirus *Ambystoma tigrinum* virus (ATV) and barred tiger salamanders (*Ambystoma mavortium*, formerly considered part of the *Ambystoma tigrinum* complex). One salamander population was highly resistant to lethal infections by all ATV strains, including its own strain, and mortality rates differed among ATV strains according to salamander population. Mortality rates in novel pairings of salamander population and ATV strain were not predictable based on knowledge of mortality rates when salamander populations were exposed to their own ATV strain. The underlying cause(s) for the differences in mortality rates are unknown, but local selection pressures on salamanders, viruses, or both, across the range of this widespread host–pathogen system are a plausible hypothesis. Our study highlights the need to minimize translocations of amphibian ranaviruses, even among conspecifc host populations, and the importance of considering intraspecific variation in endeavors to manage wildlife diseases.

Keywords: intraspecific variation, pathogen, host, translocation, amphibian decline, ranavirus

INTRODUCTION

Habitat loss, environmental contamination, and climate change can interact with pathogens in ways that intensify the effects of infections on host populations (Daszak et al., 2000; Hess et al., 2002; Smith et al., 2009). Infectious diseases can also emerge as new threats to host populations when novel combinations of hosts and pathogens are unintentionally brought together, as a result of commercial trade or wildlife management tools such as relocations and

Correspondence to: Danna M. Schock, e-mail: dschock@ucalgary.ca

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Contribution to DAO Special 4 'Chytridiomycosis: an emerging disease'

Amphibian chytrid fungus and ranaviruses in the Northwest Territories, Canada

Danna M. Schock[1,*], Gregory R. Ruthig[2,8], James P. Collins[2], Susan J. Kutz[1], Suzanne Carrière[3], Robert J. Gau[3], Alasdair M. Veitch[4], Nicholas C. Larter[5], Douglas P. Tate[6], Glen Guthrie[7], Daniel G. Allaire[5], Richard A. Popko[4]

[1]Department of Ecosystem and Public Health, Faculty of Veterinary Medicine, University of Calgary, 3330 Hospital Drive NW, Calgary, Alberta T2N 4N1, Canada

[2]School of Life Science, Arizona State University, Tempe, Arizona 85287-4501, USA

[3]Department of Environment & Natural Resources, Government of the Northwest Territories, PO Box 1320, Yellowknife, Northwest Territories X1A 2L9, Canada

[4]Department of Environment & Natural Resources, Government of the Northwest Territories, PO Box 130, Norman Wells, Northwest Territories X0E 0V0, Canada

[5]Department of Environment & Natural Resources, Government of the Northwest Territories, PO Box 240, Fort Simpson, Northwest Territories X0E 0N0, Canada

[6]Nahanni National Park Reserve, Parks Canada, 10002 – 102 St, Fort Simpson, Northwest Territories X0E 0N0, Canada

[7]Sahtu Renewable Resources Board, PO Box 134, Tulita, Northwest Territories X0E 0N0, Canada

[8]*Present address*: Department of Biology, Grinnell College, 1116 8th Ave, Grinnell, Iowa 50112-1690, USA

ABSTRACT: Pathogens can cause serious declines in host species, and knowing where pathogens associated with host declines occur facilitates understanding host-pathogen ecology. Suspected drivers of global amphibian declines include infectious diseases, with 2 pathogens in particular, *Batrachochytrium dendrobatidis* (*Bd*) and ranaviruses, causing concern. We explored the host range and geographic distribution of *Bd* and ranaviruses in the Taiga Plains ecoregion of the Northwest Territories, Canada, in 2007 and 2008. Both pathogens were detected, greatly extending their known geographic distributions. Ranaviruses were widespread geographically, but found only in wood frogs. In contrast, *Bd* was found at a single site, but was detected in all 3 species of amphibians in the survey area (wood frogs, boreal chorus frogs, western toads). The presence of *Bd* in the Northwest Territories is not congruent with predicted distributions based on niche models, even though findings from other studies at northern latitudes are consistent with those same models. Unexpectedly, we also found evidence that swabs routinely used to collect samples for *Bd* screening detected fewer infections than toe clips. Our use and handling of the swabs was consistent with other studies, and the cause of the apparent lack of integrity of swabs is unknown. The ranaviruses detected in our study were confirmed to be Frog Virus 3 by sequence analysis of a diagnostic 500 bp region of the major capsid protein gene. It is unknown whether *Bd* or ranaviruses are recent arrivals to the Canadian north. However, the genetic analyses required to answer that question can inform larger debates about the origin of *Bd* in North America as well as the potential effects of climate change and industrial development on the distributions of these important amphibian pathogens.

KEY WORDS: Ranavirus · *Batrachochytrium dendrobatidis* · Amphibian declines · *Rana sylvatica* · *Pseudacris maculata* · *Bufo boreas* · Nahanni National Park Reserve · Taiga Plains

*Email: dschock@ucalgary.ca

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Ranaviruses in European Amphibians

A. Duffus

Division of Mathematics and Natural Sciences, Gordon College, Barnesville, GA, 30204 (email: aduffus@gdn.edu)

Ranaviruses are emerging infections in amphibian populations on nearly a global scale. In Europe, the first documented large scale morbidity and mortality events associated with ranaviruses in amphibians occurred in the late 1980s and early 1990s. Adult common frogs (*Rana temporaria*) in the southeast of the United Kingdom began to experience mass mortalities associated with pox virus-like cellular inclusions which was later identified as an iridovirus. After the initial outbreaks in common frogs, ranavirus outbreaks were identified in common toads (*Bufo bufo*) and determined to be caused by frog virus 3 (FV3) – like ranavirus. Since the initial confirmed reports of ranavirus associated mortality and morbidity events in the UK, both the number of species affected and the number of countries reporting infections/disease associated with the ranavirus in Europe have increased. The emergence of ranavirus infection and disease in UK common frogs provides the longest temporal data set documenting ranavirus infections and their effects in any amphibian species. Here we will examine the current state of knowledge of ranavirus infections in European amphibians. A conclusive summary of amphibian species known to be infected by ranaviruses in Europe will be presented and the current infection status, past morbidity and/or mortality events, potential reservoirs of the virus and where appropriate, discussions on disease dynamics. Future research directions should include structured infection surveillance, increased vigilance for mortality and morbidity events, and cooperative multidisciplinary investigations into the causes of these events.

Biosketch: Dr. Amanda Duffus is currently an Assistant Professor of Biology in the Division of Mathematics and Natural Sciences, Gordon College, Georgia. She completed her Ph.D. in England in 2010, which examined many aspects of ranavirus transmission and ecology in common frogs (*Rana temporaria*), including experimental and mathematical models, at the Institute of Zoology, Zoological Society of London and Queen Mary, University of London. She completed her MSc. in 2006, at Trent University, Peterborough, ON, Canada, where she examined ranavirus transmission dynamics in native North American amphibian species. In 2004, she graduated from Queen's University, Kingston, ON, with a subject of specialization in Biology. During her undergraduate degree, most of her research activities focused on plant evolutionary biology and aquatic ecology of acidified systems. Dr. Duffus has held many prestigious scholarships such as the Queen Mary, University of London, Research Studentship; Overseas Research Studentships; a three year Doctoral Natural Science and Engineering Council of Canada Scholarship; and the Richard Ivey Memorial Scholarship. Dr. Duffus looks forward to active involvement with SEPARC, developing her own ranavirus research program, taking an inclusive community approach, as well as developing new collaborations. One of her current projects is to help organize an international ranavirus reporting system in conjunction with the US Forest Service and Imperial College, London, UK.

Amanda Duffus (Ph.D.)

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Review article

Major disease threats to European amphibians

Amanda L.J. Duffus[1,2] & Andrew A. Cunningham[1]

[1]Institute of Zoology, Zoological Society of London, UK
[2]School of Biological and Chemical Sciences, Queen Mary, University of London, UK

Disease threats to amphibians in Europe are generally poorly understood. The effects that disease can have on amphibian populations can range from minimal to local extirpation. Currently, two infectious agents are emerging as disease threats to European amphibian populations: *Batrachochytrium dendrobatidis* (Bd), which is the causative agent of amphibian chytridiomycosis, and ranavirus(es). Both pathogens are listed by the World Organization for Animal Health (OIE). The incidence of other infectious diseases, such as amphibiocystidium, might also be increasing. In this review, we discuss known and potential disease threats to European amphibians, including their current and potential impact on amphibian populations, and factors driving their emergence and spread. We provide recommendations on how to proceed with investigations into cases where disease is thought to be involved in mortality or decline. We also stress that a multidisciplinary approach to these investigations is required.

Key words: amphibiocystidium, *Batrachochytrium dendrobatidis*, chytridiomycosis, ranaviral disease, ranavirus

INTRODUCTION

Infectious diseases can cause catastrophic population declines, local extirpations, or even global species extinctions of wildlife (de Castro & Bolker, 2005), but even in the absence of such obvious effects, parasites are important determinants of host population dynamics (Anderson & May, 1979). Key factors that contribute to the impact of infectious disease include host population size, the transmission dynamics of the pathogen (density and/or frequency dependence) and the ability of the pathogen to utilize alternative, or reservoir, hosts or to remain viable in the environment (de Castro & Bolker, 2005; Ryder et al., 2007). Even large, robust populations, however, can be negatively impacted by disease, especially if the pathogen is new to the host in evolutionary terms (Cunningham et al., 2003), and this is of growing concern to conservation biologists (see Scott, 1988, for a discussion of the importance of the impacts of disease on populations).

Added to this, in Europe there appears to be a lack of knowledge regarding many aspects of amphibian biology (Pasmans et al., 2006). This is cause for concern, since it has been estimated that by 2050, depending only on predicted climate alterations, 31% of amphibians (n=42) in Europe will have experienced range contractions (Araújo et al., 2006). Compounded with other factors, such as reduced water availability (Araújo et al., 2006), land use change, environmental pollution and infectious disease, this could lead to marked declines and range restrictions even for amphibian species that are currently considered to be widespread and common in Europe (see Acevedo-Whitehouse & Duffus, 2009, for a discussion of the impacts of environmental change and wildlife health).

In this article, we discuss three major categories of infectious disease threat to amphibians in Europe: 1) amphibian chytridiomycosis, 2) ranavirus disease and 3) other infections for which the impact is currently unknown. We review the biology of the causative agent, clinical signs of disease, mode of transmission and spread, and known impacts on the distributions of amphibians in Europe. We provide starting points for investigations into cases where disease is thought to be responsible for, or to have played a role in, mortality and/or population declines, and we stress the importance of a multidisciplinary approach.

BATRACHOCHYTRIUM DENDROBATIDIS

Batrachochytrium dendrobatidis (*Bd*) is a non-hyphal, zoosporic chytridiomycete fungus that is the causative agent of the amphibian disease chytridomycosis (Berger et al., 1998). At first linked to amphibian mortality events and declines in both Australia and Central America (Berger et al., 1998), it has since been associated with mass mortality events and, in some cases, population declines and species extinctions affecting amphibians in six continents, including several European species (Bosch et al., 2001; Bosch & Martínez-Solano, 2006; Skerrat et al., 2007; Garner et al., 2009; Bielby et al., 2009). *Bd* is classified as an emerging infectious agent in amphibians (Daszak et al., 1999), is considered to be a pandemic pathogen (Pasmans et al., 2006) and, in 2008, was listed as a notifiable pathogen by the World Organization for Animal Health (OIE, 2008). Although the origin of *Bd* remains unknown, a series of genetic studies has demonstrated remarkably low genetic diversity amongst isolates from disparate parts of the world (Morehouse et al., 2003; Morgan et al., 2007; James et al., 2009) pointing to a rapid, clonal pandemic spread of the organism.

Correspondence: A.L.J. Duffus, Division of Mathematics and Natural Sciences, Gordon College, Barnesville, GA, USA.
E-mail: aduffus@gdn.edu

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FROG VIRUS 3-LIKE INFECTIONS IN AQUATIC AMPHIBIAN COMMUNITIES

A. L. J. Duffus,[1,5] B. D. Pauli,[2] K. Wozney,[3] C. R. Brunetti,[4] and M. Berrill[4]

[1] Watershed Ecosystems Graduate Program, Trent University, Peterborough, Ontario K9J 7B8, Canada
[2] Canadian Wildlife Service, National Wildlife Research Centre, Carleton University, 1125 Colonel By Drive, Ottawa, Ontario K1A 0H3, Canada
[3] Natural Resources DNA Profiling and Forensics Centre (NRDPFC), Trent University, Peterborough, Ontario K9J 7B8, Canada
[4] Department of Biology, Trent University, Peterborough, Ontario K9J 7B8, Canada
[5] Present Address: Institute of Zoology, Zoological Society of London. Regent's Park, London, NW1 4RY, UK
[6] Corresponding author (email: amanda.duffus@ioz.ac.uk)

ABSTRACT: Frog virus 3 (FV3) and FV3-like viruses, are members of the genus *Ranavirus* (family *Iridoviridae*), and they have been associated with infectious diseases that may be contributing to amphibian population declines. We examined the mode of transmission of an FV3-like virus, and potential hosts and reservoirs of the virus in a local amphibian community. Using the polymerase chain reaction to detect infected animals, we found an FV3-like virus in south-central Ontario, Canada, amphibian communities, where it infects sympatric amphibian species, including ranid and hylid tadpoles (*Rana sylvatica, Hyla versicolor*, and *Pseudacris* spp.), larval salamanders (*Ambystoma* spp.), and adult eastern-spotted newts (*Notophthalmus viridescens*). The high prevalence of FV3-like infections in caudate larvae suggests that salamanders are likely to be both hosts and reservoirs. In laboratory FV3 challenges of *R. sylvatica*, the rate of infection was dependent on the amount of virus to which the animals were exposed. In addition, although vertical transmission was suspected, horizontal transmission through exposure to infected pond water is the most likely route of infection in tadpoles. Based on our observations, a simple model of FV3/FV3-like virus transmission postulates that, in aquatic amphibian communities, transmission of the virus occurs between anuran and urodele species, with ambystomatid salamanders the most likely reservoir for the ranavirus in our study.

