DOI: 10.7589/2015-05-113 *Journal of Wildlife Diseases*, 52(2), 2016, pp. 242–252

RANAVIRUS CAUSES MASS DIE-OFFS OF ALPINE AMPHIBIANS IN THE SOUTHWESTERN ALPS, FRANCE

Claude Miaud,[1,6] Françoise Pozet,[2] Nadine Curt Grand Gaudin,[3] An Martel,[4] Frank Pasmans,[4] and Sophie Labrut[5]

[1] Unité Mixte de Recherche, 5175 Centre d'Ecologie Fonctionnelle et Evolutive, Ecole Pratique des Hautes Etudes, Biogéographie et Ecologie des Vertébrés, 34293 Montpellier, France
[2] LDA39, 59 rue Vieil Hôpital, 39802 Poligny, France
[3] Unité Mixte de Recherche, 5553 Université Savoie Mont Blanc, Laboratoire d'Ecologie Alpine, 73376 Le Bourget du Lac, France
[4] Department of Pathology, Bacteriology and Avian Diseases, Faculty of Veterinary Medicine, Ghent University, 9820 Merelbeke, Belgium
[5] Université Nantes Angers Le Mans, Oniris, Plateforme d'Analyse et de Service en Anatomie Pathologique, Nantes, F-44307, France
[6] Corresponding author (email: claude.miaud@cefe.cnrs.fr)

ABSTRACT: Pathogenic fungi and viruses cause mortality outbreaks in wild amphibians worldwide. In the summer of 2012, dead tadpoles and adults of the European common frog *Rana temporaria* were reported in alpine lakes in the southwestern Alps (Mercantour National Park, France). A preliminary investigation using molecular diagnostic techniques identified a *Ranavirus* as the potential pathogenic agent. Three mortality events were recorded in the park, and samples were collected. The amphibian chytrid fungus *Batrachochytrium dendrobatidis* was not detected in any of the dead adult and juvenile frogs sampled (n=16) whereas all specimens were positive for a *Ranavirus*. The genome sequence of this *Ranavirus* was identical to previously published sequences of the common midwife toad virus (CMTV), a *Ranavirus* that has been associated with amphibian mortalities throughout Europe. We cultured virus from the organs of the dead common frogs and infecting adult male common frogs collected in another alpine region where no frog mortality had been observed. The experimentally infected frogs suffered 100% mortality (n=10). The alpine die-off is the first CMTV outbreak associated with mass mortality in wild amphibians in France. We describe the lesions observed and summarize amphibian populations affected by *Ranaviruses* in Europe. In addition, we discuss the ecologic specificities of mountain amphibians that may contribute to increasing their risk of exposure to and transmission of *Ranaviruses*.

Key words: Altitude, amphibian disease, *Batrachochytrium dendrobatidis*, experimental infection, *Rana temporaria*, ranavirus.

INTRODUCTION

Infectious diseases such as chytridiomycosis and diseases caused by *Ranavirus* infection—listed by the World Organization for Animal Health (Schloegel et al. 2010)—have cause outbreaks of high mortality in wild amphibians (Wake and Vredenburg 2008; Miller et al. 2011; Martel et al. 2013). *Ranaviruses* (Iridoviridae: Ranavirus) are large, double-stranded DNA viruses that are pathogens of ectothermic vertebrates such as fish, reptiles, and amphibians (Gray and Chinchar 2015).

Ranaviruses have been associated with amphibian mortality events in wilderness areas of North America (Bollinger et al. 1999; Docherty et al. 2003), South America (Fox et al. 2006; Stark et al. 2014), and Japan (Une et al. 2009).

In Europe, outbreaks of *Ranavirus* disease have been recorded in native amphibian populations in the UK (Cunningham et al. 1996; Teacher et al. 2009), Spain (Balseiro et al. 2009; Price et al. 2014), Portugal (Alves de Matos et al. 2008), Denmark (Ariel et al. 2009), and the Netherlands (Kik et al. 2011). In Belgium, *Ranavirus* infection was found in tadpoles of a population of the introduced American bullfrog *Lithobates catesbeianus*, without observed mortality (Sharifian-Fard et al. 2011), and, in Germany, one live water frog (*Pelophylax* kl. *esculentus*) caught in the field was found to be infected (Stöhr et al. 2013). In addition, captive red-tailed knobby newts (*Tylototriton kweichowensis*) imported by a Belgian pet shop were diagnosed as *Ranavirus*-positive and suffered mass mortality (Pasmans et al. 2008). In Europe, the *Ranavirus*

TABLE 1. Field observation and sampling of common frog (*Rana temporaria*) mortality in the southwestern Alps (France). Two cutaneous swabs were collected per individual, one for chytrid (*Batrachochytrium dendrobatidis*) and the other for *Ranavirus* detection.

