

**Estimating photolyase activity for seven amphibian species from
south-central Ontario**

**A thesis submitted to the Committee of Graduate Studies in Partial Fulfillment
of the Requirements for the Degree of Master of Science in the Faculty of Arts
and Science**

Trent University

Peterborough, Ontario, Canada

© M. Alex Smith 1998

Watershed Ecosystems M.Sc. Program

June 1999



National Library
of Canada

Acquisitions and
Bibliographic Services

395 Wellington Street
Ottawa ON K1A 0N4
Canada

Bibliothèque nationale
du Canada

Acquisitions et
services bibliographiques

395, rue Wellington
Ottawa ON K1A 0N4
Canada

Your file Votre référence

Our file Notre référence

The author has granted a non-exclusive licence allowing the National Library of Canada to reproduce, loan, distribute or sell copies of this thesis in microform, paper or electronic formats.

The author retains ownership of the copyright in this thesis. Neither the thesis nor substantial extracts from it may be printed or otherwise reproduced without the author's permission.

L'auteur a accordé une licence non exclusive permettant à la Bibliothèque nationale du Canada de reproduire, prêter, distribuer ou vendre des copies de cette thèse sous la forme de microfiche/film, de reproduction sur papier ou sur format électronique.

L'auteur conserve la propriété du droit d'auteur qui protège cette thèse. Ni la thèse ni des extraits substantiels de celle-ci ne doivent être imprimés ou autrement reproduits sans son autorisation.

0-612-40487-0

Canada

Abstract

Estimating photolyase activity for seven amphibian species from south-central Ontario

M. Alex Smith

There are strong indications that stratospheric ozone (O₃) depletions are causing significant increases in the UVB (280-320 nm) portion of the solar radiation complement. Organisms may currently be exposed to significant amounts of ambient UVB, and if stratospheric ozone levels continue to decline, those already damaging levels will increase. It is important to understand the current effects of UVB on aquatic organisms in order to accurately predict what effects increasing UVB fluence might elicit. Accurate assays of the photoenzymatic repair qualities of these organisms play a role in this process.

I assayed photolyase activities of embryo tissue from seven amphibian species from south-central Ontario. The experiments completed here revealed the following trends. Using the bacterial transformation assay I demonstrated significant differences in photolyase activity among the species tested and these differences are not correlated with expected natural UVB exposure. There are significant between-species differences in jelly mass absorbance of UVB light and at 280 nm these differences can be positively correlated with photolyase activity. There is no relationship between photolyase and jelly absorbance at 320 nm.

There is a positive relationship between photolyase activity and ambient UVB fluence for the wood frog. This relationship is positively correlated with the ambient UVB fluence that these embryos were exposed to *in vivo*.

Future estimations of an amphibian's photolyase activity need to acknowledge the differences this study has elucidated. *In vivo* conditions can drastically alter an *in vitro* estimation of photolyase activity.

Preface

This thesis has been completed in the manuscript format. The first chapter is a brief review of elementary UVB biology, how this has had implications on amphibian populations and how these implications have been measured.

The second chapter has been submitted to Conservation Biology under the authorship and title,

Smith M. A., and M. Berrill. Comparing photoenzymatic repair and UVB jelly absorbance of several amphibian species from south-central Ontario . Conservation Biology.

The third chapter is an as yet unpublished manuscript under the authorship and title;

Smith M. A., and C. Kapron. *In vivo* induced photolyase activity of the cell free extract from wood frog (*Rana sylvatica*) embryos.

The fourth chapter outlines the relevance of the results reported here in the larger field of UVB and amphibian biology.

The first appendix is an as yet unpublished technical note under the authorship and title;

Smith M. A. Problems utilizing enzyme sensitive site assays for photorepair of exogenous DNA with cell free extracts made from amphibian embryos.

Acknowledgments

Many people have been a tremendous help to me over the past two years as I've wrestled with this beast.

The supervision, friendship and support that I've received from Michael Berrill over my past seven years at Trent has been awesome. Thank you.

The support, advice and supervision of Carolyn Kapron during this thesis has had an enormous effect on the work completed here.

The fine work of the thesis committee has molded a "quirky" thesis into a more streamlined form. Thank you Jim Sutcliffe, Tom Moon and David Lean.

Much of the work now in the appendix was completed in the lab of Michael Bidochka and I owe him a lot of thanks for continual support and advice.

For food, wine, beer, hack laughs and advice I could always count on Dave and Lynn, Steve and Monica, Scott and Sue, Eric and Sheena, Dave Steer Anthony and Ramsey. Thanks to you all, especially for putting up with my furrowed brows and frantic pace.

My mom and dad, Elaine Bazinet Smith and Clark Smith have been such an enormous support to me forever. It meant a lot to me that my parents and Laura from down from North Bay, and Adam from the muskeg of Labrador could be there to see my thesis defense and hear a fine presentation of what it is that I do now. Thank you all for your support, encouragement and love.

My largest thanks go to my lifetime supporter who isn't here right now, but whose encouragement and support and love I can feel even from the other side of the equator. Mirielle, chtm tant.

Alex Smith. December 22 1998.

Table of Contents

Chapter 1 Estimating the effects of UVB (280-320 nm) on the amphibians of Ontario through an analysis of photolyase activity, *in vitro* photolyase induction, and UVB absorbance of embryo jelly

1	Abstract	1
1.0	Introduction	3
1.1	Water surface and sub-surface UVB	4
1.2	Cyclobutane pyrimidine dimers and photolyase	6
1.3	Amphibian populations and ultraviolet-B radiation	8
1.4	Adaptive hypotheses	9
1.5	The work completed here	14

Chapter 2 Comparing photoenzymatic repair and UV-B jelly absorbance of several amphibian species from south-central Ontario

2	Abstract	16
2.0	Introduction	17
2.0.1	Brief history of recent amphibian/UV-B literature	17
2.0.2	Aims of work reported here	19
2.1	Methods	20
2.1.1	Egg and embryo collection	20
2.1.2	Between species differences in photolyase	21
2.1.3	Protein purification	21
2.1.4	Skin protocol	23
2.1.5	Transformation assay	23
2.1.6	Transformation efficiencies	24
2.1.7	Between-species comparison of jelly absorbance of ultraviolet-B light	25
2.2	Results	26
2.2.1	Between species patterns of photolyase activity	26
2.2.2	UVB damage of plasmid DNA	29
2.3	Discussion	36
2.3.1	Among species comparison of photorepair ability determined through transformation efficiencies.	36
2.3.2	Jelly absorbance at 280 and 320 nm	36
2.3.3	Mean depth of oviposition and dimer repair	38
2.3.4	Species comparison	38
2.3.4.1	Wood frog	38
2.3.4.2	Leopard frog	38
2.3.4.3	Green frog	39

2.3.4.4	Yellow and Blue spotted salamanders	40
2.3.4.5	Chorus frog	40
2.3.4.6	Gray tree frog	42
2.3.4.7	African clawed frog	43
2.3.4.8	Spring peeper	43
2.3.5	Future considerations	43

Chapter 3 *In vivo* induced photolyase activity of the cell free extract from wood frog (*Rana sylvatica*) embryos.

3	Abstract	45
3.0	Introduction	46
3.1	Methods	47
3.1.1	Egg and embryo collection	47
3.1.2	Induction of wood frog photolyase through exposure to ambient UV	47
3.1.3	Between-species differences in photolyase	48
3.1.4	Protein purification	48
3.1.5	Transformation assay	50
3.1.6	Transformation efficiencies	51
3.2	Results	52
3.2.1	Transformation efficiency	52
3.2.2	Induced photolyase activity of the wood frog	52
3.3	Discussion	58
3.3.1	History of photolyase induction research in the literature	58
3.3.2	Factors which can't be discounted as possible agents of photolyase induction	60
3.3.3	Speculations on the mechanism of induction	62
3.3.4	Importance of results demonstrated here	62

Chapter 4 A general discussion of amphibian photorepair

4.0	A general discussion of amphibian photorepair	65
4.1	Comparing enzymatic photorepair and UV-B jelly absorbance of several amphibian species from south-central Ontario	65
4.2	<i>In vivo</i> induced photolyase activity of the cell free extract of from wood frog (<i>Rana sylvatica</i>) embryos	66
4.3	The importance of <i>in vitro</i> methods of determining	

	photolyase activity in amphibian tissue	67
4.4	The importance of microbiological and ecosystem studies on the role of UV-B in temperate, mid-latitude humic waters.	68
4.5	Future research	69
4.6	Summary of results	70
Literature cited		
	Literature cited	72
Appendix 1 Problems utilizing enzyme sensitive site assays for photorepair of exogenous DNA with cell free extracts made from amphibian embryos		
	Background	83
	Methods	85
	Egg and embryo collection	85
	Protein purification	85
	Enzyme sensitive site (ESS) assays	87
	Methods of <i>Hind</i> III ESS assay	87
	Results of <i>Hind</i> III ESS assay	89
	Methods of T4 Endo V ESS assay	91
	Results of T4 Endo V ESS assay	91
	H3 immunoassay methodology	96
	Results of H3 immunoassay	97
	General results	100
	L-cysteine de-jellying	102
	Discussion	103
	ESS assays (<i>Hind</i> III and T4 Endo V)	103
	H3 immunoassay	103
	Using 2% L-cysteine as a de-jellying agent	103
	General conclusions	104
	Future analyses	104
Appendix 2		
	UVB absorbance of embryo jelly from seven amphibian species.	106

List of Tables

Chapter 1

Table 1	Predictions for the CBPD removal characteristics of CFE from eggs of various amphibian species collected in the spring and summer of 1997.	11
Table 2	Factors which either <i>in vivo</i> affect ambient UV-B exposure an amphibian embryo experiences or alter photolyase activity/concentration <i>in vitro</i> .	13

Chapter 2

Table 1	Bacterial transformation assay for cyclobutane pyrimidine dimer removal in exogenous DNA: Tissue history and repair estimation for eight amphibian species	28
---------	--	----

Chapter 3

Table 1	Bacterial transformation assay for cyclobutane pyrimidine dimer removal in exogenous DNA: Tissue history and repair estimation	53
---------	--	----

Appendix 1

Table 1	Enzyme sensitive sites (ESS) and immunoassays for cyclobutane pyrimidine dimers in exogenous DNA: Tissue history and repair estimation	101
Table 2	Differential effects of 2% L-cysteine as an agent to remove amphibians from protective jelly.	102

List of Figures

Chapter 2

Figure 1	Species comparison for photolyase activity (\pm SE) of CFE made from skin or embryo of nine amphibian species.	31
Figure 2a	Comparison of the jelly absorbance characteristics of amphibian embryo jelly and photolyase activity (\pm SE) of cell free extract from embryos of the same species.	32
Figure 2b	Comparison of the jelly absorbance characteristics of amphibian embryo jelly at 280 nm with photolyase activity of cell free extract from embryos of the same species.	33
Figure 3	Importance of <i>in vitro</i> temperature on photolyase activity (\pm SE) determination for the green frog (<i>Rana clamitans</i>)	34
Figure 4	Examination of the relationship between mean depth of oviposition and photolyase activity (\pm SE) of embryos from seven amphibian species.	35

Chapter 3

Figure 1	Change in photolyase activity characteristics of Wood Frog (<i>Rana sylvatica</i>) cell free extract (CFE) as exposed to increasing ambient UV-B fluence	55
Figure 2	Relationship between the fluence received by wood frog embryos exposed to ambient UV-B light in Peterborough ON. Canada	56
Figure 3	The proposed use of wood frog induction data to predict between-species differences in the context of ambient UV-B fluence.	57

Appendix 1

Figure 1	1% agarose gels containing UV-irradiated pBR322 plasmid DNA linearized by treatment with <i>Pvu</i> II.	90
Figure 2	1% agarose gels containing UV-irradiated pBR322	

	plasmid DNA treated with purified photolyase (lanes 4-5) or CFE from leopard frog (lanes 6-7) prior to incubation with T4 Endo V.	93
Figure 3	1% agarose gels containing UV-irradiated pBR322 plasmid DNA treated with gray tree frog CFE.	94
Figure 4	1% agarose gels containing UV-irradiated pBR322 plasmid DNA treated with wood frog CFE.	94
Figure 5	Estimations of percent photorepair utilizing the T4 Endo V ESS assay	95
Figure 6	Antibody detection of UV-B irradiated DNA on a neutral nylon membrane.	98
Figure 7	Preliminary H3 immunoassay estimation of UV-B damage to pBR322 DNA by cell free extracts of amphibian embryos	99

Appendix 2

Figure 1	UVB absorbance of chorus frog jelly between 275 and 320 nm.	106
Figure 2	UVB absorbance of gray tree frog jelly between 275 and 320 nm.	107
Figure 3	UVB absorbance of blue spotted salamander jelly between 275 and 320 nm.	108
Figure 4	UVB absorbance of yellow spotted salamander jelly between 275 and 320 nm.	109
Figure 5	UVB absorbance of green frog jelly between 275 and 320 nm.	110
Figure 6	UVB absorbance of wood frog jelly between 275 and 320 nm.	111
Figure 7	UVB absorbance of leopard frog jelly between 275 and 320 nm.	112

Chapter 1

Estimating the effects of UV-B (280-320 nm) on the amphibians of Ontario through an analysis of photolyase activity, *in vitro* photolyase induction, and UVB absorbance of embryo jelly

1 Abstract

Ultraviolet-B radiation (280-320 nm) is a wavelength range that has the potential to cause amphibian deformities, and may be linked to declines in some amphibian populations. Ultraviolet radiation is present across geographic areas undergoing radically different forms of anthropogenic influence, and is thus an especially intriguing environmental stress. Researchers have known since the 1920's (Higgins and Sheard 1926) that amphibian eggs are vulnerable to periods of enhanced UV. For many amphibians, the egg/embryo stages of their life history are the most vulnerable to UV damage because of oviposition sites that are exposed to sunlight for long periods of time (Ovaska 1997). In Eastern Canada, this problem is exacerbated by a correlation between a spring time UVB peak (Kerr and McElroy 1993) and the principal time of oviposition for many amphibian species (*Rana sylvatica*, *Ambystoma laterale*, *Ambystoma maculatum*, *Pseudacris triseriata*, *Rana pipiens*, *Bufo americanus* and *Hyla crucifer*).

The primary damage associated with UVB radiation is the cyclobutane pyrimidine dimer (CBPD) on pyrimidine base pairs in DNA (Malloy *et al.* 1997, Mitchell 1995). DNA is the major cellular target of UVB radiation due to the fact that DNA has a maximum absorbance at 260 nm (Mitchell 1995). CBPDs are repaired by a photoreactivated enzyme called photolyase (EC 4.1.99.3). Using four assays, which indirectly estimate cellular concentrations of photolyase (through the removal of CBPDs), I tested the hypothesis that eggs of species which oviposit at the water's surface would have greater photolyase activity than eggs of species which lay beneath the surface. I also examined the hypothesis that changes in photolyase concentration may be induced by exposure to

ambient UV radiation. The *in vitro* substrate for repair enzyme activity was UVB damaged plasmid DNA (pBR322). To determine whether the UV-damaged plasmid DNA was repaired by amphibian egg cell free extract containing photolyase, I compared four assays for photolyase activity: bacterial transformation efficiency, enzyme sensitive site assays (ESS) of plasmid digested by Hind III restriction enzyme or T4 endonuclease V Restriction enzyme, and an immunoassay using the monoclonal antibody H3 specific to CBPDs. All assays displayed large variance, and the amphibian cell free extract caused rapid signal loss. This loss was particularly evident using ESS assays. The immunoassay was not examined in the same detail, but displayed a signal worthy of analysis, and should be investigated in the future. The bacterial transformation assay produced the clearest signal and indicated the following trends:

- 1) Photolyase activity differed between species. This difference was not significantly related to proximity to the water surface of the primary site of egg deposition, counter to my initial adaptive hypothesis. In fact, the non-significant trend was for increasing photolyase activity in embryos from species which lay their eggs deeper under water.

- 2) Wood frog (*Rana sylvatica*) embryos exposed to different amounts of ambient UVB displayed significantly different photolyase activity. There was a non-significant positive correlation between cellular photolyase activity for the wood frog and total ambient UV fluence. These results are indicative of induced changes in cellular photolyase concentration.

- 3) There were significant between-species differences in the ultraviolet B absorbance of the embryo jelly. The absorbance was positively correlated with the embryo tissues photolyase activity at 280 nm, but not at 320 nm.

These trends indicated important intra- and inter-species characteristics which must be appropriately tested prior to making conclusions regarding an

entire species from the analysis of the photolyase activity of one brood. Researchers must determine previous UV exposure, the UVB transmittance of the protective jelly which surrounds the embryo, and the activity of other mechanisms of UVB damage repair when making estimates of both an amphibian species' ability to repair UVB damage, and the amount of UV damage a species is likely to undergo.

1.0 Introduction

Reported incidences of amphibian population decline are among the most contested controversies in conservation biology (Reed and Blaustein 1995, Pechman *et al.* 1994, Blaustein 1994, Wilbur 1994,). Conflicting results have been produced recently regarding the importance of UVB (280-320 nm) in amphibian population change. In some cases, current levels of ambient UVB restrict embryo success (Anzalone *et al.* 1998, , van de Mortel *et al.* 1998, Blaustein *et al.* 1997, Blaustein *et al.* 1996b, Blaustein *et al.* 1994), in other cases enhanced UVB levels restrict embryo success (Lizana and Pedraza 1998), and in yet other cases UVB (ambient or enhanced) has no effect on embryo success (Berrill *et al.* in prep, Corn 1998, Lizana and Pedraza 1998, Ovaska *et al.* 1997, Grant and Licht 1995). In spite of conflicts regarding experimental methods, statistical analysis and personal interpretation (Licht 1995a, Blaustein *et al.* 1995b, Licht 1996, Blaustein *et al.* 1996b), and perhaps in part because of them, UVB is considered to be an important variable due to its ubiquity across areas of radically different anthropogenic influence. Anticipated synergistic effects of the negative feedback between UVB and dissolved organic carbon (DOC) (Schindler *et al.* 1996, Yan *et al.* 1996), and demonstrated synergistic interactions between UVB and various pathogens (Long *et al.* 1996, Kiesecker and Blaustein 1995), have also been discussed and documented in the literature. Thus DNA damage repair ability is

important to understanding broad environmental pressures, such as UVB, in an ecological context where an environmental stress is simultaneously affected by multiple trophic levels and biochemical and geochemical processes in negative and positive ways.

1.1 Water surface and sub-surface UVB

Cloud opacity, vertical ozone (O₃) abundance, and solar elevation (in turn described by latitude and season (Booth *et al.* 1997)), are the most important determining factors in ground level UV irradiation levels (Lubin and Jensen 1995). Severe reductions in vertical ozone abundance have been reported worldwide (Farman *et al.* 1985, Gleason *et al.* 1992, Kerr and McElroy 1993). Such reductions lead to enhanced UVB. Little change in UV-A (320-400 nm) is expected, since these wavelengths are not absorbed by ozone (Smith 1989, Madronich *et al.* 1995). Not only is UVB the most biologically damaging wavelength (Mitchell 1995, Calkins 1980), but it is the wavelength whose surface level fluence rate will increase in the future.

Attenuation of UVB is often quite rapid in freshwater ponds and humic lakes compared to clear montane lakes and salt water bodies (Lean *et al.* submitted, Schindler *et al.* 1996, Scully and Lean 1994). The rate of attenuation increases relative to the dissolved organic carbon concentration (DOC) (Scully and Lean 1994). Therefore, wide differences are expected between attenuation curves in water sources grossly disparate in DOC (see Table 2).

At middle latitudes and lower elevations, such as Peterborough (44°21'N, 78° 17'W, 100m), typical mid-day summer UVB fluence rate is approximately 1.9-2.2 W/m² (Lean *et al.* submitted). These levels are markedly reduced as this fluence passes through the humic waters in which amphibians of this area oviposit. Five centimeters beneath the surface, the fluence rate is reduced to 2.07% of its surface intensity (Berrill *et al.* in prep). This incredible pattern of

attenuation would suggest that for most species examined here, UVB is not part of the light package they receive. Species which oviposit at or near the water's surface, (*Pseudacris triseriata*, *Hyla crucifer*, *Bufo americanus*, *Rana clamitans* and *Rana catesbeiana*, occasionally *Rana sylvatica*) could be exposed to unattenuated UVB. They would also be exposed to the indirect effects of toxic photochemicals caused by the interaction of DOC and UVB.

In addition to the direct effects of UV on an organism's physiology, aquatic organisms also suffer very damaging indirect effects of ambient or increasing UVB. UV irradiance of standing water high in DOC can cause the photochemical production of biologically damaging reduced-oxygen species such as hydrogen peroxide, hydroxide radicals and superoxides (Lean *et al.* 1994). These photochemicals are perhaps damaging enough that currently they may be a larger environmental stress to the amphibian embryo than the ambient UVB which stimulated their production. But in humic water DOC exists primarily in the form of aromatic compounds that resist bacterial decomposition. One interesting synergistic effect of the UVB-DOC interaction is that the photolytic byproducts of the interaction are more available for bacterial decomposition (Rietner *et al.* 1997, Schindler *et al.* 1996, Lindell *et al.* 1995). As UVB is attenuated by water and DOC, the DOC compounds are rendered to a form more available to decomposers (Schindler *et al.* 1996). Therefore DOC concentration of the water is reduced, and UVB reaches an increased depth before being attenuated. Thus the UVB-DOC system exists in a negative feedback loop where increasing UVB may indirectly cause the reduction of DOC. Clearly, UVB is an environmental variable that is important to the future of aquatic species characteristic of the humic mid-latitude waters of south-central Ontario.

1.2 Cyclobutane pyrimidine dimers and photolyase

UVB radiation causes the formation of multiple damaging photoproducts including cyclobutane pyrimidine dimers (CBPDs) and (6-4) photoproducts which kill cells by preventing transcription and replication and by causing a mutation if the DNA polymerase passes over the photoproduct (Kim *et al.* 1996, Mitchell and Karentz 1993, Hart *et al.* 1977). (6-4) photoproducts are repaired both by nucleotide excision repair and by (6-4) photolyase (Kim *et al.* 1996).

UVB radiation forms dimers in DNA between adjacent or non-adjacent pyrimidine monomers (Kim and Sancar 1995). These CBPDs form 70-90% of the damage that ultraviolet radiation has upon DNA (Tevini 1993, Malloy *et al.* 1997). CBPDs prevent transcription and replication by "kinking" the phosphate backbone of the DNA (Husain *et al.* 1988, Sancar 1994) and are known carcinogens and mutagens (Sancar and Sancar 1988). There are several DNA repair enzymes which repair dimers. These include nucleotide excision repair (NER) where the nucleotides containing the dimer are cut out of the strand or light-catalyzed enzymatic photorepair by an enzyme called photolyase (Kim and Sancar 1995). NER is a process which utilizes several proteins and repairs a wide range of DNA damage (Sancar and Sancar 1988). Related to the number of enzymes associated with NER is the resulting complexity of any investigation into rate of repair of animals from natural populations where concentrations of all enzymes are low, or may be absent. Photolyase is the primary repair enzyme which catalyses the breakdown of CBPDs (Sancar and Sancar 1988). In the presence of white light, it catalyses the entire process of dimer removal. This, "one enzyme-one result" relationship is experimentally attractive due to the relative simplicity of demonstrating its effects *in vitro*.

Photolyase occurs in an apparently unpredictable manner throughout pro- and eukaryotes (Sancar 1994) and interestingly, the reaction mechanism appears quite conserved where it has been described (Eubacteria, Archaeobacteria

and eukaryotes) (Kim and Sancar 1992). Photolyase is a monomeric Michaelis-Menten protein (approximately 60 kDa) that contains stoichiometric amounts of two non-covalently bound cofactors. The enzyme is present in very low cellular concentrations, and binds to dimers in DNA with great specificity and affinity (Kim and Sancar 1992). This specificity is dependent upon the backbone structure of the DNA at the binding site which is bent by the pyrimidine dimer (Sancar 1994).

The reaction mechanism of photolyase has been extensively documented (Kim and Sancar 1993, Sancar 1994, Hearst 1995, Park *et al.* 1995, Sancar 1996). Briefly, independent of light and dependent upon both dimer formation and DNA phosphate backbone deformation, photolyase binds to the CBPD in the DNA substrate. The chromophore cofactor (either methenyltetrahydrofolate (MTHF) or 8-hydroxy-5-deazaflavin) acts as a photoantenna and captures energy from visible light (350-450 nm). These captured photons allow excitation energy transfer to the flavin adenine dinucleotide (FADH-) cofactor. FADH- then donates an electron to the dimer, which forms an anion radical and splits into two monomer pyrimidines. The electron is then back transferred, the flavin is returned to a catalytically active state and the enzyme disassociates from the DNA (Sancar 1994, Park *et al.* 1995). There is no net change in electrons between enzyme or substrate. Photolyase has also been shown to stimulate the repair of chemical DNA damage in the absence of visible light (Ozer *et al.* 1995, but see Fox *et al.* 1994).

DNA repair enzymes are present in very small quantities, typically between 20 and 100 molecules per cell (Sancar 1988) and photolyase may only be present in concentrations of 10-20 molecules per cell (Sancar pers. comm., Snapka and Sutherland 1980, Kim and Sancar 1993). Such a low concentration makes for challenging purification protocols. Purified photolyase was not produced in a

laboratory until a *tac-phr* plasmid was developed which greatly overproduced photolyase (Sancar *et al.* 1984), and thus detailed studies of photolyase action have been delayed until relatively recently (Sancar 1990). Isolating purified photolyase is therefore impractical for most animals from natural populations (Sancar pers. comm.). In the place of photolyase purification, cell free extracts (CFE) are made of the tissue in question, and this enzyme slurry is then exposed to irradiated DNA where dimers have been formed. Due to the specificity of photolyase, if dimer removal is observed subsequent to incubation of the irradiated DNA with the CFE under photoreactivating light, an indirect measure of photolyase is possible.

1.3 Amphibian populations and Ultraviolet-B radiation

Amphibian population predictions for the future in Ontario are in part dependent upon determining how rises in UVB radiation, associated with either low elevation and stratospheric ozone depletion affects amphibian embryos. This work reports efforts at estimating a representative photolyase activity for seven Ontario amphibian species, and placing that estimation in a natural context of inducible changes in photolyase concentration and likely UV absorbance through humic water and protective jelly.

Surface levels of UVB are not accurate indicators of what UV stress levels organisms under water receive. The determination of these sub-surface levels is compounded by a strong ($r^2 = 0.97$) relationship between UVB attenuation and dissolved organic carbon (Scully and Lean 1994). Hypotheses of ambient exposures for amphibian embryos laid in different parts of the water column, and in different bodies of water, must consider this rapid attenuation. Hypotheses concerning ambient UVB exposure of developing amphibian embryos in a natural environment should also incorporate the amount of UVB that is absorbed by the protective mucopolysaccharide/mucoprotein jelly that

surrounds the developing embryo. This capsule, or envelope, protects the embryo from fungal infection, ingestion and rapid temperature change (Duellman and Trueb 1992). It also completely surrounds the developing embryo, and must therefore interact with incoming UVB radiation. The percent transmission of UVB through this protective jelly have recently been reported as ranging from 7% in the wood frog and 14% in the american toad, to 60-70% in the yellow spotted salamander (Grant and Licht 1995).

1.4 Adaptive Hypotheses

Ultraviolet radiation has been present throughout the evolution of multicellular organisms (Tevini 1993, Yasui and McCready 1998), and toxic effects of UV radiation may well have slowed terrestrial or water surface rates of evolution until the development of the stratospheric ozone layer (Williamson 1995, Yasui and McCready 1998). The ubiquity of ultraviolet exposure through evolutionary time therefore leads to an attractive prediction that the concentrations of DNA repair mechanisms can be explained through potential exposure when comparisons are made between species groups. Others have reported a general correlation between expected sunlight exposure and photolyase levels (Blaustein *et al.* 1995a).

This adaptive hypothesis is attractive, yet when one compares species with high levels of environmental UVB exposure to species with low levels of exposure, there is often no correlation between environmental exposure and the ability to repair such damage (Ozer *et al.* 1995). For instance, the enteric bacterium *Escherichia coli*, has quite high levels of the repair enzyme, photolyase, while *Bacillus subtilis*, the soil bacterium, has low photolyase levels (Sancar 1995). Placental mammals, including humans, have generally been considered to have no photolyase activity (Li *et al.* 1993, but see Sutherland and Bennett 1995).

It has long been known that enhanced UVB radiation can cause deformations during amphibian development (Higgins and Sheard 1926), and that subsequent exposure to radiation greater than 320 nm will mitigate that damage (Worrest and Kimeldorf 1975, Worrest and Kimeldorf 1976). As a parsimonious starting point, an adaptive correlation between expected exposure, and amounts of various repair mechanisms is sufficient. Ontario amphibian species with surface egg deposition were expected to repair UVB damage more efficiently than species with sub-surface egg deposition (Table 1). Spring breeding species might also be expected to have higher levels of photolyase as these species are exposed for longer periods of time (due to temperature dependent rates of development), and during the spring the UVB fluence is higher than in other times at this latitude (Kerr and McElroy 1993).

Table 1: Predictions for the CBPD removal characteristics of CFE from eggs of various amphibian species collected in the spring and summer of 1997. Predictions were based upon the position, timing and depth of egg deposition for each species.

