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EFFECTS OF ULTRAVIOLET LIGHT AND METHOPRENE ON SURVIVAL AND DEVELOPMENT OF *RANA PIPIENS*

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Abstract—Recently a suite of relatively specific hindlimb deformities have been observed in several anuran species in North America. These deformities include ectopic and supernumerary limbs and missing limbs, limb segments, or digits. The objective of this study was to assess two stressors hypothesized as responsible for limb malformations in amphibians: methoprene, an insect growth regulator that, through interaction with the retinoic acid signaling system, could possibly cause limb deformities, and ultraviolet (UV) light. Northern leopard frogs (*Rana pipiens*) were exposed to several different concentrations of methoprene both in the absence and presence of UV light designed to mimic the UV wavelength spectrum present in sunlight. Exposures were initiated at early embryonic stages (newly fertilized eggs) and continued through emergence of the forelimbs of the frogs. At the highest methoprene concentration tested, both in the absence and presence of UV light, severe developmental effects were observed, with all organisms dying within 12 to 16 d of test initiation. However, exposure to the pesticide did not cause limb malformations. Irrespective of methoprene treatment, a very high percentage (~50%) of animals held under the UV light for 24 d developed hindlimb malformations. These malformations usually were bilateral and sometimes completely symmetrical, and consisted of missing limb segments and missing or reduced digits. A complete proximal to distal representation of the deficiencies occurred, ranging from missing or malformed femurs to the absence of single digits or digit segments. The developmental period of greatest sensitivity to UV light occurred during very early limb bud development, corresponding with formation of the apical ectodermal ridge. The significance of these findings in terms of deformed frogs in the field is uncertain. Although the deformity types observed (i.e., missing limb segments and digits) were similar to those seen in some field specimens, the UV light treatment did not cause the full range of malformations observed in animals from the field (e.g., supernumerary limbs, nonbilateral deformities). Furthermore, although the artificial light spectrum utilized mimicked the relative UV spectrum present in sunlight, it did not match full sunlight intensity, and did not accurately mimic visible wavelengths. Finally, the relationship of the UV light dose used in the laboratory to that actually experienced by amphibians in the field is uncertain. Despite these questions, our findings suggest that UV light should be further considered as a plausible factor contributing to amphibian malformations in field settings.

Keywords—Amphibian Limb Malformations Methoprene Ultraviolet light

INTRODUCTION

Recent reports of increases in the occurrence of severely deformed frogs and toads representing several species, including the northern leopard frog (*Rana pipiens*), have received a significant amount of attention both from scientists and the lay community [1]. Observed deformities include missing limbs and digits (ectromelia, ectrodactyly), supernumerary limbs and digits (polymelia, polydactyly), ectopic limb growth, and eye and central nervous system malformations. A great majority of the malformations have been in the hindlimbs. Confirmed and anecdotal reports of these types of deformities have been relatively widespread in North America, including several states within the United States and the province of Quebec in Canada, at sites ranging from agriculturally impacted wetlands to state forests [1,2; J. Tietge, www.im.nbs.gov/naamp3/papers/59df.html]. Malformations in field-collected amphibians, in particular the presence of supernumerary digits and limbs, are not a new phenomenon, with episodic, localized outbreaks of polymelia and polydactyly reported worldwide [3–9]. Although the occurrence of limb malformations in amphibians from the field has been noted previously, the seemingly broad prevalence of these recent observations has intensified concern.

Chemical contaminants have received significant attention as a possible causative agent of amphibian limb malformations [2], in part because a plausible mechanistic argument can be made for xenobiotic-induced malformities of the type observed [10; J. Tietge, www.im.nbs.gov/naamp3/papers/59df.html]. Specifically, administration of exogenous retinoic acid (RA) can induce limb defects that are similar to some of the malformations observed in frogs from the field in a variety of vertebrate models, including amphibians. Retinoic acid, a derivative of vitamin A, is metabolized endogenously to several retinoid isoforms that exert their influence through one or more receptors that are members of the nuclear hormone receptor superfamily [11]. The RA system controls processes related to cellular differentiation, pattern development, and the establishment of embryonic polarity [12,13]. Administration of excess RA at specific developmental stages can cause defects in brain, central nervous system, and craniofacial structures, as well as abnormal limb pattern development [14–17]. Hence, it is reasonable to hypothesize that there may be chemical contaminants that act as agonists of the retinoid receptor(s).

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In fact, a recent report by Harmon et al. [18] suggests that a metabolite of the widely used insecticide methoprene, an insect growth regulator, binds to a least one of the retinoid receptors and activates gene transcription in vitro.

Other environmental stressors also have been proposed as possibly responsible for the observed amphibian malformations (J. Tietge, www.im.nbs.gov/naamp3/papers/59df.html). For example, trematode cysts are suspected to cause supernumerary limbs in frogs and salamanders through physical disruption of the developing limb bud field [9], so biological agents are a plausible explanation for at least some of the observed deformities. Several lines of circumstantial evidence also compel consideration of ultraviolet (UV) light as a possible factor contributing to the abnormalities (G. Ankley, www.im.nbs.gov/naamp3/papers/deformuv.html). First, concomitant with seeming temporal increases in the incidence of malformations, recent increases have been documented in the intensity of the UVB component of natural sunlight at various locations around the world [19,20]. Moreover, some of the largest relative increases in UVB light have been shown to occur in late spring and early summer [21], a period that coincides with reproduction and critical windows of development of many amphibian species in northern latitudes. Finally, regional and global changes in UV light offer plausible explanation from the perspective of the seemingly widespread and noncontiguous nature of locations where malformed animals have been observed.

The purpose of the present study was to investigate the effects of two potential environmental stressors on survival and development (in particular, limb development) of *R. pipiens.* Partial life-cycle experiments were conducted in which animals were exposed to methoprene both in the absence and presence of UV light. This particular design was utilized, in part, because of recent reports that methoprene can be photomodified by UV light to structures more teratogenic than the parent molecule [22].

MATERIALS AND METHODS

Design overview

The general design of the study consisted of exposure of early *R. pipiens* embryos to five concentrations of methoprene, plus a clean water control, with and without supplemental UV/visible light. To assess the possibility of differential sensitivity at various developmental stages, subsets of organisms from the primary test system were removed after 6, 15, and 24 d of UV light and/or methoprene exposure, and placed in a clean water grow-out system with no supplemental UV light. Animals were sampled upon emergence of their forelimbs, assessed for developmental abnormalities, and preserved. The test was terminated after 113 d.

Specific experimental procedures

One male and two female adult *R. pipiens* were collected from an undeveloped stretch of shoreline on a recreational lake approximately 45 km west of Duluth, Minnesota, USA, in early May 1997. The frogs were held in the laboratory at 20°C under a 16:8 h light:dark photoperiod for about 48 h, at which time a pair was observed in amplexus. The resultant egg mass (~5,000 eggs) was treated for 3 min with a buffered (pH 8.1) 2% cysteine solution to remove the gelatinous coat, and groups of 100 eggs were randomly assigned, in groups of five, to 1 of 12 glass crystalizing dishes containing 800 ml of the test solutions described below. Exposures were initiated within 2 to 3 h of fertilization; at this time most of the eggs were in the two to four cell stages.

Water used for the assay was from Lake Superior, and had been treated with UV light and filtered to remove pathogens and sediment prior to use. Water quality characteristics were monitored routinely during all phases of the test, using standard methods [23]. With the exception of dissolved oxygen (DO), water quality varied little over the course of the experiment or between the different test systems (see below for a physical description of the systems). Mean ± SD water quality values included: hardness, 50.6 ± 5.3 mg/L as $CaCO_3$; alkalinity, 45.7 ± 5.8 mg/L as $CaCO_3$; and conductivity, 71 ± 7 μS/cm. The mean (range) pH was 7.73 (7.35–8.15). Mean (±SD) DO concentrations in the continuous exposure tanks were slightly lower than those in the grow-out tanks, 5.9 ± 1.3 versus 8.4 ± 0.4 mg/L, respectively, but remained within acceptable limits in both systems. The tests were maintained at a water temperature of 20 ± 1°C through use of an external water bath. After hatch, the animals were fed a mixture of Tetrafin® (Tetra Sales, Blacksbury, VA, USA), Silver Cup Trout Starter® (Nelson & Sons, Murray, VT, USA), and *Spirulina* algae (Aquatrol, Anaheim, CA, USA) up to three times daily (twice on weekends); when the frogs were approximately 10 d old, this ration was supplemented with <24-h-old brine shrimp (Bio-Marine, Hawthorne, CA, USA). Feeding was essentially ad libitum, but to ensure maintenance of adequate water quality, care was taken to supply no more than the animals would consume.