Location	Latitude	Longitude	Altitude (m)	Field observation	Field sampling	Swabs and tissue collection
Lake des Prals	44°4′55″N	7°22′30″E	2,278	Dead and dying adults and juveniles	2 adults, 3 juveniles	10 cutaneous swabs; liver, kidney, brain in 2 adults
Lake Verdet	44°19′55″N	6°46′32″E	2,520	Dead and dying adults and juveniles	10 adults, 5 juveniles	30 cutaneous swabs; liver in 10 adults
Lake des Merveilles	44°3′58″N	7°26′29″E	2,290	Hundreds of dead tadpoles (some alive)	None	None
				A dead adult desiccated on a stone	1 adult	2 cutaneous swabs; liver, brain in 1 adult

most responsible for mass mortality is the common midwife toad virus (CMTV), which appears to be causing major loss of amphibian diversity (Kik et al. 2011; Price et al. 2014).

We describe the first mass mortality event associated with *Ranavirus* infection in wild amphibians in France. To establish a causal link between the clinical signs and the presence of a *Ranavirus*, experimental infection was carried out. Macroscopic and microscopic examinations of the internal organs of adult common frogs (*Rana temporaria*) were conducted to describe lesions and compare them to those found in the systemic hemorrhagic form of *Ranavirus* disease in adult amphibians.

Ranaviral diseases can be multifactorial; comparing the outbreak events in Europe is the first step toward understanding the causal factors (e.g., Gray and Chinchar 2015). Because a number of these mortality events have occurred in alpine amphibians (e.g., in Spain, Portugal, and France), we also discuss the demographic and ecologic characteristics that may contribute to the risk of exposure to and transmission of *Ranaviruses* in these populations.

MATERIALS AND METHODS

In the summer of 2012, hundreds of dead and dying common frogs, adults, juveniles, and tadpoles were observed in three lakes within the Mercantour National Park (southwestern Alps, France; Table 1). We collected 21 dead specimens (adults and juveniles, no tadpoles) from these three lakes (Table 1) and swabbed each 10–20 times with two swabs (M01-MW100, Kitvia, Labarthe Inard, Gironde, France) across the ventral skin, thighs, and abdomen (the pelvic patch) and the interdigital webbing of the hind limbs. A sample of liver, kidney, and/or brain was collected from 13 adults (Table 1).

We screened for the chytrid fungus, *Batrachochytrium dendrobatidis* (Bd), in one of the two skin swabs using real-time PCR (Boyle et al. 2004; Hyatt et al. 2007). Each sample was tested in two replicates. Reactions yielding 0.1 or more genomic equivalents (untransformed value) in both replicates were considered Bd-positive, allowing individuals to be assigned as either "infected" or "not infected" with Bd.

The presence of a *Ranavirus* was assessed in the second skin swab and in small pieces (1–2 mm^3) of dead adult frog organs using a similar extraction protocol as used for Bd detection. The PCR amplifications were performed with the supernatants using PCR Master Mix (Promega, Madison, Wisconsin, USA). A first PCR run was performed with primers 1 and 2 according to Mao et al. (1997), and 2 μL of this run were used as template for a nested PCR with primers 5′nMCP (5′-GCAGCAGTTTTCGGTCGGCG-3′) and 3′nMCP (5′-CGCTTGGCCTCTGGCATGGT-3′) designed in our laboratory. Thermal cycling parameters were 94 C for 5 min, followed by 40 cycles (94 C for 30 s, 59 C for 15 s, 72 C for 15 s). *Ranavirus* DNA was used as a positive control.

Genome sequencing of the 530-base-pair PCR fragment obtained with primers (Mao et al. 1997) was performed using the BigDye Terminator Cycle Sequencing kit (PE Biosystems, Thermo Fisher Scientific, Bleiswijk, the Netherlands) on an ABI Prism 3100 (Applied Biosystems, Foster City, California, USA). The electrophoregram was exported and converted to Kodon (Applied Maths, Sint Martens Latem, Belgium), and the

sequences were compared with the BLAST program in GenBank.

For the experimental infection, 25 adult male common frogs were caught at a breeding site in the northwestern Alps (45°41′34″N, 6°40′10″E; 1,832 m) on 28 May 2013. No amphibian mortality had been reported in this region. The frogs were transferred to the laboratory, where they were housed five per tank (40×30×23 cm). The water was changed every 2–3 d, and the ambient temperature was constant at 18 C.

