Current Trends in Microbiology
Vol. 7, 2011

Original Communication

Chytridiomycosis in three species of *Rana* genus from northeastern Poland

Bazyli Czeczuga*, Adrianna Semeniuk, and Ewa Czeczuga- Semeniuk

Department of General Biology, Medical University, Mickiewicza 2C, 15-222 Białystok, Poland

ABSTRACT

In Poland, *Batrachochytrium dendrobatidis* has been first reported in 2009. Here, we present the results of the study planned to obtain an overview of its distribution on two areas of northeastern Poland. During the summer 2009 and 2010 the skin samples of *Rana* kl. *esculenta* and *Rana lessonae* were collected from Mazury Lakeland and in 2010- specimens of those two species and *Rana temporaria* from Suwalki Lake District. The occurrence of *Batrachochytrium dendrobatidis* sporangia in the skin of investigated amphibians was tested using histological techniques. We identified a fungal skin infection in 11 of 46 specimens of all three species.

KEYWORDS: chytridiomycosis, *Rana*, Poland

INTRODUCTION

A chytrid fungus, *Batrachochytrium dendrobatidis* is a fungal pathogen in amphibians, which has been implicated in numerous and global amphibian population declines [1]. It was first discovered in dead and dying frogs in Queensland, Australia in 1993. This pathogen infected at least 14 families and over 300 species of amphibians in five continents [2]. The last cases were described in China [3]. The species of the amphibians in which the presence of this chytrid fungus was found in particular continents, was reviewed by the authors [4]. In those papers, the authors were signaling for the first time in 2009 the fact of the occurrence of *Batrachochytrium dendrobatidis* in the amphibians in Poland. It was a corroboration with other investigators [5]. The aim of the present work is to evaluate the presence of *Batrachochytrium dendrobatidis* in wild specimens belonging to 3 species of *Rana* genus captured in two areas of the northeastern Poland.

*bazzylio@poczta.onet.pl

MATERIAL AND METHODS

46 wild specimens belonging to 3 species of *Rana* genus collected in summer 2009 and 2010 were studied (38 live and 8 dead specimens killed by road traffic):

- *Rana* kl. *esculenta* (syn. *Pelophylax* kl. *esculentus*) Linnaeus, 1758 (edible frog)- collected in litoral area of the Lake Krakszty in Mazury Lakeland and Lake Blizno in Suwałki Lake District.
- *Rana lessonae* (syn. *Pelophylax lessonae*) Camerano, 1882 (pond frog)- collected just as *Rana* kl. *esculenta.*
- *Rana temporaria* Linnaeus, 1758 (common or grass frog)- collected in wet habitats near Lake Blizno in the village Ateny and dead specimens from the road.

The Mazury Lakeland is located in the Mazury Lowland, average values of total solar radiation is 23186 J cm^{-2} per year and average air temperature ranged from minus 4 (January) to 18.0°C (July). Average precipitation is about 600 mm per year. Lake Krakszty is located on 53°47'N- 22°07'E, altitude 121.2 m., area 50.5 ha, max. depth 8.4 m., mean 2.8 m. South part of the Lake is shallow,

subaqueous plants- grown. These wild flood waters are suitable conditions. Bed is muddy. The amphibians from this part of the lake were used for the investigation.

The Suwalki Lake District is located in the two coldest regions of the Poland: Suwalki region and West Augustowski region. It is located in the middle part of Czarna Hańcza River catchment area. Average value of total solar radiation is 21215 J cm^{-2} per year and long- term average air temperatures ranged from minus 9.2°C (February) to 22.4°C (July). Average precipitation comes to 581 mm a year. Lake Blizno is located in the Augustów Forest, at 53°57.6'N- 23°04'E. The altitude is 133.2 m and area- 238.5 ha, max. depth 28.8 m, mean 10.0 m. South- western shores are surrounded by extensive coniferous woods, while the northern shores- by the village Ateny. The specimens of amphibians living on the side (shores) of the lake were used. The bed is sand and muddy.

