Current Biology *24*, 2586–2591, November 3, 2014 ©2014 The Authors http://dx.doi.org/10.1016/j.cub.2014.09.028

Report

Collapse of Amphibian Communities Due to an Introduced *Ranavirus*

Stephen J. Price,[1,2,7,*] Trenton W.J. Garner,[1]
Richard A. Nichols,[2] François Balloux,[3] César Ayres,[4]
Amparo Mora-Cabello de Alba,[5] and Jaime Bosch[6]
[1]Institute of Zoology, Zoological Society of London,
Regents Park, London NW1 4RY, UK
[2]The School of Biological and Chemical Sciences, Queen Mary
University of London, London E1 4NS, UK
[3]UCL Genetics Institute, University College London,
Gower Street, London, WC1E 6BT, UK
[4]Asociación Herpetológica Española, José Gutiérrez
Abascal 2, 28006 Madrid, Spain
[5]Parque Nacional Picos de Europa, Avenida Covadonga 43,
33550 Cangas de Onis, Asturias, Spain
[6]Museo Nacional de Ciencias Naturales, CSIC, José Gutiérrez
Abascal 2, 28006 Madrid, Spain

Summary

The emergence of infectious diseases with a broad host range can have a dramatic impact on entire communities and has become one of the main threats to biodiversity [1–4]. Here, we report the simultaneous exploitation of entire communities of potential hosts with associated severe declines following invasion by a novel viral pathogen. We found two phylogenetically related, highly virulent viruses (genus *Ranavirus*, family *Iridoviridae*) causing mass mortality in multiple, diverse amphibian hosts in northern Spain, as well as a third, relatively avirulent virus. We document host declines in multiple species at multiple sites in the region. Our work reveals a group of pathogens that seem to have preexisting capacity to infect and evade immunity in multiple diverse and novel hosts, and that are exerting massive impacts on host communities. This report provides an exceptional record of host population trends being tracked in real time following emergence of a wildlife disease and a striking example of a novel, generalist pathogen repeatedly crossing the species barrier with catastrophic consequences at the level of host communities.

Results and Discussion

We have been monitoring amphibian communities located in the Picos de Europa National Park (PNPE) since 2005, when we first recorded ranavirus infection, disease, and mass mortality. We began demographic surveys in 2007, making annual counts of target species at 15 sites across the park (Figure 1). This predates the first published records of lethal ranavirus infections (occurring in September 2007) in the PNPE in two of our study species [5, 6]. Ranaviruses are large, double-stranded DNA viruses of the family *Iridoviridae*, which are emerging pathogens with broad geographical and host ranges. They infect and cause disease in fish and reptiles but are noted for their ability to cause lethal disease in amphibians in the Americas, Europe, Asia, and Australia [5, 7–17]. Despite *Ranavirus*'s broad geographic distribution and documented multihost epizootics in amphibian communities [18], quantitative evidence for amphibian demographic decline due to ranavirosis has previously been reported for only a single amphibian host species [19].

Disease and Mass Mortality

We have continued to record mass mortality events consistent with ranavirosis affecting amphibian communities at four locations in the PNPE (Áliva, Ercina, Lloroza, and Moñetas [ALIVA, ERC, LLOR, and MON], Figure 1). During annual field surveys, we encountered numerous dead and dying adult, juvenile, and larval caudate and anuran amphibians, including all six common species inhabiting the park. Ranavirus infections may be subclinical [20, 21] but are more typically associated with overt, distinctive disease in the form of systemic or ulcerative syndromes [6, 7] and accompanied by host mass mortality. Sick and dead animals in the PNPE exhibited superficial and ulcerating skin lesions, internal hemorrhages, and severe limb necrosis, all gross signs typical of lethal ranavirosis (see Figure S1A available online). Additionally, since 2010 we have been observing mortality associated with signs of ranavirosis at a location 200 km west of the PNPE in Galicia, affecting two caudate amphibian species that are rare or absent in the PNPE and one squamate reptile (Figure S1B).

Molecular diagnostics have confirmed that infection with *Ranavirus* is associated with disease at all five of these locations (Table S1). We also screened for *Batrachochytrium dendrobatidis* (*Bd*), a fungal pathogen commonly associated with amphibian die-offs in Iberia [22], at eight locations in the PNPE. *Bd* was present at two sites (25%; Table S1B), occurring in the absence of observed disease and mortality at La Güelga (LAG, a ranavirus-negative site, Figure 1) and at low prevalence in 2005 only at an artificial pond (near Áliva refuge). A more widespread survey for bacteria and viruses was also undertaken as part of the first published account of ranavirus infection and mass mortality in the PNPE [5] (at Igüedri [IGU], Figure 1). That mass mortality event affected some of our study species, which exhibited the same suite of lesions observed in this study. There were no other pathogens found besides the *Ranavirus Common midwife toad virus* (CMTV). We consider it unlikely that other environmental factors, such as pollution, are contributing to the observed mass mortality events, given the pristine nature of the PNPE and the heterogeneity among study sites in terms of the type and situation of water bodies.

We successfully amplified a suite of ranavirus loci (six partial open reading frames and an intergenic region) from three of the host species sampled at locations in the PNPE and all three species sampled in Galicia. Sequences from amplification products of all loci were aligned against whole genomes covering known global amphibian-like ranavirus diversity and joined to form a final concatenated alignment 2,274 bp in length. Phylogenetic assessment of the concatenated sequences derived from seven diseased animals show that the ranaviruses associated with disease and mortality in northern Spain were all related or near identical to the genome of CMTV.

---

[7]Present address: UCL Genetics Institute, University College London, Gower Street, London, WC1E 6BT, UK
*Correspondence: s.price@ucl.ac.uk

