# THE DEVELOPMENT OF INTEGRATIVE APPROACHES FOR EFFORTS IN AMPHIBIAN CONSERVATION

By

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Chapter 1

# DETERMINATION OF CAUSES FOR AMPHIBIAN DECLINES: AN INTEGRATIVE ECOTOXICOLOGICAL APPROACH

## Introduction

With the meeting of herpetologists at Irvine, CA, on February 1990 to discuss the accumulating reports of extinctions in local amphibian populations world wide, a call was made for long term population censusing (Wake, 1991). Since then, reports have indicated that many populations are in trouble (Crump et al. 1992; Walls et al. 1992; Fellers and Drost, 1993; Kagarise Sherman and Morton, 1993). Suggested causes for the declines include habitat destruction, introduction of exotic species, pollution, disease, global warming, and ozone depletion. However, the significance of the declines have been questioned due to the non-equilibrium population regulation that typifies amphibian natural history (Pechmann et al., 1991; Pechmann and Wilbur, 1994). Pechmann and Wilbur (1994) point out that the natural population fluctuations that amphibians undergo can easily be mistaken as population declines and, therefore, question both the utility of using amphibians as bioindicators and the significance of this biodiversity problem. Further, a review (Carey and Bryant, 1995) indicates that only a few anthropogenic factors, such as DDT (Kirk, 1988), UV radiation (Blaustein et al. 1994a; 1995), and habitat destruction (Vial and Saylor, 1993), have been shown to limit amphibian survival in the field.

Historically, the causes for stresses in ecosystems have been investigated from two perspectives: top-down (begin with an overall understanding of the problem before searching for the cause) or bottom-up (begin with an understanding of the factors that affect the simplest levels of biological organization and then relate them to more complex levels of biological organization) (Munkittrick and McCarty, 1995). The majority of studies that investigate the causes for the disappearance of amphibian populations take a "top-down" perspective (Pechmann et al., 1991; Corn and Vertucci, 1992; Blaustein et al., 1994a; 1995; Corn and Folgeman, 1984). This top-down perspective has resulted in a focus on the proper interpretation of amphibian population statistics, i.e., when it can be determined if a population is declining (Blaustein et al., 1994b; Pechmann and Wilbur, 1994). Biologists have largely failed to develop the proper methodological approaches required to causally link anthropogenic factors, such as pollution or UV-B radiation, to amphibian declines. The determination of cause-and-effect relationships with respect to exposure to such factors is complicated. These relationships are most easily observed in the laboratory. However, the ecological significance of these factors can only be determined under natural field conditions, the complexity of which precludes elucidation of cause-and-effect relationships (Munkittrick and McCarty, 1995). The emerging field of ecotoxicology strives to understand the ecological effects of pollutants by mixing top-down and bottom-up approaches so that cause-and-effect relationships can be addressed at multiple levels of biological complexity (Hoffman et al., 1995). Therefore, in order to determine if xenobiotics or UV-B radiation are contributing to amphibian declines, more integrative studies must be conducted.

Below, we critically review studies which have attempted to find causal mechanisms underlying global declines in amphibian populations. Further, we suggest a detailed framework for a research program, based on the principles of ecotoxicology, that offers a more rigorous and potentially more informative approach.

## Historical Concerns and Modern Problems

Conservation-minded herpetologists feel strongly that long term population data are needed in order to determine if human impacts are indeed adversely affecting amphibian populations (Pechmann and Wilbur, 1994). The determination of causality based on amphibian population data is complicated by the lack of base line data for many populations, which are needed to determine how much fluctuation is natural for species that follow non-equilibrium population dynamics (Pechmann and Wilbur, 1994). Non-equilibrium (specifically, metapopulation) dynamics involve the stochastic extinction of local populations in habitat patches and the migration of individuals to these patches from other regions (Blaustein et al., 1994b; Pechmann and Wilbur, 1994). In other words, metapopulation (regional) persistence is maintained via emigration and immigration of individuals between suitable habitat patches. Pechmann and Wilbur (1994) suggest that under this type of population regulation, amphibians may be drastically affected by anthropogenic impacts resulting in extinction vortices (e.g., pollution may limit or stress amphibian populations so that when natural climatic factors such as drought occur, the population is unable to survive). However, assuming that a pair of reproductive adults can reach the newly opened habitat patch, a viable population can be produced during a few

breeding seasons due to their high fecundity (McCoy, 1994). In order to account for such metapopulation dynamics, Corn (1994) points out that the determination of presence or absence of amphibians should not be based on single surveys, and that surveys into new areas should be conducted at the same time.

In general, it is well accepted in the herpetological community that amphibians can serve as good bioindicators of anthropogenic impacts (Power et al., 1989; Blaustein, 1994). This contention is based on the observations that amphibians possess highly permeable eggs, gills, and skin and have complex life cycles, placing them in a variety of habitat types and trophic niches (Vitt et al., 1990). It is usually assumed that the earliest life history stages are the most vulnerable to anthropogenic stress, and, therefore, eggs and larvae are the focus of many studies (Cook, 1983; Corn and Vertucci, 1992; Bantle et al., 1994; Berrill et al., 1994; Blaustein et al., 1994a; Mahaney, 1994; Blaustein et al., 1995; Grant and Licht, 1995; Kiesecker and Blaustein, 1995; Long et al., 1995; Materna et al., 1995). However, the successful extrapolation of effects on eggs and larvae to changes in populations has not yet been made and given the r-selected, complex nature of amphibian life history strategies, this provides a formidable research challenge. Further, there is a paucity of toxicological studies that examine adult responses to xenobiotics, especially sublethal responses. Given the high reproductive capacity of adult amphibians and the naturally high mortality rates in egg clutches (e.g., 85 to 95 % mortality is not uncommon (Carey and Bryant, 1995)), sublethal impacts of xenobiotics on adults could be more ecologically significant than tadpole/egg survival.

The above concerns are not usually taken into account when investigations into the causes for amphibian declines are conducted. For example, probably the most heavily

studied case that used an ecological risk assessment approach to search for a cause for amphibian declines, assessed the impact of episodic acidification on amphibian populations in the West (Corn and Vertucci, 1992; Vertucci and Corn, 1996). Corn and Vertucci (1992) were unable to correlate LC50 data for pH in amphibian eggs to presence/absence data of adult amphibians, pH, and Acid Neutralizing Capacity at breeding sites. Corn and Vertucci (1992) recognized that extrapolation of their laboratory results to the field was difficult, and that tadpoles may have been subjected to sublethal and indirect effects from the acidic deposition. However, they concluded that acid deposition posed little risk to the amphibians in the Rocky Mountains. In support of their conclusion, Corn and Vertucci (1992) point out that the acidic pulse associated with the snowmelt occurs before amphibians move to the open waters for breeding. However, this suggests that the acidic pulse occurs when adult amphibians are emerging from hibernation, a physiologically stressful time period for adults. The altered water, urea, and electrolyte balance, and the occurrence of blood acidosis, from the hypometabolic state of amphibians in torpor (Pinder et al., 1992), suggest that a drop in environmental pH could interfere with the maintenance of, or arousal from, torpor. Thus, the risk posed to amphibian populations in the Rocky Mountains from acidic deposition has not been adequately assessed. This is just one example of a potential negative impact that still warrants research.

Another study that attempted to relate laboratory findings to field observations of declining amphibian populations was conducted by Blaustein et al. (1994a; 1995). This group used *in vitro* assays to determine activities of photolyase (the enzyme responsible for photorepair of DNA damage from UV-B radiation) in amphibian eggs and then conducted field manipulations of UV-B exposures with eggs of four species used in the

laboratory work. Three of the four species with the lowest photolyase activities suffered significant mortality when exposed to natural UV-B radiation (Blaustein et al., 1994a; 1995). Of the anuran species shown to be sensitive to UV-B radiation, two populations have been shown to be declining, while the status of the third is currently unknown (Dept. of Interior, 1991; Fellers and Drost, 1993; Blaustein et al., 1995). Populations of *Hyla regilla*, the species shown to have high levels of photolyase, are apparently not declining (Blaustein et al., 1994a). From an ecotoxicological perspective this study was flawed since it made no attempt to measure UV-B damage at the molecular level of organisms exposed in the field. This extra step would have provided an important link between UV-B radiation damage and individual mortality under natural conditions.

