Combined exposure to ambient UVB radiation and nitrite negatively affects survival of amphibian early life stages

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Abstract

Many aquatic species are sensitive to ambient levels of ultraviolet-B radiation (UVB) and chemical fertilizers. However, recent studies indicate that the interaction among multiple stressors acting simultaneously could be contributing to the population declines of some animal species. Therefore, we tested the potential synergistic effects between ambient levels of UVB and a contaminant, sodium nitrite in the larvae of two amphibian species, the common European toad *Bufo bufo* and the Iberian green frog *Rana perezi*. We studied *R. perezi* from both mountain and coastal populations to examine if populations of the same species varied in their response to stressors in different habitats. Both species were sensitive to the two stressors acting alone, but the interaction between the two stressors caused a multiplicative impact on tadpole survival. For *B. bufo*, the combination of UVB and nitrite was up to seven times more lethal than mortality for each stressor alone. In a coastal wetland, the combination of UVB and nitrite was four times more toxic for *R. perezi* than the sum of the effect on mortality for each stressor alone. One mg/L of nitrite killed half the population of *R. perezi* at Gredos Mountains at day 10 in the absence of UVB. In the presence of UVB, 50% of the tadpoles from the same experiment died at day 7. Similar toxic response were found for *R. perezi* in two highly contrasted environments suggesting this synergistic interaction can be a widespread phenomenon. The interaction of excess chemical fertilizers and manure with ambient UVB radiation could be contributing to the global decline of some amphibian species. We suggest that potential exposure to UVB radiation be accounted for when assessing water quality criteria regarding nitrite pollution.
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1. Introduction

The influence of solar ultraviolet radiation on the toxicity of environmental contaminants in aquatic ecosystems has long been recognized (Larson et al., 1979; Oris and Giesy, 1985; Long et al., 1995). How- ever, the toxic effects of contaminants have been traditionally investigated by conducting studies in the absence of ultraviolet radiation. Testing contaminants alone (Sih et al., 2004) or in the absence of sunlight can underestimate the risk posed to wildlife. For example, many amphibians have suffered declines in their populations and reductions in their ranges (Houlahan et al., 2000; Stuart et al., 2004). Habitat destruction, emerging diseases, enhanced ultraviolet-B (UVB) radiation, global climate change and environmental contamination have all been implicated as contributing

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to the causes of these amphibian population declines (Lannoo, 2005). Furthermore, it has recently been emphasized that combinations of stressors may be enhancing the negative effects of each stressor alone (Blaustein and Kiesecker, 2002). Indeed, synergistic interactions among several factors have been documented for an increasing number of combinations of stressors affecting amphibians (e.g. Ankley et al., 1998; Hatch and Blaustein, 2000; Zaga et al., 1998; Kiesecker and Blaustein, 1995; Pahkala et al., 2001; Bridges and Boone, 2003). One example of such synergistic effects is the combined exposure of frog larvae to non-toxic levels of UVB and nitrate pollution. However, the combination of these two stressors has complex effects that may vary with the species, population and life stage. For example, both factors together cause a decrease in activity in Cascades (*R. cascadae*) frogs, significant mortality in a high elevation population of *Pseudacris* (=*Hyla*) *regilla* and altered larval development in the lowland population of *P. regilla*. However, no apparent negative effects of both stressors together were found in *Ambystoma macrodactylum* (Hatch and Blaustein, 2003).

UVB radiation is a well known stressor on many amphibian species and has been proposed as a contributor to global amphibian declines alone or combined with other environmental stressors such as acidification, some policyclic aromated hydrocarbons (PAHs) or nitrate (Blaustein et al., 2001). Nitrate contamination may be especially relevant to amphibians. The addition of chemical fertilizers and manure to crops or the leaching of ammonium from farms increases environmental nitrogen levels in aquatic ecosystems (Vitousek et al., 1997). An excess of nitrogen in water bodies near crop fields or livestock farms can be seriously harmful to aquatic wildlife (Camargo and Ward, 1992). Amphibians may be especially sensitive to nitrate water pollution due to their semipermeable skin and gills The decline of water quality has been proposed as one factor contributing to the global decline of amphibian populations (Stuart et al., 2004; Wake, 1991). Fields are often fertilized in spring, at the same time as amphibian eggs and larvae develop. Experimental tests of environmentally realistic ammonia or nitrate concentrations have been shown to damage amphibian eggs and larvae of certain species (Hecnar, 1995; Marco et al., 1999; Rouse et al., 1999). Levels of nitrite (a toxic form of reactive nitrogen) in natural aquatic habitats are usually low but under some circumstances and in specific areas, such as shore sites with high contents of organic matter, nitrite concentrations can rise to toxic levels (McCoy, 1972; Russo, 1985). Some inshore sites that contain rotting

vegetation and algae can have nitrite concentrations over 5 mg/L $N–NO_2^-$ (McCoy, 1972). High levels of environmental nitrite up to 4 mg $N–NO_2^-/L$ are often found in fish hatcheries. Some authors have observed that low levels of nitrite are highly toxic to newly hatched larvae of some anuran (frogs and toads) species (Marco et al., 1999; Huey and Beitinger, 1980; Smith et al., 2004). In the presence of nitrite the larvae of some amphibians larvae reduced feeding, swam less vigorously, showed disequilibrium and paralysis, suffered anatomical and physiological abnormalities and frequently died. These effects increased with exposure and concentration.

