Journal of Wildlife Diseases, 48(3), 2012, pp. 835–839

Road-killed Common Toads (*Bufo bufo*) in Flanders (Belgium) Reveal Low Prevalence of Ranaviruses and *Batrachochytrium dendrobatidis*

An Martel,[1,5,6] Mojdeh Sharifian Fard,[1,5] Pascale Van Rooij,[1] Robert Jooris,[2] Francine Boone,[3] Freddy Haesebrouck,[1] David Van Rooij,[4] and Frank Pasmans[1] [1] Department of Pathology, Bacteriology and Avian Diseases, Ghent University Salisburylaan 133, B-9820 Merelbeke, Belgium; [2] Natuurpunt–Hyla Coxiestraat 11, 2800 Mechelen, Belgium; [3] Nachtegaalstraat 91, 1501 Buizingen, Belgium; [4] Renard Centre of Marine Geology, Department of Geology and Soil Science, Ghent University Krijgslaan 281 S8, B-9000 Ghent, Belgium; [5] These authors contributed equally to this work; [6] Corresponding author (email: An.Martel@ugent.be)

ABSTRACT: Road-killed common toads (*Bufo bufo*; $n=1{,}740$) from Flanders, Belgium, were tested for ranavirus and *Batrachochytrium dendrobatidis* (Bd) using polymerase chain reaction. Both infections were present at a very low prevalence (<0.2% with a confidence interval of 95% for ranavirus and 0.63% for Bd).

Healthy amphibians can be asymptomatic carriers of diseases responsible for devastating amphibian population declines. Most importantly, ranavirus and *Batrachochytrium dendrobatidis* (Bd) are primary pathogens of amphibians, and disease outbreaks are believed to be driven by environmental factors (Walker et al., 2010). A thorough risk analysis to predict future scenarios in indigenous amphibian species should include the identification of principle pathogen reservoirs. The difficulty in identifying amphibian, clinically healthy reservoirs for both agents is that asymptomatically infected animals often have low-grade infections. Although noninvasive sampling (e.g., skin swabs) enables the detection of both pathogens, tissue samples provide a higher detection probability. However, conservation concerns hamper tissue sampling of wild amphibians.

Traffic kills many amphibians on roads yearly, especially during spring migration (Elzanowski et al., 2009). These road victims could provide a good opportunity for monitoring ranaviruses and Bd in native amphibians. In Flanders (Belgium), the common toad (*Bufo bufo*) provides the most obvious choice to monitor both infections for four reasons: Common toads are commonly killed by traffic during spring migration; migrating toads are counted on numerous sites in Flanders, enabling an estimate of population trends; the common toad is a suitable host for both ranaviruses and Bd (Bosch and Marinez-Solano, 2006; Cunningham et al., 2007); and toad populations have been found to be declining in several regions (Loras et al., 2011). Our goal was to assess ranavirus and Bd infections in common toads using tissues of road-killed specimens during the migration season.

Between 27 February 2011 and 7 April 2011, 1,740 road-killed common toads were collected from 104 migration sites in all five Flemish provinces (Fig. 1). Each toad was placed in a plastic bag that was sealed and labeled. For detection of Bd DNA, a quantitative polymerase chain reaction (qPCR; Boyle et al., 2004) was performed on a skin sample of each toad. For detection of ranavirus infections, a liver sample was examined by PCR using the primers MCP 4 and MCP 5 (Mao et al., 1997). To assess whether the presence of one or both pathogens in a toad population coincides with obvious population declines, if available, numbers of migratory toads of 2011 and previous years were extracted using the database obtained from the working group Natuurpunt Hyla (Hyla Werkgroep, 2012).

None of the samples were PCR-positive for ranaviruses. At nine sites (three in the province of East Flanders, three in Flemish Brabant, and three in Limburg), 11 Bd-positive toads were found (0.63%

