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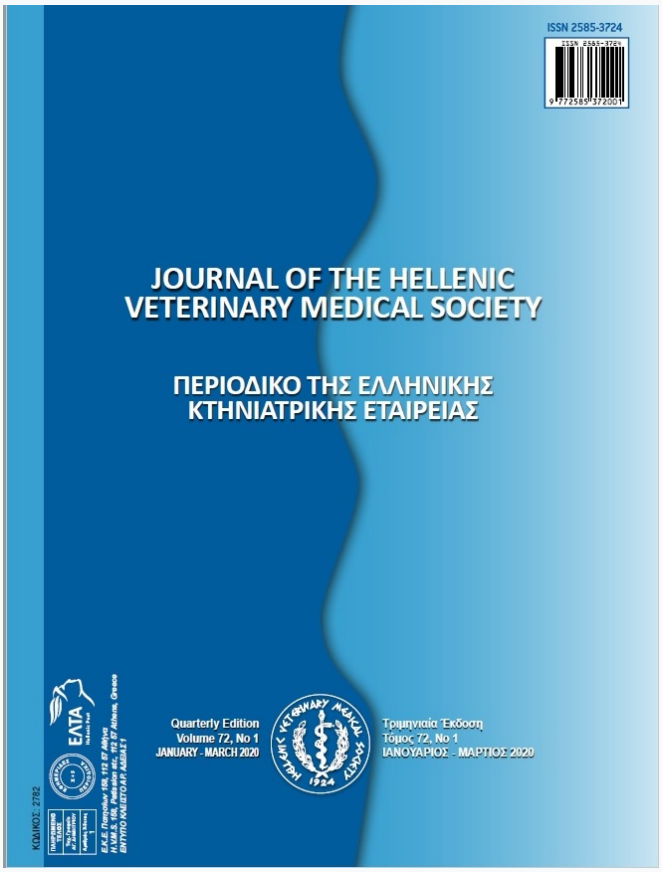

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Research article
Ερευνητικό άρθρο

Mass mortality of unknown etiology in alpine newts (*Ichtyosaura alperstris veluchiensis*) in an alpine lake in Greece

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ABSTRACT: A mass mortality in alpine newts (*Ichthyosaura alpestris veluchiensis*) was observed in May/June 2013, in Drakolimni lake on Smolikas Mountain, Northwest Greece. 1300 alpine newts were found dead in two events. In 1998 a similar incident was recorded in the nearby lake of Timfi Mt. Newts of every stage and sex were affected, presenting incoordination and inability to float evenly. Ten animals were submitted for complete pathological examination. Field environmental measurements (water temperature, oxygen saturation, pH, conductance, nitric/phosphate concentration) and samples (water, snow, benthos) were collected for ecotoxicological and quality analysis. Necropsy, microbiology (parasitology, bacteriology, mycology), histopathology, molecular investigations (*Ranavirus spp*, *Batrachochytridium dendrobatidis, Batrachochytridium salamandrivorans)*, quality and ecotoxicological examinations did not indicate a causative source for the mortality. To the author´s knowledge this is the biggest mortality of unknown etiology reported in free-living alpine newts in Europe.

Keywords: *alpine newt, mass mortality, Greece, incoordination, unknown etiology*

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INTRODUCTION

This short communication describes the investigation of a mass mortality incident in alpine newts in Greece. The Balkan Peninsula is considered to be a major hotspot for European biodiversity (Griffiths et al., 2004). Balkan alpine lakes are not well studied, due to their small size and difficult access. Greek alpine lakes are small, often fishless and located at high-altitude areas above 1900m, near the summit of mountains. They are called "dragon-lakes" (Drakolimnes in Greek) because their main vertebrate inhabitant, the alpine newt (*Ichthyosaura alpestris*), is reminiscent of the shape and appearance of a small dragon. *Ichthyosaura alpestris* (initially referred to as *Triturus alpestris* and more recently as *Mesotriton alpestris*) is a small animal with a total length of about 80 to 100 mm for males and up to 120mm for females. The Greek alpine newt was classified as a subspecies named *Ichthyosaura alpestris veluchiensis* (Denöel, 2004). Although *I. alpestris* specimens in northwestern Greece are found in various water bodies above 1190m such as ponds, drinking troughs and watering basins, large populations inhabit only the alpine lakes of the region (Denöel and Schabetsberger, 2003).