The specimens were euthanized and fixed in the field in 10% neutral buffered formaldehyde. Strips of skin (approximately 5 x 10 mm) from 4 sites of the ventral part of the body (abdomen, thighs and toe clips) were collected for the examination [6]. A calibrated eyepiece micrometer was used to make a variety of measurements on 2,5 mm long sections at the center of each strip, including the number of sporangia [6]. Standard histological techniques for light microskopy were applied, the samples were sectioned at 6 μm, stained with haematoxylen and eosin, and examined at 60x and 100 magnification [7]. The presence of the sporangia of *Batrachochytrium dendrobatidis* Longcore *et al.* (1999) in this section was noted. The procedures described by Berger *et al.* [8] and Pessier *et al.* [9] were followed to identify the chytrid. Chemical environmental parameters were determined using standard methods [10].

RESULTS

The water from lakes from which the amphibians were collected in littoral sites, contained the most of oxygen and the fewest of all forms of nitrogen, phosphates, sulphates and chlorides (Table 1).

Table 1. Chemical and physical properties of water in particular water bodies (mg L^{-1}).

Specification	Lake Blizno	Lake Krakszty
Temperature (^{0}C)	18.6	19.8
pH	7.92	7.12
OD	16.84	14.84
BOD_5	2.68	2.96
COD	4.12	5.08
CO_2	6.46	7.14
Alkalinity in $CaCO_3$ (mval L^{-1})	2.38	2.48
$N\text{-}NH_3$	0.224	0.208
$N\text{-}NO_2$	0.005	0.004
$N\text{-}NO_3$	0.028	0.032
$P\text{-}PO_4$	0.162	0.178
Sulphates	15.02	17.06
Chlorides	15.14	16.00
Total hardness in Ca	40.38	42.12
Total hardness in Mg	12.36	13.44
Fe	0.12	0.10
Dry residue	192.0	210.0
Dissolved solids	149.0	186.0
Suspended solids	43.0	24.0

Table 2. The presence of *Batrachochytrium dendrobatidis* in investigated species of *Rana* genus from Poland.

Species	Stage	Sex	No. investigated specimens	No. positive infection	Average no. sporangium mm^{-1} skin
			Mazury Lakeland, 2009		
Rana kl. *esculenta**	adult	female, male	8	2	88.4
Rana lessonae	adult	female, male	9	2	64.2
			Suwałki Lake District, 2010		
Rana kl. *esculenta*	adult	female, male	8	2	74.6
Rana lessonae	adult	female, male	9	3	62.8
Rana temporaria	adult	female, male	12	2	41.6
Total number			46	11	

*It is a hybrid of *Rana lessonae* and *Rana ridibunda* in this hybridogenetic complex.

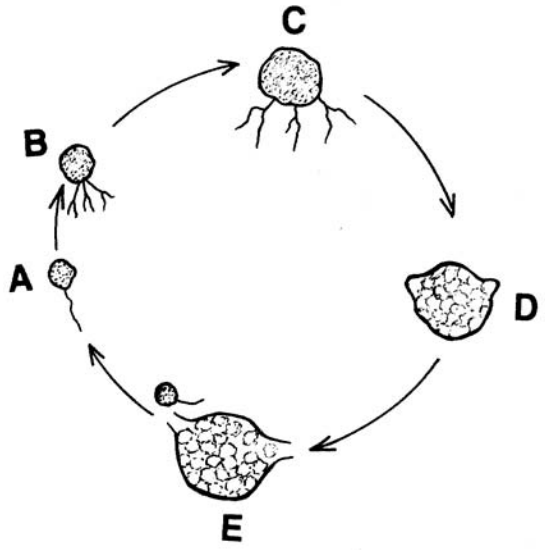

Fig. 1. Life cycle of *Batrachochytrium dendrobatidis*.
(A) Zoospore before encysting. Post propagation in water, zoospores are encysting in *stratum corneum* of amphibian epidermis, flagella resorption forming of the cell wall, origin of sporangium.
(B) Sporangium with rhizoids. At the beginning the sporangia grow in live cells of epidermis and complete their growth in keratinized dead cells of surface of amphibians' skin.
(C) Sporangium after 4-5 days maturing and transformating (mitosis) in multinuclear zoosporangium.
(D) Zoosporangium with 2 discharged papille (tubes).
(E) Zoospores discharging through 2 papillae. Zoospores leave the zoosporangia at suitable humidity conditions of amphibian skin.

