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Assessment of the Risk of Solar Ultraviolet Radiation to Amphibians. I. Dose-Dependent Induction of Hindlimb Malformations in the Northern Leopard Frog (*Rana pipiens*)

GERALD T. ANKLEY,*
STEPHEN A. DIAMOND,
JOSEPH E. TIETGE, GARY W. HOLCOMBE,
KATHLEEN M. JENSEN,
DAVID L. DEFOE, AND RYAN PETERSON

U.S. Environmental Protection Agency, National Health and Environmental Effects Research Laboratory, Mid-Continent Ecology Division, 6201 Congdon Boulevard, Duluth, Minnesota 55804

A number of environmental stressors have been hypothesized as responsible for recent increases in limb malformations in several species of North American amphibians. The purpose of this study was to generate dose−response data suitable for assessing the potential role of solar ultraviolet (UV) radiation in causing limb malformations in a species in which this phenomenon seemingly is particularly prevalent, the northern leopard frog (*Rana pipiens*). Frogs were exposed from early embryonic stages through complete metamorphosis to varying natural sunlight regimes, including unaltered (100%) sunlight, sunlight subjected to neutral density filtration to achieve relative intensities of 85%, 75%, 65%, 50%, and 25% of unaltered sunlight, and sunlight filtered with glass or acrylamide to attenuate, respectively, the UVB (290−320 nm) and UVB plus UVA (290−380 nm) portions of the spectrum. The experiments were conducted in a controlled setting, with continual monitoring of UVB, UVA, and visible light to support a robust exposure assessment. Full sunlight caused approximately 50% mortality of the frogs during early larval development; no significant treatment-related mortality occurred under any of the other exposure regimes, including 100% sunlight with glass or acrylamide filtration. There was a dose-dependent ($p < 0.0001$) induction of hindlimb malformations in the frogs, with the percentage of affected animals ranging from about 97% under unaltered sunlight to 0% in the 25% neutral density treatment. Malformations were comprised mostly of missing or truncated digits, and generally were bilateral as well as symmetrical. Filtration of sunlight with either glass or acrylamide both significantly reduced the incidence of malformed limbs. The estimated sunlight dose resulting in a 50% limb malformation rate (ED50) was 63.5%. The limb ED50 values based on measured sunlight intensities corresponded to average daily doses of 4.5 and 100 $Wh \cdot m^{-2}$ for UVB and UVA, respectively. Exposure to sunlight also resulted in increased eye malformations in *R. pipiens*; however, the dose−response relationship for this endpoint was not monotonic. The results of this study, in conjunction with measured or predicted exposure data from natural settings, provide a basis for quantitative prediction of the risk of solar UV radiation to amphibians.

* Corresponding author phone: 218-529-5147; fax: 218-529-5003; e-mail: ankley.gerald@epa.gov.

Introduction

The occurrence of malformed amphibians in several regions in North America has resulted in significant attention in both scientific and public sectors (*1*). The most commonly observed malformations have been associated with the hindlimbs of a number of ranid species (*2*−*5*). Although there has been a tendency to focus on relatively striking examples of polymelia and polydactyly (extra limbs, digits), the most prevalent types of malformations have been deletions or truncations of various bones in the hindlimbs (*2*−*4*, *6*). Several environmental stressors have been suggested as responsible for the limb malformations. Most attention has focused upon potential chemical exposures that adversely affect some aspect of early development or metamorphosis in amphibians. Specifically, it has been speculated that chemicals which disrupt retinoic acid signaling pathways may be responsible for limb defects in native anurans (*4*). Some reports suggest the presence of developmental toxicants at sites corresponding to observations of malformed amphibians (*7, 8*); however, evidence of a chemical etiology for the limb malformations remains equivocal (*9, 10*). Another hypothesis that may explain the malformations involves physical disruption of the developing limb bud field of amphibians by parasitic trematodes (*11*); recent research with Pacific tree frogs and western toads experimentally infected with trematodes (*Ribeiroia*) has demonstrated the plausibility of this hypothesis (*12*, *13*). There is significant uncertainty, however, as to the spatial and temporal occurrence of trematodes relative to malformations observed in ranid species of concern.

