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Widespread bacterial infection affecting *Rana temporaria* tadpoles in mountain areas

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Abstract. Periodic mass die-offs of *Rana temporaria* tadpole populations have occurred in the ponds of prealpine mountain areas of Brescia (northern Italy) since the early 2000s. The author reports some observational data and analytical results from three sites: tadpoles from mortality events had erythema, especially on the legs, suggestive of septicemia. Bacterial culture of these tadpoles revealed *Aeromonas hydrophila* and *Aeromonas sobria*, two organisms often associated with Red leg disease. Egg mass counts from 29 pastureland ponds did not revealed breeding activity declines over five years in the Monte Guglielmo area. *Aeromonas hydrophila* and *Aeromonas sobria* usually behave as opportunistic bacteria that can become pathogenic after suppression of the immune system by endogenous or exogenous stressors. Thus, a plurality of environmental factors may contribute to mortality events; some of them are discussed, including loss of high altitude breeding ponds resulting in overcrowding and poor water quality in remaining ponds and the presence of other pathogens.

Keywords. mass die-offs, Red leg, *Aeromonas* sp., *Rana temporaria*, tadpoles.

INTRODUCTION

Since 1990, an increasing number of studies have reported declines in amphibian populations (Wake, 1991), and several contributing factors have been proposed (Gardner, 2001), including disease (Berger et al. 1998; Daszak et al., 2000; Kiesecker et al., 2001; Kiesecker et al., 2004). Therefore a lot of conservation efforts have been created by several organizations attempting to understand and monitor the decline. The need of taking full account of each episode of abnormal mortality has been emphasized.

In the prealpine mountain areas of Brescia (Southern Italian Alps), mass die-offs among *Rana temporaria* tadpoles have been reported since the early 2000s. The purpose of this text is documenting the occurrence of these mortality events using retrospective data from Bagolino and Odeno and new data from Monte Guglielmo Massif. Therefore,

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from 2005 to 2009, in order to highlight any effect of mass die-offs on populations, *Rana temporaria* breeding activity has been monitored in the Monte Guglielmo area by counting egg masses in 29 ponds and collecting biological samples, including live and dead tadples, cloacal swambs on adult animals and water samples.

MATERIAL AND METHODS

Study area. All the samples have been collected in high altitude breeding ponds. Usually the ponds are artificially dug in high altitude pastures for watering cows; ponds persistence depends on human maintenance. The studied ponds are shallow (from a few centimeters to just over one meter) and subject to strong daily and seasonal variations of chemical and physical parameters; because of cattle presence the ponds are regularly subject to nutrient pollution in summer time. The studied areas have a typical mountain climate and the ponds freeze during the winter; in a few cases, they dry at the end of summer.

Samples come from three distinct prealpine areas of Brescia (northern Italy): Bagolino, Odeno and Monte Guglielmo (Fig. 1). The sampling sites cover a wide longitudinal range in the prealpine area of Brescia. Bagolino (Lat.: 45°49'35"; Long.: 10°27'38"; Alt.: 769 m a.s.l.) is a village in the valley of the river Caffaro on the right side of Sabbia valley; Odeno (Lat.: 45°44'50"; Long.: 10°20'12"; Alt.: 917 m a.s.l.) is a hamlet of Pertica Alta in the Sabbia valley; Monte Guglielmo is a rather isolated mountain range that straddles the watershed between Trompia valley and Camonica valley. There are no further information on the exact location of sampling site from Bagolino and Odeno, while accurate data are available for Monte Guglielmo as shown in Fig. 1 and Table 2.

