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Detection of the emerging amphibian pathogens *Batrachochytrium dendrobatidis* and ranavirus in Russia

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ABSTRACT: In a population of the European common toad *Bufo bufo* from a rural pond in the region of Lake Glubokoe Regional Reserve in Moscow province, Russia, unexplained mass mortality events involving larvae and metamorphs have been observed over a monitoring period of >20 yr. We tested toads from this and a nearby site for the emerging amphibian pathogens *Batrachochytrium dendrobatidis* (*Bd*) and ranavirus (Rv). Both pathogens were detected, and at the rural pond site, with the above-noted losses and decline in toad breeding success, 40% of *B. bufo* metamorphs were *Bd* positive, 46% were Rv positive and 20% were co-infected with both pathogens. Toad metamorphs from a neighbouring water body were also *Bd* and Rv positive (25 and 55%, respectively). This is the first confirmation of these pathogens in Russia. Questions remain as to the origins of these pathogens in Russia and their roles in documented mass mortality events.

KEY WORDS: Amphibians · Chytridiomycosis · European common toad · Population fluctuations

INTRODUCTION

Two amphibian diseases with disproportionately large global impacts are chytridiomycosis caused by the chytrid fungus *Batrachochytrium dendrobatidis* (*Bd*) and infection with ranavirus (Rv) (Schloegel et al. 2010). These pathogens have nearly global distributions, existing on all continents with amphibians, and have been associated with amphibian mass mortality and drastic population declines in several regions of the planet (e.g. Skerratt et al. 2007, Gray et al. 2009). While both pathogens exist in Europe (Garner et al. 2009, Gray et al. 2009), to date there has only been limited testing for *Bd* in Russia, and the pathogen was not detected (in *Rana temporaria* and *R. amurensis*; Ouellet et al. 2005). Recently, *Bd* sampling in far-eastern regions of Russia has also yielded negative results (samples from *Bufo gargarizans*, *R. dybowskii* and *Salamandrella schrenkii* [*keyserlingii*]; Civis et al. 2013). We are not aware of any previous attempts to test Russian amphibians for Rv.

Our study was focused on the European common toad *Bufo bufo*, which is susceptible to both *Bd* and Rv (Bosch & Martínez-Solano 2006, Cunningham et al. 2007). Our objectives were to report on unexplained cyclical mass mortality of larvae and metamorphs in Moscow province, Russia, observed over a 20 yr period, 1994 to 2013, and to examine toads for *Bd* and Rv in order to determine whether either pathogen was present.

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MATERIALS AND METHODS

Monitoring of toad breeding success

Amphibian breeding sites have been monitored annually since 1994 in the Ruza district of Moscow province, including Pond 13, at which *Bd* and Rv sampling was conducted in 2011. Using a random shoreline dip-net approach, surveys were conducted in May to June, during development of toad larvae. Densities of pre-metamorphic (stages 37 to 46; Gosner 1960) larval amphibians were assessed based on the number captured relative to the volume of water filtered by dip-netting and given a categorical relative abundance score ranging from 0 to 3: a score of 0 for no animals per cubic metre of water, a score of 1 for 1 to 8 animals m^{-3}, a score of 2 for 9 to 40 animals m^{-3} and a score of 3 for >40 animals m^{-3} (Reshetnikov 2003). This scale allows detection of the principal fluctuations in relative abundances, reflecting breeding success (assuming recruitment of metamorphs) and ignoring incidental, small-scale, within-year and between-year deviations.

Water bodies sampled

We sampled *Bufo bufo* for *Bd* and Rv at 2 water bodies. The Pond 13 site (mean depth ≈ 1 m; area ≈ 1570 m^2; 55.7183° N, 36.4942° E), a typical rural cattle pond excavated in the 1980s by a local cattle farm, was sampled on 19 June 2011. The second site, Lake Glubokoe (mean depth ≈ 33 m; area ≈ 590 000 m^2; 55.7530° N, 36.4572° E), is of natural origin, located 3.5 km N–NE of the pond at the centre of the Lake Glubokoe Regional Natural Reserve (Smirnov 1986), and was sampled on 30 June 2011. We collected larvae (n = 15) and metamorphs (n = 35) from Pond 13 and metamorphs (n = 20) from Lake Glubokoe. Specimens were euthanized with ether and fixed in 95% ethanol. All were tested for presence and relative abundance of Rv; however, fewer toads were used for estimation of *Bd* zoospore relative abundance (14, 31 and 18 specimens of larvae and metamorphs from Pond 13 and metamorphs from Lake Gubokoe, respectively).

