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Molecular response to climate change : effect of temperature on repair of UVR-induced DNA damage in Daphnia

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Molecular Response to Climate Change:

Effect of Temperature on Repair of UVR-induced DNA damage in *Daphnia*

by

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A Thesis

Presented to the Graduate and Research Committee

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in

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Master of Science.

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DEDICATION

I would like to dedicate this work to the people who were most influential in making it a

success through their daily support: Craig Williamson, Gabriella Dee, Shawna Gilroy and

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ABSTRACT

As global warming is raising global surface temperatures, depletion of the stratospheric ozone layer is also allowing increased levels of ultraviolet radiation (UVR) to reach the earth's surface. These increases in global temperature and ambient UVR are likely to simultaneously affect life at all levels. UVR induces damage to DNA at the biomolecular-level, which kills cells and organisms. While the two repair mechanisms which exist to mediate this damage are likely temperature dependent, UVR-induced damage of the DNA molecule is independent of temperature, suggesting that increased ambient temperatures may facilitate repair in exotherms; however, few studies have directly addressed the question of temperature dependence of repair of UVR-induced DNA damage. In this study, *Daphnia*, the water flea, is used as a model organism for studying the temperature dependence of repair. This "cosmopolitan" planktonic crustacean is the most widespread and abundant genus of crustacean zooplankton in lakes in the northern hemisphere, and is one of the dominant primary consumers in freshwater lakes worldwide. While previous studies have examined the behavioral responses of *Daphnia* to visible light, and recent work has focused on the behavioral responses of *Daphnia* to the ultraviolet portion of the solar spectrum, no studies have quantified the molecular responses of *Daphnia* to UVR. I tested the molecular responses of UVB-exposed *Daphnia* to two variables with known positive effects on *Daphnia* survival: 1) repair-stimulating radiation and 2) higher temperatures. Experiments were conducted to isolate the effects of longer wavelength photorepair radiation (PRR) from shorter wavelength, damaging UVB radiation. This study demonstrates that net DNA damage is

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temperature dependent, both when PER is active and when PER is inactive. Opposite

effects of temperature are exhibited depending on whether PER is active or inactive. I

proposed that different mechanistic explanations, which depend on whether PER is active

or inactive, can be used to interpret the findings. This study represents one of the first

quantitative studies of repair of UV-induced DNA damage in zooplankton.

PART I: INTRODUCTION AND LITERATURE REVIEW

INTRODUCTION:

Climate change is a suite of environmental stressors that affects life at all levels, from the molecule to the ecosystem. As global warming is raising global surface temperatures, depletion of the stratospheric ozone layer is also allowing increased levels of ultraviolet radiation (UVR) to reach the earth's surface. These increases in global temperature and ambient UVR are likely to simultaneously affect the survival and reproduction of organisms.

The ecological implications of UVR-induced DNA damage and its repair are not well understood at any scale. The objective of this study is to understand the ecology of these molecular responses to climate variables by experimentally testing these responses under a variety of ecologically relevant conditions, using *Daphnia*, the water flea, as a model organism.

While previous studies have examined the behavioral responses of *Daphnia* to visible light, and recent work has focused on the behavioral responses of *Daphnia* to the ultraviolet portion of the solar spectrum (Leech and Williamson 2001), no studies have quantified the molecular responses of *Daphnia* to UVR. Furthermore, few studies have related molecular responses to organismal responses for UVR stress in any species. Thus, this link between molecular and organismal-level responses to UVR continues to be a gap in our understanding of how organisms respond to climate variables across multiple scales of biological organization. The need for such across-scale studies was

recently identified by the IPCC as a high research priority (IPCC, WG2 TS Report, 2001). Quantifying the impacts of UVR-temperature interactions on repair mechanisms in *Daphnia* will contribute to our understanding of how large-scale climate changes can be expressed through small-scale molecular responses.

BACKGROUND ON UVR INDUCED DNA DAMAGE AND ITS REPAIR BY PHOTOLYASE:

Induction of DNA damage by UVR

The ultraviolet (UV, 100-320 nm) portion of the solar spectrum of radiation directly affects organisms at the molecular level. It has been recognized for over 35 years that biological molecules, including deoxyribonucleic acid (DNA), absorb radiation strongly in the UVB range (290-320 nm) (Giese 1957). The most germicidal wavelength of solar radiation is 260 nm, which is the peak absorbance of DNA (Atlas and Bartha 1998). Organisms are protected from the most damaging solar radiation, UVC (100-290 nm), by the earth's ozone layer. Of the wavelengths of solar radiation that reach the earth's surface, UVB radiation causes damage to DNA (Atlas and Bartha 1998). UVA radiation (320-400 nm) can have both beneficial and detrimental effects on organisms.

Ultraviolet radiation (UVR) adversely affects cells in direct and indirect ways. Direct damage caused to DNA by UVB radiation occurs primarily in pyrimidine nucleotide bases, especially thymine and cytosine, because the peak irradiative absorbance for pyrimidines falls within the UVC to UVB range (245 nm, Mitchell and Nairn 1989). This study focuses on the direct damage sustained by DNA molecules in the form of DNA dimers, for which specific enzyme-mitigated repair mechanisms exist.

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UVR causes adjacent pyrimidine nucleotide bases (thymine or cytosine) on the DNA molecule to become linked, forming a dimer. Two basic types of dimers can form, cyclobutane pyrimidine dimers (CPDs) and pyrimidine(6,4)pyrimidone photoproducts ((6-4)s). Although (6-4)s can be more cytotoxic than CPD dimers (Mitchell and Nairn 1989), CPDs account for approximately 80-90% of all dimers formed (A. Sancar 2000). The presence of either type of dimer on the DNA molecule can cause a range of biological damage, from delay of cell division to genetic mutations and cell death (Sutherland 1981).

Mechanisms for mediating UVR-induced DNA damage

To avoid genetic damage caused by UVR, organisms can employ one or more defense mechanisms; these include avoidance behavior and physiological defense mechanisms. Because such preventive measures can be energetically costly and can increase the risk of predation, from a cost-benefit perspective, an inducible defense mechanism – one that requires an environmental stimulus for activation and is selected for by previous exposure to the stimulus (Harvell 1990) – is often favored over a constitutive defense mechanism (such as photoprotective pigmentation). This is especially true when the exposure gradient is variable, (Harvell 1990), as in the case of UVR exposure. Inducible defenses against UVR-induced genetic damage are probably among the first defenses to have evolved, given the intensity and spectral composition of UVR on earth during the anaerobic early atmosphere.

Repair of UVR-induced DNA damage

This study will focus on the component of physiological tolerance that is inducible over short time scales: molecular repair. Of the three common mechanisms for repairing damaged DNA (recombination repair, nucleotide excision repair (NER) and photoenzymatic repair (PER)) two of these mechanisms, NER and PER typically repair UVR-induced DNA damage. Recombination repair has not been found to be a major mechanism for mitigating UVR damage across taxa (G.B. Sancar, personal communication), although the reason for this finding is not known.

The first mechanism, nucleotide excision repair (NER), is a multi-protein pathway powered by ATP that is found universally in all taxa but is not specific for UVR-induced DNA damage (Sancar 1994a). NER operates by splicing the damaged sequence of DNA out of the DNA and initiating the synthesis of a replacement sequence using genetic information supplied by the sister chromatid. Nucleotide excision repair can be an important mechanism for mitigating UVR stress when photoreactivation is inactive in the absence of UV-A stimulation, and in organisms that lack photoreactive capabilities.

The second mechanism, known as photoenzymatic repair (PER), is a light-driven single-enzyme system that is specific for UVR-induced DNA damage but is only exhibited by certain taxa (Sancar 1994b). When an organism with PER capability sustains genetic damage from exposure to UVB radiation, concurrent or subsequent exposure to UVA and visible radiation induces a DNA repair mechanism known as photoreactivation, or photoenzymatic repair (PER). In this process, the enzyme

photolyase harnesses photon energy and uses it to return damaged DNA molecules to their original undamaged state (Sancar 1994b).

Unlike nucleotide excision repair, which is a biologically universal repair mechanism (A. Sancar 1994a), photoreactivation has only been exhibited by certain taxa. These taxa, however, span a wide variety of organisms from archebacteria to marsupials (Kanai et al. 1997). In higher organisms, photoreactivation capability can vary with developmental stage and by tissue (Sutherland 1981). In the arctic bacterioplankton discussed below, NER accounts for only a small amount of the DNA repair that occurs during the day (Huot et al. 2000). Also, for bacteriophage, the rate of photoreactivation of viral DNA by host photolyase is many orders of magnitude greater than the rate of NER (Huot et al. 2000). NER may be an important mechanism for repairing DNA during the night, when photoreactivation does not occur (Huot et al. 2000).

Photoreactivation is controlled by a light-dependent, substrate-specific enzyme called photolyase (Sutherland 1981). There are two types of photolyase enzyme: CPD photolyase (Class I and Class II) and (6-4)photolyase. The presence of photolyase in organisms can be an important line of defense against UVR-induced DNA damage, especially since dimers are poor substrates for nucleotide excision repair (G.B. Sancar, personal communication). Photolyase may even increase the efficiency of nucleotide excision repair when there is no visible light available to stimulate photoreactivation (G.B. Sancar, personal communication).

History of photolyase research

Photoreceptive compounds, such as photolyase, have been the subjects of research for over a century. The photoreceptor for vision, rhodopsin, was discovered by Boll in 1877. By 1935, several studies had been published which demonstrated that lethal damage to bacteria by UVR could be mitigated by exposure to visible light (reviewed by Hearst 1995). The concept of photoreactivation was first introduced into the scientific dialogue in 1949, when Albert Kelner published a study describing the phenomenon of mutant strains of bacteria produced by UV irradiation being maintained in visible light with wavelengths between 350 and 400 nm (reviewed by Hearst 1995). The same year, Dulbecco demonstrated that photoreactivation was possible in bacteriophage. Dulbecco developed a dose-response curve for UV exposure and determined the temperature dependence of photoreactivation. With this observation, he further hypothesized that the mechanism of photoreactivation is an enzyme-mediated process (reviewed by Hearst 1995). Findings published by Hershey and Chase in 1925 and those published by Rupert, Goodgal and Herriott in 1956 proved that a protein component of living cells (i.e. photolyase) is necessary for photoreactivation, and that photoreactivation takes place on the DNA molecule itself (reviewed by Hearst 1995). In 1960, Rupert definitively determined that photoreactivation is an enzymatic process (reviewed by Sutherland 1981).

In 1959, Beukers, Ijstra and Berends demonstrated that the UVR-induced lesions formed on DNA molecules consist of thymine bases. Later, they determined that the lesions are cyclobutane-type thymine dimers. Further investigations showed that the

process of photoreactivation involves the reversal of pyrimidine dimers into thymine dimers. As early as 1960, the three basic steps involved in the photoreactivation mechanism were recognized and well understood. First, the enzyme binds to the cyclobutane dipyrimidine dimer. Then, the enzyme uses photon energy to reverse the reaction and form two pyrimidines. Finally, the enzyme and the DNA molecule dissociate (reviewed by Hearst 1990).

Photoreactivation research was further advanced in 1978 when Sancar and Rupert cloned the CPD photolyase gene (Hearst 1995). Sancar also published the absorption spectra of CPD photolyase in 1994 (Sancar 1994b). In 1995, Park, Sancar and Deisenhofer published the crystalline structure of the CPD photolyase enzyme (Deisenhofer 2000).

Although the existence of (6-4)photoproducts has been recognized since the mid-1980s (reviewed by Mitchell and Nairn 1989), the (6-4)photolyase was not discovered until 1993 (reviewed by Todo 1999). Coincidentally, the blue light receptor protein of plants was identified in *Arabidopsis thaliana* the same year (Todo 1999). In 1996, a homologue of the (6-4)photolyase was found in human cells (Todo et al. 1996). By that time, CPD photolyase had been isolated from 14 species and Class I and II proteins had been recognized. (6-4)Photolyase had been found only in *Drosophila melanogaster*, where the enzyme was most active in the ovary and embryo. Todo et al. (1996) recognized the functional and structural similarity of the CPD and (6-4) photolyases and the blue light receptor in plants, and proposed that they be considered a "family" of proteins.

Structure and Function of Photolyase

There are two types of photolyase: CPD photolyase and (6-4)photolyase. CPD photolyase is specific for cyclobutane dipyrimidine dimers (CPDs), while (6-4)photolyase is specific for (6-4)photoproducts, which are pyrimidine-pyrimidone dimers (A. Sancar 2000). Although the two types of photolyase act on different substrates, their structures and functional mechanisms are similar. Both types of photolyase enzyme photocatalyze reversion reactions.

Photolyase is a 55-65-kDa protein that consists of two chromophores (photoreactive pigments). The first chromophore is always FAD (in the form of FADH⁻, commonly referred to as the flavin chromophore), while the second chromophore is often pterin methenyltetrahydofolate (MTHF, commonly referred to as the folate chromophore) (A. Sancar 2000). The FAD chromophore serves as the catalytic center of the reaction and is a source of electrons for the reaction. The folate chromophore serves as the photoantenna by absorbing photon energy and transferring it the reaction center of the FAD (A. Sancar 2000). The enzyme is shaped like South America (NIGMS, Research Report, March 1996), with a hole in its center that serves as the active site for the reaction. It is believed that the close proximity of the dimer to the binding site of the enzyme accounts for the high efficiency of the reaction (G.B. Sancar, personal communication), with 0.7-1.0 reactions occurring per absorbed photon (the quantum yield of the reaction) (G.B. Sancar, personal communication, A. Sancar 2000).

Photolyase is extremely sensitive for distinguishing between damaged and non-damaged pyrimidine nucleotide bases, the latter of which are commonly present at concentrations many times higher than those of damaged pyrimidine bases (G.B. Sancar, personal communication, Vande Berg and G.B. Sancar 1998). CPD photolyase has a very high affinity for thymine-thymine dimers (quantum yield 0.6-1.0, Vande Berg and Sancar 1998), which represent 80-90% of CPD dimers formed (G.B. Sancar, personal communication). Enzyme-substrate recognition decreases successively from T-T dimers to T-C, C-T and C-C dimers. The quantum yield for C-C dimers is 20 times lower than for T-T dimers (G.B. Sancar, personal communication, Vande Berg and Sancar 1998).

Photolyase recognizes DNA damage in a specific manner (G.B. Sancar, personal communication), which recent research suggests is an interaction between the photolyase enzyme and the sugar-phosphate backbone of the DNA molecule (not the damaged oligonucleotide bases themselves, as was originally thought) (G.B. Sancar, personal communication, Vande Berg and Sancar 1998). In this light-independent step, the enzyme binds to the dimer substrate and forms a Michaelis complex (A. Sancar 2000). When the complex is exposed to UVA radiation (320-400 nm) and lower-wavelength photosynthetically active radiation (400-700 nm), the second chromophore absorbs a single photon and transfers the excitation energy to the flavin. Then, the flavin transfers a single electron to the DNA dimer, at which point the cyclobutane ring of the CPD or the oxytane ring of the (6-4)photoproduct is broken to form two pyrimidines. Finally, an electron is transferred back to the FADH, and the enzyme dissociates from the DNA molecule (A. Sancar 2000). Photolyase does not catalyze an oxidation-reduction

reaction, as both the substrate and the enzyme return to their original oxidation states after the reaction occurs (Hearst 1995).

Photolyase shares functional similarities with the photosynthetic system. Both systems have photoantennae and catalytic cofactors, and are initiated by a photoinduced electron transfer (A. Sancar 2000). The two systems differ, however, in several fundamental ways. First, photolyase is a soluble protein, while the photosystems of plants are membrane-bound. Second, photolyase has one photoantenna per reaction center while the photosystems of plants have hundreds of photoantennae per reaction center. Third, photolyase does not result in a net oxidation-reduction reaction, while photosynthesis does. The photolyase mechanism uses cyclic electron transfer, while photosynthesis requires an electron source. Another significant difference between the two systems is that photosynthesis uses energy from broad-spectrum visible solar radiation (with various photoreceptive pigments that have different absorption maxima) while photolyase uses only the blue light portion of the solar spectrum (370-420 nm for the folate class and 420-440 nm for the deazaflavin class) (A. Sancar 2000).

Photoreactivation is unique among repair pathways for two reasons. First, the repair "pathway" involves a single enzyme (photolyase), which performs a single monomerization reaction (Sutherland 1981). Second, photoreactivation does not require incision into the phosphodiester backbone of the DNA molecule (G.B. Sancar, personal communication). Because no DNA is removed or newly synthesized (Sutherland 1981, G.B. Sancar, personal communication), this system ensures that DNA repair is virtually error-free (Sutherland 1981).

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When considering the costs and benefits of this inducible defense in an evolutionary context, it is important to remember that photoreactivation does not unduly drain the energy stores of cells. Photon energy, not adenosine triphosphate (ATP), drives the reversion reaction (Sutherland 1981). Thus, energy from one portion of the sun's spectrum is used to repair damage caused by another portion of the sun's spectrum (Sutherland 1981).

Evolution of Photolyase:

There are two known classes of CPD photolyase, based on sequence homology (Vande Berg and Sancar 1998). The CPD photolyase of lower prokaryotes and eukaryotes belongs to Class I (25-43% sequence homology), while the Class II CPD photolyase (38-72% sequence homology) is generally found in higher eukaryotes. Although the two classes of enzymes have relatively low homology (10-17% sequence identity, Vande Berg and Sancar 1998; 20-30% sequence identity, A. Sancar 2000), the enzymes share a common mechanism. The conserved amino acids are largely involved in the lining of the active site cavity of the enzyme (Vande Berg and Sancar 1998). In addition, Vande Berg and Sancar (1998) found that there is a striking conservation of amino acids between the Class I and Class II CPD photolyases and the (6-4)photolyase in the protein residues of the active site cavity. Of the five residues of the Class I and II CPD enzymes that contact the CPD dimer during the photoreversion reaction, four of these residues are conserved in the (6-4)photoproduct (Vande Berg and Sancar 1998).

These findings support the theory that the CPD photolyase and the (6-4)photolyase evolved from a common ancestral gene (Vande Berg and Sancar 1998).

CPD photolyase has been studied for more years than has (6-4)photolyase and has been found in more organisms. CPD photolyase exists in bacteria, archea and eukarya (A. Sancar 1994b). While Class I CPD photolyase has been found only in unicellular organisms, including various prokaryotes and eukaryotic fungi (Todo 1999), Class II CPD photolyase has been isolated from organisms ranging from archaebacteria and eubacteria to higher eukaryotes (Todo 1999). The Class II CPD photolyase from the archaebacterium *Methanobacterium thermoautotrophicum* is the only Class II CPD photolyase to be studied extensively.

Photolyase belongs to a family of proteins that also includes two cryptochrome proteins: the (6-4)photolyase homologue in humans (animal CRY) and the plant blue light receptor (plant CRY). Much more is known about the structure and function of CPD photolyase than about the other three groups of proteins. While these CRY proteins are structurally similar to photolyase, they are functionally different. The two genes that code for animal CRY have a 41-45% sequence homology with the gene that codes for (6-4)photolyase in *Drosophila* (Todo 1999). Unlike photolyase, however, animal CRY exhibits no repair activity. Instead, it is believed to function more like the blue light receptors of plants (Todo 1999). This family of proteins can therefore be divided into two groups on the basis of functional similarity: photoreactivation proteins (Class I and II CPD photolyase and (6-4)photolyase) and photoreceptor proteins (animal CRY and plant CRY) (Todo et al. 1996, Kanai et al. 1997, Todo 1999).

Both the animal CRY and plant CRY proteins are believed to play a role in the circadian clock. The plant blue light receptor proteins are also responsible for early development signals in plants (as well as mediating phototropism, hypocotyl elongation, stomatal opening and the expression of certain genes (Kanai et al. 1997)), and use a mechanism similar to photolyase for receiving and converting photon energy (Todo at al. 1996). The role of cryptochrome in the circadian clock of both plants and animals has been examined (Somers et al. 1998, Thresher et al. 1998). A recent study by Nikaido and Johnson (2000) suggests that circadian clocks might have evolved from photolyase.

A likely scenario for the evolution of the photolyase/blue light receptor family is that the CPD Class I photolyase was replicated at least four times before the divergence of prokaryotes and eukaryotes (Kanai et al. 1997). One copy of the gene evolved to become Class II CPD photolyase. Another copy remained as Class I CPD in the prokaryotic lineage. This copy or another copy was transmitted to eukaryotes and diverged to become (6-4)photolyase, plant CRY and animal CRY (Kanai et al. 1997).

It is reasonable to assume that photolyase could be the oldest or one of the oldest mechanisms of DNA repair (Todo 1999). For the first 2.5 billion years of life on earth, early prokaryotes were exposed to extreme UVR conditions. Not until the evolution of photosynthesis, when oxygen was released into the atmosphere, was an ozone layer present to protect living cells from very high intensity damaging radiation. That it has been conserved through evolutionary time across diverse taxa (see Table 1) indicates that natural selection against the gene that codes for photolyase has not occurred, suggesting that the photolyase enzyme remains vital to cell survival.

Review of Photolyase Studies

Much of the work that has been conducted to characterize photolyase has been done using microbial species, most importantly the prokaryotic *E .coli* (bacterium) and eukaryotic *S. cerevisiae* (yeast), and it has long been recognized that UVR exposure can inhibit colony formation in bacteria (Sutherland 1981).

In addition to the species where the CPD photolyase gene has been isolated, CPD photolyase activity has been studied indirectly in many other species (Table 1).

Table 1:
Representative examples of organisms where photolyase has been isolated
(Please note: this is an incomplete list.).

CPD photolyase	(6-4)photolyase
Arabidopsis thaliana (G.B. Sancar personal communication)	*Arabidopsis thaliana* (Todo et al. 1997)
Drosophila melanogaster (GB. Sancar personal communication)	*Drosophila melanogaster* (Todo et al. 1997)
A. nidularis (G.B. Sancar personal communication)	*C. auratus*, goldfish (G.B Sancar personal communication)
E.coli, bacteria (GB. Sancar personal communication)	*Danio rerio*, zebra fish (reviewed by Todo 1999)
S. cerevisiae, yeast (G.B. Sancar personal communication)	*Xenopus laevis*, frog (reviewed by A. Sancar 2000)
Opossum (Mitchell and Nairn 1989)	Rattlesnake (reviewed by A. Sancar 2000)
	Homo sapiens (homologue, no repair function, Todo et al. 1997)

In these, and other such studies, DNA is extracted from UVB irradiated cells and CPD dimers are assayed. The concentrations of dimers and the rates of dimer formation are compared between treatments incubated in presence and absence of UVA. For many

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species, significantly lower concentrations of CPD dimers are measured when organisms were incubated under photoreactivating conditions, suggesting that CPD photolyase is present in the genome and active in mitigating DNA damage by UV-B.

Effects of temperature on UVR stress in aquatic organisms

See Part III, Introduction

INTRODUCTION TO THIS STUDY (For more comprehensive introduction, See Part III):

Understanding the ecology of DNA damage and repair is essential to predicting how organisms will respond to the altered UVR and temperature regimes of a changing climate. It is known that *Daphnia* exhibits better survival from UVB-induced damage in the presence of longer wavelength repair radiation, which indicates that PER mediates DNA damage in this species (Williamson et al. in press). This "cosmopolitan" planktonic crustacean is one of the dominant primary consumers in freshwater lakes worldwide, and is therefore both a convenient and ecologically important model study organism for understanding the ecology of DNA repair. *Daphnia* is an appropriate model study organism for PER research for three reasons: (1) it exhibits sensitivity to UVR under typical ambient surface water conditions (Williamson and Leech 2001), (2) repair accounts for a large proportion of its UVR tolerance (Grad et al. in press) and (3) it exhibits temperature-dependent survival under UVR stress (DeLange et al., in preparation).