In 1997, our laboratory conducted an experiment to investigate the effects of the juvenile growth hormone analogue methoprene on survival and development of the northern leopard frog, *Rana pipiens* (*14*). On the basis of the results of in vitro studies (*15*), we speculated that the insecticide or its metabolite(s) might produce limb malformations through activation of retinoic acid receptor(s). Because of possible conversions of methoprene by sunlight to biologically active byproducts (*16*), the exposures were conducted both with and without artificial ultraviolet (UV) radiation designed to mimic that in sunlight. Methoprene exposure did not cause limb malformations in the frogs; however, somewhat unexpectedly, treatment with UV radiation alone produced a high incidence of limb defects. The observed malformations consisted of relatively symmetrical truncations of different portions of the limb, ranging from missing digits to completely absent limbs (*14*). The period of greatest sensitivity of limb development to UV-induced defects coincided with early limb bud formation in the frogs, approximately stages 25−26 (*17*). Experimental work conducted in 1998 confirmed the 1997 data and demonstrated a dose-dependence of the phenomenon under the artificial UV radiation (*18*). Ankley et al. (*18*) also described the results of limited experiments in which *R. pipiens* exposed to filtered natural sunlight (ca. 60% of ambient) developed hindlimb malformations indistinguishable from those generated in the

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laboratory UV exposures. The purpose of the study described herein was to expand upon our earlier work and generate a full dose−response relationship for malformations in *R. pipiens* exposed to natural sunlight. This type of dose−response data is critical to assessing the potential environmental risk of UV radiation to developing amphibians (*19*).

Materials and Methods

Biological. Adult northern leopard frogs were collected from a small wetland approximately 0.4 km north of Muck Lake, WI (T45, R10W, S12), on May 5, 1999. The site is rural and not adjacent to any obvious sources of contamination. The animals were transported in a neoprene cooler containing site water to the U.S. Environmental Protection Agency (EPA) laboratory in Duluth, MN, and held under subdued fluorescent lighting at 20 °C. One pair of the frogs was in amplexus the following day, and on the morning of May 7, a mass of approximately 2000 eggs had been produced. A subsample of 20 eggs was separated from the egg mass and examined microscopically. All of the eggs appeared to be fertile, with the majority in the four-cell stage. Eggs from the remainder of the egg mass, with gel coat intact, were gently separated and randomly assigned to polyethylene containers corresponding to the different treatments until there were 40 embryos per container. The groups of embryos were then placed, within 60 min, into the treatment system described next; at test initiation, the majority of the animals were at stages 5−7 (*17*).

Solar exposures were conducted on an open wooden deck adjacent to the EPA laboratory, in concave frosted glass bowls containing about 3.5 L of Lake Superior water which was renewed at a rate of 100 mL min^{-1}. The test temperature was maintained at 20 ± 1 °C through the constant addition of freshwater and by holding the bowls in a temperature-controlled recirculating water bath. Temperature in the water bath was continually monitored; additional temperature measurements in the treatment bowls were made daily during routine maintenance of the animals. Dissolved oxygen in all the bowls was measured at least weekly and was consistently greater than 6.8 mg L^{-1}. Other routine water chemistry measurements made over the course of the assay (pH, hardness, alkalinity, conductivity) remained within ranges typical for Lake Superior water, which have been demonstrated in previous studies as adequate for the normal development of *R. pipiens* (*10*, *14*, *18*, *20*).

Under these conditions, eggs remained in a mass at the bottom of the bowls until hatching within about 7 days of test initiation. Upon hatching, the animals were fed a mixture of trout chow−algae−Tetrafin ad libitum; at about 5 days post-hatch, this mixture was supplemented with live, newly hatched brine shrimp (*14*, *18*). Feeding typically was 3 times daily Monday through Friday and once per day on weekends. Excess debris was siphoned as necessary, and the animals were evaluated daily for mortality or abnormal behavior (e.g., lethargy). Three tadpoles were removed from each treatment bowl and preserved in 4% formaldehyde in 0.1 M phosphate buffer on June 2, June 17, and July 1, 1999 (days 26, 41, and 55 after test initiation, respectively), to provide animals for staging and morphological evaluation. On the last sampling date, the density of animals in all the bowls was reduced to no more than 25 to help maintain acceptable water quality for the duration of the assay. Animals were taken from the test system upon emergence of forelimbs (stage 42); this commenced on July 12 (day 66) and continued until September 9, 1999 (day 125), when the test was ended. Upon removal, the frogs were weighed (0.01 g), measured for snout-vent length (1 mm), and examined for morphological abnormalities, including limb malformations. Animals exhibiting abnormal characteristics were digitally photographed and preserved.