To further examine the relationship between nitrate pollution and UVB radiation, we examined the survival of two amphibian species to these agents in open-air static experiments. The eggs of the two selected species usually develop in habitats with high exposure to UVB radiation. *B. bufo* usually lay their egg strings in clear water while *R. perezi* usually lay their eggs floating in the surface of permanent ponds or streams (Lizana, 2002; Egea-Serrano et al., 2005). After hatching, young tadpoles have low swimming activity and usually remain in shallow water during several days around the gelatinous matrix that surrounds the eggs. Previous studies have shown that some amphibians are especially sensitive to nitrogen pollution and UVB radiation during embryonic or early larval stages (Blaustein et al., 2001; Ortiz-Santaliestra et al., 2006; Griffis-Kyle, 2005). In this study, test animals were exposed to stressors during early life stages. We also tested the sensitivity of *R. perezi* to both stressors in two different habitats. We selected a coastal marshland in southern Spain (Doñana 10 m above sea level [m.a.s.l].) where frogs breed in late winter or early spring in shallow warm, eutrophic water. At these conditions, amphibians are exposed to lower levels of UVB compared to amphibians in the mountains but there is a greater risk of nitrite pollution at these low elevations. In contrast, we selected a mountain area in central Spain (Gredos, 1900 m.a..s.l.) where frogs breed in May and June under in colder water than at low elevation sites and where the conditions are oligotrophic. Thus, in the mounatins, UVB levels are higher and penetrates deeper but the risk of nitrite pollution is lower than at the low elevation sites.

2. Material and methods

Green frogs (*R. perezi*) were tested in the Doñana National Park (DNP) (Almonte, Huelva, Spain, 10 m.a.s.l.; N 36°59,330′/W 6°26,579′) and in the Gredos Mountains

(GRM) (Prado de las Pozas, Navalperal de Tormes, Avila, Spain, 1900 m.a.s.l.; N 40°16′34,7″/W 5°14′14,5″). Common toads (*B. bufo*) were tested in the same location in the GRM.

For Experiment 1, in DNP we collected eggs of *R. perezi* at Gosner (1960) stages 11–14 from seven different egg masses in April 2005 from a temporary pond. For Experiment 2, in GRM we collected eggs of *B. bufo* at Gosner stages 18–19 from seven different egg masses in May 2005 from a temporary pond. For Experiment 3, in GRM we collected eggs of *R. perezi* at Gosner stages 11–14 from 12 different egg masses in May 2005 from a temporary pond. Eggs were exposed to UVB in the field less than 2 days before collection. It is unlikely that amphibians were exposed to toxic levels of reactive nitrogen or xenobiotics before collection because the study area is within a National Park where the use or release of xenobiotics is prohibited.

2.1. Experimental design

Within 24 h of collection, eggs were exposed in an orthogonal bifactorial design to a series of two sodium nitrite dilutions (1 and 3 mg $N–NO_2^-/L$) and a control (no contaminant added) in combination with a series of three levels of UVB radiation (100%, 86% and 4%) for 15 days. Each treatment level was replicated three times. Tests were conducted in 70 L rigid polyethylene tanks containing 65 L of non-chlorinated tap water. Chemical analysis made water acceptable for human consumption. Because microorganisms may influence the impact of UVB and nitrite on amphibians, we inoculated enclosures with microorganisms from natural breeding ponds. In each container we added 50 ml of water (containing natural microorganisms) from the pond where eggs were collected. The nine containers (3 nitrite treatments × three replicates) of each experiment were placed together in an open-air location with a full sunlight exposure. The tanks for each experiment were randomly assigned to one of the three nitrite treatments. We used nominal concentrations of 0, 1 and 3 mg $N–NO_2Na/L$. 5 mg /L has been propossed as a limit for nitrite for warm water fishes (US EPA, 1986). LC50 values of larval stages of some amphibian species fit within 0 and 3 mg $N–NO_2Na/L$ (Marco et al., 1999). We used 10 g $N–NO_2Na/L$ stock solution prepared from sodium nitrite salt (96% purity) which was pipetted into the containers to obtain experimental concentrations.

We randomly placed three enclosures inside each container, one for each UVB treatment, so that there was maximum solar radiation (minimizing shade over the eggs). The enclosures were 18 cm in diameter, with a hemispherical shape to minimise the extension of shade within the enclosure and were made of solid stainless steel mesh to allow circulation of water and air but prevent the escape of tadpoles. Enclosures were attached to Styrofoam
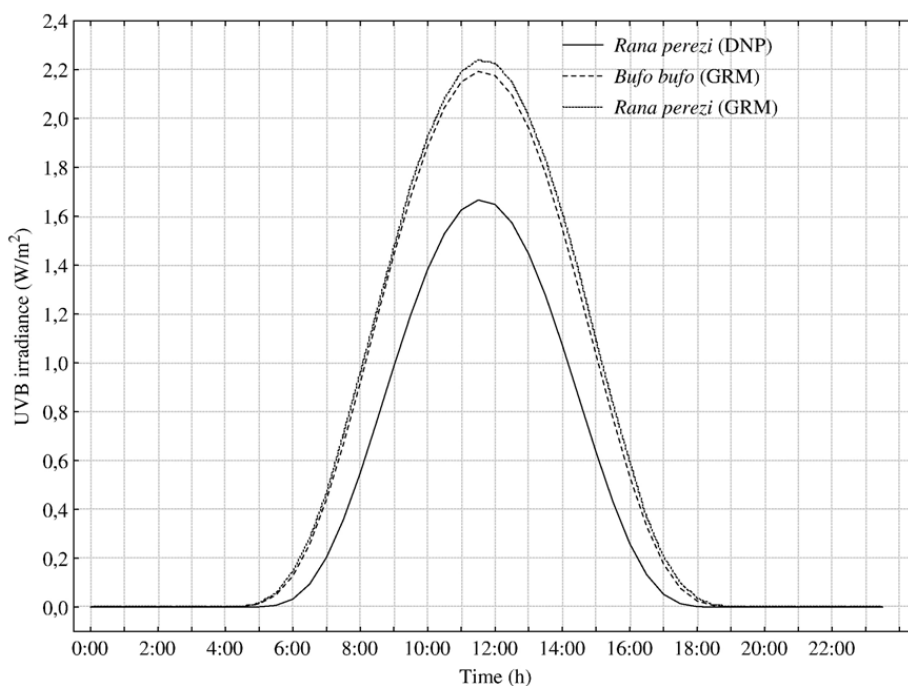