Sampling and analytical methods. In order to closely observe any sign of disease, several frogs and tadpoles were captured using a landing net or with bare hands. Some of the caught animals underwent further treatments or were sampled: five adult frogs underwent cloacal swamb sampling for bacterial examination; fecal samples were collected gently rubbing the perianal area of adult frogs using sterilized swambs with culture medium; treated animals were immediatly released. Moreover several infected tadpoles (dead or still alive) were sampled; dead tadpoles were stored in test tube, while still alive tadpoles in containers allowing their survival until the refrigeration. Finally, five water samples from the infected sites were collected for bacteriological analysis. All samples were preserved at low temperature (4 °C) and examined within one day in the IZS (Istituto Zooprofilattico Sperimentale) in Brescia. Each sample underwent bacteriological examination; parasitological and viral examinations were performed for the tadpoles sampled in Monte Guglielmo area. Details of the analytical methods are given in the following paragraphs.

Parasitological examination. Microscopic examinations were performed to identify several parasites: 1-2 drops of sample were diluted in Lugol solution to observe flagellate and ciliate protozoans; nematodes and cestodes eggs were separated by flotation in a sodium nitrate satured solution; trematodes were separated by sedimentation and centrifugation and gross specimens were observed.

Bacteriological examination. Since *Rana temporaria* is an ectothermic organism, the sample was obtained from internal tissues and organs and was directly cultured on Gassner Agar and Blood Agar plates and stored at variable temperature for 24-48 hours. Further, sub cultures were transferred from Agar plates to differential and selective media and the morphology of the colony was observed; bacterial colonies were identified at the specific level thanks to specific biochemical test. Enterobacteria and non-enterobacteria identification was performed thank to identification systems as API-20E and API-20NE.

Viral examination. Viral examination with Transmission Electron Microscopy (TEM) were performed directly on aqueous suspensions of internal organs and tissues to find mature virions. The samples were repeatedly frozen and thawed to facilitate the release of viral particles in the aque-

