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Recorded mortality in the vulnerable Alpine salamander, *Salamandra atra prenzensis* (Amphibia: Caudata), is not associated with the presence of known amphibian pathogens

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Manuscript received: 28 November 2016

Accepted: 8 April 2017 by STEFAN LÖTTERS

Batrachochytrium dendrobatidis (*Bd*), is a chytrid fungus triggering the panzootic disease chytridiomycosis, capable of infecting most of the world's amphibian species, i.e., more than 500 species across all three orders (OLSON et al. 2013). This disease causes devastating population declines and species extinctions (FISHER et al. 2009). A second chytrid pathogen known to infect only salamanders and newts has been discovered, *Batrachochytrium salamandrivorans* (*Bsal*); similarly to *Bd*, it causes lethal skin infections and is responsible for massive population declines in northwestern Europe (MARTEL et al. 2013). *Ranavirus* (*Rv*) causes amphibian die-offs by provoking haemorrhages (WHEELWRIGHT 2014); clinical signs include: lethargy, weakness, fluid accumulation under the skin, and occasionally skin ulcerations (DASZAK et al. 1999). These three agents are suggested to represent the major pathogens driving worldwide amphibian population declines (WHITTAKER et al. 2013).

Balkan populations of the Alpine salamander, described as *Salamandra atra prenzensis* MIKŠIĆ, 1969, are highly vulnerable for several reasons. They are: (i) 'sky island' populations restricted to isolated, high-altitude areas with a narrow vertical distribution (1,650–2,100 m a.s.l.; ŠUNJE & LELO 2008), (ii) extremely fragmented compared to their Alpine relatives (Fig. 1; ŠUNJE & LELO 2010), (iii) active only from June through September, which is one month less activity compared to individuals in the Alpine region (authors' unpubl. obs.), (iv) they have a long generation succession

time as they give birth to only one or two offspring after a gestation period of three years (KLEWEN 1988). It is obvious that a massive die-off event has the potential of rapidly eradicating these populations.

Presence of *Bd* infection in the Balkans region and Eastern Europe is understudied (VÖROS & JELIĆ 2011, Global *Bd* Mapping Project: <http://www.bd-maps.net>), and Alpine populations of *Salamandra atra* do not seem to suffer from *Bd* infection (LÖTTERS et al. 2012). Based on the cases of mortality presented in this paper and the poorly documented, but confirmed, spread of *Bd* towards the Dinarides, the possibility of its occurrence in the habitat of *S. a. prenzensis* is questionable.

Throughout fieldwork on Mt. Prenj (Bosnia and Herzegovina, hereafter abbreviated B & H) during the period 2008–2013, several affected (dead and diseased) *S. a. prenzensis* individuals were found and collected. It is of high importance to define if *Bd*, *Bsal* and *Rv* are the cause of observed mortality events in these populations, and if these are not, try to understand possible underlying causes, and identify whether they threaten the long-term overall population survival. We here report on the first extensive screening for *Bd* in B & H populations of *S. atra prenzensis* and our results of *Bd*, *Bsal* and *Rv* analyses of specimens found dead.

During 2013, 145 skin swabs for *Bd* analyses were collected across the entire known distribution range of *S. a. prenzensis* in B & H (Mts. Prenj and Čvrnsnica; Fig. 1A).

Swabs were taken according to the chytridiomycosis sampling protocol of BREM et al. (2007). After sampling of each individual, hands were washed with a fungicide (SEMI-DERM®) to prevent the potential spread of the fungus to other individuals. *Bd*-testing of all samples was performed using the quantitative real-time PCR (qPCR) standard protocol of BOYLE et al. (2004). To assure that qPCR would detect positive samples, we amplified dilutions (0.1, 1, 10, 100) of the *Bd* Internal Positive Control (IPC, stock solution of 1,000 zoospores in 5 µl) during each reaction.