Species	Laying Period in this area	Mean Depth (cm)	Max Depth (cm)	Min. Depth (cm)	Photolyase Activity Prediction
Chorus Frog	Late April	5	8	0	High
Spring Pepper	Late April	7.5	10	5	Lower
Wood Frog	Late April	9.25	16.75	1.75	Lower
Leopard Frog	Early May	13.1	17	9.1	Lowest
Blue Spotted Salamander	Late April	14	18	9.9	Lowest
Yellow Spotted Salamander	Late April	16.7	19.9	13.6	Lowest
American Toad	Mid-May	5	8	0	High
Gray Tree Frog	Mid-May to late July	0	5	0	Highest
Green Frog	Early June	6	12	0	Medium
Bullfrog	Mid-May to late July	6	12	0	Medium

Experiments with laboratory cell lines have illustrated that cellular concentrations of photolyase can be directly influenced by several environmental factors including temperature, UV-A, UVB and oxidative stress (Chao and Lin-

Chao 1987a, Chao and Lin-Chao 1987b, Yasuhira and Yasui 1992, and Mitani *et al.* 1996). Previous examinations of the repair abilities of species from natural populations have not accounted for such induced changes in cellular concentrations. UVB has not been measured directly (daily variation or total fluence received) by any of the recent studies relating amphibian larval success and photolyase activity (van de Mortel *et al.* 1998, Hays *et al.* 1996, Blaustein *et al.* 1996b, Blaustein *et al.* 1994). I took advantage of the BW-100 CID UV measuring Instrument (Vital Technologies Corp.), maintained at Trent University by Dr. Wayne Evans, to record ambient UVB levels on each collection day. Ambient UVB levels during an experimental exposure period designed to determine whether inducible changes in photolyase activity are observable through a transformation assay were also recorded. My findings suggest that to truly estimate repair characteristics researchers must account for previous UV exposure and jelly absorbance in the UVB range.

I would also hypothesize, but have not tested, that the number of complicating factors is certainly not limited to the two outlined here. There are other under-reported factors that are likely to be related to how much UVB exposure damages the eggs of any particular species. These *in vitro* and *in vivo* complicating factors are outlined in Table 2 and require further testing.

Table 2: Factors which either *in vivo* affect ambient UVB exposure an amphibian embryo experiences or alter photolyase activity/ concentration *in vitro*.

Factor	<i>In vivo</i>	<i>In vitro</i>
DOC concentration of water surrounding embryo	Increased rate of UVB attenuation.	N/A
Temperature of water surrounding embryo	Increased or decreased photolyase concentration	N/A
Developmental stage	Unknown effects on photolyase concentration	N/A
Biochemical composition of protective jelly	Altered attenuation of UVB	Altered actions of standard de-jellying agents such as 2% cysteine.
Debris/detritus on in water	Shielding of UVB	N/A
Concentration of unknown photolyase inhibitors in cell free extract (CFE) production.	N/A	Between-species differences in such inhibitors would dramatically alter photolyase activity estimates.
pH	-Changes in DOC composition, potentially in concert with UVB, resulting in UVB reaching deeper into humic waters. -Synergistic effects with UVB.	Unknown.
Embryonic cellular concentrations of endonucleases (Appendix 1)	N/A	Altering of accuracy of photorepair assays utilizing exogenous DNA sources
Embryonic damage by de-jellying agent (Appendix 1)	N/A	Reduction in photolyase activity estimation through embryo damage during de-jellying process.

Initial interest in determining whether UV repair abilities could be correlated with apparent population decline from areas which had undergone relatively little anthropogenic damage (or at least the damage was not apparent) was sparked by Blaustein *et al.*(1994). The work reported here, and other

amphibian/UVB studies around the world were all strongly influenced by this important study. Results from Blaustein *et al.* (1994) suggested that there are between-species differences in photolyase activity. They also documented a negative correlation between photolyase activity in amphibian eggs and natural egg mortality. In some cases, species with documented episodes of decline are those species with low photolyase activity (Blaustein *et al.* 1994). Neither Blaustein *et al.* (1994), nor numerous field studies since then (Corn 1998, van de Mortel *et al.* 1998, Anzalone *et al.* 1998) were able to report UVB conditions on the days of collection of embryos destined for *in vitro* photorepair determination. Sometimes this was due to a remote field location (Corn 1998). The location of most field collections in my study allowed me to couch my photolyase activity estimations in the context of known UVB fluences for the hours of UVB exposure on that day, prior to collection.

1.5 The work completed here

I used a standard bacterial transformation assay as a measure of photolyase activity from embryonic tissues of eight amphibian species of South-central Ontario: wood frog (*Rana sylvatica*), northern leopard frog (*Rana pipiens*), green frog (*Rana clamitans*), gray tree frog (*Hyla versicolor*), western striped chorus frog (*Pseudacris triseriata triseriata*), spring peeper (*Pseudacris crucifer*) blue spotted salamander (*Ambystoma laterale*), and yellow spotted salamander (*Ambystoma maculatum*). Methods of photorepair analysis were adapted from Sancar *et al.* (1984). Briefly, pyrimidine dimers were created in plasmid DNA (pBR322) through irradiation under UVB lights. These damaged plasmids were incubated with cell-free protein extracts (CFE) made from each amphibian species. A portion of the CFE/irradiated plasmid mixture was exposed to a period (90 min) of photoreactivating light (300-500 nm, (PRL)), and the remainder was incubated for the same period in the dark. The degree of repair of the DNA lesions in the

irradiated plasmid was estimated using a transformation assay (Sancar *et al.* 1984) which compared the relative transformation efficiencies of the damaged plasmid DNA exposed to either PRL or dark to the transformation efficiency of an unirradiated plasmid. Methods based on Dutta *et al.* (1993) and Carlini and Regan (1995) utilised the effects of CBPDs on the digestion sites of several restriction enzymes. The change in band absorbance of photoreactivated and non-photoreactivated plasmids reflected the post photorepair-dependent removal of CBPDs digestion with *Hind* III or T4 Endo V. This digestion was restricted by the number of CBPDs which was dependent upon the amount of repair the amphibian extract had caused (Dutta *et al.* 1993, Carlini and Regan 1995). These enzyme sensitive site (ESS) assays were an examination of the practicality of using non-radioactive, electrophoretic assays to determine photolyase activity faster than in the transformation assay. However plasmid DNA digesting endonucleases were apparently present in sufficient quantities in the amphibian CFE to lose the DNA signal in the electrophoretic gel (Appendix 1). Amphibian CFEs appear to contain concentrations of endonucleases that are high enough to eliminate the practical attractiveness of the ESS assays reported here (Appendix 1). The bacterial transformation assay also displayed signal loss potentially related to endonuclease digestion. The transformation assay is more sensitive than the ESS assays. Less intact plasmid DNA, (as little as one plasmid) is required to transform a competent bacterium. Much more intact DNA is required to be visible to the human eye once intercalated with ethidium bromide in an agarose electrophoretic gel.

Analyses were completed upon seven species of amphibian to determine whether photolyase concentrations differ between Ontario species, and whether these differences could be correlated with either a) total fluence of ambient UVB

the egg masses were exposed to prior to collection, or b) the percent UVB transmission of the protective jelly.

The results I report in the next two chapters document three important relationships.

The second chapter details between-species differences in cellular concentrations of photolyase. These differences are 1) not significantly related to primary depth of egg deposition (and therefore do not strictly support the hypotheses in Table 1), 2) are not related to the UVB absorbance of the protective jelly at 320 nm, but are significantly related to the absorbance of the jelly at 280 nm. CBPDs are formed with greater efficiency at 280 nm than at 320 nm (Tevini 1993). The relationships between species and between UVB fluence are complex, and efforts to correlate the amount of UVB exposure prior to collection and photolyase activity are reported.

Chapter three documents the novel finding of apparent ambient UV-induced increases in cellular photolyase activity in the wood frog. To my knowledge, this is the first reporting of inducible photorepair in tissue from animals from natural populations. This result means amphibian photolyase conclusions made in the future must attempt to incorporate the ultraviolet fluence which the egg has endured.

In Appendix one, the results of the endonuclease/DNA conformation assay are described. These results indicate that, although quick and non-radioactive, this type of assay is not appropriate for amphibian photolyase determination unless some novel method of DNase exclusion is described.

These results reinforce the need for more stringent reporting of environmental and biological conditions when documenting UV repair characteristics for any animal from a natural population.

Chapter 2

Comparing photoenzymatic repair and UVB jelly absorbance of several amphibian species from south-central Ontario

2 Abstract

Ultraviolet-B radiation (280-320 nm) is a normal part of solar radiation, yet there are strong indications that stratospheric ozone (O_3) depletions are causing significant increases in the UVB portion of the solar radiation complement. The actions of photolyase are specific to the primary form of UVB damage to DNA, the cyclobutane pyrimidine dimer (CPBD). I utilised a bacterial transformation assay to estimate the photolyase activity for oocytes, embryos and skin from seven species of amphibian from south-central Ontario. None of the populations from which the tissues were selected are currently declining. Photolyase activity was indirectly measured as the rate of CPBD removal from UVB damaged plasmid DNA by cell free extracts (CFE) created from the tissue of the species in question. *In vitro* measurements of photolyase activity for the seven species measured were not adaptively correlated with expected *in vivo* exposure to sunlight. Species which oviposit close to the surface, such as *Hyla crucifer* and *Pseudacris triseriata* displayed low photolyase activity, while *Rana clamitans* displayed high photolyase activity. Species which oviposit deeper under water displayed both high photolyase activity (*Ambystoma maculatum* and *Rana sylvatica*) and low photolyase activity (*Rana pipiens* and *Ambystoma laterale*). There was a significant positive trend demonstrated between a specie's protective jelly absorbance at 280 nm and its photolyase activity. There was no such relationship at 320 nm.

2.0 Introduction

2.0.1 Brief history of recent amphibian/UVB literature.

Previous works (Blaustein *et al.* 1994, Blaustein *et al.* 1996, Hays *et al.* 1996, van de Mortel *et al.* 1998, Lizana and Pedraza 1998, and Anzalone *et al.* 1998) have demonstrated that amphibian species differ in their sensitivity to UVB. There is also evidence of a trend of increasing photolyase activity with increasing expected ambient ultraviolet exposure (Blaustein *et al.* 1995a, Hays *et al.* 1996). Despite these trends, field experiments on UVB exposure and amphibian mortality (often utilizing the same, or closely related, species) have not equivocally supported earlier conclusions (for examples, contrast Blaustein *et al.* 1994, Blaustein *et al.* 1995a, Blaustein *et al.* 1996, Anzalone *et al.* 1998, Lizana and Pedraza 1998 with Corn 1998, Berrill *et al.* in prep, Ovaska *et al.* 1997, Grant and Licht 1995).

Ambystoma gracile embryos are adversely affected by ambient levels of UVB and are also a species with low photolyase activity (Blaustein *et al.* 1995a). Blaustein *et al.* hypothesized that, "selection pressures for evolving UV-damage DNA repair mechanisms may be much weaker for such (read "deep-water oviposition sites") species." (Blaustein *et al.* 1995a, pp742). In another study, utilizing an Ambystomid salamander with deep oviposition sites (although one from an entirely different geographic area), Berrill *et al.* demonstrated that the embryos of *Ambystoma maculatum* were not sensitive to ambient or enhanced UVB levels (Berrill *et al.* in prep).

UVB related effects on hatching success of wood frog, green frog or american toad embryos were not demonstrated by Grant and Licht (1995). A unique contribution of this study was its inclusion of data regarding reductions

in UVB transmittance by egg mass jelly. Using different methodology (more similar to Ovaska *et al.* 1997), I have expanded upon this theme.

The larval survival and rates of development for *Hyla regilla* and *Rana aurora* were tested under ambient, enhanced and blocked UVB conditions (Ovaska *et al.* 1997). *H. regilla* has been shown previously to be tolerant of high levels of UVB and to have high photolyase activity (Blaustein *et al.* 1994). Ovaska *et al.* (1997) demonstrated that current ambient levels of UV did not affect embryo survival for either species. Under enhanced conditions, both species were sensitive to reduced hatching success and larval survival. Jelly absorption of UVB was also measured for *H. regilla* and *R. aurora*.

Field exposures of *Bufo boreas* embryos between 0 and 100% ambient UVB were monitored in Rocky Mountain National Park, Colorado, USA (Corn 1998). There were no hatching success differences between the embryos treated with differing amounts of UVB in these field exposures

The embryo development and survival of low latitude and high elevation amphibian species were examined in Anzalone *et al.* (1998). As in Blaustein *et al.* (1994), neither hatching success nor developmental rate of *Hyla regilla* was affected by UVB. Their results also suggested that current levels of UVB radiation were reducing embryo survival for both *Hyla cadaverina* and *Tarisha torosa*. Each species oviposits between sea level to 1 500 m. One interesting difference between this study and most others currently reviewed in the literature, was that embryos for both hylids were experimentally fertilized. In this style of protocol, a complete history of UVB exposure could be available, or experimentally determined, and therefore such a procedure would be useful for any *in vitro* determination of repair mechanisms (Chapter 3). When the results of this study are compared with Blaustein *et al.* (1994), it illustrated intra-specific photorepair similarities for *H. regilla* across a large geographic range.

The effects of current levels of UVB on embryo mortality of *Bufo bufo* and *Bufo calamita* were examined recently by Lizana and Pedraza (1998). They reported that *Bufo bufo* displayed a marked sensitivity to UVB radiation. *Bufo calamita* were not sensitive to ambient levels of UVB, with no significant difference between embryo treatments where UVB was completely blocked, and treatments with ambient UVB.

The photolyase activities of several species of Australian tree frog were determined by van de Mortel et al. (1998). Of three species tested, the one with the lowest photolyase activity (*Litoria aurea*) also displayed non-significant sensitivity to UVB damage as measured by hatching success.

2.0.2 Aims of work reported here

It was not the aim of the work reported here to conduct field studies of larval survival under ambient or enhanced UVB regimes. Rather, I hoped to utilize already existing UVB measurement devices near Trent University, and UVB field studies (Berrill *et al.* in prep) where the majority of my embryos were collected, to estimate photolyase activity for seven amphibian species common central Ontario and to relate these estimates to UVB exposure history and patterns of UVB jelly absorbance.

Comparing trends between field studies on UVB radiation and amphibian embryo survival and mortality currently in the literature is difficult (Corn 1998). Such difficulties are also experienced when comparing the results of lab studies on between-species patterns of photolyase activity. These difficulties reflect the fact that estimating the photolyase activity of animals from natural populations is an expanding area of study that is perhaps only now beginning to include the complexity of the questions that are being posed specifically regarding the *in vivo* and *in vitro* effects of UVB on amphibian populations around the world. Not

only are there differences in experimental field design, but there are also marked differences related to treatment of embryo tissue, differences in the reporting of UVB conditions, differences in the methods of biochemical repair estimation, differences in light source, differences in light measurement and between-species differences in jelly composition. All these differences are indeed significant, but can be incorporated in future attempts to characterize the photorepair qualities of a species or population.

2.1 Methods

2.1.1 Egg and embryo collection

Eggs from each amphibian species were collected between April 23 and July 15 1997. UVB readings for each collection day (from 8:00am until 6:00pm at one minute intervals) were obtained from a BW-100 CID UV measuring Instrument (Vital Technologies Corp.) located at Trent University (44°21'N 78°17'W, 100 m elevation). These values were erythemally weighted.

Upon collection eggs were transported back to Trent University where anuran eggs were staged according to Gosner (1960), and ambystomid eggs were staged according to Harrison (1969).

2.1.2 Between species differences in photolyase

Eggs for between species comparison were collected and immediately frozen in 50 ml containers (except ambystomids which were kept in 150 ml containers) at -80 °C. Blue spotted salamander oocytes were collected from fresh road kill.

2.1.3 Protein purification

Methods of protein purification were adapted from Manly *et al.* (1980), Sancar *et al.* (1984), and Blaustein *et al.* 1994). Briefly; 50 ml egg replicates (frozen at -80°C) were warmed overnight from -80°C to 4°C . 10 ml of 2% L-cysteine was then added to each 50 ml container and embryos were shaken at room temperature for between 1 and 3 hours. L-cysteine acts as a dejelling agent with the reduction of disulphide bonds present in the jelly matrix. Shaking continued until the embryos were observed to be clustered at the bottom of the tube with no jelly surrounding and separating each embryo. Ambystomid, american toad and bullfrog embryos were observed to suffer obvious embryo damage in this treatment. Estimations of total protein concentration (Bio-Rad) of each species provided an indirect estimate of embryo damage due to cysteine exposure. Embryos were washed two times in cold Phosphate Buffered Saline (PBS (8 g NaCl, 0.2 g Na_2HPO_4 , 0.24g KH_2PO_4 in 1 l of distilled H_2O , pH 7.4)) and were then centrifuged at 3 000 rpm for 15 minutes in a KompSpin 21.50 rotor in a Beckman J2MC high speed centrifuge. The supernatant was removed and the packed cell volume (PCV) was estimated (generally less than 5.0 ml PCV resulted from 50.0 ml of egg and jelly). Four times PCV of Buffer I (10 mM Tris, 1 mM EDTA and 5 mM DTT) was added and let sit for 20 minutes on ice. Embryos were lysed using a Dyna-mix homogeniser (Fisher Scientific) set on high with 20-25 strokes, and the resulting solution was then placed in a sterile Erlenmeyer flask with a stir bar. Four times PCV of Buffer II (50 mM Tris, 10 mM MgCl_2 , 2 mM DTT, 25% sucrose w/v, and 50% glycerol v/v) was added. One times PCV of saturated DNase free ammonium sulfate was added drop by drop, while stirring, to lyse cell nuclei (Sugden and Keller 1973). Stirring was continued for 30 minutes after the last drop of ammonium sulfate was added. After stirring was completed, the solution was centrifuged at 20 000 rpm for 5 hours at 4°C . The supernatant was decanted into a sterile conical tube and measured, and then transferred to a

sterile flask where 0.33g of Sigma DNase free ammonium sulfate per 1.0 ml of supernatant was added while stirring to precipitate the protein. While stirring, 100 μ l 1N NaOH per 10 g of ammonium sulfate was added. Stirring continued for 30 minutes after the last addition of crystal. This solution was then centrifuged at 10 000 rpm for 30 minutes at 4 °C. The resulting pellet was resuspended in 1/40th the measured supernatant volume of storage/dialysis buffer (25 mM Hepes, 100 mM KCl, 12 mM MgCl₂, 0.5 mM EDTA, 2 mM DTT and 16% glycerol v/v) and dialyzed overnight in tubing of mole weight 14 000. The dialysis flask was placed in an ice bucket and covered with foil to minimize ice melting.

The dialysate was centrifuged for 10 minutes at 10 000 rpm at 4 °C. The supernatant was loaded to a Sigma chromatographic column with Blue Sepharose CL-6B, equilibrated with 0.1 M KCL and Buffer B (50 mM Tris-HCl pH 7.4, 1 mM EDTA, 10 mM 2-mercaptoethanol and 20% v/v glycerol), and washed with 0.1 M KCL and Buffer B with 0.6 g/L ATP. Fractions began to be collected after approximately forty minutes of run time. Small (20 μ l) sub-samples of each 2.0 ml fraction was added to 200 μ l Coomassie Blue stain and 780 μ l distilled water in order to determine which fractions contained the most protein. These protein rich fractions were collected, pooled, and dialyzed for 4-6 hours against photolyase storage buffer (50 mM Tris pH 7.4, 50 mM NaCl, 1 mM EDTA, 10 mM DTT and 50% glycerol). The resulting dialysate was collected, and frozen at -20 °C until used in either the transformation assay, or the ESS assay. A portion of the resulting dialysate was put aside at this point to perform a Bio-Rad assay for protein concentration.

2.1.4 Skin protocol

Upon collection from the road (04.28.1997), freshly killed spring peepers and leopard frogs were frozen at -20°C until skin removal (approximately seven days later). Upon dissection, skins were frozen at -80°C until CFE production. Skins were then thawed overnight at 4°C , cut into small pieces, shaken at room temperature for one hour in 1% SDS (sodium dodecyl sulfate) and incubated upon ice for five minutes. Skins were then homogenised with 8-15 strokes of a Dyna-mix homogeniser (Fisher Scientific) set at full speed. The supernatant was then poured into a sterile flask containing a stir bar and 4X PCV Buffer 1 was added. After this stage in the protocol, the treatment of the skin CFE was identical to the embryo protocol.

2.1.5 Transformation assay

The transformation assay is based upon Sancar et al. (1984), which was in turn based on Cohen et al. (1972), and Setlow (1968). Briefly, cell free extract (CFE) from each species (25 μg) was added to 12X CFE volume of 10X photolyase assay buffer (50 mM Tris pH 7.4, 50 mM NaCl, and 1 mM EDTA), 3X CFE volume of Tris-EDTA (TE, 10 mM Tris-Cl, 1 mM EDTA, pH 8.0) buffer, 3 μg of pBR322 DNA and EDTA and EGTA salt solutions to a final concentration of 250 mM. pBR322 DNA was obtained from Sigma, and isolated using the BioRad miniprep kit, QuantumPrep.

Pyrimidine dimers were formed in the DNA through exposure to a Photo Optic Halogen Bulb (1000W.120V.64743.Osram Corp.) at a distance of 10 cm for 60 or 90 seconds (3.3-4.0 MEDS/hr, 1MED = $21 \pm 3 \text{ mJ}/\text{cm}^2$, (Parrish *et al.* 1982) and therefore an exposure range of 11.55 - 14.00 J/m^2) in 16 mm polystyrene culture dishes at room temperature.

Replicates of this reaction mixture were placed either under photoreactivating light (+PRL = exposure to 3 GE 15W Black Lights

(1.22mW/cm²) wrapped in Saran Wrap (to remove potentially damaging lower wavelengths)) at a distance of 10 cm for 90 minutes, or in the dark for 90 minutes (-PRL, therefore approx. fluence = 6.588 J/m²). Preliminary tests indicated that this assay was linear with respect to time until 90 minutes. Photolyase deficient *Escherichia coli* CSR603, grown for three hours in LB broth and made competent through established methods (Sambrook et al. 1989), were added to the reaction mixture. The bacteria/plasmid mixture was cooled on ice for 60 minutes and heat shocked at 42 °C for 90 seconds and then cooled again on ice for five minutes. Two ml of LB with tetracycline was then added and the CSR603 was grown in a shaking incubator at 37 °C and 200 rpm for 1-2 hours to allow phenotypic plasmid expression. Bacteria were spun at 6 000 rpm for 10 minutes and the supernatant was poured off. The bacterial pellet was resuspended in 50 µl of LB + Tet and plated on selective media (LB and 20 µg/ml tetracycline), inverted and grown at 37 °C for 24-48 hours.

2.1.6 Transformation efficiencies

Resultant colonies from unirradiated plasmid (z), irradiated plasmid with no photorepair (-PRL) and irradiated plasmid with photorepair (+PRL) treatments were counted and specific transformation efficiencies for each treatment were calculated for colony forming units (CFU) per amount of DNA (CFU/µg DNA). Typically, specific transformation efficiencies for unirradiated plasmids ranged from 133 CFU per µg DNA to 832 CFU per µg DNA. For damaged and repaired plasmids, typical transformation efficiencies ranged from 0.33 CFU per µg DNA, to 10 CFU per µg DNA. This drastic reduction has been reported before by others using irradiated plasmids (Hays *et al.* 1990). Dimer repair was based on Sancar et al. (1984) and was calculated as difference between

the relative transformation efficiency for the irradiated plasmid without photorepair, and the irradiated plasmid with photorepair.

$$\text{mean dimer number per plasmid molecule} = \{-\text{Ln}(-\text{PRL}/0) - (-\text{Ln}(+\text{PRL}/0))\} \quad (1)$$

These numbers were then standardized for time of exposure to photoreactivating light, and the ratio of protein to DNA.

In the instances where the estimate for photolyase activity was negative this does not imply that the CFE has formed CBPDs in the amphibian DNA. These negative numbers might mean that the CFE is causing some alternative form of damage to the DNA, or that there is some type of dark repair going on in the treatments where photoreactivating light is blocked. It is conceivable that the cell free extract might have contained the enzyme complex of *uvrABC* gene products necessary for excision repair of the experimentally induced dimers in the plasmid DNA. This might be an explanation for instances where the -PRL CFU treatment of some species (chorus frog) were always greater than those which had been exposed to photoreactivating light.

2.1.7 Between species comparison of jelly absorbance of UV radiation

Egg masses for between species comparison of UVB transmission of protective jelly were collected in April of 1998. Egg masses were kept at 4°C in the dark until time of analysis. Embryos were dissected out of the jelly at room temperature. Jelly was then carefully loaded into a spectrophotometer cuvette (BioRad disposable, pathlength = 1 cm) to avoid the formation of bubbles. Relative arbitrary absorbance values were determined at 320 and 280 nm (N = 10). Absorbance curves encompassing the UVB range (275-320 nm) were also generated (Appendix 2).

2.2 Results

Table 1 incorporates the photolyase activity of amphibian CFE determined through the bacterial transformation assay for CBPDs in exogenous plasmid DNA. The history for each tissue and repair estimation are summarized. The DOC values are from Lean *et al.* (submitted). Un-shaded columns relate directly to work completed in this chapter. Shaded columns refer to information important to the history of the tissue assayed here, but not directly related to the work in this chapter.

2.2.1 Between species patterns of photolyase activity

Between-species patterns of photolyase activity were measured for nine species by a bacterial transformation assay. Tissue samples most frequently used were embryos which had been treated with 2% L-cysteine to remove the multiple jelly layers which protect the embryo. In addition to these embryo treatments, oocytes were collected from gray tree frogs and blue spotted salamanders and skin samples were collected from leopard frogs and spring peepers. Species for which a photolyase activity estimation was possible for more than one tissue type (gray tree frog and blue spotted salamander) exhibited a lack of significant difference between the tissue types (one-way anova for GTF= $df=1$, $F=0.3625$, $p=0.56$, and BSS= $df=1$, $F=0.3776$, $p=0.55$).

For the ten species and or tissue types where photolyase activity was compared, there were significant between-species differences ($df=9$, $F=5.100870$, $p < 0.0001$) (Figure 1, Table 1).

For eight jelly types of seven species where absorbance at 280 nm and 320 nm were compared, there were significant between species differences (320 nm

df=7, $F=331.5$, $p<0.0001$ Figure 2a), (280 nm df=7, $F=335.9$, $p<0.0001$ Figure 2b). UVB range absorbance of embryo jelly of seven species were compared (Figure 3). A correlation analysis indicated that there was no significant relationship between photolyase activity and jelly absorbance at both 320 nm (380nm: $r=0.022$, $p>0.05$), but a significant relationship at 280 nm (280nm: $r=0.88$ $p<0.05$) (Figure 2b).

Table 1 Photolyase activity of amphibian CFE determined through the bacterial transformation assay for CBPDs in exogenous plasmid DNA. The history for each tissue and repair estimation are summarized. See text for shading interpretation.

Species	Collected	Location	Mean Depth (cm)	DOC ppm	De-jelly protocol (Appendix 1)	Cell type Anuran = Gosner Stg Salamander = Harrison stg.	UV-B J/m ²	Jelly absorbance (relative arbitrary units)	<i>In vitro</i> E/S temp (°C)	Repair (dimers/3 µg DNA/25µg CFE/90 min PRL (stdev))
<i>Rana sylvatica</i>	04.29.97	Arch Pond	9.25	18.75	2% L-cysteine	11	368.44	280=2.22 320=1.26	4	0.68 (0.4)
<i>Rana clamitans</i>	06.10. 97	Turtle Pond	6	12.65	2% L-cysteine	10	38.8	280=0.58 320=0.90	15	0.73 (0.12)
<i>Hyla versicolor</i>	oocytes 04.30. 97 stg 10 06.04. 97	Kitty's Pond	0	17.6	n/a	oocyte and 16	n/a	n/a	20	-0.36 (0.37)
					2% L-cysteine		Stg. 16 unknow n	280=1.08 320=2.23	20	-0.23 (0.4)
<i>Rana pipiens</i>	05.07. 97 04.28. 97	Arch Pond Road kill	13.1	18.75	2% L-cysteine	16 Skin	unknow n	280=1.122	20	0 (0)
					n/a		unknow n	320=2.156 n/a	15	0 (0)
<i>Hyla crucifer</i>	04.28.97	Road kill	7.5	n/a	n/a	Skin	unknow n	n/a	15	0.74 (0.3)
<i>Pseudacris triseriata</i>	04.30- 05.09. 97	Near Arch. Pond	5	likel y >19	2% L-cysteine	11-17	unknow n	280=0.64 320=0.90	40+ 20	-0.3 (0.27)
<i>Ambystoma laterale</i>	04.28. 97	Road Kill	14	n/a	n/a	n/a	n/a	n/a	20	0.14 (0.09)
	05.26. 97	Kitty's Pond		17.6	2% L-cysteine	26	63.04	280=1.18 320=1.94	4	0.12 (0.16)
<i>Ambystoma maculatum</i>	05.26.97	Kitty's Pond	16.7	17.6	2% L-cysteine	16	63.04	280=1.58 320=2.97	20	0.49 (0.23)
<i>Xenopus laevis</i>	12.05.97	lab	n/a	low	Barth's	11	n/a	n/a	4	-0.09 (0.25)

For species in which there were photolyase activity differences for *in vitro* assay temperatures (green frog, chorus frog), data were pooled if there was no significant difference between the two temperatures (chorus frog 4 and 20 °C $p=0.201$, $df=1$, $F=1.86$), and kept separated if there was a significant difference (green frog 4, 15 and 20 °C Pearson's correlation coefficient, -0.62 , $p<0.05$) (Figure 4).

