EFFECTS OF PHOTINDUCED TOXICITY OF FLUORANTHENE ON AMPHIBIAN EMBRYOS AND LARVAE

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(Received 31 March 1997; Accepted 8 January 1998)

Abstract—Embryos and newly hatched larvae of three amphibian species, the spotted salamander (Ambystoma maculatum), the northern leopard frog (Rana pipiens), and the African clawed frog (Xenopus laevis), were exposed to fluoranthene and ultraviolet (UV) light in two scenarios. Embryos were exposed in a laboratory setting from an early developmental stage through hatching under artificial UV light, and newly hatched larvae were exposed outdoors in varying sunlight intensity levels. Outdoor exposures indicated greater sensitivity in the toxic response than did laboratory exposures. In the laboratory, mortality and malformation of X. laevis were the most sensitive indicators of exposure. Xenopus laevis was also the most sensitive species tested to the effects of UV light alone. Hatching success of R. pipiens was monitored outdoors and was not a useful predictive endpoint in the determination of photoinduced toxicity; however, newly hatched larvae were sensitive to the effects of photoinduced toxicity. Ambystoma maculatum and X. laevis larvae were affected by low (μg/L) concentrations of fluoranthene in sunlight. These findings suggest that low levels of polycyclic aromatic hydrocarbons could be acting synergistically with environmental factors such as UV light to place young amphibians at risk.

Keywords—Photoinduced toxicity Fluoranthene Amphibian Ultraviolet light

INTRODUCTION

Aquatic pollution is one possible cause of the apparent widespread decline of amphibian populations [1–8]. However, little experimentation into actual effects of environmental pollution on amphibians has been documented in the scientific literature. The decline may be due to the combined effects of more than one environmental factor [3,7–10]. Ultraviolet (UV) light, possibly increasing in intensity due to thinning atmospheric ozone [11,12], is apparently a significant factor in the decline. In particular, UV-B radiation appears to reduce the hatching success of embryos in a manner correlated with the species-specific capability of amphibian eggs to repair UV-B-induced damage [10,13]. The UV-repair hypothesis may explain why declines have been observed in ecosystems apparently free from human contamination or disturbance [10]. Ultraviolet light may act synergistically with other environmental factors such as pathogens or pollutants to have a profound effect on amphibian development [3,8–10]. Acid pollution and pesticide pollution are possible factors that may interact with UV light to the detriment of amphibian populations [8].

Polycyclic aromatic hydrocarbons (PAHs) are aquatic pollutants found in locations contaminated with petroleum products or urban runoff [14–16]. Polycyclic aromatic hydrocarbons are acutely toxic to aquatic organisms within the range of their aqueous solubility when biota are simultaneously exposed to UV radiation [14–18]. This phenomenon has been attributed to the production of toxic singlet oxygen when PAHs act as sensitizers in the presence of UV-A radiation [16]. Effects are dependent on both the intensity of UV radiation and the amount of PAH present in the aquatic environment [15,16,18]. Therefore, environmental conditions and physiologic and behavioral characteristics that affect exposure of aquatic organisms to UV radiation will also influence toxicity in an ecosystem contaminated with PAHs.

Few researchers have investigated photoinduced toxicity of PAHs to amphibians. Treefrogs exposed to crankcase oil in microcosms exhibited reduced growth as tadpoles and metamorphosis did not occur in the highest concentration of oil tested, which was equivalent to point-source petroleum runoff in the environment [5]. Photoinduced toxicity of PAHs to late embryonic stages (Schumway stages 24–28) of the leopard frog, Rana pipiens, was determined by exposing embryos in test solution to 30 min of sunlight, then assessing mortality 24 h later [19,20]. Under these experimental conditions, the median lethal concentration (LC50) for anthracene was 110 μg/L, the LC50 for fluoranthene was 90 μg/L, and the LC50 for pyrene was 140 μg/L [19]. In another experiment, organisms were exposed to anthracene and either 30 min or 5 h of sunlight; the LC50 for anthracene was 65 μg/L when exposed to 30 min of sunlight and 25 μg/L after exposure to 5 h of sunlight, demonstrating the effect of sunlight on toxicity [20]. Ultraviolet-A radiation enhanced the toxicity of benzo[a]pyrene to newt embryos when embryos were exposed simultaneously to benzo[a]pyrene and UV-A radiation or sunlight [21].

In this series of acute experiments, the photoinduced toxicity of fluoranthene was assessed in three amphibian species: the northern leopard frog, Rana pipiens, the spotted salamander, Ambystoma maculatum, and the African clawed frog, Xenopus laevis. In the laboratory, survival, growth, and malformations of embryos exposed to fluoranthene with UV light.
were assessed. Outdoors, hatching success of \textit{R. pipiens} eggs exposed to fluoroanthene with and without sunlight was monitored. Also in an outdoor exposure, time to death of newly hatched larvae of \textit{A. maculatum} and \textit{X. laevis} was monitored in environmentally relevant (\(\mu\)g/L) concentrations of fluoroanthene in varying levels of sunlight intensity.

