EFFECTS OF ULTRAVIOLET RADIATION ON AMPHIBIAN DEVELOPMENT: A GLOBAL PERSPECTIVE

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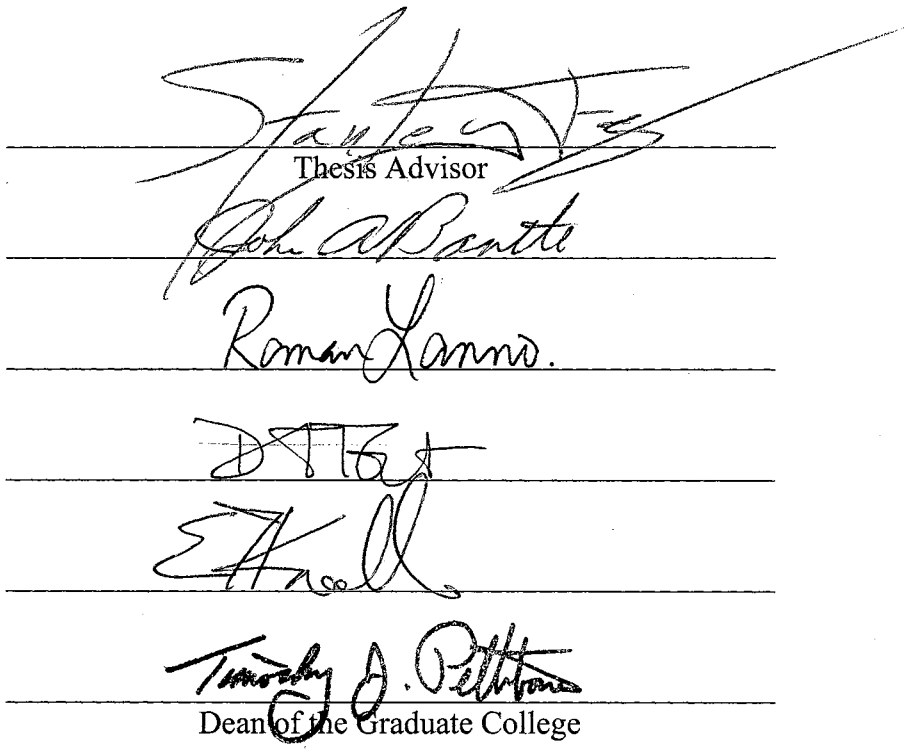

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Chapter I

A PRIMER ON THE ATTRIBUTES AND EFFECTS OF THE GLOBAL LOSS OF OZONE AND INCREASED ULTRAVIOLET-B RADIATION

Stratospheric Ozone and UV-B Radiation

The stratospheric ozone layer begins at an altitude of around 10 km and extends upwards to about 50 kilometers. This layer formed as molecular oxygen accumulated through biotic and abiotic processes (Robberecht, 1989). Statospheric ozone accounts for about 90% of total ozone with tropospheric ozone accounting for the remaining 10%. Stratospheric ozone serves to protect living systems against biologically harmful ultraviolet radiation from the sun. In its pristine condition it attenuates all radiation less than 295 nm (Robberecht, 1989), effectively excluding all UV-C (200-280 nm), which is deadly to life, and much of the UV-B (280-320 nm). There is a negative correlation between stratospheric ozone concentrations and measured UV-B at the Earth's surface. UV-B is particularly effective at causing damage to living organisms. It is even believed that life was confined to the protection of aquatic habitats until an estimated 400 million years ago when the stratospheric ozone concentration reached sufficient levels to filter enough UV radiation (Robberecht, 1989; Williamson, 1995).

There has been widespread concern about measured decreases in the concentration of stratospheric ozone at all latitudes except equatorial regions (Madronich et al., 1995;

WMO, 1999). Of particular concern is the extreme depletion of the ozone layer over Antarctica, the so-called Antarctic ozone hole. The ozone hole was first observed over Antarctica in the late 1970's to early 1980's, but not previously (Stolarski et al., 1992; WMO, 1999). It is now recognized that anthropogenic chemicals are responsible for the depletion of the ozone layer (Stolarski et al., 1992; Madronich et al., 1995; WMO, 1999). These ozone-destroying chemicals are described by the general label halocarbons and consist of various combinations of chlorine, flourine, bromine, carbon, and hydrogen. The Montreal Protocol and its subsequent amendments and adjustments have regulated the production of these compounds with the most damaging of these eliminated in developed countries in 1996 and to be eliminated by 2010 in the developing countries. Stratospheric concentrations of these chemicals are expected to peak by 2000 with recovery of the ozone layer expected over the next 50 years (WMO, 1999).

Geographic variation in UV radiation

There exists a latitudinal variation in UV irradiance. UV levels increase from the poles to the equator and are greater in the Southern Hemisphere than the Northern (Madronich et al., 1995; WMO, 1999). Moreover, the hemispherical differences are strongly weighted toward shorter, more damaging wavelengths of UV-B. Seckmeyer et al. (1995) observed erythemally-weighted UV irradiances approximately 40-50% greater in mid-latitudes of the Southern Hemisphere than the Northern. Nevertheless, it is likely that mid-latitudes of the Southern Hemisphere have always had more UV-B radiation because of less ozone and/or smaller Sun-Earth separation in the austral summer.

Temporal trends in ozone concentration and UV-B radiation since 1979

Superimposed upon this natural latitudinal variation in UV irradiance are changes in stratospheric ozone concentrations and UV-B irradiation caused by anthropogenically created ozone-destroying halocarbons. Ozone depletion has been more extensive in the Southern than in the Northern Hemisphere (Stolarski et al., 1992; Madronich et al., 1995). Total annual ozone trends from 1979-1997 indicate a 3.7% per decade decrease at high latitudes (50°-60°) in the Northern Hemisphere versus a 4.4% decrease in the Southern Hemisphere, with even larger decreases during the winter and spring of each year. This ozone depletion leads to an increase in UV-B of 3.7% per decade in the Northern Hemisphere and 9% in the Southern Hemisphere at these latitudes. Interestingly, at the mid-latitudes (30°-50°) there was a 2.8% decrease of total annual ozone in the Northern Hemisphere versus only 1.9% for the Southern Hemisphere. Despite this, there was a smaller increase in UV-B at these latitudes in the Northern Hemisphere than the Southern Hemisphere, 3% and 3.6% per decade, respectively.

Effects of UV-B Radiation on Organisms

There have been numerous studies demonstrating the effect of increased UV-B radiation on marine organisms (Smith and Baker, 1979; Smith, 1989; Hader and Worrest, 1991; Cullen et al., 1992; Smith et al., 1992; Holm-Hansen et al., 1993). There is also a plethora of studies demonstrating the effect of increased UV-B radiation on organisms inhabiting freshwater ecosystems -- including algae, freshwater zooplankton, macroinvertebrates, fish, and amphibians (Blaustein et al., 1994, 1995, 1997; Bothwell et al., 1994; Little and Fabacher, 1994; Williamson et al., 1994; Zagarese and Williamson,

1994; Fabacher and Little, 1995; Vinebrooke and Leavitt, 1996; Francoeur and Lowe, 1998). UV-B effects have also been shown in several terrestrial organisms, including plants and humans (Tevini and Teramura, 1989; Bornman, 1989, Bornman and Teramura, 1993; de Gruijl and Van der Leun, 1993; Teramura and Sullivan, 1994; Jordan, 1996; Ballaré et al., 1996; Teramura and Ziska, 1996;).

The ultimate cause of injury to organisms from UV-B radiation is damage to cellular components. The harmful effects of UV radiation at the cellular level have been studied extensively. UV-B is absorbed by proteins, lipids, and nucleic acids (Jordan, 1996). Of these, particular attention has been paid to its effects on DNA (Setlow, 1974; Achey et al., 1979; Chan et al., 1986; Mitchell and Karentz, 1993; Griffiths et al., 1998). DNA readily absorbs UV-B, resulting in strand breaks and base deletions or modifications, with potentially serious consequences for the organism such as cellular death or mutation. By far the most prominent form of DNA damage caused by UV-B is the formation of cyclobutane pyrimidine dimers (Setlow et al., 1965; Jagger, 1985; Griffiths et al., 1998). This type of damage is repaired by the enzyme photolyase (Blaustein et al., 1994; Licht and Grant, 1997; Griffiths et al., 1998). Photolyase is a photoreactivation repair enzyme that absorbs photons within the UV-A/visible range of light and uses this energy to cleave the cyclobutyl ring of the dimer (Mitchell and Karentz, 1993). This type of repair mechanism is widespread in both prokaryotes and eukaryotes, but the efficiencies of the mechanism can vary significantly. For example, Regan et al. (1982) found a 5-fold difference in the rate of photorepair in two closely related marine fishes, the tautog and cunner.

Although photolyase is an important DNA post-exposure repair mechanism, the

amount of initial photodamage depends on the absorbed dose. Organisms have evolved numerous physico-physiological mechanisms that reduce exposure of DNA and other cellular components to UV-B radiation. Many organisms have outer coverings such as bark, cuticles, skin, fur, feathers, etc. that minimize UV-B exposure. Additionally, organisms produce biochemical pigments such as melanin and anthocyanin, which absorb UV-B before it reaches internal cellular components (Takahashi et al., 1991).

Along with these UV-B filtering physiological mechanisms, organisms can also modify their behavior to avoid UV-B exposure. These behavioral adaptations exploit differences in UV-B caused by local environmental factors such as cloud cover and shade, and in aquatic systems also water depth, turbidity, and dissolved organic carbon (DOC).

Many adaptations that result in lower UV-B exposure in organisms are potentially only secondarily related or even completely unrelated to exposure and may have evolved in response to other factors. For example, shells and exoskeletons of marine invertebrates probably evolved as a defense against predation but also serve to lessen UV-B exposure. The adaptation of producing foam nests, found in many of the South American anuran species, is thought to have evolved also as a defense against predation, however a foam nest probably also blocks a significant amount of UV-B.

As a general rule, most adaptations are costly in terms of energy balance. Unnecessary ones are usually eliminated from populations because of these costs, or are never developed. Therefore, one would predict that populations have evolved to deal with the radiant conditions of the place where they are found. With respect to adaptations against UV-B, plant and animal populations should develop and retain defenses just

adequate to meet the UV-B challenges specific to the environment in which they live. However, this correlation is probably confounded by adaptations to environmental factors other than UV-B that also happen to lessen UV-B exposure. These ancillary adaptations can contribute to species differences in UV-B sensitivity. Despite these complicating factors, numerous studies with plants have demonstrated that species and populations originating from higher UV-B environments (high altitude and/or low latitude) are less sensitive to UV-B than species and populations from lower UV-B environments (Robberecht et al., 1980; Caldwell et al., 1982; Larson et al., 1990; Sullivan et al., 1992; Ziska et al., 1992; van de Staaij et al., 1995).

Effects of UV-B Radiation on Amphibians

Studies dealing with UV-B effects on amphibians have focused on early life history stages. The lethal effects of UV-B radiation on early life stages of amphibians were first noted by Worrest and Kimeldorf (1976), who warned that stratospheric ozone depletion might harm amphibian populations. Since this time, ambient UV-B radiation has been shown to cause embryo mortality and malformations in some species of amphibians but not others (a review is given below; see also Blaustein et al., 1998). UV-B has also been shown to inhibit predator-avoidance behavior in juvenile boreal toads (*Bufo boreas*), alpine newt larvae (*Taricha granulosa*), and larval cascade frogs (*Rana cascadae*) (Kats et al., 2000).

As in other organisms, the primary cause of injury to amphibians from UV-B is damage to cellular components, particularly DNA, and like most other organisms, amphibians are equipped with photolyase and other enzymes to repair this damage

(Blaustein et al., 1994; Licht and Grant, 1997). The levels of photolyase activity in amphibians vary substantially and are inversely correlated with UV-B sensitivity (Blaustein et al., 1994, 1995, 1996, 1999). It has also been observed that species which tend to hide their eggs have lower levels of photolyase than species that lay their eggs in direct sunlight (Blaustein, et al., 1994).

In addition to repair enzymes, there are other structural, physiological, and behavioral adaptations that can mitigate UV-B exposure to amphibian eggs. Structural and physiological factors such as pigmentation, nature of the jelly capsule surrounding the ova, size and shape of the egg mass, and rate of embryonic development play important roles (Licht and Grant, 1997). Behavioral defenses against UV-B include temporal changes in egg-laying, as well as spatial changes in oviposition sites, such as choosing deeper, shadier, or more turbid sites.

If increasing UV-B acts as a serious stressor on amphibian populations, these populations have to evolve rapidly to mitigate this threat or be subject to extinction. There are several reasons to think rapid adaptation via natural selection is feasible. Many species lay large numbers of eggs. For example, an individual female *Bufo americanus*, *B. variegatus*, *Rana utricularia*, or *Pleurodema bufonina* (four wide-ranging species of the central plains of North and South America) can lay hundreds or thousands of eggs in a single season. Adaptive responses to increased UV-B can be either behavioral or physiological. Behavioral responses such as increased depth of egg deposition would not require tremendously more energy (but might be limited by other factors such as increased predation, colder temperatures, or simple lack of deep water). Physiological mechanisms for mitigation of UV-B such as photolyase and melanin pigmentation may

already exist at rudimentary levels in relatively unstressed populations (most amphibians are exposed at least to some UV-B). Therefore, to compensate for increased UV-B radiation, all that is needed is an increase in the degree of expression of these adaptations. For these reasons, an entire population adapting to this stressor over the course of one or two decades is not an unreasonable proposition. An adaptive response would most likely occur in conjunction with population declines followed by recovery if secondary stressors did not overcome the smaller population.

Since the first publication by Blaustein et al. (1994) that demonstrated lowered hatching success of amphibian eggs caused by ambient UV-B, there have been various subsequent similar studies. Some of these have demonstrated similar results while many have documented a lack of ill effects caused by ambient levels of UV-B. Often results from tests conducted by the same investigators are positive for some species and negative for others. To limit confounding variables, I will examine here only field studies that examine the earliest stages of development.

Results of studies in the Northern Hemisphere

Blaustein et al. (1994, 1995, 1997) found that *Rana cascadae*, *Bufo boreas*, *Ambystoma gracile*, and *A. macrodactylum* all were negatively impacted by ambient UV-B, whereas *Rana pretiosa* and *Rana luteiventris* were not (Blaustein et al., 1999). Ovaska et al. (1997) showed that neither *Rana aurora* nor *Hyla regilla* was affected by ambient UV-B, but with supplemental UV-B of 15%-30%, a UV-B effect was shown. Langhelle et al. (1999) showed no effects on *Bufo bufo* or *Bufo calamite*. In contrast to the findings of Blaustein et al. at 44° N, Corn (1998) showed that *B. boreas* was not

affected at 40° N. Bruner et al. (2002) found that *Rana blairi* and *Hyla chrysoscelis* were unaffected by ambient UV-B. Lizana and Pedraza (1998) showed harmful effects with *B. bufo*. However, this species normally oviposits in relatively deep water (>20 cm), and the experimental design used in this experiment artificially elevated the eggs to shallow water and may have provided more UV-B than normally experienced. Anzalone et al. (1998) showed harmful effects in *Hyla cadavarina* and *Taricha torosa*, but not in *Hyla regilla*. However, this is an area that has historically been densely vegetated and only recently cleared, thus exposing eggs to intense sunlight to which the animals may not have been previously adapted. Crump et al. (1999) examined eight species *(Bufo americanus*, *Rana sylvatica*, *Rana pipiens*, *Rana clamitans*, *Rana catesbeiana*, *Hyla versicolor*, *Ambystoma maculatum*, and *Ambystoma laterale*) and found no significant negative effect caused by ambient UV-B. Starnes et al. (2000) found no differences in mortality in *H. chrysoscelis*, *Pseudacris triseriata*, *Rana sylvatica*, and *A. maculatum*, but did see a higher rate of malformed embryos in *H. chrysoscelis* and *P. triseriata*. Merila et al. (2000) found that ambient UV-B radiation caused no significant effects on mortality, malformation, nor growth in *Rana temporaria* at 69° N, while the same group of researchers (Pahkala et al., 2000) found a significant negative effect on growth (but not mortality and malformations) at both 59° N and 66° N in this species. Pahkala et al. (2001) found no negative effects of ambient UV-B on hatching success or malformations of the moor frog (*Rana arvalis*), whereas Hakkinen et al. (2001) found that ambient UV-B caused significantly reduced hatching success in *R. arvalis* but not in *B. bufo* nor *R. temporaria*.

