Vol. 103: 185–189, 2013
doi: 10.3354/dao02556

DISEASES OF AQUATIC ORGANISMS
Dis Aquat Org

Published April 11

Ranavirus infection in a group of wild-caught Lake Urmia newts *Neurergus crocatus* imported from Iraq into Germany

Anke C. Stöhr[1], Jürgen Fleck[2], Frank Mutschmann[3], Rachel E. Marschang[1,*]

[1]Fachgebiet für Umwelt und Tierhygiene, University of Hohenheim, Garbenstr. 30, 70599 Stuttgart, Germany
[2]Dr. Appia Str. 13, 63452 Hanau, Germany
[3]Exomed – Institut für veterinärmedizinische Betreuung niederer Wirbeltiere und Exoten, Erich-Kurz-Str. 7, 10319 Berlin, Germany

ABSTRACT: High mortality, in association with anorexia and skin ulcerations, occurred in a group of wild-caught Lake Urmia newts *Neurergus crocatus*, imported from Iraq in 2011. Predominant findings in the pathological examinations consisted of systemic hemorrhages and ulcerative dermatitis. Ranavirus DNA was detected via PCR in 2 of 3 dead animals, and a part of the major capsid protein (MCP) gene was sequenced. The analyzed portion of the MCP gene was 99% identical to the corresponding portion of the frog virus 3 genome. This is the first description of a ranavirus in Lake Urmia newts and in wild-caught amphibians from Iraq, as well as the first description of ranavirus infection in a urodele from the Middle East.

KEY WORDS: Iridovirus · Frog virus 3 · Urodelan · Amphibian · Major capsid protein · MCP · Middle East

INTRODUCTION

Ranaviruses (family *Iridoviridae*) are large (150 to 170 nm), icosahedral, double-stranded DNA viruses that infect ectothermic vertebrates (fish, amphibians and reptiles). They have been identified as a causative agent of multiple mass-mortality events in wild and captive amphibian populations on different continents (reviewed by Miller et al. 2011) and are considered emerging pathogens (Daszak et al. 1999, Storfer et al. 2007, Gray et al. 2009a). Although ranavirus infections have been detected in many different amphibians, the majority of reports have been in anurans, especially in the family *Ranidae* (Miller et al. 2011). In urodelans, fatal ranavirus-associated die-offs have been reported in North America in *Ambystomatidae* (e.g. Jancovich et al. 1997, Bollinger et al. 1999, Docherty et al. 2003), while subclinical infections have been detected in *Plethodontidae* (Gray et al. 2009b, Davidson & Chambers 2011) and *Salamandridae* (Duffus et al. 2008). Several cases of ranaviral disease in wild *Salamandridae* have been reported in Europe (Alves de Matos et al. 2008, Balseiro et al. 2010, Kik et al. 2011). One case of ranavirus-associated mass mortality in red tailed knobby newts *Tylototriton kweichowensis* has been described (Pasmans et al. 2008). These animals were probably imported from China. In Asian urodelans, ranavirus has been detected in farmed Chinese giant salamanders *Andrias davidianus* (Geng et al. 2011) and Japanese clouded salamanders *Hynobius nebulosus* (Une et al. 2009). In both cases, the infections caused disease with high morbidity and mortality. The only previous detection of a ranavirus in amphibians in the Middle East was in a green toad (*Pseudepidalea viridis*) tadpole in Israel (Miller et al. 2011, D. Milstein pers. comm.).

According to the latest taxonomic classification, the genus *Neurergus* (Caudata: *Salamandridae*) contains 4 species with 2 subspecies: *N. crocatus, N. kaiseri, N. derjugini* with the subspecies *N. d. derjugini* and

*Corresponding author.
Email: rachel.marschang@googlemail.com

N. d. microspilotus, and *N. strauchii* with the subspecies *N. s. strauchii* and *N. s. barani* (Fleck 2010, 2011, Schneider & Schneider 2011). *N. crocatus* (Lake Urmia newt) is listed as vulnerable on the IUCN Red List because its range is less than 2000 km^2 and is severely fragmented, and the extent and quality of its habitat is in decline (Papenfuss et al. 2009). At present, *N. crocatus* can be found only in insular habitats in Turkey and Iraq, and are no longer found at their terra typica at Lake Urmia in Iran (Schneider & Schneider 2011). Within their known range, Lake Urmia newts inhabit mountain areas between 500 and 1500 m altitude (Schmidtler & Schmidtler 1975). In spring, breeding occurs in small mountain streams. At the end of the breeding season in June, the animals return to their terrestrial habitats (Steinfartz & Schultschick 1997).

