STAGE-DEPENDENT TERATOGENIC AND LETHAL EFFECTS EXERTED BY ULTRAVIOLET B RADIATION ON *RHINELLA* (*BUFO*) *ARENARUM* EMBRYOS

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Abstract—The adverse effects of ultraviolet B radiation from 547.2 to 30,096 J/m^2 on morphogenesis, cell differentiation, and lethality of amphibian embryos at six developmental stages were evaluated from 24 up to 168 h postexposure. The ultraviolet B radiation lethal dose 10, 50, and 90 values were obtained for all developmental stages evaluated. The lethal dose 50 values, considered as the dose causing lethality in the 50% of the organisms exposed, in J/m^2 at 168 h postexposure, ranged from 2,307 to 18,930; gill circulation and blastula were the most susceptible and resistant stages, respectively. Ultraviolet B radiation caused malformations in all developmental stages but was significantly more teratogenic at the gill circulation and complete operculum stages. Moreover, at the gill circulation stage, even the lowest dose ($547.2 \ J/m^2$) resulted in malformations to 100% of embryos. The most common malformations were persistent yolk plug, bifid spine, reduced body size, delayed development, asymmetry, microcephaly and anencephaly, tail and body flexures toward the irradiated side, agenesia or partial gill development, abnormal pigment distribution, and hypermotility. The stage-dependent susceptibility to ultraviolet B radiation during amphibian embryogenesis could be explained in the framework of evoecotoxicology, considering ontogenic features as biomarkers of environmental signatures of living forms ancestors during the evolutionary process. The stage-dependent susceptibility to ultraviolet B radiation on *Rhinella* (*Bufo*) *arenarum* embryos for both lethal and teratogenic effects could contribute to a better understanding of the role of the increased ultraviolet B radiation on worldwide amphibian populations decline.

Keywords—Ultraviolet B radiation   Amphibian embryo   Stage-dependent susceptibility   Teratogenesis   Evoecotoxicology

INTRODUCTION

The rising intensities of ultraviolet B (UVB) radiation reaching Earth caused by the depletion of the stratospheric ozone layer were considered by the World Environment Conference held in Rio de Janeiro, Brazil, in 1992 as a matter of urgency, and it was recommended that research be conducted to examine the effects of increased UVB radiation on organisms [1]. The worldwide decline of amphibian populations [2,3] and the rising abnormalities observed in amphibians collected from the field [4,5] were related to the UVB radiation–induced effects on amphibians tested both in laboratory [6,7] and in ambient solar conditions [8,9]. The standard approach to evaluating the toxicity of many agents of environmental concern for early life stages of free-living embryos consists of dose–response tests with continuous treatments from the blastula stage onward, reporting lethality and teratogenic effects at the end of embryonic development. By means of short-term exposures to noxious agents in different developmental stages, relevant changes in the susceptibility of the embryos to environmental stressors was reported, providing a better understanding of their adverse effects at different stages of the

life cycle. As a general pattern, the earlier organogenic stages are susceptible to environmental agents producing oxidative stress [10]. In controlled ambient solar UVB radiation experiments, it was reported that amphibian larvae were more susceptible than embryos for lethal effects [8]. However, in preliminary studies conducted in our laboratory, it was found that certain developmental structures, such as gills, could be susceptible to UVB radiation; therefore, even low-exposure conditions could be of paramount importance for morphogenetic and cell differentiation processes during early organogenic stages.

By producing a metastable excited state or free radicals, UVB radiation exerts harmful effects on cellular DNA, proteins, and lipids by means of oxidative stress [11], which may result in cancer, malformations, suppression of immune responses various retinal effects, infection exacerbation, and death [12]. Photo repair enzymes, the principal of which is photolyase, are among the defense mechanisms against UVB radiation–induced oxidative stress [13]. Melanin, a naturally occurring pigment present in amphibians, also contributes to protection of these organisms, both as an absorber of UV light [14] and as an antioxidant molecule that prevents the formation of highly reactive hydroxyl radicals [11]. Other well-known antioxidant enzymes such as catalase, glutathione peroxidase, and superoxide dismutase also contribute to reduce UVB ra-

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diation–induced adverse effects. For instance, in *Rhinella* (*Bufo*) *arenarum* embryos, it was found that sublethal UVB doses resulted in a rapid but transient increase in superoxide dismutase activity. However, in the case of lethal doses it did not differ significantly from controls, suggesting that the response capacity was defeated [15]. On the other hand, antioxidant agents like selenium and zinc could be beneficial regarding the enhanced survival of UVB radiation–treated amphibian embryos previously exposed to these antioxidants [16].

A study of the stage-dependent susceptibility to UVB radiation could be relevant for present environmental conditions, as well as from an evolutionary perspective. Besides the abiotic inputs of physical and chemicals agents in the environment, as life and biodiversity expanded, the evolving environmental conditions were increasingly modulated by living organisms. Among others, the rise of the O_2 concentrations both in water and in atmosphere due to the achievement of photosynthetic water-splitting capacity approximately 2.4 billion years ago [17] is outstanding. This fact is usually associated with the evolution of aerobic organisms and the establishment of the ozone shelter providing protection against UVB irradiation. Living forms developed numerous defense mechanisms against UVB radiation. For instance, melanin, a well-known shelter against UVB radiation, is found from protists to mammals, reflecting the ancient incorporation of this molecule in living organisms. In the case of amphibians, the oocytes already exhibit in the animal cap, the side to be directly exposed to the sunlight after fertilization, a remarkable pigmentation produced by melanin granules. In a recent contribution focusing on environmental changes and life-features achievements during the evolutionary process, certain features of living organisms at ontogenic stages, such as metabolic changes and stage-dependent susceptibility to noxious agents, were considered as biomarkers of environmental signatures during the evolutionary process [18]. From the perspective of the evoecotoxicology, a study on the stage-dependent susceptibility to UVB radiation on amphibian embryos could be relevant because, as they are free-living organisms during the whole life cycle, they could reflect the UVB exposure conditions of their ancestors during the evolutionary process.