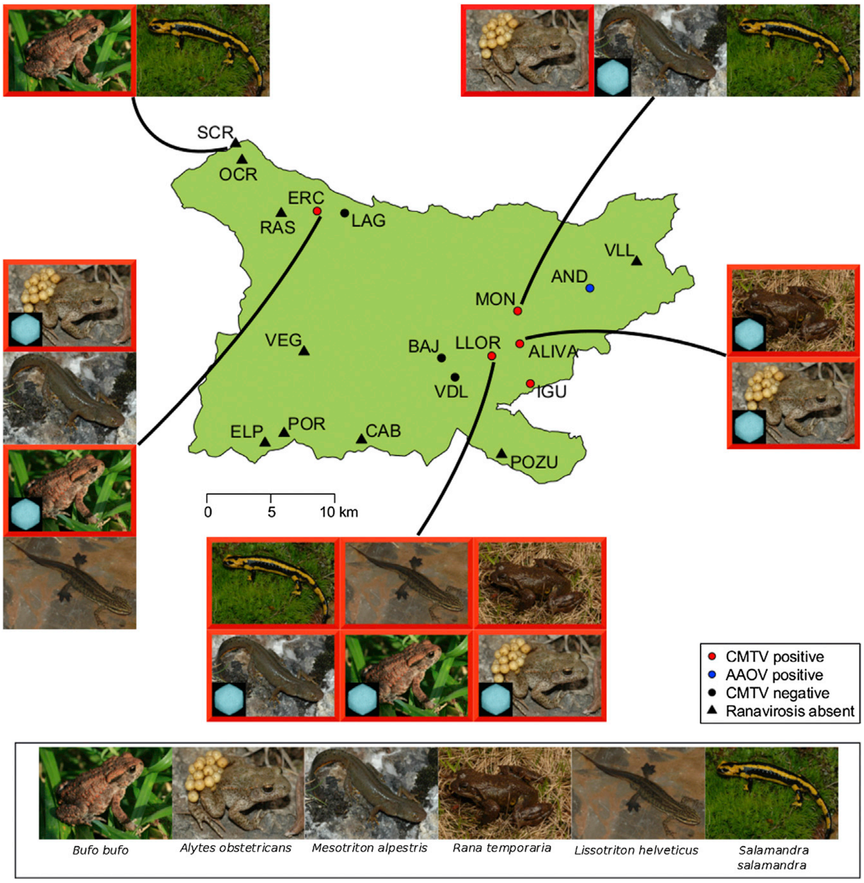

Figure 1. Ranavirus Infection and Mass Mortality among Amphibian Communities in the Picos de Europa National Park

Map shows park boundary and sites where amphibian communities have been monitored since 2005. Sites that experienced observed amphibian mass mortality events are expanded to display the amphibian community present, and affected hosts are bordered in red. Hosts with confirmed CMTV infection are denoted by a blue virus particle in bottom left corner of their image. Disease and mass mortality associated with CMTV infection at Igüedri (IGU) was reported by Balseiro et al. (2009) [5]. See also Figure S1 and Table S1.

Abbreviations key: SCR, Soto-Covadonga Road; OCR, Orandi-Covadonga Road; RAS, Rasa Pandecarmen; ERC, Ercina Lake; LAG, La Güelga; VEG, Vega Sajambre; ELP, El Pontón; POR, Pontón-Oseja Road; CAB, Charcas de Cable; BAJ, Bajero Lake; LLOR, Lloroza; MON, Moñetas; AND, Ándara Lake; VLL; Vau los Lobos; ALIVA, Áliva; VDL, Vega de Liordes; IGU, Igüedri; POZU, Pozu Llau.

This virus was originally isolated from a diseased animal sampled in 2008 in the PNPE and was associated with mass mortality and the same distinctive signs of disease (Figure 1) [6, 23]. Sequences amplified from all hosts sampled within the PNPE at sites of mass mortality exhibited 99.96% homology (varying at a single base) with the sequence of the type CMTV irrespective of what host species was sampled. Sequences from hosts sampled at the Galician site were highly similar to each other (two were identical and were 99.96% identical to the third, with variation again confined to a single base). They formed the sister clade to the CMTV cluster and are hereafter referred to as *Bosca's newt virus* (BNV; Figure 2). In contrast, an isolate from a common midwife toad at Ándara Lake, where ranavirosis and mass mortality have not been observed—*Ándaran Alytes obstetricans virus* (AAOV)—grouped with "FV3-like" viruses. We also screened animals from three other sites in the PNPE where there has been no observed disease or mass mortality, and we found no evidence of ranavirus infection. These observations support the assumption that disease and mass mortality can be used as a reliable indicator of CMTV incidence.

Multispecies Host Declines

Populations of host species in diseased communities in the PNPE have consistently experienced statistically significant, persistent, and in some cases catastrophic population declines, which was not the case at sites where disease had not been observed (Figure 3; Table S1A). The onset of decline for all species experiencing ranavirosis was contemporary with our first records of disease in the PNPE, but there is insufficient resolution of disease records to match decline and disease onset at individual locations. Our first records of mass mortality and ranavirosis in the region were at an artificial pond adjacent to Lloroza (LLOR, Figure 1; subsequently drained for other reasons) and a roadside pool at Áliva mine (ALIVA, Figure 1), both sampled in 2005.

The worst-affected species was the common midwife toad, *Alytes obstetricans*. Midwife toads experienced steep decline at both diseased sites where we monitored population trends of this host species but did not decline at two other locations where signs of ranavirosis were never detected (Figure 3; Table S1A). Alpine newts (*Mesotriton alpestris*) and common toads (*Bufo bufo*) also experienced significant declines at diseased sites (Figure 3) but did not show this dynamic at locations where we did not observe amphibians exhibiting signs of ranavirosis, with one exception (*B. bufo* at Soto-Covadonga Road [SCR], Figure 1). Amphibian species that were enumerated at disease-free locations did not generally decline from 2007 to 2012 and sometimes appeared to be increasing in numbers during the course of our surveillance (Table S1A). When all monitored host populations were considered independently, infection with CMTV was significantly associated with host declines (Fisher's exact test, p = 0.0093; raw data summarized in Figure S2). The same was true when we accounted for potential nonindependence of multiple species at the same site by using site data rather than host species data (Fisher's exact test, p = 0.022; Figure S2).

CMTV Emergence

Our findings indicate that CMTV-like ranaviruses recently emerged in northern Spain and are responsible for mortality of every amphibian species that we have sampled. Additionally, an isolate with 99.96% sequence identity to viruses isolated from amphibians in Galicia, and closely related to CMTV, was generated from esophageal tissue taken from a viperine snake (*Natrix maura*) found dead in the process