MATERIALS AND METHODS

History

A total of 11 adult Lake Urmia newts were collected near a brook (Kany Zark) from a wild population in Aqrah (Iraq) on 22 April 2011 for scientific purposes. All animals were in breeding condition and appeared healthy. Animals were transported to Germany by air cargo in plastic containers with damp moss, which was obtained at the same location as the newts. After transfer to Germany, the animals were kept by 2 owners under species-appropriate housing conditions, in groups of 5 and 6 animals, in aquaria and were fed with earthworms. One group (2 males and 3 females) was kept at temperatures ranging between 10 and 15°C at night and rising to 20°C during the day, the other group (3 males and 3 females) was kept in an identical aquarium but at a constant temperature of 15°C. The newts did not have any contact with other animals. All animals appeared healthy and ate heartily following housing in the aquaria in Germany.

On May 2, 1 female laid 30 eggs, which were transferred to a separate aquarium und developed normally. One week later, several of the wild-caught *Neurergus crocatus* stopped eating and the first signs of disease were noticed 11 d later. Some animals showed small pustules, followed by ulcerations at the end of the tail or the phalanges. All animals lost weight, some showed no other signs of disease.

Initial treatment consisted of housing half of the diseased animals on moist moss, and local therapy of the affected skin with enrofloxacine (Baytril® 2.5% injectable solution every 2 d) or iodine solution (Betaisodona®), but no improvement was observed. Bacteriological testing was not conducted. During a period of 3 wk, all imported animals developed disease. Ten animals died but 1 male recovered and did not show any clinical signs 1 yr later at the time of submission of this manuscript. Ten Lake Urmia newts, caught at the same location but 1 yr earlier in April 2010, remain apparently healthy.

Pathological examination

Two dead (1 male, 1 female) and 1 moribund animal (male) were submitted for pathological examination. All examined animals were of the same origin, but had been kept by different owners since their import from Iraq. The live male died 2 d after submission. This animal was lethargic until death. All 3 animals were in poor body condition. The tissue around the mouth and throat appeared swollen; skin ulcerations and necrosis were found on fingertips and toes (Fig. 1). Prominent capillaries were observed in the skin of the ventral tail, abdomen, arms and legs, with a tendency to hemorrhage and bleed during handling. Systemic hemorrhages were also observed. For further examination, samples from various organs (skin, lung, liver, kidney, pancreas, spleen, gonads, intestine and central nervous system) were preserved in 10% neutral buffered formalin and processed routinely. The samples were embedded in paraffin, sectioned at 2 µm and stained with hematoxylin and eosin (HE) as well as with May-Gruenwald-Giemsa stain and acid-fast staining (Ziehl-Neelson). Histological examination showed granulomatous hepatitis characterized by multifocal masses of eosinophilic granulocytes. A paravasal

Fig. 1. Ranavirus-infected *Neurergus crocatus*. Note the swollen tissue around the mouth and throat, skin ulcerations, discoloration, hyperemia and hemorrhages

accumulation of lymphocytes was also observed. The intestine showed hemorrhagic inflammation. Ulcerative dermatitis was also present. In 1 male, systemic hemorrhage was noted with necrotic foci detected in the liver (Fig. 2), spleen, and kidney.