MATERIALS AND METHODS

The incident took place in the dragon-lake of Mt. Smolikas (40°05'N, 20°54'E, 2140m a.s.l), in the wider region of Ipirus, Northwestern Greece. The lake has a rectangular shape (122m long, 61m wide) and a maximum depth of 3.7 m. Vegetation is limited to small shallow patches. The bottom is muddy, and rocks are rare (Denöel and Schabetsberger, 2003). An older incident of mass mortality was described in 1988 by one author (H. Papaioannou pers.com) in the dragon-lake of Mt. Tymphi (39°59'N, 20°47'E, 2000m a.s.l),which has a somewhat quadratic appearance (max. diameter: 100m) and a maximum depth of 4.95m. It is characterized by rich vegetation along the shoreline, consisting mostly of *Carex* sp (Denöel and Schabetsberger, 2003). The distance between Lake Tymphi and Lake Smolikas is 14.85 km. No exchange exists between newt populations inhabiting the two lakes due to impassable deep valleys that separate them (Figure 1).

A total of 1604 alpine newts were found dead in two events (Mortality rate 0.81% and morbidity rate 0.189%) in May and June 2013. Newts of every stage and both sexes were affected. Clinically affected newts were uncoordinated and unable to float evenly (Figure 2). Otherwise no other symptoms were evident upon observation. The area was searched intensively for the presence of salt, agrochemicals or other possible source of poisoning.

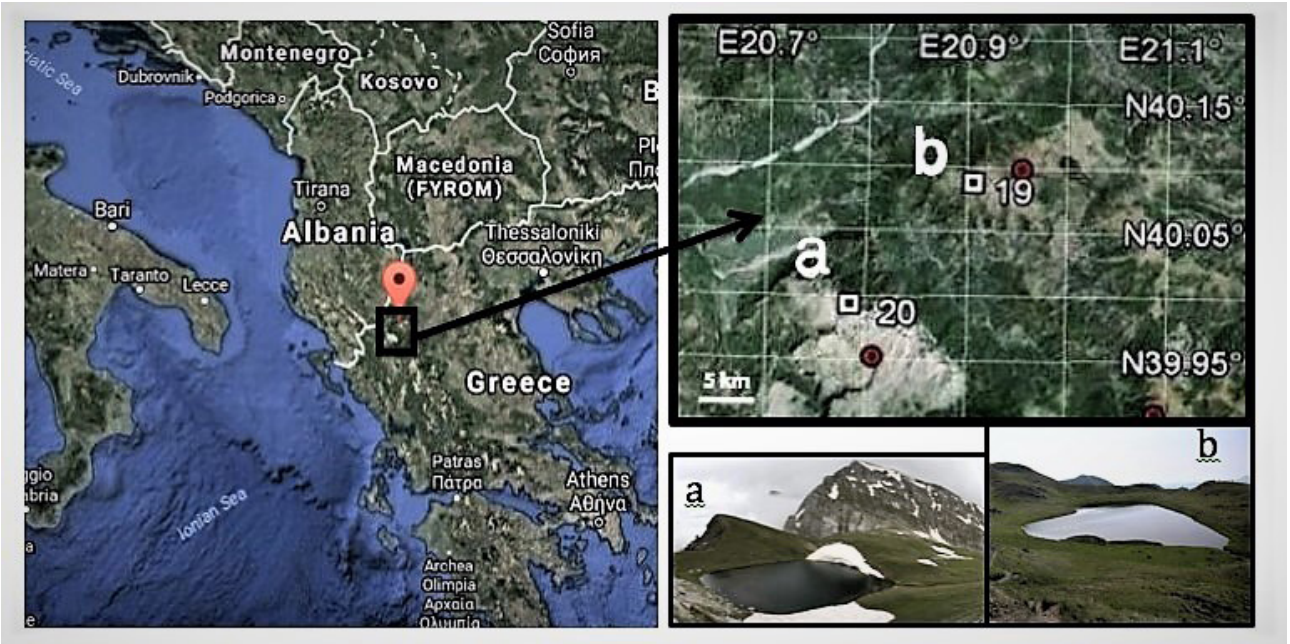

Figure 1. A satellite view of the study area in the mountains of NW Greece. The studied populations are indicated with square shape. Other known newt populations are indicated with red dots. (a: Drakolimni Tymphis, b: Drakolimni Smolika) (Satellite photos by Google-earth)