Key words: Aquatic amphibian communities, frog virus 3-like infections, transmission, vectors.

INTRODUCTION

Amphibian populations are in decline on a global scale (Stuart et al., 2004). A number of factors are thought to be contributing to amphibian population declines, including habitat loss and modification, increasing ultraviolet radiation levels, predation, climate change, environmental contaminants, and emerging infectious diseases, as well as interactions between these factors (Alford and Richards, 1999). Although emerging infectious diseases are thought to be a contributing factor to global amphibian declines, anthropogenic environmental modification is considered to be, at least in part, responsible for their recent appearance or increasing virulence (Daszak et al., 2001; Pounds et al., 2006). An emerging infectious disease is defined as a disease caused by a pathogen that is currently increasing in geographical range, is infecting an increased diversity of hosts, and/or has recently evolved (Daszak et al., 2000). Examples of diseases in amphibian populations around the world that have recently gained attention include chytridiomycosis, caused by *Batrachochytrium dendrobatidis* (Muths et al., 2003; Rachowicz et al., 2006), and the iridoviruses (family *Iridoviridae*) (Harp and Petranka, 2006). Iridoviruses have been associated with large-scale morbidity and mortality events of amphibians throughout North America and Europe. For example, of 44 amphibian mortality events studied in the United States between 1996 and 2001, iridovirus infection was the sole cause of mortality in 48% of these events, and it was thought to be a factor in 9% of the other recorded mortality events with multiple etiology (Green et al., 2002).

The distribution of iridoviruses in Ca-

Ranavirus Publications

Duffus, A. L. J., B. D. Pauli, K. Wozney, C. R. Brunetti, and M. Berrill. 2008. FV3-like infections in aquatic amphibian communities. Journal of Wildlife Diseases 44(1):109–120.

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Current Understanding of Ranaviruses in South America

R. Mazzoni

Laboratório de Diagnóstico de Doenças de Organismos Aquáticos, CPA/EV/UFG Caixa Postal 151, Campus II, 74001-970, Goiânia-GO, Brasil (email: rolo1001@gmail.com)

Knowledge on ranaviruses in South America is scarce. Ranaviruses have been detected in Venezuela, Argentina, Uruguay, and Brazil. Wild amphibians from Venezuela appear to be infected with at least two different ranaviruses, one isolated from toads (*Bufo marinus*) and the other from *Leptodactylus* frogs. In Argentina, one ranavirus has been detected in *Atelognathus patagonicus*, which showed 100% homology with FV3 and other family members within a 500 base pair fragment of the major capsid protein. In Brazil, a ranavirus was detected in morbid tadpoles (*Lithobates catesbeianus*) originally imported from North America. For this isolate, the sequences for the complete MCP coding region, and partial regions of the RNA polymerase DNA dependent gene, and of the immediate early protein-ICP 18 were highly homologous to FV3. These results suggest that importation of *L. catesbeianus* may have introduced ranavirus into Brazil. Despite detection of ranaviruses in captive and wild amphibians in some South American countries, no ranavirus infections or disease have been reported in fish and reptiles, but few pathogen surveillance programs exist. A major research need in South America is to understand the current distribution of ranaviruses and their threat to native ectothermic vertebrate populations. Controlled studies also are needed that challenge native species with ranavirus isolates known to occur in South America.

Biosketch: Dr. Rolando Mazzoni is currently employed at the Veterinary School, Federal University of Goiás (UFG), Brazil. He finished his degree in Veterinary Science studies at the University of Uruguay in 1981 and a PhD on frog diseases in 2006 at the UFG. He has been working with aquaculture and diseases of aquatic organisms as Assistant Professor at the Fisheries Research Institute in Uruguay (1981–2006) and was in charge of the Diseases of Aquatic Organisms Diagnostic Laboratory as part of a Post-doctoral research project. Since 2000 he has been working with amphibian emerging infectious diseases (Chytridiomycosis and Ranavirus) particularly in farmed American bullfrogs in South America.

Rolando Mazzoni (DVM, Ph.D.)

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Ranavirus detection by PCR in cultured tadpoles (*Rana catesbeiana* Shaw, 1802) from South America

Leonardo Galli [a], Alfredo Pereira [b], Alejandro Márquez [b], Rolando Mazzoni [a,b,c,*]

[a] *Instituto de Investigaciones Pesqueras, Facultad de Veterinaria. UDELAR. Tomás Basáñez 1160, CP 11300 Montevideo, Uruguay*
[b] *Laboratorio de Bioquímica de Organismos Acuáticos, Dirección Nacional de Recursos Acuáticos, MGAP, Constituyente 1497, CP 11200 Montevideo, Uruguay*
[c] *Universidade Federal de Goias, Programa de Pos-graduação em Ciência Animal, Escola de Veterinária, CPA, Campus Samambaia, Caixa Postal 31, CEP 74001-970, Goiânia-GO, Brazil*

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Abstract

Diseases in farmed tadpoles (*Rana catesbeiana*) are a common event, being an economically important threat for Uruguayan and Brazilian farms. Based on clinical signs and epizootiology, pathogens belonging to the Family *Iridoviridae* were suspected as the possible etiology. Although these viruses have already been widely incriminated affecting aquatic organisms including frogs, their presence in Brazil and Uruguay was never mentioned so far. The objective of this work was to detect the presence of ranaviral agents in affected tadpoles using Polymerase Chain Reaction (PCR) technique as a primary approach to the study of the disease. Primers were designed based on highly conserved iridoviral sequences. Major Capsid Protein (MCP) and Immediate Early Protein (IE) genes were the selected targets. A positive PCR result was obtained for both genes when sick tadpoles from Brazil and Uruguay were analyzed. To confirm the amplification of an *Iridoviridae*, PCR products were purified and sequenced. Amplified products showed high degree of homology with several members of the *Iridoviridae*, mostly with those belonging to the genus *Ranavirus*. Obtained sequences were registered in the GenBank with accession nos. AY585203, AY585204 and AY744387. This report indicates that *Ranavirus* should be considered into the aquatic organism disease etiologies throughout this geographical region.

Keywords: PCR; Iridovirus; Ranavirus; Frog

1. Introduction

Frog culture (*Rana catesbeiana*) is an expanding activity in several Latin American countries, mainly Brazil, Argentina, Ecuador and Uruguay (Mazzoni, 2000a; Mazzoni et al., 2003). Improvements obtained

* Corresponding author. Instituto de Investigaciones Pesqueras, Facultad de Veterinaria, UDELAR, Tomás Basáñez 1160, CP 11300 Montevideo, Uruguay. Tel.: +598 5562 992256138; fax: +598 5562 2137060.
E-mail address: rolo@pes.fvet.edu.uy (R. Mazzoni).

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DISEASES OF AQUATIC ORGANISMS
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Mass mortality associated with a frog virus 3-like *Ranavirus* infection in farmed tadpoles *Rana catesbeiana* from Brazil

Rolando Mazzoni[1,*], Albenones José de Mesquita[2], Luiz Fernando F. Fleury[3], Wilia Marta Elsner Diederichsen de Brito[4], Iolanda A. Nunes[2], Jacques Robert[5], Heidi Morales[5], Alexandre Siqueira Guedes Coelho[6], Denise Leão Barthasson[4], Leonardo Galli[1], Marcia H. B. Catroxo[7]

[1]Instituto de Investigaciones Pesqueras, Facultad de Veterinaria, Universidad de la República, Tomás Basañez 1160, Montevideo 11300, Uruguay

[2]Centro de Pesquisa em Alimentos, Escola de Veterinária, Universidade Federal de Goiás, Campus Samambaia (Campus II), Caixa postal 131, CEP 74001-970, Goiânia-GO, Brazil

[3]Departamento de Patologia e Imaginologia, Faculdade de Medicina, Universidade Federal de Goiás, 1ª Avenida s/n, Setor Universitário 74605-050, Goiânia-GO, Brazil

[4]Laboratório de Virologia Animal, Setor Microbiologia, Instituto de Patologia Tropical e Saude Pública, Universidade Federal de Goiás, Rua 235 s/n, Setor Universitário 74605-050, Goiânia-GO, Brazil

[5]Department of Microbiology and Immunology, University of Rochester Medical Center, 601 Elmwood Avenue, Rochester, New York 14642, USA

[6]Escola de Agronomia e Engenharia de Alimentos, Universidade Federal de Goiás, Campus Samambaia, CEP 74001–970, Goiânia-GO, Brazil

[7]Laboratório de Microscopia Eletrônica, Centro de Pesquisa e Desenvolvimento de Sanidade Animal, Instituto Biológico, Av. Cons. Rodrigues Alves 1252, CEP 04014-002, São Paulo-SP, Brazil

ABSTRACT: Ranaviruses (*Iridoviridae*) are increasingly associated with mortality events in amphibians, fish, and reptiles. They have been recently associated with mass mortality events in Brazilian farmed tadpoles of the American bullfrog *Rana catesbeiana* Shaw, 1802. The objectives of the present study were to further characterize the virus isolated from sick *R. catesbeiana* tadpoles and confirm the etiology in these outbreaks. Sick tadpoles were collected in 3 farms located in Goiás State, Brazil, from 2003 to 2005 and processed for virus isolation and characterization, microbiology, histopathology, and parasitology. The phylogenetic relationships of *Rana catesbeiana* ranavirus (RCV-BR) with other genus members was investigated by PCR with primers specific for the major capsid protein gene (*MCP*) and the RNA polymerase DNA-dependent gene (*Pol II*). Sequence analysis and multiple alignments for *MCP* products showed >99% amino acid identity with other ranaviruses, while *Pol II* products showed 100% identity. Further diagnostics of the pathology including histology and transmission electron microscopy confirmed the viral etiology of these mass deaths. As far as we know, this is the first report of a ranaviral infection affecting aquatic organisms in Brazil. Additionally, our results suggest that American bullfrogs may have served as a vector of transmission of this virus, which highlights the potential threat of amphibian translocation in the world distribution of pathogens.

KEY WORDS: Iridovirus · FV3 · *Ranavirus* · *Rana catesbeiana*

INTRODUCTION

Brazilian frog farming is a flourishing industry that mainly focuses on producing the American bullfrog *Rana catesbeiana* Shaw, 1802. Ranaculture has been recently plagued by mass mortality events. These acute outbreaks were identified as a severe condition in tadpoles at early developmental stages and resulted in significant economic loses (Galli et al. 2006, Mazzoni 2006). The clinical signs of disease were apparently

*Email: rolo@pes.fvet.edu.uy

Ranavirus Publications

Galli, L., A. Pereira, A. Márquez, and R. Mazzoni. 2006. Ranavirus detection by PCR in cultured tadpoles (*Rana catesbeiana* Shaw 1802) from South America. Aquaculture 257:78–82.

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Emergence of Ranaviruses in Japan

Y. Une

Labratory of Veterinary Pathology, School of Veterinary Medicine, Azabu University, 1-17-71 Fuchinobe, Chuo-ku, Sagamihara, Kanagawa, Japan (email: une@azabu-u.ac.jp)

Ranavirus was discovered in *Rana catesbeiana* (Rc) larvae in a mass die-off in October 2008 in Japan. By 2010, five outbreaks in wild Rc were discovered within a 35-km radius. Mortality events involving Rc occurred between the end of September and the beginning of October and continued for several weeks. Fish mortality was documented at two sites and ranavirus was detected in one of these cases. Additionally, an outbreak occurred in a protected colony of 80 *Hynobius nebulosus* after the introduction of newly collected animals; the entire colony was annihilated in two weeks. These ranaviruses were registered as Rc ranavirus (RCV-JP) and *H. nebulosus* ranavirus (HNV) based on sequences of the MCP gene. Subsequent surveillance of 1,200 wild amphibians revealed RCV-JP infections in Rc (larva), *Cynops ensicauda* (adult), *Hyla japonica* (adult), and *Rhacophorus arboreus* (larva). A third ranavirus TFV was found in *Fejervarya limnocharis* (adult). All infected animals appeared healthy except for *H. japonica*. In ranavirus challenge experiments using 13 native species (8 salamanders and 5 frogs, n = 486 individuals), the mortality rates of RCV-JP were 100% in salamanders and 33–100 % in frogs. The mortality rate of HNV was 0–100 %, with high mortality in all salamander species except *H. nigrescens*. Additionally, mortality was greatest at elevated temperatures. The two ranaviruses reported here could pose a threat to native amphibian species in Japan. More studies are needed investigating the threat of these isolates to other Japanese species and the prevalence of ranaviruses in wild populations.

Biosketch: Dr. Yumi Une has been employed at the School of Veterinary Medicine, Azabu University (Kanagawa Pref. near Tokyo, Honshu) as a faculty member since 1983. She graduated from Azabu University in 1977. She is a diplomate of the Japanese College of Veterinary Pathologists and has over 28 years of experience in diagnostic pathology. She conducts necropsies of all types of animals, big and small. She specializes in wild animals, exotic animals, and infectious diseases. Dr. Une has several research projects, for example, studies on the cheetah (amyloidosis, Alzheimer's disease, helicobacter infection, etc.), monkeys (yersiniosis, hyperosteosis, toxplasmosis, encephalitozoonosis, and some bacterial infections), and reptiles and amphibians. She discovered chytridiomycosis for the first time in Asia in December, 2006. She also discovered an outbreak of ranaviral disease, and has found cutaneous metacercariosis in Hynobiid salamanders. Her studies have shown expansion of the range of these parasites and a rise in prevalence. Her future work will evaluate reasons behind these changes in expansion and prevalence.