Some ecologists have questioned the significance of the study by Blaustein et al. (1994a) because they felt it was not based on an intimate understanding of the natural system (see Roush, 1995). This criticism stems from the fact that Blaustein lacked long term UV-B radiation data for his sites, even though long term UV-B radiation data at the geographic resolution required does not exist in the United States. These ecologists, who held "revisionist's" views, criticized their colleagues for placing too strong an emphasis on the use of experimental manipulations to assign cause-and-effect relationships to factors that affect natural processes (Roush, 1995). They stated that unless the experimental design and hypothesis are generated by long term field observations and the experimental system is sufficiently complex to simulate a natural ecosystem, then any conclusion drawn from such studies are without meaning (Roush, 1995). Their concerns are warranted if the system is only analyzed from the level of the organism up, since trends observed at local scales or over ecologically short time intervals may not result in larger scale

responses and may be noise or have unrelated variations (Root and Schneider, 1995). These "revisionists" recommended establishing longer observational periods, introducing more complexity, and allowing for multiple causality in experimental systems. Progress made under such a system would obviously be very slow and expensive, and the system also would invalidate any study that takes a bottom-up approach. For instance, if an area is known to be heavily impacted by some anthropogenic threat (i.e., pollution or UV-B radiation) and amphibian populations are known to inhabit these areas, but no historical records exist of population trends, then research efforts into the effects of these anthropogenic factors on the amphibian populations are not warranted if the "revisionist" view is followed.

However, what these revisionists must realize, in our opinion, is that the golden age of observational ecology occurred when the significance of human impacts on ecosystems was not well understood and molecular biology and physiology were almost solely focused on the biomedical field. Significant advances have occurred such that endpoints addressing molecular and cellular damage specific to the stressor in question can be measured in a surprising array of organisms. Employing these techniques in both laboratory and field settings greatly facilitates the determination of cause-and-effect relationships with respect to xenobiotic or UV exposure.

The emerging field of ecotoxicology strives to promote the analysis of anthropogenic impacts at multiple levels of biological complexity (i.e., from subcellular to population). However, this integrative approach still has not been applied with regard to declining amphibian populations. Few ecotoxicologists work with amphibians, which may be due inpart to the propensity of this class of organisms to undergo population

fluctuations and to have complex life history characteristics. Their complex life history pattern subjects a single organism to major shifts in dietary, habitat, and physiological requirements throughout their ontogeny. This results in a class of organisms that experience a far greater diversity of factors that can modify xenobiotic accumulation, transformation, excretion, and effects (i.e., its bioavailability) than most other vertebrates. Essentially, the life history characteristics that theoretically make amphibians good environmental sentinels, also complicate the determination of cause-and-effect relationships at multiple levels of biological organization. Authors have debated the usefulness of amphibians as bioindicators (Blaustein, 1994; Pechmann and Wilbur, 1994), while ignoring the importance of these factors, even though an understanding of the factors that modify a contaminant's bioavailability is the cornerstone of any ecotoxicological study (Sprague, 1985).

It appears that in spite of all the "armchair" biology and press coverage discussing the amphibian decline phenomenon, the proper experiments are not being conducted nor are we promoting proper methodological approaches to understand the mechanisms behind the problem. Researchers interested in linking causes and effects to conserve biological diversity must take a truly integrative approach, mixing top-down with bottom-up approaches (i.e., conduct experiments that address the impact of environmental stressors at multiple levels of biological organization under varying degrees of environmentally realistic conditions). The approaches and techniques of ecotoxicology are ideally suited to such an endeavor.

## An Ecotoxicological Approach for Understanding the Mechanisms Behind Amphibian Population Declines

To gain an understanding of the ability of amphibians to serve as environmental sentinels and to determine causes for the declines, biologists must drastically alter their current approaches. Munkittrick and McCarty (1995) offer a conceptual framework in which a hierarchical approach is taken to link cause-and-effect relationships across multiple levels of biological organization (i.e., individual, population, and community). This approach is centered around using "currency", which is a response of an organism that can be measured at two levels of biological organization, for linking cause-and-effect relationships across increasing levels of biological complexity. They define three types of effects: 1) direct, an effect resulting from chemical stress or radiation exposure; 2) nondirect, an effect generated from habitat loss or disruption; and 3) indirect, an effect generated from biological sources (competition or predation). Studies using this hierarchical approach must be driven by hypotheses examining direct effects on natural systems, i.e., pollution or UV-B radiation. Because the amphibian decline phenomenon has focused on anthropogenic impacts resulting in population level changes, our discussion will not extend to the community level. We will show that this conceptual framework does provide an excellent platform for the development of ecotoxicological studies that account for the complex natural history of amphibian populations while allowing for multiple causality.

Proper usage of this approach facilitates extrapolation of laboratory results to field observations and requires researchers to translate biochemical response to changes at the

organismal level. Therefore, this approach requires the use of biomarkers, defined as biochemical or physiological responses indicative of xenobiotic stress (for a comprehensive discussion on the benefits and limitations of a wide range of biomarkers see McCarthy and Shugart (1990), the biomarker concept has recently been extended to UV-B exposure in amphibians, see Bruggeman (1997)). In order for this hierarchical technique to work, biochemical responses must affect performance at the organismal level, i.e., growth, survival, development, or reproduction must be affected, and changes at the organismal level must affect changes in population size (Munkittrick and McCarty, 1995). If changes at one level do not affect changes at the next level, then the "currency" is said not to translate across the levels of biological complexity. This may result if indirect or nondirect effects are stronger in the natural system then the direct effect at a given exposure dose. Further, antagonistic relationships may be present with another direct factor or nondirect or indirect factors. However, if stronger organismal effects are observed in the field at a given exposure dose than in the laboratory, then additive or synergistic relationships with other direct, nondirect, or indirect factors may be involved. Both observations would result in a re-focusing of the study.

In order to implement this approach, as much historical information on the direct effect in question must be obtained, from laboratory settings to studies of its effects and behavior in the field. Historical information on the species, population, and geographic area in question are equally important. However, because toxicological responses of herpetiles is a lightly studied area, and baseline data for many amphibian populations do not exist, a paucity of information in either the biological, chemical, or physical component should not prevent the initiation of a study. The selection of a proper reference site (i.e., a

geographica area with similar habitat features, but lacking direct factors) should also be made using this historical information. Depending on the nature of the direct factor in question, the use of a reference site may not be possible (e.g., UV-B radiation). Other factors, such as historic or current landuse patterns, will likely complicate the identification of a reference site with similar habitat features, but one should be used if possible. We recommend a three-tiered approach for linking cause-and-effect relationships from a direct factor's effect on an organism to changes in the population, using the principles put forth by Munkittrick and McCarty's hierarchical paradigm.

In the first-tier, microcosm/benchtop experiments should be conducted with eggs, larvae, and adults in a controlled laboratory environment. These life stages should be exposed to the direct factor at environmentally relevant doses through the natural medium of exposure (i.e., the soil, water, or an exposed food source). The source of exposure, either directly to the species of interest or to the food source, should be from soil or water collected at impacted and reference sites. Samples should be taken from the sites at predetermined time intervals, with the greatest concentration of sampling occurring during the breeding season. The adults of many amphibian species are known to occupy different habitats depending on the season. Therefore, it is important for adults to be exposed in the medium where they are found when the field sample is taken. Exposures in the laboratory should also include a spike (i.e., a chemical-grade source of the xenobiotic or classical inducers of the organ systems the xenobiotic is known to affect) so that proper positive controls are in place. The usage of all life stages in the laboratory is needed to determine the ontogenetic stage that is most vulnerable to the factor in question. A potential problem with this approach is the effective long term animal husbandry needed to

house adults for extended periods of time. However, this is an important step in light of the prevalence of environmental estrogens that are known to alter sexual development (Palmer and Palmer, 1995). These first-tier benchtop experiments should also be designed to link biomarker response to organismal endpoints (i.e., growth, survival, terata, sexual development, and/or reproductive ability). This funneling of biochemical responses to organismal endpoints is needed to use the "currency" concept correctly, so that any negative effects can be translated to the next level of biological complexity (Munkittrick and McCarty, 1995).

The second-tier should involve experimental manipulations of exposure to putative direct factors in aquatic mesocosms or terrestrial enclosures at impacted and reference field sites, and should focus on the life stage that was determined to be the most sensitive in the laboratory. Again, growth, mortality, terata, sexual development, and reproduction can be examined at this tier. At the end of the predetermined exposure period the organisms can be harvested for analysis of the biomarkers chosen at the laboratory tier. This facilitates the estimation of exposure dose at the field site. At this tier antagonistic, synergistic, or additive effects may become evident that were not involved in the benchtop experiments. This may result if the contaminant mixture or chemical undergoes chemical speciation or photolysis under natural conditions, or if other factors (direct, nondirect, or indirect) are affecting the organisms in the study. The estimation of exposure dose via the biomarkers is an important tool in determining if other factors are clouding the system. For example, if the biomarker was to show no response but significant mortality was observed compared to the reference site, this may indicate that the direct factor was poorly chosen, or is modified to another compound in the field.