Results of studies in the Southern Hemisphere

There have been far fewer studies conducted in the Southern Hemisphere. Experiments by van de Mortel and Buttemer (1996) showed no significant UV-B effect in *Litoria aurea*, *Litoria dentata*, and *Litoria peronii*. However, a smaller experiment did show negative effects in *L. aurea* but the authors say this may have been due to predation and fouling of the water by birds in the uncovered treatments. They concluded that their data did not support a UV-B effect. Broomhall et al. (2000) found a negative effect on survival in both *Crinia signifera* and *Litoria verreauxii*, with *L. verreauxii* being affected significantly more. Fox (in prep) found no UV-B effect in *Pleurodema bufonina* nor *Bufo variegatus*.

Project Overview

The second chapter of this thesis describes a series of controlled outdoor experiments designed to assess the effects of ambient UV-B radiation alone and in conjunction with groundwater contaminated with landfill leachate. These experiments were undertaken as an extension of my research at the closed municipal landfill near Norman, Oklahoma (Bruner *et al.*, 1998). As is shown, I found no significant effects caused by ambient UV-B alone. This finding, in conjunction with the myriad of conflicting results obtained by other researchers, led me to wonder if there were some geographical pattern to these observed results or if it was simply a matter of interspecific and intraspecific differences in sensitivities. Another variable very likely producing some of the differing results obtained in these field studies is the specific ambient UV-B conditions at the time and place of the experiments.

An emerging hypothesis is that those populations of animals inhabiting areas of high natural levels of UV-B would have adapted to be more resistant. In order to completely understand the UV-B sensitivities of different species and populations, more controlled tests would be needed. This would preclude the use of field studies because of the inability to exactly control the dose received (the results of field experiments are dependent upon the specific ambient UV-B conditions at the time and place of the experiments). I therefore designed the experiments described in the third chapter to try to answer this question.

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Chapter II

DEVELOPMENTAL EFFECTS OF AMBIENT UV-B LIGHT AND LANDFILL LEACHATE IN *Rana blairi* AND *Hyla chrysoscelis*

Introduction

There have been numerous recent reports of grossly malformed frogs over large portions of North America (Sessions and Ruth, 1990; Schmidt, 1997; Ouellete, *et al.*, 1997). Potential causes for these malformations include parasites (Sessions and Ruth, 1990), xenobiotics (Ouellete, *et al.*, 1997; La Clair *et al.*,1998), and ultraviolet radiation acting alone or synergistically (Ankley *et al.*, 1998; La Clair *et al.*,1998). The reports of malformed frogs come amidst a growing concern by scientists that many amphibian populations are declining worldwide in both disturbed and relatively pristine environments (Blaustein and Wake, 1995; Phillips, 1990; Wake, 1991; Crump *et al.*, 1992; Carey, 1993; Blaustein, *et al.*, 1994a,b,c; Pechmann and Wilbur, 1994; Drost and Fellers, 1996; Fisher and Shaffer, 1996; Laurence *et al.*, 1996; Lips, 1998; Pounds *et al.*, 1998a,b). A variety of hypotheses and possible explanations for the declines in amphibian populations have been proposed, including climatological change (Pechman and Wilbur, 1994; Pounds and Crump, 1994; Beebee, 1995; Pounds *et al.*, 1998b), environmental acidification (Dunson *et al.*, 1992), environmental xenobiotics (Carey and Bryant, 1995; Fort *et al.*, 1995), disease (Blaustein, 1994; Laurence *et al.*, 1996), introduction of exotic species (Hayes and Jennings, 1986) and habitat loss (Wyman,

1991; Blaustein and Wake, 1995). Unfortunately, the causes for these malformations and the observed population declines remain unproven despite increased research efforts. Causes may vary from one location to another, but because these are global problems and a number of different species are being affected, even in relatively pristine environments, there has been speculation that some ubiquitous mechanism, such as global increases in UV-B, may be responsible (Carey, 1993; Blaustein *et al.*, 1994c).

Ambient ultraviolet-B (UV-B) radiation and artificial UV has been shown to cause embryo mortality and to induce malformations in some species of amphibians (Ankley *et al.*, 1998; Blaustein *et al.*, 1998). Synergistic effects have also been shown with UV-B and acidic conditions (Long *et al.*, 1995) and UV-B and pathogens (Kiesecker and Blaustein, 1995). Hatch and Burton (as reported in Blaustein *et al.*, 1998) observed synergistic effects between UV-B and flouranthene in *Xenopus laevis* and *Ambystoma maculatum*. Zaga *et al.* (1997) found that photoproducts of the insecticide carbaryl induced greater mortality and inactivity in *X. laevis* and *Hyla versicolor* embryos. La Clair *et al.* (1998) found that purified photoproducts of the common insecticide methoprene caused developmental abnormalities in *X. laevis* larvae, whereas Ankley *et al.* (1998) found no synergistic effects between UV-B and methoprene in *Rana pipiens* when both agents acted on embryos.

Most of the field experiments mentioned previously were carried out at higher latitudes than Oklahoma. However, due to a combination of solar angles being closer to zenith and a natural latitudinal gradient of atmospheric ozone, ambient UV-B intensities are generally higher at the lower latitude of Oklahoma (Caldwell *et al.*, 1980).

Consequently, native amphibians in Oklahoma may be better adapted either behaviorally or physiologically to higher levels of UV-B exposure.

As part of a larger study to assess the effects of multiple stressors on amphibian populations at a closed municipal landfill in Norman, Oklahoma, I assessed the effects of ambient UV light exposure and water contaminated with landfill leachate on frog embryo development. Water samples for these experiments were taken from a shallow well adjacent to and downgradient from the landfill. Only 20-30 meters downgradient from this well, a significant quantity of this same groundwater surfaces as a seep. This entire area has an abundant community of amphibians, including the two species used in this study (personal observation). The samples were collected from the well to ensure consistency of contaminant levels and lack of previous UV exposure.

Previous researchers have identified more than 40 semi-volatile and non-volatile compounds in the groundwater at the landfill (Dunlop *et al.,* 1976). Thirty-five volatile compounds (Table 2.1) were identified in water samples taken in September, 1993, by the United States Geological Survey (USGS), using gas chromatography/mass spectrometry (EPA method 8240) (Scott Christenson, USGS, personal communication; Bruner *et al.*, 1998). Many of these chemicals are known carcinogens and xenobiotics. Low levels of dissolved oxygen and elevated concentrations of hydrogen sulfide and methane were also found (Gibson and Suflita, 1986; Beeman and Suflita, 1987; Beeman and Suflita, 1990). A preliminary Toxicity Identification Evaluation (TIE) has also been performed on groundwater samples. Results indicated that elevated toxicity resulted, in part, from high concentrations of ammonia and metals (personal communication, J.A. Bantle).

I previously used the Frog Embryo Teratogenesis Assay-*Xenopus* (FETAX) (Dumont *et al.*, 1983) to evaluate the ground and surface water toxicity at the landfill site. FETAX is a 96-hr whole embryo assay for developmental toxicants that uses the embryos of the South African clawed frog, *Xenopus laevis*, and is thus particularly useful in studies dealing with impacts on amphibians. Groundwater samples downgradient from the landfill were highly toxic, with toxicity diminishing as distance from the landfill increased. I concluded that the toxicity was due to a leachate plume exuding from the landfill (Bruner *et al.*, 1998). This conclusion was further supported by an electromagnetic survey conducted by the USGS that assessed the apparent conductivity of the alluvium (Lucius and Bisdorf, 1995). Surface water samples were also analyzed using FETAX and my analysis indicated that they had elevated toxicity. Because these samples were taken from the same locations multiple times over the course of a year, I was able to correlate temporal changes in toxicity to changes in weather conditions (Bruner *et al.*, 1998). Weather data were collected by an automated Mesonet weather station installed at the landfill site (Crawford *et al.*, 1992). My analysis indicated elevated toxicity during periods of high solar radiation. One possible cause for this correlation is that solar radiation is low during rain events when toxicants are being diluted, whereas during periods of high solar radiation evaporation increases, concentrating toxicants. Because UV has been shown to act synergistically with (and enhance toxicity of) a wide variety of agents, another possibility is that photochemical reactions increased toxicity by converting less toxic compounds to more toxic derivatives (Bruner *et al.*, 1998).

Here, I examine the effects of ambient UV light exposure and water toxicity on the development of embryos of two native species of anurans during their normal breeding season in central Oklahoma. The objective of this study was to test the following three null hypothesis: 1) there are no differences in mortality, malformations, or growth of developing native anurans attributable to ambient UV exposure, 2) native frog embryos are not developmentally affected by groundwater taken from the Norman landfill study site, and 3) there is no interaction between water toxicity and natural UV light exposure that affects native frog embryo development.

Materials and Methods

General methods for all experiments

I conducted experiments at a suitable flat and open site on private property 16 km northwest of Oklahoma State University during the period of March 25 to May 8, 1998. Eggs of the Plains Leopard Frog (*Rana blairi*) and the Grey Treefrog (*Hyla chrysoscelis*) were collected from breeding ponds near Stillwater, OK, a few kilometers away from the experimental site. Prior to the experiments, eggs were sorted for viability in the laboratory, and placed in 60-mm Petri dishes with FETAX solution (50 eggs per dish). Only viable eggs that retained their individual jelly coats were used. Eggs were then transported to the field site for the experiments.

Petri dishes containing eggs were submerged without their lids in the center of small plastic experimental tubs (one dish per tub). All eggs were in late blastula stage (approximately stage nine as per Gosner [1960]) when the experiments were started. The tubs were 30 cm x 15 cm x 10 cm, made of clear plastic so as to minimize heat

absorption, and reflected UV light. The experimental tubs were filled with either FETAX solution or a mixture of reagent grade water and leachate collected from the USGS Well 0 (Bruner *et al.*, 1998) sampling location at the Norman landfill. Water quality parameters including temperature, pH, conductivity, salinity, turbidity, and dissolved oxygen of the leachate were monitored over the course of several months using *YSI 6000* (YSI Incorporated, Yellow Springs, OH) multiparameter water quality sondes installed in the stream directly downgradient of the Well 0 sampling location. This is an area where a large seep of groundwater mixes with water in the stream and thus does not represent the leachate at 100% concentration, however the experiments I conducted also used diluted leachate. These data are presented to show that ambient water quality could sustain normal growth and development of amphibians in the absence of xenobiotics. Values for pH ranged from 6.45 to 8.79. Conductivity ranged from 0.48 to 1.91 mS/cm. Salinity values ranged from 0.00 to 1.31 ppt at the landfill. Dumont (unpublished) showed that *Xenopus* embryos developed and grew normally in artificial seawater (*Instant Ocean*®) at concentrations up to 1% (10 ppt). Ammonia content for the landfill water was between 0.20 and 3.40 mg/L. Turbidity fluctuated from 0.00 to 867.0. These data suggest that the standard water quality variables (exclusive of toxicants) were within acceptable ranges for FETAX and probably did not affect the growth and development of test embryos (Bantle, 1995).

Petri dishes were supported on glass and plastic platforms elevated to within 3 cm of the surface of the water in each tub to achieve maximum UV-B exposure (Figure 2.1). Reagent grade water was added daily to each tub as needed to maintain water levels and solute concentrations. Water in the tubs was not changed during the course of the

experiment because the large volume in each experimental tub precluded the buildup of high concentrations of waste products. The experimental tubs were then placed in water baths to stabilize temperatures and minimize extreme temperatures from sunlight exposure. Water baths consisted of 2000-l (2.5-m diameter) wading pools filled with non-toxic water. The experimental tubs were elevated on blocks in the water baths to a depth whereby only the lower 3-4 cm of the tubs were submerged. Individual tubs were covered with opaque blue plastic tarp, mylar, acetate, or left uncovered. The opaque tarp was used to block UV-B, UV-A, and most of the direct visible light. The transmission characteristics of the mylar and acetate filters were determined using a spectroradiometer. The mylar used in this study blocked approximately 90% of the UV-B while allowing the transmission of UV-A. The acetate blocked approximately 40% of the UV-B while allowing the transmission of UV-A. The covering material was attached to the tubs in such a way as to form an inverted U-shaped roof with the center elevated 10-15 cm above the top of the tub to allow air exchange and prevent condensation and heat buildup (Figure 2.2). Filters were replaced every four days. Maximum-minimum recording thermometers were placed randomly in one of each of the different treatment tubs for temperature measurement.

Petri dishes were checked daily and dead embryos were noted and removed. The experiments were stopped after the majority of embryos had reached stage 25 (Gosner, 1960). Stage 25 was chosen because primary organogenesis was complete and feeding was unnecessary prior to this point. Embryos were then anesthetized with MS-222 and fixed in a 5% formalin solution for later determination of growth and malformations.

Experiments with Rana blairi

Four experiments were conducted using eggs of *Rana blairi*. Eggs were taken from a single clutch for each experiment and randomly assigned to each of the different treatments in that experiment. In the first experiment, water from Well 0 was diluted with FETAX solution to a concentration of 25%. Experiments two and three used this water at a concentration of 10%, and the fourth experiment used a concentration of 5%. Each experiment also utilized a FETAX control solution. The first three experiments continued for eight days while experiment four lasted only five days because of warmer weather and water temperatures.

I used a 2x4 factorial arrangement of treatments in a randomized complete block design. Each water bath contained eight tubs and represented one complete replicate of each type of filter treatment for both the FETAX solution and toxic water. Three of these replicates (water baths) were used in each experiment.

Experiments with Hyla chrysoscelis

One experiment lasting five days was conducted with eggs of *Hyla chrysoscelis*. Several egg masses were collected from the same breeding pond. Eggs from these egg masses were separated, then all eggs were mixed together and randomly assigned from this mixture to each of the different treatments. For this experiment only FETAX solution was used in order to examine the effects of UV-B alone. This was done because my primary objective was to examine the effects of UV-B and I had limited numbers of eggs of this species. Each water bath contained eight tubs and represented two complete replicates of each type of filter treatment. Thus, there were six replicates of each of the

four filter treatments.

Radiation measurements

The UV-B radiation at the experimental site in Stillwater was estimated based on the relationship between solar radiation and UV-B radiation (Table 2.2). A simple linear relationship was previously derived between UV-B radiation and solar radiation using data from a GUV-511C radiometer in Norman, OK (Biospherical Instruments Inc., San Diego, CA), and a solar pyronometer (LiCor Model 200) installed on a Mesonet weather station approximately 4 km distant. Daily cumulative radiation in the UV-B channels (305 nm and 320 nm) was plotted against the solar radiation observed from February 1997 to May 1998 (Figure 2.3). The linear equations derived for the two channels were then used for transforming the solar radiation data recorded at another Mesonet weather station located approximately 5 km from the experimental site in Stillwater. Table 2.2 shows the estimated UV-B radiation at the study site compared to the actual UV-B measured by the GUV-511C radiometer in Norman (110 km distant) for the same time periods.

Results

Mortality, malformation, and growth data for all experiments are given in Table 2.3. With the exception of treatments containing 10% and 25% concentrations of landfill leachate, which killed all the embryos, mortality was low across all experiments. This low mortality (ranging from 0 - 11.1% in the *Rana* experiments and from 7.7 -13.3% in the *Hyla* experiment) indicated that the vast majority of eggs used in this experiment

were viable. *Rana* malformations were elevated in some of the filter treatments caused by the landfill water, but were relatively low in the FETAX solution treatments (0.7 - 11.1%). Malformation rates for the *Hyla* were also quite low (0 - 0.7%). The length of the developing embryos was typical of stage 25, with the *Rana* larvae being larger than the *Hyla* larvae. However, the *Rana* larvae in the landfill water treatments were stunted and significantly smaller than normal, averaging 0.68 cm versus 0.86 cm in the FETAX solution treatments.