PCR testing

Given the minute size of the metamorphs (all were <10 mm snout–vent length) and evidence that swabs collected from a climatically similar region in North America detected fewer *Bd* infections than tissue samples (Schock et al. 2010), we conducted whole-tissue assays for *Bd*. The keratinized mouthparts of the larvae and legs of the metamorphs were removed using flame-sterilized scissors and forceps and used for extraction of *Bd* DNA for quantitative real-time polymerase chain reaction (qPCR) assays (using the protocol described by Boyle et al. 2004). The *Bd* qPCR was run in duplicate. If 1 replicate returned a positive result, the sample was run again in duplicate to confirm the positive result. The rest of the bodies (without legs) of metamorphs or tail clips of larvae were used for the Rv qPCR assays (Brunner & Collins 2009) following Homan et al. (2013). The Rv samples were run in triplicate on plates with a standard spanning 10^2 to 10^7 plaque-forming units (PFU; equivalent to infectious virus particles) of a frog virus 3 (FV3)-like ranavirus. Samples with amplification in ≥2 wells were scored as positive, and those without amplification in any wells were scored as negative. Ambiguous samples (1 positive well) were re-run, and if ≥1 well showed amplification the sample was scored as positive. The titers are reported as the mean across all wells for positive samples. From samples of a tadpole from Pond 13 and a metamorph from Lake Glubokoe, we sequenced, in both directions, a ~530 base pair region of the major capsid protein (between primers MCP 4 and 5; Mao et al. 1999) of 2 positive samples with an ABI 3730 (Applied Biosystems) and compared the results with published ranavirus sequences on GenBank using a BLAST search. We follow Bush et al.'s (1997) definitions of prevalence, abundance and infection intensity. The Wilcoxon matched pair test and χ^2 test were used for comparison of samples, with a significance level at $p \leq 0.05$.

RESULTS

Fluctuating breeding success of *Bufo bufo* was evident at Pond 13 from 1994 to 2013 (Fig. 1). In some years, larval toads in Pond 13 successfully developed and generated recruitment of numerous metamorphs. In such years (e.g. 2004), Pond 13 larvae developed almost synchronously with larvae from other nearby toad populations. For example, on 1 July 2004 the larval body lengths in Pond 13 and in Lake Glubokoe were 25.6 ± 0.5 mm (20.5–29 mm) and 25.6 ± 0.6 mm (20–32 mm), respectively, and Gosner (1960) ontogenetic stages were 37.3 ± 0.5 (32–41) and 37.5 ± 0.5 (33–40), respectively. The differences were not significant (Wilcoxon: n = 20, $t = 92.5$, $Z = 0.47$; and n = 20, $t = 77$, $Z = 0.37$, respectively).

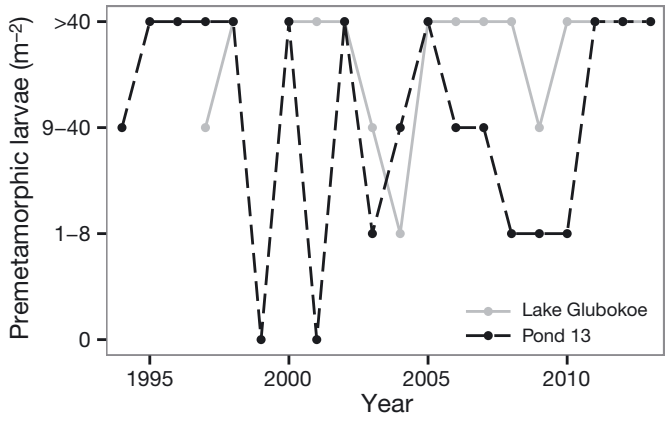

Fig. 1. Categorical reproductive success of the common toad *Bufo bufo* in 2 water bodies (Moscow province, Russia) in May to June over a 20 yr period

