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No detection of chytrid in first systematic screening of *Bombina variegata pachypus* (Anura: Bombinatoridae) in Liguria, northern Italy

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Abstract. The Apennine Yellow-bellied toad *Bombina variegata pachypus*, a small anuran endemic to peninsular Italy, has been declining throughout its range over the last 30 years. Although mortality by chytridiomycosis, caused by the fungus *Batrachochytrium dendrobatidis*, was first reported for the species in 2004, its role in the decline has not yet been assessed. Between 2011 and 2012 we sampled eight populations of *B. v. pachypus* in Liguria, northern Italy, swabbing 86 and 143 individuals respectively, corresponding to between 24 and 80% of the estimated individuals within each population. We did not detect chytrid in any the samples collected. For the three largest populations in the region, we can rule out infections of prevalence greater than 10% with at least 98% confidence. Research at a larger scale is urgently needed to clarify the role of *B. dendrobatidis* in the decline of this and other amphibians in Italy.

Keywords. *Bombina*, chytrid, confidence, Liguria, monitoring, population size, prevalence.

The amphibian chytrid fungus *Batrachochytrium dendrobatidis* is the main pathogen driver of the global amphibian decline (Fisher et al., 2009). Die-offs and extinctions have occurred in all continents (Berger et al., 1998; Conradie et al., 2011; Swei et al., 2011) including several cases in Europe (Garner et al., 2005). In Italy, infection by chytridiomycosis has been documented for several species in the last decade (reviewed in Tessa et al., 2013): further information on the distribution of *B. dendrobatidis* in Italy is needed to understand the causes of local and regional declines and inform conservation efforts.

The Apennine yellow-bellied toad *Bombina variegata pachypus* (Anura: Bombinatoridae) was the first amphibian species in Italy with confirmed mortality by chytridiomycosis, reported by Stagni et al. (2004) on captive individuals caught from three populations in the north-eastern Apennines. Recent evidence suggests chytrid is present in populations of *B. v. pachypus* along the Italian peninsula, with infections dating back as early as the late 1970s (Canestrelli et al., 2013). *B. v. pachypus* has declined steeply over the last 30 years, with widespread local disappearances: although habitat loss is believed to represent the main driver of the decline (Mirabile et al., 2009; Canessa et al., 2013), chytridiomycosis is accepted as a potential threat to the species (see for example its Red List assessment in IUCN, 2011). However, to our knowledge, no systematic assessment of the spread of chytrid among populations of *B. v. pachypus* in Italy has been undertaken to date.

In order to clarify this issue at least in part, we conducted surveys throughout the northern Italian region of Liguria in 2011 and 2012. A recent assessment of the status of *B. v. pachypus* in this area showed that over 50% of the known populations disappeared between 2001 and 2010: habitat loss relating to the abandonment of traditional farming practices has been suggested as the main cause of decline (Canessa et al., 2013). A small-scale survey (five individuals tested) carried out in 2005 at a single site in the region failed to detect any presence of *B. dendrobatidis* (Adams et al., 2008).

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In 2011, between 31 July and 4 August we visited all seven known breeding sites of *B. v. pachypus* in Liguria, located within the catchments of the Lavagna and Vara rivers in the eastern part of the region, encompassing an area of approximately 800 km^2. In 2012, we repeated our visits at five of these seven populations: the three sites believed to host the largest populations were sampled three times during the 2012 season in order to maximize the overall proportion of the population that could be tested (22 June, 24 July and 26 July). We also tested all the animals present at a small captive breeding centre within the study area: these individuals had been caught in the wild and kept in a semi-natural enclosure with no contact with other amphibians during the study period.

We captured all detected individuals by hand and photographed them to record their unique ventral pattern: this allowed us to calculate the number of unique individuals tested in addition to the total number of swabs collected at sites that were visited multiple times. To minimize the chance of spreading pathogens, we followed recommendations by Speare et al. (2004) when handling individuals, and sites within different catchments were never sampled during the same day. We collected samples as described by Hyatt et al. (2007) using cotton-tip swabs and then released individuals at the point of capture. Samples were stored at –4 °C until further analysis. We only tested post-metamorphic individuals: although pathogen prevalence can be higher in tadpoles, facilitating detection, we chose to avoid any impact to the few remaining populations of the species in the region that could result from applying lethal or non-lethal sampling to larvae. Swabbing is less sensitive than other methods, and can still damage tadpole mouthparts and facilitate infections in addition to stress resulting from handling (Retallick et al., 2006).

