Available online at www.sciencedirect.com
SCIENCE DIRECT®
Biological Conservation 120 (2004) 115–119
BIOLOGICAL CONSERVATION
www.elsevier.com/locate/biocon

Presence of an emerging pathogen of amphibians in introduced bullfrogs *Rana catesbeiana* in Venezuela

Rhea Hanselmann [a], Argelia Rodríguez [b], Margarita Lampo [c,*], Laurie Fajardo-Ramos [c], A. Alonso Aguirre [d], A. Marm Kilpatrick [e], Jon Paul Rodríguez [c], Peter Daszak [e,*]

[a] *Tufts University School of Veterinary Medicine, 200 Westboro Road, North Grafton, MA 01536, USA*
[b] *Facultad de Ciencias, Núcleo La Hechicera, Universidad de los Andes, Mérida 5101, Venezuela*
[c] *Centro de Ecología, Instituto Venezolano de Investigaciones Científicas, Apartado 21827, Caracas 1020-A, Venezuela*
[d] *Wildlife Trust, 61 Route 9W, Palisades, NY 10964, USA*
[e] *Consortium for Conservation Medicine, Wildlife Trust, 61 Route 9W, Palisades, NY 10964, USA*

Received 7 October 2003; received in revised form 5 February 2004; accepted 9 February 2004

Abstract

Chytridiomycosis is an emerging fungal disease of amphibians responsible for mass mortalities and population declines globally. One hypothesis for its recent emergence is anthropogenic introduction of the causative agent *Batrachochytrium dendrobatidis* through trade in amphibians for pets, food and biocontrol. In this study, we examined histological samples from apparently healthy American bullfrogs *Rana catesbeiana* that have been introduced into the Venezuelan Andes. *B. dendrobatidis* was present in 96% (46/48) of the individuals examined. In contrast to cases of chytridiomycosis outbreaks, the majority (44/46) of frogs had few, small lesions consistent with little or no clinical disease and no unusual mortality was observed. These findings have implications for amphibian declines in Venezuela and elsewhere. First, the high prevalence of *Batrachochytrium* but lack of clinical signs or chytridiomycosis-related mortality suggests that *R. catesbeiana* may be a good reservoir for this parasite in Venezuela. Second, the presence of this emerging pathogen in an introduced amphibian species suggests that trade and introduction of amphibians should be monitored and controlled to halt the spread of this pathogen nationally and internationally.

Keywords: Amphibian declines; Emerging diseases; Host–parasite ecology; Chytridiomycosis; Bullfrog

1. Introduction

Amphibian populations have been declining globally over the past few decades (Halliday, 1998; Houlahan et al., 2000). Among the hypothesized causal factors are habitat loss, unsustainable harvesting, environmental contaminants, increasing UV radiation, climate change, introduced predators, and emerging diseases (Young et al., 2001; Collins and Storfer, 2003). Until recently, the cause of population declines in montane amphibian species in temperate and tropical regions of North, Central and South America and Australia remained enigmatic. In 1998, an emerging disease, chytridiomycosis, caused by a non-hyphal zoosporic (chytrid) fungus *Batrachochytrium dendrobatidis* was identified as the cause of amphibian mass mortality events associated with these declines (Berger et al., 1998; Longcore et al., 1999; Daszak et al., 2003). Chytridiomycosis has since been reported causing amphibian mass mortalities and population declines in North America (Muths et al., 2003), Europe (Bosch et al., 2001) and New Zealand (Waldman et al., 2001), and as the cause of at least one species extinction (Daszak et al., 2003). In South America, it has been reported from native amphibians in Ecuador (Ron and Merino, 2000) and Venezuela (Bonaccorso et al., 2003), and from farmed bullfrogs (*Rana catesbeiana*) in Uruguay (Mazzoni et al., 2003).

Venezuela has diverse amphibian fauna, with more than 300 species, seven of which are listed as threatened (critically endangered or endangered) and four more

* Corresponding authors.
E-mail addresses: mlampo@ivic.ve (M. Lampo), daszak@conservationmedicine.org (P. Daszak).