In contrast, at Pond 13 in other years, we observed numerous *B. bufo* larvae at early Gosner (1960) stages in May and early June, but only a few larvae at late stages, with live metamorphs subsequently absent (1999, 2001) or rare (2003, 2006, 2008, 2009 and 2010) (Fig. 1). For example, in 2001, an initially numerous cohort of toad larvae appeared to be entirely extirpated before reaching metamorphic stages. Many toad larvae were emaciated and moved very slowly. In June of that year tadpoles from Pond 13 were significantly shorter in body length than those in Lake Glubokoe (mean body length = 7.4 ± 0.3 mm, range 6–9 mm vs. 10.5 ± 0.2 mm, range 10–11 mm; Wilcoxon, n = 10, $Z = 2.8$, $p < 0.01$) and were less developmentally advanced (mean Gosner stage: 27.9 ± 0.8, range: 26–35 vs. 41.0 ± 0.0; Wilcoxon, n = 10, $Z = 2.8$, $p < 0.01$). In that year the relative abundance of toad larvae in Pond 13 had decreased from approximately 800 individuals m^{-3} water between May and June to 100 individuals m^{-3} on 17 July and, again, to 10 individuals m^{-3} on 22 July, and larvae were absent on 28 and 30 July. No metamorphs were detected on the shore of this pond in 2001. In other years (e.g. 2003), larval toads developed with a remarkably long delay, but some reached metamorphic stages. In those cases, we observed both larval and metamorph mortalities of this amphibian species in Pond 13. For example, on 1 July 2003, 5% of collected larvae and 50% of metamorphs were dead.

Bd and Rv were detected in *B. bufo* from Pond 13 and the neighbouring Lake Glubokoe. From Pond 13, 3 of 15 larvae and 14 of 35 metamorphs were *Bd* positive and 7 of 15 larvae and 16 of 35 metamorphs were Rv positive (Table 1). The ~500 bp of the MCP gene of the 2 sequenced viruses were 100% identical to published sequences of FV3 (Tan et al. 2004). While *Bd* was twice as common in metamorphs as in conspecific larvae in the pond, this difference was not significant (Yates corrected $\chi^2_1 = 1.09$, $p = 0.30$). The abundances of both *Bd* zoospores and Rv virions (in terms of PFU, see 'Materials and methods') in infected animals were, on average, 1 to 2 orders of magnitude higher in larvae than in metamorphs in the pond (Table 1). From the lake, 5 of 20 metamorphs were *Bd* positive and 11 of 20 were Rv positive. Co-infection (proportion of host individuals which were positive for both pathogens) varied from 0 to 20% (Table 1), but did not deviate substantially from the null expectation that these 2 infections are independent of one another (binomial test, not significant for any of the 3 comparisons).

DISCUSSION

The causes of mass mortality events observed in the *Bufo bufo* population at Pond 13 are unknown. It is well known that seasonal or cyclic mortality in larval amphibians can be caused by pond drying, toxic cyanobacterial blooms, hypoxia, introduced species, disease outbreaks and other factors (Rowe & Dunson 1995, Brady & Griffiths 2000, Fischer & Dietrich 2000, Bosch et al. 2001, Reshetnikov 2003).