Stock solutions of methoprene were generated in liquid–liquid saturators containing about 9 L of Lake Superior water, and 2 to 3 ml of methoprene (98% purity, Chem Service, Westchester, PA, USA) that were allowed to equilibrate on a stir plate for 48 h in the dark. Under these conditions, the steady state methoprene concentration in the saturator was about 500 μg/L. Through dilution with appropriate volumes of Lake Superior water, five nominal (target) concentrations of methoprene were generated: 1.95, 7.8, 31.3, 125, and 500 μg/L. Methoprene test solutions, as well as the clean water controls, were placed in duplicate crystalizing dishes prior to introduction of the *R. pipiens* eggs. After addition of the embryos, one replicate set of treatments was held under normal laboratory fluorescent light, whereas the other set received supplemental lighting that included UV light. The supplemental lighting consisted of additional fluorescent light from Vitalight® bulbs (Duro-Test, Fairfield, NJ, USA) to produce more intense visible wavelengths than afforded by the normal laboratory regime, and UV light provided by UVA-340 lamps (The Q-Panel Company, Westlake, OH, USA), designed to simulate the relative irradiance of sunlight at wavelengths ranging from 295 to 370 nm [24]. The background laboratory lighting was supplied by fluorescent F40CW Cool White® bulbs (General Electric, Blue Oak, OH, USA). The light regime for all test chambers was 16:8 h light:dark; the supplemental UV/visible light was provided for 12 h during the 16-h daily light period.

To maintain acceptable water quality, it was necessary to modify our dosing/test system at several points during the test. Initially, the 800-ml test solutions in the crystalizing dishes were renewed every 48 h. On test day 8, the volume of the renewal solution was increased from 800 to 1,600 ml, and on day 24 we began to employ daily renewals. As biomass in the test system increased, it was necessary on test day 30 to switch

to a flow-through system, using an electronic dilutor. During this phase of the test, the organisms were held in 5-L glass tanks containing 4 L of test solution, with 2 tank volume turnovers/d through day 36, when flow was increased to provide 5 turnovers/d until completion of the assay. Methoprene solutions for the flow-through system also were generated using the liquid–liquid saturators, with four nominal test concentrations of 0.78, 3.12, 12.5, and 50 μg/L (the fifth concentration had been eliminated because of complete mortality at the highest methoprene level earlier in the test; see Results). These target concentrations were chosen as the approximate median of those likely experienced by the organisms during the static-renewal phase of the test, where almost a total loss of the parent methoprene occurred between the 48-h renewal periods (see Results).

One of the objectives of the test was to ascertain if specific life stages were exceptionally sensitive to possible developmental effects of methoprene and/or UV light. Thus, tadpoles were removed from each exposure chamber at various times during the assay and placed for the remainder of their development in a clean water system with no supplemental UV light. Twenty-five and 20 animals, respectively, were moved from each treatment on test days 6 and 15. On day 24, all but 25 animals were removed from the exposure system. Animals removed from the exposure system were held in 8-L glass tanks containing 6 L of Lake Superior water, which was continuously supplied to the system at a rate of 182 ml/min. The remaining 25 animals were held under the various treatment regimes until forelimb emergence or test termination at 113 d.

Individual frogs were sampled from the different treatment and holding tanks upon complete emergence of their forelimbs, typically at stages 42 to 44 [25], which commenced on test day 66 and continued relatively steadily through termination of the assay. They were killed by immersion in a 1.5 g/L solution of MS-222 (Argent Chemical Laboratories, Redmond, WA, USA), weighed, examined for gross external abnormalities, and preserved in 95% ethanol. Any specimens exhibiting gross deformities were imaged and archived using Image Pro Plus® (Media Cybernetics, Silver Spring, MD, USA). A subset of these were further examined via skeletal preparations following the bone–cartilage double-stain techniques described by Miller and Tarpley [26].

On test day 113, the experiment was terminated; at this point the remaining animals (77 across all treatments) were at various stages of metamorphosis prior to forelimb emergence. Those that were advanced enough to discern complete hindlimb development (~66%) were included in cumulative estimates of deformity rates. Animals at earlier developmental stages were used only for calculation of survival rates.

UV light measurements and estimation of dose

Broadband intensities of UVB (280–320 nm) and UVA (320–400 nm) at the air–water interface were measured routinely over the course of the test, using an IL1700 radiometer (International Light, Newburyport, MA, USA). Full-spectrum irradiance in the exposure system was characterized at test completion using a SD2000 photodiode array spectrometer (Ocean Optics, Dunedin, FL, USA).

To evaluate the experimental light doses relative to natural light levels, estimates of ambient UVB, UVA, and visible light were made using the FORTRAN radiative transfer computer code, SBDART (Santa Barbara DISORT Atmospheric Radiative Transfer [27]). These spectral irradiance values were modeled for April 23, May 3, May 12, May 22, and May 31, as representative of a typical *R. pipiens* breeding season in northern Minnesota. Total UVB, UVA, and visible doses were calculated from these estimates by integrating over appropriate wavelengths. These values then were averaged among the modeled breeding days to determine the mean estimated daily dose of UVB, UVA, and visible light at the surface of the water in the vicinity of Duluth. Because no effort was made during modeling to account for cloud cover, these values represent theoretical maxima. Therefore, coarse estimates of cloud cover were made using National Renewable Energy Laboratory (NREL) measurements of total sunlight from 1961, 1965, 1970, 1975, 1980, 1985, and 1990 in Duluth for the dates of April 23 through May 31. These data comprise hourly measurements of sunlight irradiance (300–3,000 nm). To characterize typical cloud cover in Duluth these data were sorted so that the highest values for each hourly measurement, among the years examined, could be used as an estimate of no-cloud irradiance levels. The sorted low values were then assumed to represent maximal cloudy conditions. The goal was to utilize the proportion of maximal sunlight present on the days of lowest irradiance intensity as an estimate of the proportionate effect of cloud cover, and to use the mean of all years as an estimate of typical cloud cover effects. It must be stressed that these calculations should be regarded only as estimates of the UV dose that might occur, in that they do not consider aspects of light attenuation in the aquatic environment, nor do they incorporate aspects of animal behavior that might limit exposure to UV light.

Methoprene measurements

Methoprene water concentrations were measured routinely over the course of the bioassay. Aliquots (50–100 ml) of saturator or test vessel water were extracted with hexane (2–5 ml) for 1 h, and a portion of the hexane layer removed and placed in an amber vial at 4°C. Methoprene in this fraction was analyzed with a Hewlett-Packard gas chromatograph–mass spectrometer (GC-MS). One-microliter injections were made at 225°C onto a 30-m DB-5 column, which was held at 40°C for 1 min and ramped linearly at 10°C/min to 275°C, where it was held for an additional 10 min. The GC-MS transfer line temperature was 280°C. Mass spectral data were acquired in the single ion monitoring mode, and the calibration and quantitation of methoprene was made using the base ion (73 *m*/*z*) and a quadratic curve fit, with an external quantitation method. Duplicate analyses, analyte spike recoveries, and procedural blanks were performed with each sample set. In addition to methoprene (GC retention time, 21 min), a compound with a retention time of 11 min was routinely monitored in the sample extracts. The peak area associated with the compound increased coincident with temporal decreases in methoprene concentrations in the saturator and/or water samples from the test tanks. The electron impact spectra of this additional peak exhibited a type of fragmentation (a tertiary methyl ether moiety dominated the mass spectra) where relatively little structural information could be obtained by examining the spectra. The results of a chemical ionization–mass spectral analysis using isobutane revealed a molecular ion consistent with 7-methoxy-3,7-dimethyl octanol. This compound has been previously identified as a degradation product in aqueous solutions of methoprene [28]. However, because of a lack of

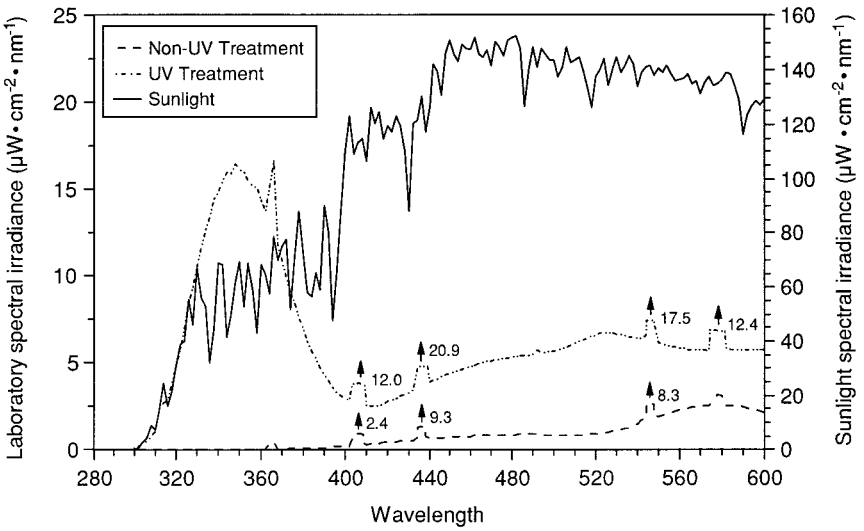

Fig. 1. Measured laboratory spectral irradiance and the modeled sunlight spectrum representing average midday intensity during *Rana pipiens* breeding season (April 23–May 31). Intensity spikes due to elemental emissions associated with laboratory fluorescent light have been truncated, and associated peak heights from the original spectrum are indicated. Note scaling difference on the *y* axes.

a suitable quantitation standard, the concentration of this compound in the extracts could not be accurately determined.

Statistical analyses

This experimental design ideally would be analyzed using two-way analysis of variance (ANOVA) with appropriate interaction terms; however, resource limitations precluded replication of discrete treatments. Because of this lack of replication, comparisons among treatments were made using one-way ANOVA or Student's *t* test. Arcsine square-root transformations were applied to percent data prior to analyses. Mean separations after one-way ANOVA were determined by Tukey's multiple comparisons test. All statistical analyses were performed using SYSTAT 7.0 for Windows® (SPSS, Chicago, IL, USA). Statistical significance was defined as $p < 0.05$.