In the third-tier, populations are monitored at impacted and reference sites. The researcher should monitor all possible life stages of the species of interest in the field. The naturally high mortality rates at early stages of development (a natural bottle-neck typically occurs at hatching and/or metamorphic climax) supports the need for a diverse monitoring program, so that natural mortality can be divorced from human-induced lethality. This diverse monitoring program would refute or support the conclusions of benchtop experiments, which indicated the most vulnerable life stage. Admittedly, the diversity of breeding habitats and egglaying, tadpole, and adult behaviors observed in Amphibia do not make the task easy (McDiarmid, 1994). Mark and recapture techniques are time- and effort-intensive and can be applied most easily to adult amphibians (Donnelly and Guyer, 1994). The application of these techniques to earlier stages of amphibians is impractical. Therefore, the estimation of population size and demographic information (i.e., sex ratio, recruitment, survival, and migration) can only be obtained for adults. Estimates of relative abundance and/or density can be obtained for larval amphibians (Shaffer et al., 1994). Reproductive activity can be estimated by the number of amplexing adults and the number of eggs laid in masses not hidden by vegetation (if applicable). Organismal endpoints of growth, survival, terata, and reproductive activity are still the currency linking this tier to the other two. If these organismal endpoints were evident at the second-tier, but are absent from this tier, then other direct, nondirect, or indirect factors may be confounding the analysis. Such a situation is most likely to occur at the third-tier, since the degree of ecological complexity and environmental reality have increased (i.e., increasing the probability of natural stochastic variation clouding the responses of the system).

This three-tier approach using the "currency" concept increases the confidence with which biologists can elucidate cause-and-effect relationships pertaining to the mechanisms behind the global decline of amphibian populations. This approach also allows for multiple causality at varying degrees of ecological complexity (Munkittrick and McCarty, 1995). For instance, this method may indicate whether metapopulation dynamics are maintaining a persistent adult presence in spite of poor habitat patch quality. Such a conclusion would follow if the results from the microcosm and mesocosm experiments would show high mortality directly related to the direct effect, as indicated by the biomarker. Further, the abundance of adults at the impacted site would be comparable to the reference site, while the abundance of the earlier stage organisms differed across sites. If a reference site is not used, this causation can still be determined, but with a lower degree of confidence. Without a comparative reference site, based on microcosm, mesocosm, and demographic information, low recruitment would be observed at the earlier life stages attributable to the direct factor. Further, the demographic information from adult population monitoring would indicate the absence of juveniles (metamorphs). Other approaches which address population changes without organismal and biomarker effects, or organismal and biomarker effects without population changes, may overlook environmentally significant interactions, especially over ecologically short time scales.

## Conclusion

No experimental evidence has yet been published that indicates amphibians can serve as reliable bioindicators at higher levels of biological organization. The paucity of

ecotoxicological theory and methods has precluded this determination and has spurred debate in the literature. In light of the apparent rates of declines in amphibian biodiversity, new approaches must be implemented in the immediate future. The hierarchical, three-tiered approach we advocate, will allow the scientific community to determine with greater confidence whether amphibian population fluctuations and/or declines are a function of the biotic and abiotic histories of their local habitat, or if anthropogenic factors are the cause.

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Chapter 2

# LINKING DNA PHOTODAMAGE, TERATOGENESIS, AND GROWTH TO ARTIFICIAL UV-B RADIATION DAMAGE IN ANURAN LARVAE

## Introduction

Conservative atmospheric models predict that maximal ozone depletion will occur by 1998, and a 50-year recovery period is expected before ozone levels return to normal (Madronich et al., 1995). Organisms which have had a long evolutionary history with naturally higher levels of UV-B radiation have adaptations to mitigate that stress (Robberecht et al., 1980; Sullivan et al., 1992). However, due to wavelength specific changes in the solar spectrum that results from reduced ozone levels, the ratio of UV-B (280-315 nm) to UV-A (315-400 nm) is significantly altered such that the UV-B component increases with the greatest change at the shorter wavelengths and without a concomitant increase in the UV-A component (Molina and Molina, 1986). This distortion in solar radiation reaching the earth's surface increases the biologically damaging effects of UV-B radiation for two reasons. First, Setlow's DNA action spectrum, which calculates the carcinogenic effectiveness of UV-B as a function of wavelength, indicates that greater molecular damage per photon occurs at the shorter wavelengths (Setlow, 1974). Secondly, an organisms ability to mitigate this damaging effect on DNA through photorepair, which is dependent on the enzyme photolyase absorbing a photon of UV-A

or near visible radiation, is not enhanced because the photons in this region have not increased (Molina and Molina, 1986). Williamson (1995) has termed this "solar ambush" since organisms that are unable to detect changes in UV-B radiation and/or are unable to behaviorally respond to changes in the solar spectrum will be selected against given a decrease in stratospheric ozone levels. Amphibian eggs laid in the open, upper surface layers of freshwater ecosystems exemplify this vulnerability to "solar ambush".

Worrest and Kimeldorf (1976) were among the first to examine the effect of environmentally relevant UV-B doses on early life stages of anurans. Their study illustrated that artificial UV-B can induce malformations and mortality in *Bufo boreas* tadpoles. It is important to note that after hatching under the simulated UV radiation environment, Worrest and Kimeldorf removed 70% of the eggs originally exposed, leaving only the most vigorous individuals for the remainder of the study, thus ignoring the impact of UV-B radiation on the earliest stages of development. Significant responses were not observed until after 55 days of irradiation (Gosner stage 35). Blaustein et al. (1994; 1995) illustrated the lethal effects of UV-B radiation at the hatching stage under field conditions with three species of amphibians, but found a fourth species to be tolerant to UV-B. In addition, they assayed the eggs of ten amphibian species for constitutive levels of photolyase. Among the four species they used in their studies, the one with the highest level of photolyase had the highest survivorship when ambient UV-B was not blocked, and the other three with lower photolyase levels experienced significantly more mortality in this same treatment. (Blaustein et al., 1994; 1995). Two of the anuran species shown to be sensitive to UV-B radiation (*B. boreas* and *Rana cascadae*) are experiencing declines (Blaustein et al., 1994). Further, research by Kiesecker and Blaustein (1995) and Long et

al. (1995) showed that UV-B radiation can act synergetically with fungal infections and low pH, respectively, to significantly raise mortality higher than the effect of UV-B, fungal infection, or low pH alone. However, Grant and Licht (1995) only found significant mortality and malformations in the embryonic and larval stages of anuran species when exposed to unshielded artificial UV-B lamps, but not with environmentally relevant wavelengths and doses of UV-B radiation. The significance of contradictory results with UV-B exposures with amphibian species may be elucidated by more complex studies. For example, Hays et al. (1996) outlines an experimental approach for determining if light conditions under which females mature affect photolyase activities in eggs and oocytes in three anuran species. The study by Hays et al. (1996) is a long term project that will aid in determining if anurans are capable of adapting to changes in light conditions in their environment.

Organisms mitigate the damaging effect of UV-B radiation through protective outer coverings, pigmentation, behavioral avoidance through habitat selection, and/or by enzymatic repair of DNA photoproducts (Mitchell and Karentz, 1993). The latter is particularly well studied, since DNA is the primary chromophore for ultraviolet light (Setlow, 1974; Achey et al., 1979; Chan et al., 1986; Mitchell and Karentz, 1993). The most common form of DNA damage resulting from a UV insult is the cyclobutane pyrimidine dimer, since this photoproduct is readily induced at the relatively longer UV-B wavelengths found in the environment (Mitchell and Karentz, 1993). Achey et al. (1979) devised a simple technique to illustrate the differential effects that UV-C (0-280 nm) and UV-A radiation have on cellular DNA. Single cells derived from thyroid tissue of the Amazon Molly (*Poecilia formosa*) were irradiated with UV-C followed by differential

irradiation with UV-A. The DNA was then extracted from the cells and incubated with a pyrimidine dimer specific (UV) endonuclease (purified from *Micrococcus luteus*), and subjected to alkaline agarose gel electrophoresis. Molecular lengths on the alkaline gel were positively correlated with the amount of UV-A exposure, after insult with the UV-C. Therefore, the average nucleotide length for the single stranded DNA is inversely proportional to the frequency of pyrimidine dimers. Freeman et al. (1986) further developed this assay by deriving a mathematical model which determines the endonuclease sensitive sites per kilobase (ESS/Kb) based on the average molecular lengths for a sample run in parallel (i.e., DNA treated and untreated with the *M. luteus* endonuclease). ESS/Kb can be used as a direct estimate for the frequency of cyclobutane pyrimidine dimers. D'Surney et al. (1993) used this assay to illustrate the importance of UV-B absorbing pigments in the leaves of two soybean cultivars. The cultivar with the limited ability to produce UV-B absorbing pigments had reduced total biomass and a higher frequency of ESS/kb. Currently, researchers at Oak Ridge National Laboratory are using this assay to examine the impact of enhanced UV-B radiation in high northern latitudes on the DNA of sea urchin embryos (C. Theodorakis, personal communication).