The water temperatures for each experiment are given in Table 2.4. The mean daily minimum temperatures for all experiments ranged from 11.4 to 18.2 °C. The mean daily maximum temperatures ranged from 21.8 to 30.3 °C. These temperatures are within expected limits for what would be encountered in a normal field situation and should not have negatively influenced the experiments. Temperature differences among the different filter treatments were not significantly different statistically for any of the experiments (ANOVA p's>0.14). However, the mylar filter treatments were consistently warmer than the other treatments. Generally, the acetate treatments were slightly cooler, followed by the tarp and uncovered treatments, which were very similar in temperature. The landfill water treatments were slightly warmer than the FETAX solution treatments, but certainly not enough to negatively impact the embryos.

Rana Experiments 1-3

The embryos in the 10% and 25% concentrations of landfill water experienced 100% mortality in all filter treatments. I combined the results for the FETAX solution treatments and used a one-way ANOVA to test for differences among filter treatments. I

found no significant differences in mortality ($F_{3,32} = 0.86$, $p = 0.470$) or malformations ($F_{3,32} = 0.36$, $p = 0.782$) among the filter treatments for the FETAX solution. However, there was a significant difference in growth ($F_{3,32} = 3.08$, $p = 0.041$). The mean length of embryos in each filter treatment closely followed the mean maximum and minimum temperatures of the filter treatments, with the warmer treatments generally having the larger embryos. This occurred even though there were no statistically significant temperature differences among filter treatments. Had I tracked the temperatures more closely rather than taking maximum and minimum measurements once per day, it is almost certain that significant temperature differences would have been observed.

Rana Experiment 4

I used a two-way ANOVA to examine the interaction and effects of water toxicity and filter treatment. For mortality I saw no significant interaction ($F_{3,14} = 0.15$, $p = 0.925$) or differences due to water toxicity ($F_{1,14} = 3.06$, $p = 0.102$) or filter treatment ($F_{3,14} = 0.15$, $p = 0.925$). However, for malformations I observed a significant interaction between water toxicity and filter treatment ($F_{3,15} = 7.79$, $p = 0.002$). There was a significant toxic effect caused by the 5% concentration of landfill water for all filter treatments (p's < 0.01) except the uncovered treatment ($p = 0.534$). There was also a significant filter effect for the landfill water treatment ($F_{3,15} = 14.07$, $p = 0.0001$), but not for the FETAX solution treatment ($F_{3,15} = 0.03$, $p = 0.993$). As the UV-B exposure increased for the different filter treatments, the rates of malformation decreased in the landfill water treatment. The rates of malformation in the landfill water ranged from 43% in the lowest UV-B exposure to less than 1% in the highest. Figure 2.4 shows a

normal tadpole from a landfill water-uncovered treatment (a) and a tadpole from a landfill water-tarp treatment (b). The embryo in Figure 2.4b demonstrates the typical malformations observed in this study. This tadpole shows delayed development, abnormal gut coiling, and head, face, and eye malformations.

For growth there also was a significant interaction between water toxicity and filter treatment ($F_{3,14} = 11.22$, $p = 0.0005$). For each filter treatment separately, the embryos in the landfill-water replicates were shorter than the embryos in the FETAX solution replicates (p's < 0.01). This was in spite of slightly warmer temperatures in the landfill-water treatments. There was a significant filter effect for both the landfill water treatment ($F_{3,14.1} = 7.55$, $p = 0.003$) and the FETAX treatment ($F_{3,14} = 3.90$, $p = 0.032$). The length of embryos under different filter treatments in the FETAX solution followed the same general pattern observed in the previous experiments, which coincided with the temperature trends for the filter treatments. However, the length of embryos under different filter treatments in the landfill water did not follow this trend. As the UV-B exposure increased, there was an increase in length and this occurred without regard to the temperatures of the different filter treatments (Figures 2.5 and 2.6).

The embryos in one of the landfill water-tarp replicates were noticeably different than the others. These embryos were larger, better developed, and very few were malformed. They closely resembled the embryos from the landfill water-uncovered treatment. I had noted during the experiment on two separate days that the tarp had blown off this tub, allowing full sunlight exposure for an undetermined time (not more than one day on each occasion). Therefore, I removed this replicate from the analysis.

Hyla Experiment 1

For this experiment I used only FETAX solution and tested the effects of UV-B alone. I used a one-way ANOVA to test for significant differences among filter treatments. I found no significant differences in mortality ($F_{3,20} = 0.38$, $p = 0.765$) or malformations ($F_{3,20} = 1.41$, $p = 0.269$) among the filter treatments. Like the first three *Rana* experiments, there was a significant difference in growth ($F_{3,13.5} = 7.40$, $p = 0.004$) among the filter treatments. Here again, the length of embryos closely followed the temperatures of the filter treatments.

Discussion

For these experiments I attempted to eliminate all the factors found in nature that could decrease the amount of UV exposure received by the developing embryos (Licht and Grant, 1997; Blaustein *et al.*, 1998). This ensured that they received a higher UV dose than wild amphibians at this latitude and elevation. The FETAX solution was transparent, there was no vegetation or other overhead shading, the eggs were submerged but elevated to near the surface of the water in a single layer with only the individual egg jelly coat to block UV (Grant and Licht, 1995).

Water temperature determines the speed of embryo development (Licht and Grant, 1997; Blaustein *et al.*, 1998). Lower temperature lengthens development, thereby increasing UV exposure to each stage of development. Additionally, there is the possibility that DNA repair mechanisms are slowed in cooler temperatures (Grant and Licht, 1995). I believe that the range of temperatures found in my experiments were representative of actual field conditions.

No differences were found in mortality, malformations, or growth between treatments attributable solely to ambient solar UV-B exposure. These results indicate that *Rana blairi* and *Hyla chrysoscelis* eggs are tolerant of current ambient UV-B levels, even at the maximum UV-B intensity likely to be experienced in nature. I cannot say, however, that no damage was done to the embryos, as effects may have appeared at later developmental stages had I allowed the embryos to develop further (Blaustein *et al.*, 1998). Additionally, UV-B exposure during later stages may lead to adverse effects (Ankley *et al.*, 1998; Ankley *et al.*, 2000). Other researchers have conducted UV experiments lasting through various developmental stages with various results. Some have stopped their experiments at hatching (this being the most common; e.g. Blaustein, *et al.*, 1994b,c; Merila *et al.*, 2000; Pahkala *et al.*, 2000), while others have gone completely through limb formation (e.g. Ankley *et al.*, 1998; Ankley *et al.*, 2000). Ankley *et al.* (1998) reported no increase in mortality or malformations of *Rana pipiens* post-hatching at 6 days test duration attributable to artificial UV (which mimicked the UV spectrum present in sunlight but at different intensities, all less than the estimated average ambient UV intensity). However, larvae exposed through hind limb bud formation (stage 26 and greater) had higher rates of hind limb malformations than control (no UV exposure) frogs.

Groundwater taken from the Well 0 location at the Norman landfill site caused 100% mortality of *R. blairi* at concentrations of 10% and 25%. It also caused increased malformations relative to FETAX solution controls at 5% concentration. There were significant differences between filter treatments that indicated that ambient solar UV-B decreased the toxicity of this water. As UV-B exposure increased with different filter

treatments, the rate of malformations decreased and the length of embryos increased. While other studies have reported synergism between UV-B and other chemicals (Zaga *et al.*, 1997; Ankley *et al.*, 1998; La Clair *et al.*,1998), to my knowledge there are no studies describing antagonistic effects. I believe that the most likely cause of this neutralization was photodegradation of contaminants. Many compounds are phototransformed directly by sunlight and many types of reactions are catalyzed by energy from the sun (Hearst, 1995; Zaga *et al.*, 1997; Little et *al.*, 2000). With such a complex mixture of chemicals as is found in this leachate, it is difficult to determine exactly which components are most critical for causing toxicity and teratogenicity and which, at low concentrations, were detoxified by UV-B.

The negative interaction I observed between UV-B exposure and water toxicity runs counter to the results obtained by Bruner *et al.* (1998). In an analysis of water toxicity at the same Norman landfill site with respect to weather parameters, they observed that water toxicity increased with solar radiation. That is, during periods of little or no rainfall (abundant sunshine), water toxicity, as measured by FETAX, increased. I conclude that the positive correlation observed by Bruner *et al.* (1998) between solar radiation and toxicity was attributable to secondary factors that surpassed any detoxifying effects of UV-B radiation as observed in this present work. The most likely of these is the negative correlation in nature between rainfall, when toxicants are being diluted, and periods of high solar radiation. During periods of cloudy weather with significant rainfall, toxicity decreases as a result of dilution, but during periods of no rain and abundant solar radiation, toxicants are concentrated due to elevated evaporation of water. This contrast in results between the two studies illustrates the need for more

detailed, multifactorial experiments when relationships between stressors are unclear.

Conclusions

Results of this study indicated that the early developmental stages of *Rana blairi* and *Hyla chrysoscelis* are resistant to the harmful effects of current levels of UV-B radiation at the latitude and elevation of my study site in central Oklahoma. Groundwater taken from the leachate plume of the closed municipal landfill near Norman, Oklahoma, was developmentally toxic to these species even when diluted to concentrations as low as 5%. However, exposure to ambient UV-B light reduced this toxicity.

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Table 2.1 Compounds Identified in Water Samples Collected in September, 1993, by the USGS, using Gas Chromatography / Mass Spectrometry (EPA Method 8240).

benzene	1-methyl-4-propylbenzene
toluene	1,3-dimethyl-5-ethylbenzene
ethylbenzene	1,2-diethylbenzene
m,p-xylene	1-methyl-2-propylbenzene
isopropylbenzene	1,4-dimethyl-2-ethylbenzene
n-propylbenzene	1,3-dimethyl-4-ethylbenzene
1-ethyl-3-methylbenzene	1,2-dimethyl-4-ethylbenzene
1-ethyl-4-methylbenzene	1,3-dimethyl-2-ethylbenzene
1,3,5-trimethylbenzene	1,2-dimethyl-3-ethylbenzene
1-ethyl-2-methylbenzene	1,2,4,5-tetramethylbenzene
1,2,4-trimethylbenzene	1,2,3,5-tetramethylbenzene
2-methylpropylbenzene	1,2,3,4-tetramethylbenzene
1,2,3-trimethylbenzene	chloroform
1-methyl-3-isopropylbenzene	1,1,1-trichloroethane
1-methyl-4-isopropylbenzene	vinyl chloride
1,3-diethylbenzene	trichloroethane
1-methyl3-propylbenzene	1,4-dichlorobenzene
o-xylene	

Table 2.2 Estimated cumulative UV-B at the study site and actual UV-B at Norman, Oklahoma, for the periods of the experiments.

	Estimated UV-B		Actual UV-B	
Experiment	305 nm ($J/m^2/nm$)	320 nm ($J/m^2/nm$)	305 nm ($J/m^2/nm$)	320 nm ($J/m^2/nm$)
Rana 1	4,641.0	44,227.3	5,269.4	42,279.2
Rana 2 & 3	6,618.8	58,379.3	7,024.7	53,029.1
Rana 4	3,837.4	34,881.0	4,490.8	32,531.8
Hyla 1	3,631.5	33,336.5	4,361.3	31,424.1

Table 2.3 Mortality, malformation, and length of tadpoles (± 1 SE) exposed to different UV filter and water treatments (FETAX and landfill leachate at 5% concentration).

Experiment	UV Filter Treatment	Water Treatment	Mean Mortality (%)	Mean Malformations (%)	Mean Length (cm)
Rana 1, 2, & 3	Tarp	FETAX	7.33 ± 1.8	7.71 ± 2.4	0.76 ± 0.014
	Mylar	FETAX	7.55 ± 2.0	7.64 ± 2.1	0.81 ± 0.026
	Acetate	FETAX	10.67 ± 2.7	8.40 ± 2.6	0.76 ± 0.012
	Uncovered	FETAX	11.11 ± 2.1	11.10 ± 3.6	0.73 ± 0.022
Rana 4	Tarp	FETAX	1.33 ± 1.3	2.00 ± 1.2	0.88 ± 0.006
	Mylar	FETAX	1.33 ± 1.3	1.33 ± 1.3	0.88 ± 0.005
	Acetate	FETAX	1.33 ± 0.7	0.68 ± 0.7	0.86 ± 0.008
	Uncovered	FETAX	1.33 ± 1.3	2.03 ± 1.2	0.81 ± 0.006
	Tarp	Landfill	0.00 ± 0.0	43.00 ± 15.0	0.64 ± 0.024
	Mylar	Landfill	0.67 ± 0.7	22.11 ± 3.0	0.65 ± 0.029
	Acetate	Landfill	0.00 ± 0.0	17.33 ± 3.5	0.69 ± 0.028
	Uncovered	Landfill	0.67 ± 0.7	5.36 ± 1.3	0.74 ± 0.015
Hyla 1	Tarp	FETAX	7.67 ± 3.9	0.34 ± 0.3	0.65 ± 0.004
	Mylar	FETAX	8.67 ± 6.2	0.00 ± 0.0	0.69 ± 0.012
	Acetate	FETAX	8.00 ± 3.0	0.00 ± 0.0	0.65 ± 0.005
	Uncovered	FETAX	13.33 ± 3.3	0.68 ± 0.4	0.64 ± 0.009

Table 2.4 Mean minimum and maximum water temperatures (±1 SE) under different UV filter and water treatments.

Experiment	UV Filter Treatment	Water Treatment	Mean Maximum (°C)	Mean Minimum (°C)
Rana 1, 2, & 3	Tarp	FETAX	23.3 ± 0.7	11.3 ± 1.3
	Mylar	FETAX	25.4 ± 0.7	11.8 ± 1.2
	Acetate	FETAX	23.7 ± 0.8	11.8 ± 1.1
	Uncovered	FETAX	23.1 ± 0.8	11.7 ± 1.1
Rana 4	Tarp	FETAX	28.0 ± 0.6	17.5 ± 0.9
	Mylar	FETAX	29.8 ± 0.5	17.3 ± 0.9
	Acetate	FETAX	28.4 ± 0.6	17.7 ± 0.9
	Uncovered	FETAX	27.8 ± 0.7	17.2 ± 0.9
	Tarp	Landfill	28.2 ± 0.6	18.1 ± 0.8
	Mylar	Landfill	29.1 ± 0.7	18.2 ± 0.8
	Acetate	Landfill	30.0 ± 0.6	17.9 ± 0.9
	Uncovered	Landfill	29.3 ± 0.7	17.4 ± 1.0
Hyla 1	Tarp	FETAX	27.9 ± 0.5	16.8 ± 0.8
	Mylar	FETAX	29.3 ± 0.6	17.9 ± 0.8
	Acetate	FETAX	29.7 ± 0.6	17.8 ± 0.9
	Uncovered	FETAX	29.8 ± 0.7	16.9 ± 0.5

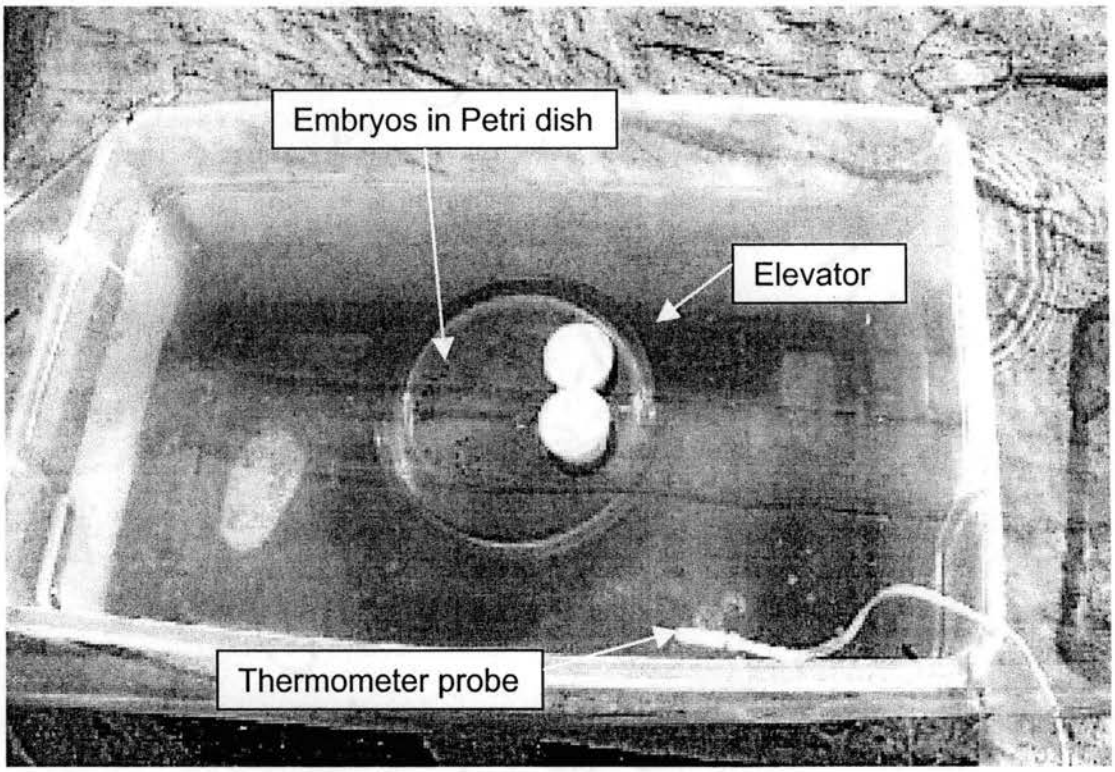


Figure 2.1 A single experimental tub in the water bath with eggs in Petri dish, submerged but elevated to near the surface. (*Note:* The round, white objects are integrating UV-B dosimeters recently developed by OSU. Additional work on the dosimeter was required before reliable results were obtained.)