RESULTS

UV exposure

Figure 1 indicates the wavelength spectra and associated relative intensities to which the developing *R. pipiens* were exposed, as well as the spectrum generated using the SBDART model, for full sunlight in the vicinity of Duluth (latitude 47°N). The UV spectrum from approximately 300 to 360 nm in the test system receiving supplemental UV and visible light, although less intense than sunlight, mimicked the relative distribution of wavelengths present in sunlight reasonably well. However, the higher (>360 nm) UVA wavelengths, as well as the visible portion of the sunlight spectrum, were not reflective of sunlight. Mean UVB, UVA, and visible (400–600 nm) light intensities in the test system receiving supplemental light were 44, 707, and 583 μW/cm², respectively. No UVB was detectable under the background laboratory flourescent light, and the mean UVA and visible light intensities were about 3 and 143 μW/cm², respectively.

Although the majority of UV light measurements were at the air–water interface of the tanks, in an attempt to better define actual exposure of the animals, we also performed a subset of measurements at a mid-water depth in the test tanks (~3 cm). The intensity of UV light associated with these measurements was about 76% of that measured at the water surface, probably due to attenuation of the UV light by dissolved organic carbon present in the Lake Superior water or emanating from the food used for the frogs.

When used in conjunction with one another, the NREL monitoring information and spectral data derived from the SBDART model facilitate putting the laboratory intensity data in a relative context of what might occur in the environment. Specifically, if estimates of cloud cover based on measurements in the range of 300 to 3,000 nm are assumed to correspond proportionally to effects in the region of 280 to 700 nm, then the NREL data can be used to derive estimates of light attenuation associated with weather patterns. Based on 7 years of data during the period of April 23 to May 31, if every day were cloudy, only about 54% of incident solar radiation would penetrate to the surface of the earth. Of course, assuming that all days would be cloudy is not realistic, so in generating comparative estimates of dose, we assumed that average relative light intensities could be represented by the mean of 54 and 100% (maximal light intensity). Based on this, and the SBDART model, we computed that the average light doses for 5 d during April 23 to May 31 at the air–water interface would be 10.2, 235, and 1,788 Wh/m² for UVB, UVA, and visible light, respectively. When these doses are divided by the average day length for the breeding period (14.8 h) and converted to μW/cm², the corresponding fluxes are 50, 1,157, and 8,816 μW/cm², respectively. Therefore, laboratory intensities represent 88, 61, and 10%, respectively, of calculated UVB, UVA, and visible light fluxes in northern Minnesota. It must be stressed, however, that these are only crude estimates of dose and do not reflect, for example, light attenuation in the aquatic environment. Because of this, quantitative extrapolation of dose–response estimates generated in these laboratory studies to the field is, at present, premature.

Methoprene exposure

Methoprene was routinely measured in the saturator water (which in the initial phase of the bioassay corresponded to the highest test concentration), and in the exposure tanks at periodic intervals during the test. Methoprene concentrations in saturator water were close to projected water solubility, and

Table 1. Stability of methoprene during static-renewal phase of the bioassay. Initial concentrations were calculated based upon methoprene measured in the saturator solution, in conjunction with dilutions prepared for the test tanks. Final concentrations indicate measured values in the test tanks prior to the 48-h renewal. Concentrations are in μg/L

Test day	Treatment[a]	Initial	Final	Loss (%)
5	0	ND[b]	ND	—
5	UV0	ND	ND	—
5	1	1.78	ND	—
5	UV1	1.78	ND	—
5	2	7.14	ND	—
5	UV2	7.14	ND	—
5	3	28.6	ND	—
5	UV3	28.6	2.00	93
5	4	115	5.20	95
5	UV4	115	5.50	95
5	5	458	10.9	98
5	UV5	458	20.1	96
14	0	ND	ND	—
14	UV0	ND	ND	—
14	1	1.90	0.125	93
14	UV1	1.90	ND	—
14	2	7.61	0.209	97
14	U2	7.61	ND	—
14	3	30.5	0.738	98
14	UV3	30.5	0.384	99
14	4	122	1.92	98
14	UV4	122	0.751	99
14	5	488	23.0	95
14	UV5	488	4.48	99

[a] Notation refers to treatments with or without supplemental ultraviolet (UV) light, varying from control (0) to the highest (5) methoprene test concentration.

[b] ND = not detectable. Detection limits for test days 5 and 14 were 1.9 and 0.12 μg/L, respectively. The lower detection limit on day 14 was achieved through concentration of larger volume water samples.

were reasonably consistent over the first 29 d of the test (the static-renewal phase); the mean ± SEM methoprene saturator concentration during this period was 538.2 ± 48.3 μg/L (n = 19). Once in the test system, however, the parent compound proved to be relatively unstable. For example, during the static-renewal phase of the experiment, on the two sampling dates for which relatively complete sets of analyses were performed (test days 6 and 14), methoprene concentrations declined by more than 95% by 48 h after introduction of the test solution (Table 1). Based on one set of 24-h analyses, degradation of the parent compound appeared to be relatively linear over the 48-h period (data not shown). The day 14 data suggest that UV light treatment may have enhanced degradation of the methoprene, but this trend was not seen in the day 6 data (Table 1).

A summary of the methoprene exposure information from the flow-through portion of the assay is presented in Table 2. In the flow-through system the organisms would theoretically be exposed to a much more consistent dose of the parent compound than in the static-renewal phase of the test. Concentrations of methoprene often were below method detection/quantitation limits at the lower two test concentrations; however, successful measurement of the pesticide was routinely achieved at the two highest concentrations. Concentrations of methoprene in the exposure tanks tended to decrease, in particular toward the end of the test, from close to the target concentrations to about one third of these values (Table 2). This was, in part, due to an increased flow of Lake Superior water through the liquid–liquid saturators, caused by the need to increase water turnover rates in the test tanks to maintain acceptable water quality. No consistent influence of UV light on methoprene concentrations was apparent during this phase of the test.

Biology

Viability of the *R. pipiens* eggs was high; inspection of newly hatched embryos on day 6 indicated 89 to 95% viability

Table 2. Methoprene water concentrations during the flow-through phase of the bioassay. Data in parentheses were not measured, but were predicted based upon measurements of methoprene in water from the highest test concentration in the treatment and appropriate correction for dilution. Concentrations are in μg/L

Test day	Treatment[a]									
	0	1	2	3	4	UV0	UV1	UV2	UV3	UV4
33	ND[b]	ND (0.59)	ND (2.34)	7.30	37.5	ND	ND (0.37)	ND (1.49)	6.60	23.9
41	ND	ND (1.00)	ND (4.03)	9.50	64.4	ND	ND (0.80)	ND (3.21)	8.60	51.3
48	ND	ND (0.32)	ND (1.29)	15.8	20.7	ND	ND (0.30)	ND (1.19)	15.7	19.0
55	ND	ND (0.45)	3.70	6.10	29.0	ND	ND (0.43)	3.70	6.10	27.8
62	ND	ND (0.36)	ND (1.44)	4.70	23.0	ND	ND (0.30)	ND (1.19)	3.80	19.0
69	ND	ND (0.30)	ND (1.19)	4.60	19.0	ND	ND (0.36)	ND (1.44)	5.90	23.0
76	ND	ND (0.38)	ND (1.54)	8.00	24.6	ND	ND (0.45)	ND (1.79)	8.50	28.6
83	ND	ND (0.23)	ND (0.91)	8.00	14.6	ND	ND (0.31)	ND (1.23)	8.20	19.7
90	ND	ND (0.25)	3.00	5.10	16.3	ND	ND (0.22)	2.90	4.00	14

[a] Notation refers to treatments with or without supplemental ultraviolet (UV) light, varying from control (0) to the highest (4) methoprene treatment concentrations.

[b] ND = not detectable. Detection limit was 1.9 μg/L.

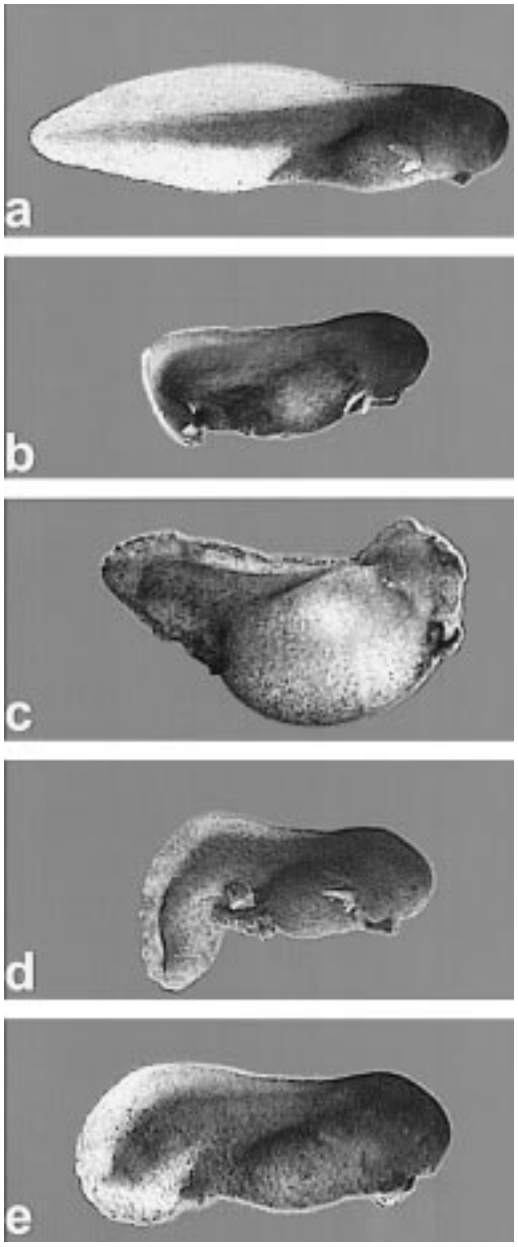

Fig. 2. Effects of methoprene on developing *Rana pipiens* at 6 d of development. Panel (**a**) depicts normal development in a larvae from the lowest methoprene concentration. Shown are typical effects of methoprene (**b, c, e**) and methoprene in the presence of ultraviolet light (**d**) on larval development. Methoprene especially affected caudal and rostra axial development.