In this study, the effects of temperature on UV-induced DNA damage were examined in *Daphnia*. Like other enzyme-catalyzed reactions, DNA repair mechanisms may be sensitive to temperature. While the temperature dependence of UV-induced DNA damage and repair in *Daphnia* are unknown, Malloy et al. (1997) have suggested that the rate of PER increases with temperature in Antarctic and temperate fishes, as well as in krill.

RESEARCH QUESTIONS:

In order to address the question of how UVR tolerance might be affected by changing environmental conditions, a series of experiments were conducted to better characterize photoenzymatic repair (PER) in *Daphnia*. PER is known to increase survival in *Daphnia*, but several key questions need to be answered before we can predict how *Daphnia* might respond to changing UVR regimes. These questions include:

1) What is the wavelength-specific impact of UVR on damage and repair in *Daphnia*?

2) What are the relative contributions of NER and PER to the UVR tolerance of *Daphnia*?

3) What is the temperature dependence of PER in *Daphnia*?

To conduct this study, two standard techniques for measuring UVR stress, one molecular and the other organismal, were integrated to develop a novel approach for assessing the impacts of UVR exposure on living organisms under ecologically relevant

experimental environments (See Part II). This avenue of research furthers our

understanding of the interaction of DNA damage and repair from the ecological

perspective.

RATIONALE:

The rationale for designing an organismal study that uses molecular metrics for

evaluating UVR stress is that it addresses a disconnect in the current UVR literature. The

literature is predominantly focused on characterizing dimer induction and repair either *in*

vitro, or *in vivo* with microbes. It has been demonstrated, however, that higher organisms

also respond to UVR. *Daphnia*, for example, have been shown, through whole-organism

studies, to have tremendous photorepair capability. In the absence of repair wavelengths,

Daphnia exposed to damaging UVB radiation survive only minimal exposure intensities.

When repair wavelengths are present, *Daphnia*'s survival following UVB exposure is

greatly enhanced. Photorepair of UVR-induced DNA damage by photolyase is the only

known mechanism that can explain this tremendous difference in survival under

controlled laboratory conditions (i.e. any positive effect of UVA and visible light on

Daphnia's phytoplankton food source is isolated from *Daphnia*'s physiological

response). The role of dimers in the life history of *Daphnia*, however, is not well

understood. Because *Daphnia* species are key components of aquatic food webs and

nutrient cycles, and are nearly ubiquitous in freshwaters in the Northern Hemisphere, it

would be valuable to better understand their molecular responses to UVR stress.

PART II: METHODOLOGICAL INVESTIGATION AND RESULTS OF EXPERIMENTS

This study – one of the first such studies in a freshwater invertebrate zooplankton species – advances the practice of climate change biology by integrating two well-established techniques for assessing UVR stress: one molecular and one organismal. The standard lamp phototron apparatus (Williamson et al., 2001) was used to isolate the light dependent from the light-independent responses of *Daphnia* to different UVR and temperature conditions. An RIA for DNA damage (Mitchell, 1996) was used to quantify the molecular impacts of UV and temperature manipulations on *Daphnia*.

Because the scales of the organismal and molecular assays are so different, the appropriate balance had to be achieved in the experimental design so that the sensitivities and detection limits of the two techniques were not over or under-shot, while maintaining ecologically relevant experimental conditions (i.e. not resorting to extreme conditions to produce treatment responses).

This series of experiments reflects adjustments in several aspects of the methods: the number of *Daphnia* per dimer sample, the number of replicate samples from a given treatment and the timing of sampling (12 or 24 hours after start of exposure). Note that 12-hour samples were collected immediately following the end of the 12-hour period of UVB lamp exposure.

The standard protocols for the lamp phototron and the dimer analysis are presented below. Throughout the course of this study, the UVR exposure levels of the UVB lamp in the lamp phototron were adjusted to develop a protocol that has yielded

valuable data. Specific details of the methods used for each experiment are included in an appendix to this document. The appendix includes a brief description of each experiment, with objectives, hypotheses, preliminary data, concise protocol of methods, results and significance.

GENERAL METHODS:

Experiments were conducted using adult female (egg-bearers or equivalent size) *Daphnia pulicaria* from Dutch Springs (Bethlehem, PA), a spring-fed quarry. *Daphnia* were collected from Dutch Springs (off steel pier to 20 meters) with a 48/202 um bongo net 1-3 days prior to the experiment. Collected organisms were filtered through a 202 or 363 um mesh to isolate larger adults. The isolated adults were incubated in 4 L aquaria (with either 0.2 um filtered spring water or 37 um-filtered water from Dutch Springs), and fed with one of two cultured algae: 1) *Cryptomonas reflexa* (C.E. Williamson lab culture, origin: White Acre Pond, Saucon Valley , PA) or 2) *Ankistrodesmus sp.* (R.E. Moeller lab culture, origin S. Kilham lab culture).

The solar and lamp phototrons (described below) were used to conduct experiments to address the research questions presented in Part 1 (repeated below):

1) What is the wavelength-specific impact of UVR on damage and repair in *Daphnia*?

2) What are the relative contributions of NER and PER to the UVR tolerance of *Daphnia*?

3) What is the temperature dependence of PER in *Daphnia*?

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QUESTION 1:

<u>Solar phototron and biological weighting functions (BWFs) (Williamson et al. 2001):</u>

To assess the effects of spectral composition and irradiance of UVR on DNA repair in *Daphnia*, experiments were designed, according to the protocol described by Williamson et al. (2001), in order to generate independent biological weighting functions (BWFs) for damage and repair. A BWF is a function that determines the wavelength-specific responses of an organism to different portions of the solar spectrum, taking into account both the quality (spectral composition) and quantity (irradiance) of the solar radiation.

The solar phototron apparatus located on the roof of Lehigh University's Williams Hall (Bethlehem, PA) was used to expose organisms to natural solar radiation. Organisms were placed in 40 mL pyrex petri dishes, the outsides of which were painted black to admit only light from directly overhead; Organisms receive all wavelengths of solar radiation from above the dishes. Quartz disks and Schott filters on the tops of the dishes are used to manipulate the wavelengths transmitted. The dishes are placed in a shallow tray of water connected to a circulating water bath to maintain a constant temperature. A PUV 501 (Biospherical Instruments Inc., BSI, San Diego, CA) was used to continuously measure irradiance during the experiment for PAR (400-700 nm) and UVR at 4 wavelength bands (305, 320, 340, 380 nm).

QUESTION 2:

<u>Lamp phototron (Williamson et al. 2001):</u>

The lamp phototron apparatus allows the investigator to manipulate the intensity

of damaging UVB radiation in the presence and absence of longer wavelength

repair radiation, or photoreactivating radiation (PRR), which induces

photoenzymatic repair (PER). The standard lamp phototron uses a level of PRR

that approaches the saturation level for *Daphnia* (Williamson, unpublished data).

In all experiments, the intensity of the PRR was held constant, while the intensity

of the damaging UVB radiation was manipulated to adjust the sensitivity of the

experiment for the survival response.

The lamp phototron apparatus is located inside a temperature and light controlled

growth chamber set to a specified temperature and kept in the dark. The

phototron apparatus consists of a horizontal black acrylic plankton wheel (2 rpm),

which rotates above a lightproof box (foil-covered foam). A UVB lamp

(Spectronics XX15B) is suspended 24 cm above the plankton wheel as a source of

UVB radiation. The UVB lamp is covered with a new sheet of cellulose acetate

prior each 12-hour exposure period to exclude wavelengths of UVB shorter than

295 nm. The box below the plankton wheel houses 4 fluorescent bulbs (2 40W

cool white bulbs and 2 40W Q-panel 340 bulbs) situated 32 cm from the bottom

of the dishes. The box containing the bulbs is ventilated with a thermostatically

regulated fan to prevent heat build-up during the exposure period. 40 holes are cut in the plankton wheel to allow 40, 40 mL quartz petri dishes to rest on the wheel. A hole is cut in the box below the plankton wheel to allow light from below the plankton wheel to reach the dishes inserted in the wheel. A black felt skirt surrounds the plankton wheel and prevents stray radiation from escaping the box. Black disks can be inserted into the holes in the plankton wheel to block repair radiation from reaching selected dishes. A 205 cm high black PVC collar is placed around each quartz petri dish to exclude radiation from the sides of the dishes. Stainless steel mesh screens are placed on top of the quartz lids of the dishes and are used to manipulate the intensity of the UVB radiation that reaches the dishes from above. Fine and medium mesh screens are used to allow known amounts of radiation from the UVB lamp to be transmitted to the dishes. Different mesh sizes are used together to prevent Moiré effects. Control dishes are placed in the incubator adjacent to the plankton wheel and are kept in the dark throughout the duration of the experiment.

QUESTION 3:

Temperature manipulations in the Lamp Phototron:

To examine the effects of temperature on DNA damage and repair, experiments were conducted at a range of ecologically relevant temperatures, using the lamp phototron (see above).

GENERAL METHODS, CONTINUED:

For lamp and solar phototron experiments, approximately 25-50 *Daphnia* were included in each quartz dish intended for dimer sampling and approximately 10-30 *Daphnia* were included in each quartz dish intended for scoring survival.

In addition to the *Daphnia*, DNA dosimeters were concurrently exposed in the lamp phototron to determine the maximum potential for DNA damage in the absence of either photoprotection or repair enzymes. The DNA dosimeter consisted of raw DNA from salmon testes (Carolina Biological Supply) dissolved in sterile 1x SCC buffered solution (10x SCC buffer: 44.5 g citric acid trisodium salt and 90 g NaCl). The dosimeter was prepared according to the protocol developed by Dr. Wade Jeffery (University of West Florida, Center of Environmental Diagnostics and Bioremediation), and demonstrated by Diana Dutt (Lehigh University, Department of Molecular Biology, Bethlehem, PA), at a concentration of approximately 100 ug/mL. Concentration was determined using a spectrophotometer (as demonstrated by Robert Moeller, Lehigh University Department of Earth and Environmental Science).

Organismal data (survival) and/or molecular data (dimer accumulation) were obtained from each experiment. Survival was scored every 24 hours using a dissecting microscope; numbers of live and dead individuals were recorded. An organism was scored as "live" if a heartbeat was observed during 10 seconds of observation at 30x magnification. If no heartbeat was observed after 10 seconds, the individual was scored as "dead" and removed from the experiment.

At appropriate the time intervals (12 and/or 24 hours), individuals were collected

from the experiment to use in samples for dimer analysis. Dead individuals were not

included in dimer samples. Dead individuals were removed and discarded, except when

dead individuals outnumbered live individuals, in which case the dead individuals were

collected and used to make an additional sample with only dead individuals.

Organisms were removed from quartz dishes using a pipette and placed in a 2 mL

microcentrifuge tube. Water was removed from the tube as needed with a small-end

pipette. After all individuals for a given sample were placed in the tube, the remaining

water was removed from the sample using a small-end pipette. The sample was

immediately frozen to preserve DNA.

Following the experiment, samples were sent to Dr. David Mitchell (University of

Texas, M.D. Anderson Cancer Center, Smithville, TX) for photoproduct analyses.

Concentrations per megabase DNA of cyclobutane-pyrimidine dimers (CPDs) and, in

some cases, pyrimidine(6-4)pyrimidone photoproducts ((6-4)s) were quantified in the

Daphnia samples. CPDs were quantified in the DNA dosimeter samples.

RIA for DNA damage analysis (Mitchell 1996):

CPDs and (6-4)s were quantified using a radioimmunoassay (RIA). An RIA is a

competitive binding assay between a radiolabeled antigen ("probe") and an

unlabeled competitor ("standard") for binding to an antibody raised against an

antigen. RIAs are useful for detecting genotoxic DNA damage, and are often

used in human cancer research and for applications in environmental toxicology.

The amount of antigen bound to the antibody is determined by separating the antigen-antibody complex from the free antigen. The amount of the antigen-antibody complex in the presence of a known amount of standard is used to quantify the amount of unknown samples present in the reaction.

The sensitivity of the RIA is determined by the affinity of the antibody, as well as the activity of the antigen. By using a high affinity antibody and probe labeled to a high specific activity, the reaction can be limited so that extremely low levels of damage can be detected in a DNA sample.

DATA ANALYSIS AND INTEGRATION:

Before analyzing survival or dimer data, the data were corrected for their respective dark control values. Therefore, for survival data from a given dish at a given time point, any mortality in the dark controls that occurred at the time point was averaged among the control dishes, and the average was subtracted from each treatment value. Likewise, for the dimer data, background damage measured in the dark controls was subtracted from the treatment values.

JUSTIFICATION OF APPROACH (ECOLOGICALLY RELEVANT EXPOSURE CONDITIONS):

One of the advantages of the lamp phototron apparatus is that it uses exposure conditions that are ecologically relevant, both in terms of the duration and the intensity of

the exposure. The cumulative exposure conditions (with full exposure to the UVB lamp and PRR) are roughly equivalent the cumulative exposure conditions that an organism would receive in the surface waters of a lake at northern temperature latitudes, around summer solstice. See Grad et al. 2001, Figure 2, for exposure spectra of lamp phototron (UVB lamp and PRR bulbs) compared to ambient solar radiation. The exposure intensity in the lamp phototron is constant for the duration of the 12-hour exposure period, instead of varying throughout the day, as with ambient solar radiation.

In contrast, clinical studies of PER typically use short-term (~5 minutes) high intensity exposures to damaging radiation (as UVC), followed by incubations under different conditions for test for repair. UVC radiation from the sun does not reach the earth's surface, and under experimental conditions, it tends to induce (6-4)s with higher frequency than does the damaging UVB radiation from the solar spectrum. In addition, this "pulse-and-response" exposure technique does not allow for simultaneous damage and repair, as would occur under ambient conditions.

For conducting ecological studies, the use of ecologically relevant exposure conditions is key to being able to extrapolate experimental findings to natural systems.

METHODOLOGICAL GOALS:

The integration of the organismal and molecular assays for UVR stress produced a methodological question that required attention throughout the course of the study. This question was: What UVB radiation intensity induces dimers above background

levels, without inducing complete mortality in the experiment before samples are collected?

The goal was to design experiments with UVB lamp exposures high enough to induce DNA damage in *Daphnia* above background levels, and also maintain close to 100% survival of the *Daphnia* until the time that samples were collected (12 and 24 hours following the beginning of the 12-hour exposure period). Only live individuals were sampled to ensure that any damage that was induced, was induced in the presence of the repair processes. The goal was to strike a balance between dimer induction and survival that would achieve two objectives: 1) To allow for treatment effects in the DNA damage samples to be distinguishable from the background levels and 2) To prevent discrimination among *Daphnia* with different amounts of damage within a given treatment (i.e. to prevent DNA damage samples being comprised of only individuals with highly efficient repair, if individuals with less efficient repair were dead and therefore not included in the samples), which would have resulted in an overestimation of repair efficiency.

Because no previous studies had been performed that examine the relationship between survival and DNA damage in *Daphnia*, a range of exposure levels were tested in order to strike the necessary balance between survival and dimer accumulation.

This balance between dimer induction and survival proved difficult to achieve due to the relationship between dimer induction and survival in *Daphnia*. Initial experiments demonstrated that low concentrations of damaged sites are present in *Daphnia* at background levels. Accumulation of dimers above background levels appears to result in

Daphnia mortality. This threshold effect, and its ecological significance, is discussed

further in the section <u>Synthesis of Methodological Development</u>.

In addition to UVB exposure levels, sample size and amount of damaged sites per

sample were taken into consideration in the experimental design. The sensitivity of the

RIA was appropriate for measuring DNA damage in *Daphnia*, as the RIA was sensitive

enough to quantify damaged sites in *Daphnia* DNA at extremely low background levels.

The assay can detect low concentrations of dimers in larger samples (~60

Daphnia/sample), or high concentrations of dimers in smaller samples (~20

Daphnia/sample). Therefore, very low concentrations of DNA damage could by

quantified with the RIA, as long as there were sufficient *Daphnia* DNA in the samples.

In this study, measured background dimer concentrations ranged from around 8 CPDs/mb

DNA to around 34 CPDs/mb DNA with an average of 15 CPDs/mb DNA, while the

highest amount of damage measured in living *Daphnia* was 507 CPDs/mb DNA

(measured in a +PRR sample exposed to 52 KJm^2 UVB, from Exp. 20).

SURVIVAL STUDY (EXP 14/15):

A survival study of the Dutch Springs *Daphnia* pulicaria population was

conducted in order to obtain curves for survival vs. UVB exposure for +PRR and –PRR

treatments. While standard survival experiments have been conducted with the lamp

phototron for *Daphnia catawba* from Lake Giles, the same studies had not been

conducted with *Daphnia pulicaria* from Dutch Springs.

Results and Discussion of Exp 14/15:

This study provided a range of survival responses, from 0% in the treatments with the highest UVB intensities (52 KJm^2 for +PRR and 6 KJm^2 for –PRR), to 71% and 86% survival in the treatments exposed to the lowest UVB intensities (+PRR, exposed to 14 KJm^2 UVB; –PRR exposed to 3 KJm^2 UVB).

Methodological Development from Exp 14/15:

The range of survival responses produced by Exp 14/15 provides information that is valuable for designing future experiments on DNA damage and repair.

As proposed, a series of experiments were conducted to better characterize photoenzymatic repair (PER) in *Daphnia*, in an effort to address the question of how UVR tolerance might be affected by changing environmental conditions. Before beginning dimer experiments with *Daphnia*, a dosimeter test was conducted (Exp 3, see Table 3) that demonstrated that the dosimeter provided data (DNA not contaminated or degraded). A total of twenty completed experiments address the research questions presented in Part 1. A summary table (Table 3) is included that provides a snapshot of each experiment. An appendix to this document provides detailed information that is specific to each experiment, and presents the results with figures. The results are presented and discussed below, in order of research question.

QUESTION I: What is the wavelength-specific impact of UVR on PER?

With the goal of developing BWFs for DNA damage and repair in *Daphnia*, 3

solar Phototron experiments were completed. In these experiments, the solar phototron

apparatus was used to expose *Daphnia* to specific wavelengths of solar radiation. In each

of the experiments, *Daphnia* were exposed to full spectrum radiation for 4-5 hours in the

morning, filters were placed over the dishes in the afternoon to cut off radiation below a

certain wavelength, and the *Daphnia* then received an additional 3-4 hours of incubation

under filter treatments.

Results/Discussion/Methodological Development, Exps 10/13/18:

> Of the three solar phototron experiments conducted, only Exp 10 was analyzed
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> for DNA damage. The data suggest that the exposure levels were too low to
>
> induce differential damage among treatments, or to allow for differential repair
>
> among the treatments. Due to the lag time between the completion of experiment
>
> and the analysis of the samples for DNA damage by the Mitchell lab, an
>
> additional two experiments (Exps 13 and 18) were conducted before data from
>
> Exp 10 were received.

Synthesis of Findings for Question 1:

> Future experiments to test BWFs for damage and repair in *Daphnia* should

attempt to induce levels of dimers high enough to allow for differences in repair

efficiency to be distinguished among treatments. See section Future Directions for

further discussion.

QUESTION 2: What are the relative contributions of NER and PER to the UVR tolerance of *Daphnia*?

The four experiments (Exp 6/7 and 8/9) were conducted to address this question and are discussed below.

In the first set of lamp phototron experiments designed to separate light-independent repair (NER) from light-dependent repair (PER) (Exp 6/7), *Daphnia* were exposed to UV-B radiation (25 KJm^2) for 12 hours in the presence and absence of photorepair radiation (PRR). For an additional 12 hours following the UV-B exposure, half of the organisms from each treatment received additional PRR, while the other half were incubated in the dark. Survival and DNA damage (CPDs) were analyzed in Experiment 7.

Discussion of Exp 6/7:

In previous studies we examined the relative importance of PER and NER to *Daphnia* survival. Preliminary findings also suggest that dark repair (NER) can repair CPDs in *Daphnia*. Because this finding is based on a single, unreplicated sample, however, more extensive experimentation is necessary to determine whether this is a significant finding. While NER and PER may both repair damaged DNA in *Daphnia*, PER appears to repair UVR-damaged DNA more effectively than NER, and therefore contributes more to the UV tolerance of *Daphnia* than does NER. In addition, the preliminary findings suggest that, in

Daphnia, PER may confer approximately a 7-9-fold UV-B tolerance advantage over dark repair and photoprotection alone.

The results also indicate that repair of UV-induced DNA damage in *Daphnia* may occur over a relatively short time-period. Following the 12-hour exposure, the CPD load in *Daphnia* incubated with additional repair radiation did not return to background levels, suggesting that damage would remain unrepaired for the life span of the *Daphnia*. In these experiments, the simulated "twilight" conditions (UV-A radiation + PAR, following a full-spectrum exposure) did not mediate the low level of damage accumulated. There are several possible explanations for this finding. One is that the duration of the experiment was too short for the *Daphnia* to fully recover from the damage sustained, in contrast to a published study in larval anchovy (Vetter et al. 1999), where dimer recovery was followed for four days. However, due to the short life-span of *Daphnia* relative to other higher organisms studied (i.e. fish larvae), 24 hours is likely sufficient for repair to be fully expressed. Another possible explanation for this result is that damage recognition is low when dimers are at a low concentration per mb DNA. An experiment that induces higher amounts of damage might help to resolve this issue.

Methodological development from Exp 6/7:

Due to the fact that there was no difference in CPDs at 24 hours in treatments exposed with additional PRR and those incubated in the dark, the treatments with

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additional PRR from 12-24 hours were eliminated from the design of future experiments.

In an effort to increase CPD concentrations of the +PRR treatments in future experiments, the UVB lamp exposures in the +PRR were increased in from 25 KJm^2 to 32 KJm^2 (Exp 9) and 52 KJm^2 (Exp 8).

In addition, due to the poor survival at 24 hours of the –PRR treatments, in the next set of experiments, the UVB lamp exposures in the –PRR treatments were decreased from 25 KJm^2 to 9 KJm^2 (Exp 9) and 15 KJm^2 (Exp 8).

This follow-up study (Exp 8/9), conducted at higher UV-B lamp exposure levels for the treatments with PRR and lower exposure levels for the treatments without PRR, included analyses of both CPDs and (6-4)s. The experiments were designed to address the more specific questions of 1) whether *Daphnia* utilizes (6-4)photolyase in addition to CPD photolyase and 2) whether dark repair acts on CPDs and (6-4)s in *Daphnia*.

Discussion of Exp 8/9:

NER does not appear to be a significant component of *Daphnia*'s UVR tolerance under the conditions of these experiments. The exposure levels were likely

appropriate to induce enough damage for repair to be exhibited by NER from 12-24 hours, if any such repair were to occur.