In spite of the seminal work by Worrest and Kimeldorf (1976) and the field work by Blaustein et al. (1994; 1995), researchers have not developed a dose-response relationship between UV-B radiation and teratogenesis in the early stages of anurans. The ecological significance of increased levels of UV-B radiation and the current decline in amphibian biodiversity provide a strong impetus for examining this dose-response relationship and the ASTM (1992) FETAX protocol could provide a means. *Xenopus laevis* embryos could be exposed to different doses of UV-B and UV-A radiation during

the first 96 hr of development and developmental effects could be moniored. In addition, the work of Achey et al. (1979), Freeman et al. (1986), and D'Surney et al. (1993) indicated that the frequency of cyclobutane pyrimidine dimers can be measured in DNA from a variety of organisms and tissue cultures irradiated with UV-B. However, the technique has not been applied previously to amphibians and has potential for revealing molecular adaptations to UV-B radiation. Because cellular death or mutation may result from the presence of cyclobutane pyrimidine dimers during transcription or replication of DNA (Hearst, 1995), it is likely that the frequency of ESS/Kb can be correlated with the incidence of terata in *Xenopus laevis*. Thus, the objective of the present study was to develop sublethal endpoints indicative of UV-B radiation exposure, so that more diverse and informative biological information can be obtained from UV exposure studies conducted with larval anurans. Two experiments were conducted using the same fundamental methods.

## Materials and Methods

### *Xenopus* exposure to UV radiation

The FETAX ASTM (1992) guide was followed except for the following modifications. Stage 8-11 eggs (collected from *X. laevis* bred in the laboratory) were irradiated under a light carriage, consisting of five 15-watt ultraviolet fluorescent bulbs (Ultraviolet Products Inc., San Gabriel, CA, U.S.A.), 3 UV-A (peak emittance at 365 nm) and 2 UV-B (peak emittance at 302 nm). These lights were suspended with ropes above a

cardboard mat sectioned into a grid (5x10 cells). The irradiance rate in each cell was measured using a UVX radiometer (Ultraviolet Products Inc., San Gabriel, CA, U.S.A.) equipped with a UV-B and/or UV-A broad band probe covered with a cellulose triacetate or Mylar cutoff filter. Based on measured irradiance, the dishes containing the embryos were arranged below the lamps to minimize variance in UV-B and UV-A irradiances between the treatments (Bjorn and Teramura, 1993). This step was required due to the heterogeneity in irradiances that are generated by fluorescent tube arrays (Bjorn and Teramura, 1993).

Plastic cut-off filters were used to modulate the UV-B dose received by the embryos. All plastic UV cutoff filters were preirradiated for 24 hr prior to use in order to obtain a stable transmittance, and were discarded after 200 hr of irradiation due to the decrease in transmittance that occurs with solarization (Steeneken et al., 1995). Based on a spectral scan performed by a spectrophotometer, after irradiating the plastic for 24 hr, Mylar (0.18 mm, Hilcor Plastics, Baldwin Park, CA, U.S.A.) excludes all wavelengths less than 315 nm (i.e. exposure to UV-A only) and the cellulose triacetate (acetate) (0.19 mm, Hilcor Plastics, Baldwin Park, CA, U.S.A.) excludes all wavelengths less than 290 nm (i.e. exposure to both UV-A and UV-B, while excluding UV-C).

The ratio of UV-A to UV-B present under the light carriage and as perceived by the amphibian eggs was determined by the following equation:

$$(IR_{\text{UV-B acetate}} - IR_{\text{UV-B Mylar}})/IR_{\text{UV-A Mylar}} = \text{UV-B (290-315nm):UV-A (315-400nm)} \quad (1)$$

Where $IR_{UV\text{-}B\ acetate}$ is the irradiance rate observed when the UV-B probe is covered with acetate; $IR_{UV\text{-}B\ Mylar}$ is the irradiance rate observed when the UV-B probe is covered with Mylar; and $IR_{UV\text{-}A\ Mylar}$ is the irradiance rate observed when the UV-A probe is covered is covered with Mylar. The resulting ratio is a 1:5 relationship of UV-B to UV-A. Under natural ozone conditions a 1:10 ratio is typically observed (Molina and Molina, 1986). However, since a doubling in UV-B irradiance can be expected from a 10% decrease in ozone column thickness, the UV light regime present in this experiment is not unlikely given current atmospheric conditions (Frederick et al., 1989).

Five treatments were applied as follows: 8 hr black plastic (control, transmitting no light); 8 hr Mylar; 4 hr acetate, 4 hr Mylar; 6 hr acetate, 2 hr Mylar; and 8 hr acetate. This treatment structure was chosen based on observations by Worrest and Kimeldorf (1976) that indicated the duration and placement of UV-A radiation with respect to the UV-B radiation had a tremendous impact on the survivability of *B. boreas* tadpoles to stage 35. These data indicated that the presence of only UV-A, after the UV-B insult, greatly increased the survival of the tadpoles, thus supporting the role of UV-A radiation in the mitigation of UV-B photodamage at the genomic level (Mitchell and Karentz, 1993). Therefore, the treatment structure used in this study allows for differential photorepair of UV-B damage. Further, the total UV-B daily doses used (see Table I) are less than the total daily, mid-summer UV-B dose for temperate North America, 37.5 $KJ/m^2$ (calculated based on data from R-B UV-B Meters given in Worrest and Kimeldorf, 1976). Each treatment was applied to two Petri dishes (25 embryos each) by covering them with black plastic, Mylar, acetate, or acetate and then Mylar at different time intervals.

The control treatment, consisting of two replicates of two Petri dishes each were always placed at the bottom of the grid where the black plastic was permanently taped in place. The position effect precludes placing the controls in the same arrangement as the light treatments. All other treatments were blocked by randomizing two Petri dishes in each half of the exposure chamber. The temperature was maintained at 24 ± 1°C. Throughout the duration of the exposure, the temperature and UV-B irradiance rate were monitored four times daily. The UV-A irradiance was monitored only at the start and finish of each exposure. Brown lights (Kodak Safelight filters 6B, Rochester, NY, U.S.A) were used when removing dead organisms or changing the FETAX solution in order to provide illumination while excluding wavelengths less than 560 nm (i.e., preventing photoreactivation of pyrimidine dimers outside of the light treatments). Each day dead embryos were removed and the FETAX solution was renewed. At the end of 96 hr (stage 46, appearance of the hindlimb bud; Nieuwkoop and Faber, 1994) the tadpoles were anesthetized with MS-222, placed in 3% formalin, scored for terata and mortality, and the length was measured using Sigma Scan software (Jandel Scientific, Corte Madera, CA, U.S.A).

Measuring DNA Photodamage

The frequency of pyrimidine dimers in DNA (Achey et al., 1979) was determined in 48-hr larvae (stage 32-34, hatching; Nieuwkoop and Faber, 1994) collected immediately after the UV lights were turned off at the end of day two from the teratogenic exposures. Ten *Xenopus* tadpoles from each treatment (five from each dish, leaving 20) were placed

in an amber microfuge tube, the majority of the FETAX solution was removed, 250 µl of DNA extraction buffer (0.25 M NaCl, 0.1 M EDTA, 0.1 M Trizma base, pH 8.0) was added, and the tadpoles were frozen at -80° C. The tadpoles were homogenized with a plastic pestle, and 30 µl 10% Sarcosyl was added to lyse the cells. The homogenate was incubated with RNase (Sigma Chemical Co., St. Louis, MO, U.S.A)(0.67 µg/µl; for 20 min. at 37°C) followed by Proteinase-K (Promega, Madison, WI, U.S.A.)(0.06 µg/µl; 30 min. at 37°C). A phenol/chloroform DNA extraction was performed, followed by one additional extraction, and a final 24:1 chloroform:isoamyl alcohol extraction. The DNA was precipitated with 100% ethanol and stored at -20° C at least over night. The DNA was pelleted and resuspended in TE buffer (0.01 M Trizma base, 0.001 M EDTA) pH 6.5 (the optimum pH for *M. luteus* endonuclease activity). The purity of DNA was determined by spectrophotometry by examining $A_{260}/A_{280}$ ratios. The concentration of DNA was estimated by $A_{260}$ values, where 1 $A_{260}$ = 50 µg DNA/ml (Sambrook et al., 1989).