**MATERIALS AND METHODS**

**Test organisms**

Eggs of \textit{X. laevis}, \textit{R. pipiens}, and \textit{A. maculatum} were obtained for testing. Spring water diluted to a hardness of approximately 170 to 185 mg CaCO\(_3\)/L was used in the maintenance of eggs in the laboratory prior to testing, in the culture water of adult \textit{X. laevis}, and for preparing all test dilutions. Eggs of \textit{X. laevis} were obtained by breeding adults in the laboratory. Prior to breeding, cultures of adult \textit{X. laevis} were fed a variety of food, including frozen beef, worms, and dry pellet food three times per week with a water change the day following feeding. Adults were induced to breed in the laboratory via injection with human chorionic gonadotropin (HCG) [22]. Eggs were removed from the bottom of the breeding tank and assigned randomly to test treatments within 2 to 4 h of being laid.

Eggs of \textit{R. pipiens} and \textit{A. maculatum} were obtained by collection. Egg masses from \textit{R. pipiens} were collected locally from a wetland site that was known not to be impacted by pollution. Eggs of \textit{R. pipiens} were separated manually from the egg mass in the laboratory and assigned to test chambers in a random fashion. Three masses of eggs of \textit{A. maculatum} were obtained from a commercial supplier (Carolina Biological Supply, Burlington, NC, USA). Upon receipt in the laboratory, eggs of \textit{R. pipiens} and \textit{A. maculatum} were kept in aerated diluted spring water until testing began.

Amphibian embryonic development can be described in stages. Although the numbers of the stages differ between species, understanding the physiology of the embryo at each stage allows for comparisons to be made among species regarding the developmental conditions of the embryos at exposure. For the three species of amphibians tested in these experiments, detailed descriptions for embryonic staging have been published. Exposures of \textit{R. pipiens} in the laboratory and outdoors began at Gosner stage 11 and continued until Gosner stage 25 [23]. Throughout this period embryos feed on the nutritive egg yolk [23,24]. At Gosner stage 11, the blastopore has just formed but the embryos have not yet developed a neural plate. The embryo feeds on the yolk until stage 25, has just formed but the embryos have not yet developed a nutritive egg yolk [23,24]. At Gosner stage 11, the blastopore develops immediately before the blastopore develops [26]. At Nieuwkoop and Faber stage 46, the hind limb bud is visible, the intestine is tightly coiled, and independent feeding begins [26]. Time for development from Nieuwkoop and Faber stage 1 to 46 is approximately 96 h at 24°C [22]. Thus, laboratory exposures of all three species and the hatching experiment of \textit{R. pipiens} involved exposure of different species of larvae at similar developmental stages for the duration until hatching occurred. The time to death experiment involved exposure of newly hatched larvae of \textit{X. laevis} and \textit{A. maculatum} at similar stages of development.

Amphibian eggs are surrounded by several jellylike layers. The jelly layers vary in thickness and viscosity depending on the species and may affect exposure of the developing embryos to UV radiation or to contaminants in the water. Eggs of \textit{A. maculatum} are surrounded by a thick (1.5–2.0-cm) viscid jelly. Before placing embryos in test chambers, this jelly was removed by scraping gently with a spatula. The capsule surrounding the embryo was left intact. Removal of the jelly facilitated the random assignment of embryos to test chambers and treatments. Embryos of \textit{X. laevis} and \textit{R. pipiens} were not dejellyed because of concern from preliminary experiments that this had caused mortality of embryos and was not representative of the potential exposure of the embryos to UV light and pollutants in nature. Light penetration through \textit{A. maculatum} jelly was measured outdoors by placing the jelly on a glass petri dish and measuring UV-A and UV-B levels with and without the jelly so that the amount of UV radiation absorbed by the dish alone could be subtracted. One layer of jelly, or the thickness that would shield an embryo on one side, was measured.

Newly hatched larvae of \textit{X. laevis} and \textit{A. maculatum} were exposed outdoors. The larvae were within 12 h of hatching at initiation of tests. To obtain these larvae, eggs were maintained in aerated diluted spring water of the same source as the water used for test dilutions. Water was changed daily and organisms were not fed. Laboratory tests lasted for the duration of development over which organisms do not require an external food source. Outdoor time to death experiments were of acute duration (24 h) so feeding was not required.

**Laboratory lighting**

Laboratory lighting was designed to include UV light. The laboratory lighting setup was designed to simulate sunlight at an approximate ratio of 100 units visible (fluorescent) light to 10 units of UV-A light to 1 unit of UV-B light using two fluorescent bulbs, one UV-A bulb, and one UV-B bulb. The UV light bulbs (40-W size; Wesco Distributors, Dayton, OH, USA) were placed alongside fluorescent light bulbs. Acetate was used to screen wavelengths below 290 nm in the experiments with \textit{R. pipiens}, \textit{A. maculatum}, and \textit{X. laevis}. The final intensity of UV-A radiation was 62 to 68 \(\mu\)W/cm\(^2\) and UV-B intensity ranged from 2 to 5 \(\mu\)W/cm\(^2\). A second experiment was performed with \textit{X. laevis} in which UV-B levels were reduced by replacing the UV-B bulb with a Vita-lite bulb (Fairfield, NJ, USA) providing UV-B levels of 0.5 to 1.5 \(\mu\)W/cm\(^2\). The UV-A and UV-B intensities were measured with a Macam 103 radiometer (Livingstone, Scotland, United Kingdom) daily during laboratory testing and every 3 h during outdoor experiments.