Virological examination

Samples from the 3 adult Lake Urmia newts were submitted for virological testing. Skin and mixed organ samples (liver and kidney) were taken from each animal and submitted in 3 ml Dulbecco's modified Eagle's medium (DMEM) (Biochrom) supplemented with antibiotics. The samples were sonicated, centrifuged at low speed (2000 × *g*, 10 min), inoculated onto Iguana heart cells (IgH-2, ATCC: CCL-108) and incubated at 28°C. IgH-2 have been used previously for the isolation of iridoviruses from both reptiles and amphibians (Alves de Matos et al. 2008, R. E. Marschang unpubl. data).

Tissue cultures were observed twice a week for cytopathic effects (CPE). Cultures showing no CPE after 2 wk of incubation were frozen to −20°C, thawed and reinoculated for a second passage.

DNA was extracted from the supernatant of the original sample using a DNeasy Kit (Qiagen) and polymerase chain reaction (PCR) for the detection of ranaviruses was performed according to Mao et al. (1997) and Marschang et al. (1999) using the sense primer Ol T1 (5′-GAC TTG GCC ACT TAT GAC-3′) and the antisense primer Ol T2R (5′-GTC TCT GGA GAA GAA GAA T-3′), targeting a 500 bp portion of the major capsid protein (MCP) gene.

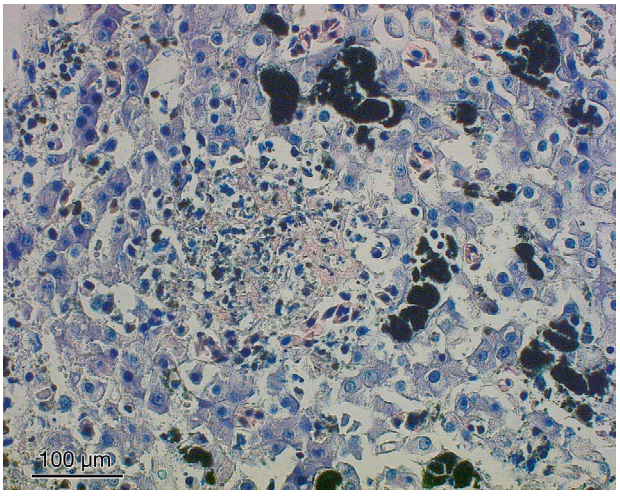

Fig. 2. *Neurergus crocatus.* Ranavirus-infected liver. Note the necrotic focus surrounded by erythrocytes. May-Gruenwald-Giemsa stain

PCR products from each positive animal were agarose gel purified (peqGOLD gel extraction kit, Peqlab Biotechnologie) and submitted to MWG Biotech for sequencing from both directions. The sequences were edited and compared using the STADEN Package version 2003.0 Pregap4 and Gap4 programs (Bonfield et al. 1995). The sequences were then compared to those online in GenBank (National Center for Biotechnology Information; www.ncbi.nih.gov) using BLASTN.

RESULTS AND DISCUSSION

Skin and tissues from 2 animals returned positive for ranavirus by PCR; the MCP gene sequence fragments (494 nucleotides) from the 2 Lake Urmia newts were 100% identical to each other and showed 99% identity to the corresponding sequence of frog virus 3 (FV3) (AY548484, Tan et al. 2004), the type species of the genus *Ranavirus*. No ranaviruses were isolated in cell culture.

This is the first description of a ranavirus infection in Lake Urmia newts and the first detection of ranavirus in wild-caught amphibians from Iraq, as well as the first description of ranavirus infection in a middle eastern urodele. In urodelans, various clinical and pathological findings (e.g. anorexia, necrotizing, vesicular and ulcerative dermatitis, hemorrhages of the skin, subcutaneous and intramuscular edema, gastrointestinal ulceration and hemorrhages, necrosis of hepatic, splenic, renal, lymphoid and hematopoietic tissues) have been reported in association with ranaviral disease; acute death with no prior clinical signs has also been described (Bollinger et al. 1999, Docherty et al. 2003, Pasmans et al. 2008). The clinical and pathological signs observed in the infected newts were very similar to those described in the literature for urodelans.