Table 1. *Batrachochytrium dendrobatidis* (*Bd*) and ranavirus (Rv) prevalence (*P*: number of infected individuals/total sampled × 100%), relative abundance (*A*: *Bd* zoospore or Rv count of plaque-forming units for all sampled animals regardless of whether or not the host was infected, mean ± SE), individual intensity of infection (*I*, range), and the co-infection rate (%) of larval and metamorphic common toads *Bufo bufo* from a pond and a lake site in Moscow province, Russia

Locality	Stage	*B. dendrobatidis*			Ranavirus			Co-infection
		P (%)	*A*	*I*	*P* (%)	*A*	*I*	(%)
Pond 13	Larvae	20	1034.84 ± 1031.01	50–14438	47	679.84 ± 515.40	10–7677	0
Pond 13	Metamorph	40	51.56 ± 15.52	78–457	46	15.87 ± 7.30	2–243	20
Lake Glubokoe	Metamorph	25	95.53 ± 75.97	86–1259	55	13.98 ± 5.72	2–112	10

Our study sites are permanent water bodies that did not experience a reduced hydroperiod, so this cannot explain the observed cyclical mortality. Also, while both study sites have had fish introduced, *B. bufo* is resistant to fish predation (Manteifel & Reshetnikov 2002), so the presence of fish is not a likely explanation for this mortality. Nor did we observe heavy bacterial blooms in Pond 13. The presence of *Bd* and an FV3-like Rv in tadpoles and metamorphs at 2 sites in this region may help explain these episodic mass mortalities of larvae and metamorphs in Pond 13.

Metamorphs tended to be more frequently infected with *Bd* than were conspecific larvae from the same water body, which may reflect biologically relevant increases in chytridiomycosis during ontogenetic development of toads (Garner et al. 2009). *Bd* infects keratinized structures. Whereas only mouthparts of *B. bufo* larvae are keratinized during late larval development, metamorphs have well-keratinized skin (Wells 2010). When infected by *Bd*, mortality is usually highest in larvae approaching metamorphosis or in metamorphs (Wells 2010). Our results coincide with this observation; although *Bd* prevalence tended to increase during *B. bufo* ontogenetic development in our samples from the pond, the relative abundance of *Bd* in metamorphs was lower (Table 1). This may reflect that individual toads highly infected by *Bd* died before metamorphosis. On the other hand, ranaviral infection is not limited by specific tissue tropism, and we found a similar percentage of Rv-infected *B. bufo* larvae and metamorphs (Table 1).

Toad reproductive success through annual larval survival and metamorphosis varied remarkably from year to year at the pond site and, to a lesser degree, in Lake Glubokoe where *Bd* and Rv were also detected (Fig. 1). The presence of these pathogens alone is not likely to be sufficient to eliminate an entirely new toad generation every year, and other unknown factors are probably influencing the toad populations and disease dynamics at these sites (Blaustein et al. 2011). Presently, adult *B. bufo* are still common during breeding at our study pond. Monitoring is warranted to assess potential cumulative population effects of recruitment losses over time.

Bd is often assumed to be an alien invasive organism for Europe (Fisher et al. 2009, Sztatecsny & Glaser 2011). This pathogen has been detected in samples from Europe since 1997 (Fig. 2a), and was