The main purpose of the present study is to report the stage-dependent susceptibility to UVB radiation on *R.* (*Bufo*) *arenarum* embryos, focusing in lethality and adverse effects on morphogenesis. The results are discussed from ecotoxicological and evoecotoxicological perspectives.

MATERIALS AND METHODS

Rhinella (*Bufo*) *arenarum* adults weighing 200 to 250 g were collected in Moreno, Province of Buenos Aires, Argentina. Living organisms were handled in accordance with institutional guidelines of animal welfare. Ovulation was induced by means of an intraperitoneally injection of a macerated homologous hyphophysis. Oocytes were fertilized in vitro, with a sperm suspension in 10% Amphitox solution [19]. Jelly coats were removed by a 2% solution of thyoglicolic acid in Amphitox solution neutralized with NaOH. The development of embryos was staged according to the table of Del Conte and Sirlin [20]. Groups of 10 embryos by replicate, at the developmental stages of blastula (S.9), gastrula (S.11), tail bud (S.17), gill circulation (S.20), opercular fold (S.23), and complete operculum (S.25), were placed in uncovered glass Petri dishes with 40 ml of Amphitox solution (depth 7 mm) 10 cm below an UVB Transilluminator (UVP, Upland, CA, USA),

model M-20, and irradiated with UVB at 302 nm with 4.56 watt/m² of irradiance. In this condition, the UVB lamp emits a dose of 273.6 J/m² per minute; the range of UVB doses tested was from 547.2 to 30,096 J/m². A total of 10 experimental conditions for each developmental stage were conducted, varying the exposure time from 2 to 110 min. For each developmental stage, the range of the doses employed was adjusted based on preliminary studies on their UVB susceptibility. Thus, the ranges of treatments were 547.2 to 30,096 J/m² for S.9; 547.2 to 30,096 J/m² for S.11; 547.2 to 8,208 J/m² for S.17; 547.2 to 3,830.4 J/m² for S.20; 547.2 to 4,377.6 J/m² for S.23; and 547.2 to 4,924.8 J/m² for S.25. A study was conducted on effects of UVB radiation on morphogenetic processes of external organs—specifically, on the development of the external gills and the operculum. Groups of 10 embryos by replicate at muscular response (S.18) and tail circulation (S.22) stages were treated with doses ranging from 820.8 to 3,009.6 J/m². In this case, five experimental conditions for each developmental stage were conducted, varying the exposure conditions from 3 to 11 min. Control embryos, 10 organisms by replicate at the different developmental stages evaluated, were maintained in glass Petri dishes in Amphitox solution. Lethality and malformations were evaluated from 24 up to 168 h postexposure for each developmental stage. The lethal doses (LD) and the effective doses (ED) 10, 50, and 90 by means of probit analysis were estimated 168 h postexposure. Malformations were determined using optic and scanning electron microscopy; for that purpose, control and experimental embryos were fixed at the end of the experiment with glutaraldehyde acid in phosphate buffer and dehydrated in acetone; after the critical point technique, specimens were coated with gold–palladium [10] and observed with an XL microscope operated at 10 kV (Philips, Eindhoven, the Netherlands). Abnormal behavior such as alterations in motility were observed and recorded. The teratogenic risk associated with UVB radiation was estimated for each developmental stage, where possible, with the teratogenic index, which is calculated by dividing the LD50 by the ED50. According to the standard guide for conducting the frog embryo teratogenesis assay *Xenopus* [21], a compound with a teratogenic index greater than 1.5 signifies that it has a large separation of the mortality and malformation concentration ranges and, therefore, a great potential for all embryos to be malformed in the absence of significant embryo mortality.

To test for differences between the LD50 values among the developmental stages, statistical significance was established when the division between the major LD50 and the minor LD50 exceeds the critic value established by the American Public Health Association [22,23].

RESULTS

The stage-dependent susceptibility of *R.* (*Bufo*) *arenarum* embryos to UVB radiation is represented in Figure 1 and Table 1. From the blastula up to the gill circulation stage, a diminution in the resistance to UVB was observed. It was followed with an increase in the resistance to UVB from gill circulation onward. Compared with gill circulation (S.20), the resistance to UVB (expressed as LD50 values at 168 h postexposure) for blastula (S.9), gastrula (S.11), tail bud (S.17), opercular fold (S.23), and complete operculum (S.25) was 8.2, 4.4, 2.2, 1.3, and 1.2 times higher, respectively.