The experimental design included treatments with unaltered sunlight, treatments that attenuated sunlight to the same relative degree across the full wavelength spectrum (neutral density filtration), and exposures that reduced energy associated with specific regions of the UV portion of the spectrum. Layers of Nitex and stainless steel screen were utilized to achieve the neutral density filtration and generate relative sunlight intensities of 25%, 50%, 65%, 75%, and 85%. Glass (AFG Industries, Kingsport, TN) and acrylamide (Rohm and Haas, Philadelphia, PA) filters were used to attenuate radiation in the UVB (290−320 nm) and UVB plus UVA (290−380 nm) portions of the spectrum, respectively. The glass and acrylamide filters were used both under full sunlight and in conjunction with the neutral density filtration at 25%, 75%, and 85% of ambient sunlight. All treatments were conducted in duplicate.

Light Measurement and Dose Estimation. Full spectrum (290−700 nm) solar irradiance was characterized for all filter treatments using a photodiode array spectrometer (model S2000; Ocean Optics, Dunedin, FL). Measurements were made on cloudless days at the bottom of an exposure chamber containing a volume of water equivalent to that used during the bioassays. The quantification process involved measuring the sunlight spectrum with and without each filter in its experimental position. These measurements were made in rapid succession (within 30 s) to eliminate the effect of sun position and repeated 3 times for each treatment. The three filtered values were averaged and then divided by averaged, unfiltered values to yield a proportion of unfiltered irradiance transmitted to the bottom of the exposure bowl, in 1-nm increments, from 290 to 700 nm.

Terrestrial solar irradiance was monitored continuously throughout the experiment using a photosynthetically active radiation (PAR) sensor attached to a data logger (sensor model LI190SB, data logger model LI-1000; Li-Cor, Lincoln, NE) mounted on the roof of a nearby building and a radiometer (model IL1700; International Light, Newburyport, MA) fitted with a broad-band UVA sensor mounted in a shade-free location, approximately 2 m above the level of the outdoor exposure facility. Irradiance was averaged over 15 and 10 min intervals, respectively, for the two instruments. The daily terrestrial irradiance measured with these two instruments was compared to confirm consistency in their response to atmospheric variation (primarily cloud cover) and consistency in the ratio of their values over the relatively long monitoring period. Ultimately, UVA and UVB values were calculated as percentages of the PAR monitoring data (10.37% and 0.47%, respectively). These percentages were derived from spectrophotometric data collected during previous experiments (*18*), as well as predictions from radiative transfer models (*21*). Hourly values were summed to estimate daily dose and total dose for discrete exposure periods. The proportionate transmission for each treatment filter (measured under water) was then used to estimate daily light dose in exposure bowls and, ultimately, to calculate a total dose for each treatment.

Data Analysis. Statistical analyses were performed using SYSTAT 7.0 for Windows (SPSS, Chicago, IL). Survival, growth, and malformation data were subjected to ANOVA, followed by Tukey's multiple comparisons test. Percentage data were arcsine square-root transformed before analyses. Results were considered significant at $p \leq 0.05$. Probit analysis was used to derive dose−response relationships (SAS Institute, Cary, NC).

Results

Light Measurements and Dose Estimation. The neutral density filters reduced solar radiation intensity in a reasonably uniform manner across the UVB, UVA, and visible wavelength ranges (Table 1; Figure 1a). The greatest deviation from true

TABLE 1. Measured Solar Radiation Transmittance for UVB, UVA, and Visible Light for Different Filter Combinations[a]

wavelength range	treatment													
	100	100G	100A	85	85G	85A	75	75G	75A	65	50	25	25G	25A
UVB	100	6	5	74	5	5	65	4	3	59	53	17	2	1
UVA	100	70	23	74	52	19	70	31	17	63	53	21	14	6
visible	100	90	94	79	79	68	73	64	69	67	55	25	22	23

[a] Values in header column indicate target (percent) transmittance based on neutral density filtration (with Nitex screen) without or with additional glass (G) and acrylamide (A) filtration. Data are expressed as percentages relative to unfiltered sunlight.