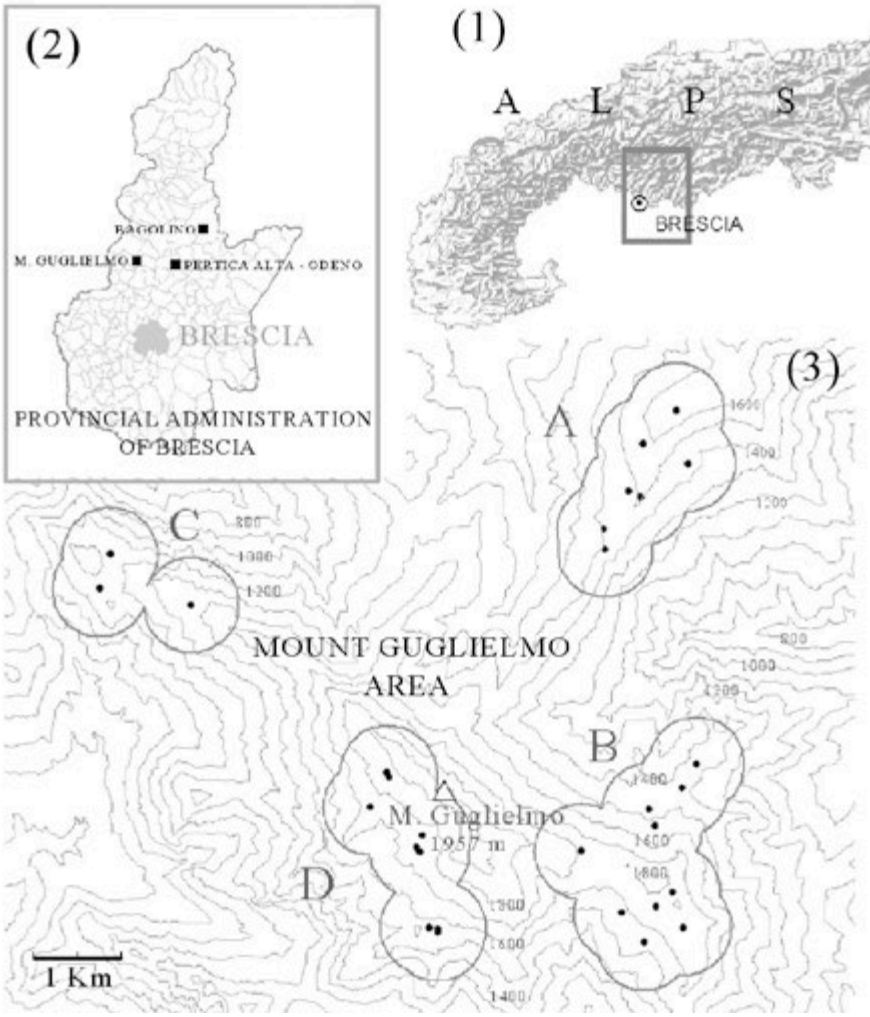

Fig. 1. Study area; (1): position on the alpine range; (2): position in the provincial administration of Brescia; (3): studied ponds and groups of ponds (A-D) in Monte Guglielmo area.

ous suspension. The suspension was centrifuged twice at low speed (6000 and 10000 rpm, for 20 minutes) to remove coarse debris; a small amount of the second supernatant was added to microtubes containing formvar-coated copper grids for TEM and underwent ultracentrifugation (80000 rpm). The grid was drawn and coloured with 2% sodium phosphotungstate (NaPT) for 1.5 minutes and finally examined by TEM.

Data analysis. Since 2005 to 2009, the author had been looking for the effects of mass die-offs on the abundance of *Rana temporaria* populations. Thus *Rana temporaria* breeding activity in the Monte Guglielmo massif was monitored by counting egg masses in 29 ponds. The presence of 4 metapopulations of *Rana temporaria* (A-D) was assumed to test for population declines; each metap-

opulation was sustainded by a group of ponds less than one kilometer far away one from another (Fig. 1). Three different Linear Mixed-Effects model (LME) fitted by Restricted Maximum Likelihood had been used to explore the effects of time on the number of egg-masses layed in the ponds (Eggs), as a dependent variable. A time variable (Year) and a binary variable standing for *Triturus carnifex* presence (Tricar) (which looked likely to be important in determining *Rana temporaria* breeding sites) had been added as fixed effects. The group of ponds (Group) had been added as random effect and each pond (Pond) as nested random effect. The models were compared each other using a Log-Likelihood Ratio Test and the accuracy of the last model was verified by testing the normality of the residuals.

RESULTS

Early signs of disease were observed in the advanced stage of larval development and rarely before the appearance of hind legs. Initial signs of disease included pettechia and ecchymosis within the caudoventral and lateroventral body and tail. In the later stages of the disease, larvae swam slowly (lethargy) even when pursued by for capture. In the terminal stage of the disease, moribund individuals hung listlessly at the surface or at the bottom of the pond. The disease was highly contagious, often involving the entire tadpole population in the infected pond and most of the ponds, even in large areas. Nevertheless adult *Rana temporaria* or other anuran (*Bufo bufo*) and urodele (*Triturus carnifex*) species,

Table 1. Summary table of examination results. MG: samples from Monte Guglielmo area; O: samples from Pertica Alta – Odeno area; B: samples from Bagolino area; CFU: Colony-Forming Units; en dash: examination has not been performed.

	Sample	Date	Area	Bacterial Examination	Viral examination	Parasitological examination
1	6 dying tadpoles	26 June 2006	MG	Negative	Negative	Negative
2	6 died and 16 dying tadpoles	26 June 2006	MG	*Aeromonas Hydrophila* infection	Negative	Negative
3	1 dying tadpole	03 July 2006	MG	Negative	Negatine	Negative
4	2 dying tadpoles	03 July 2006	MG	*Aeromonas sobria* infection	Negative	Negative
5	5 anal swambs	03 July 2006	MG	Negative	-	-
6	Water	03 July 2006	MG	*Aeromonas* sp. 52000 CFU	-	-
7	Water	03 July 2006	MG	Negative	-	-
8	3 dying tadpoles	17 June 2003	O	Negative	-	-
9	2 dead tadpoles	17 June 2003	O	Negative	-	-
10	2 dead tadpoles	17 June 2003	O	*Aeromonas Hydrophila* infection	-	-
11	10 dying tadpoles	31 May 2004	O	*Aeromonas* sp. Infection	-	-
12	10 helthy tadpoles	31 May 2004	O	Negative	-	-
13	Water	26 May 2003	O	*Aeromonas Hydrophila* 160000 CFU	-	-
14	Water	26 May 2003	O	*Aeromonas Hydrophila* 31000 CFU	-	-
15	Tadpoles	04 June 2003	B	*Aeromonas* sp. Infection	-	-
16	Water	04 June 2003	B	*Aeromonas Hydrophila* 13000 CFU	-	-
17	Water	04 June 2003	B	*Aeromonas sobria* 16000 CFU	-	-