across the various treatments. The developing embryos appeared normal, except at the highest methoprene concentration, both with and without UV light. All of the embryos from the high methoprene treatments were grossly deformed, exhibiting severe axial distortion, as well as craniofacial and caudal abnormalities (Fig. 2). These animals were relatively immobile, and did not exhibit feeding behavior. By test day 12, tadpoles from the highest methoprene treatment (in the absence of UV light) began to die at a high rate; by day 13 all organisms in this treatment were dead or completely moribund. Mortality of animals from the high methoprene treatment under UV light was delayed somewhat compared to the non-UV light treatment, but by test day 16 these animals also had all died. Similarly, the animals removed from the highest methoprene treatments on days 6 or 15, and placed in clean water, exhibited a similar pathology and all were dead or moribund by day 24 of the test, indicating that adverse effects caused by methoprene likely occurred during relatively early (i.e., <6 d) development.

Table 3. Percent survival of *Rana pipiens* at various exposure durations. Animals from the intermediate intervals (6, 15, and 24 d) were transferred to clean water following exposures and held until forelimb emergence or test termination at 113 d. Numbers in parentheses represent percent survival during this postexposure period

Methoprene treatment[a]	Exposure duration (d)			
	6[b]	15[c]	24[d]	113[e]
0	94 (100)	94 (100)	94 (94)	41[f]
1	95 (92)	95 (100)	95 (94)	83
2	93 (96)	93 (100)	93 (100)	81
3	92 (100)	92 (95)	92 (100)	82
4	94 (100)	94 (95)	94 (100)	90
5	90 (0)	0 —	0 —	0
UV0	93 (91)	93 (95)	93 (100)	87
UV1	94 (91)	94 (100)	94 (94)	94
UV2	88 (91)	88 (95)	88 (100)	84
UV3	91 (100)	91 (100)	91 (100)	91
UV4	90 (95)	90 (88)	90 (100)	90
UV5	90 (0)	41 (0)	0 —	0

[a] Notation refers to treatments with or without supplemental ultraviolet (UV) light, varying from control (0) to the highest (5) methoprene test concentration.
[b] Transferred 25 organisms/treatment.
[c] Transferred 20 organisms/treatment.
[d] Transferred 8 to 17 organisms/treatment.
[e] Initial sample size of 92 to 100 organisms; final value adjusted for routine subsampling.
[f] Unexplained mortality occurred in this treatment on test days 26 through 28, with only four organisms surviving until test completion.

With one exception, survival of *R. pipiens* from the other treatments over the course of the test was high, and was statistically comparable (Table 3). On test days 26 through 28, 72% of the remaining animals (i.e., those not sampled on days 6, 15, and 24) in the control (no methoprene) tank without supplemental UV and visible light died, in an event that seemed associated with either disease or inadvertent contamination of the test system. Cumulative survival of the animals (based on the initial 100 per treatment) ranged from 81 to 94% across the other treatments, and was not related to the methoprene or UV light treatments (Table 3). Similarly, survival of the frogs moved from the exposure system and placed in the grow-out tanks was high (Table 3).

The median time to emergence of the forelimbs of animals exposed to UV light for the entire test was 90 d versus 94 d in animals not exposed to the supplemental light; however, this difference was not statistically significant (Fig. 3). But, perhaps as a consequence of this, UV-exposed animals weighed slightly, but significantly, less at emergence of their forelimbs than the non-UV-treated animals (Table 4). Animals

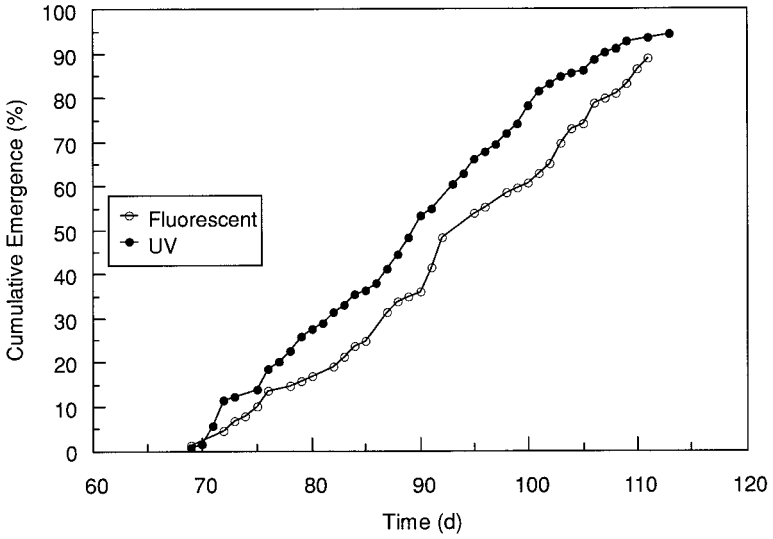

Fig. 3. Cumulative emergence over time of forelimbs in *Rana pipiens* exposed to ultraviolet (UV) light versus those that were not exposed to UV light.

from the non-UV-treated control tank were much larger than other organisms in the test. However, this likely was due to density-dependent factors, as only four organisms survived the unexplained mortality that occurred on test days 26 to 28 in this treatment. No statistically significant effects were found related to methoprene or UV light treatment on developmental rate or weight of animals held in the clean water grow-out tanks (Table 4), or lengths of any of the animals from the test (data not shown).

Table 4. Mean (±SD) weight (g) of *Rana pipiens* at the time of forelimb emergence. Number of animals sampled is given in parentheses

Methoprene treatment[a]	Exposure duration (d)			
	6	15	24	113[b]
0	2.3 ± 0.6 (19)	2.5 ± 0.5 (20)	3.1 ± 0.9 (15)	4.0 ± 1.6 (4)
1	2.4 ± 0.5 (18)	2.7 ± 0.8 (17)	3.2 ± 1.0 (15)	2.5 ± 0.5 (19)
2	2.7 ± 0.7 (16)	3.1 ± 1.1 (15)	4.1 ± 1.7 (10)	2.3 ± 0.5 (20)
3	2.5 ± 0.5 (18)	2.7 ± 0.6 (17)	3.3 ± 0.8 (13)	2.4 ± 0.7 (20)
4	2.3 ± 0.6 (18)	2.7 ± 0.5 (17)	3.2 ± 0.9 (15)	2.3 ± 0.7 (21)
5	—	—	—	—
UV0	2.5 ± 0.8 (19)	2.7 ± 0.8 (18)	2.9 ± 0.7 (15)	2.0 ± 0.5 (23)
UV1	2.7 ± 0.6 (15)	2.5 ± 0.8 (20)	3.2 ± 0.9 (13)	2.1 ± 0.6 (22)
UV2	2.8 ± 1.0 (18)	2.9 ± 1.0 (16)	4.1 ± 1.8 (8)	2.0 ± 0.6 (23)
UV3	2.5 ± 0.7 (16)	2.4 ± 0.4 (17)	3.6 ± 1.0 (12)	2.1 ± 0.6 (22)
UV4	2.6 ± 0.9 (19)	2.8 ± 0.5 (15)	3.8 ± 1.4 (12)	2.0 ± 0.5 (23)
UV5	—	—	—	—

[a] Notation refers to treatments with or without supplemental ultraviolet (UV) light, varying from control (0) to the highest (5) methoprene test concentration.

[b] Ultraviolet treatments significantly different from non-UV treatments (treatment 0 was excluded from the analyses because of small sample size).

When examining the animals, we focused primarily upon three different types of gross malformations: optic or craniofacial abnormalities, lordosis or scoliosis, and limb or digit deformities. Gross optic or craniofacial abnormalities were not observed. Some degree of scoliosis occurred in several of the developing frogs; this ranged from slight tail flexure to clearly bent spines, with the majority of the observations in the former category. The overall incidence of scoliosis in the test organisms was about 10%; no relationship was apparent in the frequency of occurrence or severity of this condition to either the methoprene or UV light treatments (data not shown).

The most notable malformations observed were in the hindlimbs of organisms from the latter phases of the UV light exposure. In the animals not exposed to UV light, a small incidence of hindlimb abnormalities occurred; however, these malformations consisted solely of missing digit segments. Conversely, in animals held for 24 d under UV light, limb and digit deficiencies were significantly increased relative to animals not exposed to the UV light, irrespective of methoprene treatment. The percent occurrence of malformations in these animals differed significantly from that in organisms not exposed to UV light. Figure 4 indicates the across-treatment incidence of all hindlimb malformations, and also delineates the degree to which the deficiencies were bilateral and/or symmetrical. For the purpose of this categorization, animals that had the same bones on both legs affected in the same fashion (albeit, perhaps, not to the same degree) were assigned to the symmetrical category, whereas those with dissimilar pathologies of the two limbs were categorized as bilateral.