TABLE 2. Survival, Growth, and Time (Days) Required to Achieve 50% Forelimb Emergence (TE_{50}) of *Rana pipiens* during Exposure to Different Sunlight Regimes[a]

treatment	cumulative survival (%)			length (mm)[b]	weight (g)[b]	TE_{50}
	0–12 days	13–48 days	49–125 days			
100[c]	97.5 ± 3.5	57.5 ± 1.3*	56.0 ± 0.9*	32 ± 1.4	3.57 ± 0.71	90 ± 1.4
100G	100 ± 0.0	98.6 ± 1.9	98.6 ± 1.9	28 ± 0.7	2.63 ± 0.04	94 ± 3.5
100A	96.2 ± 1.8	92.3 ± 3.5	98.4	29	2.85	90
85	100 ± 0.0	94.9 ± 3.7	93.8	31	3.19	86
85G	96.2 ± 5.3	94.9 ± 7.2	92.9 ± 4.4	28 ± 2.1	2.68 ± 0.51	93 ± 0.0
85A	100 ± 0.0	94.9 ± 0.1	91.2	28	2.67	89
75	96.2 ± 1.8	92.4 ± 3.7	95.0	30	3.04	93
75G	97.5 ± 3.5	97.5 ± 3.5	97.5 ± 3.5	28 ± 0.8	2.61 ± 0.10	97 ± 4.2
75A	97.5 ± 3.5	97.5 ± 3.5	94.1 ± 4.3	28 ± 0.0	2.55 ± 0.11	95 ± 1.4
65	98.8 ± 1.8	97.4 ± 0.1	97.4 ± 0.1	28 ± 0.7	2.56 ± 0.01	96 ± 3.5
50	92.5 ± 3.5	92.5 ± 3.5	95.0	28	2.75	88
25	97.5 ± 3.5	97.5 ± 3.5	97.5 ± 3.5	28 ± 0.7	2.73 ± 0.39	94 ± 0.7
25G	95.0 ± 0.0	92.4 ± 0.2	92.4 ± 0.2	28 ± 1.4	2.59 ± 0.41	96 ± 0.7
25A	100 ± 0.0	98.8 ± 1.8	97.5	29	2.87	93

[a] Data represent mean ± SD from duplicate tanks except for values without a SD ($n = 1$). Each tank contained 40 animals at test initiation. The two asterisks indicate a significant ($p < 0.01$) difference compared to other treatments. [b] Measured at complete metamorphosis (forelimb emergence). [c] Treatment numbers refer to nominal relative percentage of sunlight exposure achieved via neutral density filtration. Several treatments were conducted in conjunction with filtration with glass (G) or acrylamide (A) designed to attenuate the UVB and UVB plus UVA portions of the spectrum, respectively.

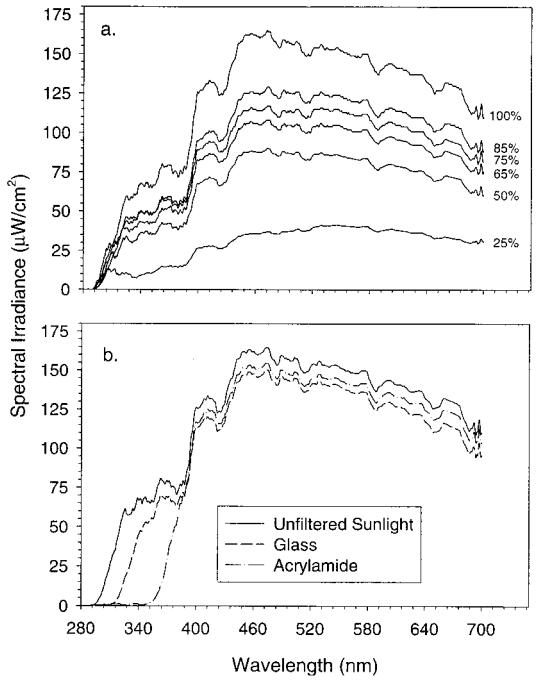

FIGURE 1. Spectral irradiance for (a) unaltered sunlight and treatments with neutral density filtration designed to achieve relative intensities of 85%, 75%, 65%, 50%, and 25% sunlight; and (b) unaltered sunlight and treatments with glass and acrylamide filtration.

