The Absence of Zoonotic Agents in Invasive Bullfrogs (*Lithobates catesbeianus*) in Belgium and The Netherlands

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Abstract: Exotic invasive bullfrogs (*Lithobates catesbeianus*) are considered to exert a considerable negative impact on native amphibian communities. This can be due to competition and predation, but they are also a notorious source of the infectious diseases chytridiomycosis and ranavirosis, affecting amphibian populations globally. Little is known regarding their carriage of other microbial agents that might be transferred to humans or other animals. In this study we determined the occurrence of the amphibian pathogens *Ranavirus* and *Batrachochytrium dendrobatidis* and of the zoonotic agents *Coxiella burnetii*, *Neospora caninum*, *Leptospira* sp., *Toxoplasma gondii*, *Mycoplasma* sp., *Campylobacter* sp., *Salmonella* sp. and extended-spectrum beta-lactamase producing *Escherichia coli* in 164 bullfrogs from three populations in Belgium and The Netherlands. Although

*B. dendrobatidis* was present at a high prevalence of 63%, mean infection loads were low with an average of 10.9

genomic equivalents (SD 35.5), confirming the role of bullfrogs as *B. dendrobatidis* carriers, but questioning their role as primary reservoirs for *B. dendrobatidis* transmission to native amphibian communities. All tested samples were negative for the other infectious agents examined. These results suggest a limited role of bullfrogs as carrier of these pathogens.

Keywords: amphibian, *Lithobates catesbeianus*, zoonosis

Bullfrogs (*Lithobates catesbeianus*) have been accidentally introduced in several European countries and have estab- lished substantial breeding populations in France, Italy, Germany, Greece, Belgium and The Netherlands (Ficetola et al. [2007](#_bookmark12); Veenvliet [1996](#_bookmark29)). They are considered to exert a considerable negative impact on native amphibian com- munities by competition and predation (Johnson et al.

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[2011](#_bookmark24); Kiesecker and Blaustein [1998](#_bookmark28); Kupferberg [1997](#_bookmark30); Pearl et al. [2004](#_bookmark18)). Additionally, bullfrogs are a notorious source of the notifiable infectious diseases chytridiomycosis and ranavirosis, affecting amphibian populations globally (Garner et al. [2006](#_bookmark14); Sharifian-Fard et al. [2011](#_bookmark22)).

Besides their impact on amphibian populations, bull- frogs could be a source of microbial pathogens for other animals or humans. Bullfrogs in Belgium and The Neth- erlands are often found in ponds created for recreational

fishing and gardens, rendering indirect contact with hu- mans or animals through water likely (Jooris [2002](#_bookmark27); Veenvliet [2009](#_bookmark31)).

On several occasions, amphibians have been implicated as a source of *Salmonella* infections to humans (Chambers and Hulse [2006](#_bookmark2); Clarkson et al. [2010](#_bookmark3); Everard et al. [1979](#_bookmark4); Sharma et al. [1974](#_bookmark25); Singh et al. [1979](#_bookmark26)). *Leptospira* has also been isolated from amphibians, suggesting that amphibians may promote *Leptospira* infections in aquatic environments (Diesch et al. [1966](#_bookmark5), [1970](#_bookmark6); Gravekamp et al. [1991](#_bookmark19); Everard et al. [1988](#_bookmark9), [1990](#_bookmark10)). However, little is known regarding amphibians carrying the zoonotic agents *Coxiella burnetii*, *Neospora caninum*, *Toxoplasma gondii*, *Mycoplasma* sp. or thermotolerant *Campylobacter* sp.

The environment, including wild birds and mammals (Guenther et al. [2011](#_bookmark20); Garmyn et al. [2011](#_bookmark13)), constitutes an important reservoir of extended-spectrum beta-lactamase (ESBL) producing *Escherichia coli* isolates. ESLBs are en- zymes which are responsible for resistance to Beta-lactam antibiotics like penicillins, cephalosporins and carbapen- ems. The emergence and wide dissemination of these ESBLs constitute a serious public health concern (Savard and Perl [2012](#_bookmark21)). Whether amphibians play a role as a reservoir of these strains is not known.