Figure 2.2 A water bath with experimental tubs having each of the different filter and water treatments.

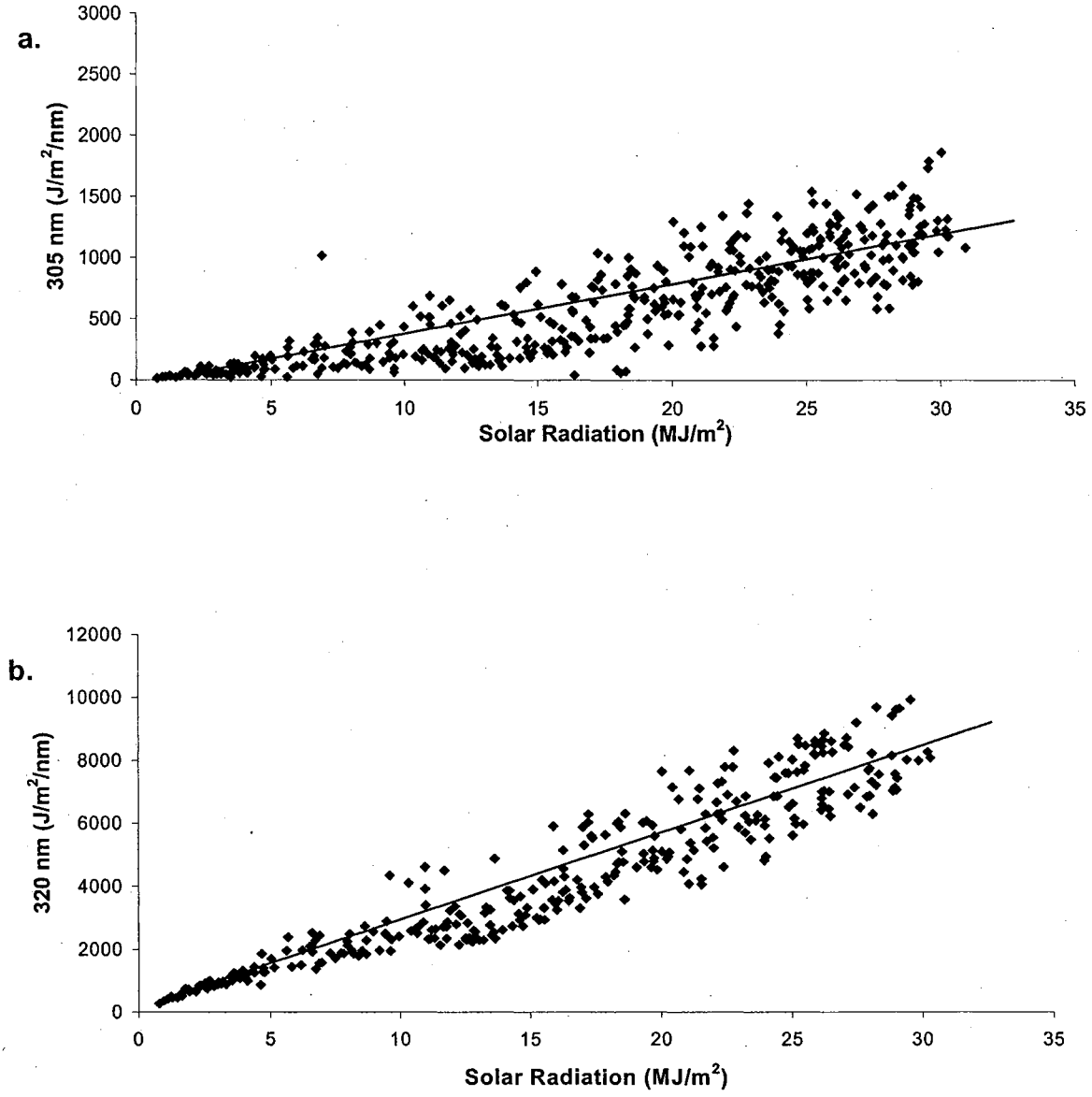
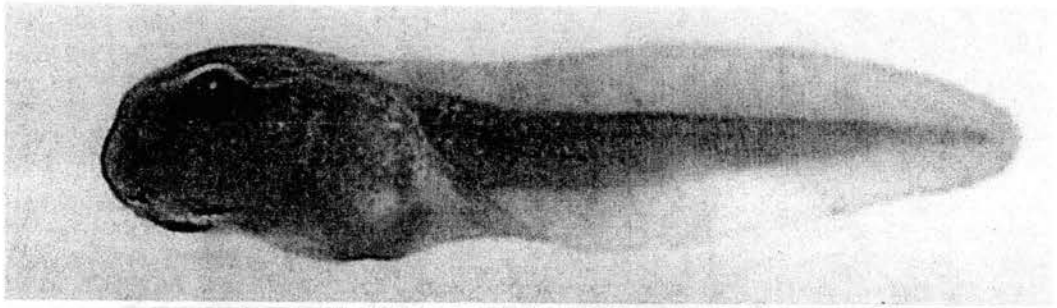


Figure 2.3 Relationship between UV radiation measured by a GUV 511 radiometer versus solar radiation measured by a Lycor solar pyranometer 4 km distant.

a) 305 nm UV versus solar radiation

b) 320 nm UV versus solar radiation

a.



b.

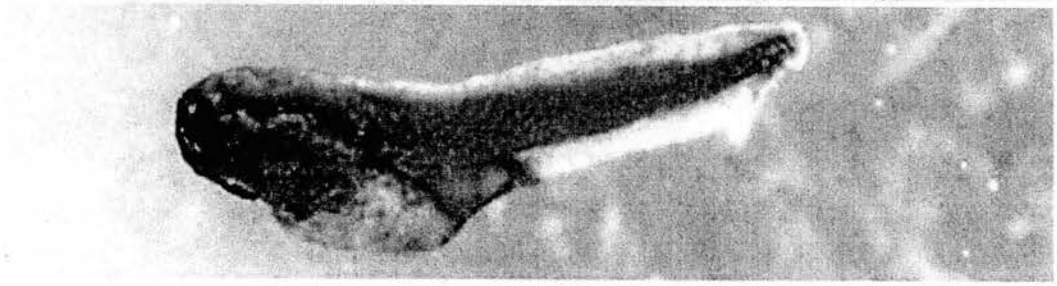


Figure 2.4 Effects of the interaction of UV and landfill leachate on the morphology of *Rana blairi* embryos (Experiment 4).

a) Tadpole from landfill water-uncovered treatment.

b) Tadpole from landfill water-tarp treatment.

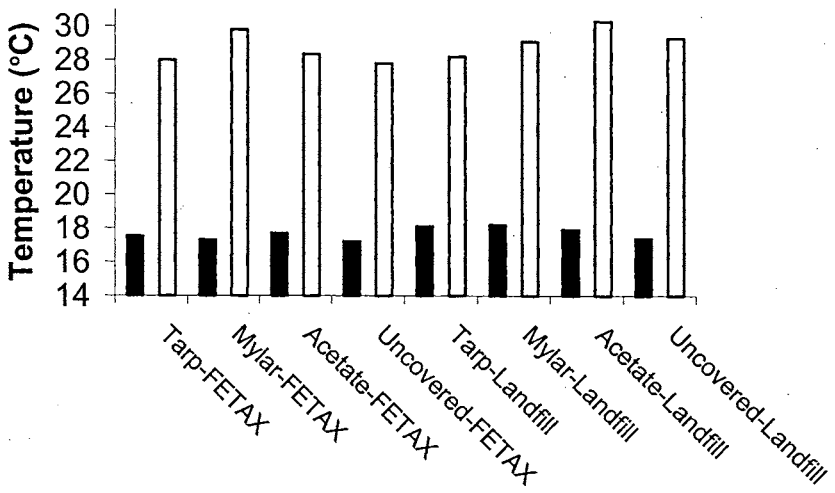


Figure 2.5 Mean daily minimum and maximum temperatures of the different filter and water treatments for *Rana* Experiment 4.

(closed=minimum, open=maximum)

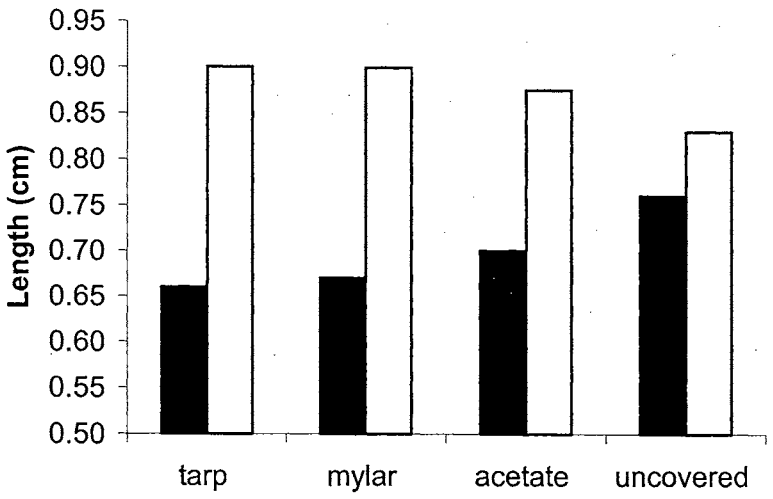


Figure 2.6 Mean length of embryos in *Rana* Experiment 4.

(closed = landfill water, open = FETAX solution)

Chapter III

SENSITIVITY OF DEVELOPING AMPHIBIANS TO ULTRAVIOLET-B RADIATION: INTERSPECIFIC AND LATITUDINAL COMPARISONS

Introduction

There is a growing concern by scientists and others that many amphibian populations are declining worldwide in both disturbed and relatively pristine environments (Phillips, 1990; Wake, 1991; Crump et al., 1992; Carey, 1993; Blaustein et al., 1994a,b,c; Pechmann and Wilbur, 1994; Blaustein and Wake, 1995; Drost and Fellers, 1996; Fisher and Shaffer, 1996; Laurence et al., 1996; Lips, 1998; Donnelly and Crump, 1998; Pounds et al., 1998a,b; Young et al., 2001). A variety of hypotheses and possible explanations for the declines in amphibian populations have been proposed, including climatological change (Pechman and Wilbur, 1994; Pounds and Crump, 1994; Beebee, 1995; Pounds et al., 1998b), environmental acidification (Dunson et al., 1992), environmental xenobiotics (Carey and Bryant, 1995; Fort et al., 1995), disease (Blaustein, 1994; Laurence et al., 1996), introduction of exotic species (Hayes and Jennings, 1986; Knapp and Matthews, 2000; Matthews et al., 2001) and habitat loss (Wyman, 1991; Blaustein and Wake, 1995; Dellis et al., 1996).

There have also been numerous recent reports of grossly malformed frogs over large portions of North America (Sessions and Ruth, 1990; Ouellete, et al., 1997;

Schmidt, 1997). Potential causes for these malformations include parasites (Sessions and Ruth, 1990), xenobiotics (Ouellete, et al., 1997; La Clair et al.,1998), and ultraviolet radiation acting alone or synergistically (Ankley et al., 1998; La Clair et al.,1998; Little et al., 2000).

Unfortunately, the causes for most of these outbreaks of malformations and observed population declines remain unproven despite increased research efforts. Causes may vary from one location to another, but because these are global problems and a number of different species are being affected, even in relatively pristine environments, there has been speculation that some ubiquitous stress, such as global increases in UV-B, may be responsible for some cases of malformations and declines (Carey, 1993; Blaustein et al., 1994c).

Changes in stratospheric ozone concentrations and UV-B irradiation are being caused by anthropogenically created ozone-destroying halocarbons. Stratospheric ozone serves to protect living systems against biologically harmful ultraviolet radiation from the sun. In its pristine condition it attenuates all radiation less than 295 nm (Robberecht, 1989), effectively excluding all UV-C (200-280 nm), which is deadly to life, and much of the UV-B (280-320 nm). There is a negative correlation between stratospheric ozone concentrations and measured UV-B at the Earth's surface.

Since the first publication by Blaustein et al. (1994c) that demonstrated lowered hatching success of amphibian eggs caused by ambient UV-B, there have been various subsequent similar studies both in a laboratory setting with artificial UV-B (Ankley, et. al., 1998; Langhelle et al., 1999; Ankley, et. al., 2000; Pahkala et al., 2001) and in the field (see review by Blaustein et al., 1998; Blaustein et al., 1999; Crump et al., 1999;

Broomhall et al., 2000; Langhelle et al., 1999; Ankley, et. al., 2000; Merila et al., 2000; Pahkala et al., 2000; Starnes et al., 2000; Pahkala et al., 2001; Hakkinen et al., 2001; Bruner et al., 2002). Some of these have demonstrated similar results while many have documented a lack of ill effects caused by ambient levels of UV-B. Often results from tests conducted by the same investigators are positive for some species and negative for others. Some results by different investigators examining the same species are in conflict. To date, no one has been able to interpret this variation other than to ascribe it to interspecific and intraspecific variation of sensitivity to UV-B, or possibly to differing ambient UV-B conditions during the different experiments.

An emerging hypothesis is that those populations of animals inhabiting areas of high natural levels of UV-B would have adapted to be more resistant. Populations of amphibians living in disparate locations can be exposed to vastly different UV-B regimes. UV-B levels increase with elevation and there is a natural latitudinal variation in UV irradiance. UV levels increase from the poles to the equator and are greater in the Southern Hemisphere than the Northern (Madronich et al., 1995; WMO, 1999). In addition to the natural latitudinal variation in UV-B, ozone depletion has been more extensive in the Southern than in the Northern Hemisphere (Stolarski et al., 1992; Madronich et al., 1995). Total annual ozone trends from 1979-1997 indicate a 3.7% per decade decrease at high latitudes (50°-60°) in the Northern Hemisphere versus a 4.4% decrease in the Southern Hemisphere, with even larger decreases during the winter and spring of each year. This ozone depletion leads to an increase in UV-B of 3.7% per decade in the Northern Hemisphere and 9% in the Southern Hemisphere at these latitudes.

In order to better understand the relationship between sensitivity to UV-B and local ambient levels of UV-B, I examined the sensitivity of anuran species from various latitudes (35° - 51°) of the Northern and Southern Hemispheres to a range of UV-B insult. I focused on species along a latitudinal gradient with similar life history strategies, egg laying behaviors, and ecological niches, but in geographically disparate sites. Eggs of these species were exposed to varying degrees of UV-B radiation using a solar simulator. A controlled laboratory setting was used because field studies are unable to control exactly the UV-B dose eggs receive, as this is dependent upon the specific ambient UV-B conditions at the time and place of the experiments. The results of these tests were then compared to the average ambient UV-B levels that would be encountered by these species during their normal breeding season.