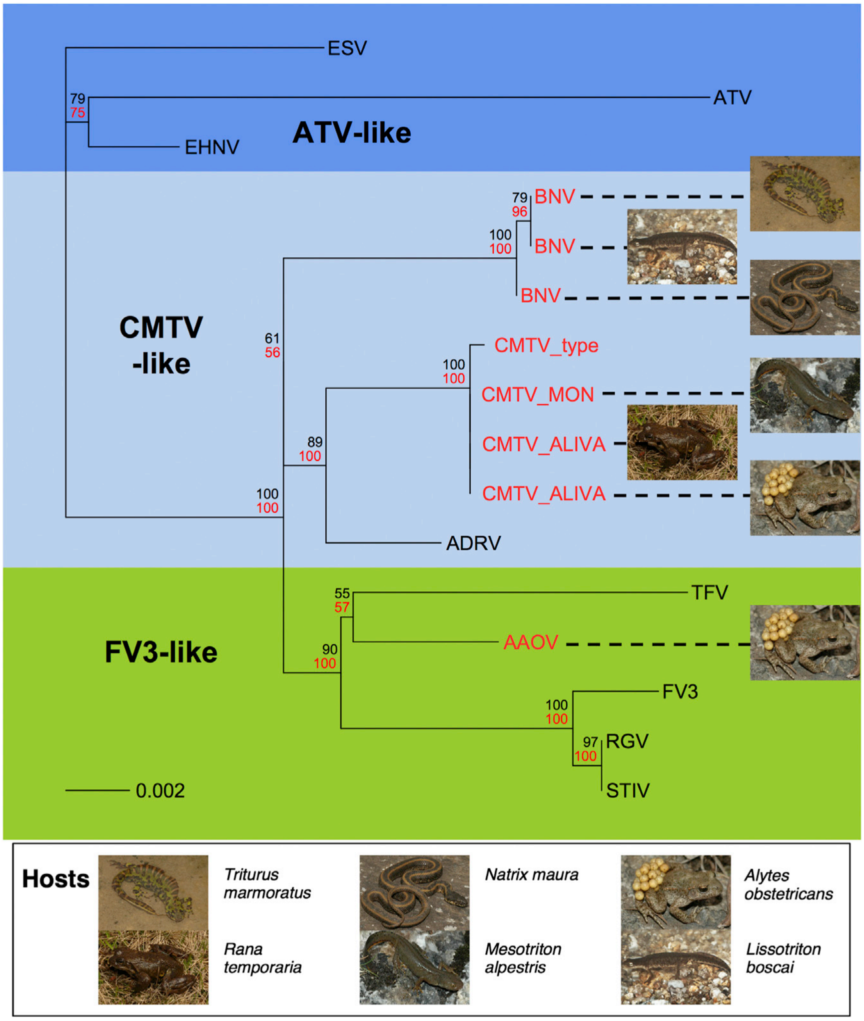

Figure 2. Phylogenetic Relationships of Spanish Ranaviruses

Ándaran Alytes obstetricans virus (AAOV) is a member of the "FV3-like" viruses, which form a monophyletic group; *Bosca's newt virus* (BNV) and *Common midwife toad virus* (CMTV) are considered "CMTV-like." The tree was constructed from seven concatenated multiple sequence alignments (details of loci are included in Experimental Procedures). Node support values are annotated on the best maximum-likelihood tree and were calculated using maximum-likelihood (bootstraps, black) and Bayesian inference (posterior probabilities, red) under a GTR model of molecular evolution. Scale of branch lengths is in nucleotide substitutions per site. Additional sequences included are *Frog virus 3* (FV3, GenBank accession number AY548484), *Tiger frog virus* (TFV, AF389451), *Ambystoma tigrinum virus* (ATV, AY150217), *Epizootic hematopoietic necrosis virus* (EHNV, FJ433873), *Soft-shelled turtle iridovirus* (STIV, NC012637), *Rana grylio virus* (RGV, JQ654586), and *European sheatfish virus* (ESV, JQ724856).

of ingesting diseased amphibians [24] and with ulcerating lesions along its gullet. The ability of FV3-like ranaviruses (the sister clade to CMTV-likes; Figure 2 [23]) to be transmitted among both closely related and highly diverged cold-blooded vertebrate taxa is well documented [25, 26]. However, recurrent epizootics caused by FV3-like viruses in the Americas, the United Kingdom (UK), and Southeast Asia have only been linked to host population decline in UK common frogs (*Rana temporaria*), and UK FV3-like viruses appear limited in their ability to cause disease and significant mortality in other native UK amphibian species [19]. We have also detected FV3-like ranaviruses cocirculating in the PNPE (at Ándara Lake [AND], Figure 2) that caused morbidity without lesions in *A. obstetricans* but with no evidence of mass mortality or population declines. Reports of amphibian mass mortality events associated with ranavirosis are rapidly accruing across Europe, and CMTV-like forms are implicated: a partial sequence of the major capsid protein gene of the virus isolate responsible for the death of thousands of pool frogs (*Pelophylax lessonae*) and smooth newts (*Lissotriton vulgaris*) in the Netherlands reported by Kik et al. exhibited 100% sequence similarity to CMTV [9], as did sequences from the same locus derived from a *Ranavirus* isolated from North American bullfrog larvae sampled from invasive populations in Belgium [10]. The accumulating body of evidence indicates that CMTV-like ranaviruses may be in the process of emerging in amphibian host communities across Europe, with the capacity to infect and cause significant disease and death in a wide range of hosts.

Community-Level Effects

Infection and disease frequency are both known to decrease with increasing host community species richness (see e.g. [27]), even if the relationship is not always straightforward [28]. This is because diverse host communities present a range of barriers to infection, and universal pathogen strategies for overcoming host barriers are rare [29, 30]. Accordingly, pathogens typically exhibit significant variation in their ability to infect and cause disease across host species and must evolve novel traits to exploit a broader host range [31–33]. It is therefore unusual for an emerging pathogen to exploit a broad range of host species and extremely rare for multiple host species to suffer synchronous mass mortality and decline when infection emerges. For the rare exceptions, the results for hosts can be notably catastrophic (e.g., West Nile virus emergence in North America [1], *Batrachochytrium dendrobatidis* emergence in the Neotropics and Australia [2], and white-nose syndrome in North American bats [4]).

We see no evidence of increased host species diversity hampering the ability of a novel pathogen to exploit a host community in northern Spain. At the most species-rich site in the PNPE, all six amphibian species are experiencing mortality associated with signs of disease and have done so since disease was first observed. Instead of being inhibited by host species diversity, it appears that a single strain of CMTV has the capacity to exploit multiple host species de novo. This hypothesis is supported by an almost complete lack of variation at seven loci located across the type CMTV genome and recovered at multiple points in space, time, and host species—spanning the width of the PNPE, several years, and diverse hosts. The synchrony of host declines across sites, beginning shortly after disease was first detected in the park, provides further support. Taken together with the obstacles to rapid amphibian dispersal presented by the rugged, mountainous terrain of the PNPE, all of the evidence points to a single introduction of CMTV at multiple locations within the park and affecting all amphibian species. Such an introduction could have occurred via human translocations of infectious materials along with equipment or livestock.

Worryingly, species declines in the PNPE show no sign of rebound over five years, and in many cases species are all but extirpated from some locations. The availability of multiple susceptible hosts at a site increases extinction risk, while small relict populations that have suffered declines are left vulnerable to stochastic events [34]. This same pattern of decline without rebound was observed in common frog populations experiencing persistent ranavirosis in the UK, where 83% median host population declines were sustained over several frog generations [19]. Selection imposed on host populations experiencing high rates of mortality due to infectious disease is expected to favor genotypes capable of either resisting or tolerating infection, and UK frog populations affected by ranavirosis do exhibit the genetic signature of directional selection at immunocompetent loci [19, 35]. However, selection has not been accompanied by demographic recovery, and debilitating ranavirosis continues to increase in scope in common frog populations across the UK. If CMTV in Spain has similar capacity to proliferate despite host adaptive responses, amphibian communities, not just single species, will suffer.