A pool of organs (liver, kidney, and spleen) from 10 common frogs collected at Lake Verdet (Table 1) was used for virus culture. The cell cultures were established on Epithelioma papulosum cyprini (EPC) cells at 20 C and bluegill fibroblast, *Cyprinus carpio* brain (CCB), and rainbow trout gonad (RTG_2) cells at 14 C (Wolf and Quimby 1962). A supernatant of homogenized organs diluted at 1:10 and 1:100 was inoculated into the cell cultures, maintained with MEM (Glasgow medium, Sigma Aldrich, St. Quentin Fallavier, France) supplemented with 10% fetal bovine serum. Viral cytopathic effect was observed daily the following week. The viral culture supernatant was analyzed by the previously described *Ranavirus* DNA detection. Three plates of EPC (75 cm^3) were inoculated with 1 mL of supernatant. After 4 d of incubation, when cytopathic effect was observed on 80% of the EPC cells, the virus was frozen at –80 C, and then thawed at room temperature and the viral culture was centrifuged at 3,000 × G. The supernatant was stored in micro-tubes at –80 C. The viral titration used was the 50% tissue-culture infectious dose ($TCID_{50}$). The viral supernatant (diluted with MEM supplemented with 10% FBS from 10^{-3} to 10^{-10}, 10 replicates per concentration) was injected into 80 cups of EPC. After 10 d, the destroyed EPC cups were counted after fixation with formalin solution 1:10 for 1 h and staining with crystal violet (6.5 g crystal violet in 500 mL 96% ethanol and 2 L water for 1 h). The titration was estimated using Reed and Muench's formulae (Reed and Muench 1938). The titration value was 7.4×10^5 $TCID_{50}$/mL.

The experimental frogs were inoculated with the cultured virus by intraperitoneal injection (0.5 mL) as follows: five frogs were injected with an undiluted viral suspension (7.4×10^5 $TCID_{50}$/mL), five frogs were injected with a 10^{-2} viral dilution (7.4×10^5 $TCID_{50}$/mL), five frogs were injected with virus-free MEM, and 10 frogs were used as controls (no injection). Health of the frogs was checked twice a day. The frogs were manipulated by hand to verify normal body movements such as escape behavior. If a frog died, its internal organs were collected and dissected (see next section). The frogs were observed for 21 d and euthanized by prolonged immersion in anesthetic (an overdose of 10 mL/L of phenoxyethanol; Gentz 2007). Samples of internal organs (liver, kidney, and spleen) were then collected in the dead and five control frogs and tested for virus using the EPC cell culture and molecular techniques described above. Tissue samples from all major organs were fixed in 10% neutral-buffered formalin, embedded in paraffin wax, and routinely processed for histology.

RESULTS

We did not detect Bd in any of the dead frog samples from the three lakes in Mercantour National Park. However, all the samples showed a clear amplification signal of *Ranavirus* DNA. Specifically, these positive detections occurred in three samples (swab, liver, and brain) of the dead common frog collected at Lake des Merveilles (n=1), in four samples (swab, liver, kidney, and brain) from frogs collected at Lake des Prals (n=2) and in the skin swabs of juveniles (n=5) and two samples (swab and liver) of adults (n=10) collected at Lake Verdet (Table 1).

The cultures for *Ranavirus* were positive only on EPC cells (negative on BF, RTG2, and CCB). Sequencing of the PCR amplicons confirmed 100% homology between the isolated virus and the virus found in dead frogs collected in the field. The five DNA sequences (514 pb) obtained from *Ranavirus* DNA amplified from the five liver samples of dead common frogs from Lake Verdet were identical. The sequence was 100% identical to the common midwife toad *Ranavirus* (GenBank accession number JQ231222) isolated from a common midwife toad (*Alytes obstetricans*; Balseiro et al. 2009) and an alpine newt *Ichthyosaura alpestris* in Spain (Mavian et al. 2012).

There was no mortality among control frogs (not injected or injected solely with MEM) during the 21-d experiment. Frogs injected with undiluted viral suspension and with 10^{-2} viral dilution suffered similar mortality, with the first death observed at day 10 and 100% mortality by days 14–15 (Fig. 1). These mortalities appeared suddenly and without external clinical signs. Some slight external lesions were observed on dead specimens, such as