**Laboratory test design**

Embryos of \textit{R. pipiens}, \textit{A. maculatum}, and \textit{X. laevis} were exposed to fluoroanthene and UV light in the laboratory. Mortality, malformation, and growth of the embryos were assessed. All three species were exposed from early in the life cycle until hatching. \textit{Xenopus laevis} were exposed for 4 d (reached Nieuwkoop and Faber stage 46); \textit{A. maculatum} were exposed...
for 12 d (reached Harrison stage 46); and *R. pipiens* were exposed for 4 d (reached Gosner stage 25). Two replicates of 20 individuals were used per treatment in the exposures with *R. pipiens* and *X. laevis*; two replicates of 10 individuals were used in the laboratory exposures with *A. maculatum*.

Fluoranthene (98% purity, Aldrich Chemical, Milwaukee, WI, USA) was used in all experiments in the laboratory and outdoors. To obtain acute toxicity within the duration of the laboratory protocol, it was necessary to test concentrations greater than the solubility of fluoranthene in water. This was accomplished by dissolving the chemical in high-performance liquid chromatography (HPLC)-grade acetone. The final volume of acetone in all spiked concentrations and in the acetone control was 0.05% (v/v). Controls in the laboratory experiment consisted of UV and non-UV controls and the acetone control. In all laboratory experiments the highest concentration of fluoranthene tested (625 or 1,250 μg/L) was replicated without UV radiation. In the experiments with *R. pipiens* and *X. laevis* all fluoranthene treatments were replicated without UV radiation. Dilution water was prepared and all treatments were renewed daily.

At the end of the experiment, survival, growth, and the presence of any malformations of the embryos were determined. Larvae were preserved in a solution of 3% formalin for microscopic measurement of growth and determination of malformations. Head–tail length was measured to assess growth of the embryos [22]. Teratogenesis was measured by determining whether the embryos were malformed. In the case of embryos of *X. laevis*, this determination was made following the frog embryo teratogenesis assay—*Xenopus* (FETAX) guidelines [22]. Malformations of *R. pipiens* were determined using published descriptions of the normal staging of this species as a pictorial guide [23,27]. Similarly, deformities of *A. maculatum* were assessed using published descriptions of the normal staging of this species [25].

**Hatching experiment**

Experiments outdoors tested the effect of fluoranthene and sunlight on the hatching success of *R. pipiens*. Embryos of *R. pipiens* (Gosner stage 11) were exposed outdoors in early April. Hatching success of *R. pipiens* at 0, 5, 25, and 125 μg/L fluoranthene with and without UV radiation was monitored, and larvae were observed until 2 d posthatching. *Rana pipiens* were exposed with three replicates of 10 individuals each. Test solutions were renewed daily.

Outdoor experiments involved a different method of spiking water with fluoranthene than that described above in the laboratory experiments. In the hatching and time to death experiments, fluoranthene was tested at concentrations within its aqueous solubility. Therefore, no carrier solvent was used in the preparation of water for exposure in the outdoor tests. Stock solutions were made by shell-coating a glass flask with a known amount of fluoranthene dissolved in acetone, allowing the acetone to evaporate overnight, then adding Nanopure® water (Sybron/Barnstead, Boston, MA, USA) to the flask and stirring periodically over 3 to 5 d [16].

Exposure chambers were designed for the hatching experiment and the time to death experiments that would permit simultaneous, randomized exposure of test chambers blocking UV light and of test chambers allowing UV light to penetrate through them. Borosilicate glass chambers were used with a water depth of approximately 5 cm and were then covered to filter out different intensities of UV light and placed in a water bath. Therefore, all experimental UV intensity treatments varying from full sunlight to shade were conducted concurrently in the same temperature conditions. The test series receiving full sunlight was not covered. The test series receiving no sunlight was coated with sunscreen (Coppertone® Sport, SPF 30), allowed to air-dry, and then covered with clear plastic wrap to prevent the sunscreen from washing off the beaker in the water bath. These chambers, designed to completely block UV-A and UV-B rays, were covered with black plastic over the top after addition of the test solution and larvae. Black plastic shielded most of the UV light; the sunscreen coating reduced incident UV radiation entering through the sides of the beaker. Ultraviolet radiation did penetrate these chambers on occasion, with intensities ranging from 2 to 10% UV-A and 4 to 12% UV-B. The entire water bath was covered with a layer of clear plastic wrap that allowed 100% of UV light through but kept insects and predatory birds out. The temperature of each outdoor experiment was kept within a 5°C range, and exposure chambers were brought indoors at sunset to prevent them from undergoing a large shift in temperature.