cohabitating the same ponds, did not display clinical evidence of disease. Five anal tampons were collected as confirmatory test on apparently healthy adult frogs and no pathological bacteria were found.

The examination of died or dying tadpoles, from all the study sites, always highlights a negative response for viral and parasitological examination, while the bacterial examinations often pointed out septicaemic infections caused by motile *Aeromonas* group (*Aeromonas hydrophila* and *Aeromonas sobria*) (Table 1). Similarly, the same pathogen was

Table 2. Geographical data of ponds, *Rana temporaria* egg masses counting (Eggs) and *Triturus carnifex* presence (Tricar) in M. Guglielmo area, since 2005 to 2009. Lat: latitude; Long: Longitude; Alt: altitude; na: not available data; x: *Triturus carnifex* presence.

POND	Lat	Long	Alt (m)	2005		2006		2007		2008		2009	
				Eggs	Tricar	Eggs	Tricar	Eggs	Tricar	Eggs	Tricar	Eggs	Tricar
A1	45°47'26"N	10°11'57"E	1434	0	x	13	x	0	x	0	x	0	x
A2	45°46'58"N	10°11'14"E	1389	3		40		7		6		0	x
A3	45°47'16"N	10°11'29"E	1472	14		30	x	53		30		70	
A4	45°47'14"N	10°11'33"E	1505	6		15		48		115		73	
A5	45°47'33"N	10°11'36"E	1562	27		6		99		22		79	
A6	45°47'33"N	10°11'36"E	1652	na		na	x	20		1		28	
A7	45°47'03"N	10°11'11"E	1371	na		na		37		30		35	
B1	45°45'43"N	10°11'59"E	1313	60		162		45		12		10	
B2	45°45'35"N	10°11'56"E	1309	9		22		26		14		50	
B3	45°45'28"N	10°11'36"E	1373	136		70		15		17		13	
B4	45°45'22"N	10°11'39"E	1408	45		na		4		na		28	
B5	45°44'43"N	10°11'30"E	1535	0		5		na		2		5	
B6	45°44'53"N	10°11'21"E	1569	0		7		2		0		15	
B7	45°45'14"N	10°11'02"E	1681	na		10		4		na		35	
B8	45°44'55"N	10°11'38"E	1674	0		4		0		0		0	
B9	45°44'60"N	10°11'47"E	1672	5		16		34		24		57	
B10	45°44'48"N	10°11'52"E	1664	10		16		3		199		53	
C1	45°46'46"N	10°07'11"E	1166	0	x	0	x	0	x	0	x	0	x
C2	45°46'57"N	10°07'16"E	1175	0	x	0	x	0	x	0		0	x
C3	45°46'40"N	10°07'55"E	1361	0	x	0	x	0	x	0		0	x
D1	45°44'48"N	10°09'52"E	1564	17		30		0		0		1	
D2	45°44'47"N	10°09'52"E	1563	6		4		1		0		1	
D3	45°44'49'N	10°09'44"E	1577	na		0		0		1		2	
D4	45°45'14'N	10°09'44"E	1734	18		7		na		0		0	
D5	45°45'16'N	10°09'43"E	1739	50		26		18		2		0	
D6	45°45'20'N	10°09'46"E	1760	na		na		na		0		0	
D7	45°45'30'N	10°09'21"E	1749	3		na		na		6		7	
D8	45°45'40'N	10°09'30"E	1853	0		na		na		0		0	
D9	45°45'42'N	10°09'29"E	1863	0		13		9		0		7	
Tot.				409		496		425		481		569	

Table 3. Fixed effect results from mixed models testing the hypothesis that number of egg masses per pond will decrease since 2005 to 2009. The groups of pond and the ponds were included as a random effect and nested random effect in these models. NS: Not Significant.