Materials and Methods

Field Methods

The species examined were from the central plains of North and South America. All of these species share traits that tend to minimize the amount of variation in UV-B exposure received by the developing embryos, were they all to live at the same latitude. Subjects for my experiments all originated from open habitats at similar elevations. With the exception of *Bufo variegatus*, they all came from below 600 m elevation. All these species breed in temporary pools, ponds, or lagoons, and they all lay eggs in open water near the surface with only the jelly coatings to protect them (Cei, 1980; Livesey and Wright, 1947; personal observation).

Experiments were conducted using fresh eggs collected from natural breeding

ponds near the study sites. Table 3.1 provides a summary of the locations, elevations and species collected at each of these sites. In South America, experiments were conducted using four species (*Bufo arenarum*, *Bufo fernandezae*, *Bufo variegatus*, and *Pleurodema bufonina*) from four latitudes (35°S, 44°S, 49°S, and 51°S) in Argentina. In North America, experiments were conducted with three species (*Rana utricularia, Bufo woodhousii,* and *Bufo hemiophrys*) from two latitudes (35°N, and 44°N) in the U.S. Although a study area was established at 51°N in Saskatchewan, Canada, for comparison with the corresponding high latitudes in South America, because of an extended large-scale drought, no eggs were found there.

Only fresh eggs were collected and in most cases the adults were seen actually ovipositing the eggs. Egg masses were rapidly transported in plastic bags to the laboratory. During transport, eggs were kept in a dark container to prevent sunlight exposure.

Laboratory methods

Prior to the experiments, eggs were sorted for viability in the lab and placed in small glass bowls with 125 ml bottled spring water (20 eggs per bowl). Eggs were taken from a single clutch for each experiment and randomly assigned to each of the different filter treatments in that experiment (with the exception of *B. variegatus*, in which three clutches of eggs were thoroughly mixed and then randomly assigned to each filter treatment). Only viable eggs that retained their jelly coats were used. All eggs were in very early stages of development when the experiments were started (see Table 3.2).

In order to obtain a dose-response relationship, eggs were treated inside a solar

simulator with five levels of UV exposure controlled by the use of filters. Four of these filters were made of cellulose acetate of different thicknesses (0.001 in, 0.003 in, 0.005 in and 0.010 in), and one was made of mylar (0.003 in). The mylar filter blocked 95% of the UV-B and virtually all of the most damaging frequencies with wavelengths shorter than 310 nm.

Three replicates of each of the different filter treatments were used. Bowls with different filters were placed inside the simulator in a completely randomized design. Embryos were checked daily to maintain water levels and remove dead embryos. The individual replicates were also re-randomized daily to help lessen the impact of any "hot-spots" of high irradiance within the simulator. The filters were replaced every four days to help prevent potential changes in emission characteristics following solarization.

Ambient temperature is one of the principal factors affecting the rates of development of eggs and larvae, and therefore the duration of exposure to sunlight. Different species may breed in waters with vastly different thermal regimes. Also, temperatures vary through the breeding season. Since temperature has an effect on the development of anuran larvae, I chose to control the temperatures of the experiments at biologically realistic levels for each species. An appropriate way to do this was to conduct each experiment at a temperature that approximates the middle of the natural range of temperatures encountered by the particular species used in the experiment. This was accomplished based on an initial measurement of temperature at the breeding ponds, followed with modifications to this temperature based upon a judgement of such factors as current weather conditions, current interval of breeding season, likely future or past weather conditions, etc. Temperatures were monitored during the experiments with

HOBO Tidbit® temperature data loggers submerged in replicates of each of the different filter treatments. These temperature loggers took readings every ten minutes throughout the course of each experiment (Table 3.2).

The experiments were stopped after the embryos under the mylar filter treatments reached stage 25 (Gosner, 1960). Durations of each of the experiments are given in Table 3.2. Stage 25 was chosen as a stopping point because primary organogenesis was complete and feeding was unnecessary prior to this point. At the end of the experiments mortality was totaled, embryos were analyzed for malformations, and digital photos were taken. The digital photos were used with SigmaScan Pro® (Version 4.01.003, *SPSS Inc.*) image analysis software to measure length. Experiments in which embryos under mylar filters experienced greater than 10% mortality were excluded.

Solar Simulator Specifications and Emission Properties

The portable solar simulator used in these experiments was 1.0 m wide by 1.5 m long. It contained 18 flourescent lamps arranged in two layers. The top layer consisted of eight very-high-output cool white lamps. The second layer of lamps was directly below the first layer and staggered between the upper lamps. The second layer of lamps consisted of four high-output UV-B lamps and six high-output UV-A lamps arranged in alternating pairs of UV-A and UV-B. The cool white lamps and the UV-A lamps were controlled by one timer and the UV-B lamps were connected to a second timer.

The entire array of lights was suspended above a water bath made of stainless steel. The sides were covered with sheets of highly reflective specular aluminum. Small ventilation fans were used in the sides of the solar simulator to help remove excess heat

from the simulator. The temperature of the water bath was controlled using a recirculating system with an external reserve chiller and thermostatically controlled heater.

The spectral emission characteristics of the solar simulator were measured using an Optronics Laboratories (Orlando, Florida) Model OL-754 spectroradiometer at one nanometer intervals over a wavelength range of 280-700 nm. Surface intensity measurements were taken through each filter treatment from three different locations within the solar simulator. These locational measurements were combined to give an average intensity for each filter treatment within the solar simulator. These measurements were taken for each of the light-cycle regimes used in the experiments.

The results of the spectral emission measurements are shown for each of the filter treatments in Figure 3.1. Because the increase in UV levels due to loss of ozone is strongly weighted toward UV-B (without a concomitant increase in visible light or UV-A, which is used by photorepair enzymes), the levels of exposure in the solar simulator needed to mimic this phenomenon. In other words, using these filters, the levels of UV-A and visible light were not changed as much as the level of UV-B.

Light Cycle

All experiments were conducted using a light cycle of 10 h dark, 14 h of UV-A/cool white light (to simulate a midsummer day at the middle latitudes), with the UV-B being turned on for 5 h in the middle of the 14 h UV-A/cool white cycle (to simulate the more intense UV-B levels of midday). Three exceptions to this are the first *B. arenarum* experiment and the two *B. fernandezae* experiments. These were the first experiments

conducted after shipping the solar simulator to Argentina and two of the enclosed UV-A lamps were damaged and not operating. Therefore the UV-A/cool white light cycle was extended to 16 h to help make up for the difference in light intensity. The damaged lights were replaced for the other experiments.

Tables 3.3 and 3.4 provide the UV-A and UV-B irradiance levels, erythemally weighted UV-B ($UV\text{-}B_{ery}$) irradiance levels, and the $UV\text{-}B_{ery}$ cumulative daily doses for each filter treatment. The Diffey erythemal action spectrum was used to weight each wavelength based on its effectiveness at erythema (sunburn) induction. This action spectrum was chosen to compare more accurately the artificial radiation from the solar simulator with natural solar irradiance data.

Determination of Ambient UV-B Levels at Each Site

Ambient UV-B levels were obtained from the National Aeronautics and Space Administration (NASA), Total Ozone Mapping Spectrometer (TOMS) satellite data. The TOMS satellites measure total column ozone with nearly complete global daily coverage. Data are mapped to a standard grid with pixels of 1° latitude by 1.25° longitude. Data were used from the pixels congruent with the geographical coordinates of the study sites. Daily $UV\text{-}B_{ery}$ is a derived data product produced from the TOMS data for latitudes between 65°S and 65°N and is thus readily comparable with my solar simulator levels. These data are calculated at the average elevation per pixel and tend to underestimate values at high elevations, particularly in areas with greatly changing topography. All of the experiments I conducted used animals from the plains regions (with the exception of *B. variegatus*). These are large areas of relatively unchanging topography and therefore

the data should be a relatively accurate estimate.

Figure 3.2a. provides the mean daily ambient UV-B_{ery} during the spring when amphibians breed. By comparing the UV-B levels during a particular month across latitudes, a natural gradient can be observed. Levels of UV-B are higher at the lower, more equatorial, latitudes. However, as shown in Figure 3.2b, when the actual breeding times for each species are considered, these differences in UV-B levels are substantially diminished. Merila et al. (2000) demonstrated the same phenomenon with *Rana temporaria* in Europe.

Statistical Analysis

Data were percent mortality, percent malformations, and length. Trimmed Spearman-Karber analysis was used to estimate LD50, ED50 for malformations, and associated 95% confidence intervals. Linear regression analyses were performed with the length data and log-transformed percent mortality and percent malformation data to test for significance of the dose-response. Analysis of Variance (ANOVA) with Fisher's Least Significant Difference analysis for pair-wise comparisons was used within each experiment to determine the lowest observed adverse effect level (LOAEL) and the no observed adverse effect level (NOAEL) for mortality, malformation and length. The average ambient daily UV-B_{ery} for the three months of spring at each location was calculated. These values were then correlated with the LOAEL and estimated LD50 for mortality, and the LOAEL and estimated ED50 for malformation.

Results

Effects on Mortality

Figure 3.3 shows the mortality dose-response relationships for each of the experiments. The range of doses provided by the solar simulator was adequate to provide a good response curve in four of the seven species, *R. utricularia*, *B. woodhousii*, *B. arenarum* and *B. fernandezae*. Unfortunately for this project (perhaps fortunately for the species in question), *B. hemiophrys*, *B. variegatus*, and *P. bufonina* were resistant within the range of doses of the experiments and therefore did not show a well-defined response curve to 100% mortality. However, the results of the regression analysis indicate a significant dose-response relationship ($p<0.05$) in at least one experiment with each of these three species (Table 3.5). The four sensitive species showed a strong significant correlation between UV-B and mortality ($p<0.05$).

Results of the ANOVA with Fisher's LSD tests (Table 3.6) show the threshold was reached where the rate of mortality was significantly greater than mortality under the mylar filter in *B. variegatus* and in at least one experiment with *B. hemiophrys*, and *P. bufonina.* The LOAEL for *B. variegatus,* one of the experiments with *B. hemiophrys*, and one of the experiments with *P. bufonina* was 14235 J/m^2/d, which is the highest dosage given. A LOAEL was unable to be calculated in the other two experiments with *B. hemiophrys*, and *P. bufonina*. In these two experiments the eggs were simply less robust and higher mortality across many of the treatments obscured the appearance of the threshold level. In the four sensitive species, *R. utricularia*, *B. woodhousii*, *B. arenarum* and *B. fernandezae*, the LOAEL ranged from 6634 J/m^2/d to 8383 J/m^2/d.

Results of the Spearman-Karber LD50 estimates (Figure 3.4) also reflect these

differences. While LD50 estimates were not attainable for the *B. hemiophrys*, *B. variegatus*, and *P. bufonina* experiments, for comparison purposes the lower end of the 95% confidence intervals are displayed at the highest dose level, as the LD50 for these species is certainly higher than this. Estimates of LD50 for the other species show significant differences. *Bufo woodhousii* was the most resistant with an LD50 of 9902 J/m^2/d. *R. utricularia* had the next highest LD50 at 8756 J/m^2/d, which was significantly higher than the other species except for one of the *B. fernandezae* experiments. There was a significant difference between the two *B. fernandezae* experiments. The first had an LD50 of 8379 J/m^2/d while the second had an LD50 of 6991 J/m^2/d. The LD50 estimates for the two *B. arenarum* experiments (7651 J/m^2/d at 35°S and 7772 J/m^2/d at 44°S) were not significantly different from each other nor from the average of the two *B. fernandezae* experiments.

UV-B effects on malformations

Results for malformations essentially mimicked those for mortality (Figure 3.5). A good dose-response was seen in the *R. utricularia*, *B. arenarum* and *B. fernandezae* experiments, with a strong significant correlation ($p<0.05$) between UV-B and malformation (Table 3.5). For malformations, however, *B. woodhousii* appeared to be somewhat more resistant. A significant dose-response ($p<0.05$) was observed with the regression analysis, but the Spearman-Karber analysis was unable to calculate an ED50 for malformation. This is probably because 100% mortality in the highest level treatments precluded measurement of malformations.

As in the results for mortality, *B. hemiophrys*, *B. variegatus*, and *P. bufonina*

were more resistant and did not show a well defined response curve. Here again, the regression analysis indicates a significant dose-response relationship ($p<0.05$) in the *B. variegatus*, and in the better quality *P. bufonina* experiment. Unlike the results for mortality, however, no significant effect for malformations was observed in either of the *B. hemiophrys* experiments.

Results of the ANOVA with Fisher's LSD tests (Table 3.6) for malformations were the same as those for mortality in all but two experiments. In the first *B. arenarum* experiment the NOAEL (4628 J/m^2/d) and LOAEL (6634 J/m^2/d) for malformation was one dosage lower than it was for mortality. In both experiments with *B. hemiophrys* none of the treatments were significantly different than the control for malformation and thus the threshold level is higher than the dosages given.

Results of the Spearman-Karber ED50 estimates for malformation (Figure 3.6) show similar patterns. Estimates of ED50 were not attainable for the *B. hemiophrys*, *B. variegatus*, and *P. bufonina* experiments, and here again the lower end of the 95% confidence intervals are displayed at the highest dose level. Estimates of ED50 for the other species indicate they were all very similar with only the second *B. arenarum* experiment being significantly different than any of the others. This experiment showed an ED50 significantly higher ($p<0.05$) than the first *B. arenarum* experiment and the second *B. fernandezae* experiment.

Examples of the types of malformations observed in this study are shown in Figure 3.7. These examples are all from the *B. arenarum* experiments as this is a species with typical development, average size, and response to UV-B. The most common type of malformation observed in this study was a dorsal curvature of the tail, which would

suggest abnormal development of the notochord. Other frequent malformations were seen in the head, face and eyes of the embryos. Typically in the more sensitive species, both the rate of malformation and the severity of those malformations increased with increasing UV-B. Both malformed and normal embryos from exposed treatments were typically shorter than control embryos and the development of these embryos was typically delayed. This effect was also clearly more pronounced with increasing UV-B dose.

UV-B effects on length

Figure 3.8 displays the length of the embryos under the different filter treatments for each of the experiments. The results of the regression analysis (Table 3.5) indicate a strong and very significant ($p<0.001$) dose-response relationship in all of the experiments. Results of the ANOVA with Fisher's LSD tests (Table 3.6) show the LOAEL for length to be at the lowest level of UV-B beyond the mylar filter in all of the experiments except *B. variegatus*, which had a LOAEL at the next higher intensity.

Correlation analysis

A significant negative correlation ($r= -0.954$; d.f.=7; $p<0.001$) was observed between the average ambient daily $UV\text{-}B_{ery}$ and the LOAEL for mortality (Figure 3.9a). A significant negative correlation ($r= -0.974$; d.f.=6; $p<0.001$) was also observed between $UV\text{-}B_{ery}$ and the LOAEL for malformations (Figure 3.9b). There was a negative correlation ($r= -0.759$; d.f.=4; $p=0.08$) between $UV\text{-}B_{ery}$ and the estimated LD50, but it was not significant at the alpha level of 0.05. The correlation analysis indicated no

significant correlation (r= -0.265; d.f.=3; p=0.67) between UV-B_{ery} and the estimated ED50 for malformations.

Discussion

All of the analyses with the mortality and malformation results indicate that *B. hemiophrys*, *B. variegatus*, and *P. bufonina* were much more resistant to UV-B_{ery} than *R. utricularia*, *B. woodhousii*, *B. arenarum* and *B. fernandezae*. Noticeable differences were observed in the morphology and development of the different species, which may help explain the proximate causes of the biggest interspecific differences in sensitivity.

Adult *B. variegatus* are relatively small (40-60 mm), but the contribution given to each individual egg is very large in comparison with the other species of the study. The eggs of *B. variegatus* were very large and very black and only about 200 - 400 were laid in each clutch, compared with the thousands of smaller eggs laid by other species. This large investment of yolk per egg provided a lot of energy for cellular repair and maintenance of melanin levels. Melanin is considered the most effective pigment in protecting animals from UV-B and can also act to absorb heat which is then effectively retained by the jelly coating (Kollias et al., 1991; Licht and Grant, 1997). Merila et al. (2000) have noted reports of more heavily pigmented eggs in higher-latitude populations and this could be an adaptation to absorb and retain heat better at cooler, more northerly latitudes in the Northern Hemisphere.