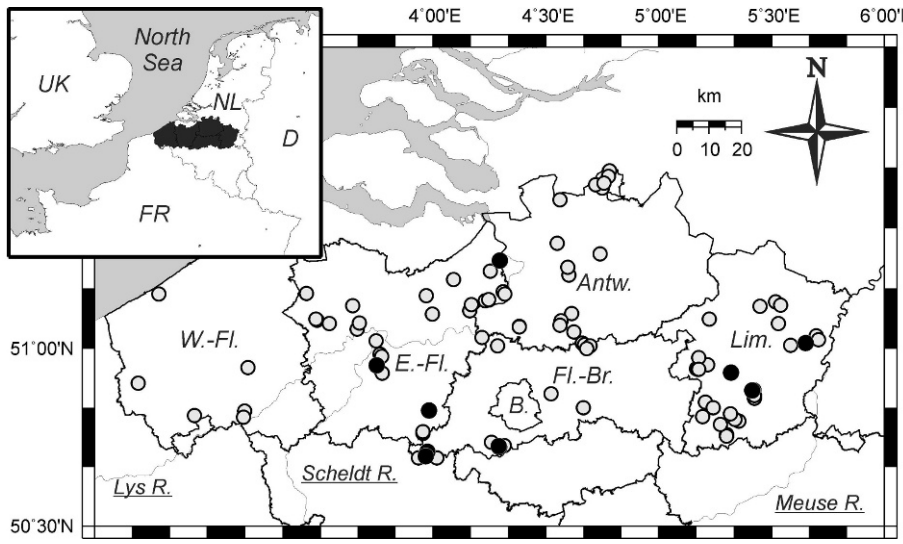

FIGURE 1. Locations in Flanders (Belgium) where road-killed common toads (*Bufo bufo*) were collected during the migration season from 27 February 2011 to 7 April 2011. Locations where *Batrachochytrium dendrobatidis* was found are indicated by black circles and negative locations by grey circles. FR=France; NL=The Netherlands; D=Germany; W.-Fl.=West-Flanders; E.-Fl.=East Flanders; Antw.=Antwerp; Fl.-Br.=Flemish Brabant; B.=Brussels; Lim.=Limburg; R.=river.

prevalence; Fig. 1). Levels of Bd in the positive samples were relatively low, with an average±SD of 0.25±0.11 genomic equivalents. Data on the number of migrating toads were available for four of the nine infected sites. For three additional sites, numbers of migrating toads were available within a region of 5 km. For all positive sites, no significant decrease in the number of migrating toads was observed during the years monitored (Table 1).

This study demonstrates that if ranavirus infections are present in adult common toads in Flanders, the prevalence is very low (<0.2% with a confidence interval of 95%). Thus, we can reject the hypothesis that, at present, adult common toads provide an important reservoir for ranaviruses in the area examined. However, these results should not be overinterpreted. First, only adult toads were sampled during the migration season, implying that these toads survived hibernation well and were healthy enough to show reproductive (migratory) behavior. Second, all samples were collected during the relatively short migration period and thus might not be representative of the entire population. Third, it is possible that the adults survived a past ranavirus infection and have developed antibodies, as was shown for *Bufo marinus* (Zupanovic et al., 1998).

Our data suggest that that ranaviruses might be absent from some native common toad populations. If true, then introduction of a ranavirus into these populations can result in disease outbreaks. Furthermore, introduction of a novel ranavirus into any population can result in mass morbidity or mortality. Neither scenario is unlikely because ranaviruses have been demonstrated in captive amphibians in Flanders (Pasmans et al., 2008), in exotic invasive bullfrogs (*Lithobates catesbeianus*) in Flanders (Sharifian Fard et al. 2011), and recently in a large ranavirus outbreak in the neighboring Netherlands (Kik et al., 2011).

The low prevalence of Bd in the study area stresses the importance of a large

TABLE 1. Yearly numbers of migrating common toads (*Bufo bufo*) at a *Batrachochytrium dendrobatidis*–positive site in Flanders (Belgium). Data from Natuurpunt Hyla (Hyla Werkgroep, 2012).[a]

Site (province)	GPS coordinates	No. positive samples/no. tested	2002	2003	2004	2005	2006	2007	2008	2009	2010	2011
Beveren (east Flanders)[a]	51°14′38.30″N 4°17′ 39.03″E	2/12	ND	609	655	852	1,204	855	1,038	817	696	1,174
Munte (east Flanders)	50°57′ 03.98″N 3°45′ 00.00″E	1/48	986	799	1,253	1,603	1,712	1,947	937	819	1,129	679
Ninove (east Flanders)[a]	50°49′ 28.73″N 3°58′ 53.58″E	1/70	ND	ND	ND	ND	ND	ND	ND	391	524	ND
Dworp (Flemish Brabant)[a]	50°43′ 19.03″N 4°17′ 31.77″E	2/144	ND	ND	ND	ND	761	731	ND	663	1,995	3,651
Diepenbeek (Limburg)[a]	50°52′ 53.76″N 5°24′ 48.86″E	1/1	ND	ND	ND	1,027	1,322	713	1,060	495	ND	ND
Dilsen-stokkem (Limburg)[b]	51°00′ 47.76″N 5°39′ 03.04″E	1/5	3,594	3,886	3,577	4,062	3,993	2,073	1,809	1,077	1,782	2,244
Hasselt (Limburg)[a,b]	50°55′ 52.72″N 5°19′ 08.83″E	1/79	ND	ND	729	708	ND	211	134	ND	168	334
Bever (Flemish Brabant)[a]	50°42′ 31.96″N 3°58′ 25.96″E	1/35	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Sint Pieters Kapelle (Flemish Brabant)[a]	50°41′ 49.95″N 3°57′ 59.56″E	1/90	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND

[a] ND=no data available

[b] Data recorded at a location within 5 km of the positive sample.