A 1.4 µg aliquot of the DNA was removed from each treatment in duplicate. One duplicate of each sample was incubated with *M. luteus* UV specific endonuclease (1.25 µg/µl, C. Theodorakis, ORNL) at 37°C for 1 hr NaOH was then added to a final normality of 0.1 to produce single stranded DNA. Two aliquots of DNA (0.80 µg) from each of the five treatments per block (i.e., the 2 original samples prepared in parallel, treated and untreated with endonuclease) were subjected to alkaline gel electrophoresis, along with molecular weight standards and positive control *X. laevis* DNA that was irradiated with 260 nm light in a quartz cuvette with a spectrophotometer for five hours,

on 0.4% agarose gel for 12 hr at 1.3 volts/cm. Upon completion of the 12 hr run, the gel pH was neutralized in 300 mM Tris-HCl, 1.5 M NaCl pH 7.6 and stained with SYBR Green II (diluted in 1X TBE). The gel was placed on a transilluminator and photographed with Polaroid 665 positive/negative film. The negative was scanned into a PC as a TIFF image, and NIH Image 6.1 was used to obtain densimetric traces of all lanes in each gel. For the standards, the peaks were identified and a standard curve was generated using nonlinear regression. The endonuclease sensitive sites per kilobase (ESS\Kb) were calculated by the method of Freeman et al. (1986).

Data Analysis

The teratogenesis and growth data were pooled for the two Petri dishes in each treatment within each block. A two-way ANOVA was performed with both endpoints to evaluate treatment and block effects. The presence of a block effect would indicate that the experiment did not adequately control for the position effect under the fluorescent lights. Fisher's LSD was employed to determine MCIG (the minimum UV dose required to inhibit growth based on the length of controls). Multiple non-linear regression was applied to the teratogenesis data in experiment 1 in order to derive a dose-response surface, since both the UV-A and UV-B dose increase across all three exposures. For experiment 2, simple linear regression was employed to determine the dose-response relationship between terata and UV-B dose.

For the genetic endpoint of ESS/Kb, a mixed model analysis of a two-way factorial treatment structure in a split-plot design was conducted. The dependent variable analyzed

was ESS/Kb. Main effects of Treatment (fixed effect), Clutch (random effect), Treatment by Clutch interaction, and Block effects were tested.

Experiment 1

Embryos/tadpoles were exposed to UV for 8 hr/day for all five days (to stage 46, appearance of the hindlimb bud) of the test with the treatment structure and daily dosages given in Table I. The light carriage was lowered after each test in order to increase the UV-A and UV-B doses to evaluate the dynamic range of the endpoints of mortality, teratogenesis, growth, and DNA photodamage. The irradiance rates chosen were 0.75, 0.95, and 1.15 J/s/$m^2$. After the tadpoles were collected and frozen for genetic analysis at the end of day 2, the remaining tadpoles were exposed for the remaining 3 days, until tadpole stage 46 was attained. At this point the tadpoles were fixed in formalin, scored mortality, malformations, and growth.

Experiment 2

This experiment differs by consisting of three UV-B FETAX exposures at the same light intensity (1.26 J/s/$m^2$). The daily doses are given in Table I. After removal of samples for genetic analysis, the remaining tadpoles were covered with black plastic (to prevent exposure to stray visible light which may enhance photorepair in the DNA adducts) and placed in the incubator (24°C) for the remaining 3 days, until stage 46 was reached. At this point the tadpoles were fixed in formalin, scored for mortality,

malformations, and growth. This methodology was adopted in order to directly relate the same total UV-B dose to terata, growth and DNA damage.

## Results

### Mortality

In experiment 1, average mortality was below 10% in all treatments and tests except for the 1c exposure (when an irradiance rate of 1.15 J/s/m$^2$ was used, see Table I). In this test, the 6 hr acetate/2 hr Mylar treatment had an average mortality of 10% and the 8 hr acetate treatment had a 23.5% average mortality. In experiment 2, mortality was not high enough to calculate an LD50, but was often higher than 10% in the following tests at the 8 hr acetate treatment: 2a, 20% average mortality; 2b, 14% average mortality; and 2c, 29% average mortality.

### Terata

Results of the two-way ANOVA indicate that no block effect was present in experiment 1 or 2 ($p > 0.05$). Significant treatments effects were observed in experiment 1 with increasing strength as UV-B irradiance rate increased (1a, $p = 0.04$; 1b, $p = 0.01$; 1c, $p = 0.001$). Therefore, terata data were pooled for each treatment at each date for multiple non-linear regression analysis. Pooling these data allows determination of the importance of UV-A dose and UV-B dose in inducing terata, since the dose of both

classes of UV changed over the three tests. The data and resulting equation is given in Figure 1. These results indicate that UV-B dose was the major factor inducing terata. Even though UV-A is shown to have a minor impact, based on autocorrelation, UV-A always increased with higher UV-B doses.

Significant treatment effects were observed in all three tests in experiment 2. Therefore, terata data for all three tests per treatment were pooled, simple linear regression was employed and the resulting equation was used to calculate an ED50 of 20,300 $J/m^2$ UV-B radiation (see Figure 2).

Figure 3 illustrates terata consistent with UV-B exposure, which includes: axial malformations (i.e. curling of the tail and kinking of the notochord), non-spherical eyes (which sometimes included pigment extending onto the optic nerve), uncoiled intestines, and blisters developed on the tail of the tadpoles.

Growth

In experiment 1, no block effects were observed with the endpoint of growth, and treatment effects were significant for the 1a and 1b exposures ($p = 0.002$ and $p = 0.005$) but not with the 1c exposure ($p = 0.074$). MCIG values varied for all three tests (Table II), 1a MCIG equaled the 4 hr acetate/4 hr Mylar treatment ($p = 0.0068$), 1b MCIG equaled the 8 hr Mylar treatment ($p = 0.039$), and 1c MCIG equaled the 8 hr acetate treatment ($p = 0.018$).

No block effect was observed in experiment 2 ($p > 0.05$). Two of the three tests in experiment 2 had significant treatment effects based on growth, 2a ($p = 0.022$) and 2c ($p =$

0.008), but no significant treatment effect was observed on 2b ($p = 0.062$). For 2a and 2c the MCIG value occurred at the 6 hr acetate/2 hr Mylar treatment ($p = 0.037$ and $p = 0.008$), and on 2b the MCIG was the 8 hr acetate treatment ($p = 0.016$).

DNA Photodamage

Background endonuclease activity was present in our controls in all tests (see Figure 4). The data for experiment 1 and 2 were analyzed in two forms: background activity subtracted from the ESS/Kb values in the light treatments, and not subtracted, therefore including the responses of Control treatments in our analysis. The results for both forms of data when subjected to the two-way mixed model ANOVA were effectively the same. Therefore, only the results for data without background subtraction are reported. For experiment 1, the two-way split-plot mixed model ANOVA indicated no Clutch effect ($p = 0.460$) (which is interchangeable with UV-B Irradiance Rate effect in these tests), no Block effect ($p = 0.167$), no Clutch by Treatment interaction ($p = 0.689$), but a significant Treatment effect was observed ($p = 0.001$). A posteriori comparisons of ESS/Kb values for treatments pooled across all three tests, showed that Control and 8 hr Mylar treatments differed significantly from the 6 hr Acetate/2 hr Mylar and Positive Control DNA treatments and that 8 hr Mylar, 4 hr acetate/4 hr Mylar, and 8 hr acetate treatments differed significantly from the Postive Control DNA. The control differs significantly from all UV-B treatment groups. The ESS/Kb value for the 8 hr acetate treatment did not differ significantly from the lower dose treatments of 8 hr Mylar and 4 hr acetate/4 hr Mylar ($p > 0.05$).

Graphical interpretation of the data for experiment 2, indicated severe variation between clutches (see Figure 5). Therefore, a Levene's test was performed to test for unequal variances among all three exposures, and found significant differences ($p = 0.0001$). The 2b exposure had higher variance than the other two, therefore, these data were excluded from the two-way mixed model ANOVA. The results for experiment 2, excluding the 2b data, indicate that no Clutch, Block, or Treatment effects were observed ($p > 0.05$). The data from exposure 2b were analyzed alone and no Block or Treatment effects were observed ($p > 0.05$).

Linking Terata to DNA Photodamage

Correlation analysis was performed on the independent variables of percent malformed and ESS/Kb for experiment 1 (see Figure 6). A significant correlation was found ($R^2 = 0.2635$; $p = 0.0037$). Influence diagnostics were performed on this correlation analysis and no points were found to be overly influential. This indicates that no points in the scatter plot are true outliers (Figure 6).