0006-3207/$ - see front matter
doi:10.1016/j.biocon.2004.02.013

listed as under lower risk (Gorzula and Señaris, 1998; Rodríguez and Rojas Suárez, 1999). Sharp population declines have been reported in *Atelopus* populations from the Venezuelan Andes (LaMarca and Reinthaler, 1991; Barrio-Amorós, 2001; Young et al., 2001). Recently, Bonaccorso et al. (2003) reported the presence of chytridiomycosis in a museum specimen of *Atelopus cruciger* from Venezuela. This specimen was one of the last two individuals of this species found (both were collected in 1986) and the authors proposed that chytridiomycosis may therefore be involved in Venezuelan amphibian population declines.

The bullfrog, *R. catesbeiana*, was first introduced as a food source into the state of Mérida, Venezuela, probably in the late 1990s. It has established dense populations in a series of natural and artificial ponds between 1800 and 2600 m altitude and has now been recorded in 14 water bodies as far as 4.3 km away from the presumed center of dispersion (Díaz de Pascual and Chacón, 2002). Recent papers have suggested that bullfrogs can be infected by *B. dendrobatidis* but do not develop chytridiomycosis, therefore can act as efficient reservoir hosts or carriers (Mazzoni et al., 2003; Daszak et al., in press). This, and their extensive introduction to new geographic regions (Kupferberg, 1997) suggests that bullfrogs may be involved in the spread of chytridiomycosis (Mazzoni et al., 2003; Daszak et al., in press). The present study involves histological examination of bullfrogs collected at the original site of their introduction into Venezuela. Histological data are reported, and the significance of these findings for conservation and disease spread is discussed.

2. Materials and methods

Live individuals of *R. catesbeiana* were collected 6–8 March 2002 from six different sampling sites around the periphery of a 30 ha pond at Hacienda Altos de Casa Vieja (08°36,872′N; 071°21,813′W) at 2370 m altitude in the area of La Carbonera in the State of Mérida, Venezuela. Bullfrogs were caught using a 6 m-long seine net. Individuals were sacrificed by pithing, classified according to their developmental stage, measured, weighed, and preserved in neutral-buffered 10% formalin.

Skin tissue was removed from the ventral abdominal and pelvic regions of adult individuals using a scalpel blade or a tissue biopsy punch (Baker's dermal punch 4 and 6 mm, J.A. Webster, Inc.). In addition, toe clips including interdigital membranes were obtained from the each individual. Tissue samples were washed in phosphate buffer, dehydrated through a graded alcohol series, embedded in paraffin wax, sectioned at 5 μm, stained with haematoxylin and eosin and examined on a light microscope (Humason et al., 1997).

3. Results

More than 1400 individuals of *R. catesbeiana* were collected in March 2002. We estimate the population at the site of introduction to be in the order of ten thousand individuals. Large numbers of larvae, recently-metamorphosed and adult bullfrogs were present at the site. Surveys conducted in surrounding areas later in the same year show that bullfrogs have spread from their original site of introduction to two further ponds: a natural pond located 900 m away and an artificial construction situated approximately 1000 m away.

Bullfrogs demonstrated no clinical signs of chytridiomycosis when collected. No unusual sloughing of the skin and no mortality was observed. Stained sections of skin from 48 post-metamorphic individuals were examined for the presence of *B. dendrobatidis* or lesions consistent with chytridiomycosis. *B. dendrobatidis* was found in 96% (46/48) individuals. The majority of these (96%, 44/46) contained only focal lesions, with less than 40% of the epithelium in any microscope section examined at low power containing *B. dendrobatidis*-infected cells. Skin sections from these individuals showed only very slight hyperplasia, with up to two keratinaceous cell layers present at the thickest part of the lesion (Fig. 1(a)). Two individuals (4% of the infected group, 2/46) showed mild hyperplasia and hyperkeratosis, with up to four layers of infected keratinaceous cells present in some regions and most (>80%) of the section skin surface infected (Fig. 1(b)). Empty or developing zoosporangia were the most commonly observed evidence of *B. dendrobatidis*, and were found in all specimens (Fig. 1(a) and (b)). Many empty zoosporangia contained septa characteristic of *B. dendrobatidis* (Fig. 1(a)). Zoosporangia containing zoospores were found in some heavily infected individuals (Fig. 1(c)).

4. Discussion

The histological findings reported here are consistent with infection by *B. dendrobatidis*, the causative agent of chytridiomycosis. This zoosporic fungus can be identified histologically by the presence of flask-shaped zoosporangia or the presence of characteristic septa in developing sporangia within the keratinaceous cells of post-metamorphic amphibian skin or within the keratinaceous mouthparts of amphibian larvae (Berger et al., 1998; Pessier et al., 1999; Green and Kagarise-Sherman, 2001).