Histological examination of skin samples showed evidence of fungal infection in some specimens of investigated species of frogs (Table 2). 11 of the 46 specimens of the investigated amphibians we swabbed were positive for presence of *Batrachochytrium dendrobatidis.* Number of infected specimens of frogs ranged from 2 (*Rana temporaria*) to 5 (*Rana lessonae*). Spherical and ovoidal zoosporangia, flash- shaped mature zoosporangia ranging from 5.5 to 9.8 μm in diameter (Fig. 1D, E), with a thin wall each and with a discharged papilla, and empty zoosporangia were present in the keratinized cell layer of the epidermis. The mature zoosporangia filled with zoospores 1 to 2 μm in diameter each had a single flagellum of 19-20 μm length (Fig. 1A). Average number of sporangia of *Batrachochytrium dendrobatidis* ranged from 41.6 (*Rana temporaria*) to 88.4 mm^{-1} skin section (*Rana* kl. *esculenta*).

DISCUSSION

The sites, on which the examinated amphibians were captured, occur on the postglacial areas. Those are well wooded, with many lakes and marshlands. Together, these are suitable conditions for the occurrence of the amphibians [11, 12]. Therefore, on this area it occurs on the newts [13], toads [14, 15], and frogs [16]. 9 species of the frogs, edible frog, pond frog and common frog are present in this area as common species [17, 18].

Batrachochytrium dendrobatidis already occurs on all continents. In Europe, the first report of the chytrid infection was described in captive bred frog- *Phyllobates lugubris* in Belgium and Germany [19]. In wild populations of *Alytes obstetricans* in Spain [20] and the Italian *Bombina pachypus* [21] the infections caused by chytrids were also recorded. Oevermann *et al.* [22] described this pathogen also in wild population of *Dyscophus antongilii* in Switzerland. Furthermore, *Batrachochytrium dendrobatidis* has been found in wild specimens of *Rana catesbeiana* and introduced in Britain [2]. Additionally, it was found in free- living individuals of *Rana* kl. *esculenta* in Italy [23,24], in Danmark [25] and in Luxemburg [26].

First evidence of this pathogen in wild population of *Rana lessonae* was found by Simoncelli *et al.* [23] in Italy and in *Rana temporaria* by Scalera *et al.* [25] in Danmark. Afterwards it was found in 2009 in wild populations of edible and pond frogs in Poland [4] and in other amphibian species in Italy and Spain. Wild specimens of *Bufo viridis* [27], *Rana lessonae* [23] in Italy and *Bufo bufo* [28, 29], *Salamandra salamandra* [28] in Spain carried this chytrid infection. Chytridiomycosis was detected in the endangered Sardinian newts *Euproctus platycephalus* [30] and on Mallorca (Spain) in individuals of *Alytes muletensis* [31]. Some other species of amphibians in Luxemburg [26] were also afflicted with this pathogen. *Batrachochytrium dendrobatidis* was found in archived samples of amphibians from as early as 1998 in 5 European countries- Great Britain, Portugal, Spain, Italy and Switzerland [32].

As known, the beginning of chytridiomycosis started from African amphibians from *Xenopus* genus, especially in African clawly frog (*Xenopus laevis*), in which it occurred as endemical disease [33]. Since 1930's this species was widely used in pregnancy tests and was exported to many countries of the world. The presence of this pathogen was described in the mountain areas of Ecuador [34], in hot Mexico [35], in cool Yukon in Canada [36] and Alaska [37]. In the Lowlands it grows in warmer months [38] and during the winter [39]. Its presence has been noted in different amphibian species on five continents from different environmental conditions, and has a various degree of the virulence and exposure to amphibians [28]. According to Fisher *et al.* [29] *Batrachochytrium dendrobatidis* is diploid of low sequence diversity and highly heterozygous, which suggests that this globalized lineage is a product of the mating between two nonidentical, but closely related heterothallic parental strains [40]. This fungus can spread from the amphibian to amphibian specimens by direct or close contact during mating, schooling of larvae or other aggregative behaviours. It may be transmitted between water bodies on everything: on amphibian limbs, insects, fishes, bird feathers (the Masurian Lakeland and Suwałki Lake District are on the migratory route of birds in autumn and spring) and even on walking shoes- similarly to other water fungus species [41]. *Batrachochytrium dendrobatidis* grows within wide range of temperatures from 4 to 25°C, optimally on 17- 25°C. Similar temperature of the water occurs in the ponds and littoral parts of the lakes on European Lowland. It grows and reproduces at pH 4-8 with maximal growth in culture at 6-7 [42] (such pH occurs in water of majority water bodies in northeastern Poland). The zoospores are discharging of zoosporangium in suitable humidity conditions. In still waters zoospores swim less than 2 cm before they encyst and infect the cells of amphibian epidermis, especially in abdominal (ventral) part of the body. Flagellum falls off, the formation of the cell wall and zoospores transformation in sporangium take place, and after 4-5 days succeeding the maturation and is called zoosporangium [42]. Out of amphibian organisms, the zoospores decline after 48 hours, and sporangia after 3-4 days. Di Rosa *et al.* [43] observed encysted zoospores with thick wall on the skin surface of *Rana lessonae* where they embody a resting spore, a saprobe or a parasitic form which is conditionally non- pathogenic. This fungus can inhabit areas without causing declines and may be present without causing disease [32, 43, 44]. About 30 species of amphibians are carrying this pathogen [45].