**Time to death experiment**

Time to death of larvae of *A. maculatum* and *X. laevis* exposed to fluoranthene and varying levels of sunlight intensity was monitored outdoors. Newly hatched larvae of *A. maculatum* (Harrison stage 46) were exposed in mid-May, whereas larvae of *X. laevis* (Nieuwkoop and Faber stage 47) were exposed in early June. *Ambystoma maculatum* and *X. laevis* were exposed at 0, 1, 5, and 25 μg/L fluoranthene in the three different UV intensity treatments described previously. *Ambystoma maculatum* and *X. laevis* were exposed with two replicates of 10 individuals at each treatment. The UV levels were checked each time mortality was assessed in the case of exposures of *A. maculatum*, and every 3 h in the case of exposures of *X. laevis*. Mortality of *A. maculatum* was quantified every 4 h, and temperature was checked hourly. The total exposure time was 24 h (14 h total daylight). The experiment was ended when larvae of *A. maculatum* in the full sun treatment appeared to be stressed (defined as a sluggish response to probing). For *X. laevis*, observations were made at first every 5 min, then every 1 h for a total exposure time of 24 h (12 h total daylight). Test solutions were renewed daily.

Exposure chambers were similar to those described above for the hatching experiment with *R. pipiens*. An additional light intensity treatment, partial sunlight, was added to the test design. The chambers in the test series receiving partial sunlight were completely covered in 16 layers of cheesecloth, permitting 15 to 30% of UV-A and 60 to 80% of UV-B radiation to pass through. Actual levels of sunlight entering the partial sunlight and no sunlight chambers were used as covariates in the statistical analysis described below.

**Water quality**

Water quality of the experiments was monitored regularly. Dissolved oxygen (DO), pH, alkalinity, hardness, and conductivity of water samples from the laboratory and outdoor experiments were analyzed following standard methods [28]. Parameters were measured in the control and high and low doses. Dissolved oxygen and pH were measured daily and alkalinity, hardness, and conductivity were measured at the beginning and end of each experiment. Temperature was measured daily in laboratory experiments and at each observation in outdoor experiments.
Table 1. Toxicity of fluoranthene to three species of amphibian embryos exposed to ultraviolet light in the laboratory

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Rana pipiens</th>
<th>Ambystoma maculatum</th>
<th>Xenopus laevis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Duration</td>
<td>96 h</td>
<td>288 h</td>
<td>96 h</td>
</tr>
<tr>
<td>LC50</td>
<td>366 μg/L (297–449 μg/L)</td>
<td>247 μg/L (CL not reliable)</td>
<td>193 μg/L (153–243 μg/L)</td>
</tr>
<tr>
<td>EC50</td>
<td>276 μg/L (CL not reliable)</td>
<td>247 μg/L (CL not reliable)</td>
<td>52 μg/L (CL not reliable)</td>
</tr>
<tr>
<td>TI</td>
<td>1.33</td>
<td>1.00</td>
<td>3.71</td>
</tr>
</tbody>
</table>

*LC50 = median lethal concentration; EC50 = median effective concentration; TI = teratogenic index (ratio of EC50 to LC50). LC50 and EC50 (malformation) calculated using the trimmed Spearman–Karber method; 95% confidence limits (CL) in parentheses.

Chemical analysis

Fluoranthene exposure concentrations were monitored by chemical analysis. Fluoranthene concentrations were measured using gas chromatophotography with flame ionization detection (GC-FID) (Carlo-Erba 6000 Vega Series, Italy) [29]. Before GC analysis, fluoranthene was extracted from aqueous samples using packed C-18 columns (J. T. Baker, Paris, KY, USA). Fluoranthene was then eluted from the column in a known amount of HPLC-grade methanol. The methanol sample was then injected into the GC-FID apparatus using a splitless injection technique. A calibration curve of five standard concentrations was used to quantify fluoranthene via linear regression. Fluoranthene concentrations were determined from samples at test initiation and termination. From laboratory tests, the highest and lowest concentrations were analyzed; from outdoor tests, the high concentration and stock solution were analyzed.

Statistical analysis

Laboratory data on survival, malformation, and growth were analyzed for significant differences among treatment groups for each species. The LC50s and median effective concentrations (EC50s) were calculated using the trimmed Spearman–Karber method (version 1.5, U.S. EPA, Cincinnati, OH, USA). The EC50 was determined for malformation of the embryos. The teratogenic index presented here is the ratio of the EC50 to the LC50 [22]. Generalized linear model procedures were used to compare solvent control to nonsolvent control and to compare UV to non-UV control (SAS® Version 6.12 for Windows, SAS Institute, Cary, NC, USA). Data were entered as a proportion of the number affected to the total number exposed and then transformed with the arcsine of the square root transformation. Analysis of variance followed by Bonferroni’s test for significant differences were used to compare treatments of the laboratory tests and to compare hatching success in the different treatments of the outdoor experiment with R. pipiens. Data from the hatching experiment with R. pipiens were also entered as proportions and transformed with the arcsine of the square root transformation.

Survival analysis procedures in SAS were required in the analysis of time to death data of larvae of A. maculatum and X. laevis [30–32]. Data were not transformed and were either interval censored (for those who died over the course of the experiment) or right censored (if survived to the final observation). Because of lack of animals, the two middle concentrations in the no sun treatments (1 μg/L and 5 μg/L) were not actually exposed and were considered missing values in the analyses.