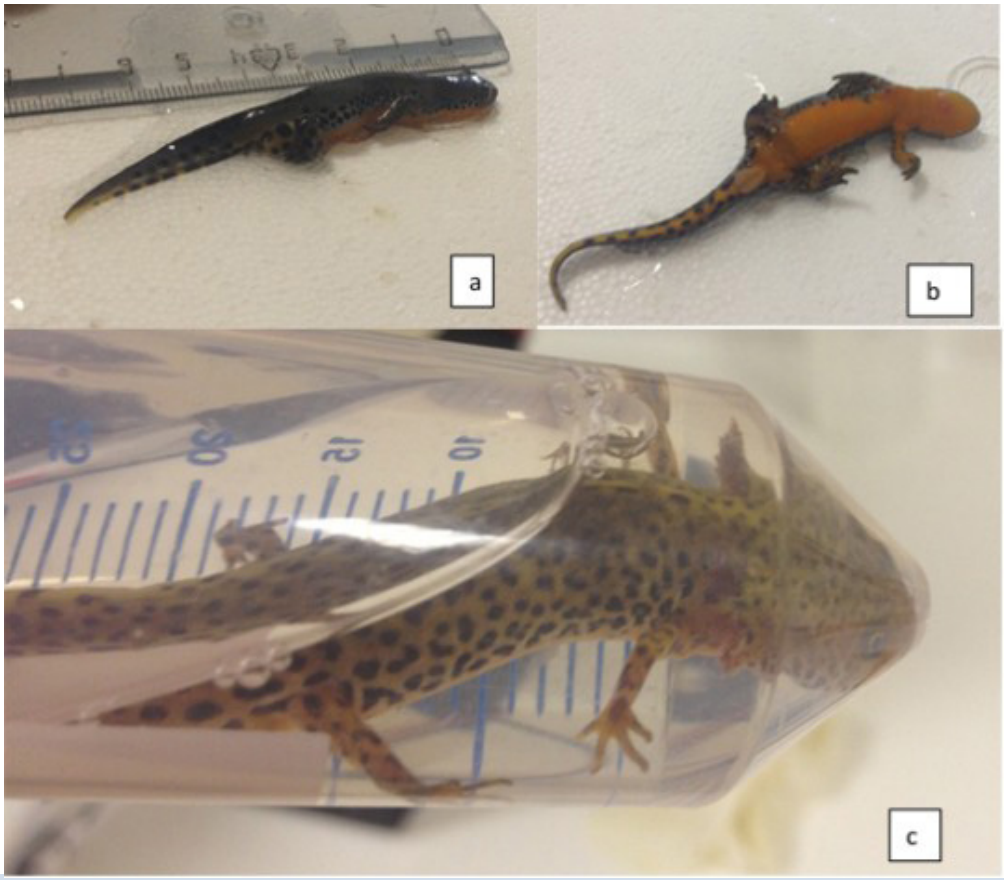

Figure 2. a) Dead male alpine newt under examination (dorsal view) b) male newt abdominal view c) neotenic newt (close up the external gills)

Environmental measurements included water temperature, oxygen saturation, pH, conductance, nitric and phosphate concentration and were performed on site while samples (water, snow and benthos) were collected using sterilized containers for further ecotoxicological and quality analysis. Samples were kept cool until they were transported to the laboratory and processed. Collected surface water and melted snow were used to detect toxicity. Thamnotox bioassay was performed according to the manufacturer's instructions (MicroBioTests Inc, Kleimoer 15, 9030, Mariakerke (Gent), Belgium). In order to check if the Thamnotox test operates properly we performed a quality control test with the toxicant potassium dichromate ($K_2Cr_2O_7$). For Thamnotox toxicity test we prepared dilutions of surface water and melted snow (6.25% -100%) and we transferred them into the multiwell plate at four replicates. Ten hatched larvae of *Thamnocephalus platyurus* were transferred into each well and the multiwell plate was incubated at 25° C in darkness for 24h. After 24h, using a dissection microscope, we counted live and dead larvae and we calculated the percentage mortality.