Several sequences of MCP genes from urodelan ranaviruses have been published in GenBank. Their similarity to our ranavirus varied between 97 and 99% (Table 1). Interestingly, the analyzed part of the MCP gene showed 100% identity to an isolate from a Hermann's tortoise *Testudo hermanni* (AF114154.1| AF114154; Marschang et al. 1999). Since the MCP gene is highly conserved and therefore not a suitable target for differentiating between ranavirus strains (Mao et al. 1997, Schock et al. 2008, Chinchar et al. 2009), more sequencing work would be necessary to understand the relationship of this virus to other ranaviruses.

Table 1. Nucleotide sequence similarity (%) of partial major capsid protein gene (494 nucleotides) from a ranavirus detected in *Neurergus crocatus* from Iraq in comparison to urodelan ranaviruses published in GenBank

Virus	Nucleotide sequence similarity (%)	Host species (scientific name)	Isolated in	Accession no.	Source
Chinese giant salamander virus	99	*Andrias davidianus*	China	JN651174	Geng et al. (2011)
Common midwife toad virus	99	*Ichthyosaura alpestris cyreni*	Spain	FM213466	Balseiro et al. (2010)
		Lissotriton vulgaris	Netherlands		Kik et al. (2011)
Hynobius nebulosus virus	98	*Hynobius nebulosus*	Japan	AB500273	
Tylototriton kweichowensis ranavirus	98	*Tylototriton kweichowensis*	Belgium (imported from China)	DQ192530	Pasmans et al. (2008)
Ambystoma tigrinum stebbensi virus	97	*Ambystoma* spp.	North America	AY150217	Jancovich et al. (2003)

Susceptibility to ranavirus infections varies among species and amphibian development stage (Haislip et al. 2011, Warne et al. 2011). Species which develop rapidly as larvae and have limited range sizes are suggested to be more susceptible to infections (Hoverman et al. 2011). Pearman & Garner (2005) demonstrated that genetically isolated *Rana latastei* populations showed increased mortality rates because of ranaviral infections. Uncommon amphibian species may therefore be more susceptible due to a reduced level of genetic diversity and the lack of a co-evolutionary history with the pathogen (Hoverman et al. 2011). It is possible that the limited and fragmented range of Lake Urmia newts could lead to a higher susceptibility for ranavirus infection and disease.

The occurrence of ranavirus-associated mass-mortality events seems to be caused by an interaction of various factors, including virus strain, host species, suppressed and naïve host immunity, anthropogenic stressors, and novel strain introduction (Gray et al. 2009a). Some species of salamander can be subclinically infected (Brunner et al. 2004). Environmental changes impact on wildlife health by influencing the immunocompetence of organisms at various levels (reviewed by Acevedo-Whitehouse & Duffus 2009). Stress-induced activation of the hypothalamus-pituitary-interrenal axis seems to mediate immune responses to ranavirus infection (Warne et al. 2011). Breeding season is considered a natural stressor for adult amphibians and is also likely a period of high viral transmission because of intensive contact among individuals (Gray et al. 2009a). The imported animals in the current study may have been immunocompromised due to the stress of capture and transport, which could have affected the clinical outcome of the ranavirus infection. Breeding (egg laying) in 1 of the females may also have influenced the clinical disease progression. Since the animals did not have contact with any other amphibians after capture, it is most likely that they were infected in their natural habitat in Iraq. At the time of capture, however, all of the animals were considered clinically healthy, and disease was only observed after transport to Germany. It remains unclear whether the disease outbreak was caused by reactivation of a chronic infection, or if the animals were newly infected in 2011.

The single animal of the 11 originally imported that survived the disease outbreak may have cleared the infection or may remain subclinically infected. Virus transmission from an inapparently infected carrier has been described even after months of quarantine in a group of *Tylototriton kweichowensis* (Pasmans et al. 2008). Therefore, the surviving animal should not be introduced into a new group of individuals. Co-housing with another species should also be avoided. The infection statuses of the progeny newts are unknown, and these animals should also remain under observation.