Fig. 1. Estimated maximum UVB (280–315 nm) daily irradiance during the three experiments obtained from the Tropospheric Ultraviolet and Visible radiation model (TUV Model, version 4.1, U.S. National Center for Atmospheric Research (NCAR)). DNP=Doñana National Park; GRM=Gredos Mountains.
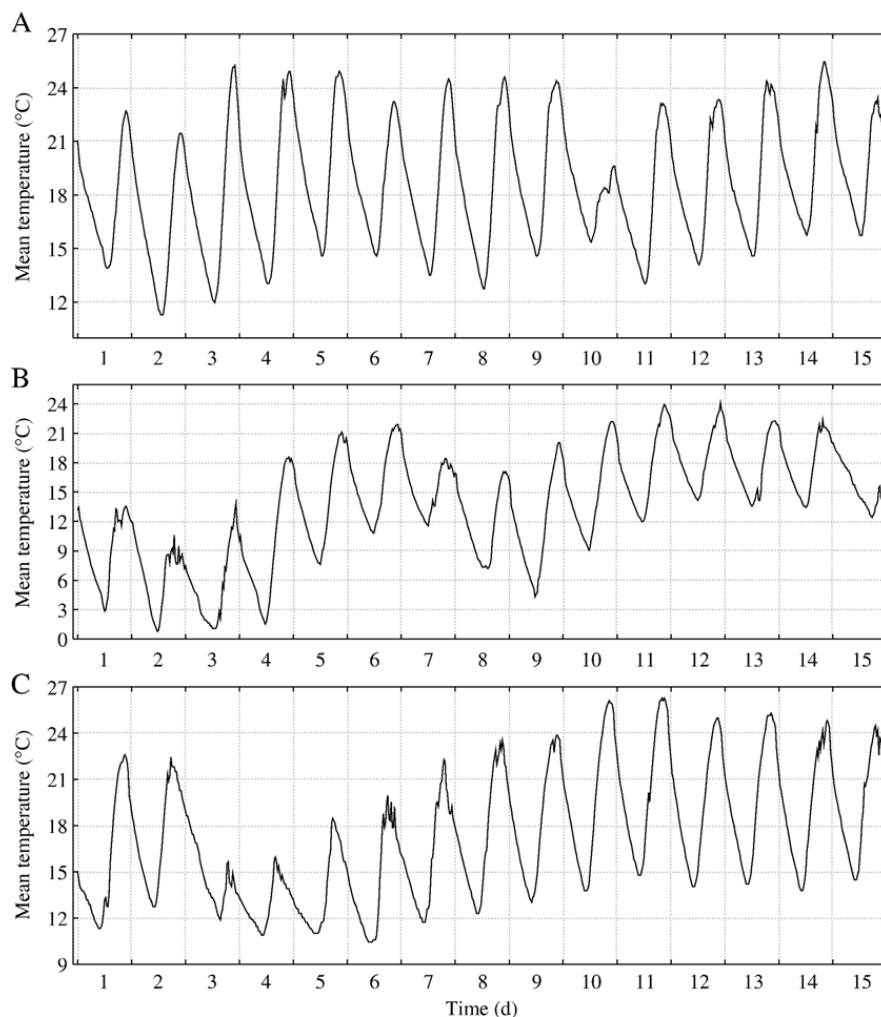
Fig. 2. Mean water temperature at the depth where eggs were developing in the experiments. Data were automatically recorded every 30 min. A. Experiment 1: *Rana perezi* in the Doñana National Park; B: Experiment 2: *Bufo bufo* in Gredos Mountains; C: Experiment 3: *R. perezi* in Gredos Mountains.

pieces that permitted permanent flotation in the water. We used 0.075 mm thick filters to expose eggs to one of three levels of UVB radiation. One set of enclosures was covered with UVB-blocking Mylar film (Dupont Teijin Films). Other enclosures were covered with UVB-transmitting cellulose acetate film (Torraspapel, S.A.

Table 1
Minimum and maximum values of water quality in experimental tanks and maximum estimated daily doses of ambient UVB (280–315 nm) and UVA (315–400 nm) radiation (estimated using the TUV Model)

Species and population	PH	Conductivity (μs/cm)	Dissolved oxygen (mg/L)	Range of temperatures (°C)	Nitrite concentration (mg N/L)	UVB (280–315 nm) (W/m2)	UVA (315–400 nm) (W/m2)
Doñana NP							
Rana perezi	8.11–8.20	410–447	4.5–14.0	11.3–25.5	0–0.024 1–0.16 3.5–2.78	1.647	55.62
Gredos Mountains							
Bufo bufo	5.85–6.70	15.3–47.9	6.2–8.4	0.8–24.1	0–0.19 1–0.79 3.5–3.08	2.174	64.24
R. perezi	6.02–6.67	13.7–46.6	6.2–7.4	10.4–26.3	0–0.007 1–0.89 3.5–3.23	2.224	64.95

Barcelona). Both Mylar and acetate filters were placed 5 cm above the water surface to permit air circulation. The remaining enclosures were not covered and were fully exposed to solar radiation. The filter characteristics of the Mylar and acetate films were tested before and after the experiments with a UV–Visible Spectrophotometer (UV-1601, Shimadzu, Kyoto, Japan) showing that the Mylar filter blocked 96% rays below 320 nm. The acetate filter permitted the passage of light over 280 nm, blocking only ca. 10–15% of the UVB radiation. The properties of both filters remained unchanged after the experiment. There are no available data about precise UVB levels in the study areas during the experiments. The maximum daily irradiances (Fig. 1) were estimated using the Tropospheric Ultraviolet and Visible radiation model (TUV Model, version 4.1, U.S. National Center for Atmospheric Research (NCAR)). The satellite data are gross estimates of UVB radiation at ground level because cannot take into account local conditions influenced by shading, cloud cover, weather patterns, water depth and dissolved organic carbon (DOC) that can affect exposure of aquatic organisms. However, in the absence of empirical data, satellite estimates can permit a rough estimate of the maximum daily irradiance of UVB during the 3 experiments.