To summarize and aid in interpretation of the data, severity scores were developed for the affected animals. We assigned a numeric value of 5 to two animals possessing the most severe malformations observed, symmetric femur deficiencies. Animals with complete femurs but symmetric tibiafibula deficiencies were assigned a value of 4 (Fig. 5 1a and 5 1b), whereas those that possessed the tibiafibula but not the tibiale and fibulare were given a severity score of 3 (Fig. 5 2a and 5 2b). Animals missing all digits (metatarsals, phalanges) were assigned a score of 2. A wide variety of less severe digit malformations were observed, ranging from a symmetrical ab-

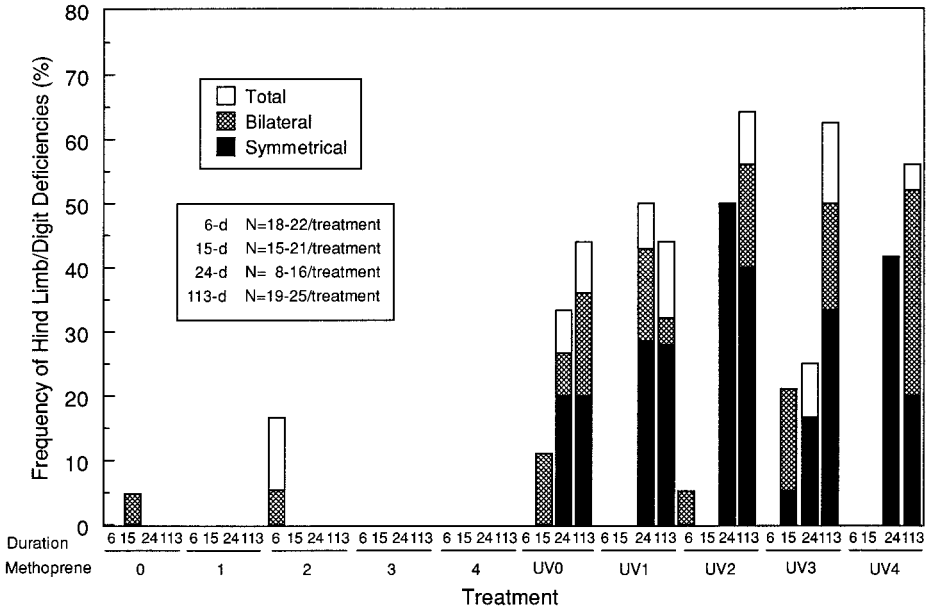

Fig. 4. Frequence of occurrence of hindlimb deformities in *Rana pipiens* exposed for various periods of time to methoprene and ultraviolet (UV) light. Treatments 1 through 4 are increasing methoprene concentrations (0 is the control) in the absence or presence of supplemental UV light. Within-treatment labels of 6, 15, 24, or 113 indicate the period of time (d) over which animals were exposed to methoprene and UV light. This figure also depicts the degree to which observed deformities were symmetrical or bilateral (see text).

sence of one or more digits (Fig. 5 3a and 5 3b) to a variety of combinations of missing or reduced digits (Fig. 5 4a and 5 4b). All animals with affected digits were assigned a severity score of 1. It is worthy of note that the various digit malformations comprised the majority of those classified as bilateral, but not symmetrical, in Figure 4. In general, the more severe the malformation the more likely it was to be symmetrical.

Figure 6 depicts the relative occurrence of the different malformation types in all animals exposed to UV light. We combined the UV data in this fashion after confirming that no statistically significant relationship existed between deformity rate and type and methoprene treatment. The onset of hindlimb abnormalities associated with UV light exposure was clearly stage-specific. Overall mean percentages of hindlimb malformations in animals held under UV light increased significantly with increasing exposure duration; specifically, animals removed from the UV light and placed in clean water on days 6 and 15 displayed little or no incidence of the pathology (Figs. 4 and 6). However, frogs removed on test day 24, as well as those held under UV light for the entire test, exhibited a high degree of the bilateral/symmetrical hindlimb segment and digit malformations (Figs. 4 and 6). Notably, the most severe deficits (categories 2–5) were completely lacking in animals removed on days 6 or 15 (Fig. 6). Of potential significance, on day 15, the majority of animals sampled were at stages 23 to 25, prior to formation of visible limb buds, whereas on day 24, most of the organisms transferred to the grow-out tanks were at stages 26 and 27, which corresponds to the very early stages of limb bud formation [24]. The total incidence of hindlimb malformations in animals removed after 24 d under UV light, compared to those exposed for the entire test, was 39 versus 54%; this difference was not statistically significant.

DISCUSSION

Effects of ultraviolet light

Our study indicates that treatment of *R. pipiens* with UV light can induce bilateral, often symmetrical hindlimb ectromelia and ectrodactyly. The observed deficit malformations were manifest in a subset of animals at each segment (proximal to distal) of the hindlimbs. The effect of UV radiation on the hindlimbs was very stage-specific, with the frogs exposed to UV light before 2 to 3 wk of age (~stage 23–25) not exhibiting a significant degree of the pathology. Our observations are relatively novel, but the full significance of these data in terms of deformities in wild frogs is uncertain. For example, although there have been observations of bilateral hindlimb deficiencies, most field-collected frogs have not displayed symmetrical dysmorphology of the hindlimbs [2; J. Helgen, personal communication]. Also, although the supplemental light used in our laboratory studies delivered realistic intensities over most of the portion of the UV spectrum, the visible spectrum that we used did not mimic sunlight either qualitatively or quantitatively. This could limit the ecological significance of our results if, for example, repair of damage caused by the UV light required a significant amount of visible light (e.g., associated with wavelengths between 380 and 450 nm) to activate latent repair pathways, such as photolyase-mediated DNA repair [29]. The ecological relevance of these results also is highly dependent upon exposure of amphibians to UV light in the field, which can be affected not only by site-specific variations in attenuation of UV light [30], but by biology and behavior of the organisms in the environment (e.g., negative phototaxis [31]). Given these uncertainties, at present, quantitative extrapolation of the UV dose–response relationship generated in those laboratory studies to field situations cannot be done reliably. Despite this, we feel that our data provide basis for further consideration of UV light as a contributing factor to the amphibian limb deformities.

Previous investigations have been conducted concerning the effects of UV light on survival and development of frogs. For example, treatment of early embryos with high-intensity, short-wavelength UV light (e.g., 254 nm) is well established to induce a host of relatively specific axial abnormalities useful for elucidation of developmental processes [32], but these