B. dendrobatidis DNA in the samples, real-time Taqman PCR assays were conducted on a CFX96 Real Time System (BioRad). Amplification conditions, primer and probe concentrations were according to Boyle et al. (2004). Due to economic constraints, samples were run in duplicate. Consistent results between the two runs were classified as correct: we considered a test result as positive where both qPCR runs gave results above 0.1 GE, with standard deviation smaller than the average of both repeats and suitable amplification curves.

After obtaining the test results, we estimated the mean prevalence (with 95% credible intervals) of the pathogen in the population using a Bayesian equal-tailed Jeffrey prior (Brown et al., 2001). To assess the sensitivity of our sampling program, we then calculated the probability of detecting 5, 10 and 30% infections with the number of individuals sampled at each site, by solving

$$C = 1 - (1 - p)^n$$

where C is the probability of detecting at least one infected individual from n tested in a population with p prevalence of the disease (DiGiacomo and Koepsell, 1986).

Additionally, for the three largest populations in the study we were able to use an existing multi-year mark-recapture database to estimate population size at the time of sampling (Canessa, *unpubl. data*). To account for open-population dynamics such as temporary emigration, often observed for *B. variegata* (Hartel, 2008), we used an open-population Jolly-Seber model (Wagner et al., 2011). We fit the model in a Bayesian framework using date- and population-dependent recapture rates (code in Kéry and Schaub, 2011). We used JAGS (Plummer, 2005) to obtain samples from three Markov chains over 100,000 iterations, after discarding the first 10,000 as a burn-in.

In 2011, we collected 81 swabs from 81 individuals in seven wild populations, corresponding to between 24 and 42% of the estimated sizes of respective populations (as estimated from 95% confidence intervals from the Jolly-Seber model): at two sites we captured only one and four individuals respectively (Table 1). In 2012, we captured and swabbed a total of 143 individuals at four of the wild locations on four separate occasions, corresponding to between 66 and 80% of the estimated sizes of the tested populations. Several individuals were re-captured and swabbed in two or three occasions in 2012, yielding a total of 224 swabs for that season. All individuals at the captive breeding centre were also caught and tested (four in 2011 and three in 2012): we did not calculate confidence intervals or sampling power for this site.

All samples tested negative for *B. dendrobatidis*. We captured more than 10 individuals only at four sites: in 2011, this resulted in upper 95% confidence intervals (CI) of prevalence between 0.09 and 0.13 (Table 1). For three of these populations, in 2012 we were able to reduce the upper CIs to 0.05 or less; at the fourth site, only seven individuals were captured in 2012, resulting in an upper CI of 0.23. For the three most intensively sampled populations, we can be at least 97% confident that chytrid would have been detected at these populations for a prevalence of at least 10%, and more than 83% confident for a prevalence of at least 5% (Table 1). For the smaller populations sampled (less than 10 individuals), we could make no reliable inference regarding the prevalence of infection, although in sites with more than one capture, the probability of having missed large infections (> 30% prevalence) was still lower than 50%.

Although reliable estimates of abundance could not be obtained for the smaller populations in the study, we

Table 1. Results for all screened populations of *B. v. pachypus* in 2011 and 2012.

		Population							
		Fav	Lo1	Lo2	LoP	Pav	Per	Pin	Tev
2011									
Total swabs[1]		4	17	21	8	17	1	13	1
Individuals swabbed[2]		4	17	21	8	17	1	13	1
Population size[3]		-	68 ± 2	67 ± 2	-	35 ± 4	-	47 ± 3	-
% tested[4]		-	25.0	31.3	-	48.6	-	27.7	-
Prevalence (95% CI)[5]		0.36	0.11	0.09	0.21	0.11	0.77	0.13	0.77
Detection	p = 0.05[6]	0.19	0.58	0.66	0.34	0.58	0.05	0.49	0.05
	p = 0.1[7]	0.34	0.83	0.89	0.57	0.83	0.10	0.75	0.10
	p = 0.3[8]	0.76	1.00	1.00	0.94	1.00	0.30	0.99	0.30
2012									
Total swabs[1]		-	106	60	-	7	-	51	-
Individuals swabbed[2]		-	55	43	-	7	-	35	-
Population size[3]		-	69 ± 2	69 ± 2	-	35 ± 4	-	48 ± 2	-
% tested[4]		-	79.7	62.3	-	20.0	-	72.9	-
Prevalence (95% CI)[5]		-	0.04	0.04	-	0.23	-	0.05	-
Detection	p = 0.05[6]	-	0.94	0.89	-	0.30	-	0.83	-
	p = 0.1[7]	-	1.00	0.99	-	0.52	-	0.97	-
	p = 0.3[8]	-	1.00	1.00	-	0.92	-	1.00	-