A correlation analysis of mean depth of oviposition and photolyase activity displayed a trend of decreasing activity with decreasing depth, but this trend was not significant ($r=0.37$, $p>0.05$). Although not significant, this counter-intuitive trend indicating increasing repair ability with increasing depth of oviposition is biologically interesting (Figure 5).

2.2.2 UVB damage of plasmid DNA

The *in vitro* fluence required to damage plasmids (11.55 - 14.00 J/m²) was lower than fluences reported earlier (99.00 J/m² in Dutta *et al.* 1993, 250 J/m² in Li *et al.* 1993, in 500 - 2500 J/m² Malloy *et al.* 1997, and 200 J/m² in Sancar *et al.* 1984, 540 J/m² in Legerski *et al.* 1987, but see Yasuhira and Yasui (1992) who used 0.12 J/m² in an assay dependant upon bacterial survival). This was in part due to lack of visible transformants when plasmid DNA was exposed to a greater fluence (data not shown). The lack of visible transformants was most likely due to the combination of three factors; 1) high concentrations of DNases in amphibian cell free extract, 2) low cellular concentrations of photolyase, and 3) greater number of dimers formed at higher fluences. I have assumed that some combination of these factors resulted in both a loss of transforming DNA through digestion, and the loss of observed transformation through the presence of un-repaired dimers. A lower fluence was utilized here, and resulted in low, but discernable, CFU. An interesting correlation, is that 14 J/m² is a biologically

relevant fluence one might expect after two hours of midday, late spring - summer irradiation at the water's surface at the latitude and elevation where the amphibians for this research were collected (Berrill *et al.* in prep).

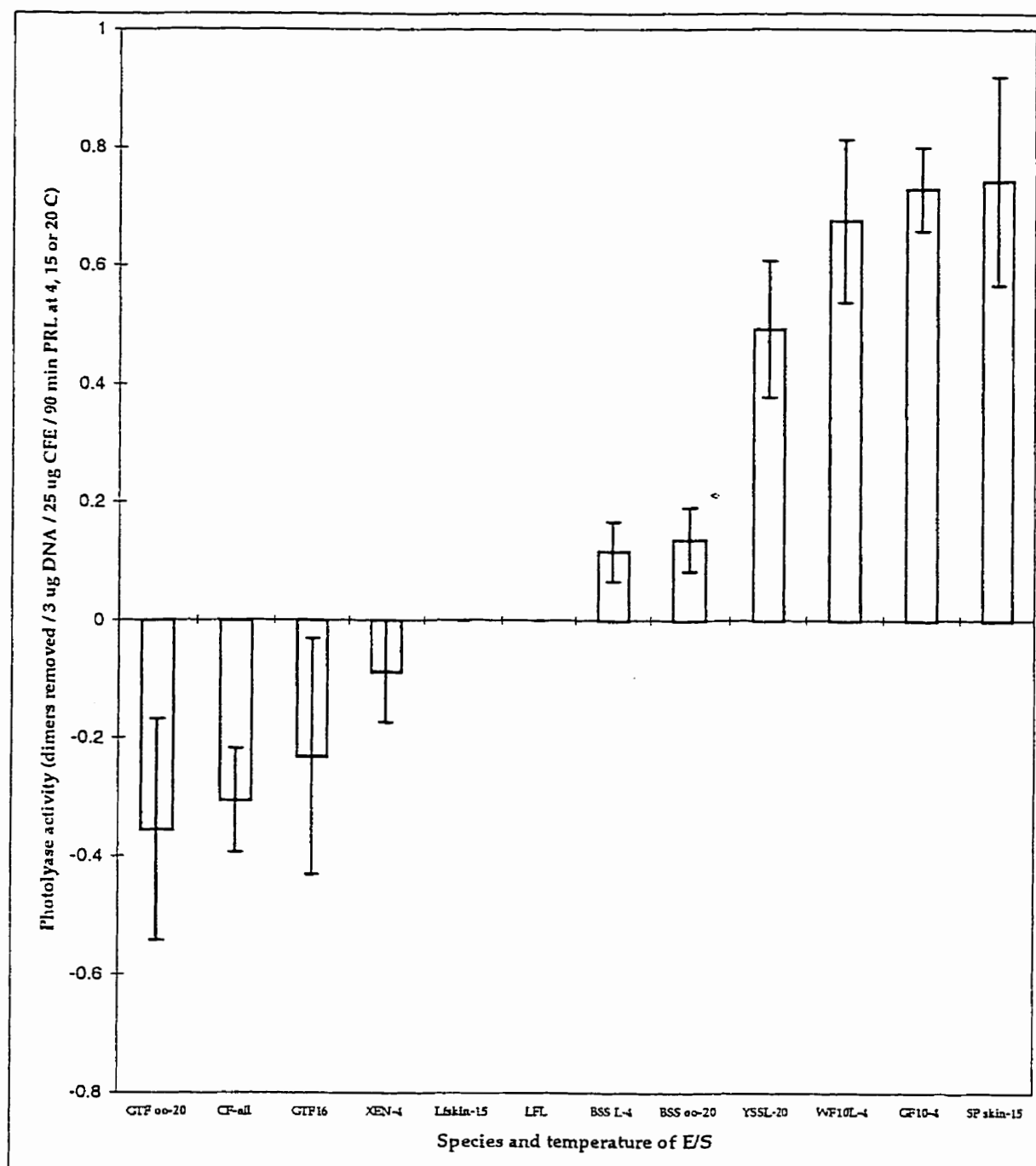


Figure 1:

Species comparison for photolyase activity (\pm SE) of CFE made from skin or embryo of nine amphibian species. Chorus frog (CF), wood frog (WF), yellow spotted salamander (YSS), blue spotted salamander (BSS), leopard frog (LF), gray tree frog (GTF), *Xenopus* (Xen), and green frog (GF).

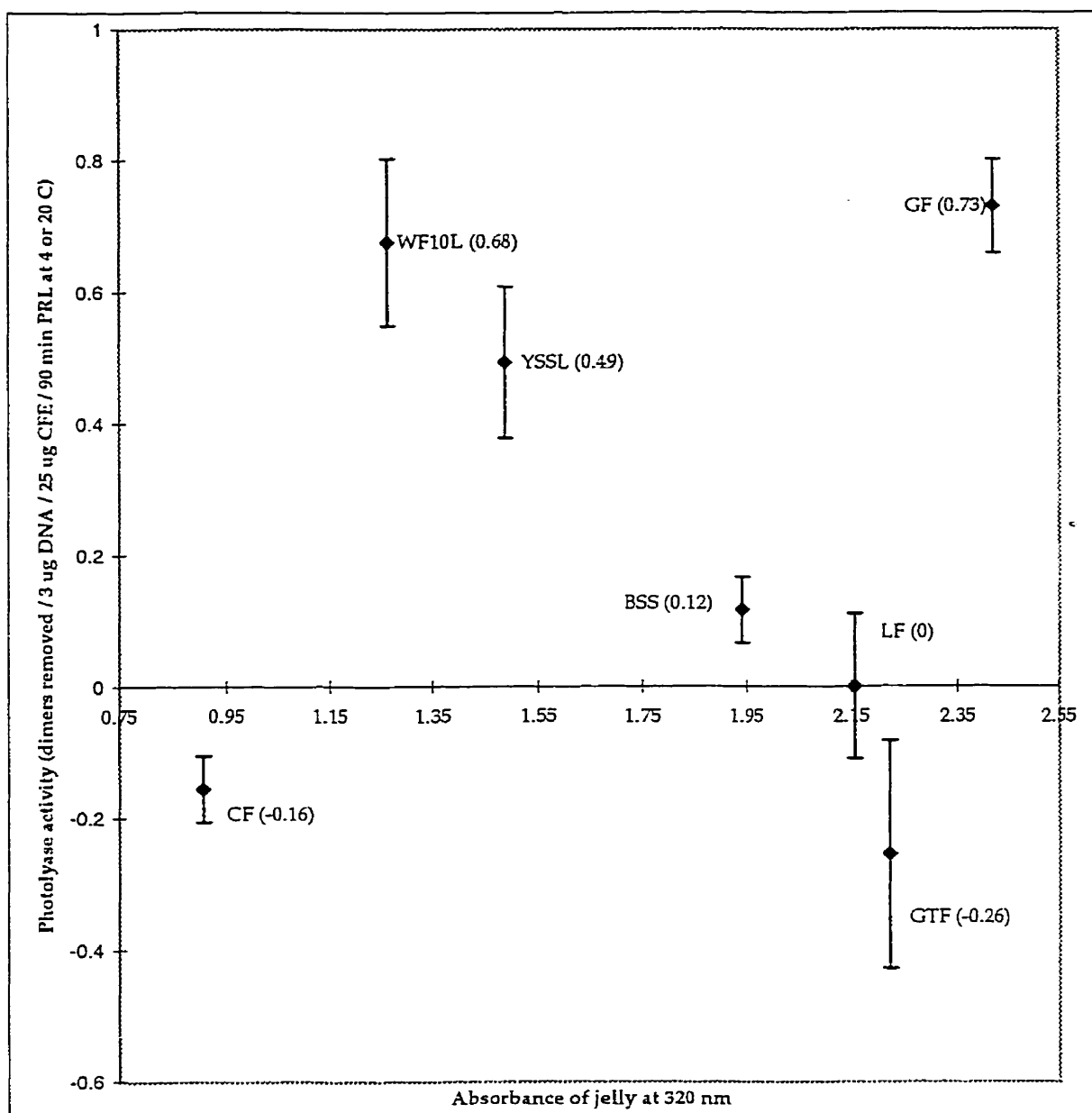


Figure 2a:

Comparison of the jelly absorbance characteristics (320 nm) of amphibian embryo jelly and photolyase activity (\pm SE) of cell free extract from embryos of the same species. Chorus frog (CF), wood frog (WF), yellow spotted salamander (YSS), blue spotted salamander (BSS), leopard frog (LF), gray tree frog (GTF), and green frog (GF). Relationship is not significant at $p < 0.05$. ($r = 0.022$). See Table 1 for further description of between species differences.

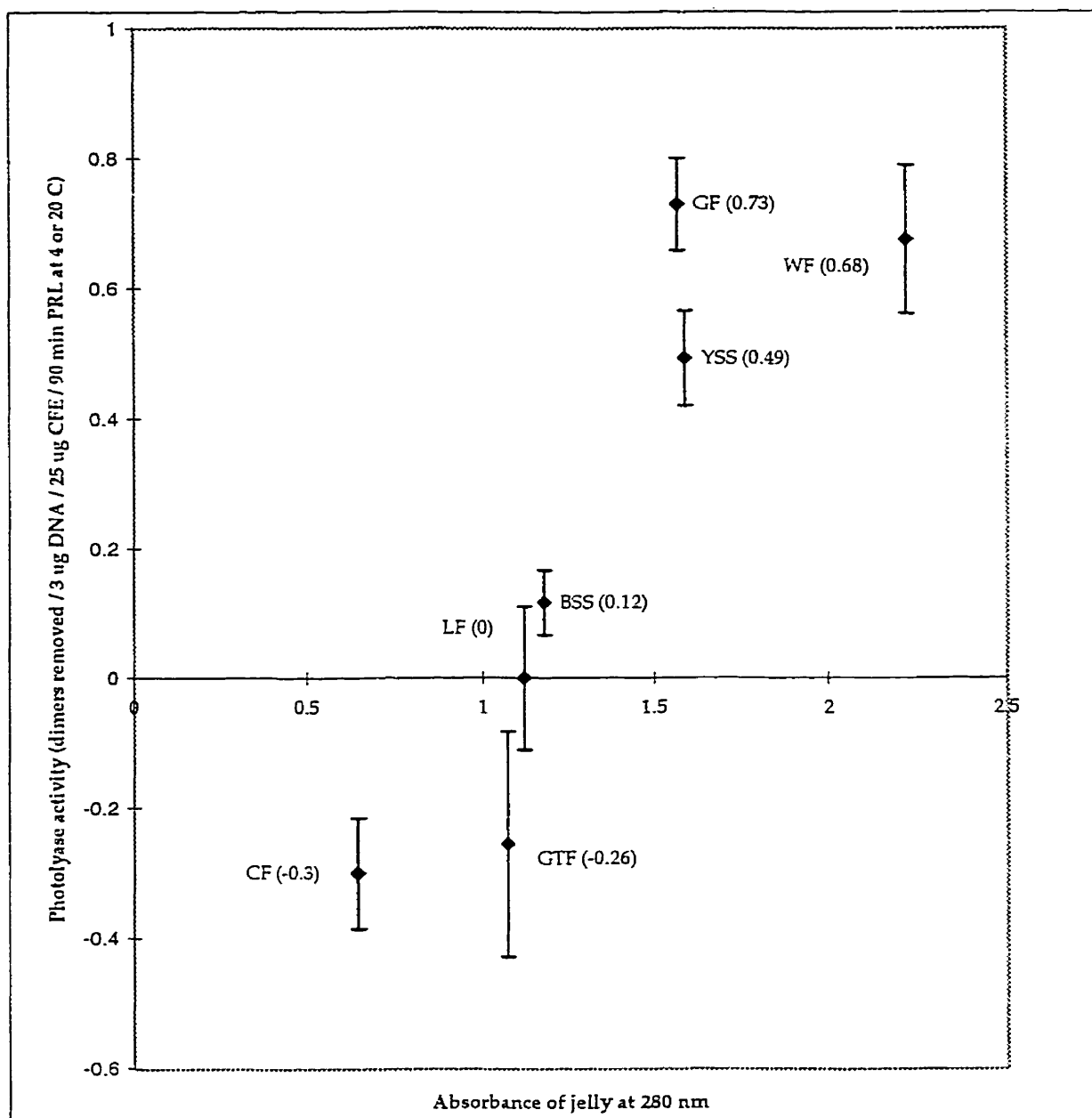


Figure 2b:

Comparison of the jelly absorbance characteristics of amphibian embryo jelly at 280 nm with photolyase activity of cell free extract from embryos of the same species. Chorus frog (CF), wood frog (WF), yellow spotted salamander (YSS), blue spotted salamander (BSS), leopard frog (LF), gray tree frog (GTF), and green frog (GF). Relationship is significant at $p < 0.05$. ($r = 0.88$). Standard error bars are shown. See Table 1 for further description of between species differences.

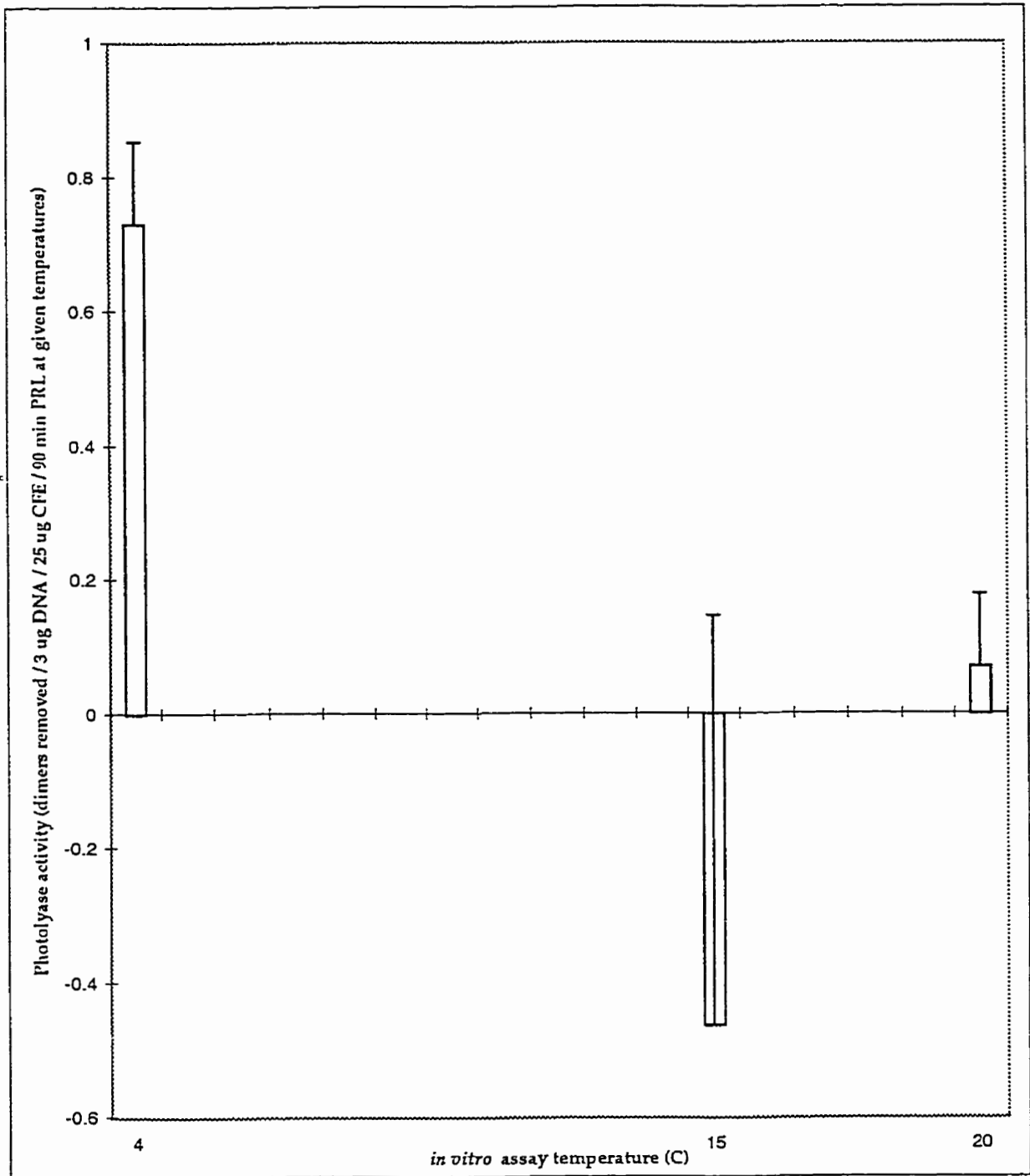


Figure 3:

Importance of *in vitro* temperature on photolyase activity (\pm SE) determination for the green frog (*Rana clamitans*) (Pearson correlation coefficient = -0.62, not significant at $p < 0.05$).

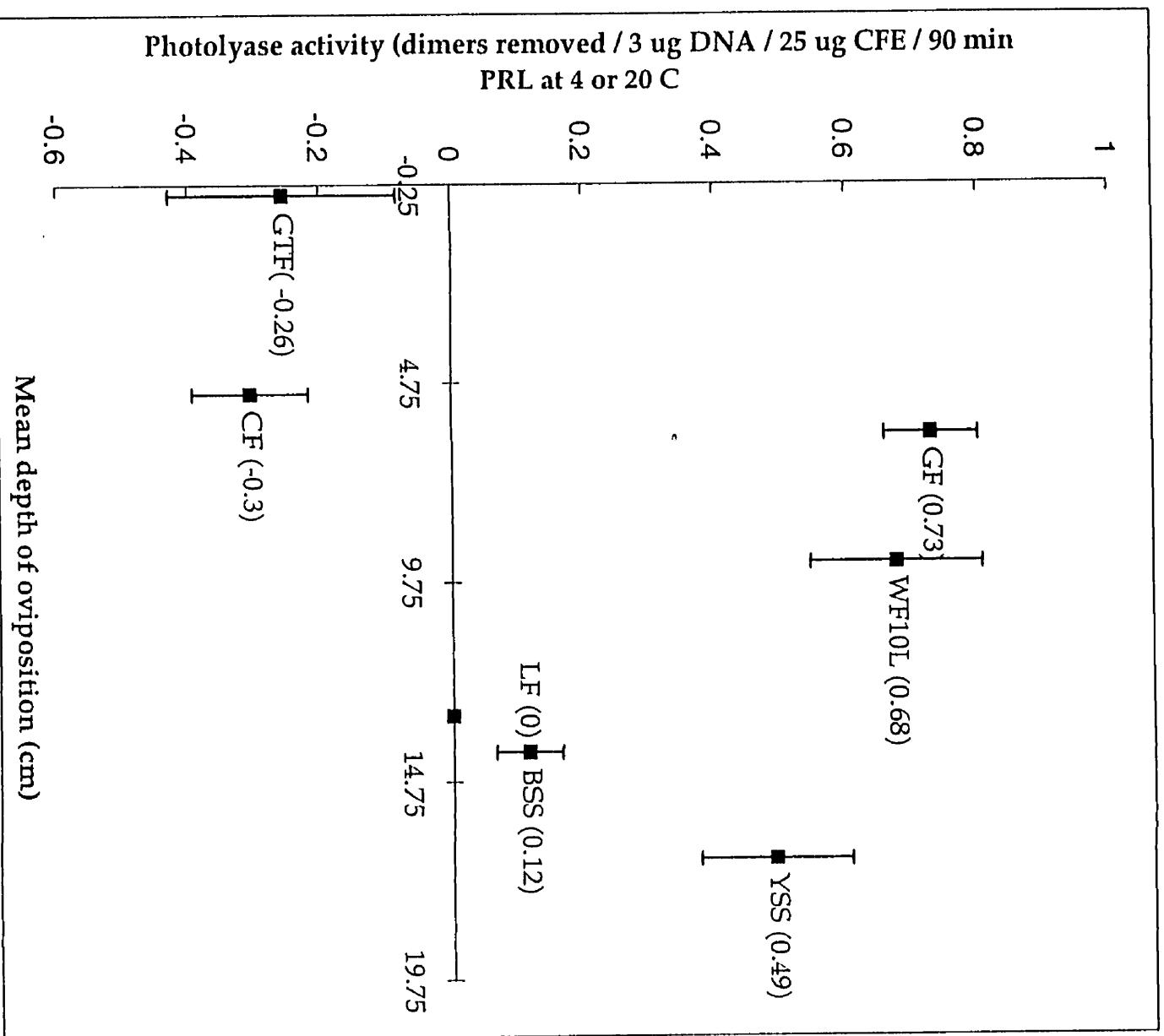


Figure 4: Examination of trends between mean depth of oviposition and photolyase activity (\pm SE) of embryos from seven amphibian species. There is a trend towards a positive relationship ($r=0.37$), but it is not statistically significant ($p>0.05$).

2.3 Discussion

A species by species interpretation of the photolyase activity assay and UVB jelly absorbance data follows a general discussion of each topic.

2.3.1 Among-species comparison of photorepair ability through transformation efficiencies

Due to the kinetics of transformation, the bacterial transformation assay is one of the most sensitive assay systems for photolyase (Li *et al.* 1993). Upon irradiation, and dimer formation in plasmid DNA, the ability to transform the bacterium is removed due to post-transformation replication difficulties with the dimer-twisted DNA (Keszenman-Pereyra 1990, Husain *et al.* 1988). Unless photolyase monomerises the dimers, the bacterium containing that DNA will not express the antibiotic resistance encoded by the plasmid as the bacterial polymerases will not replicate the damaged plasmid (Li *et al.* 1993). Thus, a single dimer is a lethal lesion on incoming plasmid DNA, and only those plasmids completely repaired by photolyase will transform the bacterium and then be apparent as colony forming units (CFU) upon plating on selective media. My results indicate significant between-species differences in photolyase activity. Because it is known that UVB can induce photolyase (Chapter 3), where possible these photolyase activity values were compared to total fluence that the waters surface would have absorbed on the day of collection (Table 1). Actual fluence received by the embryo would undoubtedly have been less than this value. UVB does induce photolyase activity, both *in vitro* and *in vivo*, but across a large range of UVB fluences, between species comparisons were possible due to the small effect of UVB on between-species comparisons.

2.3.2 Jelly absorbance at 280 and 320 nm

Amphibian embryos in south-central Ontario are enclosed in protective jelly layers. These layers are composed of differing concentrations of

glycoprotein, mucoproteins, carbohydrates and mucopolysaccharides (Salthe 1963). Although there is not a lot known regarding differences in chemical composition between jelly layers or between species, it is known that jelly envelopes of different species refract light differently (Cornman and Grier 1941, Salthe 1963) and that jelly from different species display differing patterns of UVB transmission (Grant and Licht 1995, Ovaksu *et al.* 1997). Grant and Licht (1995) found that the egg jelly reduced UVB transmission in the wood frog (7% reduction), the american toad (14% reduction), and the yellow spotted salamander (60-77% reduction). In my study, significant differences in the protective jelly absorbance at 280 and 320 nm exist between species, but there was no significant relationship between the UVB absorbance of the embryo jelly at 320 nm and photolyase activity. A significant trend exists between absorbance at 280 nm and the photolyase activity of cell free extracts made from embryos of the same species. This trend is interesting for several reasons. First it suggests that species which have jelly that absorbs more of the UVB wavelength range, therefore preventing it from reaching the embryo, have more photolyase activity than species which have jelly that is not as absorbent of this damaging wavelength. Second, it adds evidence to speculations that gray tree frogs and chorus frogs are in some type of unique situation. They are oviposited in areas which experience largely unattenuated UVB wavelengths, have jelly which does not absorb much UVB, and both have low photolyase activity. Such characteristics would suggest that if any species included in this study might be experiencing population regulation through UVB stress, these would. Yet there are no incidences of population decline in this area for either species. I would speculate that the trends displayed in Figures 2 and 3 would be characteristic of species which have a greater propensity for NER than photoenzymatic repair. NER would allow the embryo to repair oxidative damage to DNA in addition to

UVB damage. Adaptive pressures for such predominance are easy to imagine in an embryo mass suspended at or near the surface.

2.3.3 Mean depth of oviposition and dimer repair

There is a slight trend between depth of oviposition and photolyase activity indicating increasing repair ability with increasing depth of oviposition. The small r value suggests that it is not a biologically significant trend, and more than likely there is no relationship between depth of oviposition and repair ability. Yet perhaps, as I have speculated, it may perform a predictive function, for it assisted in the isolation of the chorus frog and gray tree frog as species where further research is greatly required. The gray tree frog and chorus frog egg mass, although challenging to locate, must be included in future studies of embryo sensitivity to UVB damage.

2.3.4 Species Comparisons

2.3.4.1 Wood Frogs

Wood frogs lay their eggs in a globular mass, early in the spring. They often deposit their eggs communally, and their deposition sites cover a wide range of water depths (Berven 1990). My results indicate that wood frogs have mid to high (in the context of this study) range photolyase repair. Results reported in Chapter 3 indicate that this activity is inducible, and changes throughout the course of the day. This is an extremely important result, for it implies that researchers must report the time and depth of collection if embryos are collected from natural populations. The high absorbance of the wood frog jelly, and the range of depths at which females oviposit suggest that currently the wood frog is not experiencing direct damage from UVB radiation.

2.3.4.2 Leopard Frogs

Leopard frog cell free extracts from skin and embryo both displayed next to no photolyase activity. In all assay temperatures (4, 15 and 20 °C), and at

differing concentrations of pBR322 and CFE leopard frog CFE did not give a signal which indicated that photorepair of damaged plasmid had occurred. My own *a priori* prediction was that the leopard frog would be exposed to very high levels of UVB due to its habit of basking in sunlight during summer months, and therefore CFE created from skin would have high photolyase activity. The low photolyase activity from CFE created from skin, especially when compared to the high value of spring peeper skin, was surprising. Peepers are essentially fossorial in the summer months, while leopard frogs are exposed to much higher UVB fluence as they range over wide areas and are exposed to widely disparate levels of sunlight.

The leopard frog has been utilised in UVB speculations and lab studies in the past. Ovaska 1995 speculated that the embryos in the globular egg mass of the leopard frog might be less susceptible to damage than embryos laid in a surface sheet (such as the green frog). Long *et al.* (1995) demonstrated a synergistic effect between UVB and pH on leopard frog embryo survival in the lab. Perhaps the oviposition depth and absorbance of the jelly mass preclude most UVB specific damage, and therefore there is an embryonic predominance of alternative DNA repair mechanisms. It's also possible that leopard frog skin may have sufficient dermal pigmentation to prevent DNA damage. Future investigations should make attempts at including this variable.

2.3.4.3 Green frogs

Green frogs lay eggs on the surface in a sheet of thin jelly, which Ovaska (1995) predicted would make them more susceptible to UVB damage. Green frog photolyase activity was the highest among the species measured here. Interestingly, this activity was temperature dependant *in vitro*. The assay values for green frog photolyase activity ranged from 0.73 at 4°C -0.46 at 15°C to 0.07 at 20°C.

The high absorbance of the green frog jelly, high levels of photolyase activity, and shallow depth of oviposition suggest that the green frog is likely experiencing some direct UVB radiation damage. Green frog populations are stable over large geographic areas, but like many amphibians, local populations are subject to extinction (Hecnar and M'Closkey 1997, Weller and Green 1997). The temperature dependant effects on photolyase activity, demonstrated *in vitro* here, are worthy of further *in vitro* and *in vivo* investigation. Surface temperatures of the water sources from which these embryos were collected can change rapidly during the course of embryonic development (Berrill unpublished) and an *in vivo* effect of temperature on photolyase activity is an important concern.