neutral filtration occurred in the target 75%, 65%, and 25% treatments where UVB intensity was reduced 8% more relative to the visible range intensity. The glass and acrylamide filters effectively reduced the target spectral ranges (Table 1; Figure 1b). For example, UVB was reduced 94% and 95% in the glass and acrylamide treatments under full sunlight, respectively. Intensity of UVA in the absence of neutral density filtration was reduced 30% under glass and 77% under the acrylamide. Similar relative decreases in UVB and UVA were noted when the glass and acrylamide filters were used in conjunction with neutral density filtration in the 85%, 75%, and 25% of nominal treatments (Table 1).

Average daily doses of UVB, UVA, and visible light for the entire test duration (May 7 to September 9, 1999) were 6.79, 150.8, and 1454 $Wh \cdot m^{-2}$, respectively. Average daily doses of UVB, UVA, and visible light for the period of time from test initiation to our first observation of animals with clearly malformed limbs (July 1, 1999; see the following discussion) were 6.70, 149.0, and 1437 $Wh \cdot m^{-2}$, respectively.

Biological. Tietge et al. (*20*) describe in detail the effects of this experimental regime on survival of *R. pipiens* through test day 48 (June 24, 1999). Briefly, survival was greater than 95% in all treatments prior to and immediately following hatch (ca. day 7 after test initiation). By day 13, however, episodic mortality began to occur under 100% (unaltered) sunlight, with a cumulative survival in this treatment of 57.5% by test day 48. There was no significant mortality in any of the other treatments, including the 100% exposures under glass and acrylamide, with survival ranging from about 92–99% through day 48 (Table 2).

In early July, animals in six treatment bowls started to exhibit symptoms consistent with a pathogenic infection; they typically stopped eating and became lethargic, prior to dying. This mortality did not appear to be related to sunlight exposure in that it was observed in only one of the two replicates of six quite different exposure regimes: 85%, 75%, and 50% sunlight and 100%, 85%, and 25% sunlight with the acrylamide filter. Because of low survival and to avoid any potential bias associated with multiple stressors, data from

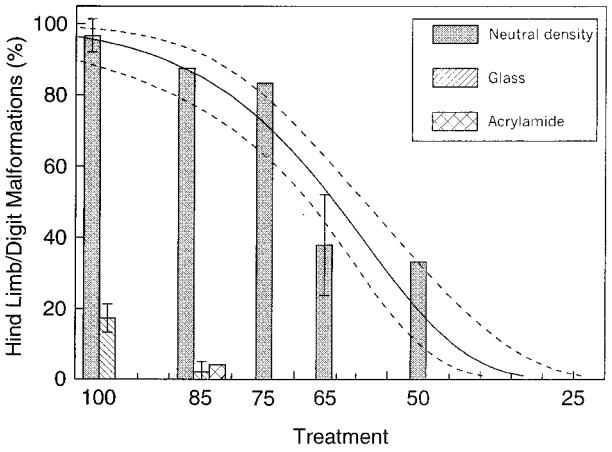

FIGURE 2. Occurrence of hindlimb malformations in *Rana pipiens* exposed to different sunlight regimes. Solid bars indicate the percentage of malformed animals under 100% (unaltered) sunlight and neutral density filtration designed to achieve relative intensities of 85%, 75%, 65%, 50%, and 25% of sunlight. Bars with diagonal-hatching indicate the percentage of malformations in animals exposed to the indicated sunlight intensity with additional glass filtration designed to remove the UVB portion of the spectrum. The bar with crosshatching indicates percentage of malformations in animals exposed to the indicated sunlight intensity with additional acrylamide filtration designed to remove both UVB and UVA portions of the spectrum. Error bars indicate the standard deviation associated with mean values from duplicate exposure containers; values without error bars were derived from one replicate only (see text for further explanation). The curve depicts the dose−response relationship (mean, 95% CI) for the unaltered sunlight/neutral density treatments derived using probit analysis.