In the course of the same year of fieldwork (2013), two diseased and three dead salamanders were found (number of affected specimens, hereafter abbreviated Na, = 5); all specimens resembled ‘mummified’ corpses. However, the clinical symptoms of the two diseased individuals were different from those of the dead individuals. They suffered from ocular keratitis, opaque eyes, dehydration, extreme lethargy, paralysis of (hind) limbs, contortion of the body, and roughening of the skin surface (Fig. 1B). The dead individuals displayed contorted bodies and cachexia. In the laboratory, the dead specimens were cut in two pieces, the first part was stored in 10% formaldehyde for histopathological *Bd* testing and the second half in 70% ethanol for molecular testing. Besides for *Bd* (qPCR and skin histopathology), they were tested for *Rv* and *Bsal* using qPCR (GREER & COLLINS 2007, BLOOI et al. 2013).

Possible relationships of environmental factors and prevalence of recorded mortality events (hereafter abbreviated Mp) were also investigated. Three data loggers with USB connectivity (PCE-HT71N) were left in the field on Mt. Prenj (for the period 2013–2016) to record the temperature and humidity data characterizing the salamanders’ microhabitat. Loggers were positioned inside deep cracks and under stones and values were recorded every hour. Collected data from all loggers were pooled and analysed for each year from May until October.

Out of a total of 10 Na, eight were dead and two were diseased (Fig. 2). Mp is expressed as a simple proportion: $Mp = \sum Na \times 100 / \text{total } N$. All 145 swab samples were negative for *Bd*. Additional histopathological and molecular analyses of dead specimens confirmed the absence of *Bd*, *Bsal* and *Rv*.

Overall prevalence of the mortality events was 2.4% and this does not seem to compromise population survival. The highest prevalence was registered for the month of June (6.8%), followed by August (2.6%), and July (2.3%) (Fig. 2). Dead individuals with the same symptoms as the ones described in this paper were also observed in Alpine populations of *S. a. atra* (V. HELFER pers. comm.), suggesting that this might be a natural mechanism of controlling population size. Mortality events are possibly related to a suppressed immune system as has been reported for amphibians right after hibernation (CAREY et al. 1999, COOPER et al. 2006, RAFFEL et al. 2006) since the highest mortality is observed in the month of June (first month of activity after hibernation, authors’ unpubl. obs.).

Although recurring mortality has thus been confirmed, we did not detect *Bd*, *Bsal* or *Rv* infections in *S. a. prenjenensis* populations in the surveyed area. The perceived absence of *Bd* infection can potentially be explained either by (1) the local/regional absence of infectious *Bd* lineages, (2) unsuitability of the area for its persistence, or (3) resistance to infection of the studied species:

(1) An increasing number of reports have been mentioning the presence of *Bd* in the Balkans (Romania: VÓROS et al. 2013; Greece: P. AZMANIS, unpubl. data; Croatia: M. LUKAČ, pers. comm.), although this region has actually been poorly screened for *Bd*. Results of RÖDDER et al. (2009) suggest that the distribution range of *S. atra* (see Fig. 1A) is only moderately suited for the occurrence of *Bd*; however, further research is warranted to clarify its distribution in this area.