The lesions we found in Venezuelan bullfrogs were not consistent with clinical or fatal chytridiomycosis, but with low intensity infection by the causative agent. Wild and experimentally-infected animals that have died of chytridiomycosis show extensive hyperkeratosis and a range of clinical signs (Berger et al., 1998; Nichols et al.,

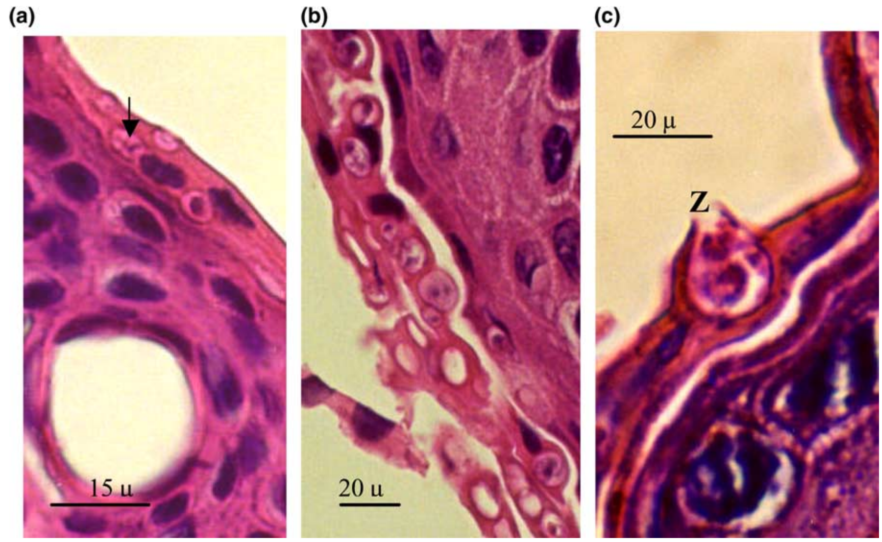

Fig. 1. Infection with *Batrachochytrium dendrobatidis* in introduced *Rana catesbeiana* from Venezuela: (a) focal lesion with slight thickening of keratinaceous cell layer. Developing zoosporangia are visible in the keratinaceous cells in the surface layer of the epithelium (black arrow); (b) developing zoosporangia and moderate hyperplasia of keratinaceous cells. The surface of the epithelium is on the left; (c) mature zoosporangium with discharge papilla (Z).

2001; Pessier et al., 1999). No clinical signs were observed in individuals collected in Venezuela, no mortality was observed and lesions were almost entirely small, focal and with little hyperkeratosis. Possible explanations for these data include: (1) the presence of an avirulent strain of *B. dendrobatidis* in Venezuela; (2) the majority of animals having recently been infected and therefore the presence of an early stage in the pathogenesis of chytridiomycosis; or (3) that bullfrogs are susceptible to infection by *B. dendrobatidis*, but are relatively resistant to chytridiomycosis. The first hypothesis is not the most parsimonious because experimental infections of bullfrogs using virulent isolates of *B. dendrobatidis* led to similar mild infections (Daszak et al., in press). The second hypothesis is unlikely because chytridiomycosis has a clinical course of around 3 weeks in susceptible frogs (Berger et al., 1998). The likelihood of sampling this population at the start of an epizootic is therefore low. Our data support the third hypothesis, that *R. catesbeiana* can act as a reservoir host for chytridiomycosis. The high prevalence of *B. dendrobatidis* at our survey site and the rapidly expanding population of the host suggests that chytridiomycosis has a negligible effect on bullfrog populations in Venezuela. Our findings are consistent with reports of *B. dendrobatidis* in wild bullfrogs in Quebec, Canada and California, USA Carey et al., 2003; captive bred bullfrogs from a supplier in Idaho, USA (Daszak et al., in press); farmed bullfrogs in Uruguay (Cunningham et al., 2003; Mazzoni et al., 2003); and experimentally-infected bullfrogs (Daszak et al., in press). In all of these cases, infection by *B. dendrobatidis* caused focal lesions with mild hyperkeratosis, no clinical signs of chytridiomycosis and no evidence of mortality or population declines due to the fungus.