We sampled 164 bullfrogs from three populations in Belgium and The Netherlands to evaluate the extent to which these frogs were hosts for the chytrid fungus *Ba- trachochytrium dendrobatidis*, *Ranavirus* and the zoonotic pathogens *C. burnettii*, *N. caninum*, *Leptospira* sp., *T. gon-*

*dii*, *Mycoplasma* sp., *Campylobacter* sp., *Salmonella* and of

ESBL producing *E. coli*. This work will help determine wether recommendations to prevent the spread of bullfrogs and to eradicate their populations are justified (Doubledee et al. [2003](#_bookmark7); Ficetola et al. [2007](#_bookmark12); Govindarajulu et al. [2005](#_bookmark16); Luja and Rodriguez-Estrella [2010](#_bookmark9)).

One hundred and sixty-four clinically healthy *L.*

*catesbeianus* were collected from 3 populations in Belgium and The Netherlands between June 2010 and October 2011. In Belgium, six animals were collected from a bullfrog population located at a private fishing pond in Hoogstraten (N51°280 E04°450) and 34 animals were collected from a private pond in Arendonk (N51°190 E05°060) (Louette et al. [2012](#_bookmark35)). In The Netherlands, animals were captured from a population in Baarlo (N51°200 E06°050).

All animals were euthanized for an invasive species eradication project (INVEXO). This eradication pro- gramme for bullfrogs was recently initiated in the frame- work of the Interreg project ‘Fighting alien invasive species

along the Dutch–Belgian border (northwest Europe)’ (INVEXO—Invasieve exoten in Vlaanderen en Zuid- Nederland, Interreg IVa-VLANED-2.31). Samples of skin, liver, heart, lung, kidney and intestines were taken imme- diately after euthanasia and stored at -20°C until further use. Intestinal content for bacterial isolation was processed immediately.

All faecal samples were analyzed using the ISO 6579:2002 method for the isolation of *Salmonella*. For the isolation of ESBL producing *E. coli*, the intestinal samples were inoculated within 4 h onto MacConkey agar plates (Oxoid Ltd., Basingstoke, UK) supplemented with ceftiofur (8 mg/L). After overnight aerobic incubation at 37°C, suspected *E. coli* colonies were purified on Columbia agar with 5% sheep blood (blood agar, Oxoid) and phenotypi- cally identified. For the isolation of *Campylobacter* sp., faecal samples were plated on modified charcoal cefope- razone deoxycholate agar (mCCDA, CM0739; Oxoid) supplemented with CCDA selective supplement (SR0155E; Oxoid) and *Campylobacter*-specific growth supplement (SR0232E; Oxoid), followed by microaerobic incubation at 42°C for 48 h.

For the molecular detection of the above-listed pathogens, DNA was extracted from skin, lung, heart, kidney and liver using the DNEasy Tissue Kit (Qiagen, Hilden, Germany). DNA from the intestinal content was prepared using the Stool kit (Qiagen). The DNA from the skin was used in the qPCR to detect *B. dendrobatidis* (Boyle et al. [2004](#_bookmark1)). All samples were run in duplicate. To control and estimate inhibition, a subset of samples (*n* = 50) were retested under the same conditions as described above, but with an exogenous internal positive control (VICTM probe, Life technologies, Austin, TX, USA) included as described by Hyatt et al. [2007](#_bookmark23). We did not find any indications for PCR inhibition. The PCR to detect *Ranavirus* using the MP4 and 5 primers described by Mao et al. ([1997](#_bookmark11)) was performed on the DNA samples obtained from the liver. On the lung samples the PCR described by Van Kuppleveld et al. ([1992](#_bookmark27)) was performed to detect *Mycoplasma* sp. The

intestinal derived DNA was used in PCRs to detect *Sal-*

*monella* sp. (Chiu and Ou [1996](#_bookmark8)) and *Campylobacter* sp. (Linton et al. [1996](#_bookmark32)). The detection of *T. gondii* (used samples: heart and liver), *N. caninum* (used samples: heart and liver), *Leptospira* sp. (used samples: liver and kidney) and *C. burnetti* (used samples: intestines and liver) was done using commercial PCR kits (Adiagene, Paris, France).