2.3.4.4 Yellow and blue spotted salamanders

Several factors suggest that these ambystomid salamanders are not currently experiencing direct UVB damage. This is probably due to the high absorbance of the yellow spotted salamander jelly, the globular egg capsule of both species, the depths at which they oviposit, and the presence of mid-high range (YSS), and mid-low range (BSS) photolyase activity. The shallow sites of oviposition, lower photolyase activity level, less absorbent jelly, and earlier spring times of oviposition indicate that if UVB levels continue to rise, the blue spotted salamander is more likely to be affected than the yellow spotted salamander.

2.3.4.5 Chorus Frogs

The chorus frog is a challenging species to interpret from the data reported here. Within this challenge lie enough contradictions to strongly suggest further study. Transformation data often incorporates numbers that are less than zero (i.e. counter to expectations, there are a greater number of transformants in the treatment without photoreactivating light, than in the

treatment with photoreactivating light), and this is usually taken to reflect random variation in a very low reading (i.e. close to zero) (A. Sancar pers. comm.). Yet, chorus frog CFE produced a very large number of transformants, and these numbers were always biased towards the -PRL treatment, and the dimer removal estimation was always less than zero. It is important to state that the negative values for both the chorus frog and the gray tree frog likely do not reflect CBPDs caused by the CFE, rather they represent one of two possible causes: 1) another form of damage caused by the CFE to the plasmid DNA that prevented transformation of the bacterium, or 2) the CFE may have contained the NER enzyme complex, and this may have repaired the -PRL plasmid treatment. I have no data to support either hypothesis.

The chorus frog also has the least absorbent jelly of any species tested here (Figure 2). The two pieces of chorus frog data generated here would then indicate that chorus frog embryos have a very transparent jelly, and not a great ability to photoenzymatically remove the principle form of damage to DNA. Is this a scenario (low repair, surface laying, low absorbance jelly) which is of relevance to conservation biology? In the specific case of the chorus frog there are probably enough other selective pressures other than direct UVB damage to DNA that apparently low photoenzymatic repair is not currently regulating population sizes. The water where chorus frogs oviposit their eggs in south-central Ontario is very shallow, very humic and littered with a large amount of detritus. UVB radiation is very quickly attenuated in water so high in DOC and such water also has a large capability for the production of hydrogen peroxide and damaging free radicals. Furthermore, chorus frog egg masses are often laid on the undersides of wide, long submerged blades of grass (personal observation). Such physical placement might largely block UVB radiation. Perhaps the chorus frog eggs, often laid beneath the depth of effective UV

penetration, have an adaptive predominance of non-photoreactivating methods of repair (ie. NER). NER would also be able to identify and excise oxidative damage in addition to UVB induced dimers. Regan *et al.* (1983) (in Mitchell and Karentz 1993) reported that there was a negative correlation between species with high levels of photoenzymatic repair, and nucleotide excision repair. Future examinations should investigate whether low-photolyase species, such as the chorus frog, have greater capacities for excision repair.

2.3.4.6 Gray tree frogs

Much of the above discussion is also applicable to the gray tree frog. In the areas where gray tree frog eggs were collected for this study, the eggs are laid in a small cluster immediately at the waters surface. Broods could often be spotted by looking for the distortions on the surface of the water caused by the egg mass actually pushing up above the surface (personal observation). Such an oviposition site is likely to receive a large UVB fluence. Interestingly, the photolyase activity of both gray tree frog oocytes and embryos was quite low, and the UVB absorbance of the jelly mass was also low at both 280 and 320 nm. There is an apparent lack of correlation between expected exposure, and photolyase activity and jelly absorbance. I have speculated on the potential, untested, role NER may play in this species, but an additional factor, specific to the gray tree frog is the dark pigmentation of the animal and vegetal pole of the gray tree frog embryo. Heavily pigmented eggs have been shown to be less sensitive to UV damage than less heavily pigmented eggs (Duellman and Trueb 1992) but this relationship is unclear, and worthy of future examination. Future analyses should focus on the adaptive nature of pigmentation in both skin and embryo amphibian tissue.

2.3.4.7 African clawed frog

Xenopus laevis was included in the study in order to have a common species between this work, and the work reported by Blaustein *et al.* (1994). Efforts were made to include species that were endemic to North America, but these efforts were unsuccessful.

In 1994, Blaustein *et al.* reported the photolyase activity of *Xenopus* as between 0.06 and 0.11 1.0×10^{11} CBPDs per hr per μg). This was the anuran species with the lowest photolyase activity reported in 1994 by Blaustein, and other work has also shown *Xenopus* to be sensitive to UVB damage (Bruggeman *et al.* 1998). A comparison of absolute values is difficult, between Blaustein *et al.* (1994), and in the work reported here. Yet in both studies, *Xenopus* is clearly demonstrated to be a species characterized by extremely low photolyase activity. Both assay protocols identified this characteristic.

2.3.4.8 Spring peeper

An insufficient number of spring peeper embryos were collected to allow for analysis of embryo photolyase activity. Analysis of CFE made from spring peeper skin revealed the second highest photolyase activity measured in this study. This again, is suggestively non-adaptive. The spring peeper is fossorial for most of the summer months, and would likely not experience damaging UVB fluence. I speculate that the high photolyase activity of spring peeper skin (which may also indicate a high embryo value (Hays *et al.* 1996)), the fossorial nature of the adult, and the depth of oviposition suggest that spring peepers are not being affected by current UVB fluence rates.

2.3.5 Future questions

Due to factors such as DOC and pH (Scully and Lean 1994, Schindler *et al.* 1996, Yan *et al.* 1996) UVB fluence at the surface of a body of water are often very different from subsurface levels. It is too early to conclude that investigating

photolyase activity for amphibians from humic environments is unnecessary due to the reduced UVR fluence these amphibians likely receive. These same humic waters are also often quite shallow, and in the case of ephemeral waters, become more shallow through time (Williams 1995). These factors result in a broad range of exposure possibilities, and the large probability of secondary UVB damage in the form of oxygen free radical formation. Developing amphibians from humic waters must still be studied due to the potential for future rises in UVB fluence and climate change resulting in feedback mechanisms which accentuate the effects of UVB (Schindler *et al.* 1996).

Previous results have indicated that species accumulate significant numbers of CBPDs throughout exposure time to UVB (Malloy *et al.* 1997). If the system is adaptive, one would expect enzymatic methods of CBPD removal would also accumulate throughout an exposure to UVB. One interesting future analysis could make use of the monoclonal antibody H3 (specific to pyrimidine dimers) and genomic amphibian DNA (Appendix 1). Another future analysis should investigate the *in vivo* effects of temperature on the photolyase activity of tissue from naturally occurring animals. The potential exists for mean temperature increases for the waters where this study was completed (Ovaska 1995) and this suggests that any potential relationship between UVB damage repair and temperature must be explored.

In conclusion, there are significant between-species differences in photolyase activity for the species tested and these differences are not correlated with expected natural UVB exposure. Where tissue was available there were no significant differences between oocyte and embryo photolyase activity. There are significant between species differences in jelly mass absorbance of UVB radiation and these differences are correlated with photolyase activity at 280 nm, yet not at 320 nm.

Chapter 3

In vivo induced photolyase activity of the cell free extract from wood frog (*Rana sylvatica*) embryos.

3 Abstract

Photolyase activity is inducible in eukaryotic cells (Mitani *et al.* 1996, Sebastian *et al.* 1990, Chao and Lin-Chao 1987b, and Fukui and Laskowski 1984) by as much as ten times (Yasuhira and Yasui 1992). The photoenzymatic removal of cyclobutane pyrimidine dimer (CBPDs) is regulated by ultraviolet-B radiation, ultraviolet-A radiation, temperature, hydrogen peroxide and the inhibition of growth (Mitani and Shima 1995, Mitani *et al.* 1996). This *in vivo* variable has not been addressed in previous examinations of organisms from natural populations (i.e. levels of ambient UVB immediately prior to the time of egg collection were not reported).

I exposed wood frog embryos to 2, 4 and 10 hours of ambient spring-time UVB and used the bacterial transformation assay to measure the photolyase activity of the embryo tissue from these exposures, and from embryos exposed to similar periods of dark. Photolyase activity of embryos exposed to ambient UVB light treatments were significantly different from each other and these differences are positively correlated with the UVB fluence each embryo mass had received *in vivo*. Embryos exposed to dark periods were not significantly different from each other.

Inducible changes in wood frog embryo photolyase activity have been demonstrated here and previous conclusions regarding dimer removal characteristics of certain amphibian species require reevaluation in the context of how much UV radiation the egg masses had been exposed to prior to collection.

3.0 Introduction

CBPD photolyase is strongly regulated by environmental factors. This regulation has been demonstrated in both prokaryotic and eukaryotic cell lines (Sebastian and Sancar 1991), but less information is available regarding induced changes in animals from natural populations. I report results here which indicate that *in vitro* measurements of the photolyase activity of wood frog (*Rana sylvatica*) cell free extracts were enhanced by *in vivo* exposure to ambient ultraviolet radiation while maintained at a constant temperature of 4°C. This study is the first attempt to quantify induced changes in photolyase activity in an organism from a natural population and has important ramifications for other *in vitro* estimations of amphibian photorepair. Most other studies utilizing animal tissues from naturally occurring species have not accommodated, or measured environmental variables, such as ultraviolet radiation, temperature and hydrogen peroxide, which are all known to alter photolyase concentrations (Mitani *et al.* 1996, Yasuhira and Yasui 1995, Sebastian and Sancar 1991). Temperature and hydrogen peroxide formation were not measured here, but ambient ultraviolet-B radiation was, and it is significantly correlated with the amplified photolyase activity of CFE from wood frog embryos exposed to greater fluences of UVB. Although the repair ability of a certain species is influenced by ultraviolet radiation, additional data (with *Ambystoma laterale* and *maculatum* embryos) indicate that photolyase activity is characterized by the species the tissue originated from, more than the amount of UV that the embryos of that species have received. This is a promising result for the future of between-species comparisons, and for reevaluations of between-species comparisons already completed.

3.1 Methods

3.1.1 Egg and embryo collection

Eggs from wood frogs (*Rana sylvatica*), were collected in April of 1997. Upon collection eggs were transported back to Trent University where the eggs were staged according to Gosner (1960). UVB readings for each collection day (from 8:00am until 6:00pm at one minute intervals) were obtained from a BW-100 CID UV measuring Instrument (Vital Technologies Corp.) located at Trent University (44°21'N 78°17'W, 100 m elevation). These values were erythemally weighted. Eggs were maintained at 4°C in the dark until experimental exposures occurred. Wood frog embryos were kept for six days at 4°C in the dark until the next "sunny" day. At the time of exposure (April 29 1997 from 10:00am until 8:00pm) wood frog embryos were approximately Gosner stage 11.

3.1.2 Induction of wood frog photolyase through exposure the ambient UV

Induction experiments with wood frog embryos were conducted on the 29th of April 1997 from 10:00am until 8:00pm. Three groups of 50.0 ml of eggs, with jelly intact, were isolated in 500 ml plastic containers containing purified river water. These egg masses were then placed either in the dark at 5°C, or were exposed to natural sunlight while maintained at 5°C. Maintaining eggs in both dark and light groups allowed the testing of whether the photolyase activity was intrinsic to the embryo, or whether exposure to ambient light would induce photolyase activity. Temperatures were reduced to ensure that eggs did not age during treatments. Wood frog egg masses were removed from dark or ambient light conditions after 2 (12:20pm), 4(2:20pm), and 10(8:20pm) hours of exposure and were then immediately frozen at -80°C until protein purification took place.

3.1.3 Between-species differences in photolyase

In an attempt to resolve difference between the UV-induced photolyase activity levels from the levels endogenous to the tissue in question blue spotted salamander and yellow spotted salamander eggs were exposed to ambient UVB light on May 26 1997 and immediately frozen at -80°C in 50 ml containers. These values were compared to the wood frog induction data.

3.1.4 Protein purification

Methods of protein purification were adapted from Manly *et al.* (1980), Sancar *et al.* (1984), and Blaustein *et al.* 1994). Briefly; 50 ml egg replicates (frozen at -80°C) were warmed overnight from -80°C to 4°C . 10 ml of 2% L-cysteine was then added to each 50 ml container for 2, 4 and ten hour light and dark treatments and embryos were shaken at room temperature for between 1 and 3 hours. L-cysteine acts as a dejelling agent with the reduction of disulphide bonds present in the jelly matrix. Shaking continued until the embryos were observed to be clustered at the bottom of the tube with no jelly surrounding and separating each embryo. Estimations of total protein concentration (Bio-Rad) of each species provided an indirect estimate of embryo damage due to cysteine exposure. Embryos were washed two times in cold Phosphate Buffered Saline (PBS (8 g NaCl, 0.2 g Na_2HPO_4 , 0.24g KH_2PO_4 in 1 l of distilled H_2O , pH 7.4)) and were then centrifuged at 3 000 rpm for 15 minutes in a KompSpin 21.50 rotor in a Beckman J2MC high speed centrifuge. The supernatant was removed and the packed cell volume (PCV) was estimated (generally less than 5.0 ml PCV resulted from 50.0 ml of egg and jelly). Four times PCV of Buffer I (10 mM Tris, 1 mM EDTA and 5 mM DTT) was added and let sit for 20 minutes on ice. Embryos were lysed using a Dyna-mix homogeniser (Fisher Scientific) set on high with 20-25 strokes, and the resulting solution was then placed in a sterile Erlenmeyer flask with a stir bar. Four times PCV of Buffer II (50 mM Tris, 10 mM MgCl_2 , 2 mM DTT, 25% sucrose w/v, and 50% glycerol v/v) was added. One times PCV

of saturated DNase free ammonium sulfate was added drop by drop, while stirring, to lyse cell nuclei (Sugden and Keller 1973). Stirring was continued for 30 minutes after the last drop of ammonium sulfate was added. After stirring was completed, the solution was centrifuged at 20 000 rpm for 5 hours at 4 °C. The supernatant was decanted into a sterile conical tube and measured, and then transferred to a sterile flask where 0.33g of Sigma DNase free ammonium sulfate per 1.0 ml of supernatant was added while stirring to precipitate the protein. While stirring, 100 µl 1N NaOH per 10 g of ammonium sulfate was added. Stirring continued for 30 minutes after the last addition of crystal. This solution was then centrifuged at 10 000 rpm for 30 minutes at 4 °C. The resulting pellet was resuspended in 1/40th the measured supernatant volume of storage/dialysis buffer (25 mM Hepes, 100 mM KCl, 12 mM MgCl₂, 0.5 mM EDTA, 2 mM DTT and 16% glycerol v/v) and dialyzed overnight in tubing of mole weight 14 000. The dialysis flask was placed in an ice bucket and covered with foil to minimize ice melting.

The dialysate was centrifuged for 10 minutes at 10 000 rpm at 4 °C. The supernatant was loaded to a Sigma chromatographic column with Blue Sepharose CL-6B, equilibrated with 0.1 M KCL and Buffer B (50 mM Tris-HCl pH 7.4, 1 mM EDTA, 10 mM 2-mercaptoethanol and 20% v/v glycerol), and washed with 0.1 M KCL and Buffer B with 0.6 g/L ATP. Fractions began to be collected after approximately forty minutes of run time. Small (20µl) sub-samples of each 2.0 ml fraction was added to 200 µl Coomassie Blue stain and 780 µl distilled water in order to determine which fractions contained the most protein. These protein rich fractions were collected, pooled, and dialyzed for 4-6 hours against photolyase storage buffer (50 mM Tris pH 7.4, 50 mM NaCl, 1 mM EDTA, 10 mM DTT and 50% glycerol). The resulting dialysate was collected, and frozen at -20 °C until used in either the transformation assay, or the ESS assay. A portion of

the resulting dialysate was put aside at this point to perform a Bio-Rad assay for protein concentration.

3.1.5 Transformation assay

The transformation assay documented here is based upon Sancar *et al.* (1984), which was in turn based on Cohen *et al.* (1972), and Setlow (1968). Briefly, cell free extract (CFE) from each species (25 μ g) was added to 12 times CFE volume of 10X photolyase assay buffer (50 mM Tris pH 7.4, 50 mM NaCl, and 1 mM EDTA), 3X CFE volume of Tris-EDTA (TE, 10 mM Tris-Cl, 1 mM EDTA, pH 8.0) buffer, 3 μ g of pBR322 DNA and EDTA and EGTA salt solutions to a final concentration of 250 mM. pBR322 DNA was obtained from Sigma, and isolated using the BioRad Quantum Prep plasmid miniprep kit.

Pyrimidine dimers were formed in the DNA through exposure to a Photo Optic Halogen Bulb (1000W.120V.64743.Osram Corp.) at a distance of 10 cm for 60 or 90 seconds (3.3-4.0 MEDS/hr, 1 MED = 21 ± 3 mJ/cm², (Parrish *et al.* 1982) and therefore an experimental fluence range of 11.55 - 14.00 J/m²) in 16 mm polystyrene culture dishes at room temperature.

Replicates of this reaction mixture were placed either under photoreactivating light (+PRL = exposure to 3 GE 15W Black Lights (1.22mW/cm²) wrapped in Saran Wrap (to remove potentially damaging lower wavelengths)) at a distance of 10 cm for 90 minutes, or in the dark for 90 minutes (-PRL) at either 4, 15 or 20 °C (therefore approx. 6.588 J/m²). Preliminary tests indicated that this assay was linear with respect to time until 90 minutes. The reaction mixture volume varied between species in relation to the concentration of the CFE. Volumes ranged from 60 μ l to 600 μ l. Photolyase/NER deficient *Escherichia coli* CSR603, grown for three hours in LB broth and made competent through established methods (Sambrook *et al.* 1989), were added to the reaction mixture. The bacteria/plasmid mixture was cooled on ice for 60 minutes and

then heat shocked at 42 °C for 90 seconds. Two ml of LB with tetracycline (20 µg/ml tetracycline) was then added and the CSR603 was grown in a shaking incubator at 37 °C and 200 rpm for 1-2 hours to allow phenotypic plasmid expression. Bacteria were then spun at 10 000 rpm for 10 minutes and the supernatant was poured off. The bacterial pellet was resuspended in 50 µl of LB + Tet and plated on selective media (LB and 20 µg/ml tetracycline), the entire transformation mixture was spread on a single plate (Sambrook *et al.* 1989). The plates were then inverted and grown at 37 °C for 24-48 hours.

3:1.6 Transformation efficiencies

Resultant colonies from unirradiated plasmid (z), irradiated plasmid with no photorepair (-PRL) and irradiated plasmid with photorepair (+PRL) treatments were counted and specific transformation efficiencies for each treatment were calculated for colony forming units (CFU) per amount of DNA (CFU/µg DNA). Typically, specific transformation efficiencies for unirradiated plasmids ranged from 133 CFU per µg DNA to 832 CFU per µg DNA. For damaged and repaired plasmids, typical transformation efficiencies ranged from 0.33 CFU per µg DNA, to 10 CFU per µg DNA. This drastic reduction has been reported before by others using irradiated plasmids (Hays *et al.* 1990). Dimer repair was based on Sancar *et al.* (1984) and was calculated as difference between the relative transformation efficiency for the irradiated plasmid without photorepair, and the irradiated plasmid with photorepair.

$$\text{mean dimer number per plasmid molecule} = \{-\ln(-\text{PRL}/0) - (-\ln(+\text{PRL}/0))\} \quad (1)$$

These numbers were then standardized for time of exposure to photoreactivating light, and the ratio of protein concentration to DNA concentration. Two high and two low outlier values were excluded from each

treatment prior to analysis of each light and dark treatment, leaving 8 or 9 replicates per treatment. These were analyzed in a one-way ANOVA.

3.2 Results

Table 1 incorporates the photolyase activity of amphibian CFE determined through the bacterial transformation assay for CBPDs in exogenous plasmid DNA. Un-shaded columns relate directly to work completed in this chapter. Shaded columns refer to information important to the history of the tissue assayed here, but not directly related to the work in this chapter.

3.2.1 Transformation efficiency

Plasmid DNA containing dimers (as few as one per plasmid) results in a marked reduction in integration of transforming DNA (Notani and Goodgal 1965). Typically, specific transformation efficiencies for unirradiated plasmids ranged from 133 colony forming units (CFU) per microgram DNA to 832 CFU per μg DNA. For damaged and repaired plasmids, typical transformation efficiencies ranged from 0.33 CFU per μg DNA, to 10 CFU per μg DNA. Drastic transformation efficiency reduction of this type has been reported for other amphibians (Hays *et al.* 1990).

3.2.2 Induced photolyase activity of the wood frog

The dimer removal characteristics of the wood frog embryos maintained at 4 °C in the dark for 2, 4 or ten hours did not significantly differ from each other (Anova, $p=0.483$, $df=2$, $F=0.7662$). In contrast, the wood frog embryos exposed to 2, 4 or 10 hours of ambient sunlight differed significantly from each

Table 1: Bacterial transformation assay for cyclobutane pyrimidine dimers in exogenous DNA: Tissue history and repair estimation (DOC values from Lean *et al.* in press). See text for interpretation of shading.

Species	Time Period Collected	Location collected	Mean Depth (cm)	DOC (ppm)	De-jelly protocol (Appendix 1)	Cell type Anuran = Gosner Stg Salamander = Harrison stg.	UV-B fluence J/m ²	Jelly absorbance (relative arbitrary units)	In vitro E/S temp (°C)	Repair (dimers/3 µg DNA/25µg CFE/90 min PRL (stdev))
<i>Rana sylvatica</i>	04.29.97	Arch Pond	9.25	18.75	2% L-cysteine	11	23.75 at 10 hours	280=2.22 320=1.26	4	0.68 (0.4)
<i>Ambystoma laterale</i>	05.26.97	Kitty's Pond	14	17.6	2% L-cysteine	26	1.81	280=1.18 320=1.94	4	0.12 (0.16)
<i>Ambystoma maculatum</i>	05.26.97	Kitty's Pond	16.7	17.6	2% L-cysteine	16	1.81	280=1.58 320=2.97	20	0.49 (0.23)

other ($p=0.001$, $df=1$, $F=9.603$) (Figure 1).

This difference was compared to the total UVB fluence that each treatment had received (Data provided by Dr. Wayne Evans at Trent University) (Figure 2). A regression analysis of wood frog photolyase activity and total UVB fluence produced a significant r value (0.99862, $p<0.05$) (Figure 2).

Two species (blue spotted salamander, yellow spotted salamander) in addition to wood frogs, were experimentally exposed to known UVB fluence (BSS, YSS). Transformation efficiency data estimating the dimer removal characteristics of the CFE from these embryos was compared with the wood frog CFE exposed to two, four and ten hours of ambient light (Figure 3). A linear fit of wood frog transformation data, and the 95% confidence intervals of that linear fit suggested that the blue spotted salamander was not significantly different from the wood frog pattern. The yellow spotted salamander likely was different from the wood frog pattern.

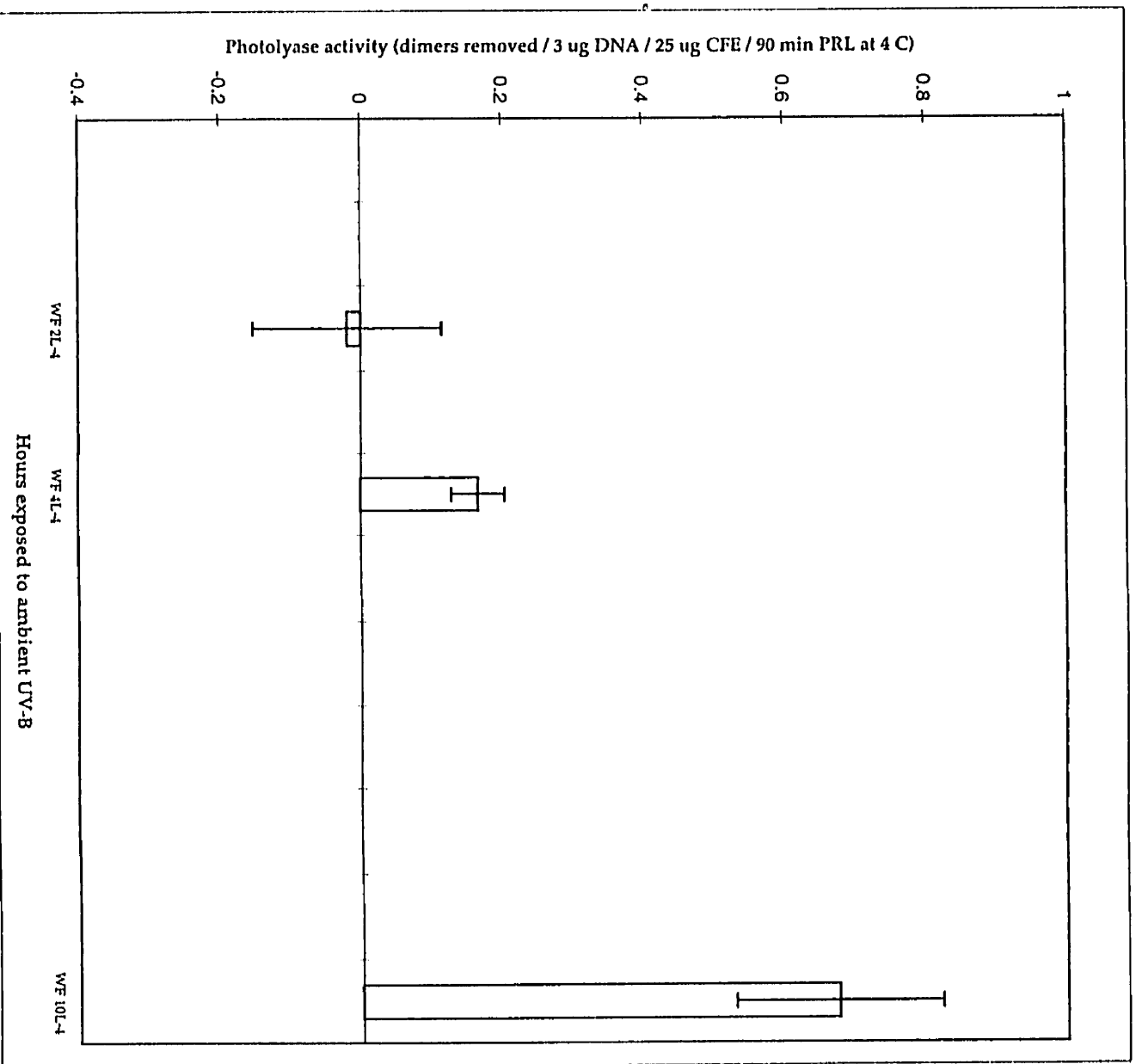


Figure 1: Change in photolyase activity characteristics of Wood Frog (*Rana sylvatica*) cell free extract (CFE) as exposed to increasing ambient UVB fluence (April 29 1997, Peterborough Ontario, 44° 21'N, 78° 17'W, 100 m elevation). The means of the three treatments are significantly different. ($F=9.6$, $p<0.001$). Standard error bars are shown.

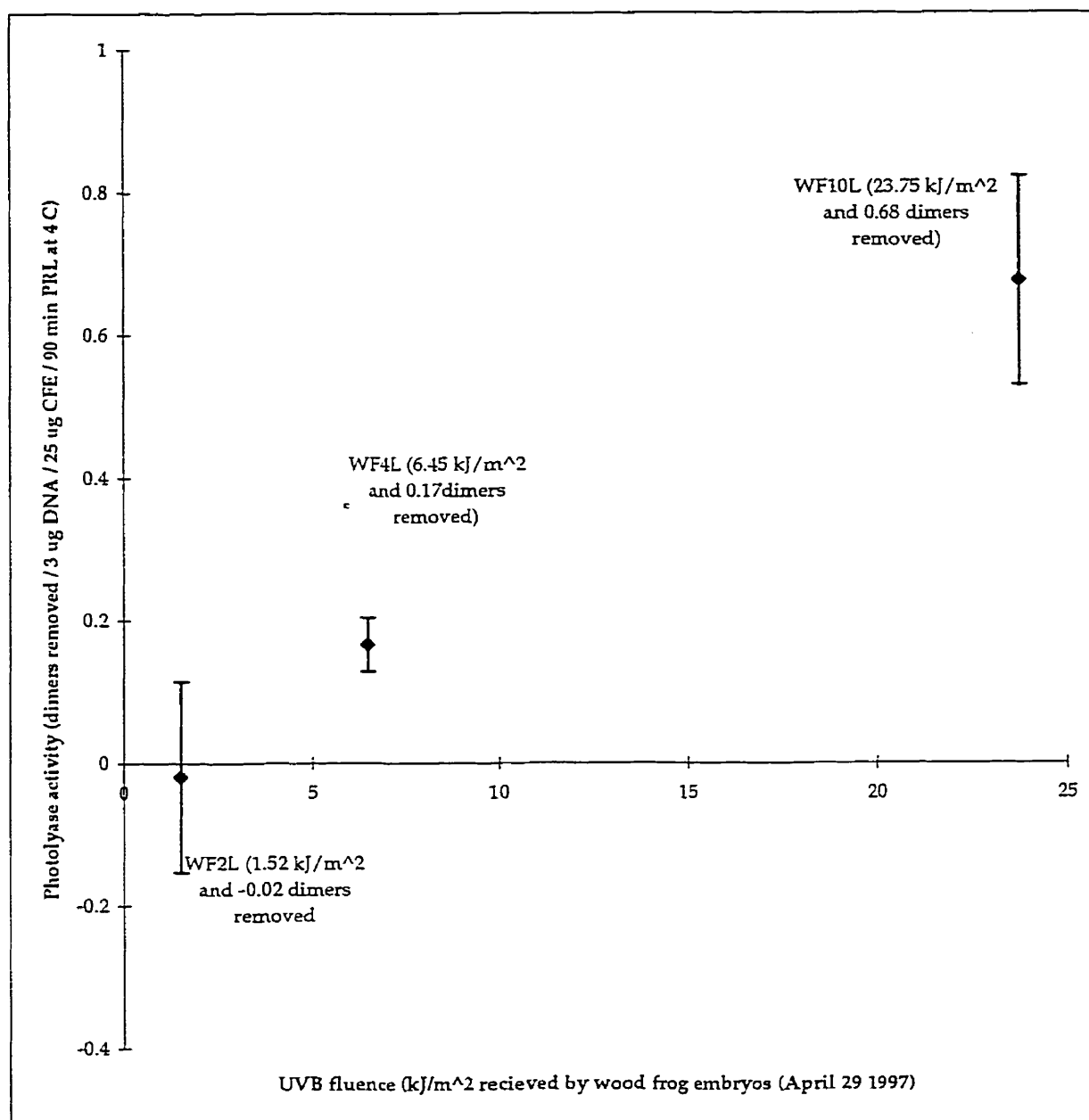


Figure 2:

Relationship between the fluence received by wood frog embryos exposed to ambient UVB radiation in Peterborough ON, Canada (44° 21'N, 78°17'W, 100 m elevation, April 29 1997) and their photolyase activity. There is a positive correlation (Pearson correlation coefficient $r=0.99862$, $p<0.05$). Standard error bars are shown.