Model	Source of variation	Beta	df	F	P
Mod1	Intercept	149.95	121	13.4	< 0.001
	YEAR	-0.07	121	0.5	NS
	TRICAR	-1.83	121	17.0	<0.001
Mod2	Intercept	116.35	96	11.5	<0.001
	YEAR	-0.06	96	0.6	NS
	TRICAR	-1.26	96	7.1	<0.01
Mod3	Intercept	147.74	96	11.7	<0.001
	YEAR	-0.07	96	1.3	NS
	TRICAR	-1.26	96	9.5	<0.01

found in pond water samples by counting the number of bacterial colonies on a culture medium (Table 1).

Testing the hypothesis that the number of egg masses per pond would have decreased from 2005 to 2009 (Table 2), no significant relationship between Eggs and Year was found (Table 3). In Mod1 we set Year and Tricar as fixed effects and Group as random effect. In Mod2, Pond has been added as nested random effect to test the effect of every pond on Eggs. Mod1 was compared with Mod2 with a Log-Likelihood Ratio Test (LRT = 18.06, $P < 0.0001$). Since the presence of *Triturus carnifex* influenced the variance structure of Mod2 ($F = 22.16$, $P < 0.0001$), Mod2 was weighted on the Tricar binary variable. The updated model (Mod3) was compared with Mod2 (LRT = 4.830825 , $P < 0.05$). The accuracy of Mod3 was verified by testing the normality of the residuals ($W = 0.99$, $P = 0.25$). Based on the models, the population of *Rana temporaria* has not significantly declined since 2005; otherwise, the importance of *Triturus carnifex* as a factor in disturbing *Rana temporaria* breeding activity is detectable in all the three models.

DISCUSSION

The only pathogens isolated in the samples were *Aeromonas hydrophila* and *Aeromonas sobria. Aeromonas* sp. occurs widely in fresh and estuarine waters (Cahill, 1990; Hazen et al., 1978; Massa et al., 2001) and is considered a primary and secondary pathogen of aquatic and terrestrial animals. These bacterial pathogens can infect anuran (Hubbard, 1981; Carey, 1993; Bradford, 1991; Marquez et al., 1995; Razzetti and Bonini, 2001; Taylor et al., 1999; Nyman, 1986; Hird et al., 1981; Sherman and Morton, 1993; Colt et al., 1984; Hayes and Jennings, 1986; Glorioso et al., 1974; Rigney et al., 1978), urodeles (Kaplan and Glaczenski, 1965; Boyer et al, 1971), fishes, reptiles, birds, and mammals, including humans (Panigrahy et al., 1981; Cahill, 1990; Gorden et al., 1979; Davis et al.,

1978). *Aeromonas hydrophila* is one of the pathogenic agents of Red leg disease. Gross signs observed during mortality events within the prealpine study sites included ettechia and ecchymosis within the caudoventral and lateroventral body and tail, lethargy and coma and were consistent with those reported for Red leg disease (Nace, 1968; Marquez et al., 1995; Nyman, 1986; Hird et al., 1981; Carey, 1993). However, *Aeromonas* sp. is not the only agent in this pathology, which is the result of a complex interaction between other gram negative bacteria including *Pseudomonas* spp., *Proteus* spp., *Flavobacterium indologenes* and *F. meningosepticum* (Hubbard, 1981; Taylor et al., 1993; Anver and Pond, 1984).