For each experiment, the 27 enclosures were submerged to a depth of 5 cm and kept afloat with polystyrene floats. At the beginning of the experiment, eggs from each clutch with the jelly matrix intact were separated and distributed among the enclosures, each one containing the same number of eggs from each of the different clutches, thus reducing the potential influence of genetic or maternal effects. On Experiment 1, 14 eggs were introduced in each enclosure and 20 eggs per enclosure were used on Experiments 2 and 3. Eggs were placed at a depth of 1–3 cm. When larvae reached Gosner stage 25, they were fed *ad libitum* with lettuce that was previously washed with water and boiled for 5 min. Every day, in each enclosure, an observer recorded egg mortality, abnormalities and embryonic developmental stage. Nitrite concentration, temperature, dissolved Oxygen, pH and conductivity (Hanna instruments), were measured periodically in each enclosure. Moreover, water temperature (Fig. 2) was recorded in one container of each nitrite treatment every 30 min during the experiment with automatic data logers (Stow Away Tidbit, Onset, USA). All enclosures were checked each day for predators, which were removed when found. To maintain the initial water level, a small amount of tap water (less than 5% of the initial water volume) was added to the containers when necessary. The experiment was completed in 15 days. Throughout the experiment tadpoles remained at

developmental stages with low swimming activity (Gosner stages below 25). In the field, tadpoles at these stages usually stay in shallow water close to the egg-laying site (Lizana, 2002; Egea-Serrano et al., 2005). At the end of the experiment all larvae were at Gosner stage 25. Mortality rate was calculated by dividing the accumulated number of dead individuals at
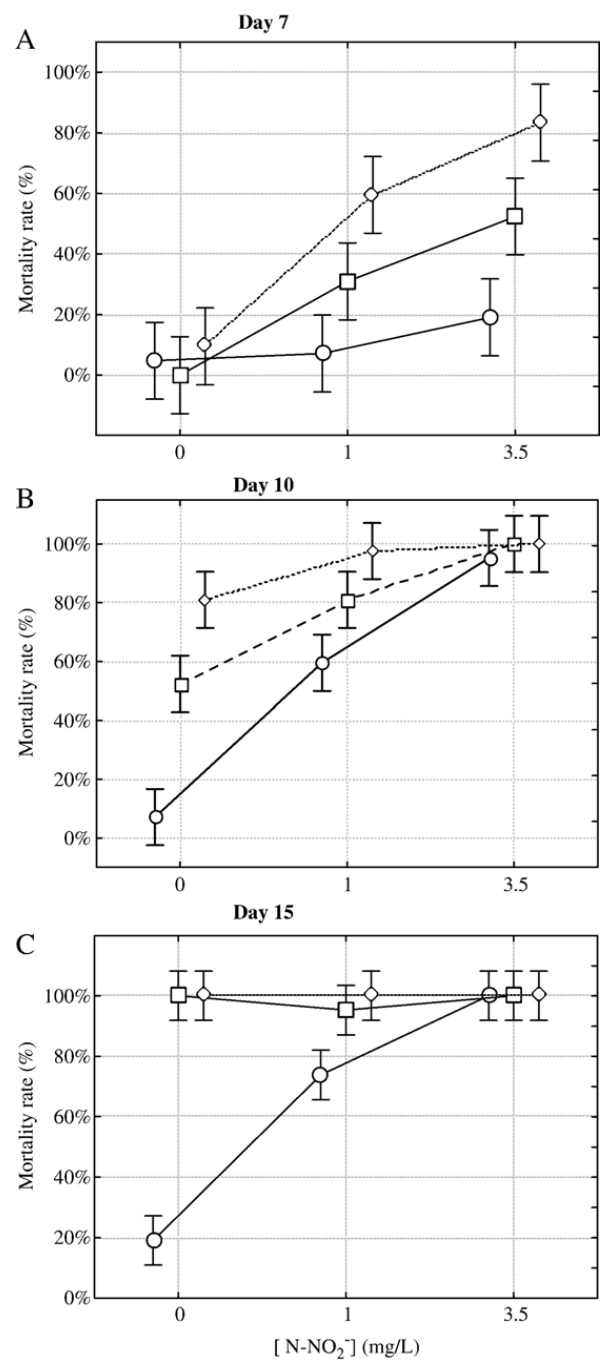

Fig. 3. Mortality of *Rana perezi* tadpoles from Doñana National Park exposed to different combinations of UVB radiation and nitrite after 7 (A), 10 (B) and 15 (C) days of exposure. Open circles indicate 4% of ambient UVB; open squares indicate 86% of ambient UVB; open diamonds indicate 100% of ambient UVB.