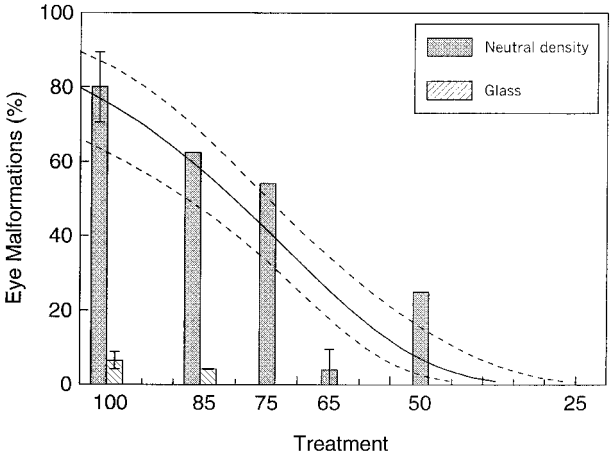

FIGURE 3. Occurrence of eye malformations in *R. pipiens* exposed to different sunlight regimes. Solid bars indicate the percentage of malformed animals under 100% (unaltered) sunlight and neutral density filtration designed to achieve relative intensities of 85%, 75%, 65%, 50%, and 25% sunlight. Bars with diagonal-hatching indicate percentage of malformations in animals exposed to the indicated sunlight intensity with additional glass filtration designed to remove the UVB portion of the spectrum. Error bars indicate the standard deviation associated with mean values from duplicate exposure containers; values without error bars were derived from one replicate only (see text for further explanation). The curve depicts the dose−response relationship (mean, 95% CI) for the unaltered sunlight/neutral density treatments derived using probit analysis.

these tanks were not used to calculate final mortality and growth values (Table 2) or malformation rates (Figures 2 and 3). When these replicates were excluded, there was no significant effect on survival in any treatment from day 48 through test completion (Table 2).

There were no significant treatment-related effects on length or weight of the frogs (Table 2). There also was no significant treatment effect on the rate of metamorphosis, expressed as time required for 50% of the animals to achieve emergence of forelimbs (TE_{50}, Table 2).

Morphological evaluation of the three animals subsampled from each replicate on test day 26 (June 2, 1999) indicated that all were at developmental stage 28; none of the animals examined had apparent malformations. The organisms sampled on June 17, 1999, were approximately equally distributed among stages 30−32. The 100% and 25% treatments were examined microscopically for pathologies in the developing hindlimbs. One of the animals from the 100% treatment had an apparent reduction in paddle development in that the distal end of the limb was tapered. The other five larvae subsampled from this treatment appeared normal, as did all six animals from the 25% treatment bowls. The animals sampled on July 1, 1999, were distributed among developmental stages 31−37. Animals from the 100%, 100% under glass, and 25% treatments were analyzed for pathologies of the hindlimbs. Four of the six larvae from the 100% exposure were affected. Malformations ranged from a complete absence of digit development in one of the animals to a combination of complete absence of some digits and reductions in the distal segments of the digits which were present. The observed malformations generally were bilateral and symmetrical. One of the six animals from the 100% treatment under glass had a reduction in distal digit segments, while the other five were normal. No adverse effects on hindlimb morphology were observed in the six larvae from the 25% sunlight treatment.

There was a significant ($p < 0.0001$) dose-dependent induction of hindlimb malformations in *R. pipiens* examined upon emergence of the forelimbs (Figure 2). The highest malformation incidence of 96.7% was associated with unaltered (100%) sunlight, and the occurrence of malformed animals decreased steadily to 87.5%, 83.3%, 38.0%, 33.3%, and 0% in the 85%, 75%, 65%, 50%, and 25% neutral density treatments, respectively. Attenuation of sunlight with either glass or acrylamide significantly reduced the occurrence of hindlimb malformations; filtration of 100% sunlight with glass reduced the malformation incidence to about 17%, while the corresponding treatment with acrylamide resulted in no animals with malformed hindlimbs (Figure 2). There was one malformed animal under both the glass and acrylamide treatments associated with 85% sunlight and no malformations under either of these filtration regimes in the 75% or 25% treatments (Figure 2).