## Summary and Conclusions

UV-B dose was positively correlated with terata in both experiment 1 and 2. However, the results obtained for growth and the genetic endpoint of ESS/Kb were not as consistent. The absence of a block effect in all analyses indicated that no position effect was present under the light carriage in any tests. The frequency of terata increased within

and across all three tests in experiment 1 based on UV-B dose, and the multiple non-linear regression analysis indicated that UV-B dose was a greater determinant of terata than UV-A dose. Growth inhibition did not show a similar response in experiment 1. Significant growth inhibition was present in the first two exposures, but not at the highest UV-B irradiance rate (exposure 1c). The MCIG value observed at a UV-B irradiance rate of 1.15 J/s/m$^2$ (1c) was at a much higher UV-B daily dose (8 hr acetate) than was observed in the other two tests (4 hr acetate and 8 hr Mylar), indicating that UV-B irradiance rate did not have a significant effect on growth. This indicated a decreased susceptibility of growth in this egg clutch at higher UV-B doses. The results for the genetic analysis in this experiment were similar, i.e., no irradiance rate or clutch effect was present, but when the data were pooled across all three clutches a significant treatment effect was found. A decrease in susceptibility to UV-B damage at higher doses can also be qualitatively observed in the histogram of DNA Photodamage (Figure 5), possibly indicating a saturation in response. The correlation analysis linking DNA photodamage to frequency of terata was significant, but at a low $R^2$ value of 0.26. Nevertheless, the analysis does generally indicate results in agreement with our predictions. Future efforts to improve the genetic analysis are warranted and potential sources of error are discussed below.

Teratogenesis was positively correlated with UV-B dose in all three tests of experiment 2. Figure 2 indicates that the frequency of malformations in the egg clutch used for 2b was less than the other two tests used in experiment 2. The lower vulnerablity of this egg clutch is supported by the growth data, since no treatment effect was observed with the endpoint of growth for 2b, unlike the results for the other two tests. The data for ESS/Kb also indicated anomalous responses for this egg clutch compared to the other

two. Figure 5 indicates that this egg clutch had higher mean response of ESS/Kb, but also with greater variability, as supported by the Levene's Test. The responses for all three endpoints in the exposures 2a and 2c are more similar to each other. Since no treatment effect was observed with the endpoint of ESS/Kb, no attempt was made to correlate DNA Photodamage to teratogenesis.

Teratogenesis appeared to be the most reliable endpoint for exposure to UV-B radiation. The terata indicative of UV-B exposure were constant across all tests, with the axial malformaties being the most prevalent. Axial malformaties in anuran larvae developing under UV-B stress have been described by other researchers (Hays et al., 1996; Grant and Licht, 1995; Worrest and Kimeldorf, 1976). The abnormal development of the eye was also described by Worrest and Kimeldorf (1976). However, abnormalities such as uncoiled intestines and blisters on tails have not been previously reported and are probably more easily observed with *X. laevis* tadpoles than with species indigenous to North America, which have historically been used in these studies. In addition, an increase in pigmentation was often observed in all UV light treatments, but varied tremendously among all individuals used in the study. The deviation of the teratogenesis data from the data for the other two endpoints (especially DNA photodamage) may be explained by the harmful effect of UV-B radiation on other macromolecules besides DNA. Protein and RNA are also known to absorb wavelengths in the UV-B range, and can be found in abundance in developing embryos (Davidson, 1976). RNA synthesis begins after the mid-blastula transition in *X. laevis* embryos, resulting in the accumulation of 10 times the amount of total RNA than DNA by stage 14, neurula (Davidson, 1976). Modification of these macromolecules, via dimerization, or the inhibition of transcription or enzymatic

activity (Smith, 1977) in developing embyros exposed to UV-B, likely has lasting effects on the organisms.

The multiple non-linear regression analysis used for the teratogenic endpoint in experiment 1 indicates a significant quadratic relationship with the UV-B axis (Figure 1). This results from fewer malformaties occurring at low UV-B doses than in the Control or Mylar treatments in the first two experiments, which may have been due to reduced bacterial contamination in the UV-B treatments. Bacterial contamination is a common problem encountered when conducting FETAX tests (Bantle, 1995). One of us (D.J.B.) has observed increased survival of *X. laevis* embryos in UV-B radiation treatments over Mylar and Control treatments when UV-B FETAX tests were unintentionally conducted with eggs heavily contaminated with bacterial and fungal agents. Both of these observations indicate that the bacterial and fungal agents found in our laboratory environment were more susceptible to UV-B radiation than the amphibian eggs. Kiesecker and Blaustein (1995) found a synergistic effect between UV-B radiation and a fungus in the field. However, our preliminary observations indicate that an antagonistic effect could also be present under certain conditions.

The inconsistent results for the genetic endpoint of ESS/Kb (i.e., the frequency of pyrimidine dimers) may be explained by three factors. Historically, this assay has been applied to tissue cultures or to epidermal layers removed from whole organisms (i. e., hairless mice, Lan et al., 1995). In our technique, we collected the hatching stage tadpoles for genetic analysis since this stage most strongly resembles a thin tissue culture (i.e., the surface area to volume ratio is high at this stage). However, based on the variability of these results it is likely that the genomic subsamples used for the assay may have been

from regions of the tadpole not directly exposed to UV-B radiation. Additional factors may explain why the range of, and variation in, response varies between different exposures. The first may be attributable to variation in the endonuclease used in each test. One aliquot of the enzyme was used for each test shortly after removal from the freezer (within 10 min.), in order to control for this potential variation since thawing time is thought to effect enzyme activity (C. Theodorakis, personal communication). However, variation between aliquots may have been present. The last factor relates to the species used in this study. Low levels of photolyase activities with significant variability have been observed in *X. laevis* pro-eggs (33% C.V.) and skin extracts from adults (values ranged from 0.03-0.08 cyclobutane pyrimidine dimers (CBPD) removed/hr/μg protein X $10^{11}$), but only a 5% C.V. was observed when eggs of *X. laevis* were assayed for photolyase activity (Blaustein et al., 1994; Hays et al., 1996). Information regarding the number of egg clutches pooled for these analyses and number of adults used for breeding would be beneficial in determining the source of this variation reported by Blaustein et al. (1994). In our study, eggs from a different set of adults were used for each exposure. Females used in experiment 1 and exposure 2c were obtained from a commercial supplier who directly imports the frogs from South Africa. The females used in the other two exposures of experiment 2 were obtained from a supplier who rears *Xenopus* indoors. Since *Xenopus* used in experiment 2 were obtained from two different commercial sources, this may have attributed to the egg clutch effect indicated by the Levene's test.

This is the first report of the determination of DNA photodamage in amphibian larvae exposed to UV-B radiation stress. This genetics assay can be a powerful tool to determine species specific repair capabilities, how repair capabilities of anurans vary

through ontogeny, or to what extent UV-B radiation plays a role in amphibian declines. The latter would rely on employing this assay as a biomarker of UV-B exposure to organisms collected in the field. However, since background nicking is commonly observed in this assay, a field exposure must have the proper controls in place. Further, since this assay only measures DNA adducts (cyclobutane pyrimidine dimers, which are transitory depending on the rate of repair and length of the cell cycle) and not mutations, the ambient UV-B and UV-A dose at the time of collection are important parameters to be noted. The maximum ratio of UV-B to UV-A occurs at local noon with UV-B irradiances dropping more rapidly than the UV-A in the afternoon (D.J.B., personal observation). Therefore, photorepair appears to be well adapted to climatic conditions and must be accounted for in field exposures attempting to measure DNA adducts.

In conclusion, we have shown that sublethal endpoints can be used as indicators of organisms exposed to harmful doses of UV-B radiation. The observation of these sublethal responses within experimental designs will aid in determining the ecological significance of UV-B radiation, by indicating adaptations designed to mitigate the UV-B damage or by indicating overt damage caused specifically from UV-B radiation exposure.

**Table I**. The treatment structure and daily UV doses in $J/m^2$ for Experiments 1 and 2.

| | Experiment 1 | | | | | | Experiement 2 | |
|---|---|---|---|---|---|---|---|---|
| Test | 1a | | 1b | | 1c | | 2a, 2b, 2c | |
| Treatment | UV-A | UV-B | UV-A | UV-B | UV-A | UV-B | UV-A | UV-B |
| 8 hr. Black Plastic | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 8 hr. Mylar | 37440 | 0 | 47520 | 0 | 56736 | 0 | 65952 | 0 |
| 4 hr. acetate/4 hr. Mylar | 37440 | 10656 | 47520 | 13392 | 56736 | 15696 | 65952 | 18144 |
| 6 hr. acetate/2 hr. Mylar | 37440 | 15984 | 47520 | 20088 | 56736 | 23544 | 65952 | 27216 |
| 8 hr. acetate | 37440 | 21312 | 47520 | 26784 | 56736 | 31392 | 65952 | 36288 |

UV-A measurements were taken with a UV-A broadband probe covered with Mylar. UV-B measurements were taken with a UV-B broadband probe covered with cellulose triacetate. Therefore, the measured dose includes some residual response of the probe to UV-A.