Methodological Development from Exp 8/9:

The next logical step in this line of inquiry would be to employ the "pulse and response" type of experimental design used by molecular and microbial biologists to characterize induction and repair of DNA damage over shorter time-scales. This approach is not typically used for multicellular organisms; however, its small size and high repair capability may make *Daphnia* a suitable organism for this approach. This type of experiment would utilize a high-intensity, short-term exposure of damaging radiation, followed by incubations in the dark or in repair radiation, with samples taken at different time points. For example, a five-minute exposure to 3 UVB lamps with approximately 50 KJm^2 output per lamp, might induce a high level of damage without immediately killing the *Daphnia*, allowing for repair to occur during the subsequent 12 hours. Data obtained from such a study would likely yield information about the maximum rates of repair by NER and PER, and could be conducted at a range of temperatures.

Synthesis of Findings for Question 2:

Based on the results of these experiments, dark repair does not appear to be a significant component of UV tolerance in *Daphnia*. The results of this preliminary study of the induction and repair of (6-4)s do not provide evidence for the utilization of (6-4)photolyase by *Daphnia*. In addition, the results do not provide much indication that

repair of CPD and (6-4) lesions by NER occurs following UV-B exposure. The results do support the findings of the initial experiments that suggest that concurrent, not sequential, exposure to repair radiation is necessary for effective PER.

QUESTION 3: What is the temperature dependence of PER?

Experiments 1 and 2 were the initial test of temperature (10 and 20 °C) on dimer accumulation in *Daphnia*. These experiments did not yield any dimer data, however, because the sample size was too small. Experiments 4 and 5 were the first successful test of this hypothesis. In this set of experiments, *Daphnia* and dosimeter were exposed to 25 KJm^2 of radiation from the UVB lamp, in treatments with and without PRR. Experiment 4 was conducted a 20 °C, while experiment 5 was conducted at 10 °C. No replicate samples of *Daphnia* were collected for dimer analysis, and no survival data were collected for the 20 °C experiment (Exp 4).

Discussion of Exp 4/5:

Despite the lack of replicate samples, the dimer data from these experiments clearly demonstrate that *Daphnia* repair CPDs using the light-dependent mechanism, CPD photolyase. The corresponding survival data also suggest that light-dependent repair of dimers is necessary for survival under these experimental conditions. While the results suggest that any temperature effect between 10 and 20 °C may be of little consequence, the relationship between temperature and dimer accumulation remains unclear after this experiment. To

better address this question, more comprehensive studies are needed that include a wider range of temperatures.

Methodological Development from Exp 4/5:

The results of these experiments confirm that phototron and CPD analysis techniques can be incorporated into a single experimental framework. Since no background information is available about magnitude or timing of the induction or repair of UVR-induced DNA damage in *Daphnia*, these data provide a starting place for designing future experiments. The results of this initial set of experiments help to address two methodological issues. First, as evidenced by the measurable levels of background dimers in the DNA of control organisms, this study has demonstrated that *Daphnia* do harbor a low concentration of dimers in their genome in the absence of any acute UVR stress. It is unknown whether these dimers were induced by past UVR exposure in the *Daphnia*'s natural habitat, or if the dimers were induced by a mutagen other than UVR, such as chemical toxin. Second, dimers can be detected, above background levels, in live *Daphnia* exposed in the lamp phototron at 10 and 20 °C, indicating that *Daphnia* are not able to repair all DNA damage as it occurs in the phototron environment, and that differential treatment responses can be obtained using the phototron exposure conditions.

Two additional sets of experiments were conducted to test the question of temperature dependence on repair.

In Exps 11/12, exposure levels of 15 KJm^2 for +PRR and –PRR were chosen in an effort to induce measurable levels of dimers while maintaining high survival at 12 and 24 hours. The experiment was designed and exposure levels chosen in order to obtain both CPD and survival data.

Discussion of Exp 11/12:

These results indicate that a lower level of UVB lamp exposure in the –PRR may be more appropriate to examine dimer formation in *Daphnia* above its mortality threshold.

Methodological Development from Exp 11/12:

The next set of temperature comparison experiments will repeat the +PRR exposure at 15 KJm^2, but decrease the –PRR exposure to a lower level, in order to maintain some survival in the treatment throughout the duration of the experiment.

The temperature comparison was repeated in an attempt to better "match" the survival responses of the +PRR and –PRR treatments.

The rationale for attempting to balance the responses of the +PRR and –PRR treatments was to define the relationship between survival and DNA damage accumulation in *Daphnia*. An experiment that induces complete mortality in all

treatments of *Daphnia* is not sensitive enough to quantify the survival response and correlate it with dimer accumulation.

Exp 11/12 was followed by a second set of experiments designed to conduct the temperature comparison. In these experiments, UVB lamp exposure levels of 15 KJm^2 (+PRR) and 7 KJm^2 (-PRR) were chosen in an effort to induce measurable levels of dimers while maintaining high survival at 12 and 24 hours.

The rationale for this decision was based on the survival of *Daphnia* in Exp 11/12 +PRR, and the survival curve for *Daphnia* determined by Exp 15.

Discussion of Exp 16/17:

Results of Exp 16/17 did not show statistical differences between 10 and 20 °C. Two interesting results, however, were obtained from this set of experiments. The first result is that CPDs in samples collected at 12 hours , while they were very low, were still higher background concentrations. CPDs in samples collected at 24 hours, however, were not separable from background. This result, in contrast to the findings of the previous experiments (Exp 8/9) suggests that NER may repair CPDs in *Daphnia*, at least at low concentrations. The high degree of variability within the treatments, however, precludes statistical significance of this finding. The variability of the results could possibly be due to the low level of damage induced in these experiments, as variability may increase as the concentrations approach the analytical detection limit. The second result

was that nearly 100% of the *Daphnia* in the treatment with repair radiation survived to day 5, despite the low levels of dimers in their DNA that had been measured at 12 hours. This result suggests that the exposure levels used in these experiments may fall just below *Daphnia's* mortality threshold.

Methodological Development from Exp 16/17:

Exposure levels of 15 and 7 KJm^2, for +PRR and –PRR, respectively, were chosen in an effort to induce measurable levels of dimers while maintaining high survival at 12 and 24 hours. This approach was not successful in providing dimer results that were statistically different between the two treatments, likely due to low levels of damage induced.

Results of Methodological Investigation (Synthesis of Methodological Development findings from Experiments 1-9, 11-12 and 14-17:

An overview of the experiments, through Exp 17, revealed several notable results. One is that the balance between survival and dimer accumulation was difficult to achieve using this combination of experimental techniques, in part because there was a long lag time between when experiments were conducted and when dimer data were available. During the lag time between data deliveries, experiments continued to be conducted, adjusting the UV exposure levels based on observed survival response.

Dimers can either be quantified in a larger number of low–exposure *Daphnia*, or a smaller number of high–exposure *Daphnia*. The lamp phototron apparatus constrains the

number of *Daphnia* used in each experiment. A single sample of approximately 60-100 large, adult *Daphnia* is necessary to yield dimer data at measurable concentrations.

Daphnia are proficient at PER, and are therefore able to maintain a low level of dimers in their genome, even under UVR exposure. It appears, however, that *Daphnia* exhibit a damage threshold; any accumulation above background levels results in mortality.

In addition to changes in UVB exposure levels, experiments reflect fine-tuning in several aspects of the methods: the number of *Daphnia* per dimer sample (sample size) and how it relates to total damage in sample, the number of replicate samples per treatment and the timing of sampling (12 or 24 hours after start of exposure).

Throughout the course of the study, I also considered whether CPD accumulation, a measure of direct damage to DNA by UVR, appeared to be a good proxy for whole-organism response to UVR (product of both direct and indirect damage by UVR). The two techniques commonly used to assess UVR-induced damage, CPDs and mortality, were compared. If CPD accumulation is found to be a good indicator of total damage by UVR, then measurements of CPDs could be ecologically relevant indicators of the UVR damage sustained by natural assemblages of multicellular organisms. A qualitative assessment of the data suggests that CPD accumulation is not a good proxy for whole-organism response to UVR. The results suggest that *Daphnia* exhibit a threshold effect for DNA damage, whereby any accumulation of dimers above background level produced mortality in the treatments. In experiments where dimers were induced at

concentrations statistically separable from background levels, mortality was evident at 24 hours (12 hours after the end of the 12-hour exposure period).

Dimers in Daphnia

The determination of the effects of CPDs on the fitness of an organism under a range of UVR conditions is necessary before the large-scale impacts of climate change can be fully understood (Malloy et al. 1997). Prior to this study, little was known about the role of dimers in the life history of *Daphnia*. A gap exists in the current literature about the ecological relevance of CPDs to mortality. The majority of studies examine either molecular or organismal responses to UVR (CPDs or (6-4)s). Few studies have incorporated both into a single experimental framework.

The question remains whether CPD measurements in organisms such as *Daphnia* are a good indicator of UVR dose in natural populations. DNA dosimeter has successfully been used as a biological endpoint for measuring UVB exposure in marine surface waters (Regan et al. 1992). Diel cycles of CPDs have been quantified in natural assemblages of marine bacterioplankton (Jeffrey et al. 1996). The levels of CPDs measured in bacterioplankton tracked DNA damage in naked DNA dosimeter, and were good indicators of UVB exposure in marine surface waters.

In contrast to the diel cycles of CPDs measured in naked DNA dosimeter and bacterioplankton, the diel cycles of CPDs measured in larval anchovy did not reflect cumulative UVR dose. In larval anchovy, CPDs were a good indicator of dose rate, but a poor indicator of cumulative dose. By the end of the day, CPDs were at their lowest

values, when UVR dose-rate was low but cumulative UVR dose was highest. This relationship is likely to be true for *Daphnia* as well as larval anchovy. *Daphnia* survival is also affected by dose-rate, not cumulative dose (Grad et al. 2001). The results of this study with *Daphnia* and the study of larval anchovy (Vetter et al. 1999) provide evidence that DNA damage measured in organisms with substantial PER capacity will not be a good metric for UVR in natural systems.

In *Daphnia*, it also appears that the relationship between dimer accumulation and survival is characterized by a threshold effect. Results of Exp. 16/17, in particular, suggest that, at 12 hours, dimer concentrations in the treatments were slightly elevated from the background levels. Samples taken 12 hours later suggest that NER may have repaired this low level of damage. This was the only experiment where the repair system appeared to be overwhelmed (made evident by dimer accumulation at 12 hours), where repair (return to background levels at 24 hours) and recovery (survival of *Daphnia* for five days) followed. The results of this experiment, compared with others where dimers were induced at higher concentrations and *Daphnia* did not survive, suggest that recovery from DNA damage is possible below some threshold concentration. In this study, dimers measured in *Daphnia* DNA represented damage in excess of repair. This unrepaired damage indicates that the repair mechanisms were overwhelmed, and mortality followed exposure by hours or days, depending on the intensity of UVB radiation used in the exposure, and the temperature of the experiment. The experiments in this study, however, were not designed to specifically test the threshold effect of *Daphnia* to UVR, and the results were not statistically significant. A recent study designed to relate

mortality to DNA damage that was conducted in sea urchin has demonstrated a strong

threshold effect (D. Mitchell, personal communication, Karentz and Mitchell, in press)

like the one that may characterize the relationship of DNA damage to mortality in

Daphnia.

Validation of Methods using Temperature Comparison:

In order to fully address the first two study questions, experimental design would

have to be altered to account for the sensitivity of *Daphnia* to dimerization in their DNA.

See section Future Directions, below, for further discussion of possible approaches for

future research in these areas.

Results of this study suggest that, if UVB exposure parameters are optimized for

maximum dimer induction, with high survival following the 12-hour exposure period, the

lamp phototron is an appropriate method for testing the temperature dependence of PER,

by exposing *Daphnia* with and without repair radiation at a range of temperatures.

In an attempt to maximize amount of dimer data returned from experiments with

Daphnia, the highest exposure levels were chosen for future studies, to allow for close to

100% survival at 12 hours. For this reason, Exps 19/20 were exposed to 25 and 52 KJm^2

UVB in the absence and presence of PRR. Results and discussion of Exp 19/20 are

presented in Part 3.

These experiments were successful primarily because a balance was achieved

between dimer induction and survival. Full mortality of exposed *Daphnia* did result from

this experiment, but nearly 100% of +PRR and –PRR were alive at 12 hours for sampling. Mortality was expressed the following day.

Problems Encountered:

The most significant problem encountered during the course of this study was striking a balance between dimer accumulation in the DNA and survival of *Daphnia*. The experiments suggest that there may be a threshold of dimer accumulation at which *Daphnia* mortality results. Identifying the UVB lamp exposure intensities that approach but do not cross this threshold can be a challenge, especially when working with field-collected *Daphnia*, which may vary seasonally in their UVR tolerance. The investigator must also be conscious of working within the detection limits of dimer assays. For example, in order to detect very low levels of damage, such as those that would likely be induced by natural solar radiation, a large sample size is necessary.

Field-collected organisms are likely more appropriate for this type of study than are lab-cultured organisms, for two reasons. First, the sample size is relatively large compared with standard lamp phototron experiments (an average of 60-120 *Daphnia* per sample, 3 replicate samples per treatment), so minor variation among individuals is less significant. Second, the large numbers of *Daphnia* required for each experiment (approximately 2000-3000 large adult *Daphnia* per experiment) make field collection much more time and resource efficient than lab culturing.

Another consideration for the investigator is the complexity of working in collaboration with another lab. While the collaboration leads to exciting exchange of

information and ideas, and enriches the study with the broader perspective that results from conversation between organismal/ecological and molecular/medical investigators, it does lead to lag-times between sample collection and analysis (see Table 1 for dates of experimentation and sample analysis) that can sometimes frustrate efforts to run follow-up experiments in rapid succession. Because communication between the labs is critical, an investigator new to this type of work would benefit from visiting the Mitchell lab and learning the techniques first-hand, in order to facilitate dialogue between the labs.

Future Directions:

Results of this study provide valuable information for conducting future studies on UVR-induced DNA damage and repair in *Daphnia*. Issues related to the third research question (What is the temperature dependence of PER in *Daphnia*?) are discussed in the discussion section of Part 3. For the first and second research questions, improved experimental approaches for follow-up studies are described below.

Question 1: What is the wavelength-specific impact of UVR on damage and repair in *Daphnia*?

Two potential approaches to BWF experiments may provide more useful information than did the current study. One would be to expose *Daphnia* in lab to several hours (standard lamp exposure, ~6-12 h) or to a pulse of intense exposure (~5 minutes) of damaging UVB radiation, then place them on the rooftop in a

solar exposure apparatus for repair. A second would be to expose *Daphnia* to UVB lamp while outdoors under natural solar radiation.

Question 2: What are the relative contributions of NER and PER to the UVR tolerance of *Daphnia*?

The logical next step in this line of inquiry would be to employ the "pulse and response" type of experimental design used by molecular and microbial biologists to characterize induction and repair of DNA damage over shorter time-scales. This approach is not typically used for multicellular organisms; however, the small size and high repair capability of *Daphnia* may make it a suitable organism for this approach. This type of experiment would utilize a high-intensity, short-term exposure of damaging radiation, followed by incubations in the dark or in repair radiation, with samples taken at different time points. For example, a five-minute exposure to 3 UVB lamps with approximately 50 KJm^2 output per lamp, might induce a high level of damage without immediately killing the *Daphnia*, allowing for repair to occur during the subsequent 12 hours. Data obtained from such a study would likely yield information about the maximum rates of repair by NER and PER, which are needed in order to calculate the relative importance of the two repair mechanisms and their roles in regulating diel cycles of DNA damage. Repair rates would provide the information to calculate the energy costs of the two repair mechanisms on a diel basis and determine the amount of damage that could be repaired in *Daphnia* given the available energy supplies of food and light. Such experiments could be conducted at a range of temperatures.

In addition to the suggestions described above for future experiments using the same basic research questions, general ideas for future research are described below.

Use of repair deficient mutants

One avenue of research that would likely lead to numerous fruitful experiments with damage and repair in *Daphnia*, and would facilitate temperature studies, would be to isolate organisms without repair capabilities (repair deficient mutants for NER and PER). Because PER can be effectively shut down in the absence of repair radiation, the NER minus mutants would be the logical first priority.

Mutants could be made by irradiation or exposure to chemical mutagens. Mutants could be determined by measuring gene activity by conducting mRNA analysis (from G. Sancar, personal communication). Because *Daphnia* can reproduce clonally, a similar procedure could be used to isolate mutants in *Daphnia*, as was used in yeast and *E. coli*.

PER deficient mutants could be looked for in dark systems, such as extremely high DOC lakes and caves. Inbreeding techniques could be used to accelerate search for mutations. If the location of PER in the genome of *Daphnia* were known, then that location of the chromosome could be targeted by radiation or a chemical mutagen.

PER deficient mutants could be used in experiments using the pulse-and-response approach described above. In fact, comparing the –PER mutants to +PER Daphnia in the presence of light and food would allow you to separate the effect of +PER (or photosynthetically active radiation, PAR) on food supply, and thus firmly reject the alternative explanation of why visible light is beneficial to Daphnia.

Threshold effect

The findings of this study suggest that the relationship between DNA damage and survival in Daphnia is characterized by a strong threshold effect; that is, accumulation above background levels results in mortality. Describing this threshold would illustrate the different rates of PER and NER needed to for survival in Daphnia and would provide the information necessary to calculate the energy requirements for each repair process in Daphnia.

Table 2: Summary of Experiments: Exposure conditions, Dates and Problems.

Experiment	+PRR (KJ/m² of UVB lamp)	–PRR (KJ/m² of UVB lamp)	Temperature (°C)	Date of experiment	Date of data analysis by Mitchell lab	Methodological and Other Problems
1	25	25	20	7/13/00	NA	Too little biomass
2	25	25	10	7/14/00	NA	Too little biomass
3	25, 52	25, 25	20	9/10/00	10/4/00	None
4	25	25	20	10/11/00	10/25/00	No replicate samples
5	25	25	10	11/1/00	11/20/00	
6	25	25	20	12/14/00	1/23/01, 2/6/01	No samples of –PRR at 24 h
7	25	25	20	12/28/00	1/23/01, 2/6/01	No replicate samples of -PRR at 24 h
8	52	15	20	5/17/01	10/16/01, 12/5/01	Poor replication between experiments
9	32	9	20	6/1/01	10/16/01, 12/5/01	
10	solar	solar	20	6/8/01	7/10/01, 7/13/01	No significant differences among treatments
11	15	15	20	6/27/01	NA	Poor survival in controls
12	15	15	10	6/27/01	NA	Poor survival in controls of companion exp (exp 11)
13	solar	solar	20	7/3/01	NA	No survival data
14	NA	1, 2, 3 and 6	20	7/12/01	NA	None
15	15, 25, 32, 52	3, 4, 5 and 6	20	7/20/01	NA	None
16	14	9	20	7/25/01	9/18/01	Low damage levels, large standard deviation
17	14	9	10	7/26/01	9/18/01	values within treatments
18	solar	solar	20	8/2/01	NA	None
19	52	25	20	11/14/01	1/4/02, 1/16/02, 1/31/02, 2/15/02	None
20	52	25	10	11/15/01	1/4/02, 1/16/02, 1/31/02, 2/15/02	None

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Table 3: Summary of Experiments: Results, Discussion and Contribution to Methodological Development.

Experiment	Results and Discussion	Contribution to Methodological Development	Appendix Page No.:	Associated Tables and Figures:
1	More biomass is needed for dimer analysis.	Larger sample size is necessary.	95	None
2				
3	Dosimeter samples will be included in all dimer experiments with *Daphnia*.	Dosimeter test in lamp phototron indicates that technique is working (DNA is not contaminated or degraded.)	98	Figure 4 – CPD damage Tables 6 and 7 – ANOVA
4	Little difference between 10 and 20 °C	Repeat temperature experiments	102	Figure 5 – CPD damage Table 8 – ANOVA
5				
6	No live individuals in -PRR for sampling at 24 hours. Additional PRR following exposure period does not affect concentration of dimers accumulated during exposure.	Higher UVB exposure for +PRR treatment and lower UVB exposure for -PRR treatment	108	Figure 6 – CPD damage Figure 7 – CPD damage Tables 9 and 10 – ANOVA
7				
8	No clear evidence for 6-4photolyase activity, no clear evidence for post-exposure NER	Future direction: pulse and response exp	115	Figures 8 and 9 – CPD damage Figures 10 and 11 – (6-4) damage Tables 11 and 12 – ANOVA
9				
10	No significant differences among treatments	Higher exposure levels for solar experiments	122	None
11	-PRR may be more appropriate to examine dimer formation in *Daphnia* above its mortality threshold.	Repeat temperature experiments	126	Figure 12 – survival
12				
13	Data not analyzed	Data not analyzed	130	None
14	A range of survival: from 0% in the treatments with the highest UVB intensities (52 KJm-2 for +PRR and 6 KJm-2 for –PRR), to 71% and 86% survival in the treatments exposed to the lowest UVB intensities (+PRR, exposed to 14 KJm^2 UVB; –PRR exposed to 3 KJm^2 UVB)	Extremely low levels of UVB are required for survival to 5 days (20 °C)	134	Figures 13, 14 and 15 – survival
15				
16	Some evidence for NER at 10 and 20 °C, variability of the results could possibly be due to the low level of damage	Repeat temperature experiments with CPD and (6-4) analyses	139	Figure 16 – survival Figure 17 – CPD damage Tables 13 and 14 – ANOVA
17				
18	Data not analyzed	Data not analyzed	144	Figure 18 – survival
19	Evidence for repair of CPDs and (6-4)s at 20 °C, and not at 5 °C; Evidence for temperature effect on damage	These exposure levels yielded good results and will be used for additional experiments at a broader range of temperatures.	149	Figure 1 – survival Figure 2 – CPD damage in dosimeter Figure 3 – CPD and (6-4) damage in *Daphnia*
20				

PART III: EXPERIMENTS 19 AND 20:

EFFECT OF TEMPERATURE ON REPAIR OF UVR-INDUCED DNA DAMAGE IN DAPHNIA

INTRODUCTION TO EXPERIMENTS 19 AND 20:

As global warming is raising global surface temperatures, depletion of the stratospheric ozone layer is also allowing increased levels of ultraviolet radiation (UVR) to reach the earth's surface. These increases in global temperature and ambient UVR are likely to simultaneously affect life at all levels, from the biomolecule to the ecosystem.

Changes in temperature and ambient UVR have already been observed at north temperate latitudes. A regional warming trend has caused lakes in the Northern Hemisphere to lose their ice cover an average of 6.5 days earlier in the spring than 100 years ago (Magnuson et al. 2000), exposing lakes to UVR closer to the late winter–early spring, when the ozone hole reaches its maximum extent. Ozone-related changes in ambient UVR remain an important factor when considering future impacts of environmental conditions on organisms. Although CFC emissions have been reduced in recent years, any recovery of the ozone hole is likely to be slow (Madronich, 1998).

While water provides a more thermally stable environment than air, even small perturbations in water temperature can have far-reaching effects on organisms in aquatic ecosystems. For example, the probability of a clear-water phase increases with lake water temperature (Scheffer et al. 2001). In north temperate lakes, temperature has risen significantly over the past decades, a phenomenon that is highly correlated with oscillations in the North Atlantic climate system. A climate-related shift in the timing of

clear water phases in the shallow lakes has been documented (Scheffer et al. 2001). Such a shift in the timing of the clear water phase likely reflects a change in the temperature and UVR exposure conditions of *Daphnia* populations, with impacts on phytoplankton and planktivorous fish, as well (Lampert et al. 1986, Luecke et al. 1990).