Two factors may heighten the role of bullfrogs on the spread and impact of chytridiomycosis in Venezuela. First, in Mérida, different developmental stages of the bullfrog were observed at very high densities in the studied pond, suggesting that adults are breeding continuously and at a high rate. This may be due to the lack of significant seasonal changes in temperature and humidity, allowing year-round bullfrog reproduction and an increase in the reproductive potential. Second, it is known that in autoclaved lake water *B. dendrobatidis* can survive saprophytically outside the amphibian host for up to seven weeks (Johnson and Speare, 2003) and therefore, in the wild, may be carried by mechanical vectors or fomites to geographically distant amphibian populations outside the range of *R. catesbeiana* dispersal.

The precise dates of introduction of *R. catesbeiana* into the Venezuelan Andes are unknown. According to a report filed in the Venezuelan Government Ministry of Environment, import permits were denied in 1993 for the settling of breeding farms in Edo. Táchira (Babarro and Trejo, 2001). The presence of *R. catesbeiana* in the Jají region was first reported in 2001 (Barrio-Amorós, 2001). The finding of *B. dendrobatidis* in a native Venezuelan amphibian collected in 1986 (Bonaccorso et al., 2003) suggests that the presence of chytridiomycosis in Venezuela may therefore precede the introduction of bullfrogs into Venezuela. Even if this is the case, it is still likely that the presence of dense populations of bullfrogs that are expanding geographically will affect native amphibian fauna by increasing the overall number of infected hosts (and therefore transmission rate) and promoting spread of this disease. A similar situation has occurred in New Zealand, where bovine TB was previously present, but the recent introduction of an efficient reservoir host for TB (the brushtail possum) has led to the re-emergence of this disease in cattle (Atkinson and Cameron, 1993). For this reason, we urge that measures be taken to rapidly eradicate this exotic species in Venezuela while the distribution is still local.

Previous authors have suggested that bullfrogs are likely involved in the anthropogenic spread of chytridiomycosis in South America and elsewhere (Mazzoni et al., 2003; Daszak et al., in press). Such a scenario is supported by molecular phylogenetic data and the pattern of geographic spread of amphibian declines (Morehouse et al., 2003; Daszak et al., 2003, 1999). Recent papers have demonstrated *B. dendrobatidis* in amphibians that are part of national and international trades as pets, food, lab animals, zoo animals and others (Cunningham et al., 2003). In South America, an increasingly intensive, international trade in bullfrogs as food animals has developed and multiple introductions of North American bullfrog stock have occurred over the last few decades (Mazzoni et al., 2003). Individuals raised for this food trade are transported within countries and internationally, with around 1 million bullfrogs imported live into the USA annually for the restaurant trade (Cunningham et al., 2003). This growing trade and the lack of concern for the negative environmental impact caused by alien species suggest that further establishment of alien populations is likely.

Bullfrogs have been introduced into the western USA, Mexico, Asia, Europe and South America (Kupferberg, 1997; Neck, 1981; Stumpel, 2003). Introduced bullfrogs compete with endemic species and perturb community structure (Kupferberg, 1997; Kiesecker and Blaustein, 1997). The potential of *R. catesbeiana* to act as an efficient carrier of chytridiomycosis adds to its capacity as an invasive species threat to amphibian populations (Kats and Ferrer, 2003). We suggest that domestic and international trade in amphibians be made subject to the veterinary surveillance and quarantine guidelines published by the International Union for the Conservation of Nature (IUCN) and Office Internationale des Epizooties (OIE) (Cunningham et al., 2001) and that stricter controls are put in place for amphibian trade. Such preventative conservation measures are likely to be less costly and more effective than future control and eradication and may help to prevent future extinction events (Leung et al., 2002; Rodríguez, 2001). Finally, our paper adds to a growing series of cases where anthropogenically-introduced diseases threaten native fauna. This previously underestimated form of environmental change (termed pathogen pollution) is responsible for a series of wildlife population declines and extinctions globally (Daszak et al., 2000; Cunningham et al., 2003). We strongly urge that conservation biologists recognize the key role that infectious diseases play as threats to global biodiversity.

Acknowledgements

This study was funded by core funding to the Consortium for Conservation Medicine from the V. Kann Rasmussen Foundation and a National Science Foundation IRCEB Grant (IBN #9977063). The authors would like to thank the following for their help and support: Mariluz Perozo, J. Celsa Señaris and Gilson Rivas (Museo de Historia Natural La Salle), Amelia Díaz de Pascual, Luisana Avilán and Amelia Díaz (Universidad de los Andes).