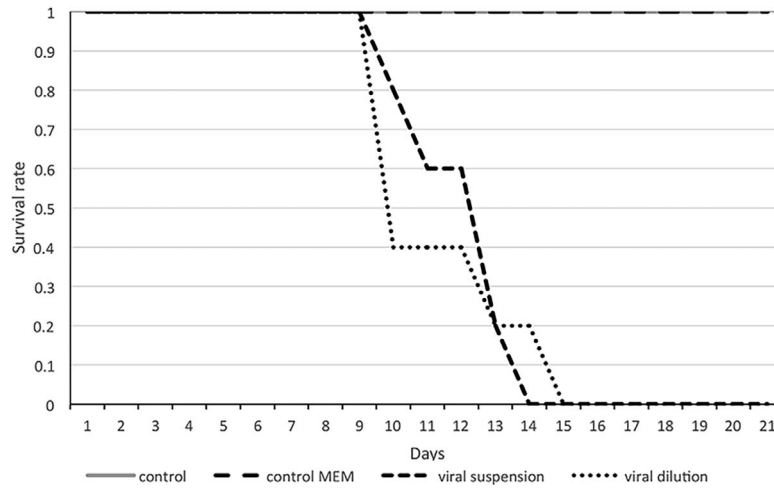

FIGURE 1. Survival curves for adult European common frogs (*Rana temporaria*) exposed to four treatments: control=frogs (n=10) not injected; MEM alone=frogs (n=5) injected with virus-free medium (MEM); viral suspension=frogs (n=5) injected with undiluted viral suspension; viral dilution=frogs (n=5) injected with 10^{-2} viral suspension. The experiment started on day 1.

hemorrhagic signs (petechiae, no ulcers) on the thighs, toes, and abdomen. The genetic sequence of the virus isolated from the experimentally infected frogs was identical to the virus isolated from the dead wild frogs.

Macroscopic internal lesions of the experimental frogs (all adult males) consisted of ascites in the abdominal cavity, petechiae or suffusions in the thigh muscles or abdominal wall, hemorrhages adjacent to the testes, and splenomegaly. Consistent acute lesions were noted in most tissue sections of all experimentally infected animals submitted to histopathologic examination. Moderate to severe lesions were noted in the skin, kidney, lung, intestinal tract, spleen, and liver, and mild lesions were noted in the testes and, in one animal, in skeletal muscle. There were no histologic lesions in the brain or heart. Lesions consisted mainly of single necrotic cells and variably sized foci of multicellular necrosis. On the skin, multifocal lesions were moderate to marked, characterized by intracellular and intercellular edema mainly of the stratum spinosum, areas of ballooning degeneration, and foci of necrosis of epithelial cells (Fig. 2a), focally progressing to erosions. These lesions were associated with numerous intracytoplasmic acidophilic cytoplasmic inclusions. Epidermal lesions were accompanied by leukocytic infiltration in the dermis with pyknosis of inflammatory cells. In the kidney, most lesions were moderate to marked and localized in the interstitium; they consisted of congestion, edema, leukocytic infiltration, and necrotic debris (Fig. 2b). Control healthy frogs had only mild and multifocal extramedullary hematopoietic tissue. Mild vacuolar degeneration of the renal tubular epithelial cells, focal tubular necrosis, and focal glomerulonephritis were also present in the kidney. In the lung, lesions involved the bronchial epithelium and lamina propria (Fig. 2c) with necrosis of ciliated epithelial cells, rare intracytoplasmic acidophilic inclusions, edema, and leukocytic infiltration (pyknotic cells) of the lamina propria. The intensity of these multifocal lesions varied from moderate to marked. In the intestinal tract, degeneration to necrosis and sloughing of the epithelium, congestion, edema, and infiltration by necrotic leucocytes of the submucosa and lamina propria were observed (Fig. 2d). In the spleen, diffuse severe acute necrosis (Fig. 2e) was a consistent lesion in all infected animals. Hepatocellular degeneration to necrosis (Fig. 2f) with a multifocal pattern and marked intensity was noted in all animals. In