Yumi Une (DVM)

Department of Health and Human Services
Centers for Disease Control and Prevention

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Ranavirus Outbreak in North American Bullfrogs (*Rana catesbeiana*), Japan, 2008

Yumi Une, Akiko Sakuma, Hiroki Matsueda, Katsuki Nakai, and Masaru Murakami
Author affiliations: Azabu University, Kanagawa, Japan (Y. Ume, A. Sakuma, H. Matsueda, M. Murakami); and Nature Conservation Division of Shiga Prefecture, Shiga, Japan (K. Nakai)
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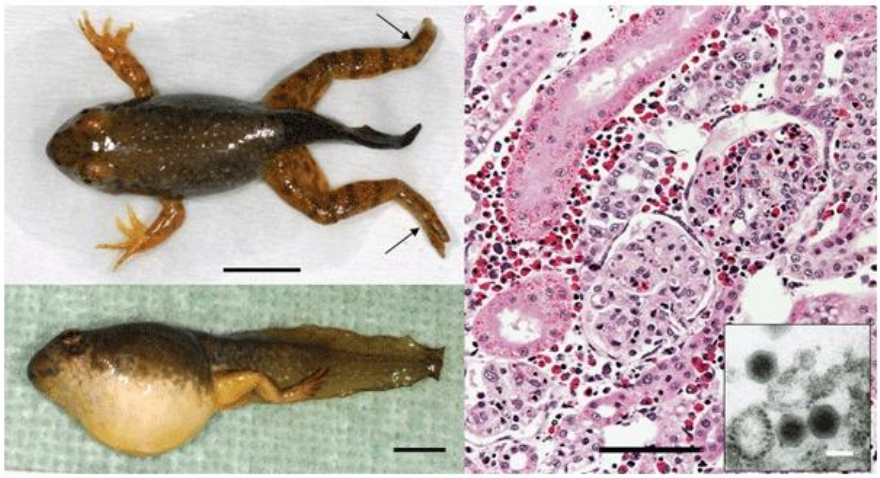

Appendix Figure. North American bullfrog (*Rana catesbeiana*) metamorphs infected with ranavirus RCV-JP. A) Necrosis of distal extremities (arrows) and mild abdominal swelling. Scale bar = 1 cm. B) Severe abdominal swelling caused by body cavity effusion. Scale bar = 1 cm. C) Kidney of an infected frog with necrosis of glomeruli and tubular hyaline droplet degeneration; hematoxylin and eosin stain. Scale bar = 100 µm. Inset shows ranavirus-like particles; scale bar = 100 nm.

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RARE CASE OF THIRD EYELID MAST CELL TUMOUR IN A HORSE

A. Śmiech*, I. Balicki† and Z. Nozdryn-Plotnicki*

**Department of Pathological Anatomy, Faculty of Veterinary Medicine, University of Life Sciences in Lublin and †Department and Clinic of Animal Surgery, Faculty of Veterinary Medicine, University of Life Sciences in Lublin, Poland*

Introduction: Mast cell tumours (MCTs) are uncommon in horses. They usually occur as benign, solitary masses on the skin of the head, neck, trunk and legs. In the present study we describe a rare case of an equine MCT located in the third eyelid.

Materials and Methods: An 18-year-old mare presented with a 4 cm diameter tumour in the left third eyelid. Other ocular examinations were unremarkable and the right eye was normal. The tumour was surgically removed with the third eyelid and fixed tissue sections were stained with haematoxylin and eosin and toluidine blue. Immunohistochemical methods for the detection of cytokeratin, vimentin and CD117 were also applied.

Results: Microscopically, tumour cells were round to ovoid with slightly pleomorphic nuclei. Fine cytoplasmic granules stained weakly with toluidine blue. The tumour cells expressed CD117, but not vimentin or cytokeratin.

Conclusions: A neoplasm in the third eyelid of an adult horse was diagnosed as a MCT according to Patnaik's classification for cutaneous MCTs. MCTs should be considered in the differential diagnosis of third eyelid tumours in horses.

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OCCURRENCE OF NEUROFIBROMA IN THE SPINAL CORD OF A GERMAN SHEPHERD DOG

A. Tavasoly and A. Malmasy

Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran

Introduction: Neurofibroma is one of the subtypes of peripheral nerve sheath tumours (PNSTs). Tumours of this type are rare in dogs and are most often observed unilaterally in the spinal nerves with high frequency in the brachial plexus and lower frequency in the lumbosacral plexus.

Materials and Methods: A 1.5-year-old male German shepherd dog with posterior paralysis was referred to the University Clinic. Using USG and MRI, a mass was observed in the vertebral column along the left side of the spinal cord between T12 and L2. The dog was humanely destroyed and the tumour was removed for gross and microscopical examinations.

Results: Macroscopically, the tumour was soft and gelatinous, white to grey and with a smooth surface. Histologically, the tumour cells were uniformly elongate and fusiform and lacked obvious cytoplasmic borders. They were arranged as bands, whorls and palisades. Immunohistochemical analysis was not available.

Conclusions: The appearance of the tumour was of a neurofibroma. This tumour should be differentiated from other neoplasms such as meningioma, fibroma and haemangiopericytoma by histological and immunohistochemical criteria.

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BIOCOMPATIBILITY AND ACTIVITY OF OXIDIZED SILICON MICROPARTICLES TREATED WITH CHITOSAN

E.G. Tirifon*, R. Anghel*, A. Popovici†, C. Mateescu*, N. Manolescu*, M.M. Nistoroiu*, I. Dumitrescu*, M. Panait*, R. Eliescu*, M. Chesnoiu*, C. Tirifon† and S. Cinca*

**Institute of Oncology Prof. Dr Alex. Trestioreanu, †Sanitary-Veterinary and Food Safety Laboratory, Bucharest and ‡University of Medicine and Pharmacy Carol Davila, Bucharest, Romania*

Introduction: The effect of oxidized silicon microparticles, treated with chitosan, was investigated in Wistar rats. Changes in skin and muscle at sites of inoculation were examined together with weight gains and survival rates. Levels of the molecular biological markers P450-cytochrome and glutathion-S-transferase (GST) were assayed.

Results: Local irritative effects at skin level disappeared 48 h after injection. After 24 h, small powdery deposits in the muscular area had an acicular and/or crystalloid appearance. These deposits were dispersed and partially resorbed at 48 h. Weight gains and survival rates were similar for both control and experimental animals, suggesting good biocompatibility of the tested microparticles. Changes in concentration of P450-cytochrome and GST-activity indicated the induction of dynamic changes resulting from activation of detoxication mechanisms.

Conclusions: This study represents an intermediary step in the development of oxidized silicon particles both as possible carriers of antitumour agents and as factors expressing synergic effects. The finding of good biocompatibility bodes well for the future use of the particles as drug carriers.

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RANAVIRUS INFECTION OUTBREAK IN THE SALAMANDER *(HYNOBIUS NEBULOSUS)* IN JAPAN

Y. Une, K. Nakajima, S. Taharaguchi, K. Ogihara and M. Murakami

Azabu University, Kanagawa, Japan

Introduction: Ranaviruses are important pathogens that are devastating amphibian populations worldwide. The first ranavirus infection (RCV-JP) in Japan was reported in a mass die-off of *Rana catesbeiana* larvae in 2008. The present study now describes ranavirus infection in *H. nebulosus*.

Materials and Methods: The outbreak occurred in a protected colony of 80 *H. nebulosus*, which had been in captivity for over 1 year. The animals began to die 15 days after the introduction of some newly collected animals and the entire colony was annihilated by the 20th day. The dead animals were examined using pathological and molecular biological methods.

Results: The animals died regardless of age. Macroscopically, immediate signs of morbidity were confined to skin ulcers. Histological examination showed extensive glomerular necrosis with renal tubular hyaline droplet degeneration and various degrees of hepatic cell degeneration and necrosis. Cytoplasmic ranavirus-like particles that were icosahedral with a diameter of about 120 nm were detected within interstitial cells of the kidneys by electron microscopy. In addition, a polymerase chain reaction technique with a pair of M153 and M154 primers successfully amplified a ranavirus-specific gene encoding the major capsid protein (MCP). The partial MCP DNA sequence analyses revealed that the present ranavirus from *H. nebulosus* differed from frog virus 3 and RCV-JP.

Conclusions: This is the first report of ranavirus infection in salamanders in Japan.

Ranavirus Publications

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Ranaviruses in Frogs and Fish in Southeast Asia

S. Kanchanakhan, J. Polchana, and W. Wongchan

Inland Aquatic Animal Health Research Institute (AAHRI), Inland Fishery Research and Development Bureau, Paholyothin Road, Chatuchak, Bangkok 10900, Thailand (email: kanchanakhan@yahoo.com)

Ranaviral disease was first documented in Asia in 1998. The disease occurred in *Rana tigrina* housed on frog culture farms located in central Thailand. The diseased frogs exhibited ulcerative lesions on the dorsal skin surfaces, similar to lesions observed in the United Kingdom. Histopathological examination revealed necrosis and chronic inflammation in skin, spleen, livers, and the gastro-intestinal tract followed by exuberant hematopoiesis. Thereafter, ranavirus surveillance was conducted on Thailand frog farms from 1998-2002, by attempting virus isolation on tissue extracts of diseased frogs. Virus was isolated from frogs in 8 of 9 provinces in central Thailand, with an overall prevalence of 65% (n = 107 individuals tested). Mortality was greatest in tadpoles, moderate in small frogs, and low in adults. All virus isolates displayed similar cytopathic effects. Sequence analysis supported a novel ranavirus: *Rana tigrina* ranavirus. In Thailand, the same or closely related ranaviruses have been isolated from diseased marble goby (*Oxyeleotis marmoratus*) in 2000 and diseased goldfish (*Carrasius arrarus*) in 2002. Other ranaviruses have been reported in ornamental fish from Japan and in cage cultured fish in Singapore. Likewise, a similar ranavirus has been isolated from frogs imported from Cambodia in 2004. The scientific findings indicate that ranaviruses can infect and cause disease in fish and amphibians in Asia, and they have the potential to negatively impact the aquaculture industry. Trans-boundary movement of ranaviruses through international trade is a major concern to the Southeast Asia region and elsewhere in the world.

Biosketch: Dr. Somkiat Kanchanakhan is a Civil Servant of the Department of Fisheries, Ministry of Agriculture and Cooperative, Thailand. He graduated from the Faculty of Fisheries, Kasetsart University, Thailand. He received his MSc in Microbiology from Oregon State University and PhD in fish virology from Stirling University, Scotland. Somkiat is a Fishery Biologist senior professional and head of the Aquatic Diseases Research Section at the Inland Aquatic Animal Health Research Institute (AAHRI). His research mainly focuses on viral diseases of fish and frogs. Dr. Kanchanakhan is an expert on Epizootic Ulcerative Syndrome of the OIE since 2001 and a regional resource expert of the Network of Aquaculture in Asia-Pacific (NACA) since 2005 and an advisory committee to NACA since 2008. Additionally, he joined the FAO emergency task force to investigate the EUS outbreak in Pakistan in 1996, koi herpesvirus outbreak in Indonesia in 2002, and EUS outbreak in Southern Africa in 2007-2008.

Somkiat Kanchanakhan (Ph.D.)

Isolation of FV3-like iridovirus from a cutaneous ulceration disease of cultured frog, *Rana tigrina* Cantor, in Thailand

SOMKIAT KANCHANAKHAN, U-MALAI SADUAKDEE, ARANYA KREETHACHAT and SUPRANEE CHINABUT
Aquatic Animal Health Research Institute, Department of Fisheries
Bangkok, Thailand

Abstract

A new frog disease occurred in frog culture farms located in Central Thailand since early 1998. The disease affects 20-100% of the frog population in affected farms. Diseased adult frogs usually exhibit ulcerative lesions on the dorsal part of the body and legs with moderate mortality (20-50%). Some frogs had red lips, ulcerated mouths and rostrums. Diseased tadpoles and small frogs appeared weak with systemic inflammation. Mortality ranged from 50-100%. Histopathological changes observed include cutaneous ulceration and systemic inflammation with exuberant hematopoiesis. No bacteria could be isolated from the kidney, spleen and liver of frogs at the early stage of the disease. Viral investigation was, therefore, conducted. Seventy virus isolates were obtained from 107 diseased frogs collected from 8 provinces using the *Epithelioma papulosum cyprini* (EPC) cell line at 25°C. One virus isolate (AV9803) was partially characterized. The virions were enveloped, possessed genomic DNA and hexagonal nucleocapsid morphology, and were ~128 nm in diameter. The virus completely lost infectivity when incubated at 56°C for 30 min, in organic solvent or buffer pH 3. These findings indicate that this frog virus belongs to the family *Iridoviridae*. DNAs of 8 virus isolates from different provinces were extracted and compared using polymerase chain reaction or PCR. Similar sized PCR products were obtained using primers that were specific to different parts of a major capsid protein gene of *Ranavirus* type genus FV3. Over 99% nucleotide homology was observed between one sequenced PCR product of AV9803 and the sequence of FV3. These findings suggest that a single virus species was isolated which is most likely a strain of *Ranavirus*. This virus strain is temporally designated as "*Rana trigrina ranavirus* (RTRV)". The RTRV seems to be associated with cutaneous ulceration. Further infection experiments and electron micrograph examinations in the diseased frog need to be done to confirm the causative agent.

Introduction

Intensive frog culture has been developed in Thailand in the early 1990s. Although bacterial disease in frogs has been reported (Somsiri and Soontornvit, 2002), there has been no report of viral isolation in frog in Thailand and in Southeast Asia. Viruses have

Kanchanakhan, S., U. Saduakdee, A. Kreethachat and S. Chinabut. 2002. Isolation of FV3-like iridovirus from a cutaneous ulceration disease of cultured frog, *Rana tigrina* Cantor, in Thailand. *In* Diseases in Asian Aquaculture IV. C.R. Lavilla-Pitogo & E.R. Cruz-Lacierda (eds.). Fish Health Section, Asian Fisheries Society, Manila.