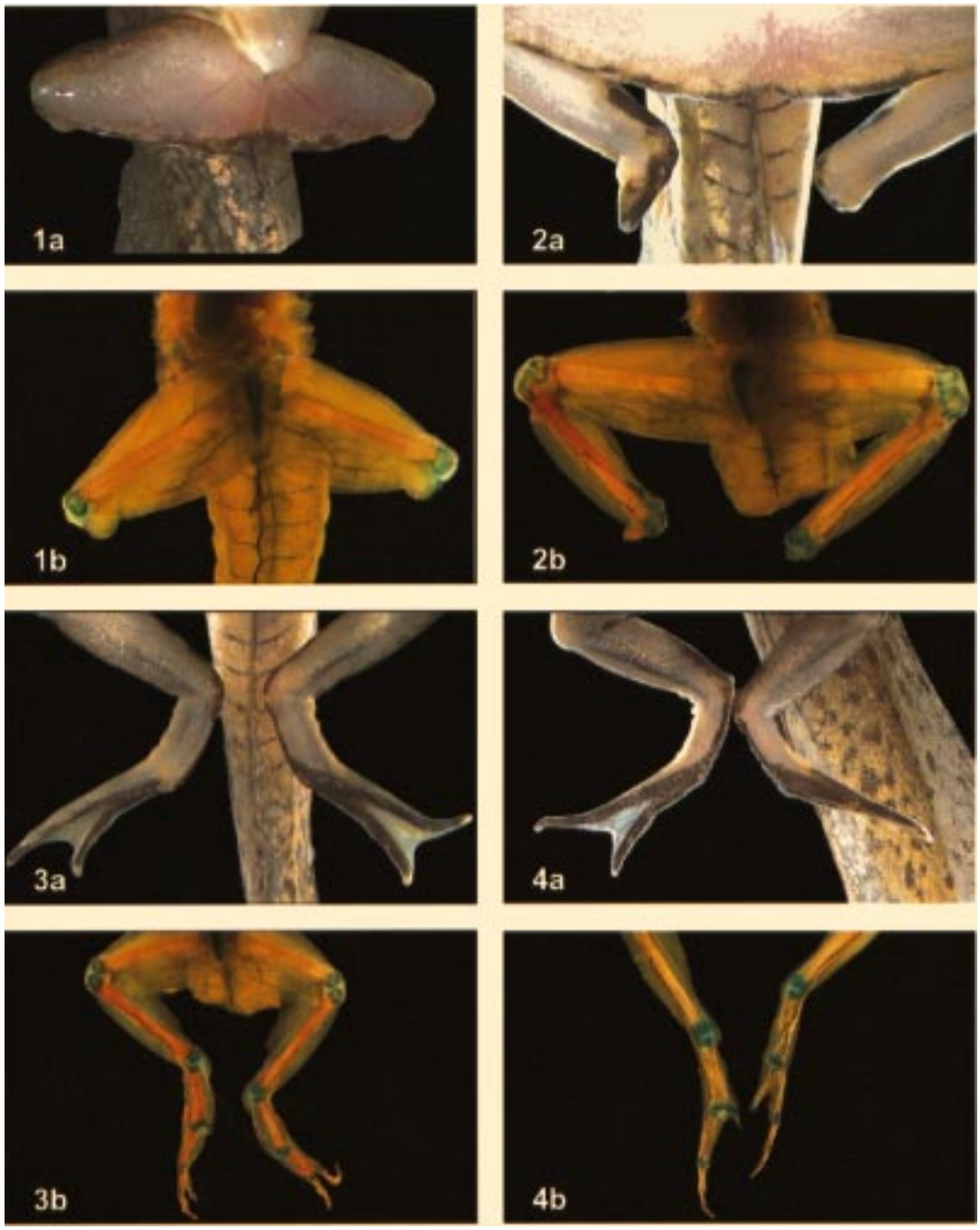

Fig. 5. Effects of ultraviolet light on developing hindlimbs of *Rana pipiens* showing typical developmental deficiencies. Images of live frogs (**a**) and corresponding skeletal preparations (**b**) demonstrate truncation of proximal to distal development at tibia/fibula (1), at tibiale/fibulare (2), and at the phalanges (3 and 4). The developmental deficiencies were symmetric (1–3) and bilateral (4). In the skeletal preparations, red is bone and blue is cartilage.

wavelengths and types of exposures are not particularly relevant from an environmental perspective. A few studies with more environmentally realistic UV intensities and wavelengths have been conducted both in the laboratory and field, but directly comparing our results to data from those studies is difficult [33–40]. For example, some of them focused almost exclusively upon endpoints related to survival or only very early development [35–37,39]. And, even in those instances where aspects of longer-term development were considered, differences in the timing and type (quality, intensity) of UV exposure and the developmental stages where effects were observed (as well as potential variations in among-species sensitivity) complicate comparisons among studies. Certain developmental abnormalities have been reported in UV-exposed animals, such as lordosis, epidermal hyperplasia, and corneal deformities, but not limb malformations [33,34,38]. However, Grant and Licht [38] used only very brief, high-intensity UV exposures (minutes to hours), and the studies by Worrest and Kimeldorf [33,34] likely were terminated too early (stages 30–35) to allow complete assessment of hindlimb abnormalities, in particular, abnormal digits. Hays et al. [40] described a study with three frog species held for relatively long periods during metamorphosis under different UV light regimes; they reported occurrence of several of the developmental abnormalities not-

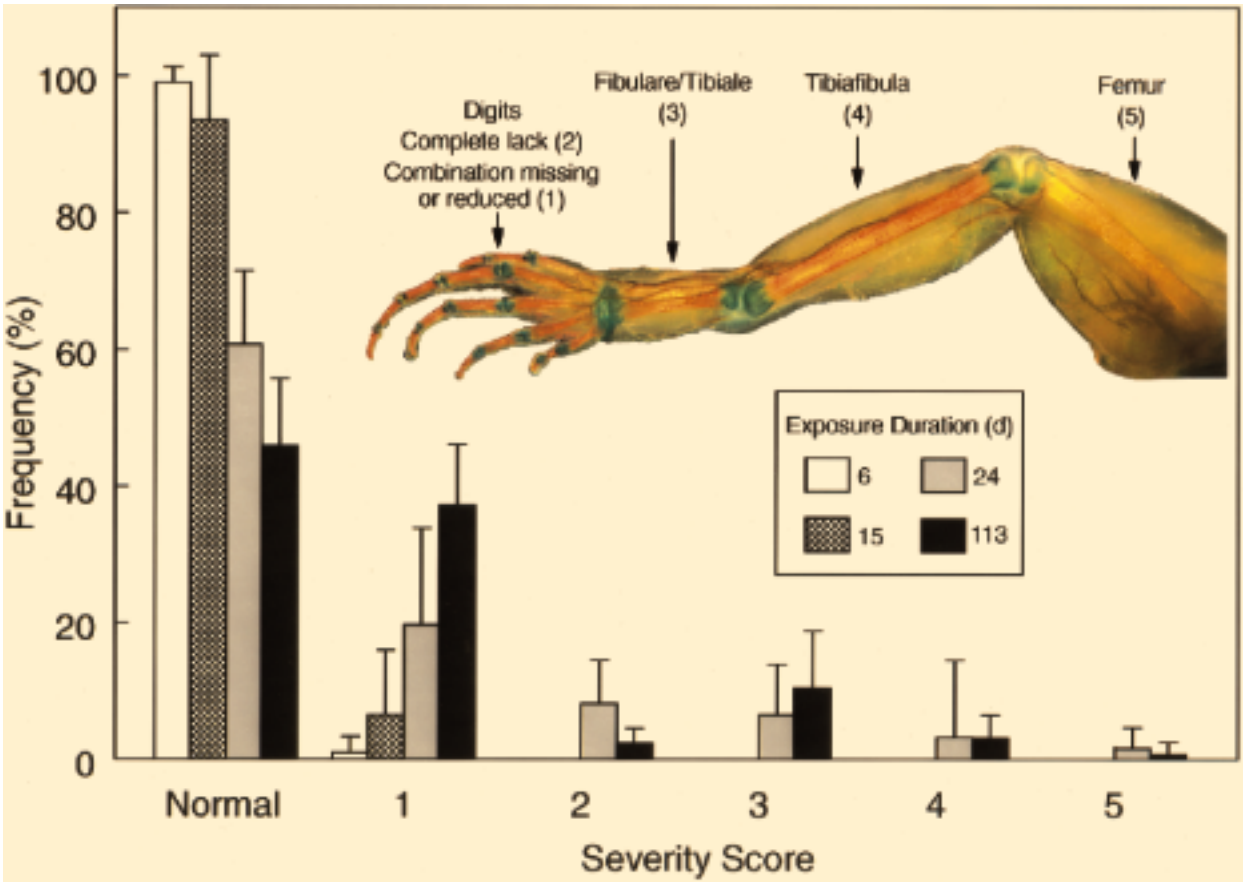

Fig. 6. Relative occurrence ($\bar{x}$, SD) of deficiencies of varying severity in *Rana pipiens* treated with ultraviolet light for 6, 15, 24, and 113 d. Numeric scores correspond to: 0 = normal; 1 = digit/digit segment deficiencies; 2 = deficiency of all digits; 3 = tibiale/fibulare deficiencies; 4 = tibia/fibula deficiencies; 5 = femur deficiencies (see Fig. 5).

ed by Worrest and Kimeldorf [33,34] and Grant and Licht [38], as well as bloating associated with certain treatments. They did not note any limb abnormalities, but in distinct contrast to our study, Hays et al. [40] reported significant mortality associated with certain of the UV exposure regimes. This suggests, perhaps, that differences may have occurred in species sensitivity, or that the UV light intensity and quality differed significantly in some manner between their study and our experiment. Finally, in a very recent study, boreal toads exposed to UV light were noted to develop an abnormally high incidence of hindlimb malformations; further comparison of those data to our results is pending (E. Little, personal communication).

Although relatively little information is available concerning the effects of UV light on limb development in anurans, some work has been conducted on the influence of UV light on limb development in urodele amphibians. For example, a number of studies have shown that localized treatment with very high-intensity UV light at wavelengths of <310 nm can affect forelimb development and regeneration in salamanders, resulting both in deficiencies and supernumerary limbs and digits [41–45]. Worthington [6] described field observations of *Ambystoma maculatum* where he found a high incidence of digit and limb abnormalities (both deficiencies and polydactyly) in salamanders collected from a shallow pond, but not in water bodies protected from sunlight. He speculated that temperature might be responsible for the malformations, but noted that differences in UV light could not be ruled out as a possible causative agent. At present, it is difficult to speculate what relationship, if any, exists between forelimb defects in urodeles induced by high-intensity UV light, and hindlimb malformations in anurans associated with lower-intensity UV treatments.

The mechanism through which UV light might have caused the observed hindlimb deformities is uncertain. But, given the bilateral nature and timing of deformities observed in the frogs, our observations strongly suggest that UV light directly affected developmental pathway(s) in early limb formation. First, the relative symmetry of the deficiencies is quite consistent with limb malformations induced in mammals by chemical teratogens that affect specific developmental pathways [46]. Further, the fact that the animals appeared to be most sensitive to UV light during initial limb bud formation suggests that there may have been some disruption of processes associated with the formation and function of the apical ectodermal ridge (AER). The AER is an area of thickened epithelium rimming the limb bud, which is a critical signaling region for limb bud growth, ostensibly through expression of various fibroblast growth factors (FGFs) (reviewed by Tickle [47,48]). Physical removal of the AER during early limb bud development can lead to limb truncation, a process that can be reversed through treatment with FGFs [49]. In addition to FGFs, a number of other signaling molecules appear to be critical to limb and digit growth and development, including retinoids (which control or act in concert with products of the sonic hedgehog gene) and products of the Wnt-7a gene [47,48]. However, assessing exactly how and where UV light might affect any of these signaling processes relative to limb de-

velopment is difficult, in part because they act in concert with one another. We currently are exploring possible mechanisms through which these developmental events could be affected by UV light.