Examination of the data using the LIFETEST procedure in SAS revealed that a plot of the negative log of the survival distribution function against the residuals was a straight line, suggesting that the assumption of the Weibull distribution was appropriate for the data. Plots of the residuals were not a straight line but were not affected by transformation of the covariates. Therefore, statistical analysis proceeded under the assumption of the Weibull distribution without transforming the covariates.

To generate the hazard model from which median times to death (MTTDs) and their associated confidence intervals were calculated, the SAS procedure PHREG was used. This procedure does not require the assumption of normality to estimate a model of the hazard function. Covariates incorporated into the models were concentration of fluoranthene (0, 1, 5, or 25 μg/L) and UV intensity treatment (no sun, low sun, or full sun). Intensity was considered a continuous variable for calculation of hazard predictions using PROC PHREG of SAS. Because UV-A is the wavelength region of interest in phototoxicity of PAHs, the average UV-A intensity reaching the exposed larvae was used in the continuous model (i.e., 6%, no sun; 22.5%, low sun; 100%, high sun).

RESULTS

Laboratory experiments

Overall, UV intensity was much higher in the outdoor exposures when compared to the laboratory exposures. The effects of this difference were evident when comparing the results of the two exposure scenarios. In the laboratory, UV-A intensity ranged from 62 to 68 μW/cm², whereas outdoor UV-A intensity ranged from 200 to 1,650 μW/cm² in the full sun treatment. Laboratory data revealed differences in sensitivity to photoinduced toxicity among the three amphibian species tested (see Table 1). Rana pipiens and A. maculatum were not significantly affected at concentrations below the aqueous solubility of fluoranthene in the presence of UV radiation (see Figs. 1 and 2). However, embryos of X. laevis appeared to be particularly sensitive to the exposure both to UV radiation alone and to photoinduced toxicity of PAHs (see Figs. 3 and 4). Survival of all three species tested was unaffected by the solvent or UV light (generalized linear model; Bonferroni’s test; alpha = 0.05). However, all embryos of X. laevis exposed under UV radiation developed a brown, speckled pigmentation.

Growth of X. laevis was sensitive to the effects of photoinduced toxicity, whereas growth of A. maculatum was not affected by photoinduced toxicity, and growth of R. pipiens was affected only at the highest concentration tested, which also resulted in significantly reduced survival. The guts of embryos of X. laevis exposed under UV radiation did not coil as tightly as did the guts of non-UV controls. Growth of embryos of X. laevis in all UV treatments was also significantly reduced in comparison to the non-UV controls (UV control, 76 ± 5% of the non-UV control; other UV treatments were...
65–72% of the non-UV control). Because of these effects in the UV control, a second series of experiments was conducted exposing *X. laevis* to fluoranthene and UV radiation; however, the UV-B intensity was lowered to approximately 0.5 to 1.5 \( \mu \text{W/cm}^2 \) UV-B in the second experiment. Although sufficient embryos were not available to permit calculation of an LC50, a noticeable difference in growth occurred in comparison to the first experiment. Growth was not significantly affected in the UV control (UV control, 100 ± 9.9% of the non-UV control). Additionally, no difference occurred in gut coiling in UV-exposed control embryos compared to non-UV control organisms, although the embryos did develop the speckled pigmentation noted in the first experiment. Thus, adverse effects were caused to *X. laevis* in the range of 1.5 to 5 \( \mu \text{W/cm}^2 \) of UV-B radiation, but not in the lower intensity range (0.5–1.5 \( \mu \text{W/cm}^2 \)).

Larvae of *X. laevis* exhibited a higher incidence of deformities than did those of the other two species (100% malformation at concentrations at and above 125 \( \mu \text{g/L} \)) (Fig. 4).

These deformities included microcephaly, axial malformations, abdominal edema, and gut malformations. For our determination of malformations, the pigmentation was not considered a deformity because it occurred in all treatments including the control under UV radiation. Also, the guts of UV-exposed embryos did not coil as tightly as the guts of embryos not exposed to UV light, although in all embryos guts coiled 2½ times, one of the criteria for attainment of Nieuwkoop and Faber stage 46. This was a difference between the UV-exposed and non-UV-exposed embryos, and not a difference from the determination of Nieuwkoop and Faber stage 46. Because the exposed embryos reached the same stage as the non-UV-exposed embryos, the gut coiling was not considered an abnormality in the determination of deformities presented in Figure 4 and for calculation of the EC50. *Rana pipiens* exhibited severely malformed guts, abdominal edema, stunted growth, and bent notochords. All of these deformities were present in all deformed larvae of *R. pipiens*. The UV controls for *R. pipiens* exhibited no malformations, although some mortality occurred in this control treatment. *Ambystoma maculatum* appeared to have a binary response at the doses and conditions tested here: they either died or survived the experiment and
posed to full sunlight and fluoranthene levels of 125 μg/L treatment died, whereas controls were unaffected. Embryos exposed to fluoranthene and sunlight at all concentrations died, whereas controls were unaffected. Embryos in this treatment hatched out from the egg but were found dead in the chambers. The hatched larvae were found dead in the chambers. The absence of UV radiation resulted in 67% survival in the 125-μg/L treatment (see Table 2).