Table 2
Results of multifactorial repeated measures ANOVA and subsequent univariate ANOVAs that analyze the effect of nitrite and UVB on survival of *Rana perezi* tadpoles from the Doñana National Park at 7, 10 and 15 days of exposure

Time (day)	Effect	df	Wilks	MS	F	p
Overall	Nitrite	8	0.0612		11.412	<0.0001
	UVB	8	0.0442		14.080	<0.0001
	Nitrite×UVB	16	0.0466		5.020	<0.0001
7	Nitrite	2		0.9837	36.3088	<0.0001
	UVB	2		0.5506	20.3184	<0.0001
	Nitrite×UVB	4		0.1140	4.2084	0.0141
	Error	18		0.0271		
10	Nitrite	2		1.4277	84.816	<0.0001
	UVB	2		0.7179	42.650	<0.0001
	Nitrite×UVB	4		0.1072	6.370	0.0022
	Error	18		0.0168		
15	Nitrite	2		0.3596	19.788	<0.0001
	UVB	2		0.9323	51.299	<0.0001
	Nitrite×UVB	4		0.3676	20.229	<0.0001
	Error	18		0.0182		

a given moment by the initial number of eggs. At the end of the experiment, healthy individuals were released in the ponds where their eggs were collected.

2.2. Analysis of data

To evaluate the independence of each enclosure within each container we used a nested MANOVA for each experiment, considering mortality rates (arcsin of square-root transformed) after 7, 10 and 15 days of exposure as dependent variables, and nitrite concentration and container nested in nitrite concentration as categorical factors. To determine the combined effect of UVB radiation and sodium nitrite on amphibians, we used a two-ways repeated measures ANOVA for each experiment, with mortality rates (Arcsin of square-root transformed) after 7, 10 and 15 days of exposure as dependent variables, and UVB radiation and nitrogen concentration as the categorical variables. To determine the time amphibians were sensitive to stressors, we conducted two-way ANOVAs with the mortality rate at that time as the dependent variable. We used post-hoc HDS Tukey tests for pair-wise comparisons. We used the software package STATISTICA version 6.0 (Stat-Soft, Inc., 2001) for all statistic analyses.

3. Results

Temperature, pH and oxygen concentration of water did not vary among tanks in any experiment (repeated measures ANOVAs: $P > 0.4$). However, nitrite concentration had a significant effect on water conductivity in the three experiments (repeated measures ANOVAs: $P < 0.001$). Especially significant differences in conductivity among tanks were found in the experiments conducted in Gredos Mountain, because of low basal

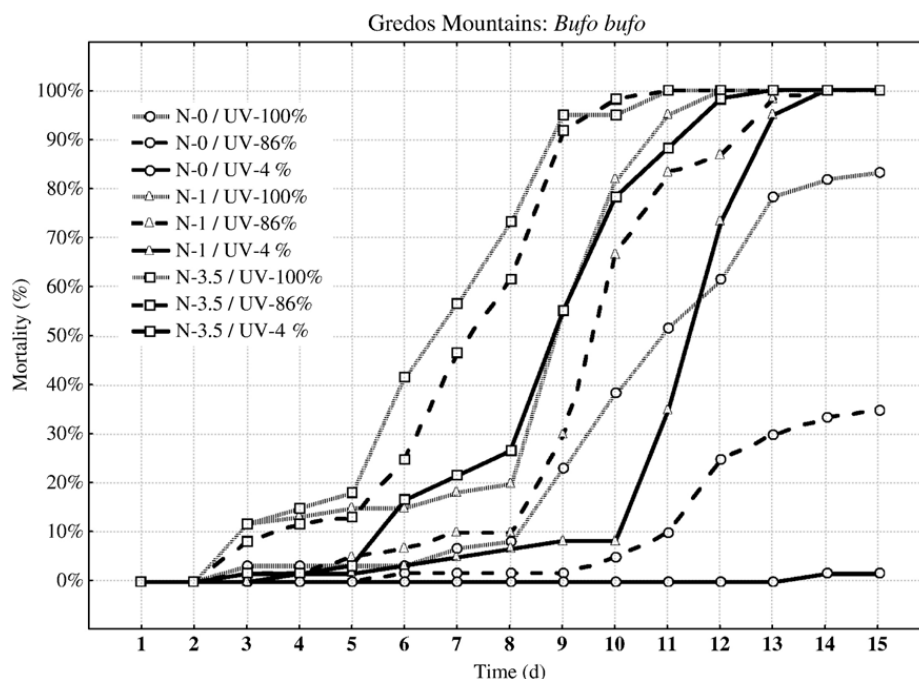Fig. 4. Mortality of *Bufo bufo* tadpoles in the Gredos Mountains exposed to three levels of UVB and two levels of sodium nitrite in an orthogonal design. Open circles indicate 0 mg N–NO$_2$/L; open triangles indicate 1 mg N–NO$_2$/L; open squares indicate 3.5 mg N–NO$_2$/L; full lines indicate 4% of ambient UVB; dashed lines indicate 86% of ambient UVB and dotted lines indicate 100% of ambient UVB.

Table 3
Results of multifactorial repeated measures ANOVA and subsequent univariate ANOVAs that analyze the effect of nitrite and UVB on survival of *Bufo bufo* tadpoles from the Gredos Mountains at 7, 10 and 15 days of exposure

Time (day)	Effect	df	Wilks	MS	F	p
Overall	Nitrite	8	0.0061		44.2926	<0.0001
	UVB	8	0.0765		9.8038	<0.0001
	Nitrite×UVB	16	0.0546		4.6170	<0.0001
7	Nitrite	2		0.8237	47.3584	<0.0001
	UVB	2		0.1597	9.1817	0.0018
	Nitrite×UVB	4		0.0120	0.6916	0.6073
	Error	18		0.0173		
10	Nitrite	2		2.5188	135.2612	<0.0001
	UVB	2		0.9130	49.0307	<0.0001
	Nitrite×UVB	4		0.0991	5.3212	0.0052
	Error	18		0.0186		
15	Nitrite	2		2.7493	169.126	<0.0001
	UVB	2		0.2979	18.328	<0.0001
	Nitrite×UVB	4		0.2979	18.328	<0.0001
	Error	18		0.0163		

levels of ion concentration. Nested MANOVAs indicate for all experiments that the container had no effect on the results (Experiment 1: Wilks value=0.008, P=0.688; Experiment 2: Wilks value=0.001, P=0.109; Experiment 3: Wilks value=0.148, P=0.958). For that reason, we can consider the results of each UV treatment within each container as independent.