Note that, although the observed developmental effects most probably were directly caused by UV light, it is not impossible that the supplemental light affected some other component of our test system, such as chemicals in the food or Lake Superior water, thus resulting in more potent teratogen(s) than in the non-UV treatments. A variety of chemicals, most prominently some polycyclic aromatic hydrocarbons, can be photoactivated by UV light to forms far more toxic than the parent molecule [50–53]. But, the presence of chemicals in our test system that could have been photoactivated or photomodified, subsequently affecting limb development, seems unlikely.

Effects of methoprene

Methoprene has been a registered pesticide for more than 20 years, and generally is considered to be of low hazard to vertebrate wildlife [54]. In our experiment, methoprene treatment at the four lowest test concentrations did not result in increased mortality or developmental abnormalities in *R. pipiens*, either in the absence or presence of UV light. In contrast, the highest methoprene concentration tested resulted in profound (and lethal) developmental effects in the embryos by about 2 weeks postfertilization. The gross pathology associated with these effects consisted of extreme axial distortion of the animals. The mechanism underlying this teratogenic effect is uncertain, but based upon the findings of Harmon et al. [18], it is tempting to speculate that methoprene or, more likely, associated metabolites might have affected the retinoid signaling pathway via interaction with one or more retinoid receptors. The retinoid system is key to defining embryonic polarity in developing vertebrates [12,13], and recent studies with methoprene have confirmed the teratogenic nature of the compound and its metabolites or degradation products in assays with the African clawed frog, *Xenopus laevis* [22]. In fact, the developmental anomalies caused by methoprene in this study were quite similar in appearance to effects observed in *X. laevis* exposed to several retinoid receptor agonists [55].

For reasons related both to exposure uncertainties and the possible role of metabolites or degradation products in causing toxicity, the potential ecological significance of teratogenic effects associated with methoprene is difficult to assess. For example, in our test system, the parent molecule was relatively unstable, with more than 95% of the methoprene disappearing over the course of 48 h during the static-renewal phase of the assay. This behavior is consistent with the fate of methoprene in the environment, where various studies have shown relatively rapid degradation of the parent chemical, in particular, in the presence of sunlight [22,28,56,57]. In any case, because of this instability, it is impossible from our data to calculate meaningful effect and no-effect methoprene concentrations. And basing effect and no-effect estimates upon the parent compound may not even be appropriate, if indeed metabolites or break-down products are responsible for adverse effects [22]. Further work concerning the mechanisms and specific chemicals responsible for the observed pathology in our study, as well as measurement of these chemicals in relevant environmental settings, is required before it is possible to estimate the potential teratogenic risk of methoprene to wildlife, including amphibians.

Limb deformities in anuran amphibians

The occurrence of supernumerary limbs and digits in anurans from the field, although relatively rare, is not without historical precedent [4,5,7–9]. The experimental induction of supernumerary limbs and digits, including homeotic transformations, has been achieved in the laboratory via treatment of anurans, as well as other vertebrate models, with retinoids [15,16]. Physical disturbance of the developing limb bud field also has been shown to result in supernumerary limbs in both frogs and salamanders [9]. As opposed to extra limbs or digits, until recently, the occurrence of missing limbs, limb segments, and digits in amphibians from the field has been a far less commonly reported phenomenon. In one older study, Merrell [58] reported the occurrence of unilateral deletions of limbs, limb segments, and digits in a population of *R. pipiens* from a small lake in southern Minnesota. In a more recent paper, Ouellet et al. [2] described a seeming elevation of ectromelia and ectrodactyly in various anuran species associated with agricultural drainages. The experimental induction of varying degrees of ectromelia and ectrodactyly in anurans in the laboratory has been achieved in studies with chemical and nonchemical stressors. For example, Muto [59] reported the occurrence of missing and shortened digits in the hindlimbs of toads reared at an abnormally high temperature. Fort and Stover [60] found that treatment of *X. laevis* with copper resulted in symmetrical hindlimb malformations consisting of an absence of the structures distal to the femur, which was similar to one of the malformations observed in our UV-exposed animals. Limb development in *X. laevis* also has been assessed after treatment with a variety of other xenobiotics but, as was the case for the copper data, none of the chemical-specific malformations observed in *X. laevis* to date have been as broad in nature (i.e., in terms of representation of the full range of proximal to distal deficiencies) as the hindlimb abnormalities associated with the UV light exposure in this study (D. Fort, personal communication).

To date, much of the public attention and scientific investigation concerning deformities in amphibians from the field has focused upon chemicals, in particular, pesticides such as methoprene. In considering the fact that a variety of chemical and nonchemical stressors can cause both extra and missing limbs and digits in amphibians, it is perhaps naive to hypothesize that the broad range of malformations observed in wild anurans can plausibly be attributed to a single environmental stressor. It seems more likely that multiple factors acting via different mechanisms, perhaps including natural causes [9], are responsible for the malformations. Hence, rather than searching for one factor that causes both supernumerary and deficient limbs and digits (as well as the eye malformations that have been reported) it might be more productive to focus upon sets of abnormalities that, through some reasonable mechanistic underpinnings, are related to one another.

SUMMARY AND CONCLUSION

In summary, we were able to induce relatively specific hindlimb malformations in *R. pipiens* by treatment with UV light. In terms of missing, as opposed to supernumerary digits and limbs, these abnormalities were superficially similar to malformations increasingly seen in some field-collected amphibians. The observed deformities were typically bilateral and seemingly quite stage-specific, which are both observations of potential utility in defining mechanisms of action through which UV light might affect amphibian development. It must

be stressed that much work remains to fully assess the significance of our results in terms of deformed amphibians from the field. This includes replication of these results, for example, using other cohorts of *R. pipiens* (the animals in this experiment came from a single pair of animals), as well as additional species of concern. To start to address the first issue, we recently completed a study with *R. pipiens* obtained from a commercial supplier (Carolina Biological, Burlington, NC, USA) that were exposed to the same UV light regime as used in the present study; preliminary results from that experiment indicate the occurrence of limb malformations similar to those described herein (unpublished data). Further work is also needed to better define dose dependence of the phenomenon both from a qualitative (light spectrum) and quantitative perspective. Research in this latter area must include consideration of UV light dose in the context of amphibian development, both in terms of where animals reproduce and develop and their behavior.

Treatment of *R. pipiens* embryos with the insect growth regulator methoprene, in the absence or presence of UV light from early embryogenesis through approximately developmental stage 44 did not result in abnormalities similar to those reported in wild amphibian populations. However, the highest concentration of methoprene tested did cause lethal developmental effects in *R. pipiens* that resemble those caused by retinoids and retinoid analogues in other species. However, the possible significance of this teratogenic effect of methoprene in the environment is uncertain.