Such ecological stress factors as chemicalization of the environment by different xenobiotics (especially by organophosphorus pesticides), eutrophication of the water bodies, ultraviolet radiation, greenhouse effect and climate changes have the influence on the development of amphibians [4]. The effects of mineral fertilization,

especially with ammonium and nitrate on larvae of amphibians were stated. The parasites of other fungus species in the spawn [46], infection of the *Ranavirus* [47] and trematode *Ribeiroia ondatrae* [48] also cause danger to amphibians. The populations of most of the amphibians, biologically weakened-by above mentioned factors, are often being attacked by fungus *Batrachochytrium dendrobatidis*.

REFERENCES

1. Berger, L., Speare, L., 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., and Parkes, H. 1998, Proc. Natl. Acad. Sci. USA, 95, 9031.
2. Cunningham, A. A., Garner, T. W. J., Aguilar- Sanchez, V., Banks, B., Foster, J., Sainsbury, A. W., Perkins, M., Walker, S. F., Hyatt, A. D., and Fisher, M. C. 2005, Weter. Rec., 157, 386.
3. Bai, C., Garner, T. W. J., and Li, Y. 2010, EcoHealth, 7, 127.
4. Czeczuga, B., Semeniuk, A., and Czeczuga-Semeniuk, E. 2011, Trends Comp. Biochem. & Physiol., 15, 17.
5. Dressen, S., Ohst, T., Plötner, J., Gräser, Y., Hoffman, A., Pruvast, N., and Reyer, H.-U. 2011, Chron. Przyr. Ojcz., 67, in press.
6. Berger, L., Speare, R., and Skerratt, L. F. 2005, Dis. Aquat. Org., 68, 65.
7. Barionneto, S. and Mangione, S. 2006, Dis. Aquat. Org., 73, 171.
8. Berger, L., Speare, R., and Kent, A. 1999, Zoos Print J., 15, 184, also available at http://www.jcu.edu.au/school/phtm/PHTM/frogs/histo/chhisto.htm [accessed September 1, 2005].
9. Pessier, A. P., Nichols, D. K., Longcore, J. M., and Fuller, M. S. 1999, J. Veter. Diag. Invest., 11, 194.
10. Standard Methods for the Examination of Water and Wastewater, 2005, American Public Health Association, Washington.
11. Beebee, T. J. C. 1996, Ecology and Conservation of Amphibians, Chapman and Hall, London.
12. Dorcas, M. and Gibbons, W. 2008, Frogs and Toads of the Southeast, Eurospan, University Presses, London.
13. Czeczuga, B. 1977, Zool. Pol., 26, 85.
14. Czeczuga, B. 1982, Amphibia- Reptilia, 3, 53.
15. Czeczuga, B. and Ruprecht, A. L. 1983, Folia Biol., 31, 349.
16. Czeczuga, B. 1980, Comp. Biochem. Physiol., 63B, 623.
17. Czeczuga, B., Czeczuga- Semeniuk, E., and Semeniuk, A. 2006, Trends Comp. Biochem. & Physiol., 12, 21.
18. Berg, L. 2000, Płazy i gady Polski. Klucz do oznaczania, PWN, Warszawa- Poznań.
19. Matschumann, E., Berger, L., Zwart, P., and Gaedicke, C. 2000, Berl. Münch. Tierärztl. Wscht., 113, 380.
20. Bosch, J., Martinez- Solano, J., and Garcia-Paris, M. 2001, Biol. Conserv., 97, 331.
21. Stagni, G., Scociannti, C., and Fuscini, R. 2002, Proc. Fourth. Congr. Soc. Herpetol. Italica, Ercolano, Italy, 22.
22. Oevermann, A., Schildger, B., Feldman, S., and Robert, N. 2005, Tier. Umsch., 4, 211.
23. Simoncelli, F., Fagotti, A., Dall'Olio, R., Vagnetti, D., Pascolini, R., and Di Rosa, I. 2005, EcoHealth, 2, 307.
24. Federici, S., Clemenzi, S., Favelli, M., Tessa, G., Andreone, F., Casiraghi, M., and Crottini, A. 2008, Herpetol. Not., 1, 33.
25. Scalerra, R., Adams, M. J., and Galvan, S. K. 2008, Herpetol. Rev., 39, 199.
26. Wood, L. R., Griffiths, R. A., and Schley, L. 2009, Bull. Soc. Nat. luxemb., 110, 109.
27. Adams, M. J., Galvan, S., Scalerra, R., Grieco, C., and Sindaco, R. 2008, Herpetol. Rev., 39, 324.
28. Bosch, J. and Martinez- Solano, I. 2006, Oryx, 40, 84.
29. Fisher, M. C., Garner, T. W. J., and Walker, S. F. 2009, Ann. Rev. Microbiol., 63, 291.
30. Bovero, S., Sotgiu, G., Angenili, C., Doglio, S., Gazzangia, E., Cunningham, A. A., and Garner, T. W. J. 2008, J. Wildl. Dis., 44, 712.
31. Garner, T. W. J., Garcia, G., Caroll, B., and Fisher, M. C. 2009, Dis. Aquat. Org., 83, 257.
32. Garner, T. W. J., Walker, S., Bosch, J., Hyatt, A. D., Cunningham, A. A., and Fisher, M. C. 2005, Immerg. Infect. Dis., 11, 1639. Also available at http://www.spatialepidemiology.net/bd/