In all experiments, embryos and tadpoles exposed to the stressors showed developmental and morphological abnormalities that included curved bodies and tails, necrosis and edema. Most of the abnormal tadpoles died within a few days. In some treatments mortality was total. Green frog (*R. perezi*) eggs from both populations that were exposed to nitrite grew slower than controls but showed a faster degradation of the jelly coat that surrounds egg masses. Water temperature and the rest of the measured water quality parameters (Table 1) (with the exception of nitrite concentration) did not vary among treatments within each experiment.

3.1. Experiment 1: Rana perezi in DNP

Mortality in control enclosures at day 15 was less than 19% (Fig. 3). The impact of UVB and nitrite increased both with time and level. After 7 days of exposure, both stressors alone caused significant mortality in green frog tadpoles (Table 2). But the combination of UVB and nitrite was four times more toxic than the sum of the effect on mortality for each stressor alone (Fig. 3). After day 7, the interaction between UVB and nitrite was highly significant (Table 2). At the end of the experiment, ambient levels of UVB killed all tadpoles. A 14% reduction of ambient UVB levels also caused total mortality. At day 15, 26% of the tadpoles survived
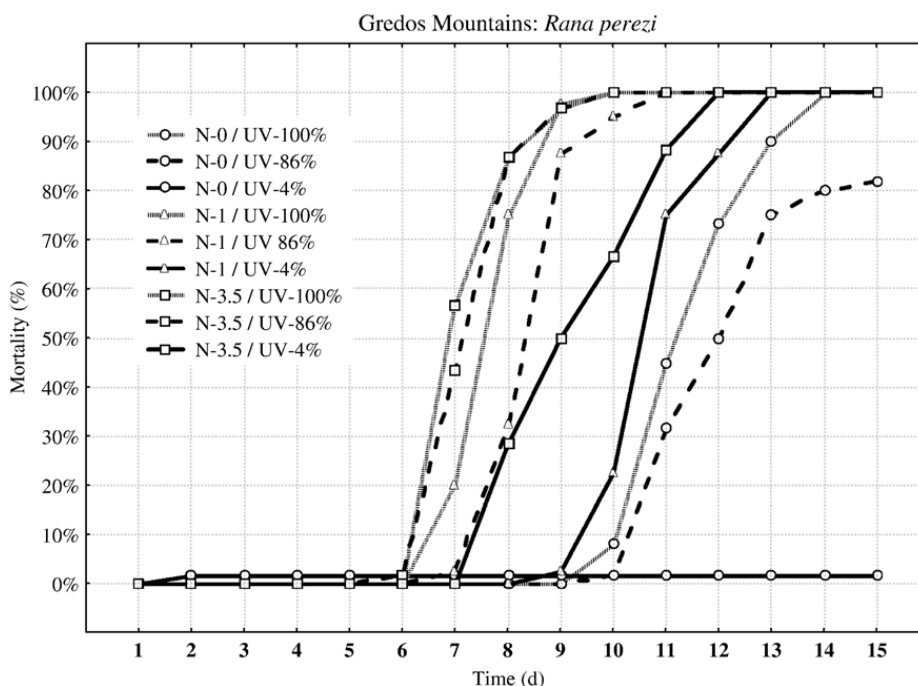

Fig. 5. Mortality of *Rana perezi* tadpoles in the Gredos Mountains exposed to three levels of UVB and two levels of sodium nitrite in an orthogonal design. Open circles indicate 0 mg N–NO$_2$/L; open triangles indicate 1 mg N–NO$_2$/L; open squares indicate 3.5 mg N–NO$_2$/L; full lines indicate 4% of ambient UVB; dashed lines indicate 86% of ambient UVB and dotted lines indicate 100% of ambient UVB.

Table 4
Results of multifactorial repeated measures ANOVA and subsequent univariate ANOVAs that analyze the effect of nitrite and UVB on survival of *Rana perezi* tadpoles from the Gredos Mountains at 7, 10 and 15 days of exposure

Time (d)	Effect	df	Wilks	MS	F	p
Overall	Nitrite	8	0.0233		16.649	<0.0001
	UVB	8	0.1081		6.126	0.0002
	Nitrite×UVB	16	0.0568		3.629	0.0006
7	Nitrite	2		0.6719	10.082	0.0017
	UVB	2		0.3214	4.823	0.0241
	Nitrite×UVB	4		0.2232	3.349	0.0378
	Error	15		0.0666		
10	Nitrite	2		3.8442	155.786	<0.0001
	UVB	2		0.8242	33.401	<0.0001
	Nitrite×UVB	4		0.1803	7.306	0.0018
	Error	15		0.0247		
15	Nitrite	2		0.9836	29.552	<0.0001
	UVB	2		0.5423	16.292	0.0002
	Nitrite×UVB	4		0.5931	17.820	<0.0001
	Error	15		0.0333		

in the lowest nitrite concentration when they were exposed to 4% ambient UVB (Fig. 3).