sample size if the presence of Bd in a seemingly healthy amphibian population needs to be excluded. Population trends in infected common toad populations, as derived from data on the migrating toads, are not suggestive of population declines over the last 5 yr. Moreover, the authors of this paper are the referral center for disease outbreaks of amphibians in Flanders, and no proven outbreak of chytridiomycosis has yet been confirmed. The only confirmed case of lethal chytridiomycosis was an isolated case in a midwife toad (*Alytes obstetricans*) in the Belgian Ardennes (Pasmans et al., 2010). The combination of low prevalence, low Bd load in infected animals, absence of obvious negative population trends, and absence of confirmed chytridiomycosis outbreaks, suggests that Bd is endemic at a low intensity in the Flemish region. However, the pathogenic potential of Bd has been demonstrated on numerous occasions, extirpating entire amphibian species (Stuart et al. 2004; Wake and Vredenburg 2008). Again, our results should be interpreted carefully. Besides the caveats mentioned above, seasonality of Bd infection dynamics is well documented. The occurrence of clinical chytridiomycosis depends on numerous factors, including strain virulence, host susceptibility, and environmental parameters. Given current climate change, epizootics might be expected in the future. Thus, vigilance systems would be advisable to monitor Bd infection dynamics.

We thank Natuurpunt volunteers who sampled road victims.

LITERATURE CITED

Bosch, J., and I. Martinez-Solano. 2006. Chytrid fungus infection related to unusual mortalities of *Salamandra salamandra* and *Bufo bufo* in the Penalara Natural Park, Spain. Oryx 40: 84–89.

Boyle, D. G., D. B. Boyle, V. Olsen, J. A. T. Morgan, and A. D. Hyatt. 2004. Rapid quantitative detection of chytridiomycosis (*Batrachochytrium dendrobatidis*) in amphibian samples using real-time Taqman PCR assay. Diseases of Aquatic Organisms 60: 133–139.

Cunningham, A. A., A. D. Hyatt, P. Russel, and P. M. Bennett. 2007. Experimental transmission of a ranavirus disease of common toads (*Bufo bufo*) to common frogs (*Rana temporaria*). Epidemiology and Infection 135: 1213–1216.

Elzanowski, A., J. Ciesiolkiewicz, M. Kaczor, J. Radwariska, and R. Urabn. 2009. Amphibian road mortality in Europe: A meta-analysis with new data from Poland. European Journal of Wildlife Research 55: 33–43.

Hyla Werkgroep. 2012. Natuurpunt Hyla. Available at http://www.hylawerkgroep.be/. Accessed March 2012.

Kik, M., A. Martel, A. Spitzen-van der Sluijs, F. Pasmans, P. Wohlsein, A. Gröne, and J. M. Rijks. 2011. Ranavirus-associated mass mortality in wild amphibians, the Netherlands, 2010. A first report. Veterinary Journal 190: 284–286.

Loras, A. F., J. Hidalgo-Vila, C. Hermosilla, G. Garcia, J. Lopez, A. L. J. Duffus, A. A. Cunningham, and V. Raco. 2011. Preliminary health screening and possible pathogen determination in a *Bufo bufo* (Linnaeus, 1758) (Amphibia: Bufonidae) population. Journal of Natural History 45: 1–14.

Mao, J., R. P. Hedrick, and V. B. Chichar. 1997. Molecular characterization, sequence analysis, and taxonomic position of newly isolated fish iridoviruses. Virology 229: 212–220.

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

———, M. Muijsers, S. Maes, P. Van Rooij, M. Brutyn, R. Ducatelle, F. Haesebrouck, and A. Martel. 2010. Chytridiomycosis related mortality in a midwife toad (*Alytes obstetricans*) in Belgium. Flemish Veterinary Journal 79: 460–462.

Sharifian Fard, M., F. Pasmans, C. Adriaensen, S. Devisscher, T. Adriaens, G. Louette, and A. Martel. 2011. Ranavirosis in invasive bullfrogs, Belgium. Emerging Infectious Diseases 17: 2371–2372.

Stuart, S. N., J. S. Chanson, N. A. Cox, B. E. Young, A. S. L. Rodrigues, D. L. Fischman, and R. W. Waller. 2004. Status and trends of amphibian declines and extinctions worldwide. Science 306: 1783–1786.

Wake, D. B., and V. T. Vredenburg. 2008. Are we in the midst of the sixth mass extinction? A view from the world of amphibians. Proceedings of the National Academy of Sciences of the USA 105: 11466–11473.

Walker, S. F., J. Bosch, V. Gomez, T. W. J. Garner, A. A. Cunningham, D. S. Schmeller, M. Ninyerola, D. A. Henk, C. Ginestet, C. P. Arthur, and M. C. Fisher. 2010. Factors driving pathogenicity vs. prevalence of amphibian pan-

zootic chytridiomycosis in Iberia. Ecology Letters 13: 372–382.

Zupanovic, Z., G. Lopez, A. E. Hyatt, B. Green, G. Bartran, H. Parkes, R. J. Whittington, and R. Speare. 1998. Giant toads *Bufo marinus* in Australia and Venezuela have antibodies against 'ranaviruses'. Diseases of Aquatic Organisms 32: 1–8.

Submitted for publication 9 November 2011.
Accepted 4 March 2012.