**Table II**. Average tadpole length in percent of Controls.

| | Treatment | | | |
|---|---|---|---|---|
| Test | 8 hr Mylar | 4 hr acetate | 6 hr acetate | 8 hr acetate |
| 1a | 98.41 | 96.08* | 95.05 | 91.34 |
| 1b | 97.98* | 96.51 | 95.26 | 94.10 |
| 1c | 99.97 | 97.28 | 96.71 | 93.04* |
| 2a | 98.52 | 94.10 | 92.23* | 85.65 |
| 2b | 99.34 | 98.87 | 95.39 | 91.57* |
| 2c | 100.00 | 97.60 | 92.52* | 89.84 |

* MCIG values (the minimum UV dose required to inhibit growth)

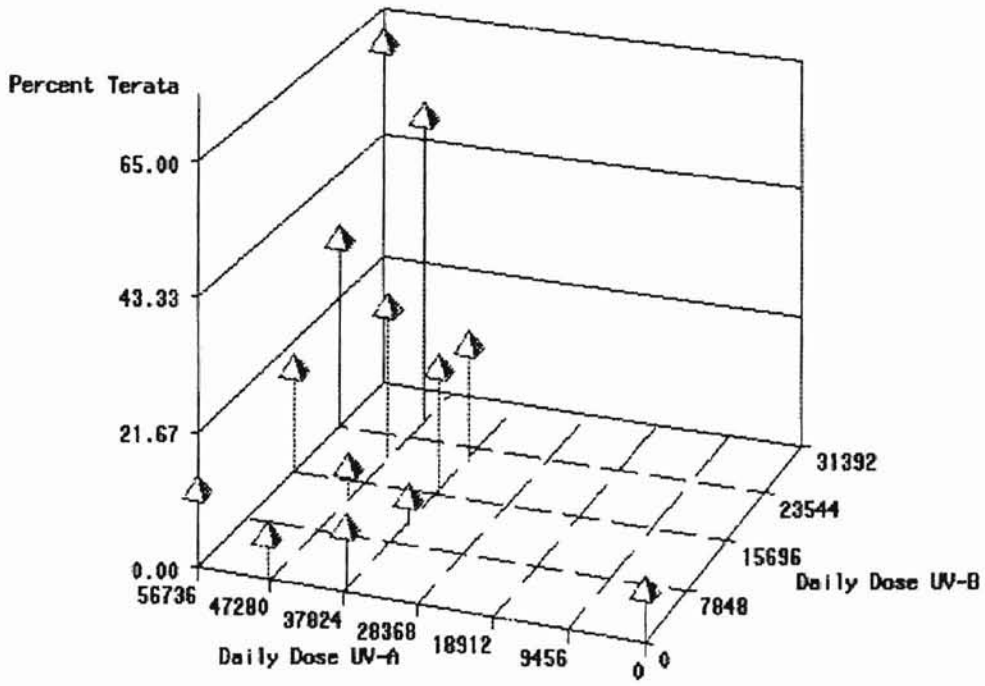


**Figure 1**. Dose-response data for percent malformed observed in Experiment 1. Average values for each of the treatments (i.e. the observations for each block were pooled and averaged), except for the 8 hr Black Plastic (Control) treatment which were averaged for all three tests, are represented. Results from the multiple regression generated the following equation: Percent Terata = 5.516 + 0.0001 (UVA) - 0.00098 (UVB) + $8.0 \times 10^{-8}$ $(UVB)^2$, $R^2 = 0.925$.

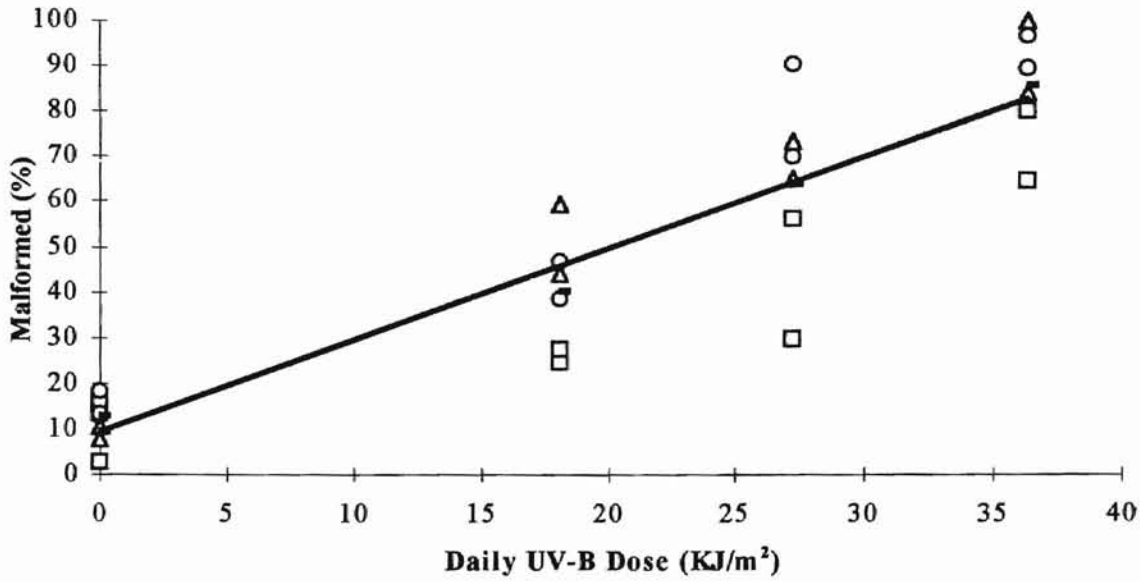


**Figure 2**. Dose-response data for percent malformed embryos observed in Experiment 2. The results for two blocks per test are represented, for 2a (open triangles), 2b (open squares), 2c (open circles), and mean response (closed rectangles). Results from the simple linear regression generated the following equation: Malformed (%) = 0.002 (UV-B) + 9.4, $R^2 = 0.856$.

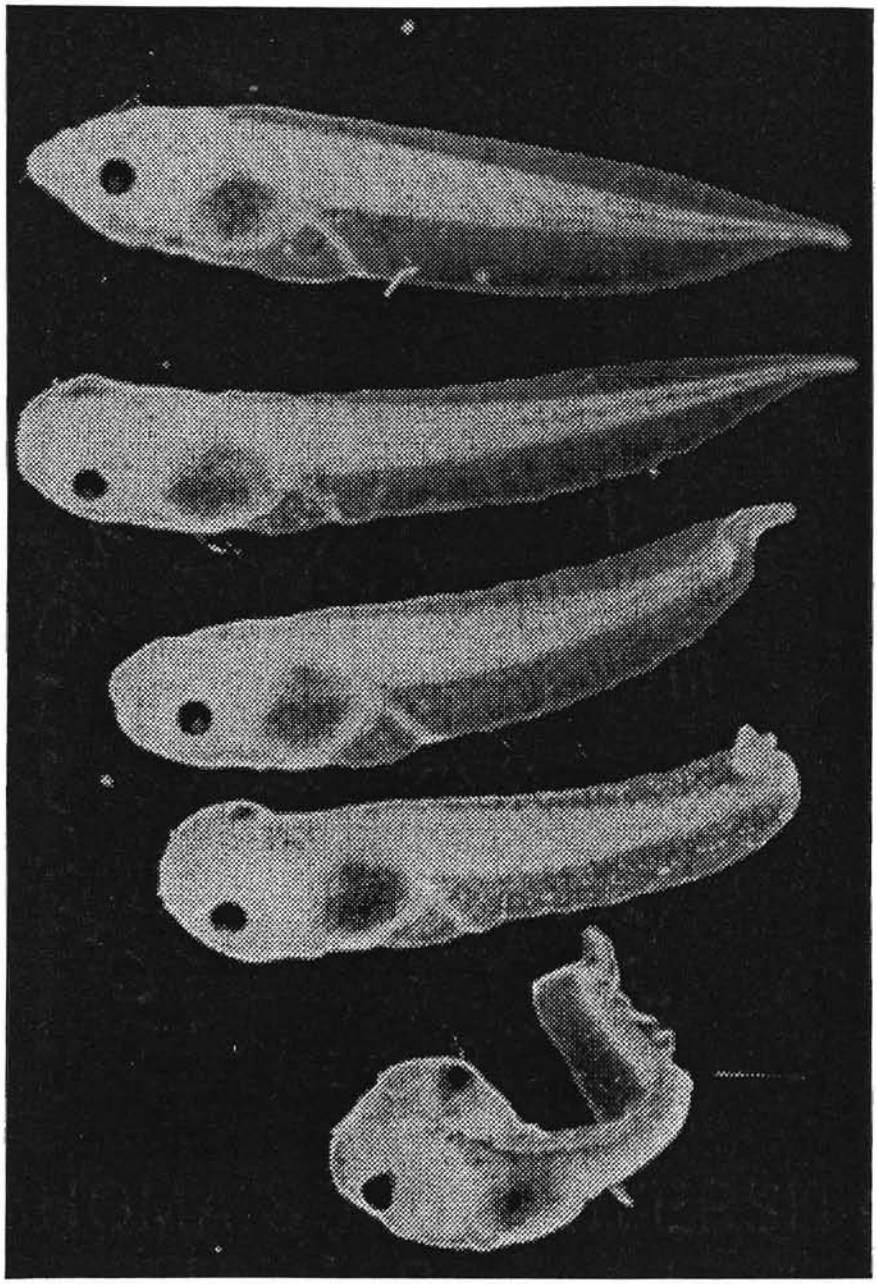

**Figure 3**. Terata consistent with UV radiation exposures. From top to bottom are representative stage 46 tadpoles in the following treatments: 8 hr Black Plastic (Control); 8 hr Mylar; 4 hr acetate/4 hr Mylar; 6 hr acetate/2 hr Mylar; 8 hr acetate.