Experimental Procedures

Sampling

All animal sampling was carried out following review by the Consejería de Medio Ambiente of Galicia and the governing body of the Parque Nacional Picos de Europa, and permits were renewed annually. We collected swabs and tissue samples from a mix of amphibian species and life stages at sites in the PNPE between 2005 and 2012, and from carcasses of two amphibian (*Lissotriton boscai*, *Triturus marmoratus*) and one reptile (*Natrix maura*) species at Pontillon (Galicia) in 2010 and 2011 (Table S1B). One of the sampled sites—Lloroza—is thought to correspond to the mass mortality event that led to the isolation of CMTV [6, 23].

Screening

Tissue samples were screened for *Ranavirus* in duplicate using a PCR of the viral MCP gene (CMTV ORF 16L; major capsid protein; AFA44920) [36]. A results summary is included in Table S1B. Swabs were screened for *Bd* using quantitative PCR [37].

Sequencing

Positive samples were subjected to additional PCR reactions to amplify partial sequences from CMTV ORFs 22L (GenBank accession number AFA44926), 58L (AFA44964), 59R (AFA44965), 81L (AFA44987), 82L (AFA44988), and a region covering a noncoding sequence and the start of 13R (AFA44917). Amplification products were submitted for Sanger sequencing, and sequences were archived in GenBank (see Supplemental Experimental Procedures).

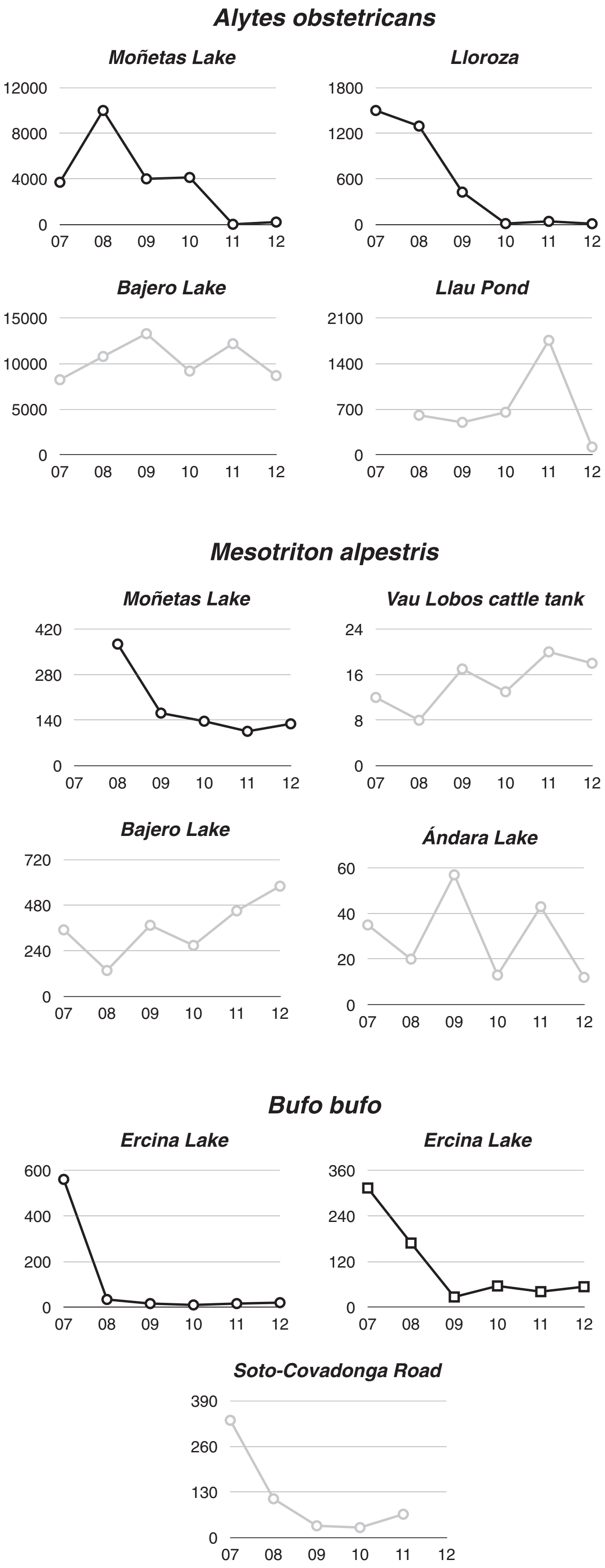

Figure 3. Population Trends for Declining Species in the Picos de Europa National Park

Common midwife toad ranavirus (CMTV) infections have been confirmed within amphibian communities at Moñetas, Lloroza, and Ercina Lake (black lines). Ranavirus infection and disease were first observed in the Picos de Europa National Park in 2005 (see Multispecies Host Declines). Trends are also shown for all other sites where monitoring of *Alytes obstetricans*, *Mesotriton alpestris*, and *Bufo bufo* has been ongoing but CMTV is assumed to be absent due to molecular screening and/or the absence of disease and mass mortality events (gray lines). Circles denote counts for adults or tadpoles; Squares denote counts for egg masses. See also Table S1.

Phylogenetics

Sequences were aligned with Prank v.100802 [38] and manually edited in Jalview 2.8 [39] to remove gaps. Additional sequences used in alignments and phylogeny construction (listed in the Figure 2 legend) were downloaded from the NCBI nucleotide database. Trees were constructed with MrBayes 3.2.2 [40] and RAxML 7.7.2 [41] using the GTR model of nucleotide substitution. Rate variation among sites was modeled by a discrete gamma distribution with four categories. Default settings were used for Markov chain Monte Carlo (MCMC) analysis in MrBayes (1,000,000 generations, 4 chains, 2 runs, sample frequency = 500, and a 25% burn-in). Twenty maximum-likelihood trees were generated on distinct starting trees in RAxML; 100 bootstrap replicates were calculated and annotated on the best maximum-likelihood tree.

Population Monitoring and Analyses

Annual counts of amphibian populations have been conducted in the PNPE (Table S1A) since 2007. Methodology and life history stage targeted varied with the size and situation of the water body but remained consistent for each site across yearly surveys. Data were analyzed for overall trends in population size using TRIM3.0 [42].

Accession Numbers

GenBank accession numbers by sample and locus are provided in the Supplemental Experimental Procedures.

Supplemental Information

Supplemental Information includes two figures, one table, and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.cub.2014.09.028.

Author Contributions

S.J.P. and J.B. carried out fieldwork in the PNPE. C.A. carried out fieldwork in Galicia. J.B. and A.M-C.d.A. organized and supervised the collection of population data, and J.B. analyzed these data. S.J.P. carried out molecular screening, processed samples for sequencing, and carried out phylogenetic analyses. R.A.N. and F.B. advised on phylogenetic analyses and interpretation. S.J.P. and T.W.J.G. prepared the manuscript, which was edited by all authors.