Additionally, ten clinical affected newts from different sex and stages (adults, juveniles, and larvae) were collected and transported alive for further veterinary investigations.After humane euthanasia (general anesthesia under isoflurane and pentobarbital injection) (Gentz, 2007),necropsy was performed immediately under standard protocols including wet mount parasitology, bacteriology, mycology and histopathology (one individual). As initial isolation media, Columbia blood agar (Oxoid, Wesel, 46483, North Rhine-Westphalia, Germany), MacConkey agar (Oxoid) and brilliant-green agar (Oxoid) were streak inoculated with the samples and then incubated at 41°C for 24 h under aerobic conditions. If after this time no or scanty growth was present the incubation time was prolonged for another 24 h. Colonies demonstrating distinctive macroscopic appearance were considered separate organisms and isolated on new plates for identification. The isolated bacteria were identified using standard microbiologic techniques including Gram-staining, morphologic characteristics, catalase and oxidase reactions and growth in limiting media. Representative Gram-negative isolates of all distinct

organisms were further identified using the multi-substrate-identification kit BBL Crystal TM E/NF (Becton–Dickinson, Franklin Lakes, 07417-1880,NJ, USA). Among Gram-positive bacteria, cocci and Bacillus were identified to the genus based on colony morphology, catalase and oxidase reactions as well as microscopic appearance after staining. Isolation of Salmonella and determination of Serotypes RV broth (Oxoid) were incubated at 41°C for 24 h, and subsequently a loopful (10 lL) of RV broth culture was streak inoculated onto a xylose lysine tergitol-4 (XLT-4) agar (Oxoid Deutschland GmbH,46483, DE) and a brilliant-green (BGA) agar plate (Oxoid Deutschland GmbH, 46483, DE), respectively. Plates were incubated for another 24 h under aerobic conditions at 41°C. All suspicious colonies were screened for Salmonella with a rapid plate agglutination test using Enteroclon Anti-Salmonella A67, omnivalent (Sifin GmbH, 13088, Berlin, DE). A mycological culture was performed on Sabouraud dextrose agar (Oxoid) and potato dextrose agar (Oxoid) and incubated in 41°C for up to 120 h. One animal was completely fixed in 4% neutral-buffered formalin. Fixed samples were embedded in paraffin, and 5 µm sections were stained with haematoxylin and eosin for histological examination by light microscopy.

Collective samples from nine newts were used for molecular investigations. Total genomic DNA was extracted using the Roche MagNA Pure 96 system with the MagNA Pure 96 DNA and viral RNA small Volume Kit (Roche, Mannheim, 68305, Germany) according to the manufacturer's instructions. A real-time PCR was used to assess the presence/absence of *B. dendrobatidis, B. salamandrivorans* and a conventional PCR to detect *Ranavirus* DNA within the extracted DNA as described previously (Marschang et al., 1999, Boyle et al., 2004, Blooi et al., 2013).

RESULTS

The field investigation by the researchers in the two visits did not reveal human activity, salt disposal or any other source of possible poisoning. The physical parameters measured on site were within the normal limits except the slightly reduced pH (Table 1). Zooplankton concentration was slightly reduced. The acute toxicity assays and the cyanotoxine measurement were negative. No pesticide traces were detected.The values from the reference test were within the limits indicating the good procedure of the bioassay. Percentage mortality of the *Tamnocephalus platyurus* in the samples was very low and comparable to controls. Complete necropsy in three newts revealed no internal or external alterations. Special attention was given to possible skin lesions indicating fungal disease. Parasitology, bacteriology, mycology was unremarkable, and histopathology showed partial autolysis, no signs of infectious process or foreign materials (i.e. sand). Skin histology from 13 sympatric newts for another study some months (October 2013) ago the mortality events, did also not reveal cancerous or skin pathology (Papaioannou et al., 2015). Real-time and conventional PCR for *Ranavirus*, *B. dendrobatidis* and *B. salamandrivorans* were negative.