33. Soto- Azat, C., Clarke, B. T., Poynton, J. C., and Cunningham, A. A. 2010, Diver. Distrib., 16, 126.
34. Bonaccorso, E., Guayasamin, J. M., Mendez, D., and Speare, R. 2003, Herpetol. Rev., 34, 331.
35. Lips, K. R., Mendelson, J. I. R., Munoz-Alonso, A., Canseco- Marquez, L., and Mulcahy, D. G. 2004, Biol. Conserv., 119, 555.
36. Slough, B. G. 2009, Herpetol. Rev., 40, 319.
37. Reeves, M. K. 2008, Herpetol. Rev., 39, 68.
38. Kriger, K. M. and Hero, J. M. 2008, Aust. Ecol., 33, 1022.
39. Bradley, G. A., Rosen, P. C., Sredl, M. J., Jones, T. R., and Longcore, J. E. 2002, J. Wildl. Dis., 38, 206.
40. James, T. Y., Litvintseva, A. P., Vilgalys, R., Morgan, J. A., Taylor, J. W., Fisher, M. C., Berger, L., Weldon, C., Preez, L. du, and Longcore, J. E. 2009, PLoS Pathog. 5(5): e1000548.doi:10.1371/journal.ppat.1000458.
41. Czeczuga, B. 2004, Rec. Res. Devel. Microbiol., 8, 121.
42. Piotrowski, J. S., Annis, S. L., and Longcore, J. E. 2004, Mycologia, 96, 9.
43. Di Rosa, I., Simoncelli, F., Fagotti, A., and Pascolini, R. 2007, Nature (London), 447 (7144), E4.
44. Bosch, J., Carrascal, L. M., Duran, L., Walker, S., and Fisher, M. C. 2007, Proc. R. Soc., B274, 253.
45. Fisher, M. C. and Garner, T. W. J. 2007, Fungal Biol. Rev., 21, 2.
46. Czeczuga, B., Muszyńska, E., and Krzemińska, A. 1998, Amphibia- Reptilia, 19, 239.
47. Teacher, A. C. F., Cunningham, A. A., and Garner, T. W. J. 2010, Anim. Conserv., 13, 514.
48. Johnson, P. T. J., Sutherland, D. R., Kinsella, J. M., and Lunde, K. B. 2004, Adv. Parasitol., 57, 191.