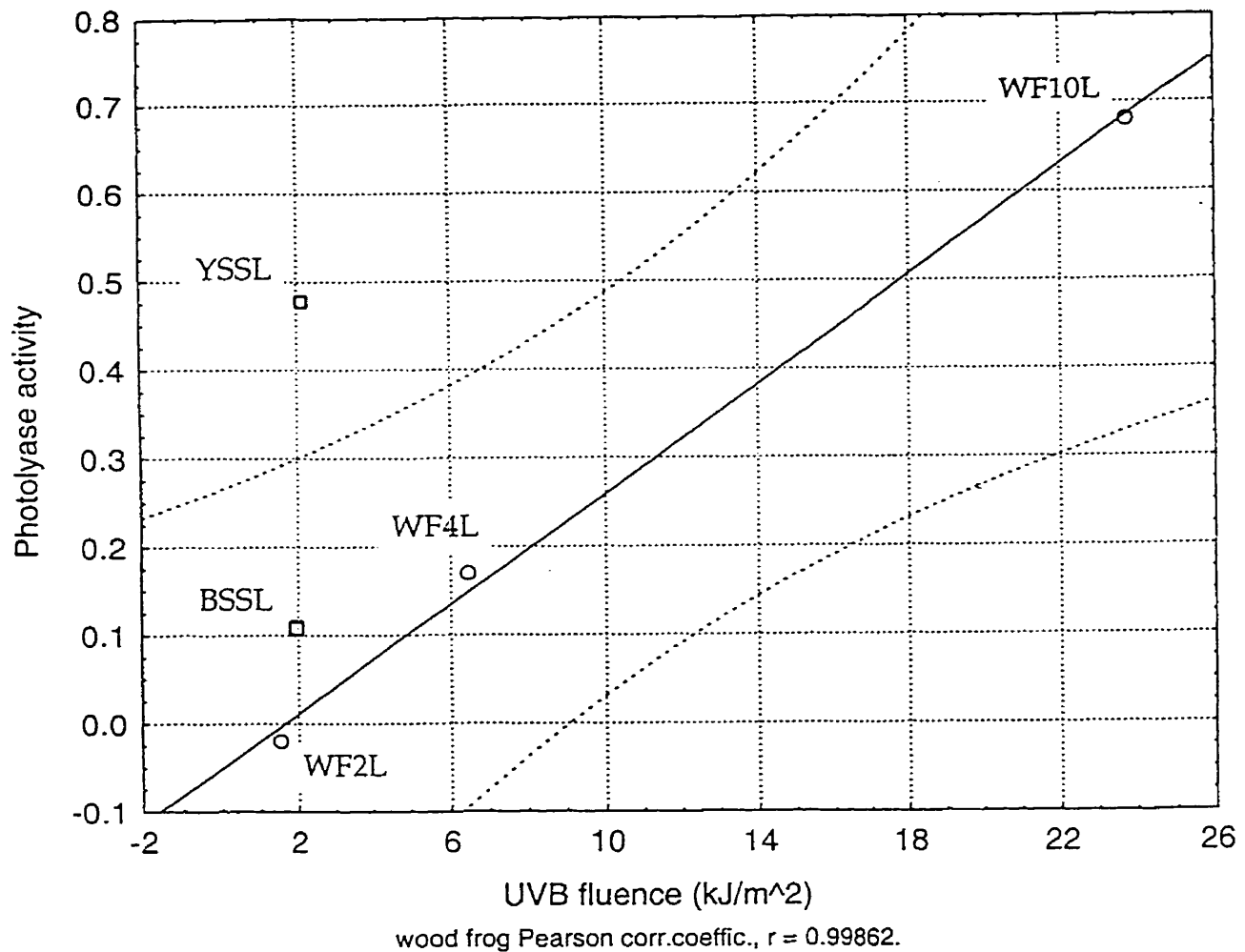


Figure 3:

The proposed use of wood frog induction data to predict between-species differences in the context of ambient UVB fluence. Photolyase activity of different species as a function of exposure to ambient UVB fluence. Yellow spotted salamander (YSS), blue spotted salamander (BSS), wood frog (WF). Relationship is compared through:

- plotting a straight line through WF induction data (Figure 2)
- plotting the 95% confidence intervals of that line
- and overlaying the plots of BSS, and YSS data. Those that fall outside the confidence limits (YSS), are significantly different from projected WF patterns of induction. Those that do not (BSS) likely are not different from the wood frog pattern.

3.3 Discussion

The efforts reported here to document ambient radiation induced changes in photolyase activity in wood frog embryos utilized crude cell free extracts, (made necessary due to the low cellular concentrations of photolyase), but yielded significant differences between ambient UV treatments of varying lengths (Figure 1) and an interesting correlation between total UVB fluence and photolyase activity (Figure 2). Such a result is important to future studies on photolyase activity of animals from natural populations. The results reported here imply that to properly estimate the photoreactivating characteristics of an animal, the researcher must estimate/measure the amount of ambient radiation that the animal has been exposed to prior to collection. The researcher must also be responsible for reporting the treatment of the tissue/animal upon collection (for example, Carlini and Regan 1995, p. 222, "*The live slugs were frozen upon arrival to the biological laboratories of the Florida Institute of Technology.*"). Specifically for amphibians, one needs to report whether embryos are maintained indoors or outdoors, under natural or florescent lights and under what temperature regime prior to freezing.

3.3.1 History of photolyase induction research in the literature

Sebastian *et al.* (1990) demonstrated that the transcription of the gene (*PHR1*) which encodes the apoprotein for yeast photolyase can be induced by exposure to UV radiation. Transcription is mediated here by a damage-responsive DNA binding protein and an upstream repression sequence (Sebastian and Sancar 1991). Sub-lethal irradiations of DNA by UV radiation have been shown to induce photorepair in fish cells (Mitani and Shima 1995). Indeed published reports dating back to 1987 have documented induced changes in photolyase concentration of amphibian cells (Chao and Lin-Chao 1987a). Despite this and more recent reports (Mitani and Shima 1995, Mitani *et al.* 1996),

studies on the photoreactivating activity of other organisms from natural populations (Blaustein *et al.* 1994, Carlini and Regan 1995, vandeMortel *et al.* 1998) have not correlated, or reported, the ambient levels of UVB radiation upon the time of collection. Furthermore, photolyase concentrations have been shown, *in vivo*, to change in cells grown at different temperatures (Fukui and Laskowski 1984). Yeast cells grown at 30 °C had photolyase concentrations that were only 13% of the same cell cultures grown at 23 °C (Fukui and Laskowski 1984). The experimental design of my study (each treatment maintained at a constant temperature) prevented the comparison of any such temperature-driven changes in concentration, but the observation raises interesting questions regarding the potential interactions of ambient water temperature, ambient ultraviolet radiation and photolyase concentration in wild populations of amphibians. Recently, van de Mortel *et al.* (1998) correctly acknowledged that differing water treatments could have altered photolyase concentrations between treatments. "Water temperature at the time of the field experiments may have affected the *in vivo* activity of photolyase." (van de Mortel *et al.* 1998, pp. 368). The van de Mortel report, and the work reported here should necessitate acknowledgement of the fact that environmental conditions (such as temperature, hydrogen peroxide and ambient UVR) experienced by amphibian embryos, *in vivo*, will significantly affect the *in vitro* estimation of photolyase activity. When the defining protocol quantifies photolyase activity via an *in vitro* assay it is crucial to remember the *in vivo* conditions.

It is also important to determine whether between treatment differences are important to a field study and a lab study of photorepair, or whether differences treated as non-significant in the field may actually be quite significant in the lab. For instance in a field study where different amphibian species are arranged in containers in the same water body, at the same time, the following

observation is quite legitimate, “When it was sunny, all treatments within a species at one lake were exposed to equivalent amounts of sunlight. When it was cloudy, all treatments were under equivalent cloud cover.” (Blaustein *et al.* 1996a p. 387, made in response to Licht 1996, regarding Blaustein *et al.* 1994). If those same embryos were utilised for *in vitro* determination of photolyase concentrations the authors should report the conditions upon collection, perhaps the total fluence received on the day of collection. It is understood that photolyase concentrations can be induced, but it is not known whether the rate of that induction differs between species, or if there are different thresholds which must be exceeded before the photolyase apoprotein is transcribed. If there are between-species differences in photolyase induction rates or threshold, then the observation listed above (Blaustein *et al.* 1996) is an integral portion of environmental variability in field experiments, but it is also a complicating variable for *in vitro* determinations of photolyase.

3.3.2 Factors which can't be discounted as possible agents of photolyase induction

UV radiation produces hydrogen peroxides when the radiation travels through humic water high in DOC (Cooper *et al.* 1994). Hydrogen peroxide can cause oxidative stress, resulting in cell lysis (Goodar *et al.* 1993), and the induction of photolyase production (Mitani and Shima 1995). Such reactions might have occurred in the plastic containers containing the wood frog embryos while they were exposed to ambient UV radiation. Therefore, I cannot discount the possibility that the changes induced in wood frog photolyase activity were actually induced by photo-induced oxidative stress. This possibility is tempered somewhat by the fact that the water surrounding the embryos was purified river water (Otonabee River). The water was low in DOC and therefore would not likely have had high oxidative production characteristics (Lean *et al.* submitted,

Scully and Lean 1994). These types of confounding factors represent indirect UVB stress to the amphibian embryo. In a natural environment, some of the intracellular effects of UV are likely oxidative, not simply CBPDs.

I cannot exclude the possibility that my experimental exposure to sunlight also induced the excision repair system. It has been speculated (Mitani and Shima 1995) that sunlight might control both enzymatic systems simultaneously. The process of cell free extract production was intended to reduce the presence of complicating enzymatic systems, but DNA repair enzymes are often of approximately the same size (A. Sancar pers. comm.), and thus, it is conceivable that the cell free extract might have contained the enzyme complex of gene products necessary for excision repair of the experimentally induced dimers in the plasmid DNA. Photolyase has also been shown to stimulate the *uvrABC* excision repair system *in vitro* (Sancar *et al.* 1984a). Another factor that might have complicated the estimation of photolyase was the fluence with which the plasmid DNA was irradiated here. Previous work has reported that "excessive" amounts of unirradiated DNA prevents the formation of the enzyme-substrate complex between photolyase and the damaged DNA (Fukui *et al.* 1981). I am prepared to discount this possibility as the fluence the plasmids were irradiated with is an expected fluence at the surface of pond water at mid-day during the summer months in the area where this study was undertaken (Berrill *et al.* in prep).

As my experimental exposure period was only for ten hours, or roughly one day-length, my data does not address what happens to photolyase activity levels during the night. I would speculate that they would decline, as the embryos maintained in the dark had very low photolyase activities. The rate of such a decline is unknown.

3.3.3 Mechanism of induction

The transcriptional control of the photolyase gene is an integral part of the *in vivo* induction of amphibian photolyase. Without further data, I speculate that transcriptional control of the photolyase gene in these wood frog embryos is responsible for the induced changes in photolyase concentration. Transcription of the photolyase gene (*PHR1*) is regulated by environmental factors in many species (Mitani and Shima 1995). It has been shown that DNA damaging agents appear to cause the release of a suppressor protein that is otherwise bound to the repression sequence upstream (URS) of the *PHR1* gene (Sebastian and Sancar 1991). Upon the release of this controlling factor, termed the photolyase regulatory protein (PRP, Sebastian and Sancar 1991), the gene is transcribed, and an increased photolyase copy number results. In the system reported here UVB, UV-A or hydrogen peroxide all might act as the environmental factor which stimulates the release of the photolyase regulatory-protein. *"At present the specific steps involved in the DNA damage signal transduction pathway are entirely unknown."* (Sebastian and Sancar 1991. p 11255.)

3.3.4 Importance of results demonstrated here

Between-species comparisons of photoreactivation characteristics are important, yet must be couched in terms of the environmental factors which regulate the cellular photolyase production. The lack of a significant relationship between total UVB fluence and photolyase activity characteristic of a species, demonstrated in Figure 3, is an important relationship. It suggests that when the total fluence an amphibian embryo receives is measured, the differences in photolyase activity can still be reflected in between-species variability. In other words, a between-species comparison of photorepair abilities should still be possible to make when that comparison accounts for environmental variables

which would alter photolyase concentration (such as ultraviolet radiation) and variables which would alter the amount of ultraviolet radiation received by an embryo. These variables are numerous and include the UVB absorbance of the embryo jelly and the UVB fluence on a particular day received by an embryo. This fluence is in turn determined by a myriad of factors including latitude, elevation, cloud cover, depth of oviposition and DOC concentration of the water. Accurate estimations of photolyase activity for a species from a natural population is a complicated process, and in the future should include attempts at quantifying the rate of induction by ambient environmental stresses.

The results reported here provide evidence of the adaptive induction of a photorepair system in an animal collected from its natural environment. The photorepair system of the wood frog was induced, either directly or indirectly, by exposure to ambient levels of late spring sunlight in south-central Ontario. This is good evidence of an adaptive response to an existing environmental stress. It is of future conservation importance as ultraviolet-B irradiance is an environmental stress that is expected to increase in severity over the next fifty years, and this may result in the reduction of DOC in areas with great UVB irradiance, thus removing a naturally occurring UVB block.

Induced changes in photorepair ability might be non-adaptive in their phylogenetic occurrence (Sancar and Sancar 1988), but appear to be adaptive (Mitani and Shima 1995) *in vivo* where they occur. Ultraviolet radiation triggers the production of the enzyme responsible for repair of DNA damaged by other wavelengths of ultraviolet radiation, and the catalysis of the initial UVB damage is dependant on longer wavelengths of ultraviolet radiation. Results from examinations of Antarctic zooplankton have indicated that species accumulate significant numbers of CBPDs throughout exposure time to UVB (Malloy *et al.* 1997). If the system is adaptive, then enzymatic methods of CBPD removal

should also accumulate throughout a period of UVB exposure. Such changes in photorepair ability have been demonstrated here *in vitro* with wood frog embryos exposed *in vivo* to different amounts of ambient ultraviolet radiation.

Chapter 4

4.0 A general discussion of amphibian photorepair

The objectives of this study were to determine whether non-radioactive methods utilizing exogenous plasmid DNA could be used in an *in vitro* quantification of photolyase activity for amphibian embryos native to the humic waters of south-central Ontario. Previous studies have investigated the photolyase activity of amphibian embryos at high latitudes, in water low in DOC, using radioactive methods of photolyase activity determination. Few studies have attempted to quantify the photolyase activity of species native to low elevation, humic water. This work is the first to attempt to utilize exogenous, non-radioactive DNA sources to determine photolyase activity in species from natural populations. In addition, this work has clearly demonstrated that *in vivo* conditions can induce changes in an *in vitro* determination of photolyase activity.

Investigating photolyase activity is important, even in species native to humic waters at low elevations where current levels of UVB might not be limiting populations. Current levels of UVB are expected to increase at this latitude, and the resulting direct and indirect photochemical effects could increase the total fluence received by amphibians in this area.

4.1 Comparing photoenzymatic repair and UVB jelly absorbance of several amphibian species from south-central Ontario

It is important to understand the role current levels of UVB plays in population and ecosystem level regulation in aquatic ecosystems. In order to accurately predict what effects increasing UVB fluence might elicit, efforts at quantifying an organism's UVB damage repair ability are the focus of this and other studies. One way to estimate this characteristic is to estimate the

concentration of the photoenzymatic repair enzyme, photolyase. Estimating the photolyase activity of a species does give an indication of how well that species is dealing with current levels of ambient UVB, but that picture is at best incomplete. True estimations of how well that species is likely to deal with potential UVB increases in the future must address alternative methods of repair, and frame photolyase activity estimations in a context that more correctly accounts for *in vivo* stressing agents.

I utilised a bacterial transformation assay to estimate the photolyase activity for oocytes, embryos and skin from seven species of amphibian from south-central Ontario. Photolyase activity was indirectly measured as the rate of CPBD removal from UVB damaged plasmid DNA by cell free extracts (CFE) created from the tissue of the species in question. *In vitro* photolyase activity measurements for the seven species measured were not adaptively correlated with *in vivo* exposure to sunlight. Species which oviposit close to the surface, such as *Hyla versicolor* and *Pseudacris triseriata* displayed low photolyase activity and low jelly absorbance. *Rana clamitans*, also a near surface ovipositor, displayed high photolyase activity and high jelly absorbance. Species where females oviposit deep under the waters surface displayed both medium-high photolyase activity (*Ambystoma maculatum* and *Rana sylvatica*) and low photolyase (*Rana pipiens* and *Ambystoma laterale*).

4.2 *In vivo* induced photolyase activity of the cell free extract from wood frog (*Rana sylvatica*) embryos.

Photolyase cellular concentrations are inducible in eukaryotic cells by such environmental factors as ultraviolet-B radiation, ultraviolet-A radiation, temperature, hydrogen peroxide and growth inhibition. These *in vivo* variables have not been addressed in previous *in vitro* examinations of animals from natural populations. Inducible changes in photolyase activity for the wood frog

were demonstrated here and previous conclusions regarding dimer removal characteristics of certain amphibian species require reevaluation in the context of how much UV radiation the egg masses had been exposed to prior to collection.

4.3 The importance of *in vitro* methods of determining photolyase activity in amphibian tissue

Ultraviolet radiation is effectively attenuated within the first 5-10 cm of the water (Berrill *et al.* in prep) column in the ephemeral ponds where most of the species examined lay their eggs. I therefore predicted inter-specific differences in the ability to repair UV damage that were correlated with the principal egg laying location in the water column. An appealing adaptive hypothesis is that species with egg deposition sites on the surface of the water would have greater ability to repair CBPDs than species which oviposit underneath the water surface. "*Levels of photolyase generally correlated with expected natural exposure of eggs to sunlight.*" (Blaustein *et al.* 1995a, pp.740). Estimates of photolyase activity for these species do not support this hypothesis. Deep water ovipositors exhibited both high and low photolyase activity. Shallow water ovipositors also exhibited a range of photolyase activities. Results reported here indicate large between-species differences in jelly absorbance, and therefore by inference, large between species differences in the damage caused by UVB radiation. These types of environmental and embryonic differences must be factored into any equation which ranks species by their ability to repair UVB damage. A species with low repair abilities and high jelly absorbance is not experiencing as dire circumstances as a low UV repair species with low jelly absorbance. It is crucial that we are able to differentiate these two scenarios.

4.4 The importance of microbiological and ecosystem studies on the role of UVB in temperate, mid-latitude humic waters.

Untangling the multiple, synergistic and interfering effects of UV radiation (UVR) on aquatic life is a complicated, and often unrewarding task. Although this study is ecological on a molecular base, it does not identify, nor include, any ecosystem level effects of UV radiation on crucial processes such as trophic level interactions, primary production, nutrient cycling or changes in community structure. Important ecosystem level processes have been shown to be affected by current UVR (Bothwell *et al.* 1994, Booth *et al.* 1997), where the balance between primary producers and consumers was significantly altered by excluding ambient levels of UVB radiation. Other trophic level interactions have been demonstrated, including reductions in marine recruitment, and therefore the transfer of productivity in zooplankton (Malloy *et al.* 1997) and bacterioplankton (Jeffrey *et al.* 1996). UVB exposure reduces nitrogen-fixation in *Anabaena flos-aquae* (Newton *et al.* 1979) and can alter primary production (Boucher and Prezelin 1996).

These types of trophic level effects might drastically affect amphibian populations. Each of these microscopic taxa are involved in nutrient cycling or other forms of community management, and will be more important to monitor than higher level consumers. Anuran larval forms often play an important role in community determination of primary producers and higher level heterotrophs, and in nutrient cycling in eutrophic bodies of water (Wassersug 1975). Therefore, while more attention should be paid to the effects of UVB on the microbiology of temperate freshwater, the attention paid to amphibians should not waver.

4.5 Future research

Currently none of the species tested here is experiencing population declines, above the local level, throughout south-central Ontario (Weller and Green 1997, Hecnar and M'Closkey 1996). The spring-breeding species represented in this study might currently be receiving UVB fluences which are greater than those experienced historically (Kerr and McElroy 1993, but see Michaels *et al.* 1994). Spring breeding amphibians also develop in colder water and since these reduced temperatures lead to reduced developmental rates it may result in a larger UVB exposure complement at time of hatching. There is also an implied likelihood of greater photo-oxidation of DOC in humic water sources. Spring breeding amphibians, especially those within 5 centimeters of the surface represent those species most likely to be experiencing largest direct and indirect effects of increased UVB fluence. Species which this work has identified as particularly deserving of future study are the gray tree frog and the chorus frog. More of the small egg masses of these species must be located, and included in future analyses of embryonic vulnerability to UVB damage.

Species that oviposit in the spring, when temperate latitudes may be experiencing UVB pulses, are also the species which might be most vulnerable to future water table changes related to global warming. If there is an increased rate of pond drying or reduced depth in ephemeral water during the spring, the species that we have coded as deep water ovipositors would be thrust to the UVB attenuating and photochemically damaging surface waters. In such a scenario, the low photolyase levels of deep water species such as the leopard frog would be a conservation concern.

One important future consideration for aquatic UV biology in Ontario involves the reductions of DOC associated with areas of reduced pH (Schindler *et al.* 1996). Where DOC is reduced, UVB photons reach greater depths before

being effectively attenuated (Schindler *et al.* 1996). Animals, including amphibians, adapted to oviposition depths where UVB is attenuated would then receive an increased UVB fluence. This could result in direct UVB damage or an increased range of the water column where the synergistic effects related to UVB and pH could occur. Synergistic interactions between UVB stress and the secondary stress of pathogens such as the fungus *Saprolegnia ferax* (Kiesecker and Blaustein 1995b), and pH (Long *et al.* 1995) have been documented. Future research should determine whether such pH mediated changes in DOC actually result in measurable damage to amphibian DNA in greater water depths.

4.6 Summary of results

Photolyase activity of cell free extract made from amphibian embryonic tissue displayed the following trends.

- 1) There are significant between-species differences in photolyase activity and these differences are not correlated with expected natural UVB exposure.

- 2) There were no significant differences in photolyase activities where more than one developmental stage was available for the gray tree frog and the blue spotted salamander.

- 3) There were significant between-species differences in jelly mass absorbance of UVB radiation at 280 and 320 nm. These differences were positively correlated with photolyase activity at 280 nm and not significantly correlated at 320 nm. Interestingly, dimers are formed with great efficiency at 280 nm, but current levels of stratospheric ozone effectively eliminate this wavelength from the compliment that reaches the earth.

- 4) There is a positive relationship between photolyase activity and ambient UVB fluence for the wood frog. Known UVB exposures for yellow and blue spotted salamander embryos were incorporated into a linear fit plot of the

wood frog induction data with 95% confidence intervals. The placement of these data points either inside (BSS), or outside (YSS) the confidence limits gave a preliminary estimate of between-species differences in photolyase activity that accounted for potentially induced photolyase activities. Thus between-species comparisons of photolyase activity remain an important photobiological and conservation tool.

Future ecological estimations of an organism's photoenzymatic activity need to acknowledge the differences this and other studies have elucidated. *In vivo* conditions can alter an *in vitro* estimation of photolyase activity. Such environmental conditions include UVA, UVB, temperature, pH, DOC and hydrogen peroxide. These interactions of these conditions can have synergistic and inhibitory effects on the UVB damage repair ability characterized in an *in vitro* assay and therefore must be accounted for. Between-species differences in endonucleases damaging to exogenous DNA substrates need to be quantified (See Appendix 1). The results of such a quantification should reflect what assay type is chosen for the species in question.

Amphibians populations around the world are declining. Understanding an environmental stress that is as ubiquitous and damaging as ultraviolet radiation is crucial to monitoring populations in decline in areas which don't appear to have undergone significant anthropogenic change. Dependable determinations of the ability to repair UVB damage to DNA remain an important factor in this process.

Literature Cited

- Anzalone C. R., L. B. Kats, and M. S. Gordon. 1998. Effects of solar UV-B radiation on embryonic development in *Hyla cadaverina*, *Hyla regilla* and *Taricha torosa*. *Conservation Biology*. 12: 646-653.
- Berrill M., and D. R. S. Lean. 1998. Problems in conducting *in-situ* experiments to evaluate the influence of UV-B radiation on amphibian eggs and larvae. *Environmental Toxicology and Risk Assessment*. 1333: 21-30.
- Berrill M., and D. Coulson, D. Crump, D. R. S. Lean, L. McGillivray, and M. A. Smith. Sensitivity of 8 species of amphibian embryos, tadpoles and larvae to enhanced UV-B radiation in natural pond conditions in Southern Ontario. *Canadian Journal of Zoology*. (in prep)
- Berven K. A. 1990. Factors affecting population fluctuations in larval and adult stages of the Wood Frog (*Rana sylvatica*). *Ecology* 71: 1599-1608.
- Blakefield M. K., and D. O. Harris. 1994. Delay of cell differentiation in *Anabaena aequalis* caused by UV-B radiation and the role of photoreactivation and excision repair. *Photochemistry and Photobiology*. 59: 204-208.
- Blaustein A. R. 1994. Chicken little or Nero's fiddle? A perspective on declining amphibian populations. *Herpetologica*. 50: 85-97.
- Blaustein A. R., and D. B. Wake. 1990. Declining amphibian populations: A global phenomenon? *Trends in Ecology and Evolution*. 5: 203-204.
- Blaustein A. R., and D. B. Wake. 1995. The puzzle of declining amphibian populations. *Scientific American*. 252: 52-57.
- Blaustein A. R., P. D. Hoffman, D. G. Hokit, J. M. Kiesecker, S. C. Walls, and J. B. Hays. 1994a. UV repair and resistance to solar UV-B in amphibian eggs: A link to population declines? *Proceedings of the National Academy of Sciences*. 91: 1791-1795.
- Blaustein A. R., R. Edmond, J. M. Kiesecker, J. J. Beatty, and D. G. Hokit. 1995a. Ambient ultraviolet radiation causes mortality in salamander eggs. *Ecological Applications*. 5: 740-743.
- Blaustein A. R., J. M. Kiesecker, and D. G. Hokit, and S. C. Walls. 1995b. Amphibian Declines and UV radiation. *BioScience* 45: 514-515.