Acknowledgments

We thank the PNPE ranger team (J.J. Alonso, I. Alonso, M.A. Bermejo, S. Casares, M. Díaz, J.E. Díez, P. Fernández, M. Fernández, S. González, S. Marquínez, J.J. Martínez, J.L. Mosquera, C. Obeso, F. Rojo, A. Tejedor, and R. Varona) for performing population monitoring surveys. The Consejería de Medio Ambiente of Galicia and the director of the PNPE extended permits for fieldwork. This work was supported by Natural Environment Research Council grant NE/G011885/1, the Systematics and Taxonomy (SynTax) research scheme administered by the Linnean Society of London, the Fundación General CSIC, Banco Santander, and European Research Council grant 260801-BIG-IDEA. We thank Florent Lasalle, Rob Knell, Steve Paterson, and three anonymous reviewers for helpful comments during manuscript preparation and Gonçalo M. Rosa for sharing high-resolution host images.

Received: May 28, 2014
Revised: August 13, 2014
Accepted: September 9, 2014
Published: October 16, 2014

References

1. LaDeau, S.L., Kilpatrick, A.M., and Marra, P.P. (2007). West Nile virus emergence and large-scale declines of North American bird populations. Nature *447*, 710–713.
2. Fisher, M.C., Garner, T.W.J., and Walker, S.F. (2009). Global emergence of *Batrachochytrium dendrobatidis* and amphibian chytridiomycosis in space, time, and host. Annu. Rev. Microbiol. *63*, 291–310.
3. Lembo, T., Haydon, D.T., Velasco-Villa, A., Rupprecht, C.E., Packer, C., Brandão, P.E., Kuzmin, I.V., Fooks, A.R., Barrat, J., and Cleaveland, S. (2007). Molecular epidemiology identifies only a single rabies virus variant circulating in complex carnivore communities of the Serengeti. Proc. Biol. Sci. *274*, 2123–2130.
4. Frick, W.F., Pollock, J.F., Hicks, A.C., Langwig, K.E., Reynolds, D.S., Turner, G.G., Butchkoski, C.M., and Kunz, T.H. (2010). An emerging disease causes regional population collapse of a common North American bat species. Science *329*, 679–682.
5. Balseiro, A., Dalton, K.P., del Cerro, A., Marquez, I., Cunningham, A.A., Parra, F., Prieto, J.M., and Casais, R. (2009). Pathology, isolation and molecular characterisation of a ranavirus from the common midwife toad *Alytes obstetricans* on the Iberian Peninsula. Dis. Aquat. Organ. *84*, 95–104.
6. Balseiro, A., Dalton, K.P., del Cerro, A., Márquez, I., Parra, F., Prieto, J.M., and Casais, R. (2010). 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. Vet. J. *186*, 256–258.
7. Cunningham, A.A., Langton, T.E.S., Bennett, P.M., Lewin, J.F., Drury, S.E.N., Gough, R.E., and Macgregor, S.K. (1996). Pathological and microbiological findings from incidents of unusual mortality of the common frog (*Rana temporaria*). Philos. Trans. R. Soc. Lond. B Biol. Sci. *351*, 1539–1557.
8. Une, Y., Sakuma, A., Matsueda, H., Nakai, K., and Murakami, M. (2009). Ranavirus outbreak in North American bullfrogs (*Rana catesbeiana*), Japan, 2008. Emerg. Infect. Dis. *15*, 1146–1147.
9. Kik, M., Martel, A., Sluijs, A.S., Pasmans, F., Wohlsein, P., Gröne, A., and Rijks, J.M. (2011). Ranavirus-associated mass mortality in wild amphibians, the Netherlands, 2010: a first report. Vet. J. *190*, 284–286.
10. Sharifian-Fard, M., Pasmans, F., Adriaensen, C., Devisscher, S., Adriaens, T., Louette, G., and Martel, A. (2011). Ranavirosis in invasive bullfrogs, Belgium. Emerg. Infect. Dis. *17*, 2371–2372.
11. Green, D.E., Converse, K.A., and Schrader, A.K. (2002). Epizootiology of sixty-four amphibian morbidity and mortality events in the USA, 1996-2001. Ann. N Y Acad. Sci. *969*, 323–339.
12. Ariel, E., Kielgast, J., Svart, H.E., Larsen, K., Tapiovaara, H., Jensen, B.B., and Holopainen, R. (2009). Ranavirus in wild edible frogs *Pelophylax* kl. *esculentus* in Denmark. Dis. Aquat. Organ. *85*, 7–14.
13. Xu, K., Zhu, D.-Z., Wei, Y., Schloegel, L.M., Chen, X.-F., and Wang, X.-L. (2010). Broad distribution of Ranavirus in free-ranging *Rana dybowskii* in Heilongjiang, China. EcoHealth *7*, 18–23.
14. Cullen, B.R., and Owens, L. (2002). Experimental challenge and clinical cases of Bohle iridovirus (BIV) in native Australian anurans. Dis. Aquat. Organ. *49*, 83–92.
15. Fox, S.F., Greer, A.L., Torres-Cervantes, R., and Collins, J.P. (2006). First case of ranavirus-associated morbidity and mortality in natural populations of the South American frog *Atelognathus patagonicus*. Dis. Aquat. Organ. *72*, 87–92.
16. Greer, A.L., Berrill, M., and Wilson, P.J. (2005). Five amphibian mortality events associated with ranavirus infection in south central Ontario, Canada. Dis. Aquat. Organ. *67*, 9–14.
17. Jancovich, J.K., Davidson, E.W., Morado, J.F., Jacobs, B.L., and Collins, J.P. (1997). Isolation of a lethal virus from the endangered tiger salamander *Ambystoma tigrinum stebbinsi*. Dis. Aquat. Organ. *31*, 161–167.
18. Gray, M.J., Miller, D.L., and Hoverman, J.T. (2009). Ecology and pathology of amphibian ranaviruses. Dis. Aquat. Organ. *87*, 243–266.
19. Teacher, A.G.F., Cunningham, A.A., and Garner, T.W.J. (2010). Assessing the long-term impact of Ranavirus infection in wild common frog populations. Anim. Conserv. *13*, 514–522.
20. Lesbarrères, D., Balseiro, A., Brunner, J., Chinchar, V.G., Duffus, A., Kerby, J., Miller, D.L., Robert, J., Schock, D.M., Waltzek, T., and Gray, M.J. (2012). Ranavirus: past, present and future. Biol. Lett. *8*, 481–483.
21. Gantress, J., Maniero, G.D., Cohen, N., and Robert, J. (2003). Development and characterization of a model system to study amphibian immune responses to iridoviruses. Virology *311*, 254–262.
22. Bosch, J., Martinez-Solano, I., and Garcia-Paris, M. (2001). Evidence of a chytrid fungus infection involved in the decline of the common midwife toad (*Alytes obstetricans*) in protected areas of central Spain. Biol. Conserv. *97*, 331–337.
23. Mavian, C., López-Bueno, A., Balseiro, A., Casais, R., Alcamí, A., and Alejo, A. (2012). The genome sequence of the emerging common midwife toad virus identifies an evolutionary intermediate within ranaviruses. J. Virol. *86*, 3617–3625.
24. Ayres, C. (2012). Scavenging in the genus Natrix. Acta Herpetol. *7*, 171–174.