3.2. Experiment 2: Bufo bufo in GRM

In this experiment, there was a great deal of cloud cover during the first 6 days, although this was not quantified. Therefore, exposure to ambient levels of UVB during the first half of the experiment may have been diminished. At day 15, mortality in control enclosures was lower than 2% (Fig. 4). The impact of UVB and nitrite increased both with time and dose. After 8 days of exposure, UVB caused significant mortality in toad tadpoles. Nitrite alone also caused significant mortality after 7 days of exposure (Table 3). Both stressors alone caused significant mortality in common toad tadpoles after day 10 (Fig. 4). But the combination of UVB and nitrite was up to 7 times more lethal than mortality for each stressor alone (Fig. 4). All the combinations of stressors caused a significant synergistic impact on common toad tadpoles. After day 10, the interaction between UVB and nitrite was highly significant (Table 3). At day 15, many tadpoles survived when exposed to UVB but in combination with 1 mg/L of nitrite, mortality was total. The impact of nitrite on common toad tadpoles was significantly accelerated in the presence of ambient and reduced UVB (Fig. 4).

3.3. Experiment 3: Rana perezi in GRM

Mortality in control enclosures at day 15 was less than 2% (Fig. 5). Tadpoles showed a similar toxic response over time under 100% of ambient UVB than under 1 mg/L of nitrite (Fig. 5). However, when tadpoles were exposed to similar levels of both stressors together the same effect was detected three days earlier. 1 mg/L of nitrite killed half the population at day 10 in the
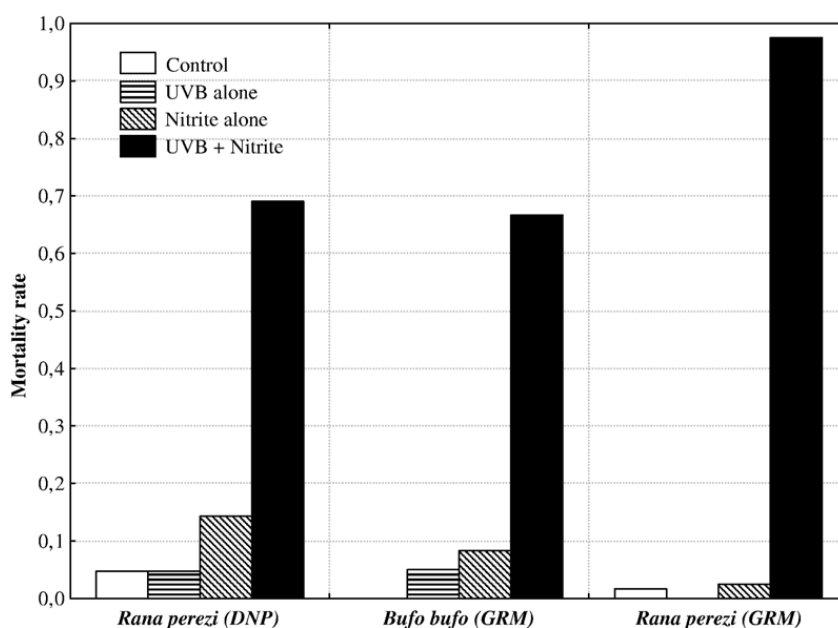

Fig. 6. Synergism between UVB radiation and nitrite: *Rana perezi* in the Doñana National Park (DNP): 6 days of exposure to 3.5 mg N–NO$_2$/L and 100% of ambient UVB; *Bufo bufo* in the Gredos Mountains (GRM): 10 days of exposure to 1 mg N–NO$_2$/L and 86% of ambient UVB; *R. perezi* in the Gredos Mountains: 9 days of exposure to 1 mg N–NO$_2$/L and 100% of ambient UVB. Open bars correspond to controls. Horizontal frame correspond to UVB alone; diagonal frame correspond to Nitrite alone; full frame correspond to UVB plus Nitrite.

absence of UVB. In the presence of UVB, 50% of the tadpoles died at day 7 (Fig. 5). Both stressors alone killed all individuals by day 13, but both stressors together killed all tadpoles by day 9 (Fig. 5). After day 7, the interaction between UVB and nitrite was highly significant (Table 4). All the combinations of stressors caused a significant synergistic impact on green frog tadpoles. Even when each stressors alone caused no mortality, the combination of both resulted in 97% of mortality (Fig. 6). At the end of the experiment, at ambient levels of UVB with no nitrite, there was no survival, but at intermediate levels of UVB about 20% of the tadpoles survived.

4. Discussion

In our study, both species that we examined displayed detrimental effects of UVB radiation and nitrite alone but when exposed simultaneously to two stressors, they died earlier and at lower contaminant concentrations. Our data suggest that the effects of aquatic contaminants are compounded when they are exposed to sunlight. Thus, simple laboratory experiments in the absence of solar radiation may not be truly representative of how the effects on wildlife are manifested under field conditions. Toxicity in aquatic organisms may vary with water quality, temperature, and the presence of other contaminants (Oris and Giesy, 1985; Pahkala et al., 2001). Furthermore, many factors influence sensitivity of animals to pollutants in the field, and it is often difficult to assess the specific factors causing particular effects under field conditions. Multifactorial experiments, where two or more factors are manipulated may be a better approach for understanding the ecological interactions between contaminants in nature (Sih et al., 2004).