Characterisation of Iridovirus Isolated from Diseased Marbled Sleepy Goby, *Oxyeleotris Marmoratus*

PONGPUN PRASANKOK, MALINEE CHUTMONGKONKUL
Department of Biology, Chulalongkorn University, Bangkok

SOMKIAT KANCHANAKHAN
Aquatic Animal Health Research Institute, Department of Fisheries, Bangkok

ABSTRACT

High mortalities of cultured marbled sleepy goby or sand goby, Oxyeleotris marmoratus, occurred in Nakonpathom province, Central Thailand in March 2000. The diseased fish had minor ulcers on the body and around the mouth. No external parasites or blood parasites were observed and no bacteria were isolated from the liver, kidney or spleen. Three diseased fish were used for virological investigation. The tissue extracts were inoculated on to *Epithelioma papulosum cyprini* (EPC) cells at 25°C inducing round plaques. Electron microscopy showed the presence of numerous icosahedral cytoplasmic particles averaging 132 ± 7.8 nm in diameter. Virus titres were over 6 log10 $TCID_{50}$/ml lower when incubated with IUdR or chloroform indicating the particles possessed a DNA genome and an envelope. The virus isolate was sensitive to heat at 56°C. These properties indicate that the new virus isolate can be classified as a virus member of the family *Iridoviridae*. This virus propagated well in fish cell lines, BF-2, EPC, FHM, BB, SSN-1 and discus tail (DT), and 2 reptile cell lines, Siamese crocodile embryo (SCE) and soft-shelled turtle embryo (STE) at 25-30°C. The highest virus titre, 9.2 $\log_{10}$ $TCID_{50}$/ml, was obtained from the BF-2 line. New virions were released from EPC cells about 15 h post-infection at 25°C. PCR amplification of the new isolate and four other previous isolates of frog iridoviruses in Thailand using specific primers designed from the major capsid protein gene of ranavirus FV-3 gave predicted PCR products of 300 bp. Sequence analysis of the PCR products found 98-99% nucleotide homology to FV-3 and *Rana tigrina* ranavirus. The marbled sleepy goby iridovirus is proposed as Oxyeleotris marmoratus ranavirus or OMRV. Virulence and pathogenicity of OMRV are yet to be clarified.

INTRODUCTION

Marbled sleepy goby or sand goby, *Oxyeleotris marmoratus*, is a freshwater fish cultured for food in Thailand and neighboring countries. It has a high commercial value and is exported to Japan, China P.R., Chinese Taipei, Hongkong China, Singapore and Malasia. In Thailand, the goby is raised in floating cages and in earthern ponds in Nakornsawan, Uthaithani, Nakonpathom, Ayuthaya and Pathumthani and elsewhere. Goby seed is mainly collected from the wild, as seed production from hatcheries is limited. A number of pathogens have been found in the fish including parasites, bacteria, and fungi that can cause great

Prasankok, P., M. Chutmongkonkul, and S. Kanchanakhan. 2005. Characterisation of iridovirus isolated from diseased marbled sleepy goby, *Oxyeleotris Marmoratus*. *In* P. Walker, R. Lester and M.G. Bondad-Reantaso (eds). Diseases in Asian Aquaculture V, pp. 197-206. Fish Health Section, Asian Fisheries Society, Manila.

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Isolation of Frog Virus 3 from Pallid Sturgeon Suggests an Interclass Host Shift

T. Waltzek,[1,2*] B. Drecktrah,[3] J. Briggler,[4] B. MacConnell,[5] C. Hudson,[6] L. Hopper,[6] S. Yun,[1] K. Malm,[1] S. Weber,[1] and R. Hedrick[1]

[1]School of Veterinary Medicine, University of California, Davis, California 95616, USA (email: tbwaltzek@ucdavis.edu)
[2]College of Veterinary Medicine, University of Florida, Florida 32610, USA
[3]Blind Pony State Fish Hatchery, Missouri Department of Conservation, Sweet Springs, Missouri 65351, USA
[4] Missouri Department of Conservation, Jefferson City, MO 65109, USA
[5] Headwaters Fish Pathology, Bozeman, Montana 59715, USA
[6]Bozeman Fish Health Center, Bozeman, Montana 59718, USA

During July – September 2009, juvenile pallid sturgeon (*Scaphirhynchus albus*) at the Blind Pony State Fish Hatchery (BPSFH) in Sweet Springs, Missouri experienced mortalities of over 500 individuals/day at water temperatures between 16–26 °C. Histological exams revealed extensive necrosis of the hematopoietic tissues. A viral replicating agent was observed in cell culture and confirmed by electron microscopy. Experimental infection studies revealed the virus is pathogenic to pallid sturgeon – a federally endangered species. Analysis of the full length major capsid protein revealed that it was identical to the type species of ranavirus, *Frog Virus 3* (FV-3), and to a previous BPSFH isolate. This suggests that recurring infections or carryover of the virus from prior groups of sturgeon may have maintained the virus at this facility. Inasmuch as the BPSFH draws water directly from nearby Blind Pony Lake without disinfection, entry of ranavirus-contaminated water into the facility cannot be ruled out. However, liver samples collected from adult and larval American bullfrogs (*Lithobates catesbeianus*) and plains leopard frogs (*Lithobates blairi*) during the fall of 2009 and 2010 in nearby wetlands were negative for FV-3. The potential for reciprocal FV-3 infections (i.e. amphibian to fish and vice versa) has only been reported in sympatric populations of threespine stickleback (*Gasterosteus aculeatus*) and red-legged frog tadpoles (*Rana aurora*). Future research will focus on discovering the source of the virus at the facility (e.g. contaminated water supply and broodstock) as well as testing the host specificity and pathogenicity of the virus across a suite of poikilothermic vertebrates.

Biosketch: Dr. Thomas Waltzek began his academic career studying marine biology at Florida State University, graduating with a B.S. in 1998. He then traveled to the University of California at Davis where he studied the functional anatomy and ecology of cichlid fishes receiving a M.S. in 2002 and completing his Veterinary Medical degree in 2009, focusing on fish health. He finished his Ph.D. dissertation on the evolution and ecology of viral diseases of poikilothermic vertebrates in October of 2010. In April of 2011, he returned to Florida as a Postdoctoral Research Associate to begin the surveillance and characterization of emerging aquatic animal pathogens. Dr. Waltzek has received many accolades for presentations at 20 national and international meetings. He has authored 20 papers on a variety of subjects from fish physiology and ecology to infectious diseases of fish. He was recently voted the newest member of the AVMA Aquatic Animal Veterinary Medicine Committee for recognition of his expertise in public health/epidemiology. He has a passion for the ocean and especially enjoys diving, fishing, kayaking, volleyball, and exploring the beach with his wife, Jenna.

Thomas Waltzek (DVM, Ph.D.)

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Systemic iridovirus infection in the Banggai cardinalfish (*Pterapogon kauderni* Koumans 1933)

E. Scott Weber III,[1] Thomas B. Waltzek, Devon A. Young, Erica L. Twitchell, Amy E. Gates, Alejandro Vagelli, Guillermo R. Risatti, Ronald P. Hedrick, Salvatore Frasca Jr.

Abstract. Iridoviruses infect food and ornamental fish species from a wide range of freshwater to marine habitats across the globe. The objective of the current study was to characterize an iridovirus causing systemic infection of wild-caught *Pterapogon kauderni* Koumans 1933 (Banggai cardinalfish). Freshly frozen and fixed specimens were processed for histopathologic evaluation, transmission electron microscopic examination, virus culture, molecular virologic testing, microbiology, and in situ hybridization (ISH) using riboprobes. Basophilic granular cytoplasmic inclusions were identified in cytomegalic cells often found beneath endothelium, and hexagonal virus particles typical of iridovirus were identified in the cytoplasm of enlarged cells by transmission electron microscopy. Attempts at virus isolation in cell culture were unsuccessful; however, polymerase chain reaction (PCR)-based molecular testing resulted in amplification and sequencing of regions of the DNA polymerase and major capsid protein genes, along with the full-length ATPase gene of the putative iridovirus. Virus gene sequences were then used to infer phylogenetic relationships of the *P. kauderni* agent to other known systemic iridoviruses from fishes. Riboprobes, which were transcribed from a cloned PCR amplification product from the viral genome generated hybridization signals from inclusions within cytomegalic cells in histologic sections tested in ISH experiments. To the authors' knowledge, this is the first report of a systemic iridovirus from *P. kauderni*. The pathologic changes induced and the genomic sequence data confirm placement of the Banggai cardinalfish iridovirus in the genus *Megalocytivirus* family *Iridoviridae*. The ISH provides an additional molecular diagnostic technique for confirmation of presumptive infections detected in histologic sections from infected fish.

Key words: Banggai cardinalfish; cytoplasmic inclusions; in situ hybridization; iridovirus; *Megalocytivirus*.

Introduction

Iridoviruses are a frequently encountered group of DNA viruses found in invertebrates, fish, and amphibians, which are associated with a range of disease presentations from skin lesions to systemic infections.[7,9,10,21,30,37,61,63] The family *Iridoviridae* contains 5 genera, 3 of which are found in fish.[8] One particular genus, *Megalocytivirus*, contains viruses associated with serious systemic infections resulting in significant mortality (up to 100%) among a growing list of marine and freshwater fishes.[2,25,43,44,50] The Red Sea bream iridovirus (RSIV), which caused major losses in Japan in 1990, was among the first megalocytiviruses described.[31] Similar viruses were circulating in populations of ornamental fish prior to the recognition of RSIV, as revealed by histologic and electron microscopic evidence from diseased orange chromide cichlids exhibiting similar clinical signs.[3] Since these initial observations, megalocytiviruses have been reported among numerous marine and freshwater fish species either endemic or originating in exports from Japan, the South China Sea, and several Southeast Asian countries.[5,8,48,49,54,59] Sequences of the entire genomes of 4 viruses in the genus *Megalocytivirus* species (i.e., Rock bream iridovirus [RBIV], *Infectious spleen and kidney necrosis virus* [ISKNV], Orange spotted grouper iridovirus [OSGIV]), and Ehime-1 strain [RSIV]) are currently known.[12,23,36,40] Sequence data from additional megalocytiviruses indicate a close relationship to the type species ISKNV, sharing up to 97% or greater identity at the deduced amino level for the major capsid protein.[8]

Wide host and geographic ranges of this pathogen coupled with the severity of infections caused by these megalocytiviruses have had a major impact on cultured fish and likely have had negative effects on wild fish populations as well.[43,62] Infections of ornamental and farmed food fish in both marine and freshwater environments can have devastating

From the New England Aquarium, Boston, MA (Weber), the Department of Medicine and Epidemiology, School of Veterinary Medicine, University of California, Davis, CA (Weber, Waltzek, Hedrick), the Department of Pathobiology and Veterinary Science, University of Connecticut, Storrs, CT (Young, Twitchell, Gates, Risatti, Frasca), and the New Jersey Academy for Aquatic Sciences, Camden, NJ (Vagelli).

[1] Corresponding Author: E. Scott Weber III, Department of Medicine and Epidemiology, School of Veterinary Medicine, University of California, Davis, CA 95616. epweber@ucdavis.edu

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IFAS Extension

Megalocytivirus Infections in Fish, with Emphasis on Ornamental Species[1]

Roy P. E. Yanong and Thomas B. Waltzek[2]

Introduction

What are the megalocytiviruses?

The megalocytiviruses are an important group (genus) of fish viruses in the family *Iridoviridae* (the iridoviruses). Megalocytiviruses cause systemic infections that can result in moderate to heavy losses in many different species of freshwater and marine fishes in both cultured and wild stocks. In some disease outbreaks, 100% losses have occurred in under one week. Megalocytiviruses have been reported in fish in the United States as well as other parts of the world, especially Asia.

Currently, nearly all isolates from diseased fish appear to be strains of the same virus species. Isolates have been divided into three major subgroups based on their genetic similarities and differences: a) infectious spleen and kidney necrosis virus (ISKNV); b) red sea bream iridovirus (RSIV); and c) turbot reddish body iridovirus (TRBIV).

Strains closely resembling ISKNV have been reported to cause disease in numerous species of ornamental freshwater and marine fishes. In the early 1990s, RSIV was first observed in Japan and since then has been reported primarily in Asian marine finfish. Today, RSIV is reportable to the World Organization for Animal Health (OIE) and the United States Department of Agriculture-Animal and Plant Health Inspection Service (USDA-APHIS). The third subgroup TRBIV has been reported predominantly in Asian flounder species. New viral isolates from other fish species are currently being evaluated by scientists to determine their relationships to these three main groups. This publication provides disease, diagnostic, and management information on megalocytiviruses in fish for producers, wholesalers, retailers, and others who work with fish and may be unaware of this disease.

Which fish species are susceptible?

Ornamental finfish species known to be susceptible to megalocytiviruses are listed in Table 1. Many popular aquarium fish are on this list, including freshwater angelfish (*Pterophyllum scalare*), other cichlids (Family Cichlidae), swordtails, sailfin mollies, and other common

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2. Roy P. E. Yanong, associate professor and extension veterinarian, Tropical Aquaculture Laboratory, Ruskin, FL 33570, Program in Fisheries and Aquatic Sciences, School of Forest Resources and Conservation, UF/IFAS, Gainesville, FL 32611; and Thomas B. Waltzek, Department of Medicine and Epidemiology, School of Veterinary Medicine, University of California, Davis, CA 95616.