This case report underlines the widespread occurrence of ranaviruses, the risk of human-enhanced spread, as well as the need to test new animals for potential infections especially when they are wild-caught. The wild populations in Iraq should be surveyed to understand the prevalence of ranavirus infections in that country and to determine threats to susceptible species.

Acknowledgements. The authors thank Dr. J. Shukri Saead from the Ministry of Agriculture & Water Resources in Arbil, Iraq, for help with the collection and export permits for the described animals.

LITERATURE CITED

Acevedo-Whitehouse K, Duffus ALJ (2009) Effects of environmental change on wildlife health. Philos Trans R Soc Lond B Biol Sci 364:3429–3438

Alves de Matos AP, Caeiro MF, Marschang RE, Papp T, Soares C, Marçal MR, Carretero MA (2008) Adaptation of ranaviruses from Peneda-Gerês National Park (Portu-

gal) to cell cultures and their characterization. Microsc Microanal 14:139–140

Balseiro A, Dalton KP, del Cerro A, Marquez I, Parra F, Prieto JM, Casais R (2010) Outbreak of common midwife toad virus in alpine newts (*Mesotriton alpestris cyreni*) and common midwife toad (*Alytes obstetricans*) in northern Spain: a comparative pathological study of an emerging ranavirus. Vet J 186:256–258

Bollinger TK, Mao J, Schock D, Brigham RM, Chinchar VG (1999) Pathology, isolation, and preliminary molecular characterization of a novel iridovirus from tiger salamanders in Saskatchewan. J Wildl Dis 35:413–429

Bonfield JK, Smith KF, Staden R (1995) A new DNA sequence assembly program. Nucleic Acids Res 23: 4992–4999

Brunner JL, Schock DM, Davidson EW, Collins JP (2004) Intraspecific reservoirs: complex life history and the persistence of a lethal ranavirus. Ecology 85:560–566

Chinchar VG, Hyatt AD, Miyazaki T, Williams T (2009) Family *Iridoviridae*: poor viral relations no longer. Curr Top Microbiol Immunol 328:123–170

Daszak P, Berger L, Cunningham AA, Hyatt AD, Green E, Speare R (1999) Emerging infectious diseases and amphibian population declines. Emerg Infect Dis 5: 735–748

Davidson SRA, Chambers DL (2011) Ranavirus prevalence in amphibian populations of Wise County, Virginia, USA. Herpetol Rev 42:214–215

Docherty DE, Meteyer CU, Wang J, Mao JH, Case ST, Chinchar VG (2003) Diagnostic and molecular evaluation of three iridovirus-associated salamander mortality events. J Wildl Dis 39:556–566

Duffus ALJ, Pauli BD, Wozney K, Brunetti CR, Berrill M (2008) Frog virus 3-like infections in aquatic amphibian communities. J Wildl Dis 44:109–120

Fleck J (2010) Beobachtungen an Bergbachmolchen der Gattung *Neurergus* Cope, 1862 und Fragen zu ihrer taxonomischen Zuordnung nach der Wiederentdeckung der ‚Forma typica' von ‚*Rhithrotriton derjugini* Nesterov, 1916'. Elaphe 18:6–14

Fleck J (2011) Weitere Erkenntnisse zur Verbreitung der Bergbachmolchart *Neurergus crocatus* mit Anmerkungen zum Geschlechtsdimorphismus. Elaphe 19:18–24

Geng Y, Wang KY, Zhou ZY, Li CW and others (2011) First report of a ranavirus associated with morbidity and mortality in farmed Chinese giant salamanders (*Andrias davidianus*). J Comp Pathol 145:95–102

Gray MJ, Miller DL, Hoverman JT (2009a) Ecology and pathology of amphibian ranaviruses. Dis Aquat Org 87: 243–266

Gray M, Miller DL, Hoverman JT (2009b) First report of *Ranavirus* infecting lungless salamanders. Herpetol Rev 40:316–319

Haislip NA, Gray MJ, Hoverman JT, Miller DL (2011) Development and disease: how susceptibility to an emerging pathogen changes through anuran development. PLoS ONE 6:e22307