As in our previous studies (*14*, *18*), the hindlimb malformations consisted exclusively of deletions and truncations. Also consistent with our past work was the observation that the malformations were bilateral and often symmetrical. The majority of malformations in the current study were comprised of missing and shortened digits and digit segments, with the most severely affected individuals missing all digits on both hindlimbs.

Exposure of *R. pipiens* to UV radiation also resulted in a significant occurrence of eye abnormalities, typified by varying degrees of micro/anopthalmia (Figure 3). The highest incidence of micro/anopthalmia (80.0%) was associated with unfiltered (100%) sunlight, and the occurrence decreased to 62.5%, 54.2%, 4.0%, 25.0%, and 0%, respectively, under the 85%, 75%, 65%, 50%, and 25% neutral density treatments. Attenuation of sunlight with either glass or acrylamide significantly reduced the occurrence of eye abnormalities; for example, filtration of 100% sunlight with glass reduced the incidence of micro/anopthalmia to 6.5%, while the corresponding treatment with acrylamide resulted in no animals with micro/anopthalmia (Figure 3).

Discussion

Our previous studies have demonstrated that UV radiation designed to mimic that of sunlight can induce hindlimb malformations in a dose-dependent fashion in *R. pipiens* in the laboratory (*14*, *18*). Ankley et al. (*18*) also reported limited data indicating that screened natural sunlight could cause similar hindlimb malformations in this species. The present study expands upon those experiments by generating a complete dose−response relationship for hindlimb malformations in *R. pipiens* exposed to varying intensities of sunlight. The incidence of hindlimb malformations ranged from none in animals exposed to a 25% neutral density filtration treatment to 97% in frogs held under full sunlight. On the basis of data from the neutral density filtration treatments, the ED50 (95% CI) for hindlimb malformations was 63.5% (58.6−67.6%) of sunlight (Figure 2). When averaged over the duration of the entire assay (May 7 to September 9, 1999), this ED50 value corresponded to average daily doses of 4.50, 100, and 964 $Wh \cdot m^{-2}$ of UVB, UVA, and visible light, respectively. If average daily doses are calculated based upon the period of time from test initiation to the first observation of abnormalities in developing limbs (July 1, 1999), UVB, UVA, and visible light values corresponding to the limb malformation ED50 are 4.26, 94.6, and 912 $Wh \cdot m^{-2}$, respectively.

An additional goal of the present study was to provide insights as to specific portion(s) of the sunlight spectrum responsible for hindlimb malformations in *R. pipiens*. When full intensity sunlight was attenuated with glass, the incidence of hindlimb malformations was decreased from 97% to 17%, while attenuation with acrylamide resulted in no animals with malformed limbs. The glass and acrylamide treatments both effectively removed about 95% of the incident UVB, while about 30% (glass) and 80% (acrylamide) of the UVA radiation was attenuated. Because glass did not completely eliminate the limb malformations, there is an indication that the UVA portion of the spectrum contributed to the observed damage. However, the lack of specificity of the two filtering materials with respect to removal of UVA complicates quantitative interpretation of the relative importance of this portion of the spectrum in producing malformations. Specifically, not only did the glass remove most of the UVB, it also attenuated 30% of the UVA, leading to uncertainty as to whether the decrease in UVB or UVA radiation was more important in decreasing the malformation rate. Additional work with filters that have precise wavelength cutoffs is required to better define the exact portion of the UV spectrum responsible for limb malformations. In terms of biological mechanism of action, this may or may not be important information; however, accurate definition of the portion of the UV spectrum responsible for adverse effects could be essential to assessing potential ecological risk in terms of determining which environmental conditions influencing UV dose have been (or could be) altered.

Recent changes in global conditions have increased the potential for exposure of aquatic animals to UV radiation through multiple mechanisms. Decreases in atmospheric attenuation of the UVB component of sunlight, in conjunction with ozone depletion caused by certain classes of halocarbons, have been well-documented (*22*−*25*). In addition, there is emerging evidence that other changes in global climate (warming, acid precipitation) may be increasing penetration, not only of UVB but also of UVA radiation in aquatic environments, predominantly through decreases in attenuation of light by dissolved organic carbon (*26*, *27*). Measures focused on reduction of halocarbon emissions indicate a wide recognition by scientific and regulatory authorities of the seriousness of increasing UVB radiation associated with ozone depletion (*28*). However, comparable attention has not been given to other alterations in global climate which could increase the penetrance of UV radiation in aquatic systems. To some extent, this is understandable in that increased penetration of UV radiation in aquatic environments does not have direct adverse human health effects. However, there could be significant consequences to aquatic biota associated with increased UV exposure. Data from the present study, for example, illustrate the fact that ambient levels of UVA radiation could produce adverse effects in developing amphibians. Further research documenting possible alterations in both UVB and UVA penetration in aquatic environments as well as the evaluation of the biological consequences of this is warranted.