References

Atkinson, I.A.E., Cameron, E.K., 1993. Human influence on the terrestrial biota and biotic communities of New Zealand. Trends in Ecology and Evolution 8, 447–451.

Babarro, R. and Trejo, V. 2001. Informe relativo al presunto establecimiento de *Rana catesbeiana* en cuerpos de agua del Estado Mérida. Dirección General de Fauna, Ministerio de Ambiente y Recursos Naturales. Caracas, Venezuela.

Barrio-Amorós, C.L., 2001. Amphibian decline in Venezuela – the state of knowledge. Froglog 47, 2–4.

Berger, L., Speare, R., Daszak, P., Green, D.E., Cunningham, A.A., Goggin, C.L., Slocombe, R., Ragan, M.A., Hyatt, A.D., McDonald, K.R., Hines, H.B., Lips, K.R., Marantelli, G., Parkes, H., 1998. Chytridiomycosis causes amphibian mortality associated with population declines in the rain forests of Australia and Central America. Proceedings of the National Academy of Science, USA 95, 9031–9036.

Bonaccorso, E., Guayasamin, J.M., Méndez, D., Speare, R., 2003. Chytridiomycosis in a Venezuelan anuran (Bufonidae: *Atelopus cruciger*). Herpetological Review 34, 331–334.

Bosch, J., Martínez-Solano, I., García-París, M., 2001. Evidence of chytrid fungus infection in the decline of the common midwife toad (*Alytes obstetricans*) in protected areas of central Spain. Biological Conservation 97, 331–337.

Carey, C., Bradford, D.F., Brunner, J.L., Collins, J.P., Davidson, E.W., Longcore, J.E., Ouellet, M., Pessier, A.P., Schock, D.M., 2003. Biotic factors in amphibian population declines. In: Linder, G., Sparling, D.W., Krest, S.K. (Eds.), Multiple Stressors and Declining Amphibian Population Declines. Society for Environmental Chemistry and Toxicology (SETAC Press).

Collins, J.P., Storfer, A., 2003. Global amphibian declines: sorting the hypotheses. Diversity and Distributions 9, 89–98.

Cunningham, A.A., Daszak, P., Hyatt, A.D., 2001. Amphibia. In: Woodford, M.H. (Ed.), Quarantine and Health Screening Protocols for Wildlife Prior to Translocation and Release into the Wild. Office International des Epizooties (OIE), Paris, France and IUCN Species Survival Commission's Veterinary Specialist Group, Gland, Switzerland, pp. 74–79.

Cunningham, A.A., Daszak, P., Rodríguez, J.P., 2003. Pathogen pollution: defining a parasitological threat to biodiversity conservation. Journal of Parasitology 89 (Suppl.), S78–S83.

Daszak, P., Cunningham, A.A., Hyatt, A.D., 2000. Emerging infectious diseases of wildlife – threats to biodiversity and human health. Science 287, 443–449.

Daszak, P., Cunningham, A.A., Hyatt, A.D., 2003. Infectious disease and amphibian population declines. Diversity and Distributions 9, 141–150.

Daszak, P., Berger, L., Cunningham, A.A., Hyatt, A.D., Green, D.E., Speare, R., 1999. Emerging infectious diseases and amphibian population declines. Emerging Infectious Diseases 5, 735–748.

Daszak, P., Strieby, A., Cunningham, A.A., Longcore, J.E., Brown, C.C., Porter, D. Experimental evidence that the bullfrog (*Rana catesbeiana*) is a potential carrier of chytridiomycosis, an emerging fungal disease of amphibians. Herpetological Journal, in press.

Gorzula, S., and J.C. Señaris., 1998. Contribution to the herpetofauna of the Venezuelan Guayana. I.A. Data Base. Scientiae Guaianae 8. FUNDACITE-Guayana, Fundación Instituto Botànico de Venezuela. Caracas, Venezuela.

Green, D.E., Kagarise-Sherman, C., 2001. Diagnostic histological findings in Yosemite toads (*Bufo canorus*) from a die-off in the1970s. Journal of Herpetology 35, 92–103.

Halliday, T., 1998. A declining amphibian conundrum. Nature 394, 418–419.

Houlahan, J.E., Findlay, C.S., Schmidt, B.R., Meyer, A.H., Kuzmin, S.L., 2000. Quantitative evidence for global amphibian population declines. Nature 404, 752–755.