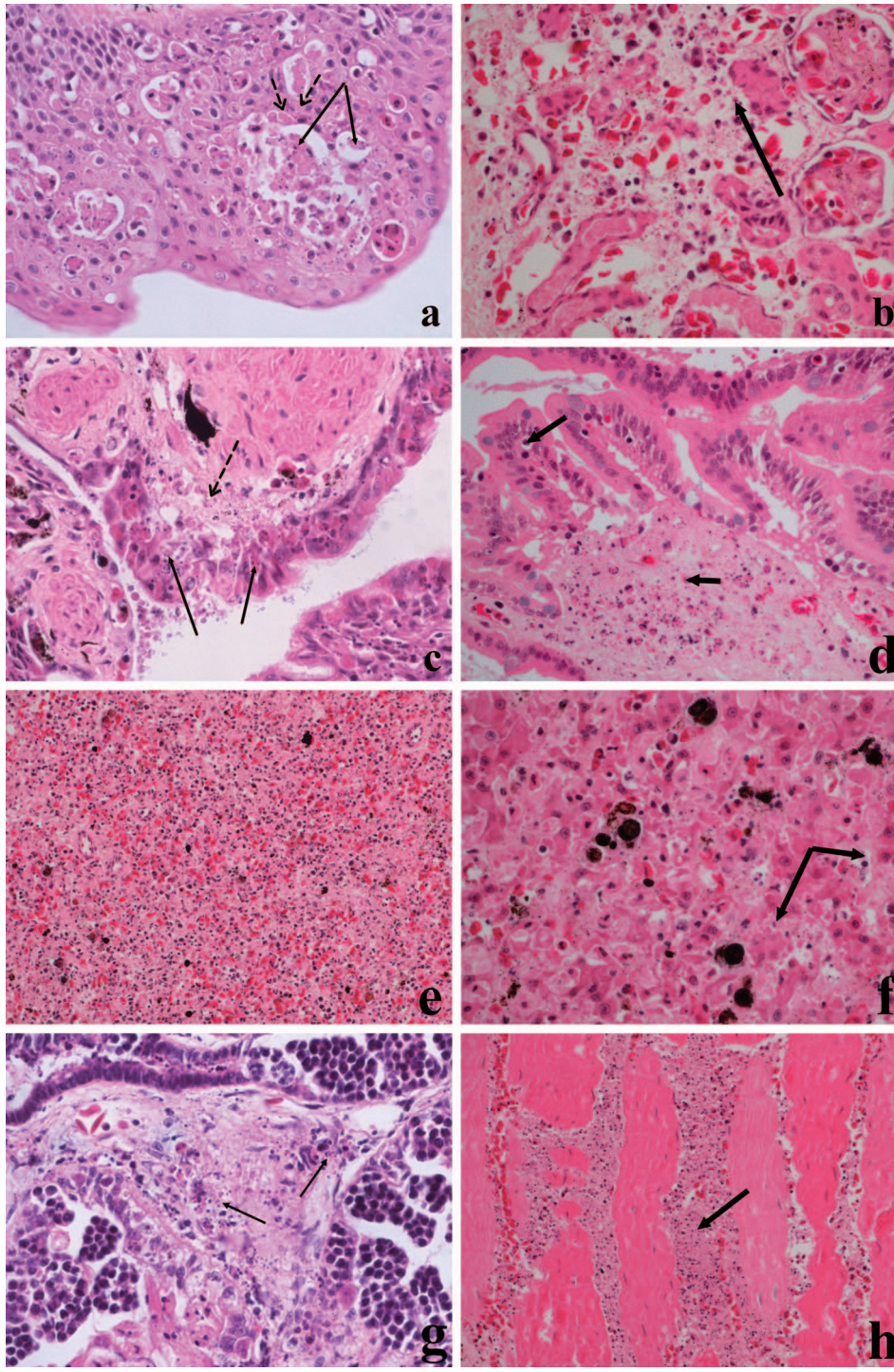

FIGURE 2. Acute lesions observed in tissue sections of infected European common frogs (*Rana temporaria*) collected following mass mortality events at three alpine lakes in the southwestern Alps (Mercantour National Park, France). All photos 400×. (a) Skin: spongiosis, multiple foci of necrotic epithelial cells (black arrows), small acidophilic. Lesions associated with small acidophilic intracytoplasmic inclusions (dashed arrows). (b) Kidney: congestion, edema, infiltration by leukocytes, and necrotic debris (black arrow) in the renal interstitium.

the testes, mild multifocal lesions were noted in all animals (Fig. 2g). These consisted of infiltration by inflammatory, often pyknotic, leukocytes in the connective tissue between seminiferous tubules. In the skeletal muscle, one animal had moderate multifocal lesions characterized by necrosis, edema, extravasated erythrocytes, and leukocytic infiltration within the endomysium (Fig. 2h).

DISCUSSION

As both chytrids and *Ranaviruses* can cause mortality in amphibian populations, we used molecular tests to detect if either pathogen was responsible for the three outbreak-affected lakes. We did not detect Bd; however, all the specimens were PCR-positive for the *Ranavirus* major capsid protein gene, and genetic sequence alignments 100% identical to the CMTV isolated from the common midwife toad (Balseiro et al. 2009). Experimental infection was used to fulfill Koch's postulates, demonstrating that this *Ranavirus* was the etiologic agent of common frog mortality in these alpine lakes—the first recorded instance of mass mortality caused by a *Ranavirus* (CMTV) in a wild native amphibian population in France.