Hatching experiment

Hatching success of R. pipiens was not affected by fluoranthene and sunlight (UV-A radiation ranging from 290 to 1,670 μW/cm²) in concentrations of 5 and 25 μg/L (see Table 2). However, 24 h after their hatching success was recorded the larvae exposed to fluoranthene and sunlight at all concentrations died, whereas controls were unaffected. Embryos exposed to full sunlight and fluoranthene levels of 125 μg/L hatched 24 h later than those in the other test treatments and the hatched larvae were found dead in the chambers. The absence of UV radiation resulted in 67% survival in the 125-μg/L treatment (see Table 2).

Time to death experiment

The outdoor time to death experiments revealed greater sensitivity than observed in the laboratory. Reasons for the greater sensitivity in this exposure scenario could include the greater UV intensity outdoors and the difference in life stage exposed. Dramatic results were observed outdoors; at 25 μg/L in full sunlight, larvae died within a matter of minutes (see Fig. 5). Trends were observed correlating risk with concentration of fluoranthene and UV light intensity (see Fig. 5). Although the middle two concentrations in the no sun treatments (1 and 5 μg/L) were not actually exposed, these treatments were treated as missing values in the statistical analysis and MTTDs for these treatments were estimated using SAS. The fact that these treatments were considered to be missing values may explain why the confidence intervals around these MTTDs are broader than the confidence intervals around the other estimates, especially the full sun treatments, in which nearly all organisms died. Also, these no sun treatments caused little mortality; most organisms remained alive at the end of the test, resulting in less information upon which to base an estimate of their life span. Overall, X. laevis appeared to be more sensitive to photoinduced toxicity of fluoranthene compared to A. maculatum. Larvae of A. maculatum were more sensitive than the other species to the effects of UV radiation alone in the outdoor exposures, contrasting with the laboratory exposures in which X. laevis exhibited the highest sensitivity to UV radiation alone.

The hazard of death of the exposed embryos as a function of fluoranthene concentration in full sunlight was estimated using survival analysis procedures. Regression models were formulated using SAS (PROC PHREG). For A. maculatum exposed to fluoranthene outdoors in the full sunlight treatment, the hazard of death increases by 13.6% for each 1-μg/L increase in concentration (b = 0.127616). For X. laevis exposed outdoors in our full sunlight treatment, the hazard of death increases by 17% for each 1-μg/L increase in concentration of fluoranthene (b = 0.157153).

Water quality

Throughout the laboratory experiments temperature varied from 22 to 25°C, and UV-A intensity ranged from 62 to 68 μW/cm². The UV-B intensity ranged from 2 to 5 μW/cm² except in the second experiment with X. laevis, in which UV-B intensity ranged from 0.5 to 1.5 μW/cm². Outdoors, UV intensity varied throughout the day and depended on cloud.

Table 2. Hatching success of embryos of Rana pipiens exposed outdoors to fluoranthene with and without sunlight

<table>
<thead>
<tr>
<th>Nominal concentration</th>
<th>0 μg/L</th>
<th>5 μg/L</th>
<th>25 μg/L</th>
<th>125 μg/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>No sun</td>
<td>73.33% (15.28)</td>
<td>60% (10.00)</td>
<td>63.33% (5.77)</td>
<td>66.67% (25.17)</td>
</tr>
<tr>
<td>Sun</td>
<td>70% (20.00)</td>
<td>66.67% (5.77)</td>
<td>80% (17.32)</td>
<td>56.67% (25.17)</td>
</tr>
</tbody>
</table>

* Values are the mean percent of the total number exposed (three replicates per treatment; n = 10 individuals per replicate). Standard deviations are shown in parentheses. No treatments are statistically significant (analysis of variance and Bonferroni’s test, p = 0.05).

Table 3. Median time to death (h) for amphibian larvae exposed to fluoranthene outdoors in full sunlight (FS), partial sunlight (PS), and no sunlight (NS). The 95% confidence limits are in parentheses.

<table>
<thead>
<tr>
<th>Fluoranthene concentration</th>
<th>0 μg/L</th>
<th>1 μg/L</th>
<th>5 μg/L</th>
<th>25 μg/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sunlight treatment</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NS</td>
<td>333.0 (9.9±656.2)</td>
<td>302.5 (16.5±588.4)</td>
<td>205.9 (31.2±380.5)</td>
<td>30.1 (15.2±44.9)</td>
</tr>
<tr>
<td>PS</td>
<td>194.7 (34.7±354.8)</td>
<td>176.8 (35.9±317.8)</td>
<td>120.4 (36.2±204.5)</td>
<td>17.6 (10.7±24.5)</td>
</tr>
<tr>
<td>FS</td>
<td>15.7 (11.3±20.1)</td>
<td>14.2 (10.4±18.0)</td>
<td>9.7 (7.4±12.0)</td>
<td>1.4 (0.6±2.3)</td>
</tr>
<tr>
<td>X. laevis</td>
<td>1,357.3 (–265.5–2,980.1)</td>
<td>1,101.7 (–171.0–2,374.4)</td>
<td>478.3 (1.4–955.1)</td>
<td>7.4 (3.7–11.1)</td>
</tr>
<tr>
<td>NS</td>
<td>641.6 (–23.3–1,306.4)</td>
<td>520.8 (2.4–1,039.2)</td>
<td>226.1 (37.0–415.2)</td>
<td>3.5 (1.9–5.0)</td>
</tr>
<tr>
<td>PS</td>
<td>19.0 (8.9–29.1)</td>
<td>15.4 (7.6–23.3)</td>
<td>6.7 (3.7–9.7)</td>
<td>0.1 (0.01–0.2)</td>
</tr>
</tbody>
</table>