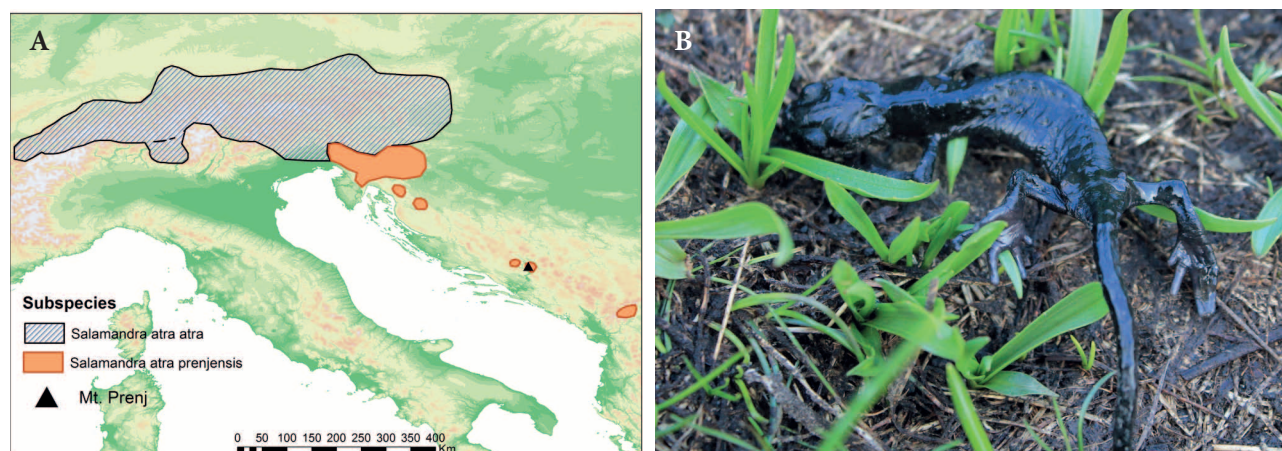


Figure 1. (A) The distribution range of the nominotypical form and the subspecies of Alpine salamanders studied in this paper. The occurrence polygon for *S. a. atra* was derived from GBIF data (www.gbif.org), HELFER (2010), and KLEWEN (1988); that for *S. a. prenjenensis* from DŽUKIĆ G. et al. (2003), KRIZMANIĆ, I. (1997), JERAN et al. (2011), RAZPET et al. (2016), and ŠUNJE, E. & S. LELO (2010). The location of the study site (Mt. Prenj) is indicated. (B) A female of the studied subspecies, *S. a. prenjenensis*, that was found sick in the field and died within 12 hours from first encountering it (29 June 2013).

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Table 1. Temperature (°C, abbreviated T) and humidity (in %, abbreviated H) values recorded by three data loggers placed on Mt. Prenj (2013–2016). Other abbreviations: SD – standard deviation; Min – minimum; Max – maximum.

Month	T mean	T SD	T Min	T Max	H mean	H SD	H Min	H Max
May	3.4	3.17	-0.1	10.40	90.84	6.76	80.2	100
June	9.6	2.39	1.6	19.1	87.69	10.49	53	100
July	12.44	3.01	7.4	28.6	89.96	9.30	40.3	100
August	14.44	3.36	8.3	27	80.89	16.17	25.20	100
September	10.08	3.39	1.9	23.5	92.29	8.6	51.00	99.8
October	7.29	3.44	- 2.7	13.8	93.63	6.49	64.60	100

(2) The optimal temperature range for the growth of *Bd* under lab conditions is 17–25°C, but it can tolerate temperatures between 4 and 28°C, and prefers water bodies for spreading (PIOTROWSKI et al. 2004). Considering that the mean temperatures on Mt. Prenj during our study never reached the values for optimal growth of *Bd*, the area does apparently not provide ‘ideal’ conditions for *Bd* survival (Table 1). Our temperature data and the landscape configuration (i.e., lack of streams and poor water sources) of the screened area may suggest that the habitat of the Alpine salamander on Mt. Prenj is quite challenging to both the occurrence and persistence of *Bd*.

(3) In terms of physiological traits, it is indeed remarkable that several terrestrial western Palearctic salamander species (e.g., genera *Lyciasalamandra* and *Speleomantes*) apparently are negative for *Bd* due to the presence or expected existence of antifungal skin secretions (GÖÇMEN et al. 2013, PASMANS et al. 2013). This might offer the possibility that *S. atra* is likewise resistant to *Bd* (cf. LÖTTERS et al. 2012). Also, *Bd* transmission from other infected am-

phibians might be expected to be minimal considering the ecology of *S. a. prenjenensis*. First, altitude might reduce the contact between the Alpine salamander and other amphibians, as only a few amphibian species are known from Alpine habitats; in our study area this is especially *Bufo bufo* (ŠUNJE & LELO 2008), a species known to be a poor *Bd* host (MARTEL et al. 2012, RASMUSSEN 2012). Second, the Alpine salamander does neither make use of aquatic habitats (where amphibians will otherwise congregate for reproduction) nor has an aquatic life stage, which additionally lowers the chances of transmission (WILLIAMS & HERO 1998). Notwithstanding, a strictly terrestrial life history does neither exclude nor even reduce the likelihood of infection with *Bd* (LÖTTERS et al. 2012, KOLBI et al. 2015).