25. Hoverman, J.T., Gray, M.J., Haislip, N.A., and Miller, D.L. (2011). Phylogeny, life history, and ecology contribute to differences in amphibian susceptibility to ranaviruses. EcoHealth *8*, 301–319.
26. Hoverman, J.T., Gray, M.J., and Miller, D.L. (2010). Anuran susceptibilities to ranaviruses: role of species identity, exposure route, and a novel virus isolate. Dis. Aquat. Organ. *89*, 97–107.
27. Johnson, P.T.J., Preston, D.L., Hoverman, J.T., and Richgels, K.L.D. (2013). Biodiversity decreases disease through predictable changes in host community competence. Nature *494*, 230–233.
28. Wood, C.L., Lafferty, K.D., DeLeo, G., Young, H.S., Hudson, P.J., and Kuris, A.M. (2014). Does biodiversity protect humans against infectious disease? Ecology *95*, 817–832.
29. Parrish, C.R., Holmes, E.C., Morens, D.M., Park, E.-C., Burke, D.S., Calisher, C.H., Laughlin, C.A., Saif, L.J., and Daszak, P. (2008). Cross-species virus transmission and the emergence of new epidemic diseases. Microbiol. Mol. Biol. Rev. *72*, 457–470.
30. Kassen, R. (2002). The experimental evolution of specialists, generalists, and the maintenance of diversity. J. Evol. Biol. *15*, 173–190.
31. Benmayor, R., Hodgson, D.J., Perron, G.G., and Buckling, A. (2009). Host mixing and disease emergence. Curr. Biol. *19*, 764–767.
32. Woolhouse, M.E.J., Taylor, L.H., and Haydon, D.T. (2001). Population biology of multihost pathogens. Science *292*, 1109–1112.
33. Bitter, W., Gerrits, H., Kieft, R., and Borst, P. (1998). The role of transferrin-receptor variation in the host range of *Trypanosoma brucei*. Nature *391*, 499–502.
34. Smith, K.F., Acevedo-Whitehouse, K., and Pedersen, A.B. (2009). The role of infectious diseases in biological conservation. Anim. Conserv. *12*, 1–12.
35. Teacher, A.G.F., Garner, T.W.J., and Nichols, R.A. (2009). Evidence for directional selection at a novel major histocompatibility class I marker in wild common frogs (*Rana temporaria*) exposed to a viral pathogen (*Ranavirus*). PLoS ONE *4*, e4616.
36. Mao, J., Tham, T.N., Gentry, G.A., Aubertin, A., and Chinchar, V.G. (1996). Cloning, sequence analysis, and expression of the major capsid protein of the iridovirus frog virus 3. Virology *216*, 431–436.
37. Boyle, D.G., Boyle, D.B., Olsen, V., Morgan, J.A., and Hyatt, A.D. (2004). Rapid quantitative detection of chytridiomycosis (*Batrachochytrium dendrobatidis*) in amphibian samples using real-time Taqman PCR assay. Dis. Aquat. Organ. *60*, 141–148.
38. Löytynoja, A., and Goldman, N. (2005). An algorithm for progressive multiple alignment of sequences with insertions. Proc. Natl. Acad. Sci. USA *102*, 10557–10562.
39. Waterhouse, A.M., Procter, J.B., Martin, D.M.A., Clamp, M., and Barton, G.J. (2009). Jalview Version 2—a multiple sequence alignment editor and analysis workbench. Bioinformatics *25*, 1189–1191.
40. Huelsenbeck, J.P., and Ronquist, F. (2001). MRBAYES: Bayesian inference of phylogenetic trees. Bioinformatics *17*, 754–755.
41. Stamatakis, A. (2014). RAxML version 8: a tool for phylogenetic analysis and post-analysis of large phylogenies. Bioinformatics *30*, 1312–1313.
42. Van Strien, A., Pannekoek, J., Hagemeijer, W., and Verstrael, T. (2000). A loglinear Poisson regression method to analyse bird monitoring data. Bird Census News *13*, 33–39.

Current Biology, Volume *24*

Supplemental Information

Collapse of Amphibian Communities Due to an Introduced *Ranavirus*

Stephen J. Price, Trenton W.J. Garner, Richard A. Nichols, François Balloux,
César Ayres, Amparo Mora-Cabello de Alba, and Jaime Bosch