Ultraviolet B radiation caused malformations in all developmental stages evaluated. Figure 2 shows the stage-dependent
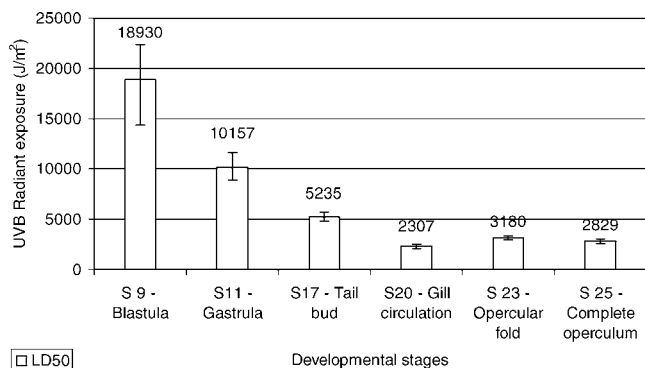
Fig. 1. Lethal dose 50 (LD50) values of ultraviolet B (UVB) radiation for *Rhinella* (*Bufo*) *arenarum* embryos at different developmental stages, 168 h postexposure.
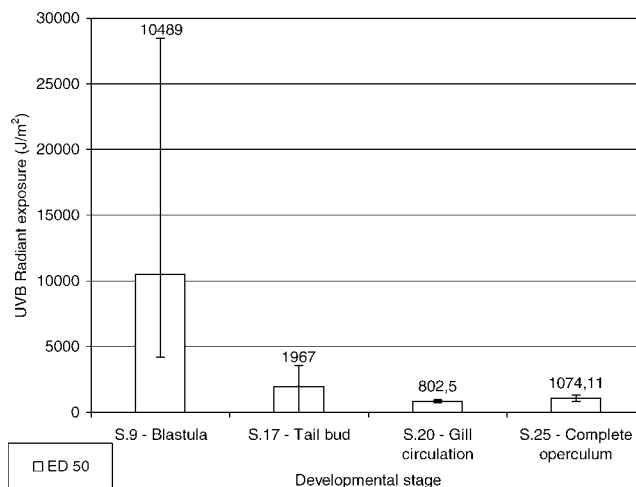

Fig. 2. Malformations plotted as effective dose 50 (ED50) values for ultraviolet B (UVB)–irradiated *Rhinella* (*Bufo*) *arenarum* embryos, 168 h postexposure at different developmental stages.

susceptibility for teratogenic effects. The ED10, ED50, and ED90 values were 8,312, 10,489.12, and 34,379 (S.9); 375, 1,967, and 4,376 (S.17); 547, 802.5, and 1,176 (S.20); and 444, 1,074.11, and 2,587 (S.25), respectively. The dose causing 50% malformations at gill circulation (S.20) was approximately 13 times lower than the dose producing the same percentage of malformations at blastula (S.9), the most resistant stage evaluated. It is noteworthy that for the opercular fold (S.23), the ED50 could not be obtained because the lowest radiation tested caused a 100% rate of malformation, while in the case of S.11 the statistical analysis used was not suitable to result in an ED50 value. As a whole, teratogenic effects result as a consequence of the impaired development of the cells directly exposed to UVB and occur at doses approximately two or three times lower than those producing lethality. Table 2 summarizes the most typical malformations obtained for each developmental stage. The teratogenic index ranged between 1.8 and 2.9 (1.8 for S.9; 2.7 for S.17; 2.9 for S.20; and 2.7 for S.25), which is indicative that UVB is a teratogenic agent producing a range of abnormalities even at very low doses, as in the case of the most susceptible developmental stages. An average of 85% of the control embryos from the stage-dependent susceptibility study developed normally. Figure 3A to C shows examples of the severity of the abnormalities caused by UVB radiation at different developmental stages at the time that the control embryos have reached S.20 (Fig. 3D). The most common adverse effects were severe delayed development and reduced body size, incurvations, microcephaly, anencephaly, prominent abdomen, underdeveloped or agenesis of gills and fin, and irregular embryonic outline, mainly in the dorsal side. It is noteworthy that more than one adverse effect was registered in all treated embryos.

Regarding epithelial tissue, it is remarkable that in embryos exposed to UVB during the blastula and gastrula stages almost normal glandular and ciliated cells were observed, except in the surroundings of severe injuries. Conversely, embryos treated at S.17 (tail bud), when ciliated cells differentiate, exhibited extensive regions of the embryonic surface only with glandular cells. The ridges corresponding to the tight junction structure were not observed in UVB-treated embryos. The main abnormalities of glandular cells were irregular apical surface in heavily affected regions of the embryo and presence of domed and small cells.

Ultraviolet B radiation on morphogenetic processes of external organs such as branchial filaments and operculum caused damage from delayed development and reduced growth up to agenesia of these structures. For example, the organisms exposed at S.18 to 820.8 J/m^2 did not develop the branchial filaments on the irradiated side; in the gill, a small and irregular ectodermal ridge formed by numerous domed and small cells was observed (Fig. 3E). On the nonirradiated side of the embryo, the branchial filaments developed normally as in control embryos (Fig. 3F). Organisms treated at S.22 with 2,983.2 J/m^2 showed severe morphological alterations in the gills of both sides of the body (Fig. 3G). In this morphogenetic territory, composed by the cells involved with the development of this structure, numerous small and domed cells were observed while no ciliated cells were seen in the affected area. In control embryos at S.22, the gills exhibit long filaments with both glandular and ciliated cells and the partial closure