Independent increases in both ambient UVB radiation and global annual mean air temperatures are projected to occur during the next 100 years; UVB radiation is projected to increase at the earth's surface due to stratospheric ozone depletion (increases in UVB radiation in the Arctic have already been reported (Kerr et al. 1996)), while climate change models predict global mean annual temperatures will increase by 1.4–5.8 °C by 2100 (IPCC WG2 TS 2001). In addition, it is very likely (90-99% confidence interval, see IPCC WG TS for explanation of model parameters and confidence intervals) that the projected temperature increase would affect three types of extreme climate events: 1) increased minimum temperatures, 2) increased maximum temperatures and 3) more intense precipitation events.

It is likely that mid-latitude continental interiors, such as the Midwestern United States, will experience increased risk of summer drought conditions (IPCC WG2 TS 2001). Such drought conditions would likely decrease transport of DOC from terrestrial to aquatic systems during summer months, when UVR is most intense.

Although terrestrial production may increase with the predicted climate changes, due to increased growth efficiency at higher temperatures, and atmospheric CO_2 enrichment has been shown to increase root exudation of DOC (Schlesinger and Lichter 2001), additions to the terrestrial carbon cycle may not translate into higher DOC retained

in aquatic systems. DOC is moved from terrestrial to aquatic systems primarily by precipitation. The higher frequency, more extreme drought and flooding conditions predicted for the north temperate latitudes may result increased DOC fluctuations, and possibly shorter retention times, in aquatic systems. Such climate-related changes in the timing and quantity of DOC transported from terrestrial to aquatic systems could lead to increased UVB penetration in aquatic systems (Williamson et al. 1999, Pienitz and Vincent 2000).

This scenario would affect the UVR attenuation depth of lakes during the summer, thereby increasing the risk of UVR-related impacts on aquatic organisms. The impact of higher ambient UVB radiation is likely to be increased by climate-related changes in water transparency. In general, models indicate that biological effects of UVR exposure may increase 2% for every 1% decrease in ozone, and above a 5% decrease in ozone, the increase in biological effects may be exponential (reviewed by Lloyd 1993). Increased UVR attenuation due to reductions in DOC may force zooplankton, such as *Daphnia*, into colder water below the thermocline, in order to avoid UVR (Williamson et al. 1996).

While climate-related changes in temperature could potentially affect the equilibrium constants of enzyme-catalyzed reactions and alter the effectiveness of DNA repair mechanisms (Hoffman and Parsons 1991), the more extensive impacts will likely come from the interaction of concurrent changes in temperature and UVR. The interaction of temperature and UVR changes is likely to intensify the impacts of climate change on aquatic systems. This interaction is likely to be system-specific and depend

largely on DOC inputs for terrestrial sources. For a lake ecosystem in the temperate

latitudes of the Northern Hemisphere, there is evidence for a seasonal lag in peak

epilimnetic (surface) water temperatures relative to peak levels of ambient UVB radiation

(Williamson et al. in review). Epilimnetic water temperatures for a north temperate lake

(based on 4-year average monthly values for Lake Giles, Blooming Grove, PA) peak in

late summer, between the end of July and the beginning of August, and then remain high

(~20-25 °C) through September, before dropping below 20 °C around October. In

contrast, irradiance of UVR (at 320 nm) peaks in June (based on modeled values using

the RT Basic radiative transfer model, Biospherical Instruments, San Diego, CA). For

example, similar UV320 irradiance levels reach the lake's surface in April and late

August, but the water temperature is significantly higher in August than in April. This

relationship between water temperature and UVR results in a much higher UV:T ratio in

the spring than in the fall.

Few studies have related molecular responses to organismal responses for UVR

stress in any species. Thus, this link between molecular and organismal-level responses

to UVR continues to be a gap in our understanding of how organisms respond to climate

variables across multiple scales of biological organization. The need for such across-

scale studies was recently identified by the IPCC as a high research priority (IPCC, WG2

Report, 2001). This study uses molecular metrics for UVR damage to explain

organismal-level responses to UVR and temperature.

UVR directly affects organisms by damaging DNA. Because DNA absorbs

strongly in the high-energy UV-B range (290-320 nm) (Giese 1957), it is vulnerable to

ozone-related changes in the solar spectrum. When UVB is absorbed by DNA, anomalous structures form in the DNA molecule. Two adjacent pyrimidine nucleotide bases (most often including at least one thymine with either uracil or cytosine) become linked, forming a dimer, also known as a photoproduct. Dimers bend the phosphate backbone of the DNA molecule, which disrupts the activity of DNA polymerase and interferes with gene transcription (reading the gene code for making proteins). This interference results in mutation and cell death. Two types of dimers are commonly induced, cyclobutane pyrimidine dimers (CPDs) and pyrimidine(6,4)pyrimidone photoproducts (6-4s), both of which are examined in this study. While CPDs account for the majority of photoproducts formed (80-90%), (6-4)s can be up to 300 times more effective in blocking DNA polymerase, and therefore more cytotoxic than CPDs (Mitchell and Nairn 1989).

To maintain the integrity of their genome under UVR exposure conditions, organisms can employ one or more defensive strategies to mediate UVR stress (Zagarese and Williamson 1994). Before UVR exposure occurs, an organism can prevent genetic damage through behavioral avoidance of UVR. During UVR exposure, organisms can use photoprotective compounds to protect their DNA. Following genetic damage avoidance, organisms can employ repair mechanisms to return damaged DNA to an undamaged state.

Two types of molecular mechanisms typically repair UVR-induced DNA damage. The first mechanism, nucleotide excision repair (NER), is a complex, multi-protein, multi-step pathway that is powered by chemical energy (ATP), and is therefore an

energetically costly process for the cell. This mechanism is found universally in all taxa, but it is not specific for UVR-induced DNA damage (Sancar 1994a). The efficiency of NER in repairing UV-induced DNA damage can vary with taxon, tissue and age (reviewed by Mitchell and Karentz 1993). The second mechanism for repairing UV-induced DNA damage is known as photoenzymatic repair (PER). In this single-enzyme system, photolyase harnesses photon energy from UV-A and visible light and uses it to power a self-sustaining light-driven reversion reaction to return damaged DNA to its original state *in situ*, without the synthesis of new DNA. PER is specific for UVR-induced DNA damage, but is not exhibited by all taxa (Sancar 1994b). Thus far, photolyase activity has been identified in a number of organisms as diverse as archebacteria and marsupials. It is lacking, however, in certain species, including several species of diatoms, a couple angiosperms, the nematode *Caenorhabditis elegans*, and probably all placental mammals, including humans (reviewed by Mitchell and Karentz 1993, G. Sancar, personal communication).

Although PER appears to be an enzymatically simple process, its functionality differs across taxa. For example, in two closely related marine fishes, rates of PER differed 5-fold (Reagan et al. 1992). These differences among organisms may be due to differences in action spectra, constitutive levels of photolyase, and cofactor concentrations. In *Euglena* and *E. coli*, for example, the peak in the PER action spectrum is around 380 nm, while in *Neurospora* and *Streptomyces*, it is closer to 440 nm. Photolyase has also been found in organisms never exposed to solar radiation, such as

soil bacteria and blind cave fish. Such incongruous results suggest that PER may have a second function, such as stimulating NER (*in vitro*) (Sancar 1994b).

Like other enzyme-catalyzed reactions, DNA repair mechanisms are sensitive to temperature. The sensitivity of enzymes to temperature was first recognized by Svante Arrhenius in 1889, and has been the subject of interest ever since. According to the basic theory of enzyme kinetics, enzyme activity increases with temperature, typically between 0 and 40-45 °C, and doubles approximately with every 10 °C (reviewed by Keeton and Gould 1996). The temperature dependence of PER and NER, in particular, have been recognized for quite some time; NER was shown to increase in yeast between 5 and 28 °C (Giese 1957), while PER in mold spores also exhibited temperature dependence (Coohill and Deering 1969). The pronounced temperature dependence of PER has been inferred from early studies using free cell extracts (Harm 1980, reviewed by Langenbacher et al. 1997). In a more recent study using ultrafast spectrophotography, the rate of the primary electron transfer that initiates the reversion reaction of photoreactivation decreased with temperature (Langenbacher et al. 1997).

While repair mechanisms are temperature dependent, UVR-induced damage of *in vitro* DNA is independent of temperature (D. Mitchell, personal communication), suggesting that increased ambient temperatures may facilitate repair in exotherms; however, few studies have directly addressed the question of temperature dependence of PER in any aquatic organism. In Antarctic zooplankton (krill, *Euphausia superba*), as well as in Antarctic and temperate ichthyoplankton (juvenile rockcod, *Notothenia coriiceps*; juvenile icefish, *Chaenocephalus aceratus*; killifish, *Fundulus heteroclitus*),

the rate of PER increased with temperature (Malloy et al. 1997). A study of *Palmaria palmata* (marine red alga) provided evidence that the temperature optimum for repair by PER is different for CPDs and (6-4)s; the temperature optimum of CPD photolyase is closer to 12 °C, while optimum of 6-4 photolyase is closer to 25 °C (Pakker et al. 2000).

In this study, *Daphnia*, the water flea, is used as a model organism for studying the temperature dependence of PER (for both CPD photolyase and 6-4 photolyase). This "cosmopolitan" planktonic crustacean is the most widespread and abundant genus of crustacean zooplankton in lakes in the northern hemisphere, and is one of the dominant primary consumers in freshwater lakes worldwide (Williamson et al. 1994). *Daphnia* promote biodiversity across trophic levels. Grazing pressure from *Daphnia* contributes to helps sustain phytoplankton diversity. In addition, *Daphnia* support the biodiversity of larger piscivorous fish, as they make up a major component of the diet of planktivorous fish, which are the prey of the larger predatory fish species (reviewed by Dodson and Frey 2001). Therefore, it is both a convenient and ecologically important model study organism for understanding the ecology of DNA repair. While previous studies have examined the behavioral responses of *Daphnia* to visible light, and recent work has focused on the behavioral responses of *Daphnia* to the ultraviolet portion of the solar spectrum (Leech and Williamson 2001), no studies have quantified the molecular responses of *Daphnia* to UVR. *Daphnia* is an appropriate model study organism for PER-temperature research for three reasons: (1) it exhibits sensitivity to UVR under typical ambient surface water conditions (Williamson and Leech 2001), (2) repair accounts for a large proportion of its UVR tolerance (Grad et al. in review) and 3)

survival under UVR stress increases with temperature from 10 to 25 °C (DeLange, in preparation).

Differential survival between *Daphnia* exposed to UVB radiation alone, and *Daphnia* exposed to UVB with UVA and visible light, has been recognized for over 30 years (Seibeck 1978). The only known explanation for this differential survival under controlled laboratory conditions (i.e. when any positive effect of UVA and visible light on *Daphnia*'s phytoplankton food source is isolated from *Daphnia*'s physiological response) is the stimulation of the photolyase enzyme by UVA and visible light. In the *Daphnia* population used for this study, the LD_{50} (exposure level that would allow for 50% survival at the end of the experiment, 5 days following exposure to UVB lamp) of *Daphnia* exposed to UVR under conditions that stimulated PER was 20 KJm^2, while the LD_{50} of *Daphnia* exposed to UVB radiation under conditions that did not stimulate PER, where photoprotection and NER are the only physiological defense mechanisms available for mediating UVB damage, was 4 KJm^2 (Williamson and MacFadyen, unpublished data). This difference in survival responses provides strong evidence that PER accounts for a much larger proportion of *Daphnia*'s UVR tolerance than do the combined contributions of NER and photoprotection (NERPP).

For this study, hypotheses tested the molecular responses of UVB-exposed *Daphnia* to two variables with known positive effects on *Daphnia* survival: 1) PER-stimulating radiation and 2) higher temperatures. Because the survival studies suggest that 1) PER mediates a large proportion of the DNA damage induced in *Daphnia*, and 2) survival of *Daphnia* under UVR stress increases with temperature (between 10 and 25

°C) we hypothesized that net damage in the presence of PER is temperature dependent (lower net damage at higher temperatures).

METHODS USED IN EXPERIMENTS 19 AND 20:

Two temperatures, 5 and 20 °C, were tested to determine the temperature dependence of DNA damage accumulation in *Daphnia*. To isolate the effect of PER on net damage accumulation from that of NER, *Daphnia* were exposed to UVB radiation with and without PER-stimulating radiation (commonly referred to as photoreactivating radiation, or PRR).

Net DNA damage was measured following exposure. Each of the measured net DNA damage values obtained from the experiments represents the difference between the total damage induced during the exposure period (an unknown value) and any DNA damage that was repaired during the experiment, either by NER (an unknown value, assumed to be low) and PER (in the case of the +PRR treatment). Net PER values (net repair in excess of concurrent net damage) can be determined be calculating the difference between the +PRR and –PRR treatments. The two experimental treatments, and the UVR-mediating mechanisms potentially available to *Daphnia* exposed to UVB under the treatments, are summarized as equations below.

+PRR:	Net DNA damage =	Total DNA damage induced	– DNA repaired by NER	
–PRR:	Net DNA damage =	Total DNA damage induced	– DNA repaired by NER	– DNA repaired by PER

Two standard techniques for measuring UVR stress, one molecular and the other organismal, were integrated to develop a novel approach for assessing the impacts of UVR exposure under different environmental conditions. In laboratory experiments, *Daphnia* were exposed to UV-B radiation for 12 hours in the presence and absence of visible light, then incubated in the dark, in order to isolate the effects of PER from NERPP. Survival and DNA damage (CPDs and (6-4)s) were analyzed.

Experiments were conducted in the lamp phototron apparatus (described in Part II, from Williamson et al. 2001) to isolate the effects of longer wavelength photoreactivating radiation (PRR) from shorter wavelength, damaging UVB radiation. The lamp phototron apparatus allows the investigator to manipulate the intensity of damaging UVB radiation in the presence and absence of the PRR that stimulates photoenzymatic repair (PER).

Experiments were conducted using adult female (egg-bearers or equivalent size) *Daphnia pulicaria* from Dutch Springs (Bethlehem, PA), a spring-fed quarry. *Daphnia* were collected on November 12, 2001, from Dutch Springs by taking several vertical tows of the water column from 0-20 m with a 202 um net. Collected organisms were filtered through a 363 um mesh to isolate larger adults. The isolated adults were incubated in 4 L aquaria of 37 um-filtered Dutch surface water, with cultured *Ankistrodesmus sp.* (green alga) as food.

In addition to the *Daphnia*, DNA dosimeter was exposed to the experimental conditions to determine the maximum potential for DNA damage in the absence of either photoprotection or repair enzymes, and test the temperature dependence of naked DNA.

The DNA dosimeter consisted of raw DNA from salmon testes (Carolina Biological Supply) dissolved in sterile 1x SCC buffered solution (10 x: SCC buffer: 44.5 g citric acid trisodium salt and 90 g NaCl). The dosimeter was prepared according to the protocol developed by Dr. Wade Jeffery (University of West Florida, Center of Environmental Diagnostics and Bioremediation).

In two experiments, one at 5 °C (November 15, 2001) and one at 20 °C (November 14, 2001), *Daphnia* and dosimeter were exposed to the UVB lamp in the lamp phototron for 12 hours. 20 dishes were exposed on the wheel to 25 KJm^2 of the UVB lamp, in the absence of PRR, and 20 were exposed to 52 KJm^2 in the presence of PRR. An additional 20 dishes were incubated in the dark alongside the phototron, as controls. 2 of the 20 dishes in each of the two treatments and the controls contained DNA dosimeter. Of the remaining 18 dishes per treatments, 10 dishes had 10 *Daphnia* each and 9 had 30 *Daphnia* each.

Immediately following the end of the 12-hour exposure period, all dishes were removed from the wheel. From each dish containing dosimeter, a 1 mL sample was taken. The dishes with 10 *Daphnia* each were put aside to score for survival. Survival was scored using a dissecting microscope; numbers of live and dead individuals were recorded. An individual was scored as "live" if a heartbeat was observed during 10 seconds of observation at 30x magnification. If no heartbeat was observed after 10 seconds, the individual was scored as "dead," removed from the dish and discarded. Survival was scored every day following the experiment for 5 days (20 °C) and 10 days (5 °C). The difference in endpoint days (last day survival scored) between the two

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experiments accounts for the difference in lag time between exposure and expression of survival response due to the different metabolic rates of *Daphnia* incubated at 5 and 20 °C. Survival data were recorded until the designated endpoint day, as long as survival in dark control organisms remained at or above 90%.

The dishes with 30 *Daphnia* each were sampled for DNA damage analysis. For each treatment and the controls, 3 dishes were combined to make a single sample with close to 90 individuals per sample. Only live individuals were included in the DNA analyses to ensure that the DNA repair processes were active for the duration of the experiment. The sample was immediately frozen at –20 °C to preserve the DNA.

Photoproduct analyses were conducted by Dr. David Mitchell (University of Texas, M.D. Anderson Cancer Center, Smithville, TX). Concentrations per megabase DNA of both CPDs and (6-4)s were quantified in the *Daphnia* samples, and CPDs were quantified in the DNA dosimeter samples using a radioimmunoassay (RIA) (Mitchell 1996). Prior to data analysis, dimer data were corrected for the average background level of dimers measured in the control samples. Due to fact that the +PRR and –PRR treatments were exposed to different levels of UVB exposure, the data are presented in terms of UVB exposure units (KJm^2 UVB) so that the effects of the two treatments could be compared. In addition, it should be noted that a small amount of damaging radiation was present in the PRR, and the data have not been corrected for this difference (Williamson et al. 2001). Therefore the magnitude of damage in the +PRR treatment may be slightly underestimated. Net DNA damage in the form of CPDs and (6-4)s is

presented in terms of damaged sites per unit DNA per unit UVB exposure (CPDs/mb DNA/KJm^2, (6-4)s/mb DNA/KJm^2).

The experiments were designed to ensure that UVB lamp exposures were high enough to induce DNA damage in *Daphnia* that was above background levels, and to ensure that close to 100% of the *Daphnia* remained alive at 12 hours. This balance of dimers induction and survival achieved two objectives: 1) To allow for treatment effects distinguishable from the controls in the DNA damage samples and 2) To prevent discrimination among *Daphnia* with different amounts of damage within a given treatment (i.e. to prevent DNA damage samples being comprised of only individuals with highly efficient repair, if individuals with less efficient repair were dead and therefore not included in the samples), which would have resulted in an overestimation of repair efficiency.

RESULTS OF EXPERIMENTS 19 AND 20:

<u>**Survival:**</u>

Survival of *Daphnia* immediately following the 12-hour exposure period was between 95 and 100% in all treatments (Table 4, Figure 1). By the respective endpoint days of the two experiments, survival in the dark control *Daphnia* remained above 90%, while survival in both the +PRR and –PRR treatments was 0% (Table 4, Figure 1).

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Table 4:
Survival of *Daphnia* following the 12-hour exposure period and on endpoint day.

		20 °C			5 °C		
		Control	-PRR	+PRR	Control	-PRR	+PRR
End of the 12-hour exposure period:	Average	100	98	95	100	100	96
	Standard deviation	0.32	4.22	5.27	0.00	0.00	5.27
Endpoint day (5 °C at 10 days; 20 °C at 5 days):	Average	91	0	0	92	0	0
	Standard deviation	8.78	0.00	0.00	8.32	0.00	0.00

Damage to raw DNA:

Damage to raw DNA was not affected by PRR or temperature (Figure 2).

Net DNA Damage:

At 20 °C, the accumulation of both CPDs and (6-4)s was significantly greater in

the absence of PRR than in its presence (Table 5, Figure 3, CPDs). The concentration of

CPD damage sustained by *Daphnia* exposed to UVB radiation without PRR was 3.69

times higher (at 20 °C) than that of *Daphnia* exposed to UVB radiation with PRR. The

response was even more pronounced in the (6-4)s, where concentration of damage was

5.82 times higher (at 20 °C) in the treatment without PRR than in the treatment with

PRR.

This pattern was not seen, however, at 5 °C where there was no difference

between the treatments with and without PRR for either CPDs or (6-4)s (Figure 3, CPDs).

As expected, net CPD and 6-4 accumulations in the presence of PRR increased

with decreased temperature from 20 to 5 °C (Figure 3, CPDs). There was approximately

a 2-fold increase in CPDs accumulated at 5 °C from 20 °C. This response was even more pronounced in the (6-4)s, which exhibited approximately a 3-fold increase from 20 °C to 5 °C. Results from the treatments without PRR, however, did not exhibit the anticipated outcomes. Instead of being higher at 5 °C—or the same at 5 and 20 °C—the CPD and (6-4) concentrations were lower by 29% and 46%, respectively, at 5 °C than at 20 °C (Figure 2, CPDs).

Net damage exhibited the same patterns in response to temperature and PER in both CPDs and (6-4)s (Figure 3, CPDs and (6-4)s).

Differences in treatment responses (+PRR or –PRR) at 5 and 20 °C depend on temperature. Results of an ANOVA (2-factor with replication) confirm this result at the $p = 0.05$ level (Table 5). No effect of temperature was found between 5 and 20 °C, when +PRR and –PRR treatments were grouped together ($p = 0.51$ for CPDs, $p = 0.31$ for (6-4)s, see Table 5). Significance was found when the interaction of temperature and PRR was tested ($p < 0.001$ for both CPDs and (6-4)s, see Table 5). In addition, a test of PRR found significance for CPDs and (6-4)s (both at $p < 0.001$).

For the DNA dosimeter (raw DNA in solution), no significant effect of temperature was found, either for temperature alone, or for the interaction of temperature and PRR (Table 5, Figure 2). This result is consistent with previous findings that direct damage to DNA by UVR is independent of temperature.

Table 5: Results of 2-factor with replication ANOVA.

	CPDs in *Daphnia*		(6-4)s in *Daphnia*		CPDs in dosimeter	
	P-value	F stat	P-value	F stat	P-value	F stat
Temperature: 5 or 20 °C	0.49	0.51	0.31	1.18	0.29	1.47
PRR: + or −	< 0.001	114.43	< 0.001	154.60	< 0.001	1004.90
Interaction	< 0.001	69.25	< 0.001	165.48	0.29	1.47

Note: 3 replicate samples were taken for each treatment (df = 11).

DISCUSSION OF EXPERIMENTS 19 AND 20:

Results indicate that *Daphnia* utilizes photolyases specific for both types of damage (CPDs and ((6-4)s), and suggest that temperature may differentially affect both damage and repair in *Daphnia*. The findings support our hypothesis that the process of DNA repair is temperature dependent for PER. While the temperature independence of DNA damage was clearly demonstrated in raw DNA dosimeter, the damage response in *Daphnia* was different from that of raw DNA. The findings suggest that DNA damage in living cells may not be independent of temperature. The mechanism for this response is not known, but potential explanations include increased NER at the lower temperature (an unlikely explanation) and increased DNA damage at the higher temperature, potentially due to a relationship between whole-organism growth-rate and damage induction in *Daphnia*. A discussion of these possibilities follows below. This study is one of the first to examine the ecological importance of different DNA repair mechanisms for UV-induced DNA damage.

Survival:

Repair processes for mediating UVR damage occur after the damage is induced and can become overwhelmed if the rate of damage exceeds the rate of repair, as may be the case with *Daphnia*, where measurable levels of dimers are measured immediately following the 12-hour exposure period, but complete mortality results several days following the exposure period.

Hypothesis: Net damage in the presence of PER is temperature dependent.