A number of researchers have assessed the effects of UV radiation on embryonic survival and early development of different amphibian species (*29*−*36*). Relatively few studies, however, have evaluated the consequences of longer term exposures to UV radiation on later life stages of amphibians. On the basis of data from our laboratory, described both here and elsewhere (*14*, *18*, *20*), it appears that larval amphibians are more sensitive than embryonic animals to UV exposure, in terms both of decreased survival and the occurrence of malformations. Further, it appears that, at least for the ranid species which we have studied, there exists a "window" of enhanced sensitivity to UV radiation that extends from hatch through developmental stages 25−30. One practical ramification of this observation is that accurate predictions of the risk of UV radiation to amphibian populations need to be predicated on estimates or measurements of UV dose experienced by the animals during potentially sensitive developmental windows. This can be challenging because (1) different amphibian species breed at different times and develop at different rates and (2) even within a species, factors such as temperature and nutrition can affect developmental rates and, hence, the period of susceptibility to UV-induced damage.

As in previous work (*14*, *18*), the predominant malformations observed in the present study were comprised of bilateral, often symmetrical, deletions or truncations of different bones in the hindlimb. Although deletions and truncations comprise the majority of limb malformations observed in increasing frequency in some populations of North American anurans, the degree of bilateral effects noted in our studies generally has not been observed in malformed animals from the field (*2*, *6*). It is not possible, however, to ascertain whether this lack of congruence between experimental and field data is due to the possibility that solar UV radiation does not contribute to hindlimb malformations in amphibians from the field or whether it is an artifact of the manner in which UV exposure occurs in the field versus our experimental systems. Specifically, the concave, frosted bowls were designed to result in an very homogeneous dose of both down-welling and reflected UV radiation regardless of position of the animal in the test container. This almost certainly does not accurately mimic the type of exposure experienced by animals in a natural environment where there is, for example, often minimal reflected light. It might be that amphibians held under a homogeneous UV exposure regime are more likely to develop bilateral limb malformations than animals exposed to a more heterogeneous dose of UV radiation in the field.

An important observation from the current study was the ability to discern UV-induced limb defects in premetamorphic (e.g., stage 31−37) animals, indicating that it should be feasible to assess populations in the field for these types of defects well in advance of complete metamorphosis. This could be of significant utility from a logistic perspective by extending the time period during which amphibians could be sampled to assess limb defects. Perhaps more importantly, sampling of earlier life stages might help to reduce bias in determination of true malformation rates. Specifically, if

animals are sampled after most of the population has undergone metamorphosis and (as is true for many ranids) dispersed into terrestrial habitats, the malformation rate could appear artificially high in that only relatively immobile (malformed) animals are collected. Conversely, if the malformations result in postmetamorphic animals being more susceptible to predation than nonaffected animals, estimates of population malformation rates could be low.

In summary, increases in the dose of UV radiation to aquatic systems, coupled with studies demonstrating that UV exposures can adversely affect survival and development, highlight the importance of more thoroughly assessing the risk of this stressor to amphibians. To achieve this, our laboratory has been pursuing research to generate the necessary high-quality dose−response data and link this to measurements or predictions of UV exposure of amphibians in the field. Tietge et al. (*20*) describe comparative dose−response relationships for embryonic and larval mortality in three ranid species exposed to natural sunlight. In this paper, we report dose−response relationships for limb malformations in *R. pipiens* exposed to differing sunlight regimes. In the following paper, Peterson et al. (*37*) focus on definition of the UV dose experienced by amphibians in natural habitats as a function of attenuation associated with water quality characteristics, and the final manuscript in this series (*19*) consolidates the biological effects and exposure data into a probabilistic assessment of the risk of UV radiation to survival and development of amphibians.

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