Ranaviruses in European Reptiles

R. Marschang[1], A. Stöhr[1], S. Blahak[2], T. Papp[1,3], M. Filomena Caeiro[4,5], A. de Matos[5,6], and J. Nazir[1]

[1]Institut für Umwelt und Tierhygiene, University of Hohenheim, Garbenstrasse 30, 70599 Stuttgart, Germany
(email: rachel.marschang@googlemail.com)
[2]Chemisches und Veterinäruntersuchungsamt OWL, Westerfeldstr. 1, 32758 Detmold, Germany
[3]Veterinary Medical Research Institute of the Hungarian Academy of Sciences, Hungaria krt. 21, H-1143 Budapest, Hungary
[4]University of Lisbon, Faculty of Sciences, Deptartment of Plant Biology, Campo Grande 1749-016 Lisbon, Portugal
[5]CESAM – Centre for Environmental and Marine Studies, Aveiro University, Campus Universitário de Santiago, 3810-193 Aveiro, Portugal
[6]Anatomic Pathology Department, Curry Cabral Hospital, R. da Beneficência 8, 1069-166 Lisboa, Portugal

In Europe, ranaviruses have been isolated from chelonians and lizards. There are three reports of ranavirus infections in tortoises in Europe, two in Hermann's tortoises (*Testudo hermanni*) and one in a leopard tortoise (*Geochelone pardalis*). All of the tortoises were kept in captivity and developed disease. In two cases, disease spread from one animal to another and one recent case has been associated with an outbreak among several different collections of tortoises and spread from Hermann's tortoises to other species. Ranaviruses have also been isolated from lizards in Europe. In one case, a virus was isolated from a gecko (*Uroplatus fimbriatus*) kept in a private collection. In another, a ranavirus was isolated from an iberian rock lizard (*Lacerta monticola*). The second is the only documented case of ranavirus infection in a wild reptile in Europe. Characterization of the ranavirus isolates obtained from these reptiles has been carried out by various methods, making a direct comparison between the European reptile ranaviruses somewhat difficult. Available sequence data show that the reptilian ranaviruses are each more closely related to various described amphibian ranaviruses than to one another. However, available restriction enzyme analysis of some of the reptilian ranaviruses does show considerable differences between these and specific amphibian isolates. Future research directions include further comparison of ranaviruses from reptiles, environmental persistence of reptilian, amphibian, and fish ranaviruses, and screening of reptiles for ranavirus infections by virus and antibody detection.

Biosketch: Dr. Rachel Marschang is a research scientist in charge of the virology laboratory of the Institut für Umwelt und Tierhygiene at the University of Hohenheim in Stuttgart, Germany. She studied veterinary medicine at the Justus-Liebig University Giessen and graduated in 1995. She did her "Doktorarbeit" on viruses of tortoises at the Clinic for Avian, Reptile, Amphibian, and Fish Medicine of the same university, finishing in 2001. She is a diplomate of the European College of Zoological Medicine (herpetology), a certified specialist in microbiology (FTÄ Mikrobiologie) and a certified specialist in reptile medicine (ZB Reptilien). Her research over the past 15 years has focused on the diagnosis and characterization of viruses in reptiles, particularly herpes, irido, paramyxo, and adenoviruses. Dr. Marschang is currently involved in research projects dealing with paramyxoviruses of snakes and tortoises, adenoviruses of lizards, picornaviruses of tortoises, ranaviruses of amphibians and reptiles, and other iridoviruses in reptiles, as well as on disinfection testing and disinfection of fish ponds. She has research cooperations with partners in many different countries including various European countries, the USA, and Australia.

Rachel Marschang (DVM, Ph.D.)

Arch Virol (1999) 144: 1909–1922

Archives of Virology

Isolation and characterization of an iridovirus from Hermann's tortoises (*Testudo hermanni*)

R. E. Marschang[1], P. Becher[2], H. Posthaus[3], P. Wild[4], H.-J. Thiel[2], U. Müller-Doblies[5], E. F. Kaleta[1], and L. N. Bacciarini[3]

[1]Institut für Geflügel- und Reptilienkrankheiten, Justus-Liebig-Universität, Giessen, Germany
[2]Institut für Virologie (FB Veterinärmedizin), Justus-Liebig-Universität, Giessen, Germany
[3]Institute of Animal Pathology, Center for Fish and Wildlife Medicine, University of Bern, Bern, Switzerland
[4]Institute of Veterinary Anatomy, Laboratory for Electron Microscopy, University of Zurich, Zurich, Switzerland
[5]Institute of Virology, University of Zurich, Zurich, Switzerland

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Summary. A virus was isolated from tissues of 2 diseased Hermann's tortoises (*Testudo hermanni*) and preliminarily characterized as an iridovirus. This conclusion was based on the presence of inclusion bodies in the cytoplasm of infected cells, sensitivity to chloroform, inhibition of virus replication by 5-iodo-2′-desoxyuridine and the size and icosahedral morphology of viral particles. The virus was able to replicate in several reptilian, avian and mammalian cell lines at 28 °C, but not at 37 °C. Restriction enzyme analysis showed resistance of the viral DNA to digestion with HpaII due to methylation of the internal cytosine at CCGG sequences. Part of the genomic region encoding the major capsid protein was amplified by PCR and subjected to sequence analysis. Comparative analysis of the obtained nucleotide sequence revealed that the isolate is closely related to frog virus 3, the type species of the genus *Ranavirus*.

Introduction

The family *Iridoviridae* is currently subdivided into five genera, the genera *Iridovirus* and *Chloriridovirus* which contain invertebrate iridoviruses, the genus *Lymphocystivirus*, the type species of which is flounder virus (LCDV-1), the genus *Ranavirus* the type species of which is frog virus 3 (FV3), and the "goldfish virus-1 like viruses" [28]. Iridoviruses are responsible for a number of diseases in fish and amphibians as well as in a wide range of invertebrates [15, 21, 38, 43]. Iridovirus-like particles have been observed in reptiles as lizard erythrocytic

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Microscopy AND Microanalysis

New Viruses from *Lacerta monticola* (Serra da Estrela, Portugal): Further Evidence for a New Group of Nucleo-Cytoplasmic Large Deoxyriboviruses

António Pedro Alves de Matos,[1,2,*] Maria Filomena Alcobia da Silva Trabucho Caeiro,[2,3] Tibor Papp,[4] Bruno André da Cunha Almeida Matos,[1] Ana Cristina Lacerda Correia,[1] and Rachel E. Marschang[4]

[1]*Anatomic Pathology Department, Curry Cabral Hospital, R. da Beneficência 8, 1069-166 Lisboa, Portugal*
[2]*CESAM—Centre for Environmental and Marine Studies. Aveiro University, Campus Universitário de Santiago, 3810-193 Aveiro, Portugal*
[3]*University of Lisbon, Faculty of Sciences, Department of Plant Biology, Campo Grande, 1749-016 Lisbon, Portugal*
[4]*Institut für Umwelt und Tierhygiene, Hohenheim University, Garbenstr. 30, 70599 Stuttgart, Germany*

Abstract: Lizard erythrocytic viruses (LEVs) have previously been described in *Lacerta monticola* from Serra da Estrela, Portugal. Like other known erythrocytic viruses of heterothermic vertebrates, these viruses have never been adapted to cell cultures and remain uncharacterized at the molecular level. In this study, we made attempts to adapt the virus to cell cultures that resulted instead in the isolation of a previously undetected *Ranavirus* closely related to FV3. The *Ranavirus* was subsequently detected by polymerase chain reaction (PCR) in the blood of infected lizards using primers for a conserved portion of the *Ranavirus* major capsid protein gene. Electron microscopic study of the *new Ranavirus* disclosed, among other features, the presence of intranuclear viruses that may be related to an unrecognized intranuclear morphogenetic process. Attempts to detect by PCR a portion of the DNA polymerase gene of the LEV in infected lizard blood were successful. The recovered sequence had 65.2/69.4% nt/aa% homology with a previously detected sequence from a snake erythrocytic virus from Florida, which is ultrastructurally different from the studied LEV. These results further support the hypothesis that erythrocytic viruses are related to one another and may represent a new group of nucleo-cytoplasmic large deoxyriboviruses.

Key words: lizard erythrocytic virus, *Ranavirus*, *Lacerta monticola*, virus isolation, PCR

Introduction

Erythrocytic viruses of heterothermic vertebrates (fish, amphibians, and reptiles) produce cytoplasmic inclusions in the infected cells (Johnston, 1975; Paperna & Alves de Matos, 1993). These are readily seen in Giemsa-stained smears and were once thought to represent protozoan parasites (Johnston, 1975). Morphologically the viruses have the traits commonly found among the nucleo-cytoplasmic large deoxyriboviruses (NCLDVs), such as a cytoplasmic virus assembly site and complex large icosahedral virions reminiscent of the *Iridoviridae* or *Asfarviridae* (Devauchelle et al., 1985; Alves de Matos & Paperna, 1993). However, early attempts to isolate erythrocitic viruses have mostly failed, and molecular data were insufficient to evaluate their phylogeny (Gruia-Gray et al., 1989, 1992). A recent study of a rattlesnake erythrocytic virus (SEV) from Florida was first to describe a sequence from the viral DNA dependent DNA polymerase using a polymerase chain reaction (PCR) procedure designed for amplification of a conserved region of the DNA polymerases encoded by a broad spectrum of DNA viruses (Hanson et al., 2006). Results suggested that the virus could represent a new group of NCLDVs, probably belonging to a novel genus and species (Wellehan et al., 2008).

In previous studies, erythrocytic viruses were found in lizard populations of *Lacerta monticola* and *Lacerta schreiberi* from Serra da Estrela, Portugal, and their interaction with the infected cells were studied (Alves de Matos et al., 2002). However, the phylogeny of these viruses remained obscure because no information on their molecular features has ever been obtained.

Other NCLDVs that have been described in reptiles are members of the *Ranavirus* genus of the *Iridoviridae* family. These viruses may induce highly lethal infections in a range of fish, amphibian, and reptilian species (Williams et al., 2005). Ranaviruses of reptiles have been found in turtles, snakes, and geckos (reviewed in Jancovich et al., 2010). Members of the insect iridoviruses (*Iridovirus* genus of the *Iridoviridae* family) have also been found to be able to infect captive lizards (Weinmann et al., 2007).

In this article, we report ultrastructural and molecular data on two viruses from *L. monticola* from Serra da Estrela Portugal. One of the viruses is a typical lizard erythrocytic virus (LEV) that has been reported elsewhere (Alves de Matos et al., 2002), but from which no molecular data were previously available. The second virus is a *Ranavirus* that was unexpectedly recovered from LEV-infected specimens

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*Corresponding author. E-mail: apamatos@kanguru.pt

Ranavirus Publications

Marschang, R. E., P. Becher, H. Posthaus, P. Wild, H-J. Thiel, U. Müller-Doblies, E. F. Kaleta, and L. N. Bacciarini. 1999. Isolation and characterization of an iridovirus from Hermann's tortoises (*Testudo hermanni*). Archives of Virology 144:1909–1922.

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Ranaviral Disease in Chelonians of North America

M. Allender

Department of Comparative Biosciences, College of Veterinary Medicine, University of Illinois, (email: mcallend@illinois.edu)

Ranaviruses have caused mass mortality events in wild fish, amphibian, turtle, and tortoise populations worldwide. However, compared to amphibians and fish, our understanding of the extent, impact, and transmission of ranaviral disease in chelonians is considerably less. In the United States, ranaviral disease has been diagnosed in seven chelonian species across thirteen states. Clinical manifestations of ranaviral infections in chelonians are not always present, but may include lethargy, dyspnea, ocular, nasal and oral discharges, oral plaques, and death. Other signs may include subcutaneous edema, hepatitis, necrotizing splenitis, conjunctivitis, and pneumonia. The duration of disease is short, and many wild animals likely die prior to their presentation at wildlife rehabilitation centers or clinics. Current diagnostic methods primarily utilize conventional PCR and histopathology, but use of an ELISA in gopher tortoises and blood smears demonstrating the presence of inclusion bodies in circulating white blood cells of box turtles are other potential tools. Ranaviral disease has been shown to be highly fatal in turtles during transmission studies. While prevalence has been investigated for gopher tortoises, little is known about the prevalence of this pathogen in other species, specifically the eastern box turtle – a species frequently observed in chelonian die-offs. Previous studies have failed to identify a mechanism of transmission; oral inoculation was not successful in red-eared sliders. Future research directions need to focus on elucidating the epidemiology of infections in wild reptiles, improving diagnostic assays, and determining the drivers and routes of transmission.

Biosketch: Dr. Matthew Allender is currently a Visiting Instructor in the Department of Comparative Biosciences/Veterinary Teaching Hospital in the College of Veterinary Medicine at the University of Illinois. Matthew received his B.S in 2000, DVM in 2004, and M.S. in 2006 from the University of Illinois. His M.S. work focused on the health and disease of two free-ranging reptile species. He completed a three-year residency program in Zoological and Wildlife Medicine at the University of Tennessee/Knoxville zoo in 2009. His current projects focus on the epidemiology of ranavirus in free-ranging turtles, the health of the massasauga rattlesnake, and hemolymph gas analysis in free-ranging sea urchins in Peru.

Matthew Allender (DVM, MS)

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Intracytoplasmic Inclusions in Circulating Leukocytes from an Eastern Box Turtle (*Terrapene carolina carolina*) with Iridoviral Infection

Matthew C. Allender,[1,5,6] Michael M. Fry,[2] Armando R. Irizarry,[3] Linden Craig,[2] April J. Johnson,[4] and Michael Jones[2] [1] University of Illinois, College of Veterinary Medicine, 2001 South Lincoln Ave., Urbana, Illinois 61802, USA; [2] University of Tennessee, College of Veterinary Medicine, 2407 River Dr., Knoxville, Tennessee 37996, USA; [3] Eli Lilly and Company, Lilly Research Laboratories, PO Box 708, Greenfield, Indiana 46140, USA; [4] University of Florida, College of Veterinary Medicine, PO Box 100126, Gainesville, Florida 32608, USA; [5] Current address: University of Tennessee, College of Veterinary Medicine, 2001 South Lincoln Ave, Urbana, Illinois, 61802, USA; [6] Corresponding author: (email: mattallender@yahoo.com)

ABSTRACT: A free-ranging adult female eastern box turtle (*Terrapene carolina carolina*) was presented to the University of Tennessee in October 2003 because of suspected trauma and blindness. Physical examination revealed lethargy, clear ocular and nasal discharges, and white oral and laryngeal plaques. Intracytoplasmic inclusions within heterophils and large mononuclear leukocytes were observed on routine blood smear examination. Postmortem findings included necrosis of epithelial and parenchymal cells with intracytoplasmic inclusions. Ultrastructurally, the leukocyte inclusions consisted of variably electron-dense granular material and viral particles consistent with the *Iridoviridae* family of viruses. The virus shared 100% sequence identity to a 420-base pair sequence of frog virus 3 (family *Iridoviridae*, genus *Ranavirus*) as determined by polymerase chain reaction and gene sequencing targeting a portion of the *Ranavirus* major capsid protein gene.