The type species of the genus *Ranavirus* was isolated from a leopard frog *Lithobates pipiens* and named Frog Virus 3 (FV3; Granoff et al. 1965). Several other species have been described (e.g., *Ambystoma tigrinum* virus in North America [Jancovich et al. 1997], *Bohle iridovirus* in Australia [Speare and Smith 1992], and CMTV in northern Spain [Balseiro et al. 2009]). In Europe, several ranaviral diseases with mass mortality have been described, involving both FV3 and CMTV (Table 2). Researchers have detected CTMV in introduced American bullfrog (*L. catesbeianus*) tadpoles in Belgium (Sharifian-Fard et al. 2011), water frogs (*Pelophylax* spp.) in the Netherlands (Kik et al. 2011), and alpine newts in Spain (Mavian et al. 2012). Common midwife toad virus is implicated in the mortality in the Picos de Europa National Park (northern Spain), which has affected the whole amphibian community since 2005 (e.g., marbled newt [*Triturus marmoratus*], palmed newt [*Lissotriton helveticus*], common midwife toad, spiny toad [*Bufo spinosus*], and the common frog) (Price et al. 2014). These incidences of *Ranavirus*—and specifically CMTV—infecting European amphibian populations confirm the wide range of their potential hosts (Table 2; Stöhr et al. 2015). Although CMTV is described in continental Europe, it has not been found elsewhere (Table 2; Duffus et al. 2015). In the southern Alps where our study was conducted, the amphibian community is composed of three rather abundant species: the common toad (*Bufo bufo*), the fire salamander (*Salamandra salamandra*), and the common frog. However, mass mortalities have been observed only in the common frog, in three lakes above 2,200 m where only the common frog is present.

Anuran eggs do not seem to be affected by *Ranaviruses*. Duffus et al. (2013) found no infected common frog eggs in a historically *Ranavirus*-endemic site in the UK, and no anuran spawn or hatchling mortality has been reported in Europe (Table 2). Tadpoles, juveniles, and reproducing adults, however, can be fatally infected (Table 2). Bayley et al. (2013) showed that *R. temporaria* tadpoles and post-metamorphs are susceptible or

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(c) Lung: necrosis of ciliated epithelial cells (bronchial epithelium; black arrows), edema, and leukocytic infiltration (pyknotic cells; dashed arrow) of the lamina propria. (d) Intestine: necrosis of the epithelium, congestion, edema, infiltration by necrotic leucocytes (black arrow) of the submucosa and lamina propria. (e) Spleen: diffuse severe acute necrosis, a consistent lesion in all infected animals. (f) Liver: hepatocellular degeneration to necrosis (black arrows) with a multifocal pattern and a marked intensity. (g) Testes: pyknotic leukocytes (black arrows) in the connective tissue between seminiferous tubules. (h) Skeletal muscle: multifocal moderate muscular lesions characterized by necrosis (black arrow), edema, extravasated erythrocytes, and leukocytic infiltration within the endomysium.

TABLE 2. *Ranavirus* mass mortality events observed in wild amphibians in Europe.

Family	Species infected	Event description[a]	Life stage affected[b]	Country	Water body
Ranidae	*Pelophylax* spp.	1	A	Denmark	Artificial pond
		2	A, M	the Netherlands	Artificial pond
	Rana temporaria	3	A, J, T, M	UK	Natural and artificial ponds
		4	A	Spain	Natural lakes
			A, T	France	Natural lakes
			T	UK	Natural and artificial ponds
Bufonidae	*Bufo bufo*	4	A	Spain	Natural lakes
	Bufo spinosus	5	T	Spain	Natural lakes
Alytidae	*Alytes obstetricans*	4	A, J, T, M	Spain	Natural lakes
			A	UK	Artificial pond
		2	A	the Netherlands	Artificial pond
Salamandridae	*Lissotriton vulgaris*	6	A	Portugal	Natural lakes
	Triturus marmoratus		A, L	Spain	Natural lakes
		6	A	Portugal	Natural lakes
	Lissotriton boscai			Spain	Natural lakes
		5	L	Spain	Natural lakes
	Ichtyyosaura alpestris	7	M	Spain	Natural lakes