Humason, G.L., Presnell, J.K., Schreibman, M.P. (Eds.), 1997. Humason's Animal Tissue Techniques, fifth ed. The John Hopkins University Press, Baltimore.

Johnson, M.L., Speare, R., 2003. Survival of *Batrachochytrium dendrobatidis* in water: quarantine and disease control implications. Emerging Infectious Diseases 9, 922–925.

Kats, L.B., Ferrer, R.P., 2003. Alien predators and amphibian declines: review of two decades of science and the transition to conservation. Diversity and Distributions 9, 99–110.

Kiesecker, J.M., Blaustein, A.R., 1997. Population differences in responses of red-legged frogs (*Rana aurora*) to introduced bullfrogs (*Rana catesbeiana*). Ecology 78, 1752–1760.

Kupferberg, S.J., 1997. Bullfrog (*Rana catesbeiana*) invasion of a California river: The role of larval competition. Ecology 78, 1736–1751.

LaMarca, E., Reinthaler, K.P., 1991. Population changes in *Atelopus* species of the Cordillera de Mérida, Venezuela. Herpetological Review 22, 125–128.

Leung, B., Lodge, D.M., Finnoff, D., Shogren, J.F., Lewis, M.A., Lamberti, G., 2002. An ounce of prevention or a pound of cure: bioeconomic risk analysis of invasive species. Proceedings of the Royal Society of London Series B-Biological Sciences 269, 2407–2413.

Longcore, J.E., Pessier, A.P., Nichols, D.K., 1999. *Batrachochytrium dendrobatidis* gen. et sp. nov., a chytrid pathogenic to amphibians. Mycologia 91, 219–227.

Mazzoni, R., Cunningham, A.A., Daszak, P., Apolo, A., Perdomo, E., Speranza, G., 2003. Emerging pathogen of wild amphibians in frogs (*Rana catesbeiana*) farmed for international trade. Emerging Infectious Diseases 9, 995–998.

Morehouse, E.A., James, T.Y., Ganley, A.R.D., Vilgalys, R., Berger, L., Murphy, P.J., Longcore, J.E., 2003. Multilocus sequence typing suggests that the chytrid pathogen of amphibians is a recently emerged clone. Molecular Ecology 12, 395–403.

Muths, E., Corn, P.S., Pessier, A.P., Green, D.E., 2003. Evidence for disease-related amphibian decline in Colorado. Biological Conservation 110, 357–365.

Neck, R.W., 1981. Probable native population of bullfrog, *Rana catesbeiana*, in South Texas. Herpetological Review 12, 68.

Nichols, D.K., Lamirande, E.W., Pessier, A.P., Longcore, J.E., 2001. Experimental transmission of cutaneous chytridiomycosis in dendrobatid frogs. Journal of Wildlife Diseases 37, 1–11.

Pessier, A.P., Nichols, D.K., Longcore, J.E., Fuller, M.S., 1999. Cutaneous chytridiomycosis in poison dart frogs (*Dendrobates* spp.) and White's tree frogs (*Litoria caerulea*). Journal of Veterinary Diagnostic Investigation 11, 94–199.

Rodríguez, J.P., 2001. Exotic species introductions into South America: an underestimated threat? Biodiversity and Conservation 10, 1983–1996.

Rodríguez, J.P., Rojas-Suárez, F., 1999. Libro Rojo de la Fauna Venezolana, second ed. PROVITA: Fundación Polar, Caracas.

Ron, S.R., Merino, A., 2000. Amphibian declines in Ecuador: overview and first report of chytridiomycosis from South America. Froglog 42, 2–3.

Stumpel, A.H.P., 2003. Succesful reproduction of introduced bullfrogs *Rana catesbeiana* in northwestern Europe: a potential threat to indigenous amphibibians. Biological Conservation 60, 61–62.

Young, B.E., Lips, K.R., Reaser, J.K., Ibañez, R., Salas, A.W., Cedeño, J.R., Coloma, L.A., Ron, S., LaMarca, E., Meyer, J.R., Munoz, A., Bolanos, F., Chaves, G., Romo, D., 2001. Population declines and priorities for amphibian conservation in Latin America. Conservation Biology 15, 1213–1223.

Waldman, B., van de Wolfshaar, K.E., Klena, J.D., Andjic, V., Bishop, P.J., Norman, R.J.deB., 2001. Chytridiomycosis in New Zealand frogs. Surveillance 28, 9–11.