The eggs of *B. hemiophrys* and *P. bufonina* developed for a longer time within the jelly coat than the other species. This was particularly noticeable in *P. bufonina*, which had a very thick, gelatinous jelly in which the embryos remained up until approximately

stage 22, whereas the other species usually hatched at stage 19 or 20. Such extended development inside the jelly coat could have reduced the embryo exposure to UV-B. Grant and Licht (1995) demonstrated that segments of jelly 3 mm thick from *Bufo americanus*, *Rana aurora*, and *Rana sylvatica* could absorb 6 - 14% of the UV-B, while an entire clear egg mass (devoid of ova) from *Ambystoma maculatum* could reduce transmission by as much as 77%.

In the four more sensitive species, there was a rapid response once a threshold dose was achieved. All of these four species had an estimated LD50 within 2100 $J/m^2/d$ of their respective LOAEL for mortality. If the three more resistant species were to follow the same pattern, then we could expect to see an LD50 for these three species between 14,250 $J/m^2/d$ and 16,350 $J/m^2/d$.

The significant negative correlations observed between ambient UV-B levels and several indicators of sensitivity (LOAEL's for mortality and malformations, and LD50's) simply do not make much adaptive sense. This result may very well be attributable to a lack of data for a sufficient number of species and the correlation may be spurious. As mentioned previously, it is also possible that the TOMS satellite data underestimated the amount of UV-B at the site where *B. variegatus* were collected, as this location was probably at a higher elevation than average and TOMS data are calculated at the average elevation per pixel and tend to underestimate values at high elevations. Even so, it is unlikely that it is so underestimated such that the geographical pattern would be obliterated or reversed (see Figure 3.2b).

The pattern was observed in both hemispheres and appears to reflect a valid relationship, though. Phylogenetic differences should not be a confounding issue in this

relationship as two of the resistant species and three of the sensitive species are bufonids of the genus *Bufo*. *Pleurodema bufonina* (a resistant species) is a leptodactylid and *R. utricularia* (a sensitive species) is a ranid. A tentative phylogenetic relationship among test species (Figure 3.10) based on Martin (1972), Greybeal (1997), and Zug *et al.* (2001) indicates that the pattern of resistance to UV-B does not display a strong phylogenetic correlation.

One potential reason for the observed pattern of results could be that the eggs of higher latitude species actually do receive more UV-B, despite the TOMS satellite data that show higher surface levels of UV-B at low latitudes. For example, if turbidity or dissolved organic carbon (DOC) levels are naturally lower at high latitudes, then frog eggs there may actually receive more UV-B compared to low latitudes. Published studies of this type provide conflicting results, and deal mainly with streams, rivers, and lakes (Webster and Meyer, 1997; Virola et al., 2001). Middleton et al. (2001) point out that DOC in the tropics is probably higher than in temperate regions due to especially abundant vegetation. However, all of the species in my study tend to lay their eggs in shallow, temporary rainwater ponds, and all except *B. variegatus* live in similar plains type habitat, and thus are probably not subject to any latitudinal gradient in turbidity or DOC levels. In fact, all of the eggs encountered in this study were found in such temporary ponds. Another possible reason that eggs at higher latitude may receive more UV-B is if these species tend to lay their eggs closer to the surface, to gain heat in generally colder water for example. This pattern, however, was not observed with the eggs collected for this study. While eggs of both *B. hemiophrys* and *P. bufonina* (two resistant species) were found in very shallow water (0 -5 cm), so were *R. utricularia* eggs

and the first clutch of *B. arenarum* (two sensitive species). A third possibility is that strong pulses of UV–B occur more frequently or more intensely at high versus low latitudes. This has been observed at very high southern latitudes, as ozone-poor air from the South Pole drifts north over the southern tip of South America (Frederick et al., 1994). Maybe a similar phenomenon occurs occasionally in the northern hemisphere as well and could be a sufficient insult for adaptations to arise. As pointed out by Middleton et al. (2001), effects attributable to differences in the timing and duration of high radiation events might overshadow the effects expected from the typical background levels of UV-B. Middleton et al. (2001) showed that extreme peaks of UV-B_{ery} since 1975 have lasted more days and were more intense at South American sites compared to Central American sites. Their South American sites were on the average at higher latitudes than their Central American sites, but all of their sites were less than 35° latitude and likely peaks of UV-B_{ery} were influenced by factors other than latitude alone.

Another possible explanation for the observed pattern is that the high latitude species have evolved adaptations in response to other selective pressures, and that these adaptations simultaneously provide a defense against UV-B, even though it is not really needed. As mentioned previously, eggs of *B. variegatus* were very large and very black, while developing embryos of *P. bufonina*, and to a much lesser extent *B. hemiophrys*, remained in the jelly coat for an extended period of development. Potentially, both of these adaptations arose to provide a thermal advantage to developing embryos in colder water at higher latitudes (Kollias et al., 1991; Licht and Grant, 1997; Merila et al., 2000). I think in proximate terms, these structural and physiological differences are the best explanations (in the absence of other data such as photolyase levels) for the observed

differences in sensitivity. However, in an ultimate sense, I am not convinced that these differences are persistently correlated with latitude and would once again caution that the observed geographical pattern may not persist once more data on more species are collected.

Analysis of the length data indicates a significant negative response at the lowest dose given in all species (except *B. variegatus*, which was significantly affected at the next highest dose). This response increased in severity with increasing intensity of UV-B_{ery}. This effect on the length of the embryos occurs at levels much lower than those needed to cause significantly higher mortality or malformations. Developing embryos probably encounter levels high enough to produce these negative effects on growth routinely in the wild. While not directly causing mortality, these levels of UV-B_{ery} probably decrease the fitness of the organisms affected as energy normally devoted to growth would be utilized repairing the damage and producing defensive proteins and pigments. Other studies that have shown no negative effect of UV-B on embryo survivorship similarly have shown a negative effect on growth (Bruggeman et al., 1998; Pahkala et al., 2000)

The LOAEL's for mortality and malformation were all above the average intensities of ambient UV-B_{ery} during the spring months. It is, however, important to realize that these levels are averages that include both sunny and cloudy days. TOMS satellite data show that UV-B_{ery} levels during very sunny days in the spring can reach as much as 7000 to 8000 $J/m^2/d$ at these locations. The more sensitive species of this study all have LOAEL's or even LD50's near or below these intensity levels. The levels of UV-B_{ery} discussed throughout this paper are those levels encountered at the land or water

surface. The transmission through the water in my simulator would be high because I used bottled spring-water, which had virtually no suspended particles or dissolved carbon. Natural bodies of water have vastly different characteristics that influence the transmission of UV-B_{ery} (Licht and Grant, 1997; Blaustein et al., 1998). If these sensitive species were to breed in particularly clear water and have sunny weather during early development, they would likely experience significant negative effects.

I believe that the different and often conflicting results observed by other scientists can be explained by an examination of my results. It appears that there is a large degree of interspecific variation in sensitivity to UV-B. Noticeable differences were observed in the morphology and development of the different species, which may help explain the proximate causes of the biggest interspecific differences in sensitivity. Some species experienced elevated mortality and malformations at levels of UV-B only slightly higher than those likely to be encountered on average in the field. Under particularly good conditions, such as artificially elevated eggs, in very clear water with low DOC, and under sunny skies, UV-B levels may in fact exceed this threshold dose, and researchers conducting field experiments under these optimal conditions would detect a significant effect. Change any one of these variables and results would be different. Thus, experiments conducted even on the same species, if it is one of these with response thresholds near the ambient UV-B levels, might show a negative effect under a given set of exposure conditions one time, and not show a negative effect under a different set of conditions another time. For that presumably large set of species with defenses closely matching average ambient levels of UV-B, one would expect the considerable variation of responses that has been reported in the literature to date. No

longer must this observed variation be interpreted as problematical.

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Table 3.1 Locations, elevations, and dates eggs were collected for the experiments.

Experiment	Latitude	Elevation (m)	Date Collected
B. hemiophrys 1	44°30'N	550	May 10, 2001
B. hemiophrys 2	44°30'N	550	May 10, 2001
R. utricularia 1	35°10'N	340	March 12, 2001
B. woodhousii 1	35°10'N	340	April 5, 2001
B. arenarum 1	34°57'S	<100	Sept. 13, 2000
B. fernandezae 1	34°57'S	<100	Sept. 23, 2000
B. fernandezae 2	34°57'S	<100	Sept. 23, 2000
B. arenarum 3	43°19'S	<100	Dec. 13, 2000
B. variegatus 1	49°13'S	850	Oct. 14, 2000
P. bufonina 1	51°37'S	<100	Nov. 3, 2000
P. bufonina 2	51°37'S	<100	Nov. 3, 2000

Table 3.2 Temperature of the ponds where the eggs were collected, average temperature of each experiment (±1 SE), initial stage of development when the experiments were started, and duration of each experiment.

Experiment	Temperature of Pond (°C)	Temperature of Experiment (°C)	Stage of Development*	Duration of Experiment (days)
B. hemiophrys 1	18.0	18.7 ± 0.05	5	6
B. hemiophrys 2	18.0	18.7 ± 0.05	5	6
R. utricularia 1	17.6	18.7 ± 0.08	14	7
B. woodhousii 1	17.2	16.2 ± 0.06	14	7
B. arenarum 1	13.2	17.0 ± 0.14	8	8
B. fernandezae 1	17.0	18.4 ± 0.15	5	7
B. fernandezae 2	17.0	18.4 ± 0.15	5	7
B. arenarum 3	19.2	17.1 ± 0.06	8	8
B. variegatus 1	11.6	14.0 ± 0.04	4	18
P. bufonina 1	16.1	16.1 ± 0.04	11	8
P. bufonina 2	16.1	16.1 ± 0.04	9	9

*Developmental stage as per Gosner (1960).

Table 3.3 Filter treatments with respective levels of irradiance, Diffey weighted irradiance ($UV\text{-}B_{ery}$), and Diffey weighted daily dose for the *B. arenarum* 1 and *B. fernandezae* 1 and 2 experiments (when two of the UV-A lights were not functioning).

	Irradiance ($\mu W\ cm^{-2}$)		$UV\text{-}B_{ery}$	$UV\text{-}B_{ery}$ Dose
Filter Treatment	UV-B	UV-A	($\mu W\ cm^{-2}$)	($J\ m^{-2}\ d^{-1}$)†
Mylar 0.003 in	25	709	1.5	291
Cellulose acetate 0.010 in	191	810	24.0	4,628
Cellulose acetate 0.005 in	220	846	34.0	6,634
Cellulose acetate 0.003 in	234	848	44.0	8,383
Cellulose acetate 0.001 in	252	858	55.0	10,500

†- Units are m^{-2} for easier comparison with TOMS satellite data.

Table 3.4 Filter treatments with respective levels of irradiance, Diffey weighted irradiance ($UV\text{-}B_{ery}$), and Diffey weighted daily dose for all experiments **except** *B. arenarum* 1 and *B. fernandezae* 1 and 2 (when two of the UV-A lights were not functioning).

	Irradiance ($\mu W\ cm^{-2}$)		$UV\text{-}B_{ery}$	$UV\text{-}B_{ery}$ Dose
Filter Treatment	UV-B	UV-A	($\mu W\ cm^{-2}$)	($J\ m^{-2}d^{-1}$)†
Mylar 0.003 in	22	1,030	1.5	301
Cellulose acetate 0.010 in	213	1,205	28.0	5,301
Cellulose acetate 0.005 in	253	1,265	41.0	7,816
Cellulose acetate 0.003 in	288	1,346	56.0	10,575
Cellulose acetate 0.001 in	338	1,479	76.0	14,234

†- Units are m^{-2} for easier comparison with TOMS satellite data.

Table 3.5 Results of regression analysis of the log mortality, log malformations and length versus UV-B dose.

		Mortality		Malformation		Length	
Experiment	Latitude	*r*	*p*	*r*	*p*	*r*	*p*
B. hemiophrys 1	44°N	0.586	0.02	0.096	0.733	0.947	<0.001
B. hemiophrys 2	44°N	0.345	0.21	0.305	0.27	0.95	<0.001
R. utricularia 1	35°N	0.901	<0.001	0.808	0.005	0.941	<0.001
B. woodhousii 1	35°N	0.856	<0.001	0.634	0.03	0.98	<0.001
B. arenarum 1	35°S	0.817	<0.001	0.742	0.006	0.931	<0.001
B. fernandezae 1	35°S	0.862	<0.001	0.88	<0.001	0.894	<0.001
B. fernandezae 2	35°S	0.836	<0.001	0.676	0.02	0.949	<0.001
B. arenarum 3	44°S	0.883	<0.001	0.857	0.001	0.962	<0.001
B. variegatus 1	49°S	0.571	0.03	0.574	0.03	0.847	<0.001
P. bufonina 1	51°S	0.382	0.16	0.01	0.97	0.823	<0.001
P. bufonina 2	51°S	0.685	0.005	0.505	0.05	0.923	<0.001

Table 3.6 No observed adverse effect levels (NOAEL)and lowest observed adverse effect levels (LOAEL) ($UV\text{-}B_{ery}$ in $J/m^2/d$) for mortality, malformations and length, determined with ANOVA and Fisher's Least Significant Difference tests at $p<0.10$.

		Mortality		Malformation		Length	
Experiment	**Latitude**	**NOAEL**	**LOAEL**	**NOAEL**	**LOAEL**	**NOAEL**	**LOAEL**
B. hemiophrys 1	44°N	10575	14235	*	*	&	5301
B. hemiophrys 2	44°N	*	*	*	*	&	5301
R. utricularia 1	35°N	5301	7816	5301	7816	&	5301
B. woodhousii 1	35°N	5301	7816	5301	7816	&	5301
B. arenarum 1	35°S	6634	8383	4628	6634	&	4628
B. fernandezae 1	35°S	4628	6634	&	4628	&	4628
B. fernandezae 2	35°S	4628	6634	4628	6634	&	4628
B. arenarum 3	44°S	5301	7816	5301	7816	&	5301
B. variegatus 1	49°S	10575	14235	10575	14235	5301	7816
P. bufonina 1	51°S	*	*	*	*	&	5301
P. bufonina 2	51°S	10575	14235	10575	14235	&	5301

* - Unable to calculate because no significant effects observed at highest dose

& - Unable to calculate because significant effects observed at lowest dose beyond controls

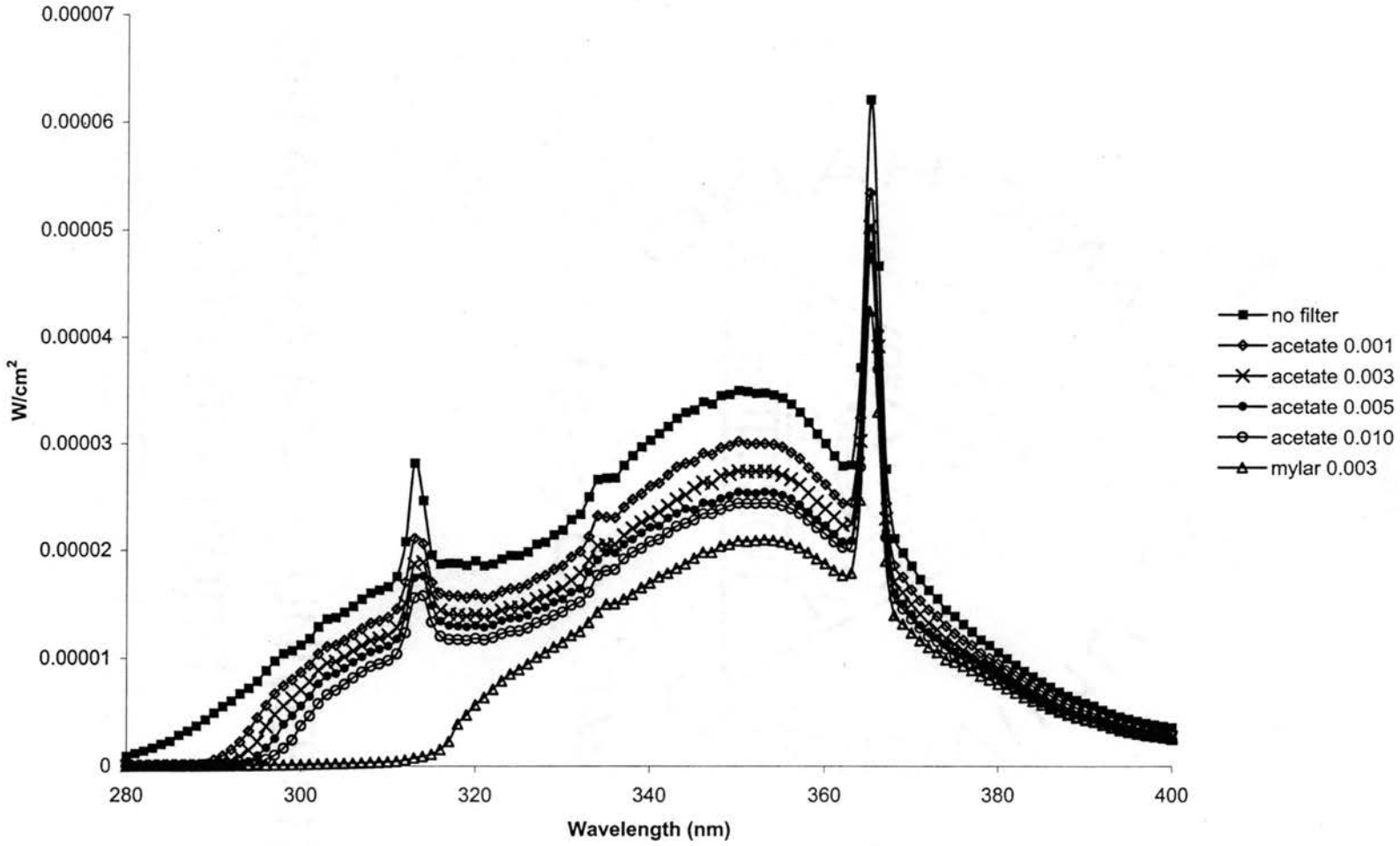


Figure 3.1 Spectral characteristics of the solar simulator and transmission characteristics of the different filter treatments.