[a] 1 = Approximately 1,200 adults *Pelophylax esculentus* died over the course of the weekend of 3–4 August 2008. Two adults were positive for *Ranavirus*. 2 = Sudden mortality in September 2010, >1,000 young, incompletely metamorphosed and adult water frogs (edible frogs and pool frogs *Pelophylax lessonae*), and at least 10 smooth newts (*Lissotriton vulgaris*) had died. 3 = Incidents of unusual and mass mortalities of the common frog (*Rana temporaria*) in the 1990s. Investigations conducted at 10 sites of unusual mortality in Britain. 4 = Mass mortality in the amphibian community of Picos de Europa National Park since 2005. 5 = First observation of midwife toad tadpoles and a newt larva (*Ichtyosaura alpestris*) found dead in an isolated, permanent water body in the Picos de Europa National Park. 6 = Mass mortality episodes in *Triturus marmoratus* in the Peneda-Gerês National Park (NW Portugal). *Ranavirus*-like particles identified in the tissues of diseased newts *Triturus marmoratus* and *Lissotriton boscai*. 7 = High mortality in midwife toad tadpoles and juvenile (larvae) alpine newts (*Mesotriton alpestris cyreni*) in August 2008, in a pond approximately 1 km from the permanent water body where the first outbreak occurred in September 2007 (Balseiro et al. 2009).

[b] A = breeding adult; J = juvenile (metamorphosed individual); T = tadpole, L = larvae; M = metamorphosing tadpole or larvae (last stage of aquatic development).

[c] CMTV = common midwife toad virus; PEV = *Pelophylax esculentus* virus (nucleotide sequence similarity of 98.3% with FV3; FV3 = Frog Virus 3.

resistant to different *Ranavirus* species, but both FV3 and CMTV lead to anuran mortality in European species (Table 2). To our knowledge, Urodela egg mortality has not been evaluated. Mortality has been observed in larvae and metamorphosing alpine newts and adult smooth newts (*Lissotriton vulgaris*), marbled newts (*T. marmoratus*), palmed newts, and Bosca's newts (*Lissotriton boscai*), involving FV3 and CMTV (Table 2).

In our experimental infection, adult common frogs suffered 100% mortality after virus injection. No external clinical signs were noted before mortality, as is usual in cases of ranaviral disease (Miller et al. 2011). Gross lesions noted during postmortem examinations included subcutaneous ascites, petechiae, or muscular suffusions in the thighs or abdominal wall. These findings, consistent with systemic disease, are frequently noted in fatal cases (Miller et al. 2011). Macroscopic cutaneous ulcerations have been sometimes associated to systemic lesions in adult anurans in the UK (Cunningham et al. 1996). In our study, they were not associated, although microscopic acute necrotic epidermal lesions with focal erosion could have progressed to ulceration over time.

TABLE 2. Extended.

Altitude (m)	Other species infected	*Ranavirus*[c]	Reference
50	*Astacus Astacus*; *Cyprinus carpio*	PEV	Ariel et al. 2009
20	*Lissotrition vulgaris*; *Gasterosteus aculeatus*	CMTV	Kik et al. 2011
<500	Not recorded	FV3 variant	Cunningham et al. 1996; Teacher et al. 2010
>1,500	*Lissotriton helveticus*; *Alytes obstetricans*; *Triturus marmoratus*; *Bufo spinosus*; *Ichtyosaura alpestris*; *Lissotriton boscai*	CMTV	Price et al. 2014
>2000	Not recorded	CMTV	This study
<300	Not recorded	FV3	Duffus et al. 2013
>1,500	cf Price et al. 2014		Price et al. 2014
>1,500	*Ichtyosaura alpestris*	CMTV	Balseiro et al. 2009
>1,500	cf Price et al. 2014	CMTV	Price et al. 2014
<300	*Rana temporaria*	FV3 variant	Duffus et al. 2013
20	*Pelophylax spp.*; *Gasterosteus aculeatus*	CMTV	Kik et al. 2011
>1,000	*Lissotriton boscai*	FV3 type	Alves de Matos et al. 2008
>1,500	cf Price et al. 2014	CMTV	Price et al. 2014
>1,000	*Triturus marmoratus*	FV3 type	Alves de Matos et al. 2008
>1,500	cf Price et al. 2014	CMTV	Price et al. 2014
>1,500	*Alytes obstetricans*	CMTV	Balseiro et al. 2009
>1,500	*Alytes obstetricans*	CMTV	Balseiro et al. 2010

The microscopic lesions of the frogs in this study were variably sized necroses throughout most affected organ systems and were similar overall to those described in the systemic hemorrhagic form of *Ranavirus* disease in frogs and other amphibians (Cunningham et al. 1996; Balseiro et al. 2009, 2010). Ranaviral inclusion bodies are most often documented as round, intracytoplasmic, basophilic inclusions; they can also appear as eosinophilic, as in our case, which can be due to the stage of the disease or vary depending on the host (Miller et al. 2011). The affected organs and tissues in all the animals we tested were the skin, kidney, lung, intestinal tract, spleen, liver, testes, and, in one animal, skeletal muscle. Microscopic lesions consisted primarily of variably sized foci of necrosis throughout most affected organ systems. There was no significant histologic lesion in the brain (although, mild multifocal meningitis has been reported in tiger salamanders; Docherty et al. 2003) or heart (although pericardial or myocardial lesions have been reported in the common frog; Cunningham et al. 1996).