Key words: Electron microscopy, frog virus 3, intracytoplasmic inclusion, iridovirus, leukocyte, PCR, *Ranavirus*, *Terrapene carolina*.

The family *Iridoviridae* consists of four genera of large, icosohedral, enveloped, cytoplasmic, double-stranded DNA (Mao et al., 1997; Marschang et al., 1999), and iridoviruses in the genera *Ranavirus* and *Lymphocystitivirus* cause infections in vertebrate hosts (Mao et al., 1997; Marschang et al., 1999; Jancovich et al., 2003; De Voe et al., 2004). Frog virus 3 (FV3) of the genus *Ranavirus* has been specifically identified as pathogenic to fish, amphibians, and reptiles (Mao et al., 1997). Round-to-ovoid intracytoplasmic inclusions associated with iridoviral infections have been reported from reptiles in gastric, intestinal, hepatic, tracheal, and pulmonary epithelial cells as well as erythrocytes (Johnsrude et al., 1997; Marschang et al., 1999; Just et al., 2001). However, to date, no intracytoplasmic inclusions have been reported in leukocytes of reptiles or amphibians infected with iridoviruses.

In October 2003, a free-ranging adult female eastern box turtle (*Terrapene carolina carolina*) from Knox County, Tennessee (35°49′N, 83°59′W) was presented to the Avian and Zoological Clinical Service of the Veterinary Medical Teaching Hospital at the University of Tennessee, Knoxsville, Tennessee, USA, because of suspected trauma and blindness. On initial examination, the animal weighed 336 g, its eyelids were closed, and it had clear ocular and nasal discharges. Supportive care was initiated with ceftazidime (17.8 mg/kg, Abbott Laboratories, North Chicago, Illinois, USA) and vitamins A and D (0.05 ml, Vedco, Inc., St. Joseph, Missouri, USA). Thoracic radiographs revealed no abnormalities. Ophthalmologic findings consisted of severe conjunctivitis.

The turtle had a packed cell volume of 13%. Absolute and differential white blood cell (WBC) counts were not performed, because the moderate number of disrupted cells noted on routine blood smear prevented an accurate determination of leukocyte populations. Based on subjective blood smear evaluation, the total number of WBCs was considered moderately increased and consisted pre-

Absence of *Ranavirus* and Herpesvirus in a Survey of Two Aquatic Turtle Species in Illinois

Matthew C. Allender[1], DVM, MS, Mohammed Abd-Eldaim[2,3], BVSc, MVSc, PhD, Andrew Kuhns[4], MS, Melissa Kennedy[2], DVM, PhD, DACVM

1. Department of Small Animal Clinical Sciences, College of Veterinary Medicine, University of Tennessee, 2407 River Drive, Knoxville, TN 37996, USA

2. Department of Comparative Medicine, College of Veterinary Medicine, University of Tennessee, 2407 River Drive, Knoxville, TN 37996, USA

3. Virology Department, College of Veterinary Medicine, Suez Canal University Faculty of Veterinary Medicine, Ismailia, Egypt

4. Institute of Natural Resource Sustainability at the University of Illinois at Urbana-Champaign, Illinois Natural History Survey, 1816 South Oak Street, Champaign, IL 61820, USA

Abstract: Infections with *Ranavirus* and herpesvirus have contributed to numerous morbidity and mortality reports in chelonians worldwide. To better understand the prevalence of these viruses in healthy and declining populations, a survey for these viruses was performed on two aquatic turtle species in 2007 using polymerase chain reaction assays. Blood and oral swabs were taken from 47 painted turtles, *Chrysemys picta*, and 58 Blanding's turtles, *Emydoidea blandingii*. Results demonstrated no positive cases using this method in these populations. The lack of positive *Ranavirus* test results may indicate that these turtles have never been exposed to virus, have been exposed but have cleared the virus, are not shedding the virus in oral swabs or blood, or that oral swabs are inappropriate samples to assess ranaviral shedding in these species. Similarly, the lack of positive herpesvirus test results may indicate that these turtles have never been exposed to virus, have been exposed but have a latent infection, are not shedding the virus in oral swabs or blood, or that oral swabs are also inappropriate samples to assess herpesviral infection in these species.

Key Words: *Chrysemys picta*, *Emydoidea blandingii*, *Ranavirus*, iridovirus, turtle, herpesvirus.

Introduction

Diseases that affect the upper respiratory tract (URT) in chelonians have been well described as a significant contributor to morbidity and mortality (Brown and Sleeman, 2002; Origgi, 2006; Wendland *et al*., 2006). Numerous pathogens have been identified in these cases, but three major pathogens have been implicated. Herpesvirus, *Mycoplasma*, and chelonian iridovirus have been reported in the literature as historic or emerging causes of URT disease (Brown *et al*., 1999; Brown and Sleeman, 2002; Allender *et al*., 2006; Origgi, 2006; Wendland *et al*., 2006; Johnson *et al*., 2007). Herpesviruses have been identified in numerous turtle and tortoise species, but traditionally reports are confined to captive individuals (Harper *et al*., 1982; Pettan-Brewer *et al*., 1996; Origgi, 2006). Recently, mortality events in both free-ranging and captive amphibians and chelonians have implicated *Ranavirus* as the causative pathogen (Westhouse *et al*., 1996; DeVoe *et al*., 2004; Johnson, 2006). Clinical abnormalities with both infections are similar and include cutaneous abscesses, oral ulcers or abscesses, respiratory distress, anorexia, and lethargy.

Ranavirus, a member of the family *Iridoviridae*, is a large, icosahedral, variably enveloped, double-stranded DNA virus (Mao *et al*., 1997; Marschang *et al*., 1999). Type species Frog Virus 3 (FV3) of the genus *Ranavirus* has been identified as pathogenic to amphibians and reptiles (Mao *et al*., 1997; Johnson *et al*., 2007). The ecology of *Ranavirus* infection is unknown; however, amphibian reservoirs have been hypothesized because of the presence of these viruses in amphibians (Johnson *et al*., 2008). A study of an iridovirus outbreak in a group of Burmese starred tortoises, *Geochelone platynota*, in Georgia found that local amphibians were infected with the same virus, and one could be a source of virus for the other (Johnson, 2006; Johnson *et al*., 2008). Therefore, the investigation into aquatic turtle species is a logical step to determine other potential carrier species.

The family *Herpesviridae* is comprised of double-stranded DNA viruses that affect numerous species of animals (Origgi, 2006). Herpesviruses have been recognized in all orders of reptiles, with mortality rates up to 100% in experimental studies (Origgi, 2006). Clinical signs most commonly associated with herpesvirus infections in tortoises are rhinitis, stomatitis, and conjunctivitis (Origgi, 2006). The implications for species conservation is unknown because no known outbreaks have occurred in nonmarine species of wild chelonians (Harper *et al*., 1982; Pettan-Brewer *et al*., 1996; Origgi, 2006). Furthermore, few studies have determined the prevalence or pathogenesis of herpesviruses in wild turtles or tortoises.

A demographic and ecological study of a freshwater turtle assemblage (phylogenetically or ecologically related organisms) comprised of painted turtles, *Chrysemys picta*, snapping turtles, *Chelydra serpentina*, and Blanding's turtles, *Emydoidea blandingii*, is ongoing in northeastern Illinois and southwestern Wisconsin. Although painted turtles and snapping turtles are considered habitat generalists that can

Ranavirus Publications

Allender, M. C., M. M. Fry, A. R. Irizarry, L. Craig, A. J. Johnson, M. Jones. 2006. Intracytoplasmic inclusions in circulating leukocytes from an Eastern box turtle (*Terrapene carolina carolina*) with iridoviral infection. Journal of Wildlife Diseases. 42: 677-684.

Allender, M. C., M. Abd-Eldaim, A. R. Kuhns, M. Kennedy. 2009. Absence of Frog Virus 3 and Herpesvirus in a PCR survey of two aquatic turtle species from a threatened habitat in Illinois. Journal of Herpetological Medicine and Surgery 19: 16-20.

Allender, M. C., M. Abd-Eldaim, J. Schumacher, D. McRuer, L. S. Christian, M. Kennedy. 2011. PCR Prevalence of *Ranavirus* in free-ranging Eastern Box Turtles (*Terrapene carolina carolina*) at rehabilitation centers in Three Southeastern US states. Journal of Wildlife Disease. (in press.)

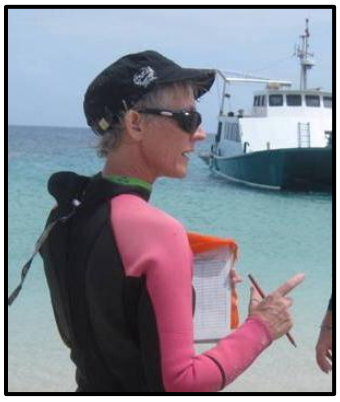
Challenge Studies of Australian Native Reptiles with a Ranavirus Isolated from a Native Amphibian

E. Ariel and L. Owens

School of Veterinary and Biomedical Sciences, James Cook University, Townsville, 4811 QLD, Australia,
(email: Ellen.Ariel@jcu.edu.au)

The capacity of ranavirus to cross species boundaries makes the epidemiology complex with potential reservoirs in many different species in any given location. Bohle iridovirus (BIV) was originally isolated from amphibians and shown to be pathogenic to fish in challenge trials. This study aimed to clarify the potential pathogenicity of BIV in six native Australian reptile species of the common aquatic and riparian fauna of northern Queensland. Animals were challenged by IC inoculation and were observed over a period of 30 days. Mortality and specific antibody response to BIV was monitored during the trials. Histopathology, immunohistochemistry and virus isolation were performed at the end of the study. Bohle iridovirus was found to be extremely virulent in hatchling tortoises (*Elseya latisternum* and *Emydura krefftii*), resulting in lesions in multiple organs and death (100 and 40% respectively). In contrast, adult tortoises, snakes (*Boiga irregularis*, *Dendrelaphis punctulatus* and *Amphiesma mairii*), and yearling crocodiles (*Crocodylus johnstoni*) were not acutely affected. Virus was re-isolated from BIV-exposed tortoise hatchlings and one *B.irregularis*. Adult tortoises survived BIV-challenge and produced antigen-specific antibodies. Thus, serological surveys of adult tortoises may be useful for determining the presence and spread of BIV in northern Australia, and help to predict the potential impact to native fauna from this pathogen.

Biosketch: Dr. Ellen Ariel is currently employed as senior lecturer in Virology at the School of Veterinary and Biomedical Sciences, James Cook University and spends much of her non-teaching time doing research into ranavirus in freshwater turtles and other viruses in marine turtles. Prior to that, she spent 11 years coordinating the European Union Community Reference Laboratory for Fish Diseases, which were mainly viral in nature. During this period she was also in the Steering group for the PANDA project (Permanent Advisory Network for Diseases in Aquaculture) and coordinator of the RANA project (Risk assessment of new and emerging systemic iridoviral diseases for European fish and aquatic ecosystems), both of which were funded by the European Commission.



Ellen Ariel (Ph.D.)

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Propagation and isolation of ranaviruses in cell culture

Ellen Ariel [a,c,*], Nicole Nicolajsen [a], Maj-Britt Christophersen [a], Riikka Holopainen [b], Hannele Tapiovaara [b], Britt Bang Jensen [a,d]

[a] Department of Fish, Poultry and Fur Animals, National Veterinary Institute, Technical University of Denmark, Hangøvej 8, 8210 Århus N, Denmark
[b] Department of Veterinary Virology, Finnish Food Safety Authority, Evira, Mustialankatu 3, Helsinki, FI-00790, Finland
[c] School of Veterinary and Biomedical Sciences, James Cook University, Townsville, QLD 4811, Australia
[d] Department of Large Animal Sciences, Faculty of Life Sciences, University of Copenhagen, Grønnegårdsvej 8, 1870 Frederiksberg C, Denmark

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ABSTRACT

The optimal *in vitro* propagation procedure for a panel of ranavirus isolates and the best method for isolation of Epizootic haematopoietic necrosis virus (EHNV) from organ material in cell-culture were investigated. The panel of ranavirus isolates included: Frog virus 3 (FV3), Bohle iridovirus (BIV), Pike-perch iridovirus (PPIV), European catfish virus (ECV), European sheatfish virus (ESV), EHNV, Doctor fish virus (DFV), Guppy virus 6 (GF6), short-finned eel virus (SERV) and *Rana esculenta* virus Italy 282/102 (REV 282/102). Each isolate was titrated in five cell lines: bluegill fry (BF-2), epithelioma papulosum cyprini (EPC), chinook salmon embryo (CHSE-214) rainbow trout gonad (RTG-2) and fathead minnow (FHM), and incubated at 10, 15, 20, 24 and 28 °C for two weeks.

BF-2, EPC and CHSE-214 cells performed well and titers obtained in the three cell lines were similar, whereas FHM and RTG-2 cells consistently produced lower titers than the other cell lines at all temperatures. The optimal temperature for propagating the isolates collectively to high titers *in vivo* was 24 °C.

Additionally, three established methods for re-isolation of virus from EHNV-infected organ material were compared. Challenged fish were sampled twice weekly and 7 organs were processed separately according to the three methods. Samples incubated on BF-2 cells at 22 °C for 2 weeks + 1 week sub-cultivation (method 1) provided more positive results than the other 2 methods and when using the EPC cell line. Virus was most frequently isolated from the kidney, followed by brain, muscle, heart, liver, gills and lastly spleen.

1. Introduction

Ranaviruses are receiving increasing attention due to the severe losses they can inflict on both wild and cultured fish and amphibians (Langdon et al., 1986; Pozet et al., 1992; Bovo et al., 1993; Cullen and Owens, 2002; Bigarré et al., 2008). Epizootic Haematopoietic Necrosis (EHN) has been listed by the World Organisation for Animal Health (OIE) (Anonymous, 2008) for more than a decade, and has recently become notifiable under EU legislation for prevention of certain Fish Diseases as well (Anonymous, 2006a,b). Ranavirus disease in amphibians has also recently been approved for listing by the OIE (Anonymous, 2008).