Table 1. Lethal dose (LD) 10, 50, and 90 values expressed as ultraviolet B radiation in joules per square meter at different developmental stages of *Rhinella* (*Bufo*) *arenarum*[a]

|	Blastula (S.9)	Gastrula (S.11)	Tail bud (S.17)	Gill circulation (S.20)	Opercular fold (S.23)	Complete operculum (S.25)
LD10	6,122	5,022	3,539	1,793	2,542	2,003
(Lower/upper CI)	(3,014/8,843)	(4,015/5,923)	(3,002/3,922)	(1,472/2,002)	(2,239/2,747)	(1,658/2,235)
LD50	18,930	10,157	5,235	2,307	3,180	2,829
(Lower/upper CI)	(14,357/22,385)	(8,907/11,640)	(4,836/5,737)	(2,079/2,519)	(2,985/3,364)	(2,600/3,075)
LD90	65,541	20,539	7,744	2,994	3,979	4,036
(Lower/upper CI)	(29,383/2,864,649)	(17,188/26,303)	(6,831/9,569)	(2,715/3,526)	(3,723/4,404)	(3,605/4,917)

[a] CI = confidence interval.
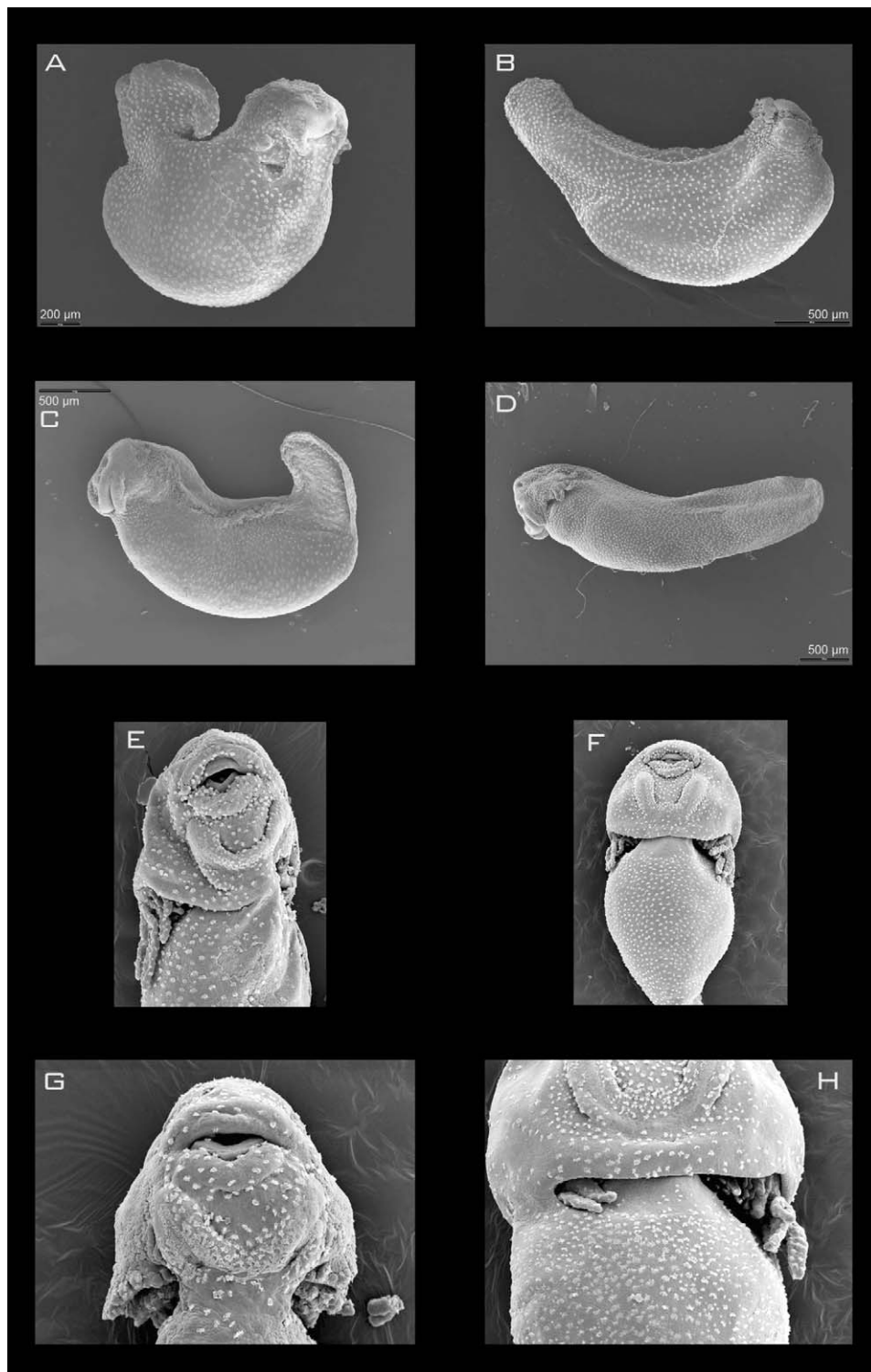
Fig. 3. (**A**) Panoramic view of a *Rhinella* (*Bufo*) *arenarum* embryo exposed to 16,416 J/m^2 of ultraviolet B (UVB) radiation at blastula stage (S.9) at the time control embryos reached S.20, exhibiting severe delayed development and axial incurvations, reduced body size, prominent abdomen, microcephaly, underdeveloped gills, and fin and irregular embryonic outline, mainly in the dorsal side. Embryonic longitude = 1,426 μm. ×62. (**B**) Panoramic view of a *R.* (*Bufo*) *arenarum* embryo exposed to 16,416 J/m^2 of UVB radiation at the gastrula stage (S.11) at the time control embryos reached S.20. Anencephaly, delayed development and dorsal incurvation, reduced body size, underdeveloped fin, gill agenesia, and prominent abdomen can be observed. Embryonic longitude = 2,231 μm. ×50. (**C**) Panoramic view of a *R.* (*Bufo*) *arenarum* embryo exposed to 8,208 J/m^2 of UVB radiation at the tail bud stage (S.17) at the time control embryos reached S.20. Severe delayed development and incurvations, reduced body size, underdeveloped gills, and abnormal epithelial tissue including cellular cordons can be noticed. Embryonic longitude = 1,941 μm. ×46. (**D**) Panoramic view of a *R.* (*Bufo*) *arenarum* control embryo at the gill circulation stage (S.20). Embryonic longitude = 3,279 μm. ×31. (**E**) Ventral view of a *R.* (*Bufo*) *arenarum* embryo exposed at S.18 to 820.8 J/m^2. The gill formation was affected on the side irradiated by UVB radiation. ×95. (**F**) Ventral view of a control *R.* (*Bufo*) *arenarum* embryo at S.18 with normal gills. ×55. (**G**) Ventral view of a *R.* (*Bufo*) *arenarum* embryo exposed at S.22 to 2,982.2 J/m^2. The gills and operculum formation were affected on both sides due to the normal position of the embryo at this developmental stage. ×110. (**H**) Ventral view of a control *R.* (*Bufo*) *arenarum* embryo at S.22. The partial closure of the right operculum can be observed. ×90.