Hoverman JT, Gray MJ, Haislip NA, Miller DL (2011) Phylogeny, life history, and ecology contribute to differences in amphibian susceptibility to ranaviruses. EcoHealth 8: 301–319

Jancovich JK, Davidson EW, Morado JF, Jacobs BL, Collins JP (1997) Isolation of a lethal virus from the endangered tiger salamander *Ambystoma tigrinum stebbinsi*. Dis Aquat Org 31:161–167

Jancovich JK, Mao JH, Chinchar VG, Wyatt C, and others (2003) Genomic sequence of a ranavirus (family *Iridoviridae*) associated with salamander mortalities in North America. Virology 316:90–103

Kik M, Martel A, Spitzen-van der Sluijs A, Pasmans F, Wohlsein P, Gröne A, Rijks JM (2011) Ranavirus-associated mass mortality in wild amphibians, The Netherlands, 2010: A first report. Vet J 190:284–286

Mao J, Hedrick RP, Chinchar VG (1997) Molecular characterization, sequence analysis, and taxonomic position of newly isolated fish iridoviruses. Virology 229:212–220

Marschang RE, Becher P, Posthaus H, Wild P and others (1999) Isolation and characterization of an iridovirus from Hermann's tortoises (*Testudo hermanni*). Arch Virol 144: 1909–1922

Miller D, Gray M, Storfer A (2011) Ecopathology of ranaviruses infecting amphibians. Viruses 3:2351–2373

Papenfuss T, Sparreboom M, Ugurtas I, Rastegar-Pouyani N and others (2009) *Neurergus crocatus*. In: IUCN 2011. IUCN Red List of Threatened Species, Version 2011.2. www.iucnredlist.org (accessed 24 April 2012)

Pasmans F, Blahak S, Martel A, Pantchev N, Zwart P (2008) Ranavirus-associated mass mortality in imported red tailed knobby newts (*Tylototriton kweichowensis*): a case report. Vet J 176:257–259

Pearman PB, Garner TWJ (2005) Susceptibility of Italian agile frog populations to an emerging strain of *Ranavirus* parallels population genetic diversity. Ecol Lett 8:401–408

Schmidtler JJ, Schmidtler JF (1975) Untersuchungen an westpersischen Bergbachmolchen der Gattung *Neurergus* (Caudata, Salamandridae). Salamandra (Frankf) 11:84–98 (in German with English Abstract)

Schneider C, Schneider W (2011) Die Bergbachmolche der Gattung *Neurergus* im Irak (Caudata: Salamandridae) [The Kurdistan newts of the genus *Neurergus* in Iraq (Caudata: Salamandridae)]. Herpetozoa (Wien) 23:3–20 (in German with English Abstract)

Schock DM, Bollinger TK, Chinchar VG, Jancovich JK, Collins JP (2008) Experimental evidence that amphibian ranaviruses are multi-host pathogens. Copeia 2008: 133–143

Steinfartz S, Schultschick G (1997) Die Gattung *Neurergus*. Reptilia 6:43–48

Storfer A, Alfaro ME, Ridenhour BJ, Jancovich JK, Mech SG, Parris MJ, Collins JP (2007) Phylogenetic concordance analysis shows an emerging pathogen is novel and endemic. Ecol Lett 10:1075–1083

Tan WGH, Barkman TJ, Chinchar VG, Essani K (2004) Comparative genomic analyses of frog virus 3, type species of the genus *Ranavirus* (family *Iridoviridae*). Virology 323: 70–84

Une Y, Nakajinma K, Taharaguchi S, Ogihara K, Murakami M (2009) Ranavirus infection outbreak in the salamander (*Hynobius nebulosus*) in Japan. J Comp Pathol 141:310

Warne RW, Crespi EJ, Brunner JL (2011) Escape from the pond: stress and developmental responses to ranavirus infection in wood frog tadpoles. Funct Ecol 25:139–146

Editorial responsibility: Alex Hyatt, Geelong, Victoria, Australia

Submitted: May 7, 2012; Accepted: November 5, 2012
Proofs received from author(s): March 28, 2013