Within the genus *Ranavirus*, the difference between EHNV which is notifiable, and other isolates in terms of pathogenicity to different hosts, has not been fully clarified. Furthermore, EHNV can only be distinguished from other ranaviruses by sequencing or restriction endonuclease analysis (REA) (Hyatt et al., 2000; Marsh et al., 2002), lending evidence to the close relatedness of the members of the genus. For these reasons it is prudent to consider the genus rather than a single species when planning for disease control.

Freedom from notifiable viral diseases of fish and detection of emerging viral diseases of fish in EU Member States is based on surveillance, including laboratory testing of samples from fish organs for isolation of virus in cell culture (Anonymous, 2001). It is therefore important to establish which cell culture techniques are required for the detection of ranaviruses.

From the group of ranaviruses, the propagation of EHNV has been tested in 4 fish cell lines at 15 and 22 °C incubation temperatures (Crane et al., 2005). Similarly, the propagation of BIV was tested in 11 mammalian cell lines and four fish cell lines at 20 to 30 °C (Speare and Smith, 1992).

However, in order to determine if the same cell lines and temperatures are appropriate to all the ranaviruses, conditions and protocols for testing must be the same. Therefore, a panel of ranavirus isolates from different hosts and geographical regions were titrated in five cell lines incubated at five different temperatures. In addition, methods for isolation of ranavirus from infected fish were compared by processing

* Corresponding author. The School of Veterinary and Biomedical Sciences, Douglas Campus, James Cook University, Queensland 4811, Australia. Tel.: +61 747 81 4123; fax: +61 747 81 6174.
E-mail address: Ellen.Ariel@jcu.edu.au (E. Ariel).

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DISEASES OF AQUATIC ORGANISMS
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Ranavirus in wild edible frogs *Pelophylax* kl. *esculentus* in Denmark

Ellen Ariel[1,7,*], Jos Kielgast[2], Hans Erik Svart[3], Knud Larsen[4], Hannele Tapiovaara[5], Britt Bang Jensen[1,6], Riikka Holopainen[5]

[1]National Veterinary Institute, Technical University of Denmark, Hangøvej 2, 8200 Århus N, Denmark
[2]Department of Biology, Section for Microbiology and Evolution, University of Copenhagen, Universitetsparken 15, 2100 Copenhagen, Denmark
[3]Danish Forest and Nature Agency, Haraldsgade 53, 2100 Copenhagen Ø, Denmark
[4]Council of Slagelse, Dahlsvej 3, Korsør 4220, Denmark
[5]Department of Veterinary Virology, Finnish Food Safety Authority Evira, Mustialankatu 3, Helsinki, Finland
[6]Department of Large Animal Sciences, Faculty of Life Sciences, University of Copenhagen, Grønnegårdsvej 8, 1870 Frederiksberg C, Denmark
[7]*Present address:* School of Veterinary and Biomedical Sciences, James Cook University, Douglas Campus, Townsville, Queensland 4811, Australia

ABSTRACT: A survey for the amphibian pathogens ranavirus and *Batrachochytrium dendrobatidis* (*Bd*) was conducted in Denmark during August and September 2008. The public was encouraged via the media to register unusual mortalities in a web-based survey. All members of the public that registered cases were interviewed by phone and 10 cases were examined on suspicion of disease-induced mortality. All samples were negative for *Bd*. Ranavirus was isolated from 2 samples of recently dead frogs collected during a mass mortality event in an artificial pond near Slagelse, Denmark. The identity of the virus was confirmed by immunofluorescent antibody test. Sequencing of the major capsid protein gene showed the isolate had more than 97.3% nucleotide homology to 6 other ranaviruses.

KEY WORDS: Ranavirus · *Batrachochytrium dendrobatidis* · *Rana* kl. *esculenta* · *Pelophylax* kl. *esculentus* · Amphibian declines · Survey · Frogs · Amphibians

INTRODUCTION

The recent listing of *Batrachochytrium dendrobatidis* (*Bd*) and ranavirus infection in amphibians by the World Organisation for Animal Health (OIE) reflects a global concern for the health of farmed and declines of wild populations of amphibians (OIE 2008). Reports on ranavirus disease in amphibians are listed in the literature (Speare & Smith 1992, Kanchanakhan et al. 2002 Zupanovic et al. 1998, Zhang et al. 2001, Green et al. 2002, Weng et al. 2002, Greer et al. 2005, Fox et al. 2006) and several publications indicate that ranaviruses are part of the cause of amphibian declines across the world (Cunningham et al. 1996, Chinchar 2002, Green et al. 2002, Docherty et al. 2003, Pearman et al. 2004, Greer et al. 2005, Jancovich et al. 2005).

The type species of the ranavirus genus, frog virus 3 (FV3), was isolated for the first time in 1965 (Granoff et al. 1965, Rafferty 1965). In Europe, ranaviruses were isolated from moribund edible frogs *Pelophylax* kl. *esculentus* (formerly *Rana* kl. *esculenta*) in Croatia (Fijan et al. 1991) and Italy (G. Bovo pers. comm.) and from wild populations of the common frog *Rana temporaria* in the UK (Drury et al. 1995). The origin of these

*Email: ellen.ariel@jcu.edu.au

Ranavirus Publications

Ariel E., J. Kielgast, H. E. Svart, K. Larsen, H. Tapiovaara, B. B. Jensen, and R. Holopainen. 2009. Ranavirus in wild edible frogs (*Pelophylax* kl. *esculentus*) in Denmark. Diseases of Aquatic Organisms 85:7–14

Ariel**,** E., N. Nicolajsen, M-B. Christoffersen, R. Holopainen, H. Tapiovaara, and B. B. Jensen. 2009. Propagation and isolation of ranaviruses in cell culture. Aquaculture 294:159–164

Ariel, E., and B. Bang Jensen. 2009. Challenge studies of European stock of redfin perch, *Perca fluviatilis* L. and rainbow trout, *Oncorhyncus mykiss* (Wallbaum) with epizootic haematopoietic necrosis virus (EHNV). Journal of Fish Diseases 32:1017–1025.

Bang Jensen, B., A. K. Ersbøll, and E. Ariel. 2009. Susceptibility of Pike *Esox lucius* to a panel of ranavirus isolates. Diseases of Aquatic Organisms 83:169–179.

Ariel, E., R. Holopainen, H. Tapiovaara, and N. J. Olesen. 2010. Comparative study of iridovirus isolates from pike-perch (*Stizostedion lucioperca*), cod (*Gadus morhua*) and turbot (*Scophthalmus maximus*) with reference to other ranaviruses. Archives of Virology 155:1261–1271.

Holopainen, R., J. Honkanen, B. Bang Jensen, E. Ariel, and H. Tapiovaara. 2011. Quantification of ranaviruses from experimentally infected cell culture and tissue samples. Journal of Virological Methods 171:225–233.

Vesely, T., K. Cinkova, S. Reschova, F. Gobbo, E. Ariel, M. Vicenova, D. Pokorova, P. Kulich, and G. Bovo. 2011. Investigation of ornamental fish entering EU for the presence of ranaviruses. Journal of Fish Diseases 34:159–166.

Bang Jensen, B., R. Holopainen, H. Tapiovaara, and E. Ariel. 2011. Susceptibility of pike-perch *Sander lucioperca* to a panel of ranavirus isolates. Aquaculture 313:24–30.

Bang Jensen, B., S. Reschova, K. Cinkova, E. Ariel, and T. Vesely. 2011. Common carp (*Cyprinus carpio*) and goldfish (*Carassus auratus*) were not susceptible to challenge with Ranaviruses under certain conditions. Bulletin of the European Association of Fish Pathologists 31:112–118.

Assessing the Risk of Introducing Exotic Ranaviruses into Europe via Imports of Infected Ornamental Fish from Asia

B. Jensen[1], A. Ersbøll[2], A. Bayley[3], S. Bergmann[4], G. Bovo[5], K. Cinkova[6], F. Gobbo[5], B. Hill[3], R. Holopainen[7], S. Ohlemeyer[4], H. Schuetze[4], H. Tapiovaara[7], T. Vesely[6] and E. Ariel[8]

[1]Norwegian Veterinary Institute, Oslo, Norway (email: britt-bang.jensen@vetinst.no)
[2]National Institute of Public Health, Copenhagen, Denmark
[3]Centre for Environment, Fisheries and Aquaculture Science, Weymouth, England
[4]Friedrich-Loeffler Institute, Insel Riems, Germany
[5]Istituto Zooprofilatico Sperimentale delle Venezie, Padova, Italy
[6]Veterinary Research Institute, Brno, Czech Republic
[7]Finnish Food Safety Authority Evira, Helsinki, Finland
[8]James Cook University, Townsville, Australia

Introduction of exotic ranaviruses is a major concern for European aquaculture and aquatic ecosystems. Project RANA was developed to increase knowledge on susceptible hosts and improve diagnostic tools, as well as assess the risk of introducing exotic ranaviruses into Europe. The risk assessment was based on World Animal Health Organisation (OIE) guidelines and expert opinion, and the outcomes were: 1) the identification of a pathway of introduction and spread of ranaviruses into Europe via importation of live infected ornamental fish from Asia, 2) a generic model for assessing the risk of introducing an exotic pathogen via importation of ornamental fish, and 3) identification of knowledge gaps. The calculations of risk, based on our model, indicate that there is: 1) a high risk of exotic ranaviruses entering into Europe, 2) a moderate risk of ranaviruses becoming established in wild populations, and 3) a low risk of ranaviruses entering an aquaculture facility. Our model provides a preliminary tool to assess risk associated with the translocation of ranaviruses via imported fish. However, the results showed a high degree of uncertainty, due to lack of knowledge. We recommend the following future research directions: (1) Investigations on the prevalence of ranaviruses in fish and amphibian populations in both exporting and importing countries (2) Survey to estimate the likelihood of release of imported ornamental fish and amphibians and (3) In-depth research on the potential for natural transmission of ranaviruses between fish and amphibians.

Biosketch: Dr. Britt Jensen graduated as a doctor of Veterinary Medicine from Copenhagen University in 2004, and started her professional career with 10 months as a diagnostician at the Community Reference Laboratory (CRL) for Fish Diseases in Denmark. This led to a PhD project on "The implications of Ranaviruses to European farmed and wild freshwater fish", and I got my PhD-degree from Copenhagen University in 2009. Dr. Jensen then worked as a project coordinator for an EU-project at the CRL, and in fall 2009 she got a position as researcher at the section for epidemiology at the Norwegian Veterinary Institute. Her main research areas include various aspects of aquatic epidemiology including disease surveillance, control strategies, risk analysis and studies of risk factors and transmission pathways.

Britt Jensen (DVM, Ph.D.)

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Susceptibility of pike *Esox lucius* to a panel of *Ranavirus* isolates

Britt Bang Jensen[1,2,*], Annette Kjær Ersbøll[1], Ellen Ariel[2,3]

[1]Department of Large Animal Sciences, Faculty of Life Sciences, University of Copenhagen, Grønnegårdsvej 8, 1870 Frederiksberg C, Denmark

[2]Department of Fish, Poultry and Fur Animals, National Veterinary Institute, Technical University of Denmark, Hangøvej 8, 8210 Århus N, Denmark

[3]School of Veterinary and Biomedical Sciences, James Cook University — Douglas Campus, Townsville, 4810 Queensland, Australia

ABSTRACT: In order to study the pathogenicity of ranaviruses to a wild European freshwater fish species, pike *Esox lucius* fry were challenged with the following *Ranavirus* isolates: epizootic haematopoietic necrosis virus (EHNV), European sheatfish virus (ESV), European catfish virus (ECV), pike-perch iridovirus (PPIV), New Zealand eel virus (NZeelV) and frog virus 3 (FV3). The fry were infected using bath challenge at 12 and 22°C. Significant mortalities were observed at 12°C for EHNV, ESV, PPIV and NZeelV. Background mortality was too high in the experiments performed at 22°C for any conclusions about viral pathogenicity at this temperature to be drawn. Viruses could be re-isolated from samples from all challenged groups, and their presence in infected tissue was demonstrated using immunohistochemistry. The findings suggest that pike fry are susceptible to EHNV, ESV, PPIV and NZeelV and can be a vector for ECV and FV3. Statistical analysis of the factors associated with positive virus re-isolation showed that the number of fish in the sample influenced the outcome of virus re-isolation. Moreover, the likelihood of positive virus re-isolation significantly differed among the 6 viral isolates. The temperature from where the sample was taken and the number of days after infection were not associated with the probability of a positive virus re-isolation.

KEY WORDS: *Ranavirus* · Pike · Epizootic haematopoietic necrosis virus · European sheatfish virus · European catfish virus · Pike-perch iridovirus · New Zealand eel virus · Frog virus 3

INTRODUCTION

Ranavirus is a genus of the family *Iridoviridae*. Infection with *Ranavirus* is characterised by a systemic disease affecting the haematopoietic organs in fish, amphibians and reptiles. Although its taxonomy is still not fully resolved, the following species within the genus are recognised: epizootic haematopoietic necrosis virus (EHNV), European catfish virus (including European catfish virus [ECV] and European sheatfish virus [ESV]), Bohle iridovirus (BIV), frog virus 3 (FV3 and several other amphibian and reptile viruses) and Santee-Cooper *Ranavirus*, which includes doctor fish virus (DFV) and guppy virus 6 (GV6) (Chinchar et al. 2005).

It is not possible to differentiate the ranaviruses based on their antigenic properties, since they share conserved group-specific antigens (OIE 2006). However, viruses within the genus can be differentiated by the size of the virions. The commonly used method for identification is PCR, followed by either sequencing or restriction endonuclease analysis, although difficulties in differentiating ESV and ECV still exist (Hyatt et al. 2000).