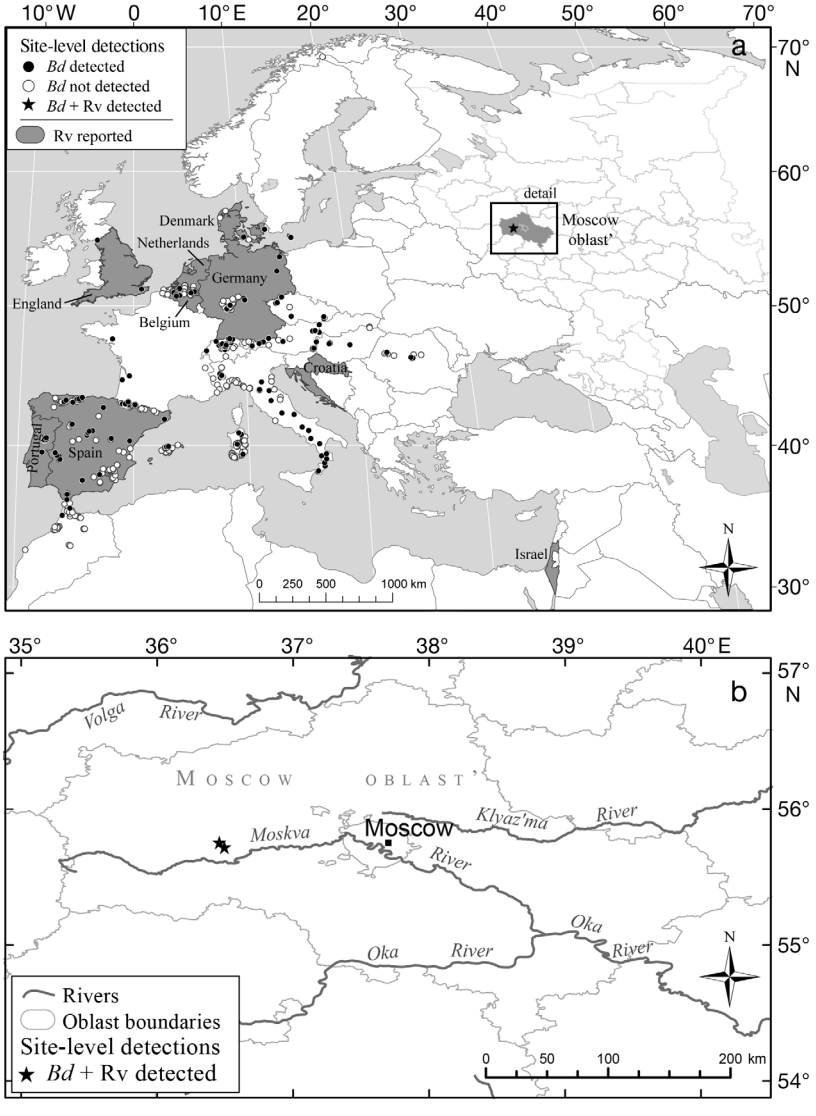

Fig. 2. (a) Current knowledge on the geographic distribution of *Batrachochytrium dendrobatidis* (*Bd*; black circles are positive and open circles are negative samples) and ranavirus (Rv) in Europe (dark grey corresponds to countries with positive samples; ranavirus data were compiled for the developing Ranavirus Reporting System; Amanda Duffus, Gordon College, Georgia, USA, pers. comm.), and the location of new records of these pathogens in Russia (asterisk, inset). (b) Location of the samples in the Moscow province (oblast')

initially found in Spain (Bosch et al. 2001). To date, the easternmost record of *Bd* in Europe was from Romania (www.Bd-maps.net; Olson et al. 2013). Our detection of *Bd* in Russia confirms its presence in European Russia; however, we are not aware of whether this pathogen is aboriginal or alien to this area. Investigation of old museum specimens of amphibians from the studied region would shed light on this issue. Additional information may be obtained using comparative DNA analysis of samples from different parts of the *Bd* ranges.

This is also the first documented evidence of a ranavirus in this region. Ranaviruses have been isolated from amphibians in many locations in Europe: Belgium, Croatia, Denmark, Israel, the Netherlands, Portugal, Spain, Switzerland and the UK (Miller et al. 2011), including from *B. bufo* in the UK (Cunningham et al. 1996), as well as in Asia (China, Japan and Thailand; Miller et al. 2011), but not, to our knowledge, in Russia. While the Rv samples are identical to the type FV3 virus first isolated in North America in the 1960s, further genetic testing should help determine the source (if it was recently introduced) or relatedness to other members of this globally distributed Ranavirus species.

Our study confirms the presence of 2 emerging amphibian pathogens in the territory of Russia (Fig. 2). Questions remain about the origin of these pathogens, previously unknown in Russia, and the role of the detected pathogens in documented mass mortality events. Globally, amphibian population losses and species extinctions are occurring at unprecedented rates and the causes for these declines are complex (Stuart et al. 2004, Hoffmann et al. 2010). Amphibian responses to disease can differ among species, life stages and populations, and responses are dependent on the interaction of multiple stressors that may drive declines (Blaustein et al. 2011). Amphibians are key constituents of ecological communities; as such, we emphasize the importance of results showing amphibian declines at the community level, with considerations of the disease involved and the interactions between cofactors that may drive population declines, when planning conservation strategies for the region.

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