When PER was stimulated, net DNA damage was significantly lower at 20 °C than at 5 °C. This finding is consistent with the expectation that the repair efficiency of PER would be greater at 20 °C than at 5 °C in *Daphnia*. This expectation was based on the basic principle of the enzyme kinetics hypothesis: within a biologically stable range (between 0 and 40-45 °C, reviewed by Keeton and Gould 1996), higher temperatures result in higher enzyme efficiencies. The relationship of PER to temperature supports our hypothesis that PER is temperature mediated in *Daphnia*, and is consistent with previous findings for aquatic organisms (Malloy et al. 1997) that demonstrated greater PER efficiency at increased temperatures in Antarctic zooplankton and fish species.

Net damage in the absence of PER is temperature dependent.

In the absence of PER-stimulating radiation, when PER was not active in *Daphnia*, net damage was higher at 20 °C than at 5 °C. This result is not consistent with

the expectation that net damage in the absence PER would be either similar at 5 and 20 °C or lower at 20 than at 5 °C, as seen when PER was stimulated.

Although NER, like PER, is an enzyme-driven process, temperature was expected to have less of an effect on the treatment with only NERPP than on the treatment with PER. This expectation was based on the previous findings with *Daphnia* survival, which suggest that only a small proportion of the DNA damage sustained by *Daphnia* is mediated by NERPP.

Therefore, while a similar effect of temperature was expected for NER and PER, the relative contributions of NER and PER to the mediation of UVR stress in *Daphnia* (based previous studies of survival) suggest that the effect of temperature on DNA damage accumulation in *Daphnia* would be greater when PER was stimulated.

The magnitude of the effect of temperature on PER was, in fact, somewhat higher than the effect on NERPP alone (% difference in net damage between 5 and 20 °C was greater for the treatment with PER than for the treatment with only NERPP), as had been expected. The direction of the effect, however, was unexpected: temperature had opposite effects depending on whether or not PER was stimulated (in the absence of PER, net damage was higher at 20 °C than at 5 °C).

Because net repair in the absence of PER represents the difference between the total damage induced in the presence of photoprotection, and any damage that was repaired by NER, this unexpected effect of temperature on net damage is likely due to the interaction of temperature with one or both of the two potential defense strategies, NER and photoprotection, or the interaction of temperature with damage. The mechanism

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responsible for the observed difference in CPDs and (6-4)s between 5 °C and 20 °C in the treatments without PRR is not known; however, there are several potential explanations. The likelihood and implications of the three possibilities are discussed below.

Temperature independence of DNA damage in raw DNA:

As expected, damage to raw DNA exhibited was independent of temperature. While this finding provides evidence that temperature does not affect DNA damage in raw DNA, it is not necessarily directly applicable to the determining the relationship between temperature and DNA damage in a living organism. In *Daphnia*, temperature dependence persisted in the absence of PER. An inverse relationship was found between net damage and temperature when PER was stimulated. When PER was not stimulated, the relationship between net damage and temperature became direct. This finding suggests that either NER has the opposite temperature dependence as PER, or that DNA damage has some temperature dependence in *Daphnia*, that is not seen in raw DNA. This idea is discussed at length in one of the sections that follow, Temperature dependence of DNA damage in living cells.

Temperature dependence of NER:

One potential explanation for this finding is that NER may differ from PER in its temperature sensitivity; NER in *Daphnia* may have a temperature optimum that is closer to 5 °C than 20 °C. Data from a study on Antarctic zooplankton (Malloy et al. 1997) suggest that PER in fish increases with increased temperature, from 6 to 25 °C, while

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NER levels off at 12 °C, and may mean that NER has a lower temperature optimum than PER in those study organisms.

There are three possible scenarios that could describe the effectiveness of NER for repairing UVR-induced damage: (1) NER and PER are complementary processes for repair of UVR-damaged DNA (i.e. NER is effective only in the presence of PER), (2) when PER is active, it inhibits NER and (3) NER is equally effective (or ineffective) in the presence or absence of PER. The relationship of NER and PER in *Daphnia* is not known, but it is possible that NER occurs at different rates in the presence and absence of PER. There is no consensus in the literature about whether PER inhibits or facilitates NER. For example, in the bacterium *Escherichia coli*, PER and NER appear to serve complementary functions. In *E. coli*, the PER driver, photolyase, increases the affinity of NER for chemically damaged sites on the DNA molecule (Ozer et al. 1995). In yeast (*Saccharomyces cerevisiae*), a eukaryote, the opposite effect was observed (Ozer et al. 2000); PER and NER appear to be exclusive processes. In yeast, the binding of photolyase to chemically damaged DNA interfered with NER and inhibited repair of the damaged sites by NER. To explain the different interactions of PER and NER observed in *E. coli* and yeast, Ozer et al. (1995) hypothesize that the cause of the differences between their study and that done in *E. coli* lies in the different NER systems of prokaryotes and eukaryotes. Results from whole-organism UVR-tolerance experiments, however, might suggest that the NER-PER interaction in higher eukaryotes is more like that observed in *E. coli*, than that observed in yeast. In a recent study of the northern anchovy (*Engraulis mordax*), repair of UV-damaged DNA occurred during the day but

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not at night, indicating that the activity of NER alone was ineffective in repairing UVR-induced DNA damage (Vetter et al. 1999). This result may suggest that PER inhibits NER. If NER is, in fact, inhibited by PER, then a suppression of PER at 5 °C could potentially release NER from inhibition.

It is unlikely that NER has a temperature optimum closer to 5 °C than 20 °C, based on the basic theory of enzyme kinetics, where enzyme efficiency tends to increase with temperature. In addition, it has been demonstrated that the enzyme activity of NER increases between 5 and 28 °C in yeast (Giese 1957).

The role of NER in mediating UVR-induced DNA damage varies among aquatic organisms. In bacterioplankton, NER is crucial to UVR tolerance (Jeffrey et al. 1996), while in Antarctic zooplankton, rates of PER may be 6-7 times higher than rates of NER (Malloy et al. 1997). In larval anchovy, NER appears to be completely ineffective in repairing CPDs (Vetter et al. 2000). It is important to note, however, that only CPDs, and not (6-4)s were quantified in these studies. NER may have higher affinity for (6-4)s than for CPDs (Sancar 1994, Roy et al. 2000), while, at least in certain regions of the yeast mini-chromosome, PER is the predominant repair mechanism for CPDs (Suter et al. 2000). It remains unclear how the affinities of NER and PER compare for the two different types of DNA lesions in *Daphnia*.

Temperature dependence of DNA damage in living cells:

A more plausible explanation for the finding that greater net damage is induced at 20 °C than at 5 °C when PER is not stimulated is: DNA may be more sensitive to damage

at the higher temperature. The mechanism for such a response could likely be explained by considering the relationship between DNA damage and DNA repair in faster versus slower growing cells. At a higher temperature, the physical protection of the DNA molecule from UVR damage may be compromised when the DNA is unwound during DNA replication, transcription, and cell division. Meanwhile, in adult *Daphnia*, PER would likely not be enhanced during rapid cell growth and division. In such a situation, faster growing cells, with less compressed, actively transcribed DNA, may be vulnerable to UVR damage. This potential for greater DNA damage to be induced at higher temperatures is supported by findings from the human genome, where induction of (6-4)s occurs at a much higher frequency in actively transcribing regions of chromatin than in non-expressed genes (reviewed by Mitchell and Karentz 1993).

This potential vulnerability of actively transcribed DNA may be counterbalanced by increased repair in proliferating cells. It has been demonstrated that, in higher organisms, photoreactivation capability can vary with developmental stage and by tissue (Sutherland 1981). In yeast, for example, photoreactivation activity increases with ploidy, as well as with cell growth rate (Sutherland 1981). Several examples of the growth-rate dependence of DNA damage and repair are documented in the literature, and while they are not directly related to variations in environmental temperature, as different factors are constraining growth-rate (i.e. environmentally controlled growth-rate vs. developmentally controlled growth-rate), all studies describe the effect of growth-rate on DNA damage and repair. The most compelling argument in favor of growth-rate dependent PER comes from a study in *C. elegans* (a nematode), where the rate of repair

of (6-4)s was much higher in younger individuals than in older individuals (Hartman et al. 1990). This finding may be due to DNA damage induced in metabolically active sites being more accessible to NER than damage induced in compacted regions of the chromosome (reviewed by Mitchell and Karentz 1993). In a similar study, UVR-induced DNA damage in rodent and human epidermis cells was repaired more efficiently in basal cells than in terminally differentiated cells (Mitchell and Hartman 1990), suggesting that actively transcribed DNA is repaired more readily than inactive DNA.

The growth-rate dependence of DNA repair may also differ between PER and NER. In fish cells, for example, PER is equally effective in repairing damaged DNA in both the active and inactive regions of the genome, while NER appears to be more sensitive to active areas of the genome than PER (Komura et al. 1991).

One fundamental difference between previously published studies on the growth-rate dependence of damage and repair, and the present study with *Daphnia*, is that in this *Daphnia* study, the high growth rate of *Daphnia* at 20 °C was a function of external, environmental control, while the other studies were conducted using subjects whose high growth rate was a function of internal, developmental control. In addition, the increased net damage in *Daphnia* was measured in whole-organism samples of adult individuals, as opposed to selected active genes, undifferentiated cells or prolific tissues (i.e. epidermis), or juveniles. So, the net damage values measured in *Daphnia* represent the sum of all damage and repair processes in the organism, regardless of tissue.

It is known that *Daphnia* has a fast metabolism and a high surface area:volume ratio (Dodson and Frey 1991). While faster metabolism may make *Daphnia* more

susceptible to UVR-induced DNA damage, it is known that, for unicellular organisms, susceptibility of DNA to UVR-induced damage increases with surface area:volume ratio (Karentz et al. 1991).

No good description of the effect of temperature on *Daphnia* growth rates has been produced (Dodson and Frey 2001). It is known that egg development time and longevity are approximately proportional to the inverse of temperature with an exponent of around 2.5, (Dodson and Frey 1991) and that the maximum sustainable temperature for *Daphnia* is around 30 °C, depending on the species (Dodson and Frey 2001). One indication of *Daphnia*'s high metabolism is the fact that they can actually starve at high food densities because their respiration exceeds their maximum feeding rate (Dodson and Frey 1991).

In *Daphnia*, damage could potentially increase with growth rate (due to vulnerability of active DNA), as was demonstrated with (6-4)s in human DNA. This increase in vulnerability to damage could occur while repair rates remained constant, due to the age (adult) and developmental stage (differentiated) of the cells from adult *Daphnia*.

Potential temperature dependence of photoprotection:

For the lamp phototron experiments conducted in this study, photoprotection was considered to be a constant among all treatments. One study with copepods, however, did suggest that protection might have some temperature dependence (Hairston et al. 1979). In that study, copepods were more pigmented in the winter and early spring than

in summer. The survival of red (pigmented) copepods depended on both exposure and temperature: survival of dark-incubated individuals was higher than survival of light-exposed individuals, and survival for both light-exposed and dark-incubated individuals was higher at 8 °C than at 20 °C. Survival of pale (non-pigmented) copepods was also higher in the dark-incubated individuals than the light-exposed individuals, but pale copepods exhibited little temperature dependence. The author concludes by saying that his results are inconsistent with the photoprotection explanation and may indicate metabolic mediation of photodamage. The suggestion of Hairston (1979), that photoprotection may be temperature dependent would be consistent with the temperature-dependence of NERPP found in this study of *Daphnia*.

Like copepods, *Daphnia* can utilize photoprotection to mediate UVR exposure. While the *Daphnia* population used for this study displayed the typical lack of visible pigmentation, darkly melanized (pigmented) *Daphnia* are found in certain high-UVR systems. The trade-off between melanization and DNA damage is energetically costly. In order to prevent genetic damage, some *Daphnia* species can produce melanin. This production of melanin is possibly associated with a decrease in reproductive potential (Dodson and Frey 2001). Increased pigmentation also increases the predation risk to *Daphnia* from visual predators, making the likelihood of finding pigmented *Daphnia* much higher in fishless systems.

CPDs vs. (6-4)s:

Results from these experiments provide the first direct evidence that *Daphnia* utilizes light-dependent enzymes to repair both CPD and 6-4 lesions in its DNA. The two repair enzymes are common, but not ubiquitous among taxa (Mitchell and Karentz 1993). Unlike nucleotide excision repair, which is a biologically universal repair mechanism (A. Sancar 1994a), photo reactivation has only been exhibited by certain taxa. These taxa, however, span a wide variety of organisms from archebacteria to marsupials (Kanai et al. 1997).

In this study, CPDs and (6-4)s were induced at different frequencies: approximately 5-8 times more CPDs were induced for every 6-4 induced. This finding is consistent with the literature, which states that CPD dimers account for a much larger proportion of the total dimers formed compared to (6-4)s. Under ambient conditions, CPDs typically account for 80-90% of dimers formed (A. Sancar 2000). While (6-4)s are less readily induced in DNA, their effect can be much more devastating, as these photoproducts can be 300 times more effective in blocking DNA polymerase than CPDs (Mitchell and Nairn 1989). Therefore, the proportion of CPDs to (6-4)s induced in this study supports our claim that the lamp phototron apparatus tests UVR tolerance within ecologically relevant parameters. Fewer CPDs were induced with the lamp phototron than are typically induced under ambient solar conditions, suggesting that the results of this study do not overestimate the cytotoxicity of UVR to *Daphnia*, and may even be an underestimation.

In the present study, the two types of photolyase appear to exhibit the similar trends in response to both PER and temperature. These results will be discussed in detail below.

The results suggest that 6-4 photolyase may be more sensitive to variations in temperature between 5 and 20 °C than CPD photolyase. For example, PER accounted for 56% reduction in net CPD damage between 5 and 20 °C, while PER accounted for a 69% reduction in net (6-4)s damage between 5 and 20 °C.

If, in fact, the temperature optimum of 6-4 photolyase is around 12 °C, and the temperature optimum of CPD photolyase is around 25 °C (Pakker et al, 2000), then the experimental temperature of 20 °C fell between these two optima, and the activity of one should not have been favored over the activity of the other.

Oxidative damage and other indirect effects of UVR:

In whole animals, including *Daphnia* (Williamson et al. 2001), and larval anchovy (Vetter et al. 1999), survival does not track absorption spectrum for DNA, as whole organisms are more sensitive to UVA radiation than raw DNA. This increased sensitivity to UVA radiation is due in part to the detrimental effects of UVA-induced oxidative damage.

In addition to the trade-off between pigmentation and predation risk, pigmentation can also increase the susceptibility of an organism to the indirect (non-genetic) effects of UV damage. For example, melanization can increase oxidative damage by UVA (D. Mitchell, personal communication). UVA induces reactive oxygen species (ROS) that

include hydrogen peroxide, superoxide and hydroxl radicals (Mitchell and Karentz 1993).

In addition to causing intracellular radical formation, UVA can also cause free radicals to

form extracellularly in an aquatic environment, such as in lake water (D. Mitchell,

personal communication). The damaging impact of ROS on cell structure and

physiology is mediated by antioxidants, and free radical scavengers and quenchers,

including: ascorbate, tocopherols, carotenoids, urate, as well as several enzymes

(reviewed by Karentz et al. 1994).

Ecological implications of UVR-temperature interactions:

The response of *Daphnia* to UVR-temperature interactions can have far-reaching

impacts beyond the physiology of the single organism. One such impact is on the trophic

structure of lake ecosystems. *Daphnia* are phenomenal grazers. A single *Daphnia* can

graze down the phytoplankton in over 1 mL of lake water every hour (Dodson and Frey

2001). *Daphnia* populations are typically present in large abundances, and even in high

productivity systems, they commonly cause a sudden depletion in phytoplankton biomass

every year (Dodson and Frey 2001). In lakes with moderate productivity, the presence of

Daphnia might exclude smaller herbivorous species from the zooplankton community

due to competition for food (Dodson and Frey 2001).

One biological interaction of *Daphnia* that would be propagated up the trophic

structure to fish would be the relative impacts of UVR and temperature on *Daphnia*,

compared with their phytoplankton food species. The same UVR and temperature

conditions would likely have differential impacts on a photosynthetic organism than they

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would have on an invertebrate. Phytoplankton may be more likely to benefit from higher UV:T conditions, than *Daphnia*. Assuming that the increase in the UV:T ratio is due to increased UVR, and not decreased temperature, the increased PAR that would be associated with increased solar exposure may be more beneficial than detrimental to photosynthetic organisms, as long as increased UVR does not result in photoinhibition of photosynthesis. Non-photosynthetic organisms, on the other hand, may be more likely to benefit from lower UV:T conditions (i.e. resulting from increased temperatures under constant UVR conditions), at temperatures within the range that does not have detrimental effects on their general physiology.

One of the ecological questions surrounding the interaction of UVR and temperature that has not received much attention is the question of how changing UV:T conditions affect the costs and benefits of the different strategies for mediating UVR stress, and how a shift in the cost-benefit balance might affect community structure.

For example, the impact of food or nutrient limitation on DNA repair in *Daphnia* and other zooplankton could be examined. In a low-productivity, low DOC system, such as an alpine lake with a forested watershed, food limitation might give species with more efficient PER a selective or competitive advantage over those with less efficient PER, because it is an energy efficient mechanism for mediating UVR-induced DNA damage. In contrast, in systems without food limitation (i.e. eutrophic systems), or with warmer waters (which would increase repair enzyme efficiency), species might be less energetically constrained. In this case, NER may be as or more efficient than PER as a UVR-mediation mechanism.

CONCLUSION:

This study demonstrates that net DNA damage is temperature dependent, both when PER is active and when PER is inactive. Opposite effects of temperature are exhibited depending on whether PER is active or inactive. I proposed that different mechanistic explanations, which depend on whether PER is active or inactive, can be used to interpret the findings. The temperature-dependent reduction in net damage with PER is consistent with the basic theory of enzyme kinetics. The temperature-dependent increase in net damage without PER in *Daphnia* can be explained with a more structural theory for DNA damage, such that the vulnerability of the DNA molecule to UVR-induced damage increases with temperature, due to increased transcription and cell division activity, which would unwind the DNA molecule and expose more of its surface to UVR.

Large-scale ecosystem changes result in part from the impacts of environmental variables on small-scale molecular processes, the effects of which are propagated through the organism, population, community and ultimately ecosystem levels. In the case of UVR, one of the important impacts of the stressor on the ecosystem is the direct damage to DNA. As this study has demonstrated, direct damage to DNA in *Daphnia* is likely to be affected by the interactive effects of UVR and temperature. Therefore, a shift in the UV:T ratio of temperate lakes in the northern hemisphere could have a significant impact on *Daphnia*. The magnitude and direction of such an impact will be, in part, a function of the UVR intensity and the water temperature and the time of damage and repair. In

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general, a lower UV:T ratio would be more beneficial to *Daphnia* than a higher UV:T ratio. Increases in UV:T are likely to have the most impact on *Daphnia* in shallow, alpine systems, at high latitudes, where water temperatures are cold and no depth refugia exist to allow for behavioral avoidance of UVR. Even in low altitude, more productive lakes at temperate latitudes, shifts in UV:T could potentially have impacts on *Daphnia*. Such impacts would most likely occur at the spring peak in UV:T, when cold surface waters coincide with high UVR irradiance, which is intensified by the occurrence of the maximum extent of the ozone hole in early spring and the clear-water phase in late spring.

This study represents one of the first quantitative studies of repair of UV-induced DNA damage in zooplankton. By continuing to study how the molecular-level processes involved in DNA damage and repair respond to the environmental variables of temperature and UVR, we will be better able to predict how these responses translate into organism and ecosystem-level responses to climate change.

Figure 1: Survival of *Daphnia* at 5°C and 20°C following a 12-hour UVB exposure with and without photorepair radiation (PRR).

Figure 2: CPDs in DNA dosimeter (raw DNA) at 5°C and 20°C following 12-hour UVB exposure with and without photorepair radiation (PRR).

Figure 3: CPDs and (6-4)s in *Daphnia* at 5°C and 20°C following 12-hour UVB exposure with and without photorepair radiation (PRR).

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EXPERIMENTS: 1 (20 °C) and 2 (10 °C)

QUESTION: What is the temperature dependence of PER in *Daphnia*?

OBJECTIVE:

To determine how temperature affects DNA damage, nucleotide excision repair (NER) and photoenzymatic repair (PER) in *Daphnia* by quantifying CPDs (cyclobutane pyrimidine dimers) in *Daphnia* adults (at 10 and 20 °C) and *Lepomis* (sunfish) larvae (at 20 °C) (+PRR and –PRR exposed to 25 KJm^2 of UVB radiation).

HYPOTHESIS:

PER is temperature sensitive: Net CPD accumulation in the presence of PER (DNA damage minus NER and PER) decreases with increased temperature (net CPD accumulation is lower at 20 °C than at 10 °C).

LITERATURE REVIEW:

Malloy et al. (1997) found that rate of PER increased as a linear function with temperature (6-25 °C) in Antarctic zooplankton (predominantly ichthyoplankton).

PRELIMINARY DATA:

Survival of *Daphnia catawba* under UVR stress increases from 15 to 25 °C, presumably due to increased activity of photolyase enzyme at higher temperatures (DeLange et al., in preparation).

METHODS:

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- *Daphnia* were collected from Lake Giles (Blooming Grove, PA). Adults were isolated in the lab at Lake Lacawac.

- *Lepomis* larvae were collected from Lake Giles (Blooming Grove, PA).

  - Organisms were incubated overnight at experimental temperatures (10 and 20 °C) under a 12:12 light:dark cycle

  - *Daphnia* were fed *Cryptomonas reflexa* (WCR) as food. *Lepomis* were not fed (had yolk sac remaining).

- Exp 1 included both *Daphnia* and *Lepomis*, while Exp 2 included only *Daphnia*.

- Lamp phototron was used to separate the effects of damage and repair processes (PER and NER) on CPD accumulation and survival.

  - PRR + treatment was exposed to 25 KJm^2 of the UVB lamp.

  - PRR– treatment was exposed to 25 KJm^2 of the UVB lamp.

- A DNA dosimeter was used for each treatment (DNA in buffered solution, salmon testes DNA from Carolina Biological Supply).

- *Daphnia* and dosimeter samples for CPD analysis were taken at 0, 2, 4, 8 and 12 hours. Only live individuals were included in *Daphnia* samples. Samples were frozen immediately after collection to prevent repair of CPDs or degradation of DNA.

- Samples were sent to Dr. David Mitchell (M.D. Anderson Cancer Center, Smithville, TX) for CPD analysis.

PROBLEMS AND OTHER COMMENTS:

- No dimer data were obtained due to small sample size.

RESULTS:

Only survival data were obtained.

DISCUSSION:

Additional experiments, with larger sample sizes, are necessary to determine the

temperature dependence of DNA damage and repair in *Daphnia*.

EXPERIMENT: 3 (20 °C)

OBJECTIVE:

To test induction of cyclobutane pyrimidine dimers (CPDs) in DNA dosimeter (raw

salmon DNA in solution) at 20 °C in lamp phototron, exposed to 25 and 52 KJm^2 UVB

radiation, with and without repair radiation.

HYPOTHESES:

- Induction of CPDs will increase with UVB lamp exposure level.

- Induction of CPDs will be comparable for +PRR and –PRR treatments at same UVB

  exposure level.

LITERATURE REVIEW:

Dosimeters provide information about the maximum about of DNA damage possible in

the absence of photorepair and photoprotection.

METHODS:

- DNA dosimeter (DNA in buffered solution, salmon testes DNA from Carolina

  Biological Supply) was prepared on July 13, 2000 in a buffered citric acid solution,

  following the protocol developed by David Mitchell and demonstrated by Diane Dutt.