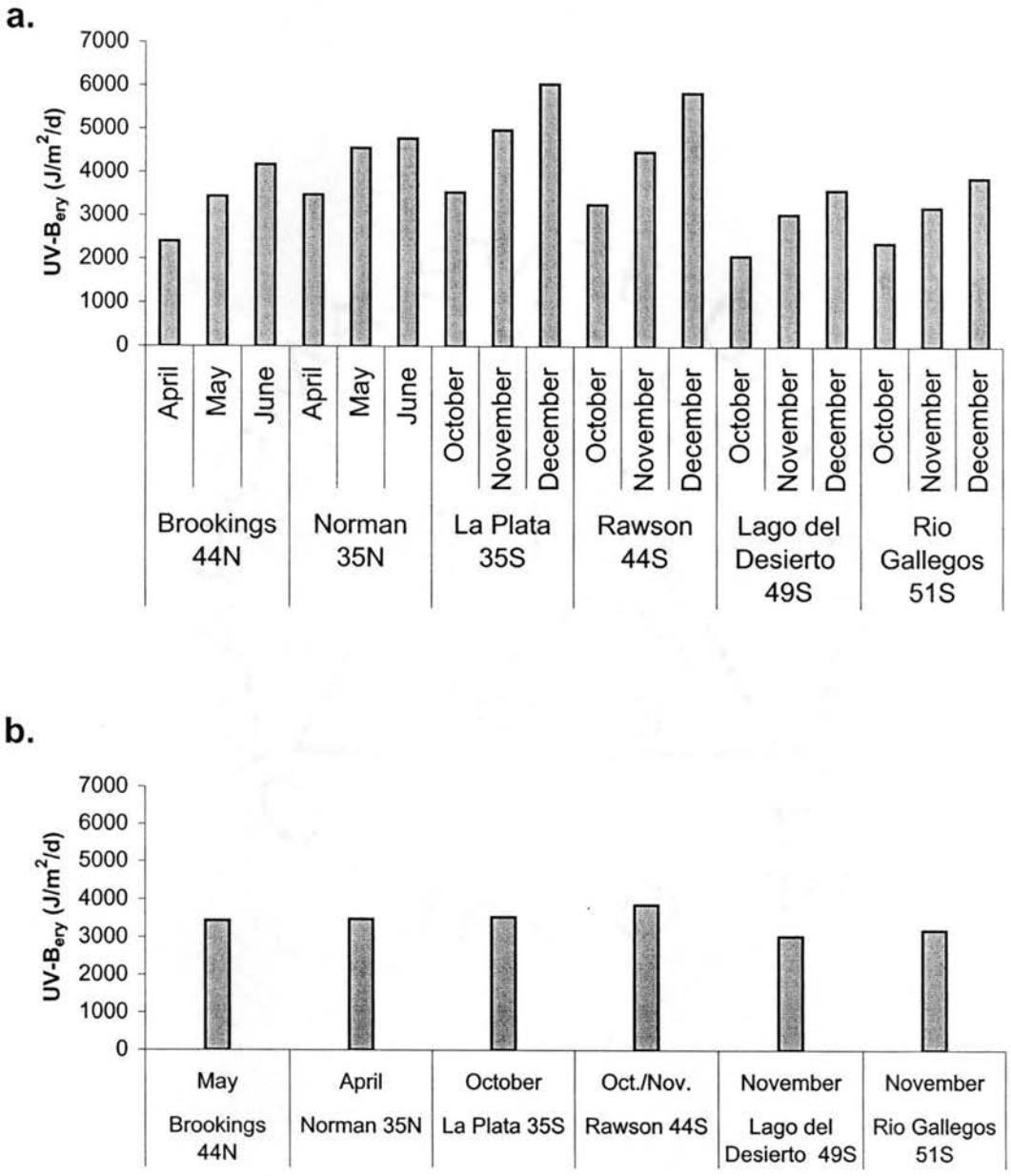


Figure 3.2 TOMS satellite data of mean daily ambient UV-B_{ery} from 1997-2001.
a. UV-B during the springtime months.
b. UV-B during the most likely breeding times.

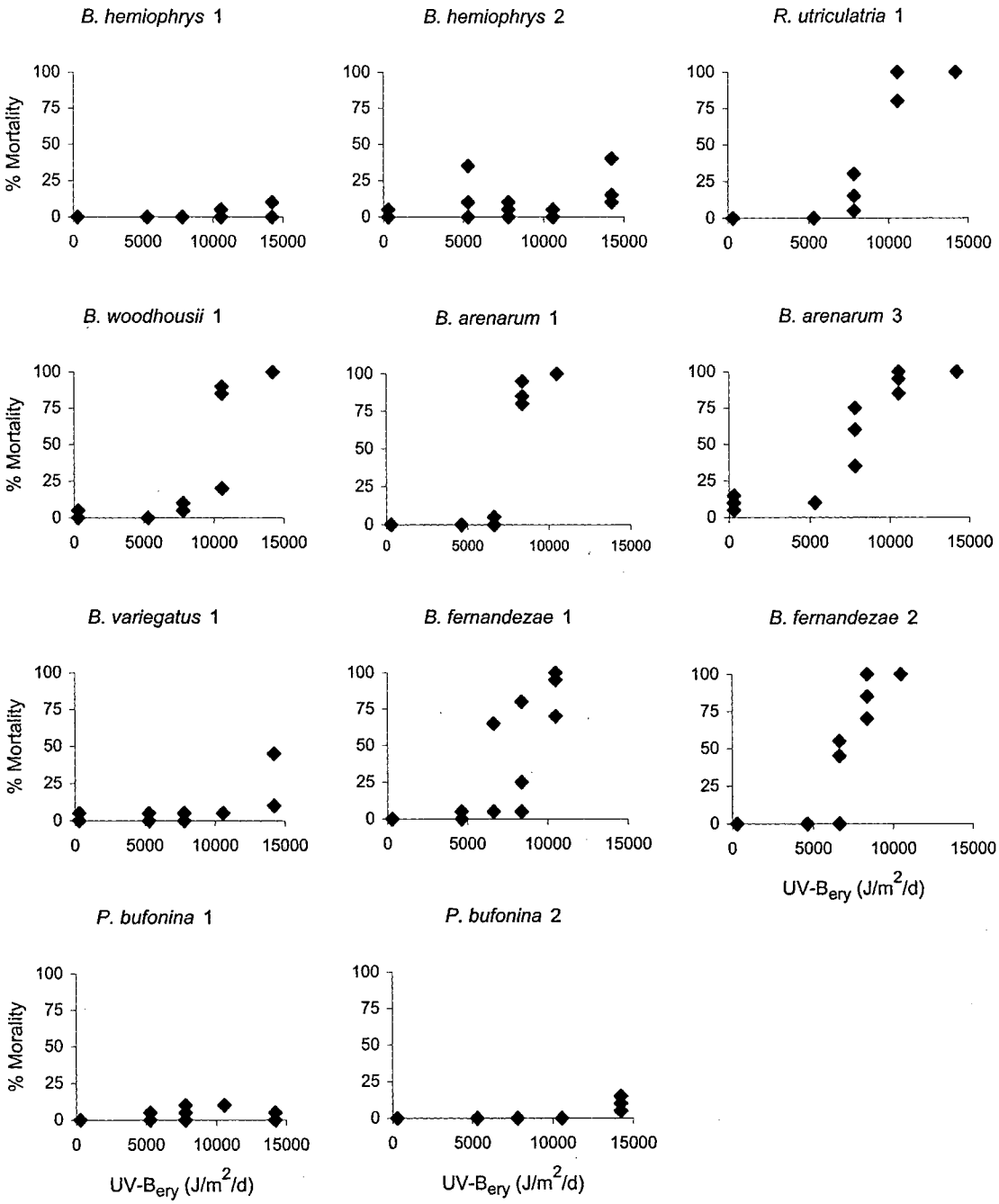


Figure 3.3 Percent mortality versus UV-B_{ery} for each of the experiments.

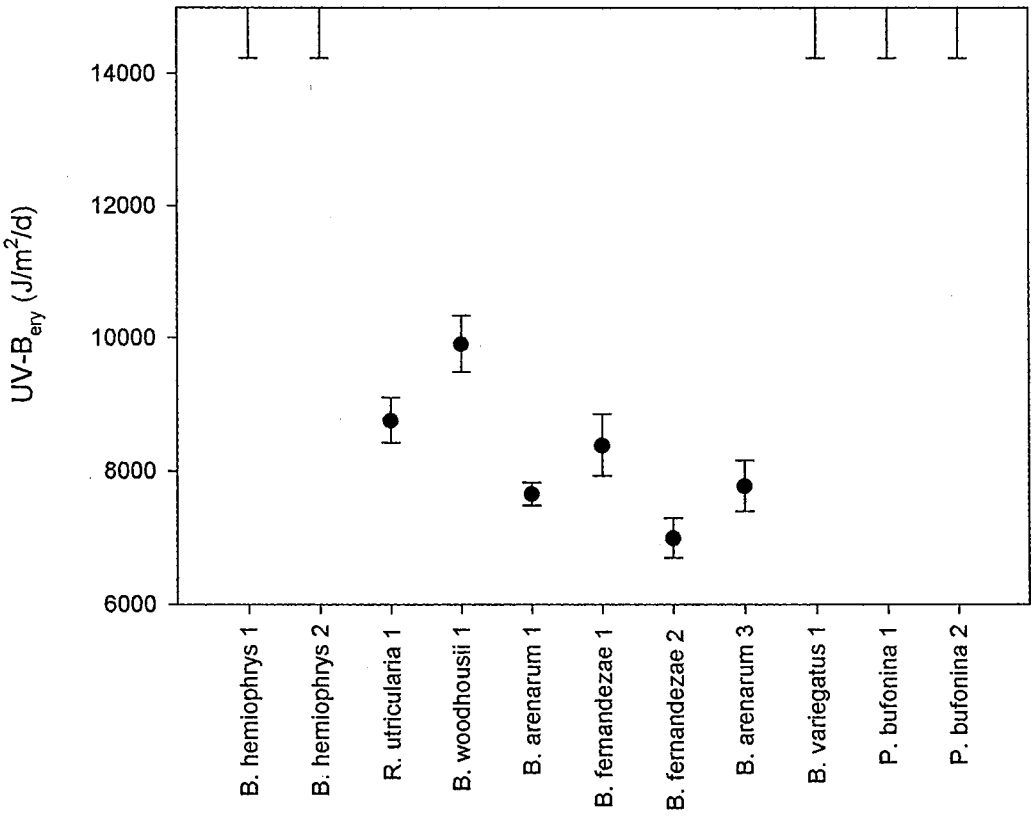


Figure 3.4 Spearman-Karber LD50 estimates. (Note: While estimates were not possible for *B. hemiophrys, B. variegatus, and P. bufonina,* the lower end of the 95% confidence intervals are shown at the highest dose.)

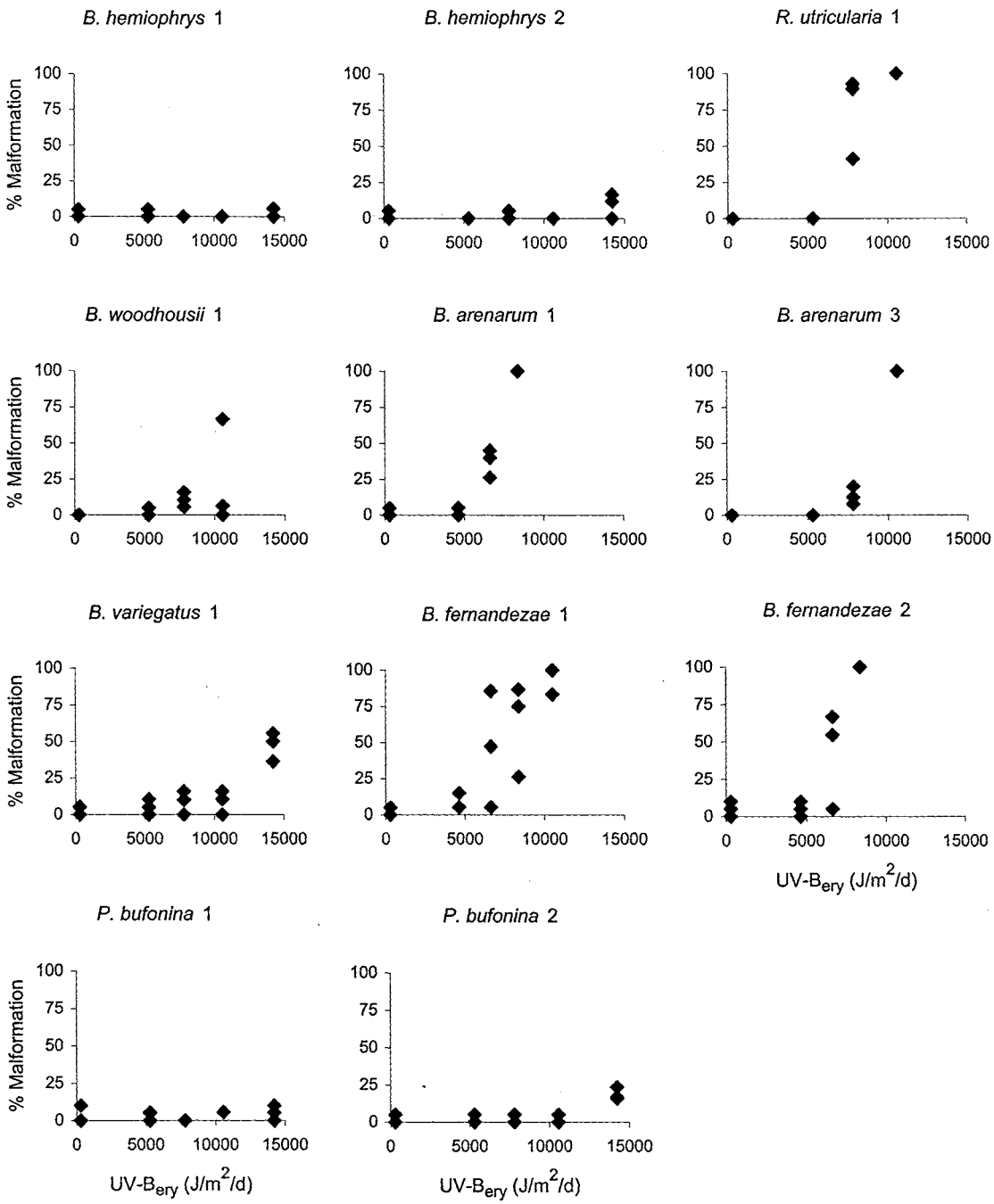


Figure 3.5 Percent malformation versus UV-B_{ery} for each of the experiments.