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REFERENCES

1. Schmidt CW. 1997. Amphibian deformities continue to puzzle researchers. *Environ Sci Technol A* 31:324–326.
2. Ouellet M, Bonin J, Rodrique J, DesGranges J, Lair L. 1997. Hindlimb deformities (ectromelia, ectrodactyly) in free-living anurans from agricultural habitats. *J Wildl Dis* 33:95–104.
3. Bishop DW. 1947. Polydactyly in the tiger salamander. *J Hered* 38:290–293.
4. Rostand J. 1958. *Les Anomalies des Amphibiens Anoures.* Sedes, Paris, France.
5. Hebard WB, Brunson RB. 1963. Hindlimb anomalies of a western Montana population of the Pacific tree frog, *Hyla regilla* Baird and Girard. *Copeia* 1963:570–572.
6. Worthington RD. 1974. High incidence of anomalies in a natural population of spotted salamanders, *Ambystoma maculatum. Herpetologica* 30:216–220.
7. Reynolds TD, Stephens TD. 1984. Multiple ectopic limbs in a wild population of *Hyla regilla. Great Basin Nat* 44:166–169.
8. Borkin LJ, Pikulik MM. 1986. The occurrence of polymely and polydactyly in natural populations of anurans of the USSR. *Amphib-Reptilia* 7:205–216.
9. Sessions SK, Ruth SB. 1990. Explanation for naturally occurring supernumerary limbs in amphibians. *J Exp Zool* 254:38–47.
10. Ankley GT, Giesy JP. 1998. Endocrine disruptors in wildlife: A weight of evidence perspective. In Kendall R, Dickerson R, Suk W, Giesy J, eds, *Principles and Processes for Assessing Endocrine Disruption in Wildlife.* SETAC, Pensacola, FL, USA, pp 349–368.
11. Schena M. 1989. The evolutionary conservation of eukaryotic gene transcription. *Experientia* 45:972–983.
12. Wagner M, Thaller C, Jessell T, Eichele G. 1990. Polarizing activity and retinoid synthesis in the floor plate of the neural tube. *Nature* 345:819–822.
13. Shimeld SM. 1996. Retinoic acid, HOX genes and the anterior–posterior axis in chordates. *Bioessays* 18:613–616.
14. Shenefelt R. 1972. Morphogenesis of malformations in hamsters caused by retinoic acid: Relation to dose and stage of treatment. *Teratology* 5:103–118.
15. Maden M. 1993. The homeotic transformation of tails into limbs in *Rana temporaria* by retinoids. *Dev Biol* 159:379–391.
16. Rutledge JC, Shourbaji AG, Hughes LA, Polifka JD, Cruz YP, Bishop JB, Generoso WM. 1994. Limb and lower-body duplications induced by retinoic acid in mice. *Proc Natl Acad Sci USA* 91:5436–5440.
17. Scott WJ, Collins MD, Ernst AN, Supp DM, Potter SS. 1994. Enhanced expression of limb malformations and axial skeleton alterations in legless mutants by transplacental exposure to retinoic acid. *Dev Biol* 164:277–289.
18. Harmon JE, Boehm MF, Heyman RA, Manglesdorf DJ. 1995. Activation of mammalian retinoid X receptors by the insect growth regulator methoprene. *Proc Natl Acad Sci USA* 92:6157–6160.
19. Kerr JB, McElroy CT. 1993. Evidence for large upward trends of ultraviolet-B radiation linked to ozone depletion. *Science* 262: 1032–1034.
20. Fioletov VE, Evans WFJ. 1997. The influence of ozone and other factors on surface radiation. In Wardle DI, Kerr JB, McElroy CT, Francis ER, eds, *Ozone Science: A Canadian Perspective on the Changing Ozone Layer.* University of Toronto Press, Toronto, ON, Canada, pp 73–90.
21. Herman JR, Bhartia PK, Ziemke J, Ahmad Z, Larko D. 1996. UV-B increases (1979–1992) from decreases in total ozone. *Geophys Res Lett* 23:2117–2120.
22. Dumont J, Bantle J, LeClair J, Fort D, Propst T, Faulkner B, Stover E. 1997. Methoprene exposed to UV light causes developmental toxicity in *Xenopus* embryos. *Abstracts,* 18th Annual Meeting, Society of Environmental Toxicology and Chemistry, San Francisco, CA, USA, November 17–21, pp 91–92.
23. American Public Health Association, American Water Works Association, and Water Pollution Control Federation. 1992. *Standard Methods for the Examination of Water and Wastewater,* 18th ed. American Public Health Association, Washington, DC.
24. Brennan P, Fedor C. 1988. Sunlight, UV and accelerated weathering. *Paint Resin* 58:17–21.
25. Gosner KL. 1960. A simplified table for staging anuran embryos and larvae with notes on identification. *Herpetologica* 16:183–190.
26. Miller DM, Tarpley J. 1996. An automated double staining procedure for bone and cartilage. *Biotech Histochem* 71:79–83.
27. Ricchiazzi PJ, Shiren Y, Gautier C. 1999. SBDART: A research and teaching software tool for plane-parallel radiative transfer in the earth's atmosphere. *Bull Am Meteorol Soc* (in press).
28. Quistad GB, Staiger LE, Schooley DA. 1975. Environmental degradation of the insect growth regulator methoprene (isopropyl (2E, 4E)-11-methoxy-3,7,11-trimethyl-2,4-dodecadienoate). III. Photodecomposition. *J Agric Food Chem* 23:299–303.
29. Kim ST, Sancar A. 1993. Photochemistry, photophysics, and mechanism of pyrimidine dimer repair by DNA photolyase. *Photochem Photobiol* 57:895–904.
30. Holm-Hansen O, Lubin D, Helbling EW. 1993. Ultraviolet radiation and its effects on organisms in aquatic environments. In Young AR, Bjorn LO, Moan J, Nultsch W, eds, *Environmental UV Photobiology.* Plenum, New York, NY, USA, pp 379–425.
31. Nagl AM, Hofer R. 1997. Effects of ultraviolet radiation on early larval stages of the alpine newt, *Triturus alpestris,* under natural and laboratory conditions. *Oecologia* 110:514–519.
32. Kao K, Danilchik M. 1991. Generation of body plan phenotypes in early embryogenisis. *Methods Cell Biol* 36:271–284.
33. Worrest RC, Kimeldorf DJ. 1975. Photoreactivation of potentially lethal, UV-induced damage to boreal toad (*Bufo boreas boreas*) tadpoles. *Life Sci* 17:1545–1550.
34. Worrest RC, Kimeldorf DJ. 1976. Distortions in amphibian development induced by ultraviolet-B enhancement (290–315 nm) of a simulated solar spectrum. *Photochem Photobiol* 24:377–382.
35. Blaustein AR, Hoffman PD, Hokit DG, Kiesecker JM, Wells SC, Hays JB. 1994. UV repair and resistance to solar UVB in amphibian eggs: A link to population declines? *Proc Natl Acad Sci USA* 91:1791–1795.
36. Blaustein AR, Edmund B, Kiesecker JM, Beatty JJ, Hokit DG.

1995. Ambient ultraviolet radiation causes mortality in salamander eggs. *Ecol Appl* 5:740–743.
37. Blaustein AR, Kiesecker JM, Chivers DP, Anthony RG. 1997. Ambient UV-B radiation causes deformities in amphibian embryos. *Proc Natl Acad Sci USA* 94:13735–13737.
38. Grant KP, Licht LE. 1995. Effects of ultraviolet radiation on life-history stages of anurans from Ontario, Canada. *Can J Zool* 73: 2292–2301.
39. Long LE, Saylor LS, Soule ME. 1995. A pH/UV-B snyergism in amphibians. *Conserv Biol* 9:1301–1303.
40. Hays JB, Blaustein AR, Kiesecker JM, Hoffman PD, Pandelova I, Coyle D, Richardson T. 1996. Developmental responses of amphibians to solar and UVB sources: A comparative study. *Photochem Photobiol* 64:449–456.
41. Rieck AF. 1954. The effects of ultraviolet, and of photorecovery, on the developing forelimb of *Amblystoma*. *J Morphol* 94:367–408.
42. Butler EG, Blum HF. 1955. Regenerative growth in the urodele forelimb following ultraviolet radiation. *J Natl Cancer Inst* 15: 877–889.
43. Butler EG, Blum HF. 1963. Supernumerary limbs of urodele larvae resulting from localized ultraviolet light. *Dev Biol* 7:218–233.
44. Butler EG, Blum HF, Schmidt SE. 1957. The localized character of ultraviolet effects on the urodele forelimb. *J Cell Comp Physiol* 50:381–388.
45. Blum HF, Butler G, Schmidt SE. 1958. Regeneration of limb abnormalities after ultraviolet irradiation. *J Cell Comp Physiol* 52:177–186.
46. Sanders DD, Stephens TD. 1991. Review of drug-induced limb defects in mammals. *Teratology* 44:335–354.
47. Tickle C. 1996. Vertebrate limb development. *Cell Dev Biol* 7: 137–143.
48. Tickle C. 1996. Genetic and limb development. *Dev Genet* 19: 1–8.
49. Fallon JF, Lopez A, Ros MA, Savage MP, Olwin BB, Simandl BK. 1994. FGF-2: Apical epidermal ridge growth signal for chick limb development. *Science* 264:104–107.
50. Bowling JW, Leversee GJ, Landrum PF, Giesy JP. 1983. Acute mortality of anthracene-contaminated fish exposed to sunlight. *Aquat Toxicol* 3:79–90.
51. Oris JT, Giesy JP. 1987. The photoinduced toxicity of polycyclic aromatic hydrocarbons to larvae of the fathead minnow (*Pimephales promelas*). *Chemosphere* 16:1395–1404.
52. Huang XD, Dixon DG, Greenberg BM. 1993. Impacts of UV radiation and photomodification on the toxicity of PAHs to the higher plant *Lemna gibba* (duckweed). *Environ Toxicol Chem* 12:1067–1078.
53. Ankley GT, Erickson RJ, Phipps GL, Mattson VR, Kosian PA, Sheedy BR, Cox JS. 1995. Effects of light intensity on the phototoxicity of fluoranthene to a benthic macroinvertebrate. *Environ Sci Technol* 29:2828–2833.
54. U.S. Environmental Protection Agency. 1991. R.E.D. facts—methoprene. EPA 738/F-91/104. Washington, DC.
55. Minucci S, et al. 1996. Retinoid receptor-selective ligands produce malformations in *Xenopus* embryos. *Proc Natl Acad Sci USA* 93:1803–1807.
56. Quistad GB, Staiger LE, Schooley DA. 1974. Environmental degradation of the insect growth regulator methoprene (isopropyl (2E, 4E)-11 methoxy-3,7,11-trimethyl-2,4-dodecadienoate). I. Metabolism by alfalfa and rice. *J Agric Food Chem* 22:582–586.
57. Schooley DA, Bergot BJ, Dunham LL, Siddall JB. 1975. Environmental degradation of the insect growth regulator methoprene (isopropyl (2E, 4E)-11 methyl-3,7,11-trimethyl-2,4-dodecadienoate). II. Metabolism by aquatic microorganisms. *J Agric Food Chem* 23:292–297.
58. Merrell DJ. 1969. Natural selection in a leopard frog population. *J Minn Acad Sci* 35:86–89.
59. Muto T. 1969. Anomalies in the hindlimb skeleton of the toad larvae reared at a high temperature. *Congenital Anom* 9:61–73.
60. Fort DJ, Stover EL. 1998. Development of short-term whole-embryo assays to evaluate detrimental effects on amphibian limb bud development and metamorphosis using *Xenopus laevis*. In Dwyer FJ, Doane TR, Himan ML, eds, *Environmental Toxicology and Risk Assessment: Modeling and Risk Assessment*, Vol 6. STP 1317. American Society for Testing and Materials, Philadelphia, PA (in press).