  The solution was determined to be at a concentration of 101 ug/mL using a

  spectrophotometer.

- Lamp phototron was used to expose DNA dosimeter.

  - PRR + treatment was exposed to 25 and 52 KJm^2 of UVB radiation.

  - PRR– treatment was exposed to 25 and 52 KJm^2 of UVB radiation.

- Dosimeter samples for CPD analysis were taken at 0, 4, 8 and 12 hours.

- Samples were frozen immediately after collection to prevent degradation of DNA.

- Samples were sent to Dr. David Mitchell (M.D. Anderson Cancer Center, Smithville, TX) for CPD analysis.

PROBLEMS AND OTHER COMMENTS:

- Experiment 3: none

RESULTS:

See Tables 6 and 7 and Figure 4. Exposure level had a slight effect on the damage accumulated (CPDs/KJm^2/hour), but PRR and length of exposure had no effect. CPDs in DNA dosimeter can be induced and quantified using lamp phototron apparatus.

DISCUSSION:

Future experiments will include DNA dosimeter.

Figure 4: Exp. 3: CPDs in DNA dosimeter at 20°C, following 12-hour UVB exposure with and without photorepair radiation (PRR): +PRR and -PRR exposed to 25 and 52 KJm^2 UVB.
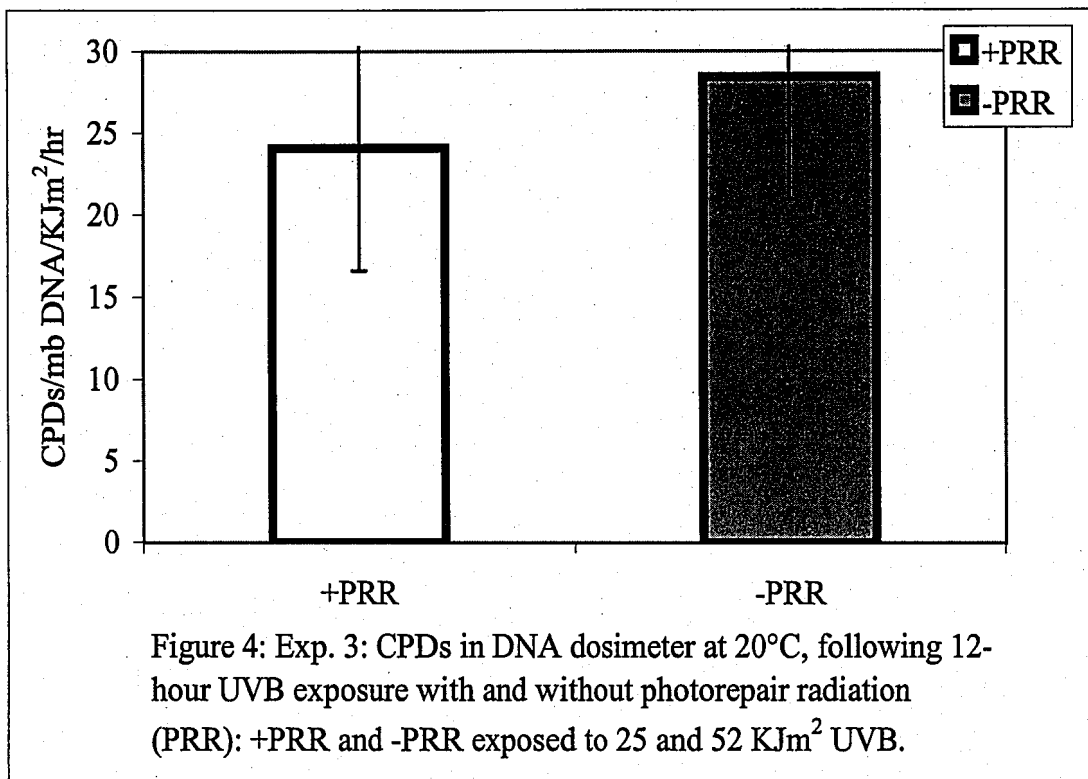
Table 6: Exp. 3: Results of single factor ANOVA for PRR.

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	56.87	1	56.87	0.97	0.35	4.96
Within Groups	583.93	10	58.39			
Total	640.80	11				

Table 7: Exp. 3: Results of two factor with replication ANOVA for exposure time and exposure level (all values were divided by exposure level and exposure time for units of CPDs/mb DNA/KJm2/h before running ANOVA.

Source of Variation	SS	df	MS	F	P-value	F crit
Exposure time	132.38	1	132.38	5.60	0.08	7.71
Exposure level	207.36	1	207.36	8.78	0.04	7.71
Interaction	82.44	1	82.44	3.49	0.14	7.71
Within	94.51	4	23.63			
Total	516.68	7				

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EXPERIMENTS: 4 (20 °C) and 5 (10 °C)

QUESTION: What is the temperature dependence of PER in *Daphnia*?

OBJECTIVE:

To determine how temperature affects DNA damage and photoenzymatic repair (PER) in *Daphnia* by quantifying CPDs (cyclobutane pyrimidine dimers) in *Daphnia* at 10 and 20 °C (+PRR and –PRR exposed to 25 KJm^2 of UVB radiation).

HYPOTHESIS:

PER is temperature sensitive: Net CPD accumulation in the presence of PER (DNA damage minus NER and PER) decreases with increased temperature (net CPD accumulation is lower at 20 °C than at 10 °C).

LITERATURE REVIEW:

Malloy et al. (1997) found that rate of PER increased as a linear function with temperature (6-25 °C) in Antarctic zooplankton (predominantly ichthyoplankton).

PRELIMINARY DATA:

Survival of *Daphnia catawba* under UVR stress increases from 15 °C to 25 °C, presumably due to increased activity of photolyase enzyme at higher temperatures (DeLange et al. in preparation).

METHODS:

- *Daphnia* were collected from Dutch Springs, Bethlehem, PA. Adults were isolated in the lab.

  - Incubated overnight (10 and 20 °C) in 4 L aquaria at experimental temperature under a 12:12 light:dark cycle

- Fed *Cryptomonas reflexa* (WCR) as food

- Lamp phototron was used to separate the effects of damage and repair processes (PER and NER) on CPD accumulation (Exps 4 and 5) and survival (Exp 5 only).

  - PRR + treatment was exposed to 25 KJm^2 of UVB lamp.

  - PRR– treatment was exposed to 25 KJm^2 of UVB lamp.

- A DNA dosimeter was used for each treatment (DNA in buffered solution, salmon testes DNA from Carolina Biological Supply).

- *Daphnia* and dosimeter samples for CPD analysis were taken at 12 hours. Only live individuals were included in *Daphnia* samples. Samples were frozen immediately after collection to prevent repair of CPDs or degradation of DNA..

- Samples were sent to Dr. David Mitchell (M.D. Anderson Cancer Center, Smithville, TX) for CPD analysis.

- No survival data were collected for Exp 4. Survival was scored for 5 days for Exp 5.

PROBLEMS AND OTHER COMMENTS:

- Experiment 4 (20 °C): CPD data analyzed 10/25/00. No replicate samples were taken.

- Experiment 5 (10 °C): CPD data analyzed 11/20/00. No replicate samples were taken.

- Due to the fact that dimer samples from the two experiments were analyzed on different days, comparison of the absolute CPD values from the two experiments is difficult. An analysis of the experiments in terms of CPDs/mb DNA/KJm^2 per CPD

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damage in dosimeter suggests that damage in both the +PRR and –PRR is slightly higher at 20 °C than at 10 °C. Lack of replication makes prevents statistical analysis.

RESULTS:

At 10 °C, the survival of *Daphnia* at 12 hours was approximately 100% in both the presence and absence of PRR (Figure 5). The survival at 10 °C in +PRR and –PRR treatments remained above 88% at 24 hours. By 48 hours, differential survival between the two treatments was expressed, when survival in the +PRR remained high (84%), but dropped to 6%. By day 5, the last day that survival data were collected, +PRR survival was 67%, while there were no survivors in –PRR.

CPD dimer data obtained from this experiment provide the first information about the differential accumulation of damage in *Daphnia* exposed to UVB with and without PRR. The –PRR treatments accumulated approximately 8-40 times more damage than the +PRR treatments.

In addition, damage in the presence of PRR was 5.79 times higher at 10 °C than at 20 °C, while damage in the absence of PRR was only 1.15 times higher at 10 °C than at 20 °C. This result may suggest that PER could be more sensitive to temperature than NER.

The magnitude of dimer accumulation, however, was only 0.21 and 0.25 CPDs/mb DNA/KJm^2 higher in at 10 °C than at 20 °C, in the +PRR and –PRR treatments, respectively, which is not a statistically significant result (Table 8).

DISCUSSION:

Additional experiments, at a broader range of temperatures, are necessary to determine the temperature dependence of DNA damage and repair in *Daphnia*.

Figure 5: Exps. 4 and 5: CPDs from *Daphnia* at 10°C and 20°C, following 12-hour UVB exposure with and without photorepair radiation (PRR): +PRR and -PRR exposed to 25 KJm^2 UVB.
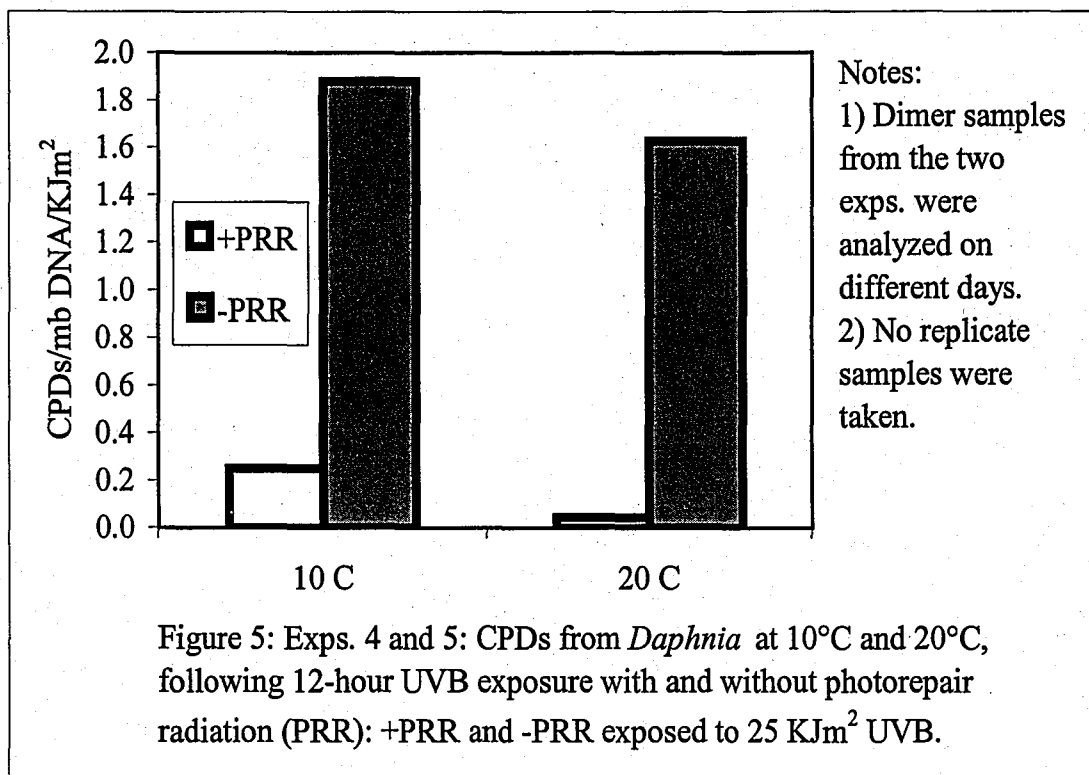
Table 8: Exps. 4 and 5: Results of two factor without replication ANOVA for effects of temperature and PER (+ or -PRR).

Source of Variation	SS	df	MS	F	P-value	F crit
Temperature	0.05	1	0.05	101.89	0.06	161.45
PRR	2.58	1	2.58	4996.53	**0.01**	161.45
Error	0.00	1	0.00			
Total	2.64	3				

EXPERIMENTS: 6 (20 °C) and 7 (20 °C)

QUESTION: What are the relative contributions of NER and PER to the UVR tolerance of *Daphnia?*

OBJECTIVE:

To determine how the two repair mechanisms for UVR–induced DNA damage (photoenzymatic repair, PER, and nucleotide excision repair, NER) interact in *Daphnia* by measuring the effects of the PER and NER on the molecular scale (cyclobutane pyrimidine dimer, CPD, accumulation) and the organismal scale (survival) (+PRR and – PRR exposed to 52 KJm^2 from UVB lamp.), following the 12 hour exposure period, and after an additional 12 h incubation, either in the dark or in additional repair radiation.

HYPOTHESIS:

- PER will contribute more to the UVR tolerance of Daphnia than NER.

LITERATURE REVIEW:

While there is no clear consensus in the current literature about how PER and NER interact, there is a suggestion that, in taxa with PER capability, the rate of PER tends to exceed the rate of NER (Pakker et al., 2000).

PRELIMINARY DATA:

Little is known about the relative effectiveness of NER and PER. No consensus exists in the current literature about how NER and PER interact. For example, in the bacterium *Escherichia coli*, PER and NER appear to serve complementary functions. In *E. coli*, the PER driver – photolyase – increases the affinity of NER for chemically damaged sites on the DNA molecule (Ozer et al. 1995). In yeast (*Saccharomyces cerevisiae*), a eukaryote,

the opposite effect was observed (Ozer et al. 2000); PER and NER appear to be exclusive processes. In this species, the binding of photolyase to chemically damaged DNA interfered with NER and inhibited repair of the damaged sites by NER. To explain the different interactions of PER and NER observed in *E. coli* and yeast, Ozer et al. (1995) hypothesize that the cause of their difference lies in the different NER systems of prokaryotes and eukaryotes. Results from whole-organism UVR-tolerance experiments might suggest that the NER-PER interaction in higher eukaryotes is more like that observed in *E. coli*, than yeast. In a recent study of northern anchovy (*Engraulis mordax*), repair of UV-damaged DNA occurred during the day but not at night, indicating that the activity of NER alone was ineffective in repairing UVR-induced DNA damage (Vetter et al. 1999). Results such as these might suggest one of two possibilities for the effectiveness of NER for repairing UVR-induced damage: (1) NER and PER are complementary processes for repair of UVR-damaged DNA (i.e. NER is effective only in the presence of PER) or (2) NER is equally ineffective in the presence or absence of PER. Data from preliminary experiments with *Daphnia* suggest that NER might occur at different rates in the presence and absence of PER.

METHODS:

- *Daphnia* were collected from Dutch Springs, Bethlehem, PA. Adults were isolated in the lab.

    - Incubated overnight (20 °C) in 4 L aquaria at experimental temperature under a 12:12 light:dark cycle

    - Fed *Cryptomonas reflexa* (WCR) as food

- Lamp phototron was used to separate the effects of damage and repair processes (PER and NER) on CPD and 6-4 accumulation (Exps 6 and 7) and survival (Exp 7 only).
  - PRR + treatment was exposed to 25 KJm^2 of the UV-B lamp.
  - PRR – treatment was exposed to 25 KJm^2 of the UV-B lamp.
- Following standard exposure period (12 h), half of each treatment (+PRR and –PRR) was incubated in additional repair radiation, while the other half of each treatment received the standard dark incubation.
- A DNA dosimeter was used for each treatment (DNA in buffered solution, salmon testes DNA from Carolina Biological Supply).
- *Daphnia* and dosimeter samples for CPD analysis were taken at 12 and 24 hours. Only live individuals were included in *Daphnia* samples. Samples were frozen immediately after collection to prevent repair of CPDs or degradation of DNA..
- No survival data were collected for Exp 6. Survival was scored at 24 hours for Exp 5.

PROBLEMS AND OTHER COMMENTS:

- Experiment 6 (20 °C): No survival data were collected. No *Daphnia* from –PRR (0-12 h) survived to 24 h.
- Experiment 7 (10 °C): A small number of *Daphnia* from –PRR (0-12), that were incubated +PRR (12-24 h) survived to 24 h, allowing for a single sample to be collected.
- Dimer samples for the two experiments were analyzed together, allowing for direct comparison of damage units.

RESULTS:

Survival at 24 hours of *Daphnia* exposed to UVR in the presence of PRR was 62%, while survival in the absence of PRR was 6%, suggesting that PER is necessary for survival.

After a 12-hour exposure to the UV-B lamp, *Daphnia* exposed in the presence of PRR had accumulated 89% (Exp 7) fewer CPDs than *Daphnia* exposed in the absence of PRR (Figures 6 and 7). *Daphnia* that had received PRR during the UV-B lamp exposure exhibited little or no reduction in CPDs after an additional 12-hour incubation, regardless of whether they were incubated with PRR or in the dark (Exps 6 and 7, Tables 9 and 10). NER may have accounted for a small amount of post-exposure repair in these treatments. CPDs in *Daphnia*, which had been exposed to the UV-B lamp in the absence of PRR, and then incubated in the dark for 12 hours, exhibited a 41% (Exp 7) reduction in CPD load following the 12-hour post-exposure incubation. Insufficient data are available to determine whether this result is significant, as the –PRR, 24 h result is based on a single un-replicated sample. If significant, however, the data suggest that PER repairs CPDs at a higher rate (6.8 CPDs/mb DNA/hour) than does NER (maximum rate of 4.4 CPDs/mb DNA/hour). In addition, no data are available for *Daphnia* exposed to the UV-B lamp in the absence of PRR, and then incubated with repair radiation for 12 hours, as all of these individuals were dead at 24 hours.

DISCUSSION:

The results of this preliminary study suggest that concurrent, not sequential exposure to repair radiation may be necessary for effective repair by PER. The results also indicate that repair of UV-induced DNA damage in *Daphnia* may occur over a relatively short time-period. Following the 12-hour exposure, the CPD load in *Daphnia* incubated with additional repair radiation did not return to background levels, suggesting that damage would remain un-repaired. In these experiments, the simulated "twilight" conditions (UV-A radiation + PAR, following a full-spectrum exposure) did not mediate the low level of accumulated damage. There are several possible explanations for this finding. One is that the duration of the experiment was too short for the *Daphnia* to fully recover from the damage sustained (compare to Vetter et al. 1999). However, due to the short life span of *Daphnia* relative to other higher organisms studied (i.e. fish larvae), 24 hours is likely a sufficient interval for repair to be fully expressed. Another possible explanation for this result is that damage recognition is low when dimers are at a low concentration on the DNA molecule (Mitchell, photo-enhanced recognition of damage). An experiment that induces higher amounts of damage might help to resolve this issue. In addition, this experiment provides preliminary molecular evidence that NER, as well as PER, may can effectively repair UV-induced DNA damage in *Daphnia*. Because this finding is based on a single, unreplicated sample, however, more extensive experimentation is necessary to determine whether this is a significant finding.

Figure 6: Exp. 6: CPDs from Daphnia at 20°C, following a 12-hour UVB exposure (25 KJm2) with and without photorepair radiation (PRR) and after an additional 12-hour incubation (12-24 h), either in the dark, or with additional PRR.

Figure 7: Exp. 7: CPDs from Daphnia at 20°C, following a 12-hour UVB exposure (25 KJm2) with and without photorepair radiation (PRR), and after an additional 12-hour incubation (12-24 h), either in the dark, or with additional PRR.
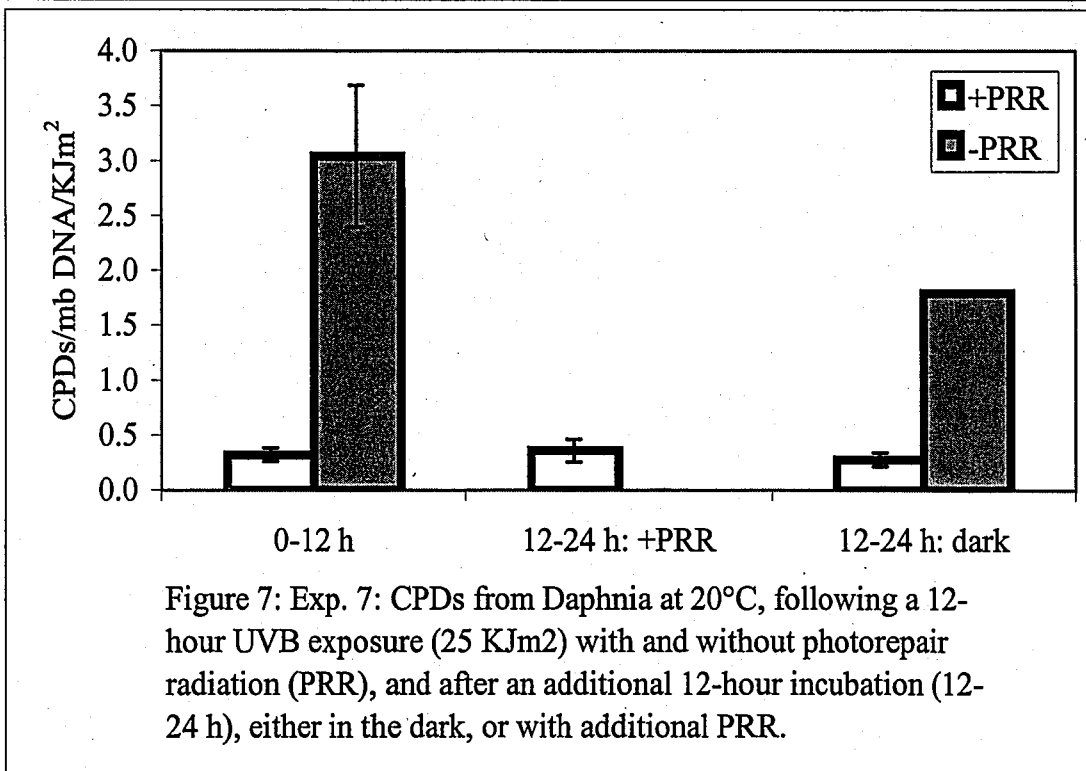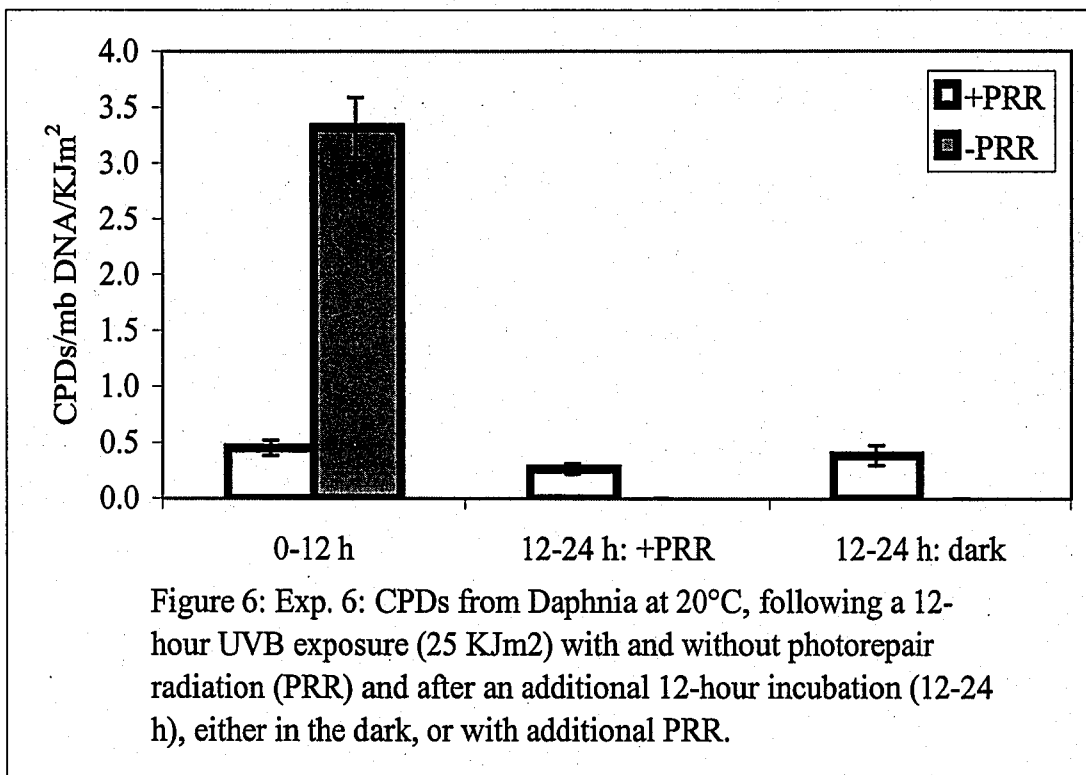
Table 9: Exps. 6 and 7: Results of single factor with replication ANOVAs the effect post-exposure PER (additional PRR from 12-24 h).