Fig. 5. Median time to death of larvae of Ambystoma maculatum and Xenopus laevis exposed to fluoranthene outdoors.
cover. In full sunlight in the water, UV-A intensity ranged from 200 to 1,650 µW/cm², and UV-B intensity ranged from 45 to 320 µW/cm². Tests conducted outdoors in April had a temperature range of 18 to 22°C; temperatures in tests conducted in late May and early June ranged from 23 to 28°C. Water quality for all of the experiments remained within reasonable limits (DO, 80–94% saturation; alkalinity, 100–140 mg CaCO₃/L; hardness, 180–220 mg CaCO₃/L; conductivity, 190–300 µmhos/cm; pH, 7.96–8.62).

Chemical analysis

Exposure concentrations measured using GC-FID are presented in Table 4. Water samples extracted via C-18 columns yielded 80 to 100% recovery of the surrogate standard. For shell-coated samples, concentrations quantified were slightly below nominal value. Concentrations obtained by spiking were slightly above the nominal value. Nominal values for concentration were used in all statistical calculations. This was warranted due to the complexity of determining the exact composition of the water sample once photodegradation had begun.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Nominal concentration (µg/L)</th>
<th>Initial concentration (µg/L)</th>
<th>Final concentration (µg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Rana pipiens</em>, laboratory</td>
<td>25</td>
<td>24.2</td>
<td>17.6</td>
</tr>
<tr>
<td></td>
<td>625</td>
<td>602.8</td>
<td>425.3</td>
</tr>
<tr>
<td><em>Ambystoma maculatum</em>, laboratory</td>
<td>25</td>
<td>21.6</td>
<td>17.2</td>
</tr>
<tr>
<td></td>
<td>1,250</td>
<td>906.1</td>
<td>690.0</td>
</tr>
<tr>
<td><em>Xenopus laevis</em>, laboratory</td>
<td>25</td>
<td>16.2</td>
<td>15.3</td>
</tr>
<tr>
<td></td>
<td>1,250</td>
<td>1,350.0</td>
<td>852.8</td>
</tr>
<tr>
<td><em>R. pipiens</em>, outdoor</td>
<td>25</td>
<td>34.6</td>
<td>22.8</td>
</tr>
<tr>
<td></td>
<td>125</td>
<td>122.0</td>
<td>63.8</td>
</tr>
<tr>
<td><em>A. maculatum</em>, outdoor</td>
<td>25</td>
<td>17.9</td>
<td>15.3</td>
</tr>
<tr>
<td></td>
<td>125</td>
<td>110.9</td>
<td>59.8</td>
</tr>
<tr>
<td><em>X. laevis</em>, outdoor</td>
<td>25</td>
<td>18.9</td>
<td>15.2</td>
</tr>
<tr>
<td></td>
<td>125</td>
<td>100.4</td>
<td>43.7</td>
</tr>
</tbody>
</table>

* Sample collected from freshly made dilution water immediately prior to water renewal.
* Sample collected from exposure chamber at time of water renewal.

DISCUSSION

Results of laboratory testing suggest that *X. laevis* are highly sensitive to the teratogenic effects of photoinduced toxicity of fluoranthene. *Xenopus laevis* were also sensitive to the effect of UV radiation alone, as evidenced by their reduced growth, incompletely coiled gut, and increased pigmentation, which may have been a defensive response to UV. Other researchers have similarly observed bronzing of newt skin exposed to UV radiation [21]. Ultraviolet-B radiation inactivated the nuclei of eggs of *X. laevis* exposed during their development [33]. Ultraviolet-B radiation appears to have a significant negative impact on development of *X. laevis* that can be distinguished from the effects of photoinduced toxicity alone. This sensitivity should be considered when designing experiments with *X. laevis* and UV light.

In contrast to the laboratory experiments, outdoor exposures indicated that survival of newly hatched larvae of *X. laevis* and *A. maculatum* was dramatically affected in a manner that highlights the importance of UV intensity in the photoinduced toxicity of fluoranthene. Developing embryos were exposed in the laboratory whereas newly hatched larvae were exposed outdoors, and this difference in life stage may explain the difference in response in the two scenarios. The egg may attenuate the effects of photoinduced toxicity to the developing embryo by absorbing UV or by interfering with the availability of fluoranthene to the developing embryo. The exposure route to the contaminant may change when the embryos hatch and the larvae are directly exposed to contaminants in the water through respiration and feeding. Although the egg yolk is the sole source of food for most amphibian embryos [24], upon hatching, anuran larvae begin to filter-feed, pumping water through specialized oral structures, whereas most salamander larvae prey on zooplankton [24,33,34].