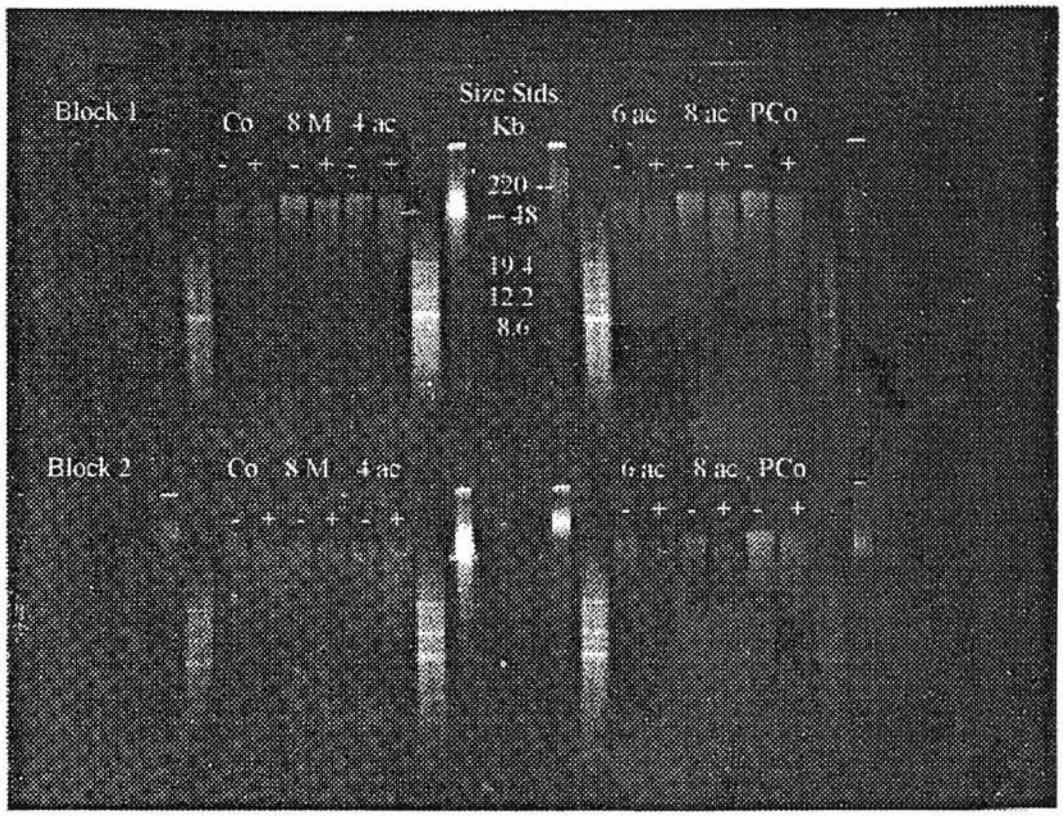


**Figure 4**. Aklaline agarose gel of *X. laevis* DNA for all five treatments exposed under UV lights incubated with and without *M. luteus* UV endonuclease run with Positive Control DNA and molecular weight standards.

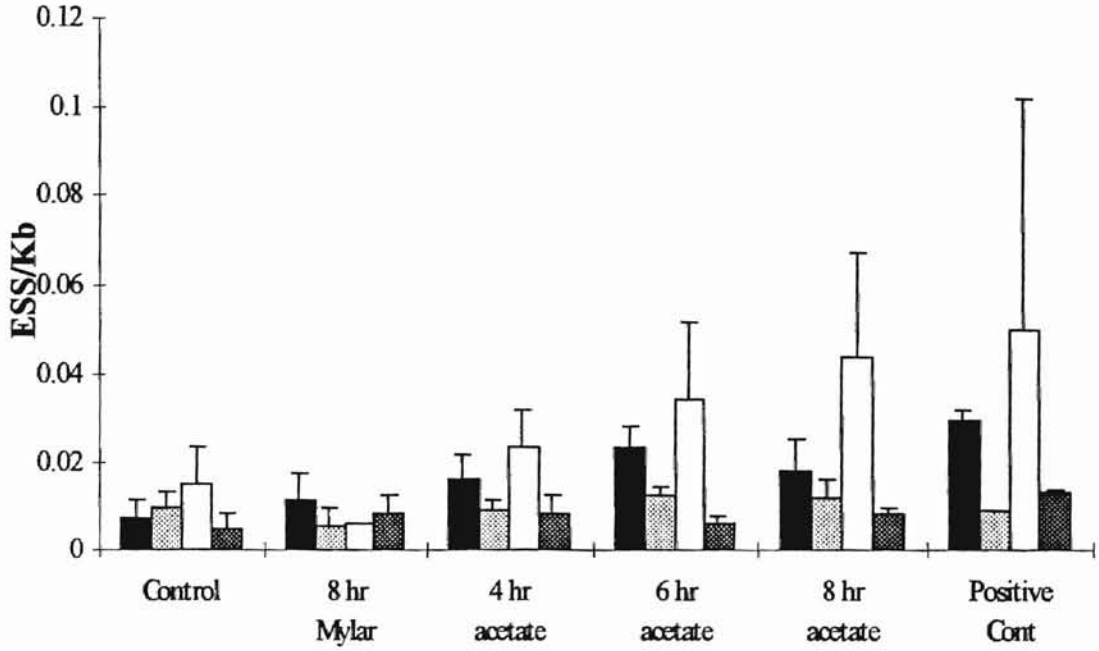


**Figure 5.** DNA photodamage in ESS/Kb for all tests. Values for experiment 1 (solid-black bars) were averaged across all three exposures, but the values for exposures 2a, (black spotted bars), 2b (open bars), and 2c (white spotted bars) were averaged across blocks.

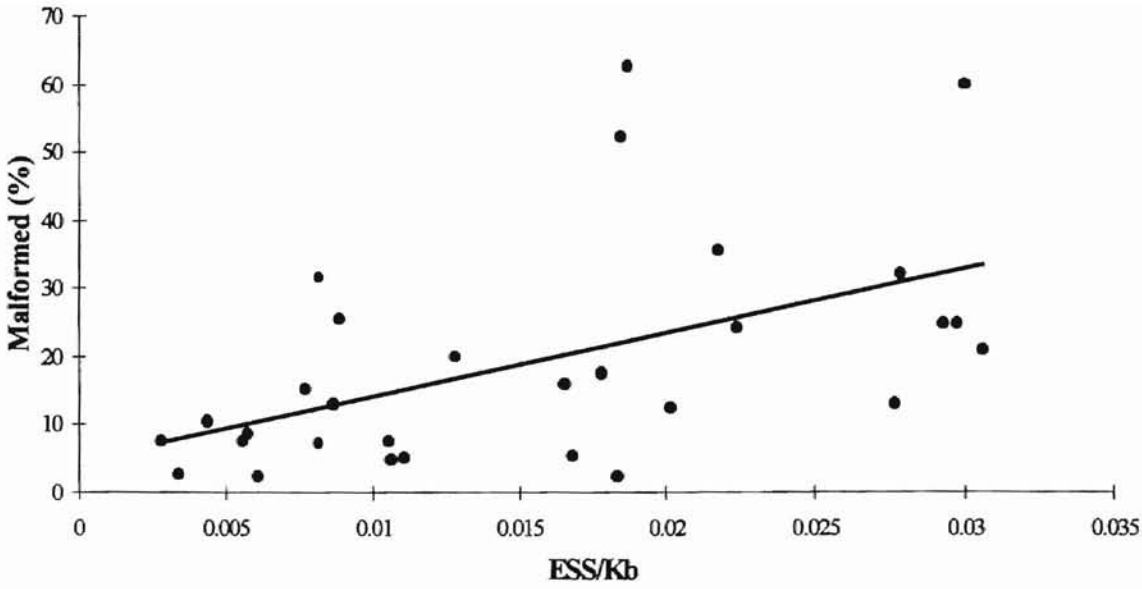


**Figure 6**. Linking Terata to DNA Photodamage (ESS/Kb) in Experiment 1. The resulting relationship, based on correlation analysis, is given by the following equation: Malformed (%) = 936.02 (ESS/Kb) + 4.91, $R^2 = 0.264$.

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