Source of Variation	SS	df	MS	F	P-value	F crit
Exp. 6						
Between Groups	42.95	2	21.48	6210.01	p < 0.001	9.55
Within Groups	0.01	3	0.00			
Total	42.96	5				
Exp. 7						
Between Groups	0.01	1	0.01	0.96	0.43	18.51
Within Groups	0.01	2	0.01			
Total	0.02	3				

Table 10: Exps. 6 and 7: Results of single factor with replication ANOVAs for effect of post-exposure NER (+PRR 0-12h, dark 12-24h).

Source of Variation	SS	df	MS	F	P-value	F crit
Exp. 6						
Between Groups	1197.25	1	1197.25	58.19	p < 0.001	4.75
Within Groups	246.89	12	20.57			
Total	1444.14	13				
Exp. 7						
Between Groups	1204.90	1	1204.90	58.57	p < 0.001	4.75
Within Groups	246.88	12	20.57			
Total	1451.78	13				

EXPERIMENTS: 8 (20 °C) and 9 (20 °C)

QUESTION: What are the relative contributions of NER and PER to the UVR tolerance of *Daphnia*? 1) Does *Daphnia* utilize (6-4)photolyase in addition to CPD photolyase? 2) Does dark repair (NER) act on CPDs at (6-4)s in *Daphnia*?)

OBJECTIVE:

To determine how temperature affects DNA damage, nucleotide excision repair (NER) and photoenzymatic repair (PER) in *Daphnia* by quantifying CPD and (6-4) formation in at 20 °C, with and without repair radiation (Exp 8: +PRR 52 KJm^2 UVB and -PRR 14 KJm^2 UVB; Exp 9: +PRR 32 KJm^2 UVB and –PRR 9 KJm^2 UVB) following a 12 hour exposure, and after an additional 12 h dark incubation. These experiments are a follow Exps 6 and 7 and were conducted at higher UV-B lamp exposure levels for the treatments with PRR and lower exposure levels for the treatments without PRR.

HYPOTHESES:

- The rate of PER will exceed the rate of NER.

- NER will be equally effective in repairing CPDs and (6-4)s.

LITERATURE REVIEW:

PRELIMINARY DATA:

Preliminary experiments suggested that NER can repair CPDs. No data are available on the repair of (6-4)s by NER.

METHODS:

- *Daphnia* were collected from Dutch Springs, Bethlehem, PA. Adults were isolated in the lab.

- Incubated overnight (20 °C) in 4 L aquaria at experimental temperature under a 12:12 light:dark cycle

- Fed *Cryptomonas reflexa* (WCR) as food

- Lamp phototron was used to separate the effects of damage and repair processes (PER and NER) on CPD and (6-4) accumulation (Exps 8 and 9) and survival (Exp 9 only).

  - PRR + treatment was exposed to 52 KJm^2 UVB (Exp 8) and 32 KJm^2 (Exp 9) of the UV-B lamp.

  - PRR – treatment was exposed to 15 KJm^2 (Exp 8) and 9 KJm^2 (Exp 9) of the UV-B lamp.

- Following standard exposure period (12 h), treatments received the standard dark incubation.

- A DNA dosimeter was used for each treatment (DNA in buffered solution, salmon testes DNA from Carolina Biological Supply).

- *Daphnia* and dosimeter samples for CPD analysis were taken at 12 and 24 hours. Only live individuals were included in *Daphnia* samples. Samples were frozen immediately after collection to prevent repair of CPDs or degradation of DNA..

- Samples were sent to Dr. David Mitchell (M.D. Anderson Cancer Center, Smithville, TX) for DNA analysis.

- Survival was scored at 12 and 24 h (Exp 9).

PROBLEMS AND OTHER COMMENTS:

- Experiment 8 (20 °C): No survival data collected.
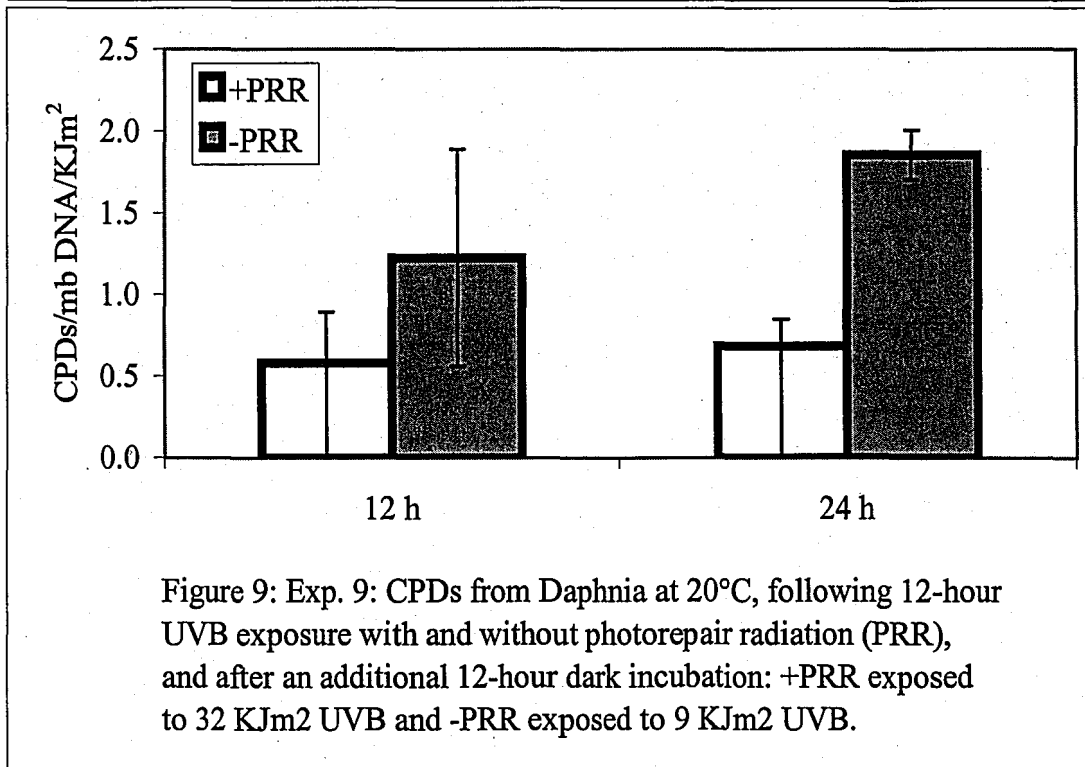
- Experiment 9 (10 °C): none

RESULTS:

Survival at 12 hours of *Daphnia* exposed to UVR in the presence of PRR (PER

and NER) was 80%, while survival in the absence of PRR (NER alone) was 39%,

suggesting that PER is necessary for survival. By 24 hours, survival had dropped to 66%

and 0%, in the +PRR and –PRR, respectively. Following the 12-hour exposure to the

UV-B lamp, *Daphnia* exposed in the presence of PRR had accumulated, on average, 49%

to 53% fewer CPDs than *Daphnia* exposed in the absence of PRR (Figures 8 and 9, Table

11). At 24 hours, the proportion of CPDs in the +PRR, compared to the –PRR, had

decreased slightly, so that *Daphnia* exposed in the presence of PRR had, on average, 33%

to 37% fewer CPDs than *Daphnia* exposed in the absence of PRR. Replication between

the two experiments, however, was poor. In Exp 8, *Daphnia* that had received repair

radiation during the UV-B lamp exposure did exhibit a 47% reduction in CPDs after an

additional 12-hour incubation in the dark. CPDs in *Daphnia*, which had been exposed to

the UV-B lamp in the absence of PRR, exhibited a 28% reduction in CPD load at 24

hours.

In Exp 9, +PRR *Daphnia* exhibited, on average, a 19% increase in CPDs after an

additional 12-hour incubation in the dark. CPDs in *Daphnia*, which had been exposed to

the UV-B lamp in the absence of PRR, exhibited, on average, a 51% increase in CPD

load at 24 hours. (6-4) data also indicate that post-exposure NER does not contribute a

great deal to the recovery of *Daphnia* from UVR-induced DNA damage (Figures 10 and

11, Table 12). There was no effect of PER or NER on (6-4) accumulation. Poor

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replication between two experiments and large error bars within experiments resulted in insufficient data to determine whether this result is significant, as large standard deviation values result in no significant difference between the data collected at 12 h and 24 h in the replicate experiment (Exp 9).

DISCUSSION:

The results of this preliminary study of induction and repair of (6-4)s do not provide evidence for the utilization of (6-4)photolyase by *Daphnia*. In addition, the results do not provide much indication that repair of CPD and (6-4) lesions by NER occurs following UV-B exposure. The results do support the findings of the initial experiments that suggest that concurrent, not sequential, exposure to repair radiation is necessary for effective PER.

Figure 8: Exp. 8: CPDs from Daphnia at 20°C, following 12-hour
UVB exposure with and without photorepair radiation (PRR),
and after an additional 12-hour dark incubation: +PRR exposed
to 52 KJm2 UVB and -PRR exposed to 14 KJm2 UVB.

Figure 9: Exp. 9: CPDs from Daphnia at 20°C, following 12-hour
UVB exposure with and without photorepair radiation (PRR),
and after an additional 12-hour dark incubation: +PRR exposed
to 32 KJm2 UVB and -PRR exposed to 9 KJm2 UVB.

Figure 10: Exp. 8: (6-4)s from Daphnia at 20°C, following 12-hour UVB exposure with and without photorepair radiation (PRR), and after an additional 12-hour dark incubation: +PRR exposed to 52 KJm2 UVB and -PRR exposed to 14 KJm2 UVB.

Figure 11: Exp. 9: (6-4)s from Daphnia at 20°C, following 12-hour UVB exposure with and without photorepair radiation (PRR), and after an additional 12-hour dark incubation: +PRR exposed to 32 KJm2 UVB and -PRR exposed to 9 KJm2 UVB.

Table 11: Exps. 8 and 9: CPDs: Results of two factor with replication ANOVAs for effects of PER (+ or -PRR) and NER (sample time 12 or 24 h).

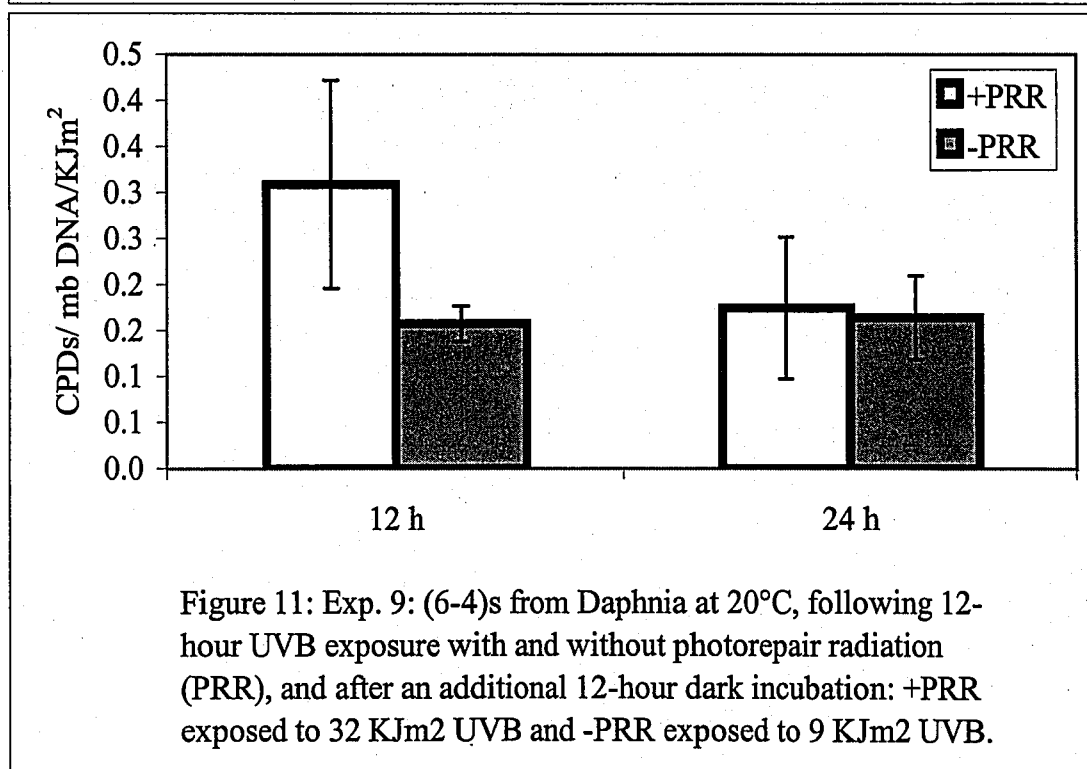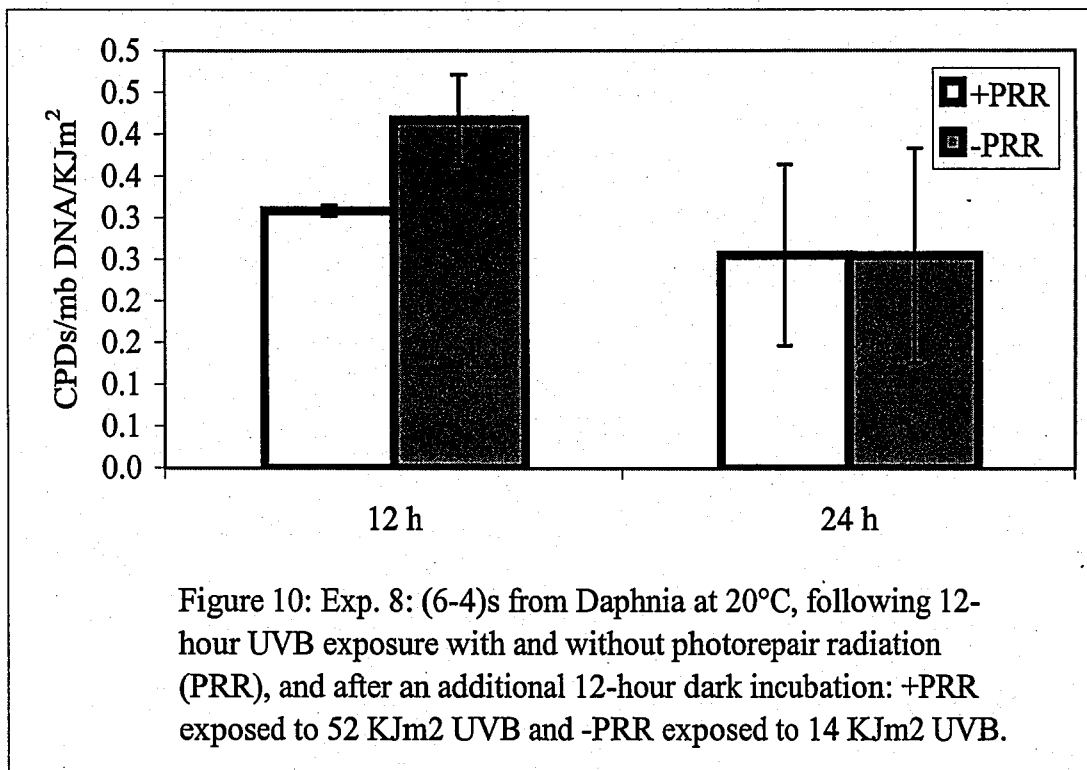| Source of Variation	SS	df	MS	F	P-value	F crit
Exp. 8						
Sample time	1.05	1	1.05	4.99	0.09	7.71
PRR	3.41	1	3.41	16.21	0.02	7.71
Interaction	0.00	1	0.00	0.00	0.97	7.71
Within	0.84	4	0.21			
Total	5.30	7				
Exp. 9						
Sample time	0.27	1	0.27	1.84	0.25	7.71
PRR	1.65	1	1.65	11.18	0.03	7.71
Interaction	0.14	1	0.14	0.94	0.39	7.71
Within	0.59	4	0.15			
Total	2.65	7				

Table 12: Exps. 8 and 9: (6-4)s: Results of two factor with replication ANOVAs for effects of PER (+ or -PRR) and NER (sample time 12 or 24 h).

Source of Variation	SS	df	MS	F	P-value	F crit
Exp. 8						
Sample time	0.02	1	0.02	2.99	0.16	7.71
PRR	0.01	1	0.01	0.75	0.44	7.71
Interaction	0.01	1	0.01	0.77	0.43	7.71
Within	0.03	4	0.01			
Total	0.07	7				
Exp. 9						
Sample time	0.01	1	0.01	1.55	0.28	7.71
PRR	0.01	1	0.01	2.50	0.19	7.71
Interaction	0.01	1	0.01	1.87	0.24	7.71
Within	0.02	4	0.01			
Total	0.05	7				

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EXPERIMENT: 10 (20 °C), solar phototron

QUESTION: What is the wavelength-specific impact of UVR on damage and repair in *Daphnia*?

OBJECTIVE:

- To determine the wavelength-specific responses of DNA damage and repair due to exposure of *Daphnia* to solar radiation by developing independent biological weighting functions (BWFs) for DNA damage and repair.

- To determine if CPDs (direct damage by UVR) are an accurate indicator of total damage (direct and indirect damage by UVR) by comparing BWFs based on survival with BWFs based on DNA damage.

HYPOTHESES:

- H1 – BWFs are similar whether they are based on CPDs or survival, which suggests that measuring direct UVR-induced damage (CPDs) is a good indicator of total UVR-induced damage (direct and indirect) in *Daphnia*.

- H2 – BWFs are different whether they are based on CPDs or survival, which suggests that measuring direct UVR-induced damage (CPDs) is not a good indicator of total UVR-induced damage (direct and indirect) in *Daphnia*.

BACKGROUND:

It is known that the damage to organisms induced by ultraviolet radiation (UVR) has 2 components: (1) direct damage to DNA, as cyclobutane pyrimidine dimers (CPDs) and (6-4)photoproducts, and (2) indirect damage to cells and tissues, through photo-oxidative damage. While the indirect effects of UVR are diverse and difficult to assay, the direct

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effects of UVR are well characterized and easily quantified. This study will determine whether direct damage is a good indicator of total damage in *Daphnia*.

PRELIMINARY DATA:

A biological weighting function (BWF) for net damage has been developed for *Daphnia* using survival as the measured data endpoint (Williamson et al. in press). Independent BWFs for damage and repair are necessary to predict how *Daphnia*, an organism without dose rate – cumulative dose reciprocity, might respond to changing UVR regimes. Preliminary data suggest that direct damage might not be an indicator of total damage in *Daphnia* (i.e. low survival despite repair of damaged DNA).

METHODS:

- Adult *Daphnia* were isolated from CEW laboratory cultures (culture origin: Dutch Springs, Bethlehem, PA, started by H. DeLange).

- Cultures were fed *Cryptomonas reflexa* (WCR, origin White Acre Pond, Saucon Valley, PA) from start of culture until 04/30/01, when the food alga was changed to *Ankistrodesmus* (ANK, culture obtained from Robert Moeller, origin Sue Kilham). Approximately 1/3 of the culture was switched from WCR to ANK each week for 3 weeks. Entire culture was fed ANK for at least approximately 3 weeks prior to experiment.

- Cultured at 20 °C in 4 L aquaria under a 12:12 light:dark cycle

- One day prior to the experiment, cultures were fed approximately double the daily amount of food to ensure that only well-fed individuals were used in the experiment.

- On the morning of the experiment, the culture was poured through a 363 um mesh to isolate larger individuals. Smaller individuals (<363 um) were returned to culture).

- Healthy-looking medium-large individuals were placed into black petri dishes (40/dish for dimer samples, 20/dish for survival).

- A DNA dosimeter was used for each treatment (DNA in buffered solution, salmon testes DNA from Carolina Biological Supply).

- Solar phototron was used to isolate wavelength-specific effects of damage and repair processes (PER and NER) on CPD accumulation and survival.

- All dishes, with the exception of dark controls (black-plastic-covered), were exposed to full solar radiation (under quartz lid) for 5 hours (from the start of the experiment at 9:45 am to 1:45 pm). At 4 hours, filters were placed above the quartz lids to cut out solar radiation below specific wavelengths. Filters with cutoffs of 305, 326, 352, 370, 404, 423, 433 and 450 nm, as well as a dark treatment, were applied to dishes of dosimeter, while filters with cutoffs of 404, 423, 433 and 450 nm and a dark treatment were applied to dishes of *Daphnia*.

- *Daphnia* and dosimeter samples for CPD analysis were taken at 0 hours, 4 hours (at the time of filter application), 7 hours (at the end of solar exposure) and at 24 hours (following 17 hours of dark incubation). Only live individuals were included in *Daphnia* samples. Samples were frozen immediately after collection to prevent repair of CPDs or degradation of DNA.

- Samples were sent to Dr. David Mitchell (M.D. Anderson Cancer Center, Smithville, TX) for CPD analysis.

- Survival data were collected through day 5.

PROBLEMS AND OTHER COMMENTS:

- Survival of *Daphnia* was variable in dishes with 40/dish.

- Dimer data did not indicate significant differences among treatments, as levels of

  dimer damage were low.

RESULTS:

Survival within treatments was too variable to detect significant differences among

treatments. CPD data do not indicate significant differences among treatments, as levels

of DNA damage were low.

DISCUSSION:

Additional experiments, at higher intensity exposures, are necessary to determine the

wavelength-specific impact of UVR on PER.

EXPERIMENTS: 11 (20 °C) and 12 (10 °C)

QUESTION: What is the temperature dependence of PER in *Daphnia*?

OBJECTIVE:

To determine how temperature affects DNA damage, nucleotide excision repair (NER) and photoenzymatic repair (PER) in *Daphnia* by quantifying CPDs (cyclobutane pyrimidine dimers) in *Daphnia* at 10 and 20 °C (+PRR and –PRR 15 KJm^2 UVB).

HYPOTHESIS:

PER is temperature sensitive: Net CPD accumulation in the presence of PER (DNA damage minus NER and PER) decreases with increased temperature (net CPD accumulation is lower at 20 °C than at 10 °C).