- Blaustein A. R., J.M. Kiesecker, and D. G. Hokit, and S. C. Walls. 1996a. Field experiments, amphibian mortality and UV radiation. *BioScience*. 46: 386-388
- Blaustein A. R., P. D. Hoffman, J. M. Kiesecker, and J. B. Hays. 1996b. DNA repair activity and resistance to solar UV-B radiation in eggs of the Red-Legged Frog. *Conservation Biology*. 10: 1398-1403.
- Blumthaler M., and W. Ambach. 1990. Indication of increasing solar ultraviolet-B radiation flux in alpine regions. *Science*. 248: 206-208.
- Booth C. R., J. H. Morrow, T. P. Coohill, J. J. Cullen, J. E. Frederick, D. P. Hader, O. Helm-Hansen, W. H. Jeffrey, D. L. Mitchell, P. J. Neale, I. Sobolev, J. VanderLeun, and R. C. Worrest. 1997. Impacts of solar UVR on aquatic microorganisms. *Photochemistry and Photobiology*. 65: 252-269.
- Bothwell M. L., D. M. Sherbot, and C. M. Pollock. 1994. Ecosystem response to solar ultraviolet-B radiation: Influences of trophic level interactions. *Science*. 265: 97-100.
- Boucher N. P., B. B. Prezelin 1996. Spectral modeling of UV inhibition of *in situ* Antarctic primary production using a field-derived biological weighting function. *Photochemistry and Photobiology*. 64: 407-418.
- Bruggeman D. J., J. A. Bantle, and C. Goad. 1998. Linking teratogenesis, growth, and DNA photodamage to artificial ultraviolet B radiation in *Xenopus laevis* larvae. *Environmental Toxicology and Chemistry*. 17: 2114-2121.
- Caldwell M. M., and S. D. Flint. 1994. Stratospheric ozone reduction, solar UV-B radiation and terrestrial ecosystems. *Climatic Change*. 28: 375-394.
- Carlini D. B., and J. D. Regan. 1995. Photolyase activities of *Elysia tuca*, *Busatell leachii* and *Haminaea antillarum* (Mollusca: Opisthobranchia). *Journal of Experimental Marine Biology and Ecology*. 189: 219-232.
- Chao C. C-K., and S. Lin-Chao. 1987a. Loss of inducible photorepair in a frog cell line hypersensitive to solar UV light. *FEBS*. 225: 133-138.
- Chao C. C-K., and S. Lin-Chao. 1987b. Regulation of photorepair in growing and arrested frog cells in response to ultraviolet light. *Mutation Research*. 192: 211-216.
- Cleaver J. E. 1983. Restriction enzyme cleavage of ultraviolet-damaged simian

- virus 40 and pBR322 DNA. *Journal of Molecular Biology* 170: 305-317.
- Cooper, W. J., C. Shao, D. R. S. Lean, A. S. Gordon, and R. E. Scully, Jr. 1994. Factors affecting the distribution of H_2O_2 in surface waters. In: Baker, L. A. (ed.): *Environmental Chemistry of Lakes and Reservoirs*. Adv. Chem Ser. 237. American Chemical Society. pp. 393-422
- Corn P. S. 1998. Effects of ultraviolet radiation on boreal toads in Colorado. *Ecological Applications*. 8: 18-26.
- Connman I. and N. Grier. 1941. Refraction of light by amphibian egg jelly. *Copeia*. 1941: 180.
- Denis H. 1974. Nucleic acid synthesis during oogenesis and early embryonic development of the amphibians. in J. Paul ed. *Biochemistry of cell differentiation* University Park Press. London.
- Duellman W. E., and L. Trueb. 1992. *Biology of Amphibians*. 2nd edition. McGraw-Hill. New York.
- Dutta K., W. S. Hejmadi, and N. C. Verma. 1993. An assay for DNA photolyases using non-radioactive DNA and restriction enzymes. *Journal of Photochemistry and Photobiology. B: Biology*. 18: 211-214.
- Farman J. C., B. G. Gardiner, and J. D. Shanklin. 1985. Large losses of total ozone in Antarctica reveal seasonal ClO_x/NO_x interaction. *Nature* 315: 207-210.
- Freeman F. B. 1968. A study of the jelly envelopes surrounding the egg of the amphibian *Xenopus laevis*. *Biological Bulletin*. 135: 501-513.
- Gleason J. F. P. K. Bhartia, J. R. Herman, R. McPeters, P. Newman, R. S. Stolarki, L. Flynn, G. Labow, D. Larko, C. Seftor, C. Wellemeyer, W. D. Kohmyr, A. J. Miller, and W. Planet. 1993. Record low global ozone in 1992. *Science*. 260: 523-526.
- Goin O. B., C. J. Goin, and K. Bachmann. 1968. DNA and amphibian life history. *Copeia*. 532-540.
- Gosner K. L. 1960. A simplified table for staging anuran embryos and larvae with notes on identification. *Herpetologica*. 16: 183-190.
- Grant K. P, and L. Licht. 1995. Effects of ultraviolet radiation on life-history stages of anurans from Ontario, Canada. *Canadian Journal of Zoology*.

73: 2292-2301.

- Hader D. P., R. C. Worrest, H. D. Kumar, and R. C. Smith. 1995. Effects of increased solar ultraviolet radiation on aquatic ecosystems. *Ambio* 24: 174-180.
- Hall K, and L. L. Larcom. 1982. Blockage of restriction endonucleases cleavage by thymine dimers. *Photochemistry and Photobiology* 36: 429-432
- Harrison R. G. 1969. Harrison stages and description of the normal development of the spotted salamander, *Ambystoma punctatum* (Linn). in R. G. Harrison (ed.), *Organization and Development of the Embryo*. New Haven. Yale University Press.
- Hart R. W., R. B. Setlow, A. D. Woodhead. 1977. Evidence that pyrimidine dimers in DNA can give rise to tumors. *Proceedings in the National Academy of Science*. 74: 5574-5578
- Hays J. B., E. J. Ackerman, and Q. Pang. 1990. Rapid and apparently error-prone excision repair of nonreplicating UV-irradiated plasmids in *Xenopus laevis* oocytes. *Molecular and Cellular Biology*. 10: 3505-3511.
- Hays J. B., A. R. Blaustein, J. M. Kiesecker, P. D. Hoffman, I. Pandelova, D. Coyle, and T. Richardson. 1996. Developmental responses of amphibians to solar and artificial UVB sources: A comparative Study. *Photochemistry and Photobiology*. 64: 449-456.
- Hearst J. E. 1995. The structure of photolyase: using photon energy for DNA repair. *Science*. 268: 1858-1859
- Hecnar S. J., M'Closkey, R. T. 1996. Regional dynamics and the status of amphibians. *Ecology*. 77: 2091-2097
- Hecnar, S. J., M'Closkey, R. T. 1997. Spatial scale and determination of species status of the Green Frog. *Conservation Biology* 11: 670-682.
- Higgins G. H., and C. Sheard. 1926. Effects of ultraviolet radiation in the early larval development of *Rana pipiens*. *Journal of Experimental Zoology*. 46: 333-343.
- Holmes K. J., and Ellis H. 1997. Simulation of halocarbon production and emissions and effects on ozone depletion. *Environmental Managment*. 21: 669-685.

- Husain I. J. Griffith, and A. Sancar. 1988. Thymine dimers bend DNA. *Proceedings of the National Academy of Sciences*. 85: 2558-2562.
- Jen J., D. L. Mitchell, R. P. Cunningham, C. A. Smith, J.-S. Taylor, and J. E. Cleaver. 1997. Ultraviolet irradiation produces novel endonuclease III-sensitive cytosine photoproducts at dipyrimidine sites. *Photochemistry and Photobiology*. 65: 323-329.
- Jeffery H. W., P. Aas, M. Maille-Lyons, R. B. Coffin, R. J. Pledger, and D. L. Mitchell. 1996. Ambient solar radiation-induced photodamage in marine bacterioplankton. *Photochemistry and Photobiology*. 64: 419-427.
- Kerr J. B., and C. T. McElroy. 1993. Evidence for large upward trends of Ultraviolet-B radiation linked to ozone depletion. *Science*. 262: 1032-1034
- Kerr J. B., and C. T. McElroy. 1994. Analyzing Ultraviolet-B radiation: Is there a trend? *Science* 264: 1341-1342
- Keszenman-Pereyra D. 1990. Repair of UV-damaged incoming plasmid DNA in *Saccharomyces cerevisiae*. *Photochemistry and Photobiology* 51: 331-342.
- Kiesecker J. M., and A. R. Blaustein. 1995. Synergism between UV-B radiation and a pathogen magnifies amphibian embryo mortality in nature. *Proceedings of the National Academy of Sciences*. 92: 11049-11052.
- Kiesecker J. M., and A. R. Blaustein. 1997. Influences of egg laying behaviour on pathogenic infection of amphibian eggs. *Conservation Biology*. 11: 214-220.
- Kim S-T., K. Malhotra, C. A. Smith, J-S. Taylor, and A. Sancar. 1994. Characterization of (6-4) photoproduct DNA photolyase. *Journal of Biological Chemistry*. 269: 8535-8540
- Lean D. R. S., W. J. Cooper, and F. R. Pick. 1994. Hydrogen peroxide formation and decay in lake waters. In: Helz G. R., Zepp R. g., and Crosby D. G.: *Aquatic and surface photochemistry*. CRC Press Inc. pp 207-214.
- Lean D. R. S., D., Coulson, D., Crump, M., Berrill, and L., Toy. Spectral irradiance in pond water; influence of water chemistry. In preparation.
- Legerski R. J., J. E. Penkala, C. A. Peterson, and D. A Wright. 1987. Repair of UV-induced lesions in *Xenopus laevis* oocytes. *Molecular and Cellular Biology*. 7: 4317-4323.

- Licht L.E. 1995. Disappearing amphibians. *BioScience*.45: 307.
- Licht L. E. 1996. Amphibian decline still a puzzle. *BioScience*. 46: 172-173
- Licht L. E. and K. P. Grant. 1997. The effects of ultraviolet radiation on the biology of amphibians. *American Zoologist*. 37: 137-145
- Lindell, M. J., W. Graneli, and L. J. Tranvik. 1995. Enhanced bacterial growth in response to photochemical transformation of dissolved organic matter. *Limnology and Oceanography* 40: 195-199.
- Long L. E., L. S. Saylor, and M. E. Soule. 1995. A pH/UV-B synergism in amphibians. *Conservation Biology*. 9: 1301-1303
- Li Y. F., S-T Kim, and A. Sancar. 1993. Evidence for lack of DNA photoreactivating enzyme in humans. *Proceedings of the National Academy of Sciences*. 90:4389-4393.
- Lizana M., and E. M. Pedraza. 1998. The effects of UV-B radiation on toad mortality in mountainous areas of Central Spain. *Conservation Biology*. 12:703-707.
- Lubin D., and E. H. Jensen. 1995. Effects of clouds and stratospheric ozone depletion on ultraviolet radiation trends. *Nature*. 377: 710-713.
- Madronich S., and F. R. de Gruijl. 1994. Stratospheric ozone depletion between 1979 and 1992: Implications for biologically active ultraviolet-B radiation and non-melanoma skin cancer incidence. *Photochemistry and Photobiology*. 59: 541-546.
- Madronich S., R. L. McKenzie, M. M. Caldwell, and L. O. Bjorn. 1995. Changes in ultraviolet radiation reaching the Earth's surface. *Ambio*. 24: 143-152.
- Marvik O. J., M. L. Isaksen, L. Roza and B. H. Lindqvist. 1997. Photoimmunodetection: A nonradioactive labeling and detection method for DNA. *BioTechniques*. 23: 892-896.
- Malloy K. D., M. A. Holman, D. Mitchell, and H. W. Detrich. 1997. Solar UVB-induced DNA damage and photoenzymatic DNA repair in Antarctic zooplankton. *Proceedings of the National Academy of Science*. 94: 1258-1263.
- Manley J. L., A. Fire, A. Cano, P. A. Sharp, and M. L. Geftter. 1980. DNA-

- dependent transcription of adenovirus genes in a soluble whole-cell extract. *Proceedings of the National Academy of the Sciences*. 77: 3855-3859
- Michaels P. J., S. F. Singer, and P. C. Knappenberger. 1994. Analyzing Ultraviolet-B radiation: Is there a trend? *Science* 264: 1341-1342
- Mitani H., and A., Shima. 1995. Induction of cyclobutane pyrimidine dimer photolyase in cultured fish cells by fluorescent light and oxygen stress. *Photochemistry and Photobiology*. 61(4): 373-377.
- Mitani H., and N., Uchida A., Shima. 1996. Induction of cyclobutane pyrimidine dimer photolyase in cultured fish cells by UVA and blue light. *Photochemistry and Photobiology*. 64(6): 943-948.
- Mitchell D. L.. 1995. Ultraviolet radiation damage to DNA. in. R. A. Meyers ed. *Molecular Biology and Biotechnology: a comprehensive desk references*. VCH Publishers Inc. New York.
- Mitchell D. L, and D. Karentz. 1993. The induction and repair of DNA photodamage in the environment. in. A. R. Young, J. Moan, L. O. Bjorn, and W. Nultsch eds. *Environmental UV Photobiology*. Plenum Press. New York.
- Mitchell D. L., J. M. Clarkson, C. C. K. Chao, and B. S. Rosenstein. 1986. Repair of cyclobutane dimers and (6-4) photoproducts in 1CR 2A frog cells. *Photochemistry and Photobiology* 43: 595-597.
- Mitchell D. L., J. Jen., and J. E. Cleaver. 1992. Sequence specificity of cyclobutane pyrimidine dimers in DNA treated with solar (UVB) radiation. *Nucleic Acids Research*. 20: 225-229.
- Nagl A. M., and R. Hofer. 1997. Effects of ultraviolet radiation on early larval stages of the Alpine newt, *Triturus alpestris*, under natural and laboratory conditions. *Oecologia*. 110: 514-519.
- Notani N. K., and S. H. Goodgal. 1965. Decrease in integration of transforming DNA of *Hemophilus influenzae* following ultraviolet irradiation. *Journal of Molecular Biology*. 13: 611-613
- Ovaska K. 1995. Vulnerability of amphibians in Canada to atmospheric changes. Canadian Wildlife Services. Ottawa. Canada.

- Ozer Z., J. T. Reardon, D. S. Hsu, K. Malhotra, and A. Sancar. 1995. The other function of DNA photolyase: Stimulation of excision repair of chemical damage to DNA. *Biochemistry*. 34: 15886-15889.
- Park J-W., S-T. Kim, A. Sancar, and J. Deisenhofer. 1995. Crystal structure of DNA photolyase from *Escherichia coli*. *Science*. 268: 1866-1872
- Reitner B., G. J. Herndl and A. Herzig. 1997. Role of ultraviolet-B radiation on photochemical and microbial oxygen consumption in a humic-rich shallow lake. *Limnology and Oceanography* 42(5): 950-960.
- Salthe S. N. 1963. The egg capsules of the Amphibia. *Journal of Morphology*. 113: 161-171.
- Sambrook J., E. F. Fritsch, and T. Maniatis. 1989. *Molecular Cloning: a laboratory manual*. 2nd edition. Cold Spring Harbour Laboratory Press.
- Sancar A. 1994. Structure and function of DNA photolyase. *Biochemistry*. 33: 2-9.
- Sancar A. 1996. No "end of history" for photolyases. *Science*. 272: 48-49
- Sancar G., B. 1990. DNA photolyases: physical properties, action mechanisms and roles in dark repair. *Mutation Research*. 236: 147-160.
- Sancar A., and G. B. Sancar. 1988. DNA Repair Enzymes. *Annual Review of Biochemistry*. 57: 29-67.
- Sancar A., K. A. Franklin and G. B. Sancar. 1984a. *Escherichia coli* DNA photolyase stimulates *uvrABC* excision nuclease *in vitro*. *Proceedings of the National Academy of Sciences*. 81: 7397-7401.
- Sancar A. , F. W. Smith, and G. B. Sancar. 1984b. Purification of *Escherichia coli*. DNA photolyase. *Journal of Biological Chemistry*. 259: 6028-6032
- Sancar G. B., F. W. Smith, M. C. Lorence, C. S. Rupert, and A. Sancar. 1984c. Sequences of the *Escherichia coli* photolyase gene and protein. *Proceedings of the National Academy of Sciences*. 259: 6033-6038
- Schindler D. W., P. J. Curtis, B. R. Parker, M. P. Stainton. 1996. Consequences of climate warming and lake acidification for UV-B penetration in North American boreal lakes. *Nature* 379: 705-708.

- Scully N. M., and D. R. S. Lean. 1994. The attenuation of ultraviolet radiation in temperate lakes. *Ergeb. Limnol.* 43: 135-144.
- Sebastian J. B. Kraus, and G. B. Sancar. 1990. Expression of the yeast PHR1 gene is induced by DNA-damaging agents. *Molecular and Cellular Biology.* 10(9):4630-4637.
- Sebastian J., and G. B. Sancar. 1991. A damage-responsive DNA binding protein regulates transcription of the yeast DNA repair gene *PHR1*. *Proceedings of the National Academy of Sciences.* 88: 11251-11255.
- Setlow R. B. 1968. Photoproducts in DNA irradiated *in vivo*. *Photochemistry and Photobiology.* 7: 643-649.
- Snapka R. M., and B. M. Sutherland. 1980. *Escherichia coli* photoreactivating enzyme: purification and properties. *Biochemistry.* 19: 4201-4208.
- Steinke J. H., and D. G. Benson Jr. 1970. The structure and polysaccharide chemistry of the jelly envelopes of the egg of the frog, *Rana pipiens*. *Journal of Morphology.* 130: 57-66.
- Strickland P. T. 1985. Immunoassay of DNA modified by ultraviolet radiation: A review. *Environmental Mutagenesis* 7: 599-607
- Strickland P. T., O. Nikaido, T. Matsunaga and J. M. Boyle. 1990. Further characterization of monoclonal antibody indicates specificity for (6-4)-dipyrimidine photoproducts. *Photochemistry and Photobiology.* 55: 723-727.
- Sugden B., and W. Keller. 1973. Mammalian deoxyribonucleic acid-dependent ribonucleic acid polymerases. *Journal of Biological Chemistry.* 218: 3777-3788
- Sutherland B. S., and P. V. Bennett. 1995. Human white blood cells contain cyclobutyl pyrimidine dimer photolyase. *Proceedings of the National Academy of Sciences.* 92: 9732-9736
- Svoboda D. L., C. A. Smith, J-S. Taylor, and A. Sancar. 1993. Effect of sequence, adduct type, and opposing lesions of on the binding and repair of ultraviolet photodamage by DNA photolyase and (A)BC excinuclease. *Journal of Biological Chemistry* 268: 10694-10700.
- Svoboda D. L., J-S. Taylor, J. E. Hearst, and A. Sancar. 1993. DNA repair by eukaryotic nucleotide excision nuclease. *The Journal of Biological*

Chemistry. 268: 1931-1936.

Szymkowski D. E., C. W. Lawrence, and R. D. Wood. 1993. Repair by human cell extracts of single (6-4) and cyclobutane thymine-thymine photoproducts in DNA. *Proceedings of the National Academy of Science*. 90: 9823-9827.

Tevini M. 1993. UV-B radiation and ozone depletion: effects on humans, animals, plants, microorganisms and materials. Boca Raton, LA Lewis Publishers

Todo H., H. Takemori, H. Ryo, M. Ihara, T. Matsunga, O. Nikaido, K. Sato, and T. Nomura. 1993. A new photoreactivating enzyme that specifically repairs ultraviolet light-induced (6-4)photoproducts. *Nature*. 361: 371-374.

van de Mortel T., W. Buttemer W., P. Hoffman, J. Hays, and A. Blaustein. 1998. A comparison of photolyase activity in three Australian tree frogs. *Oecologia* 155: 366-369.

Wassersug, R. 1975. The adaptive significance of the tadpole stage with comments on the maintenance of complex life cycles in anurans. *American Zoologist*. 15: 405-417.

Watson N. 1988. A new revision of the sequence of plasmid pBR322. *Gene* 70: 399-403.

Weller, W., F., and D., M., Green. 1997. Checklist and current status of Canadian amphibians. In: D. M. Green editor. *Amphibians in decline: Canadian studies of a global problem*. *Herpetological Conservation*. 1: 309-328.

Williamson C. E. 1995. What role does UV-B radiation play in freshwater ecosystems? *Limnol. Oceanogr.*. 40: 386-392.

Worrest R. C., and D. J. Kimeldorf. 1975 Photoreactivation of potentially lethal, UV-induced damage to boreal toad (*Bufo boreas*) tadpoles. *Life Sciences* 17: 1545-1550

Worrest R. C., and D. J. Kimeldorf. 1976. Distortions in amphibian development induced by ultraviolet-B enhancement (290-315) of a simulated solar spectrum. *Photochemistry and Photobiology* 24: 377-382.

Yan N. D., W. Keller, N. M. Scully, D. R. S. Lean, and P. J. Dillon. 1996.

Increased UV-B penetration in a lake owing to drought-induced acidification. *Nature*. 381: 141-143.

Yasuhira S., and A. Yasui. 1992. Visible light-inducible photolyase gene from the goldfish *Carassius auratus*. *Journal of Biological Chemistry*. 267: 25644-25647.

Yasui A. and S. J. McCready. 1998. Alternative repair pathways for UV-induced DNA damage. *BioEssays*. 20: 291-297

Appendix 1

Problems utilizing enzyme sensitive site assays for photorepair of exogenous DNA with cell free extracts made from amphibian embryos

Background

As stratospheric ozone levels continue to decrease (Holmes and Ellis 1997), estimating an organism's ability to repair damage from UVB (280-320 nm) radiation is of ever increasing ecological and conservation importance. Such importance is highlighted if that organism is sessile, (lacking behavioral means of UVB avoidance), or exists in habitats which tend to accentuate ultraviolet radiation (UVR) exposure. Most amphibian eggs meet the first criterion, and because of egg deposition sites on the surface of the water column or in ephemeral water sources, many also meet the second criterion. Differentiating tissues present in early development, in concert with rapidly increasing chromosomal content (Goin *et al.* 1968, Denis 1974, Worrest and Kimeldorf 1976) also increases the potential for large toxic effects of UVB on amphibian embryos. These factors indicate that a clear understanding of amphibian repair abilities is of critical import to our ability to predict future population trends.

Legarski et al (1987) demonstrated that the amount of UV damage per plasmid molecule could be determined through topoisometric shift of plasmid conformation resolved in agarose gel electrophoresis. Such a non-radioactive assay has been used (Dutta *et al.* 1993), to demonstrate the repair actions of photolyase. Enzyme sensitive site (ESS) assays utilised in this study depend on the recognition of the cyclobutane pyrimidine dimers (CBPD) by the restriction enzyme T4 Endo V, or the prevention of *Hind* III digestion by the formation of CBPDs in it's recognition sequence.

The recognition sequence of *Hind* III (Watson 1988) contains two potential dimer sites. Irradiation with UVB can cause the formation of a CBPD in either of these sites in plasmid DNA. In a solution of linearised (treated with *Pvu* II) pBR322, *Hind* III will then fail to cut the DNA leaving the plasmid substrate only partially digested (Figure 1). When a mixture of irradiated plasmid DNA and amphibian cell free extract containing photolyase is exposed to photoreactivating light (PRL) prior to electrophoresis, a more complete digestion will occur due to the catalyzed photorepair by photolyase of the CBPDs.

T4 Endo V is a viral enzyme which specifically cuts the DNA strand at CBPDs resulting in a change in conformation from closed covalent circles (Form I or supercoiled) to relaxed molecules (Form II/nicked circular or Form III/linear) (Schrock and Lloyd 1993). The enzymatic activity of T4 Endo V has been measured by the rate of loss of Form I DNA (Schrock and Lloyd 1993). Upon incubation with amphibian CFE and photoreactivating light, the dimer sites are removed and the proportion of Form I as a percent of the total DNA in each lane indicates photolyase activity. The conformation shift from Form I to Form II is evident through agarose electrophoresis of the irradiated plasmid/amphibian protein extract subsequent to time under PRL.

Both of these enzyme systems have been successfully used as quick assays to determine concentrations of purified bacterial photolyase (Dutta *et al.* 1993) and for one study of several wild sea slugs which demonstrated photolyase activity (Carlini and Regan 1995). I attempted to assay the UVB damage repair ability of seven Ontario amphibian species using both *Hind* III and T4 Endo V assays. I report results that suggest apparent high endonuclease concentrations in amphibian egg cell free extracts that cause the degradation of even high (9µg/ml) DNA concentrations. This type of signal loss is characteristic of endonuclease digestion. I speculate that high endogenous concentrations of

amphibian endonucleases appear to preclude the use of plasmid DNA/ESS assays to determine UVB repair abilities of amphibian eggs. Proper estimation of amphibian ultraviolet repair characteristics, using any assay, is reliant upon the generation of protein cell free extracts (CFE) created from amphibian embryos covered in protective jelly. The process of releasing the embryo from the glycoprotein/carbohydrate jelly ("de-jellying") is achieved by shaking the jelly mass in a solution of 2% L-cysteine (Blaustein *et al.* 1994). I report here results which demonstrate that equivalent exposure to 2% L-cysteine to different taxa results in radically different results.

To my knowledge this is the first documentation of these phenomena. These results have important implications for the production, standardization and reporting of amphibian photorepair data.

Methods

Egg and Embryo Collection

Eggs from each amphibian species were collected between April 23 and July 15 1997. UVB readings for each collection day (from 8:00am until 6:00pm at one minute intervals) were obtained from a BW-100 CID UV measuring Instrument (Vital Technologies Corp.) located at Trent University (44°21'N 78°17'W, 100m elevation). Eggs were transported back to Trent University upon collection, staged according to Gosner (1960), or Harrison (1969), and immediately frozen in 50 ml containers (except ambystomids which were kept in 150 ml containers) at -80°C.

Protein Purification

Methods of protein purification were adapted from Manly *et al.* (1980), Sancar *et al.* (1984), and Blaustein *et al.* 1994). Briefly; 50 ml egg replicates (frozen at -80°C) were warmed overnight from -80°C to 4°C. 10 ml of 2% L-cysteine

was then added to each 50 ml container and embryos were shaken at room temperature for between 1 and 3 hours. L-cysteine acts as a dejelling agent with the reduction of disulphide bonds present in the jelly matrix. Shaking continued until the embryos were observed to be clustered at the bottom of the tube with no jelly surrounding and separating each embryo. Embryos were washed two times in cold phosphate buffered saline (PBS (8 g NaCl, 0.2 g Na_2HPO_4 , 0.24g KH_2PO_4 in 1 l of distilled H_2O , pH 7.4)) and were then centrifuged at 3 000 rpm for 15 minutes in a KompSpin 21.50 rotor in a Beckman J2MC high speed centrifuge. The supernatant was removed and the packed cell volume (PCV) was estimated (generally less than 5.0 ml PCV resulted from 50.0 ml of egg and jelly). Four times PCV of Buffer I (10 mM Tris, 1 mM EDTA and 5 mM DTT) was added and let sit for 20 minutes on ice. Embryos were lysed using a Dyna-mix homogeniser (Fisher Scientific) set on high with 20-25 strokes, and the resulting solution was then placed in a sterile Erlenmeyer flask with a stir bar. Four times PCV of Buffer II (50 mM Tris, 10 mM MgCl_2 , 2 mM DTT, 25% sucrose w/v, and 50% glycerol v/v) was added. One times PCV of saturated DNase free ammonium sulfate was added drop by drop, while stirring, to lyse cell nuclei (Sugden and Keller 1973). Stirring was continued for 30 minutes after the last drop of ammonium sulfate was added. After stirring was completed, the solution was centrifuged at 20 000 rpm for 5 hours at 4 °C. The supernatant was decanted into a sterile conical tube and measured, and then transferred to a sterile flask where 0.33g of Sigma DNase free ammonium sulfate per 1.0 ml of supernatant was added while stirring to precipitate the protein. While stirring, 100 μl 1N NaOH per 10 g of ammonium sulfate was added. Stirring continued for 30 minutes after the last addition of crystal. This solution was then centrifuged at 10 000 rpm for 30 minutes at 4 °C. The resulting pellet was resuspended in 1/40th the measured supernatant volume of storage/dialysis

buffer (25 mM Hepes, 100 mM KCl, 12 mM MgCl₂, 0.5 mM EDTA, 2 mM DTT and 16% glycerol v/v) and dialyzed overnight in tubing of mole weight 14 000. The dialysis flask was placed in an ice bucket and covered with foil to minimize ice melting.

The dialysate was centrifuged for 10 minutes at 10 000 rpm at 4 °C. The supernatant was loaded to a Sigma chromatographic column with Blue Sepharose CL-6B, equilibrated with 0.1 M KCL and Buffer B (50 mM Tris-HCl pH 7.4, 1 mM EDTA, 10 mM 2-mercaptoethanol and 20% v/v glycerol), and washed with 0.1 M KCL and Buffer B with 0.6 g/L ATP. Fractions began to be collected after approximately forty minutes of run time. Small (20 µl) sub-samples of each 2.0 ml fraction was added to 200 µl Coomassie Blue stain and 780 µl distilled water in order to determine which fractions contained the most protein. These protein rich fractions were collected, pooled, and dialyzed for 4-6 hours against photolyase storage buffer (50 mM Tris pH 7.4, 50 mM NaCl, 1 mM EDTA, 10 mM DTT and 50% glycerol). The resulting dialysate was collected, and frozen at -20 °C until used in either the transformation assay, or the ESS assay. A portion of the resulting dialysate was put aside at this point to perform a Bio-Rad assay for protein concentration.

Enzyme sensitive site (ESS) assays

The ESS assays utilised here are based upon those performed in Dutta *et al.* (1993), and Carlini and Regan (1995). This report is essentially a comparison of the efficacy of three different methods of photorepair detection and therefore the methods and results sections are paired.