Table 2. Common malformations and anomalies produced by ultraviolet B radiation at different developmental stages of *Rhinella* (*Bufo*) *arenarum* embryos

Blastula (S.9)	Gastrula (S.11)	Tail bud (S.17)	Gill circulation (S.20)	Opercular fold (S.23)	Complete operculum (S.25)
Reduced body size	Reduced body size	Reduced body size	Reduced body size	Reduced body size	Reduced body size
Bifid spine	Microcephaly	Asymmetries	Asymmetries	Asymmetries	Facial hydropsy
Persistent yolk plug		Microcephaly	Tail and axial incurvations	Tail and axial incurvations	
			Cell desquamation	Cell desquamation	
			Abnormal pigment distribution	Abnormal pigment distribution	
			Gill underdevelopment	Gill underdevelopment or agenesis	
			Abnormal swimming	Abnormal swimming	
			Hypermotility		

of the right operculum is in progress (Fig. 3H). At the teratogenic doses, the rate of abnormal development for gill and operculum was 100%; 95% of the control embryos developed those structures normally.

DISCUSSION

The stage-dependent susceptibility for lethal, as well as teratogenic, effects by UVB radiation reported in the present study contributes to the understanding of the adverse effects due to the increased intensity of this agent on amphibian species [6,8,24]. From our results, we can conclude that *R.* (*Bufo*) *arenarum* embryos at early organogenic stages are susceptible for both lethal and teratogenic effects produced by UVB radiation. The external gills and the operculum were the most susceptible structures to this physical agent, and UVB radiation caused an adverse effect on the differentiation of ciliated cells, reflecting the high impact of UVB radiation on external structures. The stage-dependent susceptibility during early life stages reported for different noxious agents [8,10,25,26] suggests that this endpoint seems to be relevant for environmental protection purposes.

The experimental UVB treatment employed allowed us to obtain adverse effects by means of exposure periods from minutes to less than 2 h. In these intensive exposure conditions for the most susceptible stage, the amount of UVB radiation producing LD50 is 13 times lower than UVB daily radiation reaching the Earth's surface in Buenos Aires latitudes during spring (31,212 J/m^2 modeled with the tropospheric ultraviolet visible model 4.1 radiative transfer program, National Center for Atmospheric Research, Boulder, CO, USA), the typical season for *R.* (*Bufo*) *arenarum* reproduction [27]. It is noteworthy that UVB radiation effectively reaching amphibian embryo and larvae depends on the environmental conditions of their habitats, such as shading, dissolved organic carbon [28], and water optical transparency due to dissolved matter; their breeding behavior [29]; and seasonal and geographic selection of the ponds considering altitude and latitude [30]. On the other hand, the severity of damage among different amphibian species could be determined by intrinsic defense mechanisms against UVB radiation [13,14]. In the case of *R.* (*Bufo*) *arenarum*, its reproduction occurs mainly in uncovered ponds during the spring and summer season when solar radiation is maximum, which is suggestive that this species may rely on intrinsic defense mechanisms. The dark color of the *R.* (*Bufo*) *arenarum* eggs and embryos likely provides the main defense

mechanism against UVB radiation by the absorption of its energy by means of melanin pigment granules. It is well known that melanin is the major UV-absorbing chromophore exhibiting an extremely broad spectrum of absorption over the UVB radiation, ultraviolet A radiation, and visible ranges and moreover has antioxidant activity [31]. The assumption that melanin could have a major role against deleterious effects caused by UVB radiation on *R.* (*Bufo*) *arenarum* embryos is in line with the study reported by Perotti and Diéguez [9] in which the photoprotection against UVB radiation in three amphibian species was associated to the melanin content of the embryo. On the other hand, photolyase, one of the principal photo repair enzymes induced by UVB irradiation and antioxidant molecules such as catalase, superoxide dismutase, and glutathion peroxidase, could also contribute to the protection of UVB-irradiated embryos [15,32].