Until recently, the disease epizootic haematopoietic necrosis has only been recognized in Australia; however, similar diseases have caused gross mortalities in Europe in the last 2 decades, with subsequent isolation of ECV and ESV from the affected fish (Ahne et al. 1989, Pozet et al. 1992). This raised concern about the possibility of the introduction and spread of rana-

*Email: abrj@vet.dtu.dk

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Susceptibility of pike-perch *Sander lucioperca* to a panel of ranavirus isolates

Britt Bang Jensen [a,b,*], Riikka Holopainen [c], Hannele Tapiovaara [c], Ellen Ariel [a,1]

[a] Department of Fish, Poultry and Fur Animals, National Veterinary Institute, Technical University of Denmark, Hangøvej 8, 8200 Århus N, Denmark
[b] Department of Large Animal Sciences, Faculty of Life Sciences, University of Copenhagen, Grønnegårdsvej 8, 1870 Frederiksberg C, Denmark
[c] Finnish Food Safety Authority Evira, Veterinary Virology Research Unit, Mustialankatu 3, FI-00790 Helsinki, Finland

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ABSTRACT

The host range of ranaviruses was investigated by challenging pike-perch (*Sander lucioperca*) with the following ranavirus isolates: epizootic haematopoietic necrosis virus (EHNV), European sheatfish virus (ESV), European catfish virus (ECV), pike-perch iridovirus (PPIV), short-finned eel virus (SERV) and frog virus 3 (FV3). Pike-perch fry were bath-challenged at 12 °C and 22 °C at 5 weeks post hatching, and the challenge was repeated with EHNV and PPIV in older fish (15 weeks post hatching) at higher densities. A third batch of fish was subjected to intraperitoneal (i.p.) and cohabitation challenge with EHNV, ESV, ECV and PPIV at 16 °C. Statistically significant mortality was observed in EHNV-challenged fish at both temperatures in the five week old fish. No mortalities were seen in older fish challenged with EHNV and PPIV. High mortalities were registered in i.p.-challenged fish, but not in cohabitated fish. Virus re-isolation was possible from the youngest bath-challenged fish in all challenge trials and the i.p.-challenged fish, but not from older fish or cohabitated fish. Susceptibility of pike-perch to ranaviruses appears to be dependent on age of fish and challenge route. The study shows that pike-perch are susceptible to infection with ranavirus under certain conditions, and it is suggested that this be considered when reviewing the legislation on notifiable diseases.

1. Introduction

In 2006, epizootic haematopoietic necrosis disease (EHN) was included on the list of exotic fish diseases in the European Union (EU) legislation. The criteria for listing a disease as exotic are that the disease i) is not established in the Community and the pathogen is not known to be present and ii) it has potential for either significant economic impact if introduced or detrimental environmental impact on wild aquatic animal species (Anonymous, 2006). EHN is caused by the epizootic haematopoietic necrosis virus (EHNV), which has previously induced high mortalities in redfin perch (*Perca fluviatilis*) and morbidity in rainbow trout (*Oncorhynchus mykiss*) in Australia (Langdon et al., 1986; Langdon et al., 1988). These two species are listed as susceptible to EHN in the current legislation (Anonymous, 2006), and surveillance programmes are in place, targeting EHNV infection in these species.

In European-based challenge studies, EHNV did not appear to be as virulent for European stocks of red-fin perch and rainbow trout as reported for Australian fish stock (Ariel and Bang Jensen, 2009; Ariel et al., 2010). However, other important European fish species can be susceptible to EHNV, as has been demonstrated for black bullhead (*Ameiurus melas*) and pike (*Esox lucius*) (Bang Jensen et al., 2009; Gobbo et al., 2010).

EHNV is a member of the genus *Ranavirus* (family *Iridoviridae*), which contains viral isolates obtained from fish, amphibians and reptiles from all over the world. Most viruses in the genus are genetically very closely related in terms of the major capsid protein gene, and can only be differentiated after amplification by polymerase chain reaction (PCR) and subsequent restriction enzyme analysis (REA) or sequence analysis (Hyatt et al., 2000; Marsh et al., 2002; OIE, 2006; Holopainen et al., 2009).

EHNV has not been isolated in the EU. However, ranaviruses closely related to EHNV have been isolated in outbreaks with high mortalities in farmed sheatfish (*Siluris glanis*; European sheatfish virus, ESV) and farmed or wild black bullhead (European catfish virus, ECV) (Ahne et al., 1989; Bigarré et al., 2008; Pozet et al., 1992). A ranavirus isolate (pike-perch iridovirus, PPIV) has also been isolated from apparently healthy farmed pike-perch (*Sander lucioperca*) (Tapiovaara et al., 1998), another (Rmax) from clinically healthy turbot fry (*Psetta maxima*) and another (CodV) from free-living cod (*Gadus morhua*) with an ulcus-syndrome (Jensen et al., 1979; Ariel et al., 2010). Short-finned eel virus (SERV) was found in non-symptomatic short-finned eels (*Anguilla australis*) imported to Italy from New Zealand (Bang Jensen et al., 2009).

Previous challenge studies have shown that ECV, ESV, PPIV and SERV can be pathogenic to European stocks of pike, and that ECV and frog virus 3 (FV3), the type species of the genus, can infect pike

* Corresponding author at: Current address: National Veterinary Institute, P.O. Box 750 Sentrum, N-0106 Oslo, Norway. Tel.: +47 23216360; fax: +47 23216001.
E-mail addresses: britt-bang.jensen@vetinst.no (B. Bang Jensen), riikka.holopainen@evira.fi (R. Holopainen), hannele.tapiovaara@evira.fi (H. Tapiovaara), ellen.ariel@jcu.edu.au (E. Ariel).
[1] Current address: The School of Veterinary and Biomedical Sciences, Douglas Campus, James Cook University, Queensland 4811, Australia.

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Ranavirus Publications

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Amphibian Commerce and the Threat of Pathogen Pollution

A. Picco

United States Fish and Wildlife Service, Sacramento, California, USA, (email: Angela_Picco@fws.gov)

The global trade of amphibians has the potential to spread diseases into new areas and contribute to amphibian die-offs and declines around the world – a phenomenon known as pathogen pollution. Amphibians are widely traded as pets, food, bait, and for biomedical and research purposes. Recent studies show that ranaviruses, a group of ectothermic vertebrate pathogens, affect a variety of hosts and are common in the global amphibian trade. Studies in North America indicate that pathogen pollution is likely occurring with the translocation of larval tiger salamanders (*Ambystoma tigrinum*) used in the fishing bait industry and the sale of market bullfrogs (*Rana catesbeiana*) for human consumption. Further, strains from bait shops and ranaculture facilities may be more pathogenic than wild strains. What we do not know is the likelihood that ranaviruses are transmitted from trade to amphibians in the wild, how trade is responsible for the spread of diseases into new areas, what effects released pathogens may have on native populations, how pathogen pollution contributes to amphibian declines around the world, and what the most effective approaches are for curbing the spread of ranaviral disease into new areas. Future research on these topics is needed to help address this risk of pathogen pollution to native amphibians and to formulate intervention strategies.

Biosketch: Dr. Angela Picco is a Fish and Wildlife Biologist with the Pacific Southwest Regional Office of the U.S. Fish and Wildlife Service in Sacramento, California. Her current focus is on Endangered Species, specifically regarding the listing of species and the designation of critical habitat for listed species. Prior to working for the U.S. Fish and Wildlife Service, Dr. Picco completed her Ph.D. in Biology at Arizona State University. Her dissertation was on the movement of amphibian pathogens through trade, particularly the trade in tiger salamanders as fishing bait. Angela completed her B.S. in Evolution and Ecology at the University of California, Davis, and worked as a postgraduate researcher at the University of California, Davis, prior to starting graduate school. In addition to her work in the U.S., Angela has conducted amphibian disease research in Costa Rica and conservation genetics work in Australia. She also collaborates with fellow disease ecologists and conservation biologists to better understand the movement of diseases through trade and the effects of amphibian pathogens on populations.

Angela Picco (Ph.D.)

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Short Communication

Pathogen Host Switching in Commercial Trade with Management Recommendations

Angela M. Picco,[1,2] Abraham P. Karam,[1] and James P. Collins[1]

[1]*School of Life Sciences, Arizona State University, Tempe, AZ 85287*
[2]*Present address:* U.S. Fish and Wildlife Service, 2800 Cottage Way, Room W-2605, Sacramento, CA 95825

Abstract: Global wildlife trade exacerbates the spread of nonindigenous species. Pathogens also move with hosts through trade and often are released into naïve populations with unpredictable outcomes. Amphibians are moved commercially for pets, food, bait, and biomedicine, and are an excellent model for studying how wildlife trade relates to pathogen pollution. Ranaviruses are amphibian pathogens associated with annual population die-offs; multiple strains of tiger salamander ranaviruses move through the bait trade in the western United States. Ranaviruses infect amphibians, reptiles, and fish and are of additional concern because they can switch hosts. Tiger salamanders are used as live bait for freshwater fishing and are a potential source for ranaviruses switching hosts from amphibians to fish. We experimentally injected largemouth bass with a bait trade tiger salamander ranavirus. Largemouth bass became infected but exhibited no signs of disease or mortality. Amphibian bait ranaviruses have the potential to switch hosts to infect fish, but fish may act as dead-end hosts or nonsymptomatic carriers, potentially spreading infection as a result of trade.

Keywords: amphibian, bait, fish, ranavirus, tiger salamander, waterdog

Wildlife is traded globally for food, pet, research, education, medicinal, and bait purposes. As wildlife species are moved commercially, individuals often are released into new areas where they can establish and outcompete indigenous species (Kolar and Lodge, 2001). Additionally, the wildlife trade poses a risk of moving pathogens into naïve populations through the release of nonindigenous species, resulting in the spread of infectious diseases (Daszak et al., 2000; Smith et al., 2009). As pathogens move through trade, they may threaten human health, livestock, indigenous wildlife populations, and ecosystem functions (Karesh et al., 2005).

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Correspondence to: Angela M. Picco, e-mail: Angela_Picco@fws.gov

The anthropogenic movement of pathogens through trade is a form of pathogen pollution where pathogens move out of their geographical or ecological range as a result of human actions (Cunningham et al., 2003). Amphibians are moved commercially and are an excellent model for studying how trade in wildlife relates to pathogen pollution. Three examples of pathogen movement in amphibian commerce include the global movement of the chytrid fungus *Batrachochytrium dendrobatidis* (Bd), the movement of ranaviruses through trade in bullfrogs for food (Schloegel et al., 2009), and movement of ranaviruses and Bd through the tiger salamander bait trade in the western United States (Picco and Collins, 2008). It is not known how often these pathogens are released into new

Contributed Paper

Amphibian Commerce as a Likely Source of Pathogen Pollution

ANGELA M. PICCO* AND JAMES P. COLLINS

School of Life Sciences, Arizona State University, Tempe, AZ 85287-4601, U.S.A.

Abstract: *The commercial trade of wildlife occurs on a global scale. In addition to removing animals from their native populations, this trade may lead to the release and subsequent introduction of nonindigenous species and the pathogens they carry. Emerging infectious diseases, such as chytridiomycosis caused by the chytrid fungus* Batrachochytrium dendrobatidis *(Bd), and ranaviral disease have spread with global trade in amphibians and are linked to amphibian declines and die-offs worldwide, which suggests that the commercial trade in amphibians may be a source of pathogen pollution. We screened tiger salamanders involved in the bait trade in the western United States for both ranaviruses and Bd with polymerase chain reaction and used oral reports from bait shops and ranavirus DNA sequences from infected bait salamanders to determine how these animals and their pathogens are moved geographically by commerce. In addition, we conducted 2 surveys of anglers to determine how often tiger salamanders are used as bait and how often they are released into fishing waters by anglers, and organized bait-shop surveys to determine whether tiger salamanders are released back into the wild after being housed in bait shops. Ranaviruses were detected in the tiger salamander bait trade in Arizona, Colorado, and New Mexico, and Bd was detected in Arizona bait shops. Ranaviruses were spread geographically through the bait trade. All tiger salamanders in the bait trade were collected from the wild, and in general they moved east to west and north to south, bringing with them their multiple ranavirus strains. Finally, 26–73% of anglers used tiger salamanders as fishing bait, 26–67% of anglers released tiger salamanders bought as bait into fishing waters, and 4% of bait shops released tiger salamanders back into the wild after they were housed in shops with infected animals. The tiger salamander bait trade in the western United States is a useful model for understanding the consequences of the unregulated anthropogenic movement of amphibians and their pathogens through trade.*

Keywords: *Ambystoma tigrinum*, *Batrachochytrium dendrobatidis*, fishing-bait trade, ranavirus, tiger salamander, waterdog

El Comercio de Anfibios como una Probable Fuente de Contaminación por Patógenos

Resumen: *El comercio de vida silvestre ocurre a escala global. Adicionalmente a la remoción de animales de sus poblaciones nativas, este comercio puede llevar a la liberación y subsecuente introducción de especies no nativas y los patógenos que portan. Enfermedades infecciosas emergentes, como la quitridiomicosis causada por el hongo* Batrachochytrium dendrobatidis *(Bd) y enfermedades ranavirales, se han dispersado con el comercio global de anfibios y están ligados a las declinaciones en todo el mundo, lo cual sugiere que el comercio de anfibios puede ser una fuente de contaminación por patógenos. Muestreamos individuos de* Ambystoma tigrinum *involucrados en el comercio de carnada en el oeste de Estados Unidos para buscar ranavirus y Bd con reacción en cadena de polimerasa y utilizamos reportes orales de tiendas de carnada y secuencias de ADN de ranavirus extraídas de salamandras infectadas para determinar cómo son movidos geográficamente por el comercio estos animales y sus patógenos. Adicionalmente, aplicamos dos encuestas a pescadores para determinar la frecuencia con que utilizan salamandras como carnada y la frecuencia con que son liberadas y organizamos muestreos en tiendas de carnada para determinar si las salamandras son liberadas después de estar en las tiendas. Detectamos ranavirus en el comercio de salamandras en*

*email angela.picco@asu.edu

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