Supplemental Information

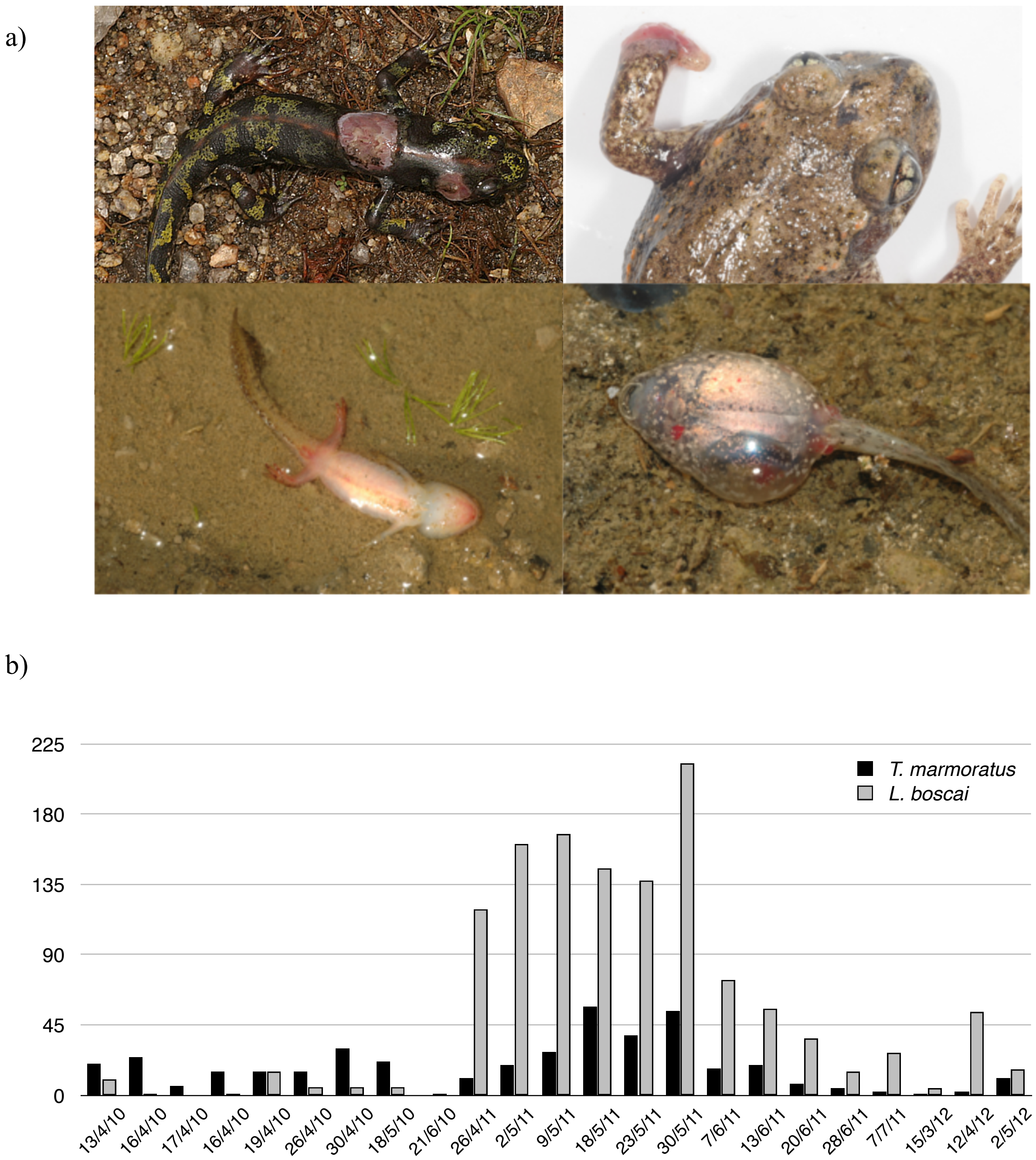

Figure S1, related to Figure 1. Ranavirosis and mass mortality in amphibians in northern Spain. a) Images of diseased animals observed at sites of mortality. (Clockwise from top-left) *Triturus marmoratus* with severe ulceration; *Alytes obstetricans* adult with severe limb necrosis; *A. obstetricans* larvae and *Mesotriton alpestris* with systemic hemorrhaging. b)

Periodic counts of carcasses of two salamander species – *Triturus marmoratus* and *Lissotriton boscai* - at Pontillon reservoir, Galicia between April 2010 and May 2012.

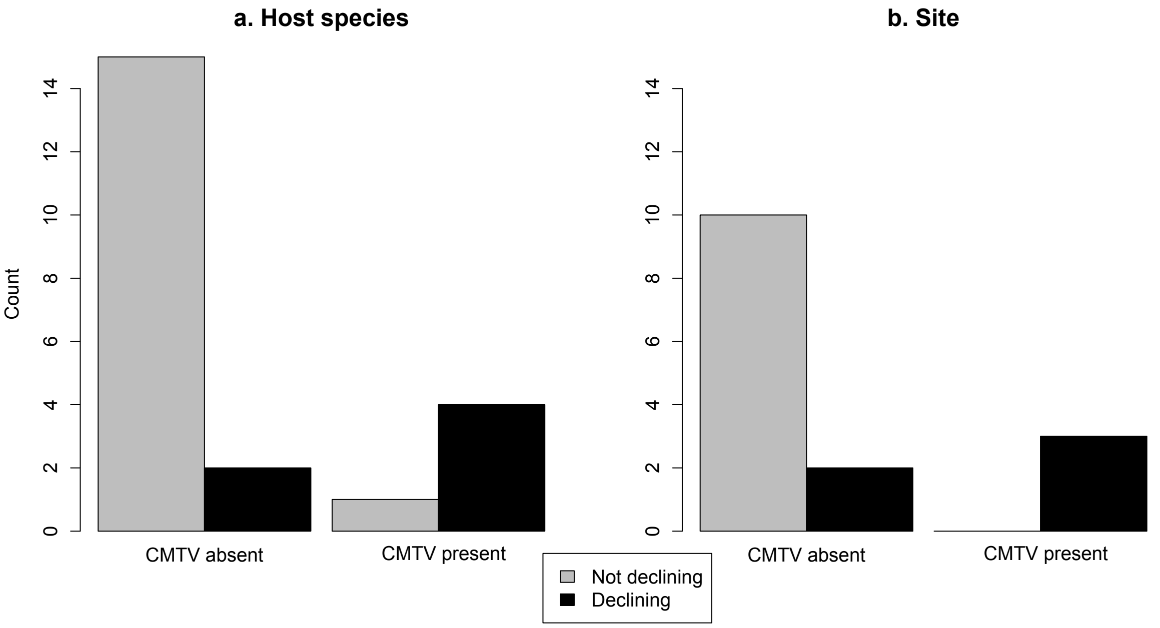

Figure S2, related to Figure 3. Association between declining population trends in the Picos de Europa and incidence of CMTV. Data summarized at the level of a) host species*, and b) site. CMTV absent hosts/sites include absences confirmed with molecular tests and those inferred through absence of disease and mass mortality (see main text for further details). *Multiple life history stages were monitored at Ercina (declining trends in *Bufo bufo* adults and egg clutches) but are considered as a single data point due to non-independence.

Table S1, related to Figure 1 and Figure 3. Amphibian population monitoring at study sites in Northern Spain: a) community composition, observations of mortality, disease and infection, and demographic trends, b) Summary of samples screened for ranavirus infection in northern Spain by site and year.

a)