The stage-dependent susceptibility reported for UVB radiation in this study corresponds with data obtained by means of different environmental agents, with oxidative stress as a major mechanism of toxicity such as cadmium [33] and lead [25] on *R.* (*Bufo*) *arenarum* embryos, pointing out that the evaluation of the stage-dependent susceptibility is of high relevance for the understanding of adverse effects produced by environmental agents and for the protection of living organisms. The greatest difference in the susceptibility of *R.* (*Bufo*) *arenarum* embryos to UVB radiation for both lethality and malformations was found between the blastula stage, as the most resistant, and the gill circulation stage, as the most susceptible. The resistance of the blastula stage seems to be related to *R.* (*Bufo*) *arenarum* embryos exhibiting a darkly pigmented animal cap that reflects abundant melanin granules in the area of the embryo directly exposed to UVB; a spherical shape that, from a geometric perspective, reduces the surface area that can be irradiated; and a high resistance to oxidative stress as shown by exposure to H$_2$O$_2$ [34].

As development advances by means of the gastrulation process, external cells from the marginal and vegetative zone invaginate while those remaining in the surface expand to cover the whole spherical embryo, resulting in a thinner layer of protective pigment granules. The diminution of pigment granules per surface area of the embryo, as well as the gradual metabolic change of the embryos toward increasing oxygen consumption [18], seems to be directly related to the gradual reduction in the resistance against UVB radiation as development advances toward the organogenic stages. It is note-

worthy that the amphibian embryos at those organogenic stages are susceptible to different oxidative stress agents [10,24].

Teratogenic effects were observed in all developmental stages evaluated, and based on the teratogenic index values obtained, UVB is a teratogenic agent with maximal effect during gill circulation, the most susceptible stage evaluated. The agenesis of entire body parts (e.g., the head or some organs such as gills) could illustrate the severity of but eventually limited damage in a specific morphogenetic field or tissue area. The flexures reported in different regions of the experimental embryos could reflect impaired growth in the irradiated area, probably as a consequence of the arrest in the G_2 phase of the cell cycle by UVB radiation as reported by van Oosten et al. [35]. The normal growth in nonirradiated tissues, concomitantly with the damage in the irradiated side, may have folded the embryo toward the affected side. Delayed development and reduced body size, which were observed in all stages evaluated, could also be related to the arrest of the cell cycle induced by UVB radiation [35]. Hypermotility reported in irradiated embryos could reflect that UVB radiation produces persistent damage in the developing neural cells. However, by the end of the embryonic development, exposure to UVB did not result in an impairment of the swimming capacity. This illustrates the vulnerability of the embryo during the cell differentiation processes at early organogenic stages.

The high susceptibility and adverse effects of UVB radiation on external structures such as the gills and operculum are remarkable, resulting in underdevelopment and even agenesia of these structures. In the case of the gills, the adverse effects are usually unilateral; for the operculum, they are bilateral due to the spontaneous position of the embryos during the UVB treatment at stages 18 and 22, respectively. It is noteworthy that UVB exposure at the blastula and gastrula stages did not interfere with the differentiation of ciliated cells, which occurs from the neurula stage onward, but that exposure of the embryos during the period in which ciliated cells are still in a process of differentiation resulted in an impairment or significant diminution of those cells in the ectodermic surface. The small and rounded cells in the affected area seems to reflect a regeneration process following a severe UVB radiation–induced injury, as was observed in other regenerative processes [36]. It is noteworthy that those teratogenic effects did not result in lethality of the embryos, at least not until the complete operculum stage (S.25), which was the last developmental stage evaluated in the present study.

Our results complement previous studies conducted with other species of amphibians, *Rana pipiens, Rana clamitans*, and *Rana septentrionalis*, that postulated that solar UVB radiation could be a factor in the increased number of malformations in amphibian limbs collected from the field [8]. Our studies are also in line with studies that used meta-analytic techniques to show that UVB is a global stressor with overall negative effects on both survival and growth that crosses life histories, trophic groups, habitats, and experimental venues [37].

Is the stage-dependent susceptibility to UVB radiation reported in this study reflecting environmental and life-features changes during the evolutionary process? In a recent contribution, ontogenic features such as stage-dependent biochemical and physiological changes, as well as susceptibility to noxious agents, have been considered as biomarkers of the coevolution between living forms' ancestors and environmental signatures during the Earth's history (evoecotoxicology)