The absence of a given pathogen was calculated using Win Episcope 2.0.

Table 1. Prevalence of *B. dendrabatidis* in three populations of invasive bullfrogs in Belgium and The Netherlands.

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| Population site | Numbers of tested animals | Prevalence of*B. dendrobatidis* (%) | Range of GE | Mean *B. dendrobatidis*load (GE) | Standard deviation |
| Hoogstraten (Belgium) | 6 | 67 | 1.7–4.3 | 1.6 | 1.6 |
| Arendonk (Belgium) | 34 | 62 | 4.4–102 | 21 | 30.3 |
| Baarlo (The Netherlands) | 124 | 68 | 1.6–368 | 8.6 | 37.4 |

*Salmonella*, *Campylobacter* and ESBL carrying *E. coli* were not isolated from any of the samples. All samples were negative in the PCRs for the detection of *Ranavirus*, *C. burnetii*, *N. caninum*, *Leptospira* sp., *T. gondii*, *Mycoplasma* sp., *Campylobacter* sp., *Helicobacter* sp. and *Salmonella*. The absence of detection of a given pathogen results in an estimated prevalence of 0% with a maximum prevalence of 39, 8.3 and 2.3% (95% confidence interval) for the three respective populations. One hundred and four out of 164 samples tested positive for *B. dendrobatidis.* Positive ani- mals were present in the three tested populations. The genomic equivalents (GE) ranged between 1.6 and 368 (mean GE 10.9, SD 35.5) (Table [1](#_bookmark0)).

The high prevalence (63%) of chytrid infection in adult bullfrogs is comparable with the results found by Garner et al.  [2006](#_bookmark14). In their study the prevalence varied between 0 and 80% in adult bullfrogs in different locations across Europe, Canada and the United States. Since efficient transmission of

*B. dendrobatidis* is promoted both by high infection loads and

high prevalence, it is unclear which role bullfrogs play in maintaining *Bd* in amphibian assemblages. However, high prevalence combined with generally low infection loads, with the exception of erratic *B. dendrobatidis*-caused mortalities (Pasmans et al. [2010](#_bookmark17)), appear to be the rule rather than the exception in amphibian assemblages under northern Euro- pean conditions (Garner et al. [2006](#_bookmark14); Martel et al. [2012](#_bookmark12)), suggesting low infection loads to be sufficient for maintain- ing *B. dendrobatidis*. Given the recent discovery of non- amphibian hosts for *B. dendrobatidis* (McMahon et al. [2013](#_bookmark15)), we hypothesize bullfrogs to be part of a complex system promoting the persistence of *B. dendrobatidis*, without adding support to their role as primary contributors to the spread of *B. dendrobatidis*, as questioned by Liu et al. ([2013](#_bookmark33)). Sharifian-Fard et al. ([2011](#_bookmark22)) reported a low prevalence (0.75%) of *Ranavirus* in bullfrog tadpoles. In our study no positive samples for *Ranavirus* were detected. 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](#_bookmark34)).

We conclude that invasive bullfrogs in Belgium and The Netherlands do not constitute a significant reservoir for the zoonotic pathogens tested. The relatively low body temperature of the ectothermic anuran host might not provide a suitable environment for these pathogens, mainly occurring in endotherm hosts or, in case of *Salmonella*, occurring in ectothermic hosts reaching relatively high body temperatures, such as many reptiles.

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