Many field studies, by numerous investigators, have shown that ambient levels of UVB radiation decreases the hatching success of some amphibian species at their natural oviposition sites (Blaustein et al., 2001). There is also an increasing number of studies showing that UVB radiation harms amphibians at later stages of development, including larvae and juvenile stages (e.g. discussion in Blaustein and Bancroft, 2007). There is, however, significant variability in sensitivity to UVB radiation among species because molecular, behavioural and morphological characteristics make some amphibians less vulnerable to UVB radiation (Blaustein and Belden, 2003; Marco et al., 2001; Palen et al., 2005). Water quality should also be considered when evaluating vulnerability of amphibians to UVB. In this study the presence of nitrite in the water significantly enhanced the impact of UVB on *B. bufo* and *R. perezi* embryos and tadpoles. Nitrate, a more abundant form of reactive nitrogen that can be toxic to amphibians also reacts synergistically with UVB radiation. Hatch and Blaustein (2000) observed that larval *R. cascadae* exposed to sodium nitrate alone had reduced activity. But with UVB and nitrate together, an initial dose of nitrate four times lower than that in the single nitrate experiment caused a similar behavioral effect. Although Hatch and Blaustein (2003) found that the effects of nitrate was influenced by the presence of other factors such as UVB radiation, the interaction between UVB and nitrate was complex and had differential effects on different species. These authors found that UVB and nitrate together lowered the survival of larval *Pseudacris regilla* in a high-elevation population and reduced its mass at both low and high elevations.

The few experiments that have been conducted to elucidate the direct toxicity of nitrite on amphibians show strong effects at low levels (Huey and Beitinger, 1980; Marco et al., 1999; Smith et al., 2004; Griffis-Kyle, 2005). In some cases, amphibian larvae were negatively affected by concentrations of nitrite below the recommended limit for drinking water (Marco et al., 1999). Along with lethal effects, nitrite can also induce a variety of sublethal effects including, a reduction in growth rates, changes in behavior and delayed metamorphosis (Marco and Blaustein, 1999). All these effects could potentially have an impact at the population level.

Strong sensitivity to nitrite is shared by aquatic species from several taxa including mollusks (Alonso and Camargo, 2003), crustaceans (Scott and Crunkilton, 2000), and fishes (Eddy and Williams, 1994). Many species within these groups are also exposed to UVB radiation in their natural habitats (Häder et al., 1998). Due to the strong synergism between UVB radiation and nitrite detected in our study, we suggest that potential exposure to UVB radiation be accounted for when assessing water quality criteria regarding nitrite pollution. This is especially important, because, as we have shown, nitrite in relatively small amounts can cause major damage to amphibians if the amphibians are exposed to nitrites and UVB radiation simultaneously.

The physiological mechanisms that are responsible for the toxic synergism we report here may be complex. Moreover, the impact of UVB on ecosystems is potentially far-reaching Bancroft et al., 2007). For example, damage by UVB to primary producers may indirectly impact amphibians. Phytoplankton includes the most important biomass producers and nitrogen consumers in the top layers of aquatic ecosystems. In

shallow water, UVB affects the uptake of ammonium and nitrate by phytoplankton, as well as macroalgae (Häder et al., 1998). UVB damage to aquatic primary producers may increase the availability of reactive nitrogen to amphibians. There could also be a significant reduction in food availability for anuran tadpoles in the field if UVB kills or damages plankton.

B. bufo has suffered significant reductions in its range in several areas of Western Europe during the past few decades (Cooke, 1972; Lizana, 2002; Carrier and Beebee, 2003). These reductions are mainly reported from agricultural areas. Earlier studies have shown that *B. bufo* is sensitive to relatively low levels of nitrate in the water (Baker and Waights, 1993; Ortiz-Santaliestra et al., 2004). Furthermore, *B. bufo* eggs and embryos from mountain populations in Central Spain are relatively sensitive to ambient levels of UVB radiation (Lizana and Pedraza, 1998). In our study, *B. bufo* showed a significant sensitivity to the presence of nitrite in the water and to ambient levels of UVB. Our data suggest that the combination of both nitrite fertilizers or livestock residues and ambient levels of UVB could be contributing to the disappearing populations of *B. bufo* from agricultural lands in Spain and other regions of Europe. In mountain populations, levels of UVB are usually higher than those in lowland areas at similar latitude. Moreover, in mountain locations amphibian breeding occurs later in the season and thus, with a higher exposure to UVB. However, levels of nitrite are usually low because land uses are less intense. However, any activity that can increase the concentration of nitrite of other form of reactive nitrogen, such as an excess of cattle or mountain meadows fertilization for increasing grass production for cattle or game species, may cause a significant impact on amphibians via synergism with UVB radiation.

R. perezi embryos and tadpoles were sensitive to the combination of a full exposure to UVB radiation and nitrite both in coastal and mountain populations. While in mountain locations these eggs are often fully exposed to UVB radiation. At lowland areas, *R. perezi* usually breed late in the season in temporal and heavily vegetated aquatic habitats. *R. perezi*, attach their eggs to aquatic plants that could partially shade eggs from UVB radiation. Moreover, aquatic plants are active fixers of nitrogen ions, thus reducing the concentration of nitrogen ions in the water. For these reasons, exposure to a combination of UVB radiation and high levels of nitrite at lowland locations is probably not common in many freshwater ecosystems. However, several factors can significantly increase the risk of toxic exposure of frog eggs to UVB and nitrite. For example, human activities or climate change can result in an increase or decrease in precipitation or evaporation rates and may influence the dynamics of hydroperiod in wetlands. These processes may expose amphibian eggs to more toxic levels of UVB radiation if the water level decreases. Moreover, increasing numbers of livestock may contribute to the problems facing amphibians in the wetlands of Mediterranean semiarid zones. Cattle heavily consuming aquatic vegetation and also contribute to the eutrophication process of fresh-water ecosystems with their nitrogen wastes.

The present study offers new clear evidence about the potential negative impact of the combination of environmental stressors on wildlife. Animals are usually exposed to cocktails of contaminants in the field that interact and increase their toxicity to wildlife. Testing stressors alone can underestimate their risk to wildlife. Levels of some stressors that are non-toxic when they are tested alone can be lethal in the field when combined with synergistic agents.

Acknowledgements

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