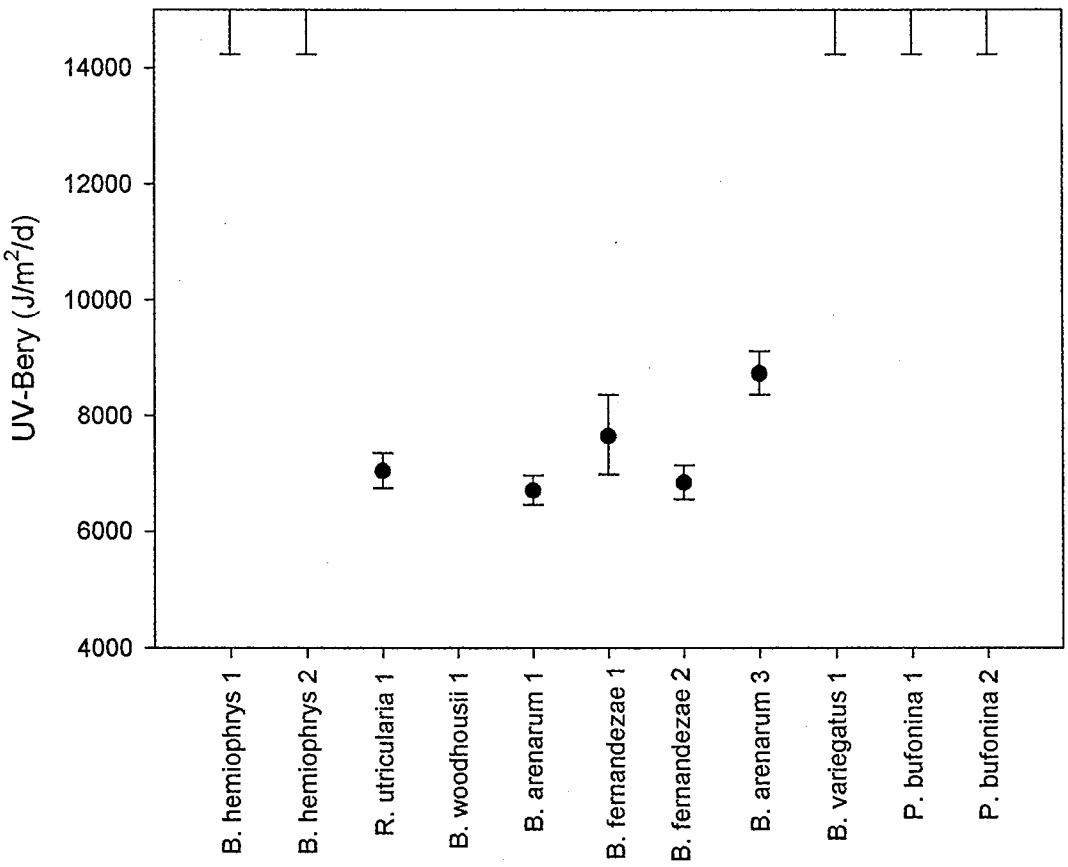


Figure 3.6 Spearman-Karber malformation ED50 estimates. (Note: While estimates were not possible for *B. hemiophrys, B. variegatus, and P. bufonina,* the lower end of the 95% confidence intervals are shown at the highest dose. Unable to calculate ED50 for *B. woodhousii* probably because high mortality precluded measurement of malformations.)

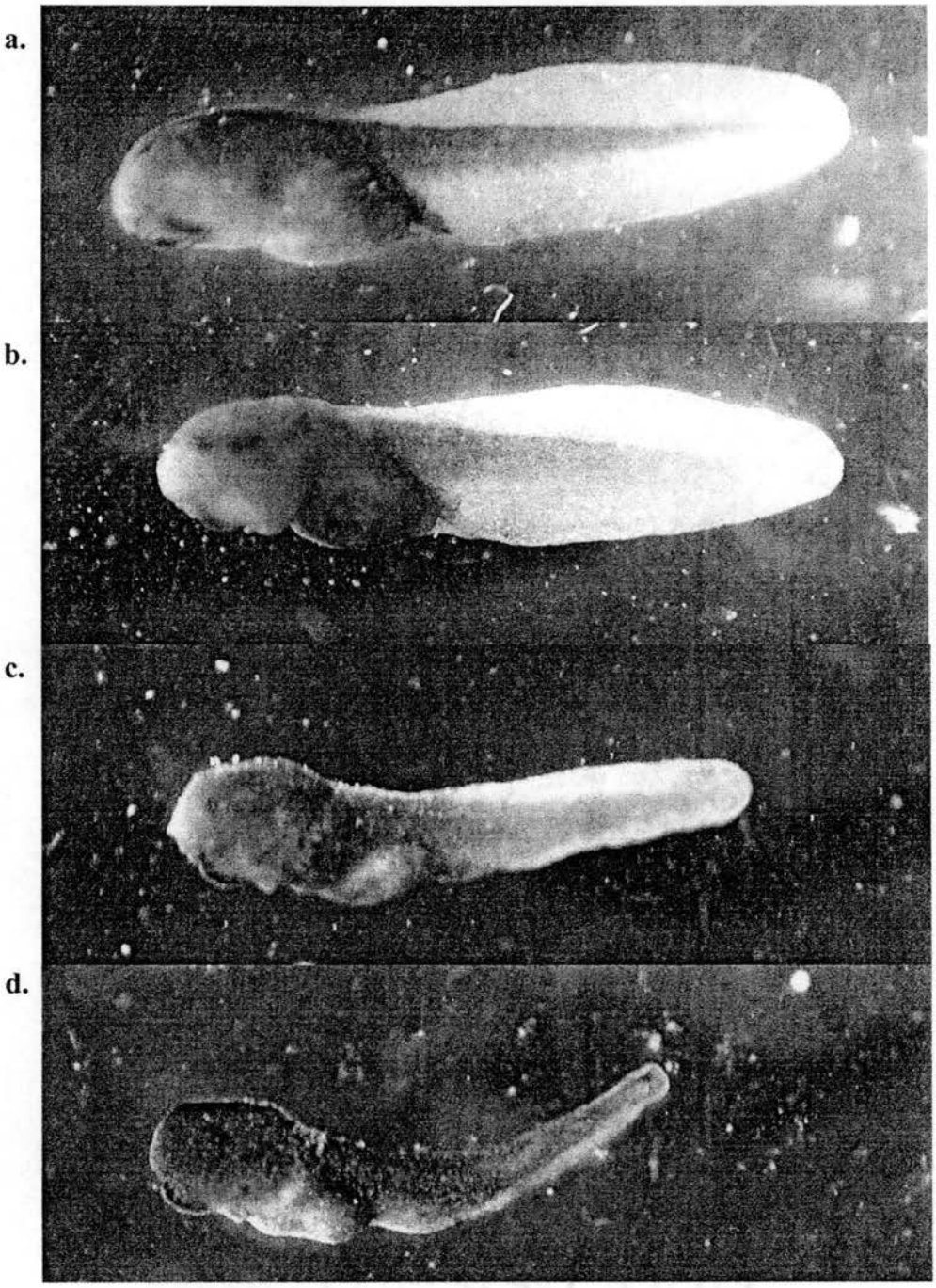
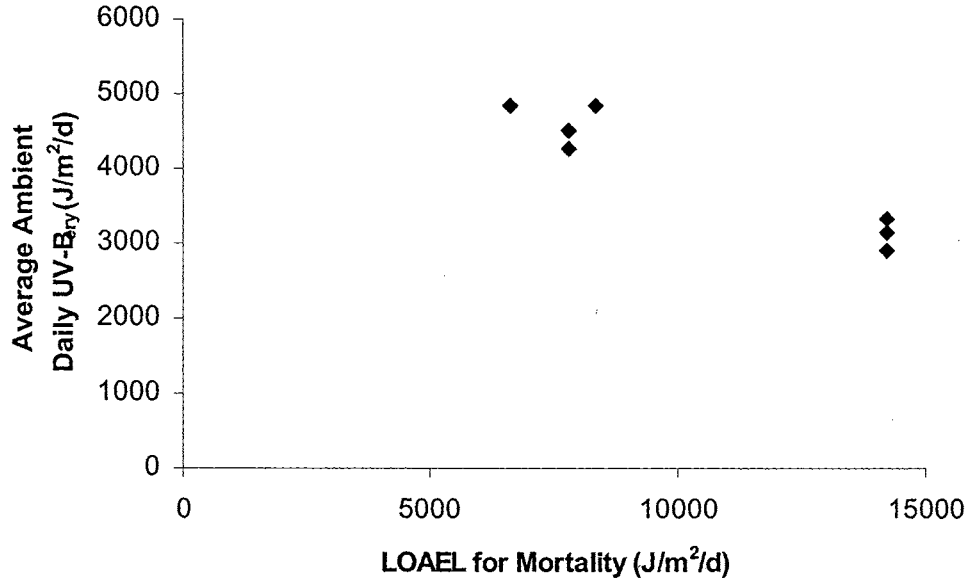


Figure 3.7 Embryos of *B. arenarum* from a series of exposure levels (**a.** 301 J/m^2/d; **b.** 5301 J/m^2/d; **c.** 7816 J/m^2/d; **d.** 10575 J/m^2/d). Typical types and severities of malformations are shown, as well as the general effects on growth.

Figure 3.8 Average length of embryos under each filter treatment for all experiments.

a.



b.

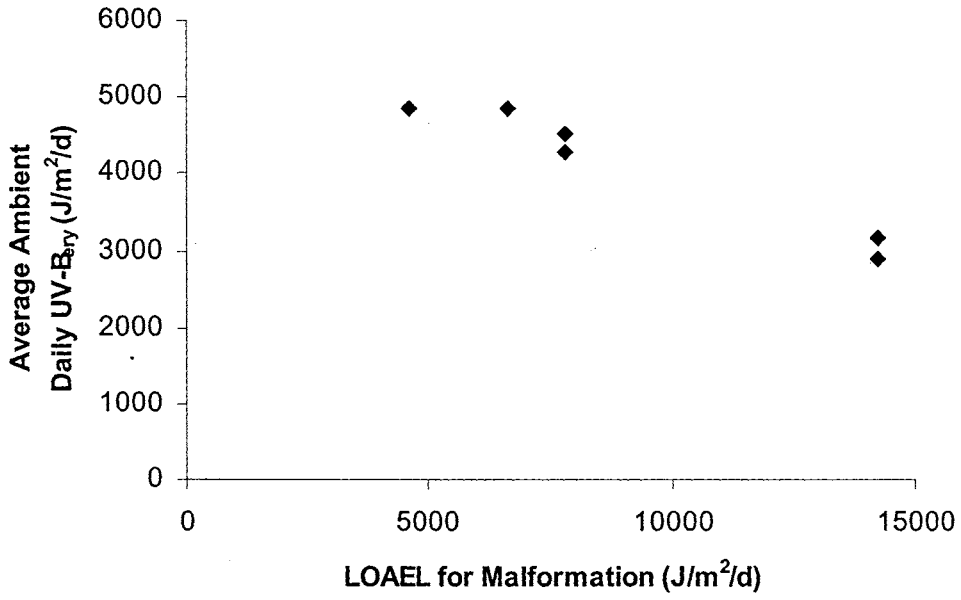


Figure 3.9 **a.** Correlation of the ambient UV-B versus the LOAEL for mortality.
b. Correlation of the ambient UV-B versus the LOAEL for malformation.

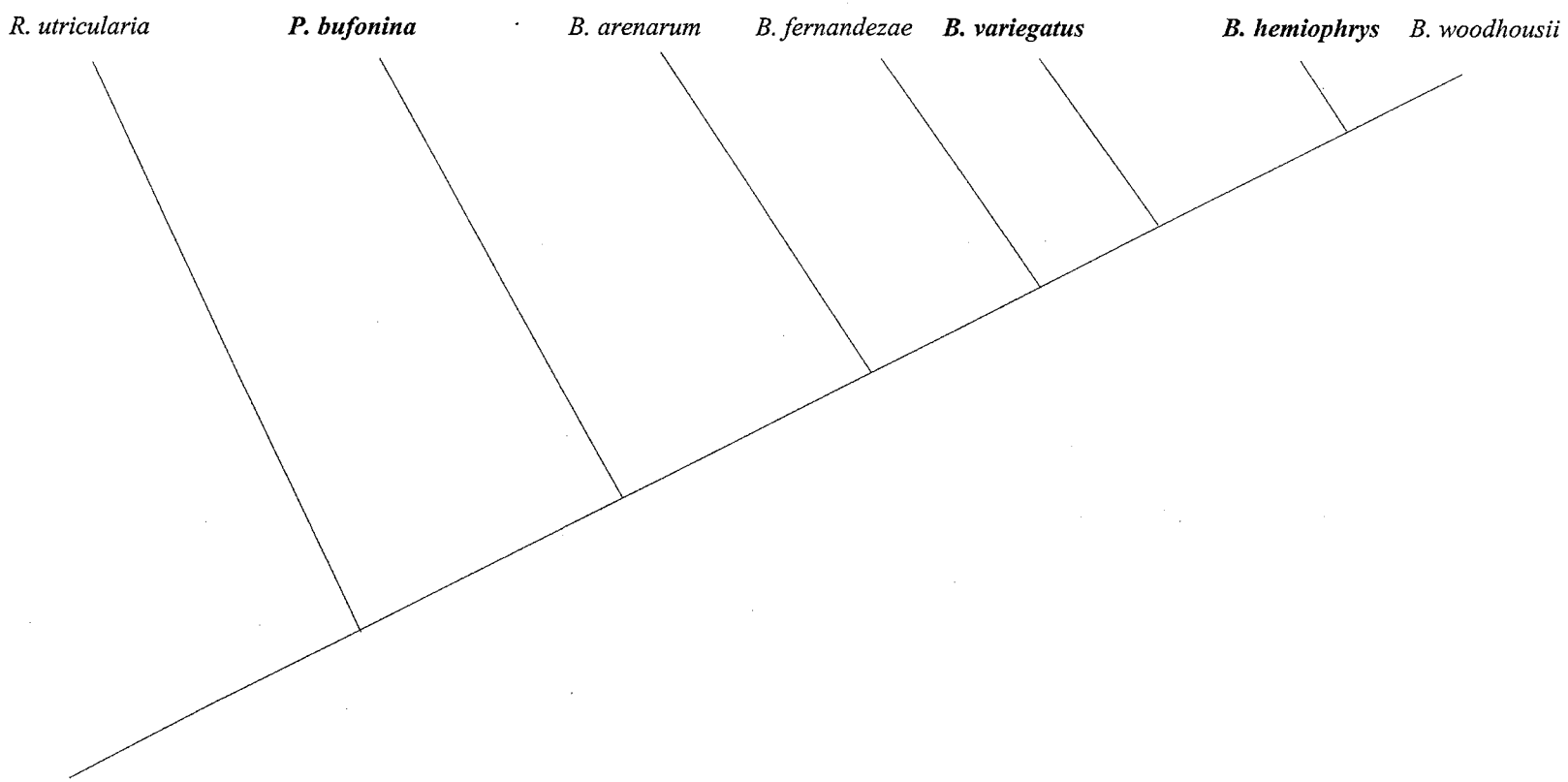


Figure 3.10 Proposed phylogenetic relationship among test species, based on Martin (1972), Greybeal (1997), and Zug et al. (2001).
(**Bold type** indicates the more resistant species.)

VITA 2

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