Site details				**Infection**				**Population monitoring**			
Locality	**ID**	**Habitat, altitude**	**Amphibian Community**	**Mortality**	**Ranavirosis**	**Screened for ranavirus?**	**Virus type**	**Target species**	**Target stage**	**Slope (mean & SE)**	**Population trend**
Orandi-Covadonga road	OCR	road, 250m	*Ss*, *Bb*, *Rt*	none	no	no	n/a	*Ss*	adults	-0.0819 (0.1230)	Uncertain
Pontón-Oseja road	POR	road, 950m	*Ss*, *Bb*, *Rt*	none	no	no	n/a	*Ss*	adults	0.0555 (0.0119)	Moderate increase (p<0.01) **
								Bb	adults	-0.0169 (0.0116)	Stable
Pozu Llau	POZU	pond, 1856m	*Ss*, *Rt*, *Ao*	none	no	no	n/a	*Ss*	larvae	-0.2003 (0.1535)	Uncertain
								Ao	tadpoles	-0.0597 (0.0346)	Uncertain
								Rt	egg clutches	-0.0553 (0.0048)	Moderate decline (p<0.01) **
Bajero lake	BAJ	small lake, 1875m	*Ma*, *Lh*, *Ao*, *Rt*	none	no	yes	none	*Ma*	adults	0.0280 (0.0130)	Moderate increase (p<0.05) *
								Ao	tadpoles	0.0009 (0.0046)	Stable
Vau los Lobos	VLL	cattle tank, 1080m	*Ss*, *Ma*, *Lh*, *Ao*, *Rt*	none	no	no	n/a	*Ma*	adults	0.0459 (0.0152)	Moderate increase (p<0.01) **
Ándara lake	AND	small lake, 1750m	*Ma*, *Lh*, *Ao*	none	no	yes	AAOV	*Ma*	adults	-0.0277 (0.0523)	Uncertain
El Pontón	ELP	pond, 1297m	*Ma*, *Lh*, *Ao*	none	no	no	n/a	*Ma*	adults	-0.0180 (0.0598)	Uncertain
La Güelga	LAG	stream pool, 1056m	*Ss*, *Ma*, *Lh*, *Ao*	none	no	yes	none	*Ma*	adults	-0.0805 (0.1322)	Uncertain
Charcas de Cable	CAB	group of ponds, 1600m	*Ma*, *Lh*, *Rt*	none	no	no	n/a	*Ma*	adults	-0.0111 (0.0320)	Uncertain
								Rt	egg clutches	0.0107 (0.0205)	Uncertain
Moñetas	MON	small lake, 1712m	*Ss*, *Ma*, *Ao*	*Ao*	yes	yes	CMTV	*Ma*	adults	-0.0482 (0.0169)	Moderate decline (p<0.01) **
								Ao	tadpoles	-0.1736 (0.0494)	Steep decline (p<0.01) **
Lloroza	LLOR	small lake, 1850m	*Ss*, *Ma*, *Lh*, *Ao*, *Bb*, *Rt*	*Ss*, *Ma*, *Lh*, *Ao*, *Bb*, *Rt*	yes	yes	CMTV	*Ma*	adults	0.0102 (0.0203)	Uncertain
								Ao	tadpoles	-0.2471 (0.0535)	Steep decline (p<0.01) **
Soto-Covadonga road	SCR	road, 80m	*Ss*, *Bb*	*Bb*	?	no	n/a	*Bb*	adults	-0.0970 (0.0502)	Moderate decline (p<0.05) *
Ercina lake	ERC	lake, 1100m	*Ma*, *Lh*, *Ao*, *Bb*	*Ao*, *Bb*	yes	yes	CMTV	*Bb*	adults	-0.1288 (0.0644)	Moderate decline (p<0.05) *
								Bb	egg clutches	-0.0734 (0.0341)	Moderate decline (p<0.05) *
Rasa Pandecarmen	RAS	pond, 1117m	*Rt*	none	no	no	n/a	*Rt*	egg clutches	0.0506 (0.0200)	Moderate increase (p<0.05) *
Vega Sajambre	VEG	pond, 1318m	*Rt*	none	no	no	n/a	*Rt*	egg clutches	-0.0275 (0.0271)	Uncertain
Áliva	ALIVA	roadside pool	Rt, Ao	Rt, Ao	yes	yes	CMTV	nd	nd	nd	nd
Igüedri	IGU	cattle tank, 1400m	Ao, Ss, Ma, Lh	Ao, Ma, Ss	yes†	yes†	CMTV†	nd	nd	nd	nd
Vega de Liordes	VDL	small ponds, 1874m	Ao, Ss, Rt, Lh, Ma	none	no	yes	none	nd	nd	nd	nd
Pontillon, Galicia	n/a	reservoir	Tm, Lb	Nm, Tm, Lb	yes	yes	BNV	nd	nd	nd	nd

† pathology, screening and sequencing reported in Balseiro et al. (2009) [5]

b)

Site	**Mortality**	**Ranavirosis**	**Host declines?**	***Bd* present?**	**Host**	**Year**	**Positives**	**Total**	**Prevalence (95% confidence interval†)**
Áliva	yes	yes	nd	nd	Ao/Bb/Ma	2005	5	22	23% (10-43)
					Ao/Rt	2011	4	4	100% (51-100)
Ercina	yes	yes	yes	nd	Ao/Bb	2011	5	24	21% (9-40)
Pontillon, Galicia	yes	yes	nd	nd	Tm/Lb	2010	12	15	80% (55-93)
					Tm/Lb/Nm	2011	10	10	100% (72-100)
Lloroza	yes	yes	yes	no	Ao/Bb/Ma	2009	13	26	50% (32-68)
					Ao/Rt	2011	0	25	0% (0-13)
Artificial pond (Áliva refuge)	yes	yes	nd	2005 only	Ao/Ma	2005	8	19	42% (23-64)
Monetas	yes	yes	yes	no	Ma	2011	1	2	50% (9-91)
Igüedri	yes	yes	nd	no	Ao/Ss	2009	0	3	0% (0-56)
Ándara lake	no	no	no	nd	Ao	2011	2	2	100% (34-100)
Bajero lake	no	no	no	no	Ao	2005	0	33	0% (0-10)
La Güelga	no	no	no	yes	Ao	2011	0	20	0% (0-16)
Vega de Liordes	no	no	nd	no	Ao	2005	0	5	0% (0-43)
Pozu Llau	no	no	no	no	n/a	n/a	n/a	nd	n/a

†Wilson's confidence interval for a single proportion

Ao=*Alytes obstetricans*, Rt=*Rana temporaria*, Bb=*Bufo bufo*, Ma=*Mesotriton alpestris*, Tm=*Triturus marmoratus*, Lb=*Lissotriton boscai*, Nm=*Natrix Maura*, Ss=*Salamandra salamandra,* Lh=*Lissotriton helveticus,* nd=not done.

Supplemental Experimental Procedures

Genbank accession numbers by sample and locus are provided in the table below.

References for loci relate to CMTV complete genome (JQ231222). nd=not sequenced.

Sample ID	Virus species	Site	Host	Accession numbers by locus (CMTV ORF ref.)						
				13R	16L	22L	58L	59R	81L	82L
GA11001	BNV	Pontillon	*Natrix maura*	KJ703145	KJ703122	KJ703154	KJ703129	KJ703137	KJ703118	KJ703163
GA11002	BNV	Pontillon	*Lissotriton boscai*	KJ703144	KJ703120	KJ703155	KJ703130	KJ703138	KJ703114	KJ703161
GA11010	BNV	Pontillon	*Triturus marmoratus*	KJ703143	KJ703121	KJ703156	KJ703131	KJ703139	KJ703119	KJ703162
PE11001	CMTV	Áliva	*Alytes obstetricans*	KJ703148	KJ703124	KJ703151	KJ703134	KJ703142	KJ703115	KJ703158
PE11004	CMTV	Áliva	*Rana temporaria*	KJ703149	KJ703126	KJ703150	KJ703133	KJ703140	KJ703116	KJ703157
PE11112	AAOV	Ándara	*Alytes obstetricans*	KJ703146	KJ703123	KJ703153	KJ703132	KJ703136	KJ703113	KJ703159
PE11114	CMTV	Moñetas	*Mesotriton alpestris*	KJ703147	KJ703125	KJ703152	KJ703135	KJ703141	KJ703117	KJ703160
PE11103	CMTV	Ercina	*Alytes obstetricans*	nd	KJ703128	nd	nd	nd	nd	nd
PE11105	CMTV	Ercina	*Alytes obstetricans*	nd	KJ703127	nd	nd	nd	nd	nd