Moreover, *Aeromonas* sp. presence by itself does not necessarily give evidence of disease; these bacteria can live in free waters, on the skin and in the digestive tract of amphibians without causing any disease (Hubbard, 1981; Carey, 1993; Hird et al., 1981). Consequently, even if *Aeromonas* sp. is also considered a primary pathogen, several stressors are often involved in outbreaks of disease and *Aeromonas* sp. is considered to behave as an opportunistic pathogen, infecting immunodepressed hosts. For example Carey (1993) reported that *Aeromonas hydrophila* infection occurred in *Bufo boreas* after the immune system of the amphibians was suppressed by environmental factors.

Natural and anthropogenic stressors, including pre-existing diseases, may be involved in the occurrence of Red leg; as a matter of fact, *Aeromonas* sp. can behave as a secondary pathogen and its irregular isolation in the samples (Table 1) might suggest the presence of other primary pathogens. Moreover, the occurrence of the same above mentioned die-offs with gross signs of systemic hemorrhagic disease are considered to be indicators of viral infection (Gray et al., 2009; Converse and Green, 2005; Cunningham et al., 1996) and although *Aeromonas* sp. can cause Red leg disease, retrospective studies showed that ranaviruses are also often present and may be the primary pathogen. Nevertheless, viral examination seems to rule out ranaviruses presence at least in the samples from Monte Guglielmo, even if no confirmatory diagnosis (PCR and Immunohistochemistry methods) (Hyatt et al., 2000; Reddacliff and Whittington, 1996) has been performed and the examined samples were few. Additionally, gross lesions, similar to the observed ones, were pointed out in diseases caused by *Batrachochytrium dendrobatidis* in adult amphibians (Green and Converse 2005). However chytridiomycosis is a fatal disease of post-metamorphic frogs infecting keratinized skin of adults and can be ruled out as primary pathogen of the observed mass die offs; since tadpoles have keratin only in mouthparts, chytrid infection on larval anurans commonly results in reduced developmental rate and foraging efficiency (Venesky et al., 2010) without causing mass die-offs.

Lots of other factors may be implicated in determing the pathogenicity of *Aeromonas* sp. For example, during the last decades farmers reduced the number of cows in the mountain areas and consequently the demand for water; mountain ponds were often abandoned and underwent a drying process. This may result in a more extreme habitat for tadpoles. Still existing ponds are subject to wider depth fluctuations and the reduced basin volume is worsened by stronger seasonal and daily variations, resulting in increased total solids, eutrophication and tadpoles crowding; everyone of these factors can potentially suppress the tadpoles immune system and facilitate the disease. For instance, the abandonment of mountain ponds can affect the immune system of tadpoles in the wild and this process is potentially linked with diurnal oxygen supersaturation which can occur in eutrophic ponds: evaluating the importance of dissolved gas, Colt et al. (1984) exposed *Rana catesbeiana* tadpoles to supersaturated water causing signs of Gas bubble disease followed by Red leg disease.

Finally, several limitations should be pointed out in the current study, since the obtained results show that it is not possible to state unequivocally whether *Aeromonas* sp. acts as a primary pathogen or whether there are pre-existing environmental or infective stressors in the studied populations. Several unexplored factors could be involved in the observed mass die-offs, weakening the immune system of tadpoles; even some short-term acute stressors can induce immunosuppression lasting for days (Ellsaesser and Clem, 1986; Pickering et al., 1982; Carey, 1993), making even more difficult to determine conclusively which environmental factor (or which combination of environmental factors) may have caused a sufficient degree of stress to induce disease in the prealpine populations. However, in Monte Guglielmo area, the number of egg masses has not significantly declined since 2005 and the consequences of the disease were not found in a reduced breeding activity of *Rana temporaria,* suggesting that this species is able to withstand high larval mortality occurred in the last years. Regardless, evidence of widespread mass die-offs in prealpine areas has been reported since the early 2000s and previous declines should not be ruled out. The measured abundance could be the result of a decline lasting for years and reducing the population to the current minimum. Finally, the extent of the phenomenon and the amplitude of the affected area should arouse the interest for other conservation issues, such as the health and the consevation status of other amphibians living in the same areas (including *Bombina variegata* and *Triturus carnifex*, listed in Annex II of the Habitat Directive) and the preservation of important habitats such as high altitude ponds.

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