The hatching experiment with *R. pipiens* supports the hypothesis that the change in exposure route that occurs at hatching may influence susceptibility to toxicants. Embryo hatching success did not differ by fluoranthene treatment except at the highest level, in which hatching was delayed by 24 h. However, the newly hatched larvae in the fluoranthene treatments died shortly after exposure to sunlight, suggesting that they were quite sensitive to photoinduced toxicity. These results suggest that larvae were susceptible to toxic effects immediately upon hatching, possibly as a result of imbibing the chemical from the water or as a result of developmental effects caused by fluoranthene uptake in the embryonic stage. Overall, hatching success was not a useful predictive endpoint of photoinduced toxicity. Similarly, a study of developing treefrogs exposed to used crankcase oil noted that hatching success was not concentration-dependent [5]. A study of fathead minnow tolerance to fluoranthene (with UV radiation) investigated hatching success and larval survivorship and noted that survival was most often and most significantly affected by exposure [35]. Hatching success was significantly affected by exposure; however, it was affected to a lesser extent than was survival [35].

*Ambystoma maculatum* appeared to be more sensitive to the high intensity of light encountered outdoors than were *X. laevis*, both with and without fluoranthene. *Ambystoma maculatum* in the full sunlight treatments were sluggish in response to probing and all treatments in the full sun series, including controls, exhibited some mortality. These experimental observations may be explained in considering the life history of this species. *Ambystoma maculatum* lay eggs in open pools of water, usually in shaded areas and less frequently in open, sunlit areas [25]. Salamanders frequently deposit eggs on twigs or vegetation near the water surface to prevent them...
from sinking to the muddy bottom of a pond where the DO levels may be lower [24]. Salamanders in general have relatively low levels of photolyase, an enzyme that repairs DNA damage caused by UV radiation [8,10,13]. Other researchers have speculated that because many salamander species lay their eggs in areas not exposed to solar radiation, selection pressure for the evolution of photorepair mechanisms such as photolyase may have been weak. Conversely, because salamanders have low photolyase levels, many species may have evolved to lay their eggs in areas not exposed to UV radiation [8,10,13].

Another possible factor in explaining the sensitivity of A. maculatum to sunlight is the potential UV-screening capacity of the jelly surrounding the eggs. The jelly removed from eggs of A. maculatum before experimentation blocked UV-A intensity by 53 to 62% and blocked UV-B intensity by 78 to 80%. The jelly surrounding salamander eggs reduced detrimental effects of UV radiation in other studies [10,21]. The jelly may protect salamander eggs lain in temporary shallow pools from desiccation [25].

Eggs of R. pipiens are typically laid near vegetation or twigs in the shallow water of temporary ponds or wetlands, and the frog may be exposed to sunlight as embryos [36]. Upon hatching, tadpoles of Rana spp. search for food near the bottom of the water, as evidenced by their physiologic adaptations for digesting plant matter [34]. Like salamanders, X. laevis also have low photolyase levels [10]. Also similar to the manner suggested for salamanders above, these low photolyase levels coupled with the general tendency for X. laevis to lay eggs in deep murky waters may suggest a lack of adaptation to development in sunlight [10]. Thus, it is possible that R. pipiens developing in nature could be exposed to sunlight in shallow ponds, but X. laevis developing in murky waters in their natural environment of South Africa would not be likely to be exposed to intense sunlight [26]. These adaptations could help to explain the greater sensitivity of X. laevis to UV light in comparison to R. pipiens.

Although all treatments and chambers were tested at the same temperature, the importance of temperature in the outdoor experiments merits discussion. Temperature possibly may have influenced the overall toxic response, as experimental temperature (23 to 28°C in the time-to-death experiments) was likely higher than that to which larvae would naturally be exposed to in a large pond; therefore, our exposure temperature may not have been realistic. This could heighten the sensitivity of the test organisms to toxic effects. Other studies have shown greater mortality from exposure to trace levels of pesticides at higher temperatures [4]. Because amphibians are poikilothermic, their metabolic activity and thus the amount of contaminants they consume will increase proportionately with environmental temperature.

We observed a clear effect of sunlight intensity on photoinduced toxicity of PAHs to young amphibians; MTTD of larvae exposed to fluoranthene outdoors increases with fluoranthene concentration and with increasing sunlight intensity (Table 3 and Fig. 5). Our hazard analysis could be strengthened by incorporating the quantitative measurements of UV-A intensity that were recorded at each observation of the larvae, possibly providing more information about the interaction between PAH concentration and UV intensity. Although this reanalysis could reveal a more precise dose–response relationship, the reanalysis would not affect our conclusions. Overall, our conclusion that UV intensity has an effect on photo-

toxicity suggests that factors that affect exposure to UV will impact photoinduced toxicity. Pollution acting in concert with other environmental factors is an important focus for further research and may help to elucidate causes of the amphibian decline.

Acknowledgement—This research was supported by funds from U.S. Environmental Protection Agency grant R-823973. Mike Kepler provided statistical analysis and interpretation of the time to death data. Michael Newman provided useful suggestions in the data analysis. We thank Andrew Blaustein and three anonymous reviewers for thoughtful comments on earlier versions of this paper.

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