[18]. This new synthesis of the evolutionary process contributes to a better understanding of the evolution of both life forms and their environments. From this perspective, the remarkable transition from (almost) anaerobic to aerobic metabolism at early developmental stages reported from invertebrates to mammals (e.g., at the gastrula stage in the case of amphibians) could reflect that multicellular blastula-like organisms flourished in the anoxic planet more than 2 billion years ago [18]. At that time, as free oxygen was not available in significant concentrations, the ozone shelter was not in place, and therefore early multicellular organisms probably had to cope with much higher UVB irradiation conditions than after free oxygen was available in high levels. Melanin is selectively distributed in the animal cap during the early developmental stages of amphibian embryos, which seems to contribute to the high resistance to UVB at those early developmental stages and implies the greater build up of an inner shelter against much higher UVB irradiation than necessary for present time conditions. Thus, the high resistance to UVB at the blastula stage could be considered a biomarker of the high UVB exposure condition that amphibian ancestors may have faced during the anoxic period of the planet. Conversely, from the gastrula stage onward, the gradual increase in the susceptibility to UVB radiation reported in the present study occurs concomitantly with a gradual increase in oxygen consumption by the amphibian embryo [38,39]. From an evoecotoxicological perspective, it reflects that the organogenic differentiation capability in the amphibian ancestors evolved in the presence of increasing levels of free oxygen and therefore with a rising benefit of an enhanced energetic budget and an ozone shelter protecting living forms from UVB adverse effects. Thus, the gradual diminution in the resistance of the amphibian embryos to UVB toward the organogenic stages could be considered a biomarker of the gradual increase in free oxygen and the concomitant ozone layer development, allowing the amphibian ancestor to diminish gradually its defense mechanisms against UVB radiation during its phylogenetic process. As it is well accepted that free oxygen levels (and therefore, presumably, the ozone shelter) varied significantly during the last 500 million years, the slight variation in the susceptibility to UVB radiation during the last developmental stages of the *R. (Bufo) arenarum* development could be related to those environmental changes. High free oxygen concentrations and the concomitant ozone shelter protection seem to be the key conditions that provided the possibility for a remarkable progress in cell differentiation and morphogenesis in living forms' ancestors as reflected, for instance, in the amphibian ontogenesis. Evoecotoxicology, among other potentials, provides a rational explanation for the stage-dependent susceptibility to adverse environmental conditions during the ontogenic process [18]. The results reported with UVB radiation in the present study are in line with those expected within the framework of this theory.

Although there is no consensus as to the role of UVB in the worldwide amphibian decline [40,41], it is noteworthy that this environmental agent, especially during organogenic stages, which, as this study shows, are the most susceptible to UVB radiation, could represent a threat for amphibians. Moreover, UVB as a worldwide environmental stressor could have significant adverse effects in synergy with other toxic agents [25,33] and on the resistance to infectious diseases.

REFERENCES

1. Kerr J, McElroy C. 1993. Evidence for large upward trends of ultraviolet-B radiation linked to ozone depletion. *Science* 262: 1032–1034.

2. Wake DB. 1991. Declining amphibian populations. *Science* 253: 860.

3. Houlahan JE, Findlay CS, Schmidt BR, Meyers AH, Kuzmin SL. 2000. Quantitative evidence for global amphibian population declines. *Nature* 404:752–754.

4. Helgen JC, Gernes MC, Kersten SM, Chirhart JW, Canfield JT, Bowers D, Haferman J, McKinnell RG, Hoppe DM. 2000. Field investigations of malformed frogs in Minnesota, 1993–1997. *J Iowa Acad Sci* 107:96–105.

5. Taylor B, Skelly D, Demarchis L, Slade M, Galusha D, Rabinowitz P. 2005. Proximity to pollution sources and risk of amphibian limb malformation. *Environ Health Perspect* 113:1497–1501.

6. Blaustein AR, Hoffman PD, Hokit DG, Kiescker K, Walls SC, Hays JB. 1994. UV repair and resistance to solar UV-B in amphibian eggs: A link to population declines? *Proc Natl Acad Sci USA* 91:1791–1795.

7. Ankley GT, Tietge JE, Holcombe GW, DeFoe DL, Diamond SA, Jensen KM, Degitz SJ. 2000. Effects of laboratory ultraviolet radiation and natural sunlight on survival and development of *Rana pipiens*. *Can J Zool* 78:1092–1100.

8. Tietge JE, Diamond SA, Ankley GT, DeFoe DL, Holcombe GW, Jensen KM, Degitz SJ, Elonen GE, Hammer E. 2001. Ambient solar UV radiation causes mortality in larvae of three species of *Rana* under controlled exposure conditions. *Photochem Photobiol* 74:261–268.

9. Perotti MG, Diéguez MC. 2006. Effect of UV-B exposure on eggs and embryos of Patagonian anurans and evidence of photoprotection. *Chemosphere* 65:2063–2070.

10. Herkovits J, Cardellini P, Pavanati C, Pérez-Coll CS. 1997. Susceptibility of early life stages of *Xenopus laevis* to cadmium. *Environ Toxicol Chem* 16:312–316.

11. Halliwell B, Gutteridge JMC. 1999. *Free Radicals in Biology and Medicine,* 3rd ed. Clarendon, Oxford, UK.

12. Gallagher RP, Lee TK. 2006. Adverse effects of ultraviolet radiation: A brief review. *Prog Biophys Mol Biol* 92:119–131.

13. Mitani H, Uchida N, Shima A. 1996. Induction of cyclobutane pyrimidine dimer photolyase in cultured fish cells by UVA and blue light. *Photochem Photobiol* 64:943–948.

14. Prota G. 2000. Melanins, melanogenesis and melanocytes: Looking at their functional significance from the chemist's viewpoint. *Pigm Cell Res* 13:283–293.

15. Herkovits J, D'Eramo JL, Fridman O. 2006. The effect of UV-B radiation on *Bufo arenarum* embryos survival and superoxide dismutase activity. *Int J Environ Res Public Health* 3:43–47.

16. Herkovits J, Pérez-Coll CS, Fridman O, D'Eramo JL, Stockert JC. 2006. Protection possibilities against oxidative stress exerted by physic–chemical agents on amphibian embryos. In Herkovits J, ed, *Salud Humana y Ambiental*. SETAC, Buenos Aires, Argentina, pp 63–65.