Methods of *Hind* III ESS assay

pBR322 plasmid DNA (Sigma) was prepared using the Bio-Rad Quantum Prep kit. This DNA was linearised by incubation with *Pvu* II for 60 min at 37 °C. Pyrimidine dimers were formed in the DNA by exposure to a Photo Optic

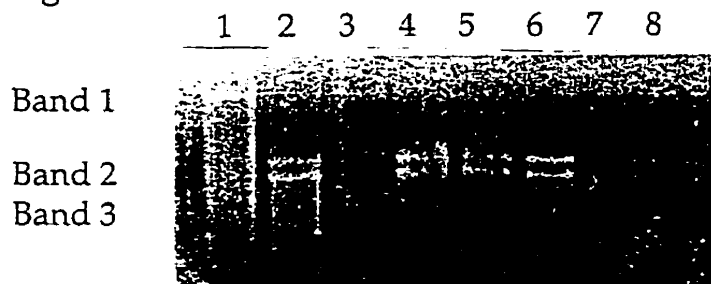
Halogen Bulb (1000W.120V.64743.Osram Corp.) at a distance of 10 cm for between 60 to 240 seconds (3.3-4.0 MEDS/hr at 60 second, (1MED = $21 \pm 3 \text{ mJ/cm}^2$) = $11.55 - 14.00 \text{ J/m}^2$) in 16 mm polystyrene culture dishes at room temperature. A reaction mixture of CFE, photolyase assay buffer, and plasmid was made and exposed to either photoreactivating light (10 cm from GE 15W Black Light lights), or dark for 90 minutes. Preliminary transformation assays indicated that this relationship was linear with respect to time until 90 minutes. After light or dark treatments, the reaction mixture was incubated with *Hind* III restriction endonuclease (Sigma) for 60 minutes at 37°C . After *Hind* III incubation, 1-2 μl of 6x tracking dye (0.25% bromophenol blue, 40% w/v sucrose) was added to each mixture, and they were loaded onto a 1.0% agarose gel of Tris-acetate EDTA buffer (TAE, 0.04 M Tris-acetate and 0.001 M EDTA) buffer with 2-4 μl of 10 mg/ml ethidium bromide. Electrophoresis was carried out in the dark for 3-5 hours at 20-45 V. The resulting banding patterns can be seen in Figure 1.

Gels were photographed using Polaroid 667 black and white film with a yellow filter (speed = 1-2 seconds, $f = 8$ or 16 depending on DNA concentration). The positive print of each photograph was scanned using a digital scanner and the absorbance of each band was calculated using the Macintosh shareware program, NIH Image by comparing an unknown band with a series of known standards. The digital image was then used to quantify the absorbance of each Band 1 from light and dark treatments. Photoreactivation of the plasmid/CFE complex allows for the monomerisation of pyrimidine dimers by photolyase in the CFE and therefore a more complete digestion of the plasmid. The difference between the two absorbances (expressed as a percentage of total absorbance for that lane) allowed for the calculation of percentage repair of plasmid DNA. This was calculated as the difference in the mean absorbance of Band 1 DNA for the

+PRL treatments and the Band 1 DNA for the -PRL treatments for each preparation.

Results of *Hind* III ESS assay

In the first lane of Figure 1, pBR322 is seen in its linear conformation following *Pvu*II digestion. Upon UVB irradiation the DNA retains this conformation (Lane 3). Upon incubation with *Hind* III, the linear plasmid DNA is cut into two lengths (Bands 2 and 3 in lanes 2 and 4). If dimers are formed in the *Hind* recognition sequence, digestion will be incomplete, resulting in the partial retention of Band 1. When plasmids are incubated with photolyase in either the presence or absence of photoreactivating light, the difference in absorbance of this band allows estimation of photolyase concentration (Dutta *et al.* 1993). In lanes 5 and 6 the bands representing the spring peeper skin treatment can be seen. Band 1 is too faint for analysis. Between June 1997 and November 1998, 30 attempts were made to determine the photolyase activity of five species with this assay. The most frequent results obtained were empty lanes (results not shown).

Figure 1:**Figure 1:**

1% agarose gels containing UV-irradiated pBR322 plasmid DNA linearized by treatment with *Pvu* II. This is called Band 1 (Lane 1=unirradiated linear DNA, Lane 3= UVB irradiated linear DNA). Lanes 2 and 4 represent the pair of bands produced by digestion of linear pBR322 by *Hind* III. UVB irradiated DNA was treated with purified photolyase (Lane 5 and 6), and 25 µg CFE from the spring peeper (Lane 7 and 8). The top band in each lane represents the DNA retaining dimers, resistant to *Hind* III digestion. This band was not sufficient for analysis.

Methods of T4 Endo V ESS assay

Plasmid DNA was isolated, irradiated, mixed with amphibian CFE, and electrophoresed as described above. T4 Endo V is a viral enzyme that cuts DNA at CBPDs. This digestion results in a break in the DNA strand which is evident in electrophoresis as a conformational change from covalent closed circular molecules (CCC, Form 1) to relaxed circular molecules (Form II), (Figure 2, lane 1).

Gels were photographed using Polaroid 667 black and white film with a yellow filter (speed = 1-2 seconds, $f = 8$ or 16 depending on DNA concentration). The positive print of each photograph was scanned using a digital scanner and the absorbance of each band was calculated using the Macintosh shareware program, NIH Image. Since Form I DNA fluoresces at 70% the intensity of other conformations, the absorbance of this band was divided by 0.7 (Carlini and Regan 1995). The percentage of the DNA in Form I conformation was determined by dividing absorbance of Form I (adjusted) by the sum of the absorbance of all three conformations and multiplying by 100. Subtracting the percentage DNA in Form I of +PRL from -PRL allowed for an estimation of photorepair (Carlini and Reagan 1995).

Results of T4 Endo V ESS assay

In treatments with purified photolyase incubated in the dark, most dimers were not been repaired, T4 digestion is more complete, and therefore most of the DNA is in Form II (Figure 2). In the same treatment, but in the presence of ninety minutes of photoreactivating light, dimers were removed, and therefore T4 digestion is less complete, and there is an increasing proportion of the total DNA in the Form I conformation visible in Lane 5. Lanes 6-7 were empty after incubation with leopard frog CFE. This result was characteristic for most

exposure of DNA to amphibian cell free extract (21 of 24 assay attempts). Banding patterns were sufficient for analysis for gray tree frogs once (Figure 3) and once for wood frogs (Figure 4) and yellow spotted salamanders (not shown). The absorbance of these bands was analyzed by the NIH Image package configured for the Windows platform (Scion Image) (Figure 5). A time series for exposure to photoreactivating light displayed a negative relationship for the wood frog. As photoreactivating light is necessary for the catalysis of the CBPD, this trend is counter intuitive. A time series for gray tree frog exposure to photoreactivating light displayed a trend of increasing repair (Figure 5, $R^2=0.05$), but all band absorbance readings were opposite what would be recorded if photorepair had occurred. That is, Form I intensities were greater in the lane of plasmid where the DNA/CFE mixture had not been photoreactivated than in the lane where the DNA/CFE mixture had been reactivated and therefore should have had fewer digestions sites.

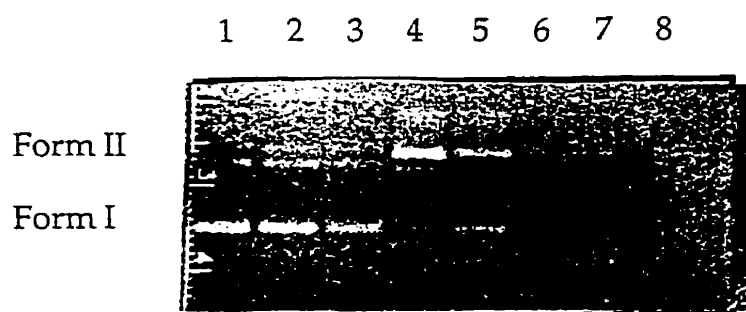


Figure 2:

1% agarose gels containing UV-irradiated pBR322 plasmid DNA treated with purified photolyase (lanes 4-5) or CFE from leopard frog (lanes 6-7) prior to incubation with T4 EndoV. DNA in lanes 1-3 was irradiated with UVB radiation. Lanes 5 and 7 were exposed to 90 minutes of photoreactivating light, and lanes 4 and 6 were placed in the dark for 90 minutes.

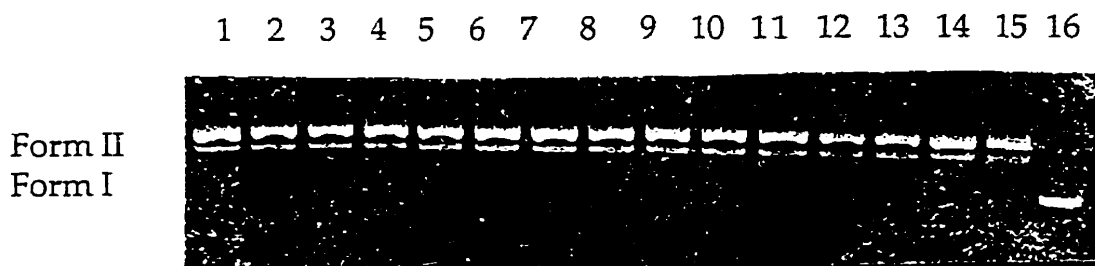


Figure 3:

1% agarose gels containing UV-irradiated pBR322 plasmid DNA treated with gray tree frog CFE. Lanes 1-2, 3-4, 5-6 were placed in the dark for 40, 60 and 80 minutes respectively. Lanes 9-10, 11-12, 13-14 were exposed to 40, 60, or 80 minutes of photoreactivating light. The top band in each lane is Form II DNA, and the bottom band is Form I DNA.

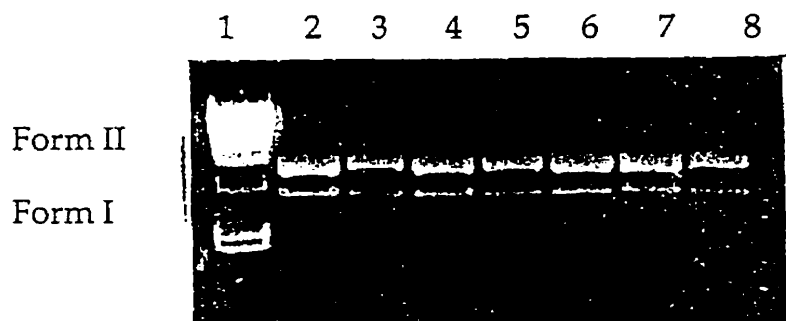


Figure 4:

1% agarose gels containing UV-irradiated pBR322 plasmid DNA treated with wood frog CFE. Lane 1 is a lambda control. Lane 2, 3, 5 and 7 were exposed to 20, 40, 60 and 80 minutes respectively of photoreactivating light. Lanes 4, 6 and 8 were placed in the dark for 40, 60, or 80 minutes. The top band in each lane is Form II DNA, and the bottom band is Form I form of plasmid DNA.

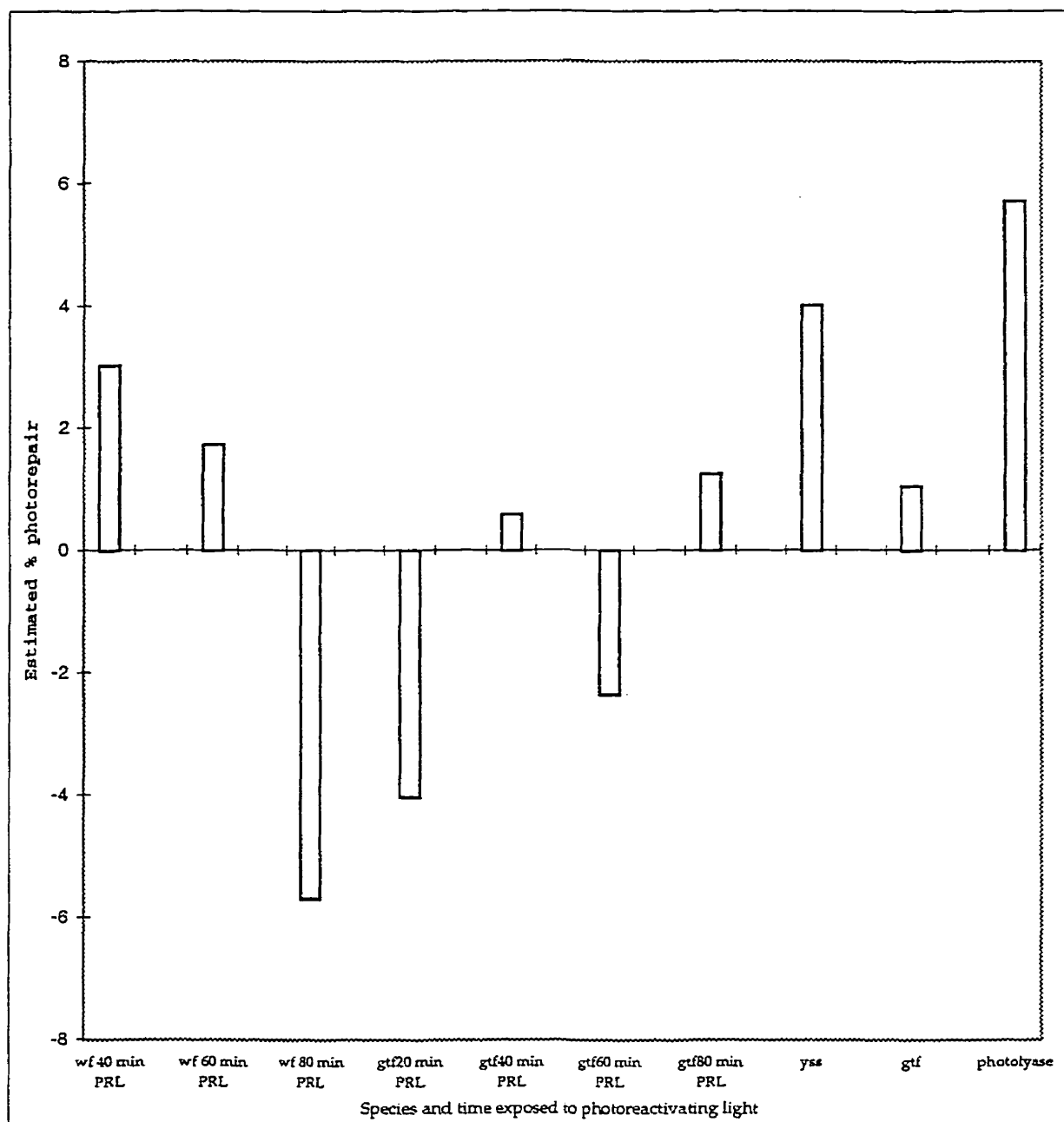


Figure 5:

Estimations of percent photorepair utilizing the T4 Endo V ESS assay (WF 40, 60, 90 min PRL exposed on 12.16.97, GTF 20, 40, 60, 80 min PRL exposed on 12.17.97, YSS exposed on 01.10.98, GTF 02.03.98, and purified photolyase 11.06.98). Bars represent standard error.

Percent photorepair = (absorbance Form I as % of all three conformations for +PRL) - (absorbance Form I as a % of all three conformations for -PRL)

H3 immunoassay methodology

Ultraviolet-B irradiated DNA solutions were diluted in 6X standard saline citrate (SSC, 3 M NaCl, 0.3 M). Fifty μ l aliquots of DNA were immobilized on a Magna-Graph neutral nylon membrane (Fisher Scientific) using the Bio Rad Dot-Blot Apparatus. Membranes were air dried and then baked at 80 °C for 1 hour. The membrane was then saturated with 5% non-fat milk in TBS-T (20mM Tris-HCl, pH 7.5, 500 mM NaCl, 0.1% Tween 20) for 1 hour at room temperature while gently shaking. After one hour of blocking, H3 monoclonal antibody (specific to cyclobutane pyrimidine dimers (Marvik *et al.* 1997) purchased from Ard Vink, Department of Molecular Toxicology, TNO Nutrition and Food Research Institute, The Netherlands) was added to the blocking buffer (1:2 000) and shaking was continued overnight at 4 °C. Upon completion of incubation with primary antibody, the membrane was washed four times (5 min) in 250 ml TBS-T and then incubated with horseradish peroxidase conjugated goat anti-mouse antibody (1:5 000) in 1.0 % non-fat milk TBS-T while gentle shaking for 90 minutes at room temperature. After secondary antibody exposure, the membrane was washed four times (7.5 min) in 250 ml TBS-T. The signal was then developed using the ECL Western Blotting analysis system (Amersham Pharmacia Biotech) following instructions contained in the kit. Kodak Biomax film exposure times ranged from 30-120 minutes.

Using the shareware program Scion Image, I quantified the intensity of each blot. When this intensity was converted to a plot of ng of DNA vs. placement on the membrane, I calculated the area under each portion of the curve representing + or - PRL. If photorepair had occurred, and dimers were removed, the +PRL blot will be less intense than the -PRL blot.

Results of H3 immunoassay

Figure 6 illustrates the dot blotted Magna-Graph neutral nylon membrane with UVB irradiated DNA fluorescing after primary binding to the monoclonal antibody H3 and secondary goat-anti-mouse-HRP binding, and exposure to Kodak BioMax film. The eighth row is of two times dilutions of genomic *Saccharomyces cerevisiae* DNA exposed to 240 seconds of the Halogen Photo-Optic UVB bulb. Using the shareware program Scion Image, I quantified the intensity of each blot for chorus frog, green frog and yellow spotted salamander + and - PRL treatments (Figure 7). The percent photorepair was estimated as, $(100 - \{(+PRL/-PRL)*100\})$.

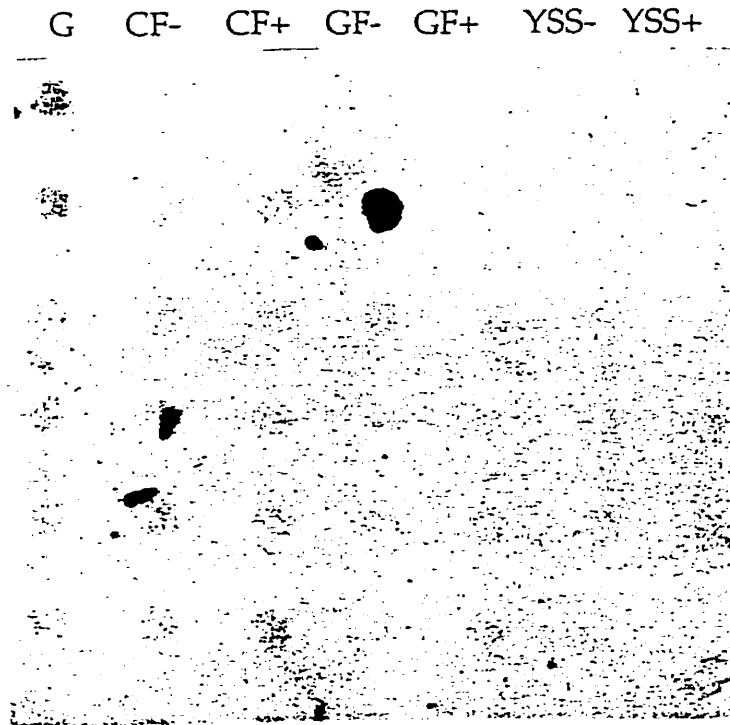


Figure 6:

Antibody detection of UVB irradiated DNA on a neutral nylon membrane. The first blot lane contains *Sacchromyces cerevisiae* genomic DNA irradiated for 240 seconds in serial 2X dilutions. The blot lanes 2 and 3 contain pBR322 DNA irradiated with 60 seconds of UVB irradiation and chorus frog CFE. Lane 2 was placed in the dark for 90 minutes (CF-) and lane 3 was exposed to photoreactivating light for 90 minutes (CF+). The same pattern is blotted in lanes 4 (GF-) and 5 (GF+) and lanes 6 (YSS-) and 7 (YSS+) with green frog and yellow spotted salamander CFE respectively.

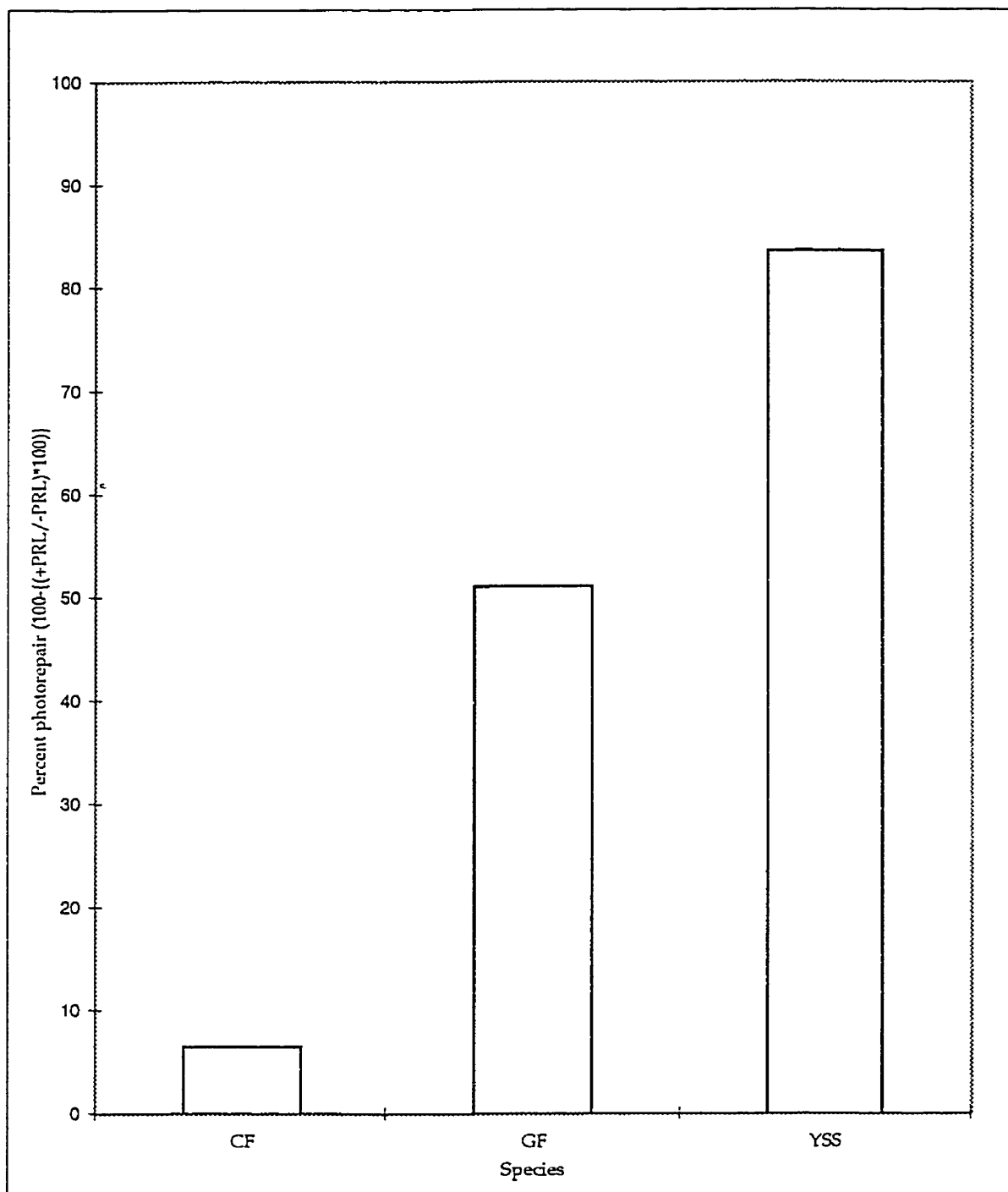


Figure 7:

Preliminary H3 immunoassay estimation of UVB damage to pBR322 DNA by cell free extracts of embryos from the chorus frog (CF), green frog (GF), and yellow spotted salamander (YSS). Estimations created with 50 μ g CFE / 6 μ g DNA / 90 min PRL. Percent photorepair (100-((+PRL/-PRL)*100))

General Results

Table 1 outlines the enzyme sensitive sites (ESS) assays and immunoassays for CBPDs for photolyase activity of amphibian CFE in exogenous plasmid DNA. The history of each tissue source and the repair estimation are included. The DOC values are from Lean *et al.* (submitted). Unshaded columns relate directly to work completed in this chapter. Shaded columns refer to information important to the history of the tissue assayed here, but not directly related to the work in this chapter.

°

Table 1 Enzyme sensitive sites (ESS) assays and immunoassays for CBPDs for photolyase activity of amphibian CFE in exogenous plasmid DNA. The history of each tissue source and the repair estimation are included. The DOC values are from Lean *et al.* (submitted). See text for interpretation of shading.

Species	Collection Time	Location collected	Mean Depth (cm)	DOC mg/L	De-jelly protocol	Tissue type	UV-B J/m ²	Jelly absorbance (relative arbitrary units)	<i>In vitro</i> E/S temp (°C)	Repair
<i>Rana sylvatica</i>	04.29. 97	Arch. Pond	9.25	18.75	2% L-cysteine	11	368.44	280=2.22 320=1.26	4	Hind III= no signal T4 Endo V= approximately 0% photorepair H3 = unknown
<i>Rana clamitans</i>	06.10. 97	Turtle Pond	6	12.65	2% L-cysteine	10	38.8	280=1.61 320=2.39	15	Hind III= no signal T4 Endo V = no signal H3= 50% photorepair
<i>Hyla versicolor</i>	oocytes 04.30. 97	Kitty's Pond	0	17.6	n/a	oocyte	oocyte= n/a	n/a	20	Hind III = no signal T4 Endo V= % photorepair = 1.15 H3=unknown
	stg 10 06.04. 97				2% L-cysteine	16	Stg. 16 = unknown	280=1.08 320=2.23	20	
<i>Rana pipiens</i>	05.07. 97	Arch. Pond	13.1	18.75	2% L-cysteine	10	unknown	280=1.122 320=2.156	20	Hind III = no signal T4EndoV=no signal H3 = unknown
<i>Hyla crucifer</i>	04.28. 97	Road Kill	7.5	n/a	n/a	Skin	unknown	n/a	15	Hind III= visible bands, not absorbent enough for analysis.
<i>Pseudacris triseriata</i>	04.30-05.09. 97	Arch. Pond	5	likely >19	2% L-cysteine	11-17	unknown	280=0.64 320=0.90	40+ 20	H3 = 6.49 % photorepair
<i>Ambystoma laterale</i>	04.28. 97	Road Kill	14	n/a	n/a	26	n/a	n/a	20	Hind III = no signal
	05.26. 97	Kitty's Pond		17.6	2% L-cysteine		63.04	280=1.18 320=1.94	4	H3 = unknown
<i>Ambystoma maculatum</i>	05.26. 97	Kitty's Pond	16.7	17.6	2% L-cysteine	16	63.04	280=1.58 320=2.97	20	Hind III= no signal T4 Endo V= signal often too faint for analysis (4.01% photorepair) H3=86.3% percent photorepair

L-cysteine de-jellying

The amphibian embryos examined here displayed differential sensitivity to 2% L-cysteine as a de-jellying agent. Ambystomid salamanders (*Ambystoma laterale* and *A. maculatum*), *Bufo americanus*, and summer laying ranids (*Rana clamitans* and *R. catesbeiana*) were susceptible to rapid embryo damage (Table 2). *B. americanus* and *R. catesbeiana* samples were damaged sufficiently to preclude their inclusion in this study. Spring laying ranids (*Rana sylvatica* and *R. pipiens*) were not as susceptible, and indeed much longer cysteine exposure times were required to remove embryos from jelly (Table 2).

Table 2: Differential effects of 2% L-cysteine as an agent to remove amphibians from protective jelly.

Species	Agitation Time Range (min)	Protein Concentration Range (mg/ml)	Comments
<i>P. triseriata</i>	1-2	1.37 - 1.45	Little exposure or agitation required to remove embryos.
<i>R. sylvatica</i>	60-120	1.51 - 7.99	Little embryo damage after great agitation and cysteine exposure
<i>R. pipiens</i>	90-120	1.5 - 5.0	Little embryo damage after great agitation and cysteine exposure
<i>A. laterale</i>	0.5 - 20	0.13 - 0.49	Rapid embryo destruction in cysteine, thus embryos dissected out of jelly capsule and briefly exposed to cysteine.
<i>A. maculatum</i>	0.5 - 20	0.03 - 1.86	Rapid embryo destruction in cysteine, thus embryos dissected out of jelly capsule and briefly exposed to cysteine.
<i>B. americanus</i>	10-60	0.09 - 0.23	Complete embryo homogenization after subsequent cysteine exposure to remove embryos from jelly. Not enough protein for photorepair analysis.
<i>R. clamitans</i>	60-360	0.06 - 0.78	Greater exposure times required to remove embryos from jelly. Some embryo degradation after cysteine exposure.
<i>R. catesbeiana</i>	7-120	none	Embryos removed as freed from jelly. Not enough protein for photorepair analysis.

Discussion

ESS assays (Hind III and T4 Endo V)

"Levels of DNase in some amphibian egg extracts are high enough to cause significant degradation of exogenous DNA substrates." Hays et al. 1996, p. 450.

Population declines, certainly not restricted to amphibian taxa, are occurring at an ever increasing rate. Some of these declines are "normal" processes of population ebb and flow, but some are more closely related to anthropogenically induced stress. UVB represents such a stress that is increasing in areas that often don't appear to be experiencing changes in environmental stress. The speed at which these changes are occurring, and the fact that their effects will be felt all over the world, require that we have a quick, inexpensive, non-radioactive method of assessing one of the abilities organisms have to repair UVB damage. Currently, ESS assays don't seem to represent this method.

H3 Immunoassay

Although not inexpensive, and although only preliminary investigations have been completed, this immunoassay may offer an interesting option for assessing the ultraviolet damage repair abilities of different species in the future.