Although *Rv* is widespread around the world, the risk of *S. atra* being infected with *Rv* is reduced since this agent prefers water bodies for transmission, higher temperatures, and poor-quality habitats (DUFFUS et al. 2015). *Bsal* is probably the most dangerous agent threatening *S. atra* (all subspecies), as it is known to likely affect all salaman-

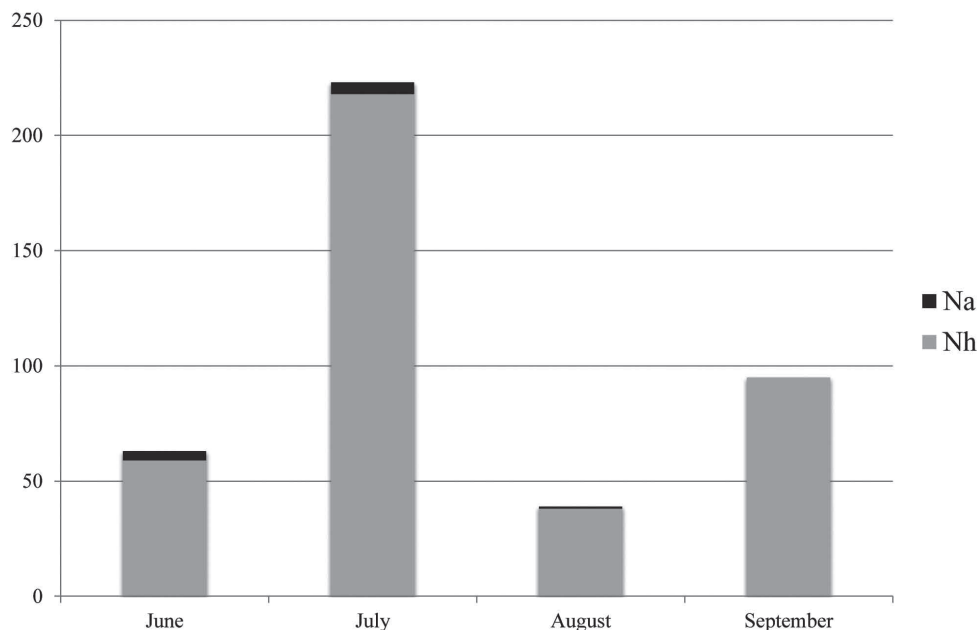


Figure 2. Prevalence of reported mortality events (Mp) on Mt. Prenj for the period 2008 through 2013. Abbreviations: Na – number of affected individuals found (diseased and dead – $\sum Na = 10$); Nh – number of healthy individuals; total N = 420.

drids (MARTEL et al. 2014). Moreover, *Bsal* has an optimal temperature range of 10–15°C for growth (MARTEL et al. 2013), which matches the temperature range during periods of activity of the Alpine salamander in the study area (Table 1). Data on the distribution of *Bsal* in Europe are scarce since it is a relatively recent discovery, but existing data confirmed its presence in Belgium, Germany and The Netherlands and has been detected in captive salamanders in the United Kingdom (SPITZEN-VAN DER SLUIJS et al. 2016).

Acknowledgements

We are very grateful to The Rufford Foundation – Small Grants for Nature Conservation for their supporting this project. We thank the members of the Herpetological Association in B & H: ATRA (www.bhhuatra.com) for their invaluable contribution in collecting samples for this study; and special thanks go to SAUDIN MERDAN and ADNAN ZIMIĆ who were main assistants in this project.

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