17. Anbar AD, Knoll AH. 2002. Proterozoic ocean chemistry and evolution: A bioinorganic bridge? *Science* 297:1137–1142.

18. Herkovits J. 2006. Evoecotoxicology. Environmental changes and life features development during the evolutionary process: The record of the past at developmental stages of living organisms. *Environ Health Perspect* 114:1139–1142.

19. Herkovits J, Pérez-Coll C. 1999. Bioassays for toxicity testing with amphibian embryos ''ANFITOX'' based on *Bufo arenarum*: Acute Test (ANFIAGU), Short chronic test (ANFICOR), Chronic

20. Del Conte E, Sirlin JL. 1951. Early life stages of *Bufo arenarum*. *Acta Zool Lilloana* 12:495–499.

21. American Society for Testing and Materials. 1994. Standard guide for conducting the frog embryo teratogenesis assay: *Xenopus*. E 1439-91. In *Annual Book of ASTM Standards,* Vol 11.04. West Conshohocken, PA, pp 1285–1296.

22. American Public Health Association, American Water Works Association, Water Pollution Control Federation. 1980. *Standard Methods for the Examination of Water and Wastewater*. American Public Health Association, Washington, DC.

23. Rodriguez EM, Lombardo RJ. 1991. Acute toxicity of parathion and 2,4-D to estuarine adult crabs. *Bull Environ Contam Toxicol* 46:576–582.

24. Bruggeman DJ, Bantle JA, Goad C. 1998. Linking teratogenesis, growth, and DNA photodamage to artificial ultraviolet B radiation in *Xenopus laevis* larvae. *Environ Toxicol Chem* 17:2114–2121.

25. Pérez-Coll CS, Herkovits J. 1990. Stage-dependent susceptibility to lead in *Bufo arenarum* embryos. *Environ Pollut* 63:239–245.

26. Ortiz-Santaliestra ME, Marco A, Fernandez MJ, Lizana M. 2006. Influence of developmental stage on sensitivity to ammonium nitrate of aquatic stages of amphibians. *Environ Toxicol Chem* 25:105–111.

27. Gallardo JM. 1974. *Amphibians from Buenos Aires Surroundings.* Universidad de Buenos Aires, Buenos Aires, Argentina.

28. Schindler DW, Curtis PJ. 1997. The role of DOC in protecting freshwaters subjected to climatic warming and acidification from UV exposure. *Biogeochemistry* 36:1–8.

29. Palen WJ, Williamson CE, Clauser AA, Schindler DE. 2005. Impact of UV-B exposure on amphibian embryos: Linking species physiology and oviposition behaviour. *Proc R Soc London Ser B* 272:1227–1234.

30. Adams MJ, Schindler DE, Bury RB. 2001. Association of amphibians with attenuation of ultraviolet-B radiation in montane ponds. *Oecologia* 128:519–525.

31. World Health Organization, United Nations Environment Programme, Ultraviolet Radiation, International Commission on Non-Ionizing Radiation Protection. 1994. Environmental Health Criteria 160. Geneva, Switzerland.

32. Smith MA, Kapron CM, Berrill M. 2000. Induction of photolyase activity in wood frog (*Rana sylvatica*) embryos. *Photochem Photobiol* 72:575–578.

33. Herkovits J, Pérez-Coll CS. 1993. Stage-dependent susceptibility of *Bufo arenarum* embryos to cadmium. *Bull Environ Contam Toxicol* 50:608–611.

34. Barbieri F, Legname C. 1962. Hydrogen peroxide action on *Bufo arenarum* eggs. *Archivos de Bioquímica Química y Farmacia* 10:59–64.

35. van Oosten M, Rebel H, Friedberg EC, van Steeg H, van der Horst GT, van Kranen HJ, Westerman A, van Zeeland AA, Mullenders LH, de Gruijl FR. 2000. Differential role of transcription coupled repair in UVB-induced G_2 arrest and apoptosis in mouse epidermis. *Proc Natl Acad Sci USA* 97:11268–11273.

36. Herkovits J. 1977. Normal and tail regeneration development on *Bufo arenarum* during embryogenesis: Ectodermal apical surface characteristics. *Medicina* 36:553–554.

37. Bancroft BA, Baker NJ, Blaustein AR. 2007. Effects of UVB radiation on marine and freshwater organisms: A synthesis through meta-analysis. *Ecol Lett* 10:332–345.

38. Legname A, Barbieri F. 1962. Breathing metabolism during the initial development of *Bufo arenarum*. *Archivos de Bioquímica Química y Farmacia* 10:31–39.

39. Herkovits J, Jatimliansky JR. 1982. Alterations in ionic calcium concentrations in the maintaining media and its influence on oxygen consumption during embryonic development in *Bufo arenarum*. *Medicina* 42:867.

40. Licht LE. 2003. Shedding light on ultraviolet radiation and amphibian embryos. *Bioscience* 53:551–561.

41. Wake DB, Vredenburg VT. 2008. Are we in the midst of the sixth mass extinction? A view from the world of amphibians. *Proc Natl Acad Sci USA* 105:11466–11473.

(ANFICRO) and Early life stages (ANFIEMB). *Ingeniería Sanitaria y Ambiental* 42:24–3043:50–55.