Comparing amphibian outbreaks in Europe can help understand risk of *Ranavirus* exposure. Although ranaviral infection seems higher in high-elevation watersheds (Gahl and Calhoun 2008), *Ranavirus* (both FV3 and CMTV) has been detected from plains to highland populations, and from garden ponds to natural lakes in Europe (Table 2), confirming the wide ecologic niche of these viruses (Gray and Chinchar 2015). Given the virulence of *Ranaviruses*, infection leading to host extinction appears possible. Monitoring of the amphibian community in Spain's Picos de Europa National Park since 2007 has revealed the dramatic decline or extirpation of several species with no sign of recovery for >5 yr (Price et al. 2014). Pathogens can drive hosts to extinction if density-independent transmission is possible. This can occur under at least three conditions: 1) there are multiple host species with variable susceptibility, 2) the pathogen persists for a long period outside the host via a reservoir, or 3) aggregation of breeding adults or larvae facilitates transmission at a low population

(McCallum et al. 2001; Miller et al. 2011). Regarding condition (1), the number of species in European amphibian communities varies with elevation, and the most common alpine species (e.g., *R. temporaria*, *A. obstetricans*, and *I. alpestris*) suffer *Ranavirus* mortality (Table 2). However, variation of susceptibility among developmental stages and host species needs to be estimated in the same watersheds. Regarding condition (2), persistence of a *Ranavirus* outside the host is possible via other amphibian species or the larval stage (reservoir). The multiyear duration of the tadpole stage of *A. obstetricans* (Tobler et al. 2012) and the larval stage of *I. alpestris* (Miaud et al. 2000) could facilitate host–pathogen system maintenance at high altitudes. Regarding condition (3), the characteristic behavior of alpine amphibians could facilitate virus transmission: *R. temporaria* adults migrate in autumn to aquatic hibernation sites where they aggregate (even in amplexus) for the duration of the winter (Miaud et al. 1999), and adult alpine newts can stay in water the whole year (Denoël and Joly 2001). During summer, tadpoles can also aggregate on the shores of lakes, where the water temperature is higher.

In the southwestern Alps, the common frog is abundant in many lakes, with similar ecological conditions, but mortalities were observed in only three. Being in a national park, our study site is under consistent observation and missing other mass mortality events is unlikely. In Spain, CMTV's genetic diversity and the temporal synchronicity of observed amphibian mortalities seem to point to its introduction via human translocations of infected materials (Price et al. 2014). Transmission of *Ranaviruses* between fish and frogs has been recorded (Moody and Owens 1994), and *R. temporaria* exposed to a *Ranavirus* originally isolated from fish experienced mortality (Bayley et al. 2013). In the phylogeny of the *Ranaviruses* infecting both reptiles and amphibians in Europe the reptilian viruses cluster closely with amphibian viruses (including CMTV), supporting the theory of host switching (Stöhr et al. 2015). Transmission through water among ectothermic vertebrates can increase the potential role of species as reservoirs for *Ranaviruses* (Currylow et al. 2014). In alpine lakes, fish stocking (mostly salmonids) for recreational fishing is widespread, but the origin of this *Ranavirus* in European alpine lakes in Spain and France remains unknown.

ACKNOWLEDGMENTS

We thank Alain Morand and Marie-France Leccia, former and current scientific directors of the Mercantour National Park, and Vincent Marty (ONEMA DIR 08), René Bonvallat (ONEMA 06), and Dominique Baril (ONEMA DIR 08) for sharing their field observations and collecting dead frogs. We also thank Amanda L. J. Duffus (Gordon State College, Barnesville, Georgia, USA) who kindly provided information about *Ranaviruses* in the UK and Stephen Price (Zoological Society, London, UK) who provided the *Ranavirus* DNA for PCR control. All the experiments in this study were submitted to and accepted by the ethics committee of the Université de Savoie-Mont-Blanc. Authorization to catch common frogs as a control was provided by the regional government (DREAL Rhône-Alpes, permit DDT-2010.880). The experimental infection was conducted at the Laboratoire départemental d'analyses in Poligny, France (agreement for animal experiment 39-0204 CSPP). We thank the reviewers and the editorial team of the *Journal of Wildlife Diseases* for their valuable and useful comments on the manuscript.

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Received for publication 4 May 2015.
Accepted 28 October 2015.