Using 2% L-cysteine as a de-jellying agent

Understanding amphibian ultraviolet repair abilities is of critical importance in the maintenance of a diverse, functional, ecosystem. Such an understanding is dependant upon the acceptance that all amphibians aren't anuran, and anurans don't represent all amphibians, and sometimes this self-evident truth is under-reported. Blaustein *et al.* (1994) describe their process of de-jellying to extract amphibian embryos, *"Pro-eggs and eggs were first dejellied by treatment for 30-120 minutes with 2% cysteine...."* Blaustien et al. 1994, p. 1792, paragraph 2). This treatment, if applied constantly across the amphibian taxa collected in this study, would have destroyed embryos of some species

(bullfrogs, blue/yellow spotted salamanders), and for others (wood frog and leopard frog) half of the embryos in one jelly mass would not have been released (Table 2). It is critical that between-species differences in response to a methodological variable are reported.

General Conclusions

Results reported elsewhere (Chapter 2) support the declaration of Li *et al.* (1993) that, "*One of the most sensitive assays for photolyase is the transformation assay.*" (p. 4393). The bacterial transformation assay was much more efficient at illustrating dimer removal from exogenous DNA than either ESS assay completed here. Infrequent, low intensity banding patterns characterized all ESS assays. Low absorbance plasmid DNA resulted after incubation with amphibian CFE and did not allow for proper estimation of photolyase activity for the embryos of that species. Results from my data for H3 monoclonal antibody to detect dimer removal indicates that this assay should be nearly as sensitive as the bacterial transformation assay to quantify photolyase activity of amphibian cell free extract.

Future analyses

Future analyses of these ESS assays with amphibian cell free extracts should attempt to estimate concentrations of substrate-damaging endonucleases for any species before utilizing the assay. I would suggest the following protocol to roughly estimate the endonuclease proportion of the CFE created from the embryos of different amphibian species.

- 1) Subsequent to the CFE protocol outlined here, and prior to any incubation with UVB irradiated plasmid DNA, incubate different concentrations of CFE with different concentrations of a DNA source of known concentration and size. Due to the probable amounts of DNA which would be utilised, and the

temporal and monetary costs of isolating this DNA, I suggest that an affordable source, such as lambda DNA could be used.

2) Amphibian CFE would be incubated with the lambda DNA at 37°C for 15, 30, 60 and 120 minutes, and then loaded onto an agarose gel.

3) When the absorbency of the lambda/CFE bands are compared to a standard of known concentration (i.e. the initial lambda concentration), a rough endonuclease estimate should be possible.

This estimate would allow a researcher to eliminate the potential use of the ESS assays *a priori* if there were between-species differences in band digestion.

Appendix 2

UVB absorbance of embryo jelly from seven amphibian species from south-central Ontario

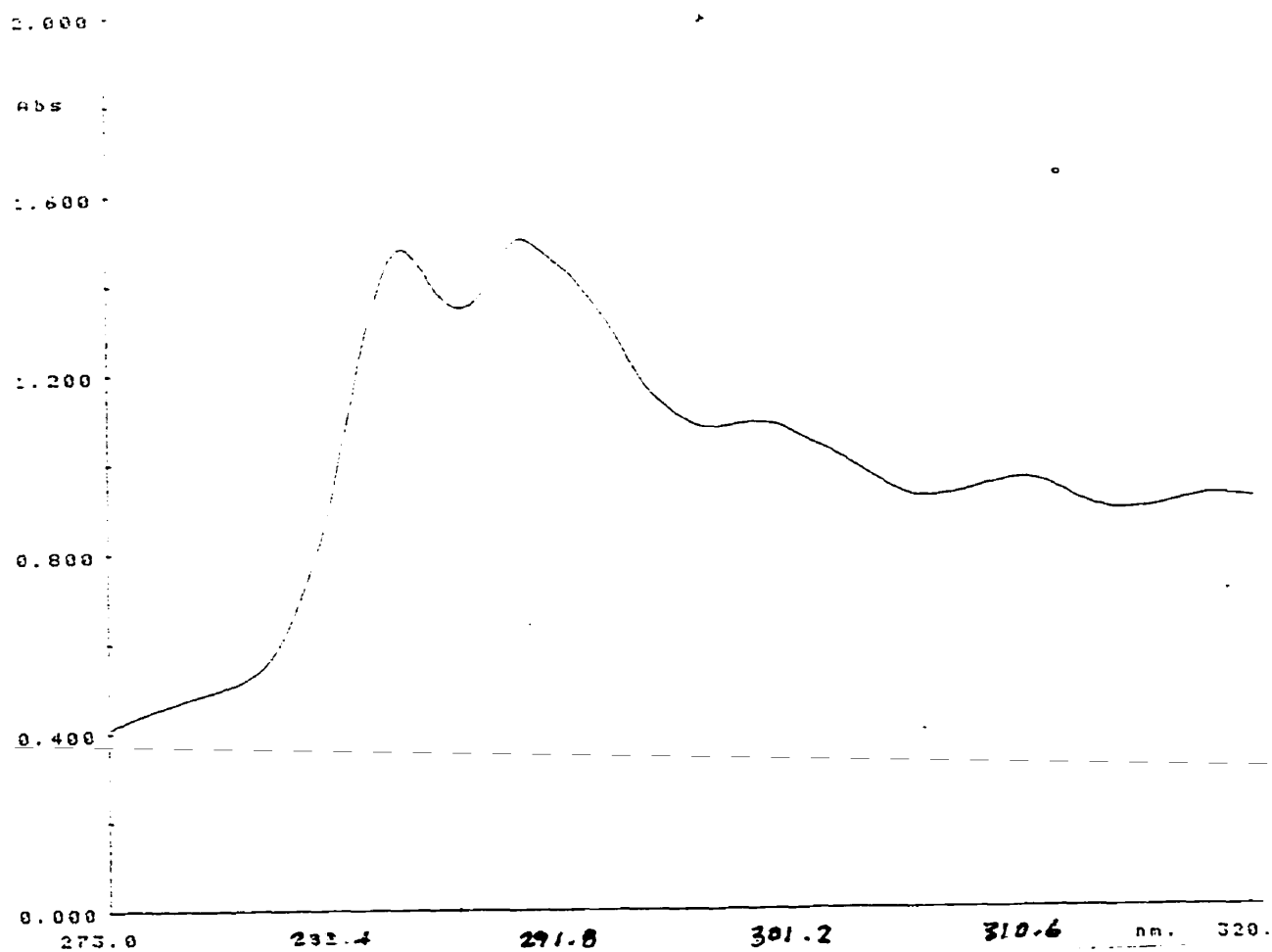


Figure 1: UVB absorbance for chorus frog embryo jelly between 275 and 320 nm.

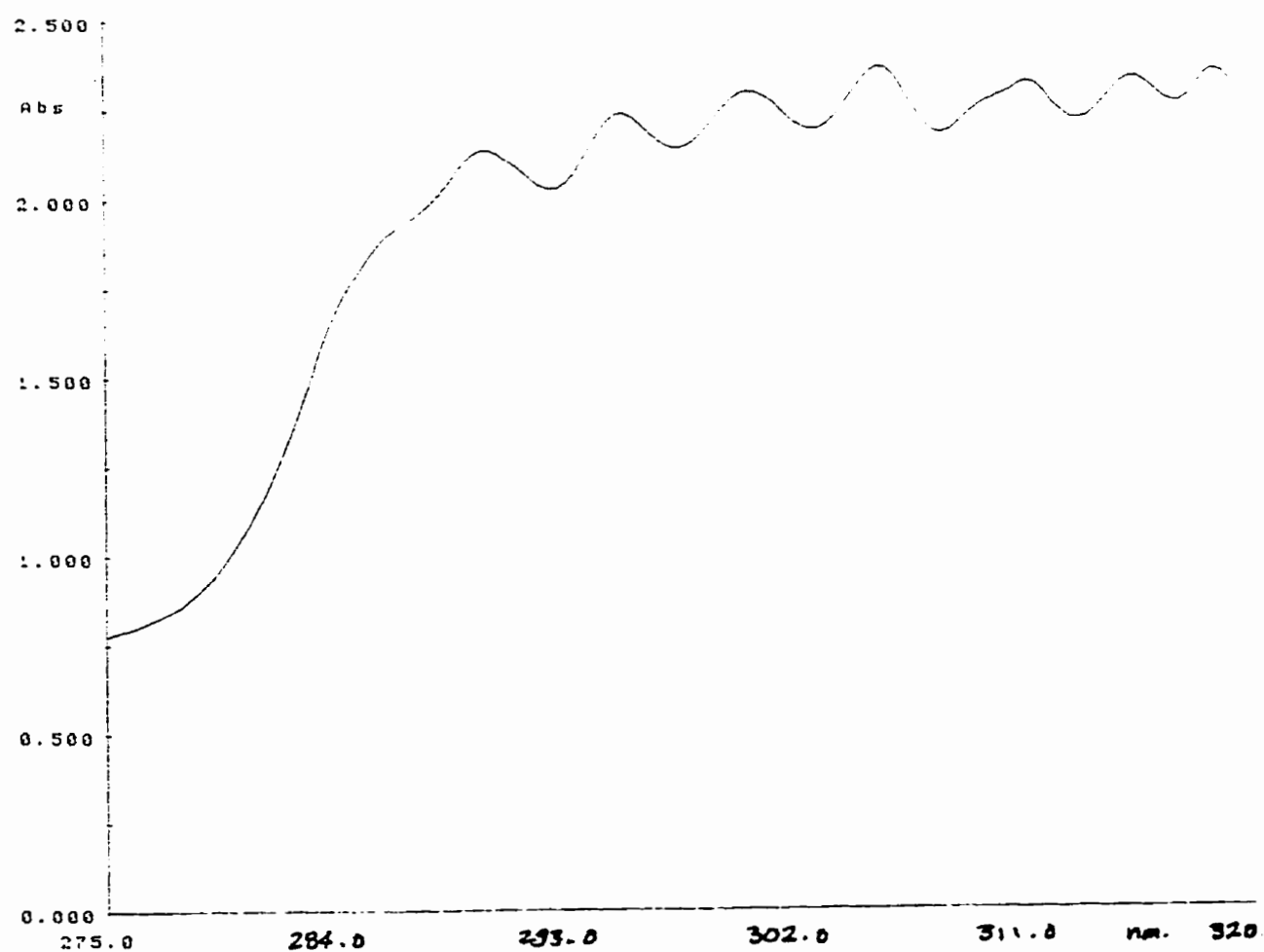


Figure 2: UVB absorbance for gray tree frog embryo jelly between 275 and 320 nm.

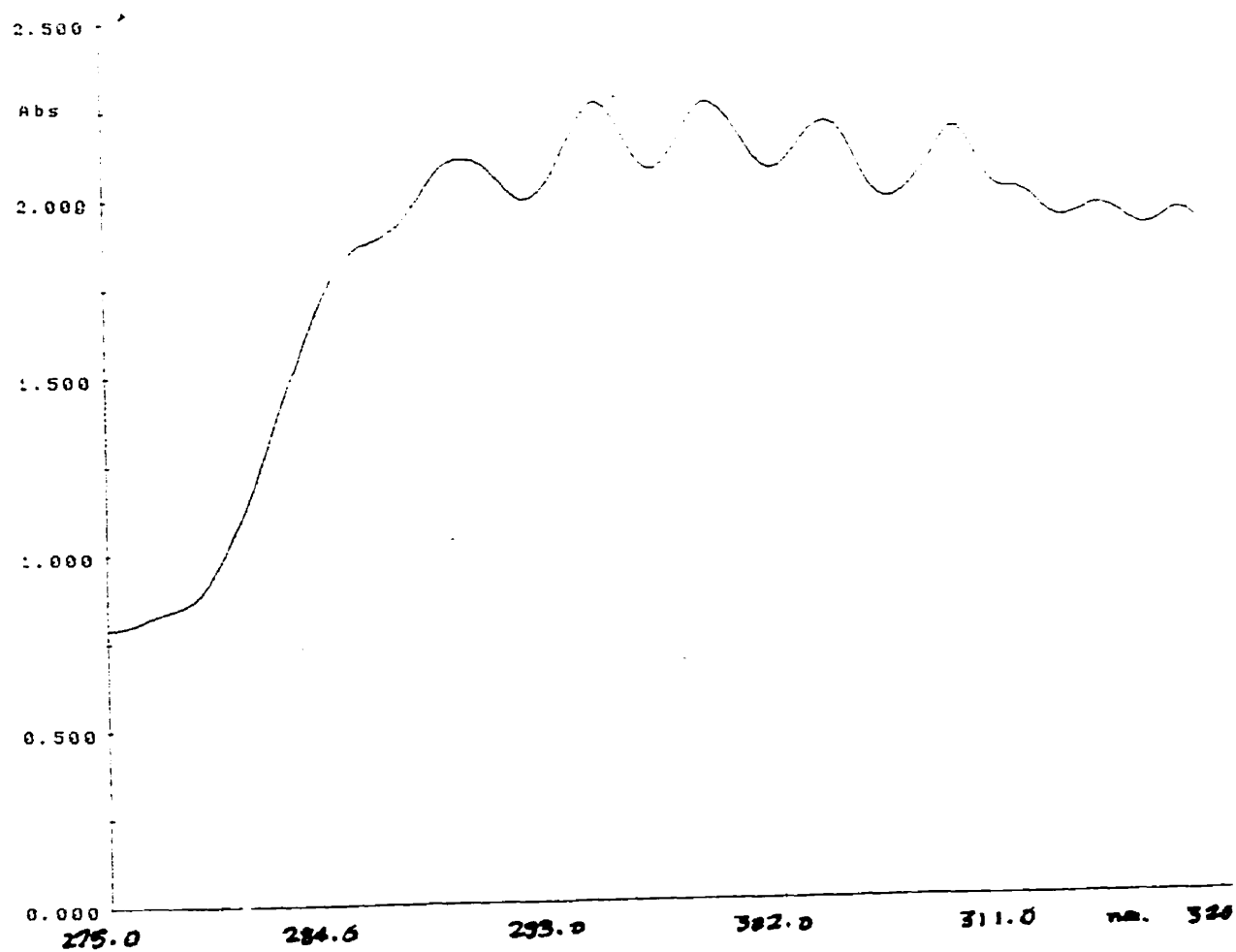


Figure 3: UVB absorbance for blue spotted salamander embryo jelly between 275 and 320 nm.

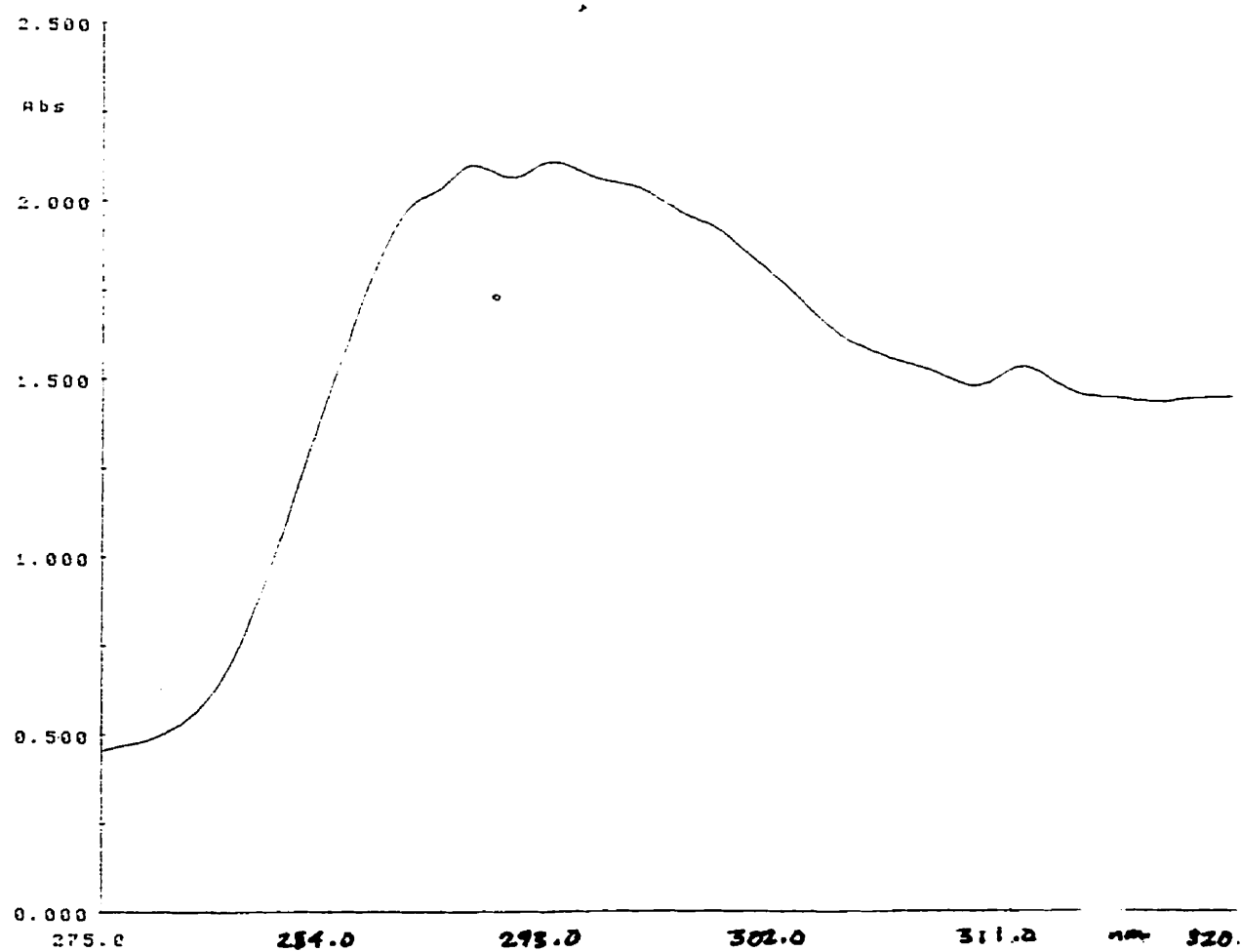


Figure 4: UVB absorbance for yellow spotted salamander embryo jelly between 275 and 320 nm.

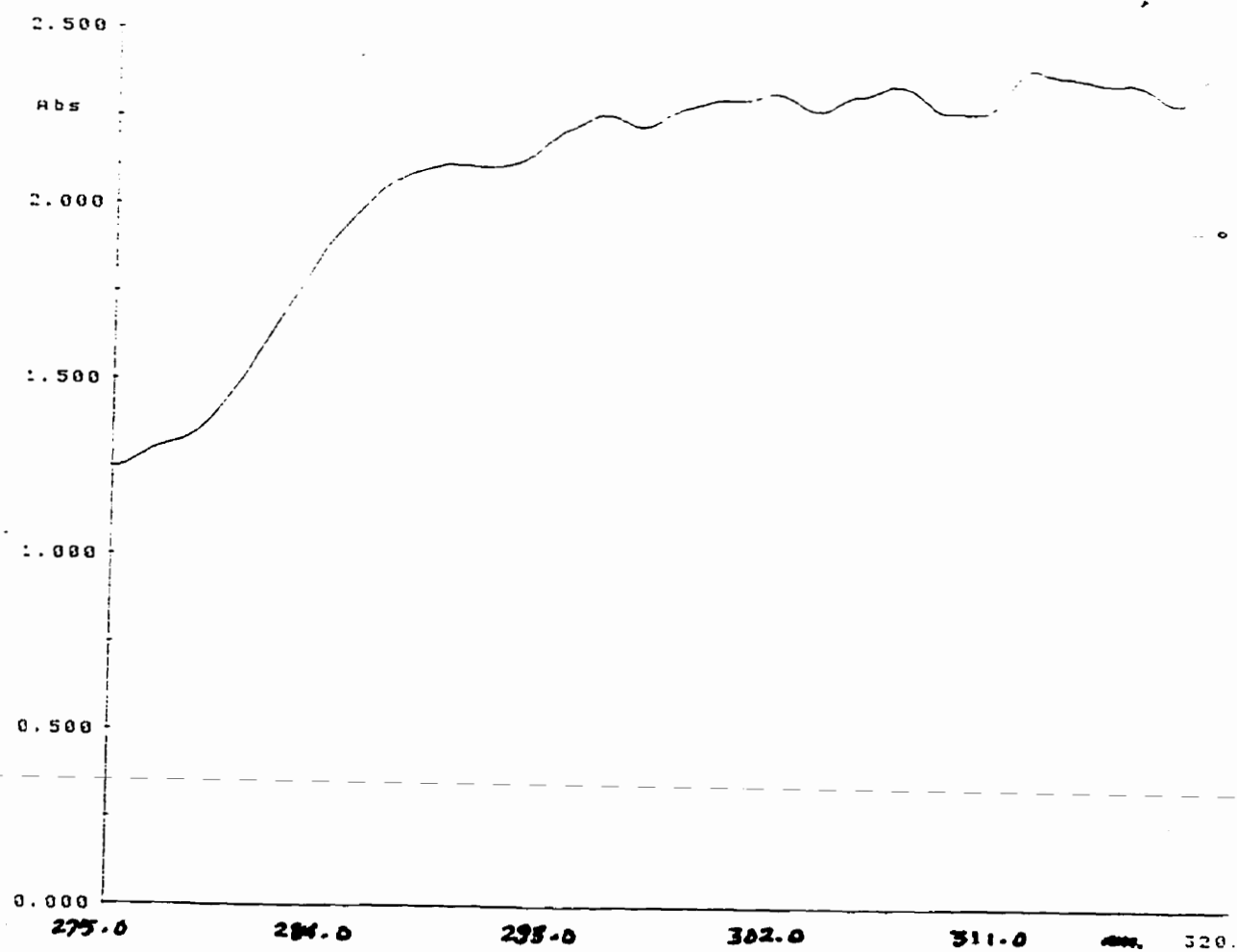


Figure 5: UVB absorbance for green frog embryo jelly between 275 and 320 nm.

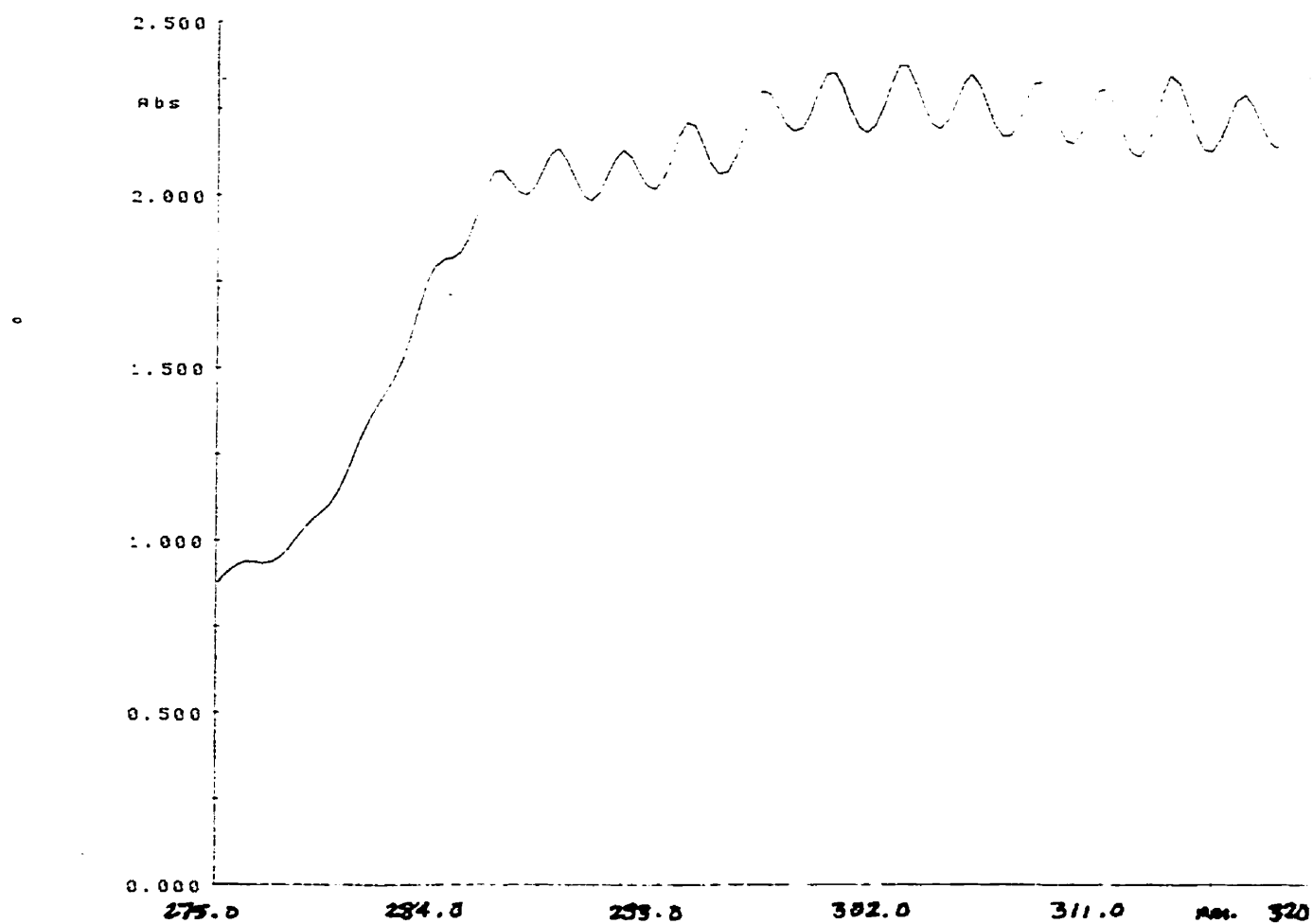


Figure 6: UVB absorbance for wood frog embryo jelly between 275 and 320 nm.

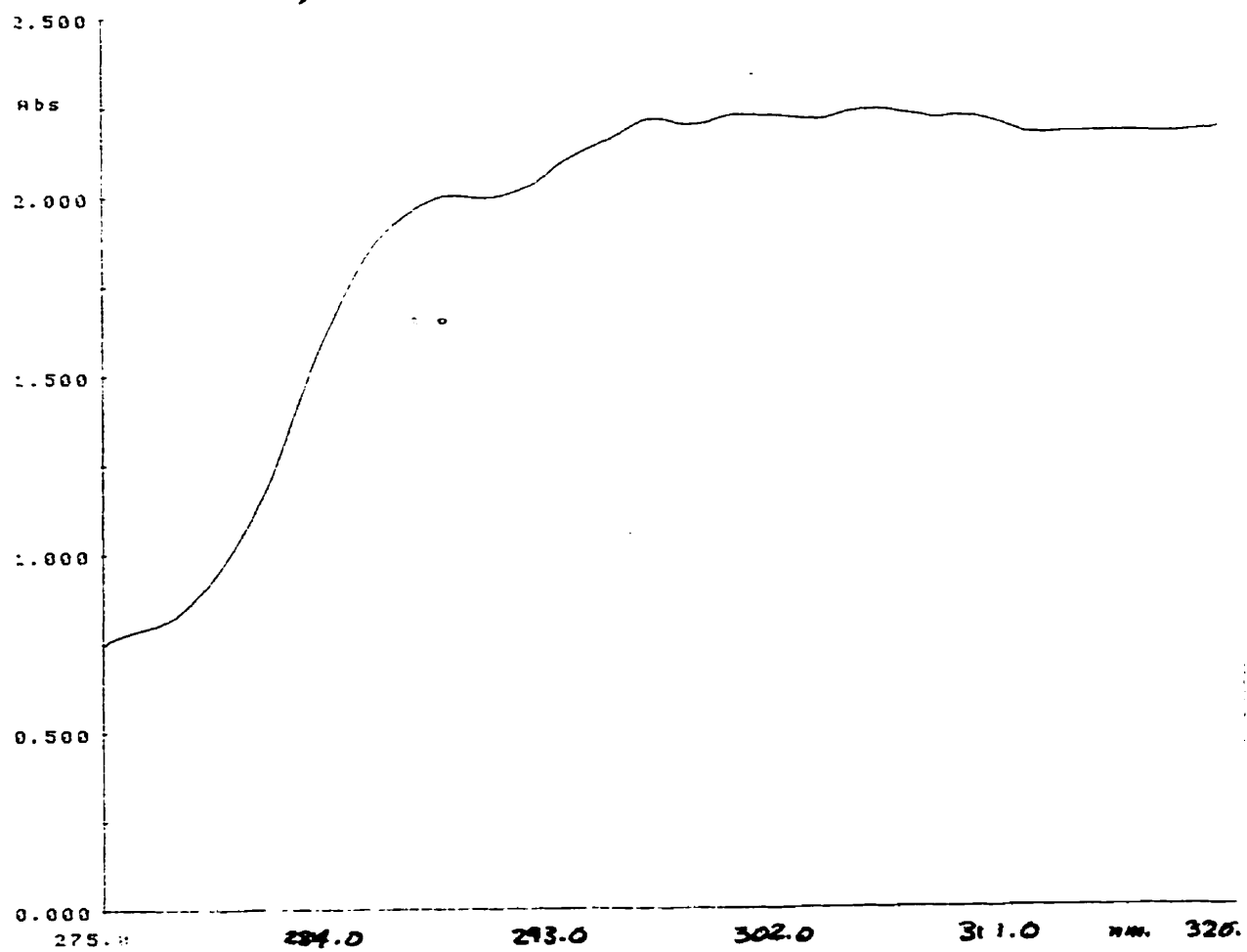


Figure 7: UVB absorbance for leopard frog embryo jelly between 275 and 320 nm.