Table 1. Physical and chemical parameters in alpine dragon-lake of Mt. Smolikas during a newt mass mortality event

Physical chemical parameters in Smolikas Dragon-lake in May 2013	
Temperature	4°C
pH	7.2
S^{2-}	Not detected
NH_4^+	0.02 mg/L
NO^{2-}	0.019 mg/L
NO^{3-}	1.2 mg/L
Ca^{2+}	2.5 mg/L
Mg^{2+}	0.46 mg/L
PO_4^{3-}	Not detected
Na, K, Cl	Not detected
O_2	8 mg/L

DISCUSSION

Mass mortality of free-living newts in Europe, has been reported infrequently with the greatest number recorded being 691 alpine newts in Austria (Sztatecsny and Hodl, 2009). The die-offs so far reported were attributed to road trespass (Mitchell, 2000), ranaviruses (Balseiro et al.,2010, Kik et al., 2011, Martinez-Silvestre et al., 2017) and salt toxicity (Duff et al.,2011).*B. dendrobatidis* and *B. salamandrivorans* were blamed for the decline of Sardinian newts (Bovero et al., 2008) and fire salamanders (Martel et al., 2013) respectively. In alpine newts, which reside in isolated highland niches, only one outbreak has been reported due to ranavirus (Balseiro et al., 2010)and Common Midwife Toad Virus (CMTV)-like ranavirus (Price et al., 2014). Mesomycetozoans fungus-like organisms like *Ichthyophonu* ssp*, Amphibiocystidium* sp cause marked changes in skin and internal organs (Raffel et al., 2008). None of the above-mentioned causes of newt mortality causing skin and internal organ alterations, was detected in the current investigated event (alive specimens, post-mortem examined

specimens and the single histopathologic examined specimen). Additionally,skin histology from 13 sympatric newts for another study five months after the mortality event, did also not reveal cancerous or skin pathology (Papaioannou et al., 2015).Nevertheless, it could not be excluded that other pathogens could be involved such as invertebrate Iridovirus IIV-6 (Stöhr et al., 2016)and Frog Virus-like ranavirus (FV3) (Peace et al.,2019, Vilaca et al., 2019) although the later viruses have been so far affecting anuran populations mostly in North America. Apart from viruses, Perkinsea protists have been associated with cryptic infections and mass mortality of anuran populations and have been found in tadpoles in UK and Czech Republic (Chambouvet et al., 2015).In another scenario biological/environmental parameters could havetriggered the event (e.g. overpopulation, quicker defrosting of the lake with abrupt temperature variation and reduced oxygen concentration). The authors also investigated the option of pneumoconiosis as subsequent to an African red sandstorm in the area. None traces of sand were detected in oropharyngeal cavity, lungs, or internal organs of the examined newts. The limited histopathological samples and the recent molecular sequencing of various rana-like viruses (which were not known at the time of the events) could also have implicated the elucidation of a specific pathogen in this event.The exact date that the mortality started remains unknown, thus the authors could not exclude the possibility that the physical parameters measured on-site could have been changed during the time of initial newt death and the arrival of the investigators. The presence of both dead and alive newts suggests that the events were not acute, but rather spread over a couple of months and if a pathogen or poisoning agent was implicated it would be still active and detectable during the sampling. At the present date of submission of this manuscript the authors have not observed any further mortality in the same alpine lake or any other alpine lake in the area.

CONCLUSIONS

Amphibian medicine can be challenging despite its progress the recent years. In this outbreak sampling was challenging due to the rugged terrain and a possible causative agent/parameter was not identified. Authors stress the importance that similar mortality events in amphibians should be elaborated interdisciplinary (chemical, biological, veterinary, meteorological data) to unveil a possible puzzle of parameters leading to such events. At the time of this manuscript no other mortalities have been reported from these lakes or other alpine lakes in Greece. The authors will further monitor these and other alpine lakes and record possible mortalities in the future.

CONFLICT OF INTEREST

None declared by the authors.

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