[1] Total number of swabs collected
[2] Total numbers of unique individuals swabbed, where repeated visits were carried out (Lo1, Lo2 and Pin in 2012)
[3] Population size at the time of sampling (± standard deviation), estimated using the Jolly-Seber open population model – a dash indicates no estimate could be made for that population/year
[4] Proportion of the estimated population size (mean) that was tested
[5] Upper 95% equal-tailed Jeffrey prior confidence interval (lower interval is 0 in all cases).
[6, 7, 8] Probability of detecting at least one infected individual if the pathogen were present in the population with a prevalence of 0.05, 0.1 and 0.3 respectively.

observed relatively high recapture rates (> 40%) for the larger populations, suggesting the small sample sizes at some sites might reflect actual abundances. Nevertheless, the estimates we present for populations with less than 10 sampled individuals can only be considered as preliminary and do not allow reliable inference. For the largest populations in the region the large sample sizes, particularly for 2012, allows us to rule out with sufficient confidence infections of prevalence greater than 10%.

Additional information about the expected prevalence of chytrid infection in *B. v. pachypus* is needed to make more precise inference: for example. Canestrelli et al. (2013) recently found that prevalence of *B. dendrobatidis* in present and historical specimens of *B. v. pachypus* across the Italian peninsula we never below 12%. Amongst populations of *B. variegata* in Hungary, the infection rate has been estimated between 12% and 29% (Vörös, pers. comm.). In the Netherlands and Flanders (Belgium), Spitzen-van der Sluijs et al. (2010) reported a prevalence of infection of 5.9% for *B. variegata* out of 255 individuals sampled. Holding this relatively high base rate as a guideline, the failure to detect chytrid in the Ligurian populations may indicate lower susceptibility due to local conditions, bias related to the date of sampling (patterns of chytrid infection are highly seasonal: see Kriger and Hero, 2007), or possibly actual absence of the pathogen from the region at present. In addition, breeding sites of *B. v. pachypus* in Liguria have been regularly monitored throughout the breeding season since 2008 and no obvious sign of chytrid infection (e.g. individual mortality or abnormal behaviour) has ever been reported (Arillo et al., 2009; Arillo et al., 2011).

It is difficult to infer whether chytridiomycosis might have had a role in past local extinctions in the region. Most disappearances in the region in recent years have occurred at sites in agricultural areas (Canessa et al., 2013). However, the existing literature suggests such sites might be less likely to host permanent foci of chytrid infection, due to their low elevation and open vegetation types, resulting in higher water temperatures (> 30 °C in August) and regular desiccation, unsuitable for the pathogen (Johnson et al., 2003). For example, Hyne et al.

(2009) found lowland species persisting without noticeable population declines in the presence of chytrid in an agricultural landscape with relatively warm climate. Conversely, particularly high impacts of chytrid worldwide have occurred in forest areas with permanent hydroperiods and cooler mesoclimates (Berger et al., 1998): all remaining large populations of *B. v. pachypus* in Liguria occur in such areas and therefore require special attention. At the moment, monitoring must continue across all known sites to ensure prompt detection of possible outbreaks. Quantifying the efficiency of monitoring (e.g. capture rates of individuals and test sensitivities) can help inform screening programs to achieve the best results for the effort invested (Lachish et al., 2012).

Although *B. v. pachypus* is recognized as an endangered species in Italy, this is, to our knowledge, the first systematic screening of any of its populations for *B. dendrobatidis*. Conservation plans for the species include habitat restoration and ex-situ initiatives (Di Cerbo and Ferri, 2002; Gentilli et al., 2002; Arillo et al., 2011). Additional information about the role of chytridiomycosis in the decline of *B. v. pachypus* is urgently needed, both to inform actions (for example, habitat restoration may not be sufficient where disease is the main threat) and to raise awareness of the need to implement safety protocols (Phillott et al., 2010). In general, further research about the distribution and prevalence of chytrid amongst amphibian species in Italy should be seen as a priority, particularly in areas of known and ongoing declines. Surveys at the local and regional scale, whilst relatively inexpensive, can provide valuable information about the spread and impact of the pathogen and help optimize conservation strategies.

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