LITERATURE REVIEW:

Malloy et al. (1997) found that rate of PER increased as a linear function with temperature (6-25 °C) in Antarctic zooplankton (predominantly ichthyoplankton).

PRELIMINARY DATA:

Survival of *Daphnia catawba* under UVR stress increases from 15 °C to 25 °C, presumably due to increased activity of photolyase enzyme at higher temperatures (H. DeLange).

METHODS:

- *Daphnia* were collected from Dutch Springs, Bethlehem, PA. Adults were isolated in the lab using a 363 um mesh.

    - Incubated overnight (10 and 20 °C) in 4 L aquaria at experimental temperature under a 12:12 light:dark cycle

- Fed *Ankistrodesmus* (ANK) as food

- Lamp phototron was used to separate the effects of damage and repair processes (PER and NER) on CPD accumulation and survival.

    - PRR + treatment was exposed to 15 KJm^2 of UVB lamp

    - PRR– treatment was exposed to 15 KJm^2 of UVB lamp

- Following standard exposure period (12 h), treatments received the standard dark incubation.

- A DNA dosimeter was used for each treatment (DNA in buffered solution, salmon testes DNA from Carolina Biological Supply).

- *Daphnia* and dosimeter samples for CPD and 6-4 analyses were taken at 12 and 24 hours. Only live individuals were included in *Daphnia* samples. Samples were frozen immediately after collection to prevent repair of dimers or degradation of DNA.

- Samples were sent to Dr. David Mitchell (M.D. Anderson Cancer Center, Smithville, TX) for dimer analysis.

- Survival was scored for 10 days following exposure.

PROBLEMS AND OTHER COMMENTS:

- Experiment 11 (20 °C): Survival endpoint was day 3 based on controls (Figure 12).

- Experiment 12 (10 °C): none

RESULTS:

Survival data suggest that 15 KJm^2 of UVB lamp exposure is sufficient to induce a moderate mortality in the +PRR, but induces complete mortality in the –PRR (Table 1, Appendix 11/12).

In these experiments, samples for dimer analysis were collected at 12 and 24 hours. Due to poor survival in the dark controls of the 20 °C experiment (Exp 11) Insufficient survival data were obtained to make temperature comparison. Dimer data were not requested from Mitchell Lab.

DISCUSSION:

Experiment will be repeated due to poor survival of controls.

Figure 12: Exps. 11 and 12: Survival of Daphnia at 10°C and 20°C with and without photorepair radiation (PRR): +PRR and -PRR exposed to 15 KJm2 UVB.
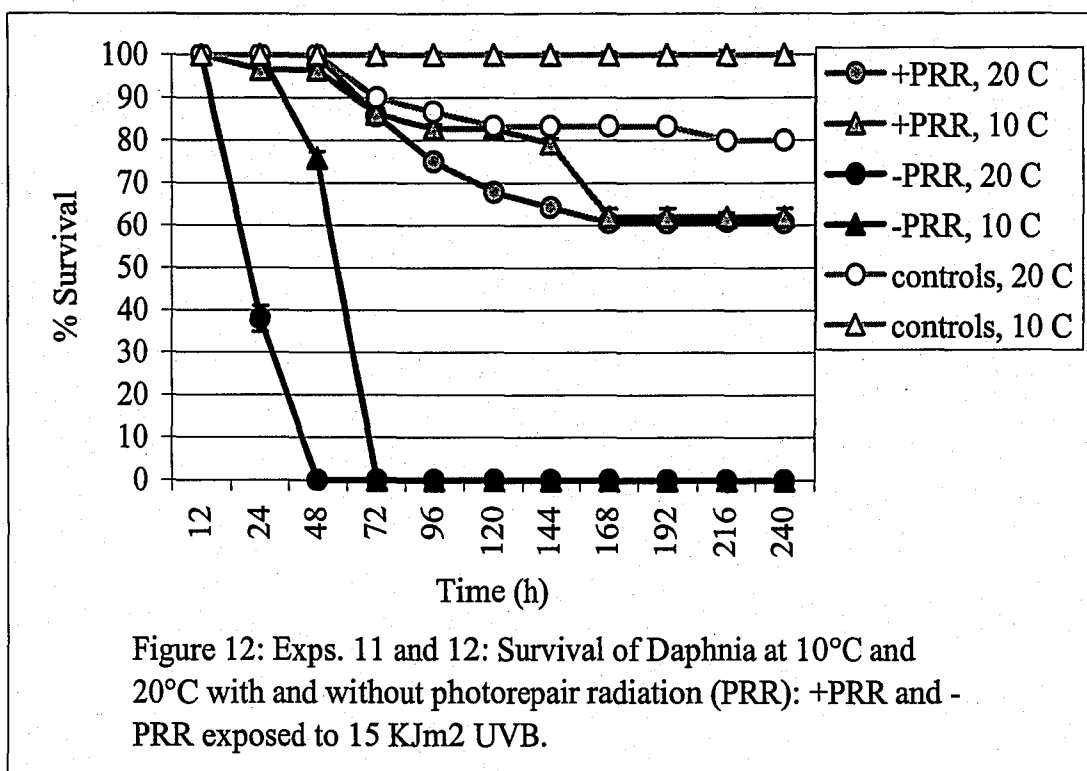
EXPERIMENT: 13 (20 °C), solar phototron (repeat of Exp 10, with modifications)

QUESTION: What is the wavelength-specific impact of UVR on damage and repair in *Daphnia*?

OBJECTIVE:

- To determine the wavelength-specific responses of DNA damage and repair due to exposure of *Daphnia* to solar radiation by developing independent biological weighting functions (BWFs) for DNA damage and repair.

- To determine if CPDs (direct damage by UVR) are an accurate indicator of total damage (direct and indirect damage by UVR) by comparing BWFs based on survival with BWFs based on DNA damage.

HYPOTHESES:

- H1 – BWFs are similar whether they are based on CPDs or survival, which suggests that measuring direct UVR-induced damage (CPDs) is a good indicator of total UVR-induced damage (direct and indirect) in *Daphnia*.

- H2 – BWFs are different whether they are based on CPDs or survival, which suggests that measuring direct UVR-induced damage (CPDs) is not a good indicator of total UVR-induced damage (direct and indirect) in *Daphnia*.

BACKGROUND:

It is known that the damage to organisms induced by ultraviolet radiation (UVR) has 2 components: (1) direct damage to DNA, as cyclobutane pyrimidine dimers (CPDs) and (6-4)photoproducts, and (2) indirect damage to cells and tissues, through photo-oxidative damage. While the indirect effects of UVR are diverse and difficult to assay, the direct

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effects of UVR are well characterized and easily quantified. This study will determine whether direct damage is a good indicator of total damage in *Daphnia*.

PRELIMINARY DATA:

A biological weighting function (BWF) for net damage has been developed for *Daphnia* using survival as the measured data endpoint (Williamson et al. in press). Independent BWFs for damage and repair are necessary to predict how *Daphnia*, an organism without dose rate – cumulative dose reciprocity, might respond to changing UVR regimes. Preliminary data suggest that direct damage might not be an indicator of total damage in *Daphnia* (i.e. low survival despite repair of damaged DNA).

METHODS:

- Adult *Daphnia* were isolated from CEW laboratory cultures (culture origin: Dutch Springs, Bethlehem, PA, started by H. DeLange).

- Cultures were fed *Cryptomonas reflexa* (WCR, origin White Acre Pond, Saucon Valley, PA) from start of culture until 04/30/01, when the food alga was changed to *Ankistrodesmus* (ANK, culture obtained from Robert Moeller, origin Sue Kilham). Approximately 1/3 of the culture was switched from WCR to ANK each week for 3 weeks. Entire culture was fed ANK for at least approximately 3 weeks prior to experiment.

- Cultured at 20 °C in 4 L aquaria under a 12:12 light:dark cycle

- One day prior to the experiment, cultures were fed approximately double the daily amount of food to ensure that only well-fed individuals were used in the experiment.

- On the morning of the experiment, the culture was poured through a 363 um mesh to isolate larger individuals. Smaller individuals (<363 um) were returned to culture).

- Healthy-looking medium-large individuals were placed into black petri dishes (50/dish: 40 to be sampled for dimers and 10 to be followed survival).

- A DNA dosimeter was used for each treatment (DNA in buffered solution, salmon testes DNA from Carolina Biological Supply).

- Solar phototron was used to isolate wavelength-specific effects of damage and repair processes (PER and NER) on CPD accumulation and survival.

- All dishes, with the exception of dark controls (black-plastic-covered), were exposed to full solar radiation (under quartz lid) for 5 hours (from the start of the experiment at 8:40 am to 1:40 pm). At 5 hours, filters were placed above the quartz lids to cut out solar radiation below specific wavelengths. Filters with cutoffs of 305, 326, 352, 370, 404, 423, 433 and 450 nm, as well as a dark treatment, were applied to dishes of dosimeter, while filters with cutoffs of 370, 404, 423, 433 and 450 nm and a dark treatment were applied to dishes of *Daphnia*.

- *Daphnia* and dosimeter samples for CPD analysis were taken at 0 hours, 4 hours (at the time of filter application), 8 hours (at the end of solar exposure) and at 24 hours (following 16 hours of dark incubation). Only live individuals were included in *Daphnia* samples. Samples were frozen immediately after collection to prevent repair of CPDs or degradation of DNA.

- Samples were sent to Dr. David Mitchell (M.D. Anderson Cancer Center, Smithville, TX) for CPD analysis.

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- No survival data were collected.

PROBLEMS AND OTHER COMMENTS:

- No survival data were collected.

- Dimer data have not been analyzed

RESULTS:

Dimer data have not been analyzed.

DISCUSSION:

After Exp 13 was conducted, data from Exp 10 were received. Results suggested that

dimer levels would be too low to quantify in samples from Exp 13.

EXPERIMENTS: 14 and 15

QUESTION: What is the UVR tolerance of *Daphnia* at 20 °C in the presence (+PRR) and absence of repair radiation (–PRR)?

OBJECTIVE:

To quantify the UVR tolerance of *Daphnia* at 20 °C, with 1) photoenzymatic repair (PER), nucleotide excision repair (NER) and photoprotection, and 2) only nucleotide excision repair (NER) and photoprotection.

HYPOTHESES:

- The survival response of *Daphnia* to low levels of UVR in the absence of repair radiation will be similar to its survival response to higher levels of UVR in the presence of repair radiation.

PRELIMINARY DATA:

Prior experiments conducted at 20 °C with field-collected *Daphnia*, demonstrated that, in the presence of repair radiation, *Daphnia* have considerable tolerance to UVR, while in the absence of repair radiation, *Daphnia* mortality results from very low UV-B exposure levels.

METHODS:

- *Daphnia* were collected from Dutch Springs, Bethlehem, PA. Adults were isolated in the lab.

    - Incubated overnight at 20 °C in 4 L aquaria under a 12:12 light:dark cycle

    - Fed *Ankistrodesmus* (ANK) as food

- Lamp phototron was used to separate the effects of damage and repair processes (PER and NER) on survival.

  - PRR + treatments were exposed to higher intensities of UV-B lamp (Exp 15: 15, 25, 32 and 52 KJm^2)

  - PRR – treatments were exposed to lower intensities of the UV-B lamp (Exp 14: 1, 2, 3, 6 KJm^2; Exp 15: 3, 4, 5 and 6 KJm^2)

- Survival was scored for 5 days following exposure.

PROBLEMS AND OTHER COMMENTS:

- Experiment 14: none

- Experiment 15: none

RESULTS:

In the first experiment (Exp 14), no treatments received PRR. *Daphnia* were exposed at 1, 2, 3 and 6 KJm^2 (Figure 13). At 5 days, survival in *Daphnia* exposed from 1 to 3 KJm^2 UVB radiation was between 97 and 99%, while survival in 6 KJm^2 treatment was 1% (Figure 14s). A second experiment (Exp 15) was conducted to characterize the survival between 3 and 6 KJm^2 (Figures 14 and 15). This experiment included concurrent exposure of *Daphnia* in the presence of PRR, exposed at higher levels UVB (14, 25, 32 and 52 KJm^2). This study provided a range of survival responses, from 0% in the treatments with the highest UVB intensities (52 KJm^2 for +PRR and 6 KJm^2 for –PRR), to 71% and 86% survival in the treatments exposed to the lowest UVB intensities (+PRR, exposed to 14 KJm^2 UVB; –PRR exposed to 3 KJm^2 UVB) (Figure x). Similar survival response curves for *Daphnia* at 20 °C result from exposure at 62% of the UV-B lamp (32

KJm^2) with repair radiation, and 9% of the UV-B lamp (5 KJm^2) without repair radiation (see attached figure).

DISCUSSION:

The results of this experiment provide comparative data for survival of *Daphnia* in +PRR and –PRR treatments and may be useful in planning future experiments.

Figure 13: Exp. 14: Survival of Daphnia at 20°C, following 12-hour UVB exposure at 1, 2, 3 and 6 KJm2 without photorepair radiation (-PRR).
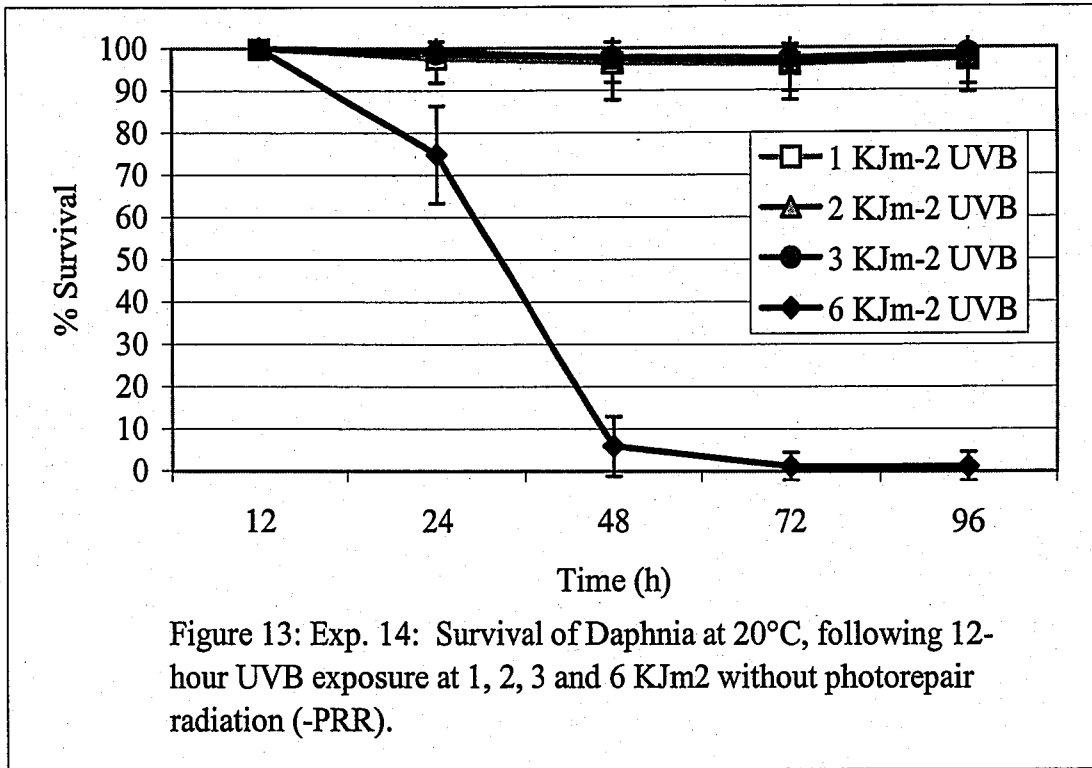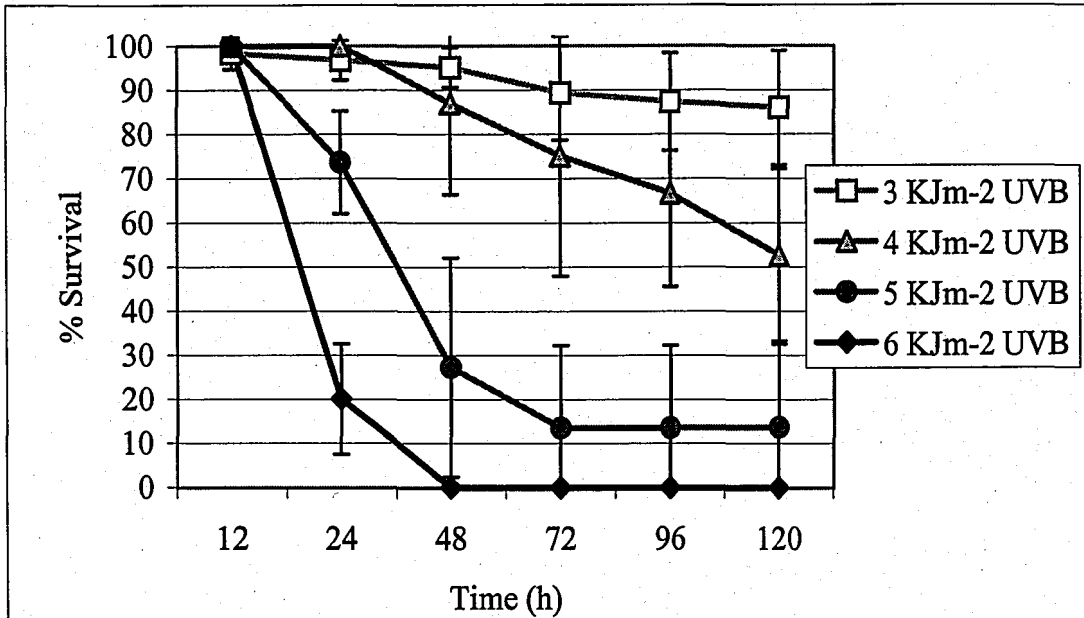
Figure 14: Exp. 15: Survival of Daphnia at 20°C, following 12-hour UVB exposure at 3, 4, 5 and 6 KJm2 without photorepair radiation (-PRR).
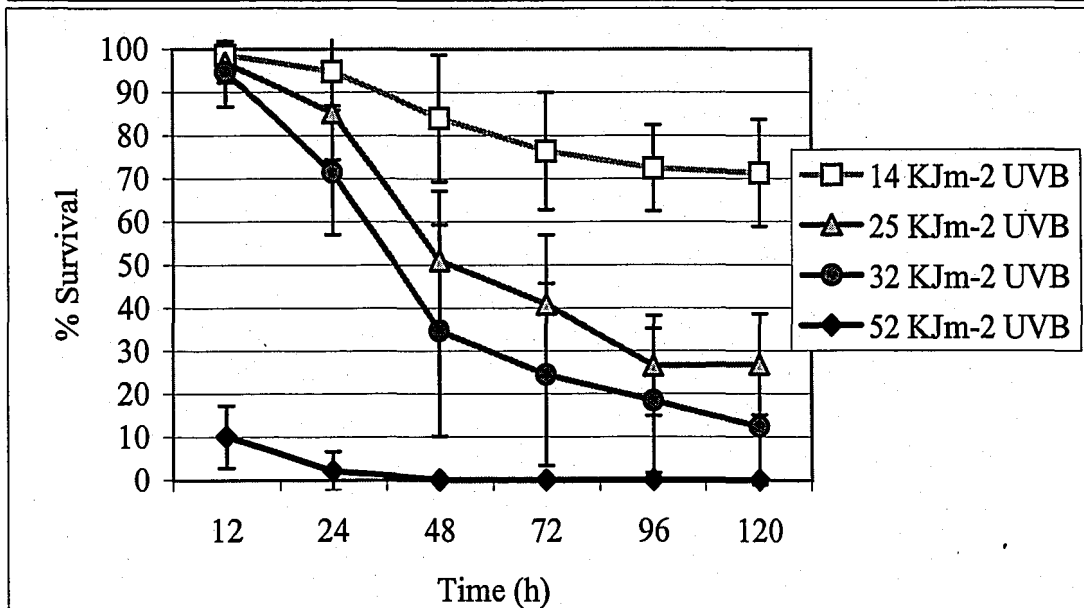

Figure 15: Exp. 15: Survival of Daphnia at 20°C, following 12hour UVB exposure at 14, 25, 32 and 52 KJm2 with photorepair radiation (+PRR).

EXPERIMENTS: 16 (20 °C) and 17 (10 °C)

QUESTION: What is the temperature dependence of PER in *Daphnia*?

OBJECTIVE:

To determine how temperature affects net DNA damage, (damage in the presence of nucleotide excision repair, NER) and photoenzymatic repair (PER) in *Daphnia*.

HYPOTHESIS:

PER is temperature sensitive: Net CPD accumulation in the presence of PER (DNA damage minus NER and PER) decreases with increased temperature (net CPD accumulation is lower at 20 °C than at 10 °C).

LITERATURE REVIEW:

Malloy et al. (1997) found that rate of PER increased as a linear function with temperature (6-25 °C) in Antarctic zooplankton (predominantly ichthyoplankton).

PRELIMINARY DATA:

Preliminary experiments were conducted at 10 °C and 20 °C with field-collected *Daphnia*, incubated overnight at the experimental temperature. Data from these experiments show no significant difference in CPD accumulation between 10 °C and 20 °C.

METHODS:

- *Daphnia* were collected from Dutch Springs, Bethlehem, PA. Adults were isolated in the lab with a 363 um mesh.

    - Incubated for 2 days (20 °C) or 3 days (5 °C) in 4 L aquaria at experimental temperature under a 12:12 light:dark cycle

- Fed *Ankistrodesmus* (ANK) as food

- Lamp phototron was used to separate the effects of damage and repair processes (PER and NER) on CPD accumulation and survival.

  - PRR + treatment was exposed to 15 KJm^2 of UVB lamp

  - PRR – treatment was exposed to 4 KJm^2 of UV-B lamp

- Samples were sent to Dr. David Mitchell (M.D. Anderson Cancer Center, Smithville, TX) for CPD analysis.

- Survival was scored for 5 days (20 °C) or 10 days (10 °C) following the exposure.

PROBLEMS AND OTHER COMMENTS:

- Experiment 16 (20 °C): Survival endpoint was day 2 based on controls. CPD concentrations too low to distinguish significant differences among treatments (see attached figure).

- Experiment 17 (10 °C): CPD concentrations too low to distinguish significant differences among treatments (see attached figure).

RESULTS:

Poor survival in the 20 °C dark controls (Exp 16), affected this experiment as well the previous experiment (Exp 11) (Figure 16).

CPD data were obtained for +PRR and –PRR samples at 12 and 24 hours. The same relationship between the presence or absence of repair radiation and relative CPD accumulation was exhibited by these experiments as was previously observed. At 20 °C, the +PRR *Daphnia* accumulated 56% fewer CPDs than did the –PRR *Daphnia* (Figure 17). At 10 °C, the same pattern was exhibited but to a lesser extent, as the +PRR

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Daphnia accumulated 36% fewer CPDs than did the –PRR *Daphnia*. This slight

difference in results between the 10 and 20 °C experiments again suggests (see Exp 4/5)

that there may be an effect of temperature on repair between these two temperatures. The

results, however, are not conclusive for temperature. The only statistically significant

result obtained from this experiment was that there is a suggestion of post-exposure NER

at 20 °C.

DISCUSSION:

Future experiment will use high exposure levels.

Figure 16: Exps. 16 and 17: Survival of Daphnia at 10°C and 20°C, following 12-hour UVB exposure with and without photorepair radiation (PRR): +PRR exposed to 15 KJm2 and -PRR exposed to 7 KJm2 UVB.
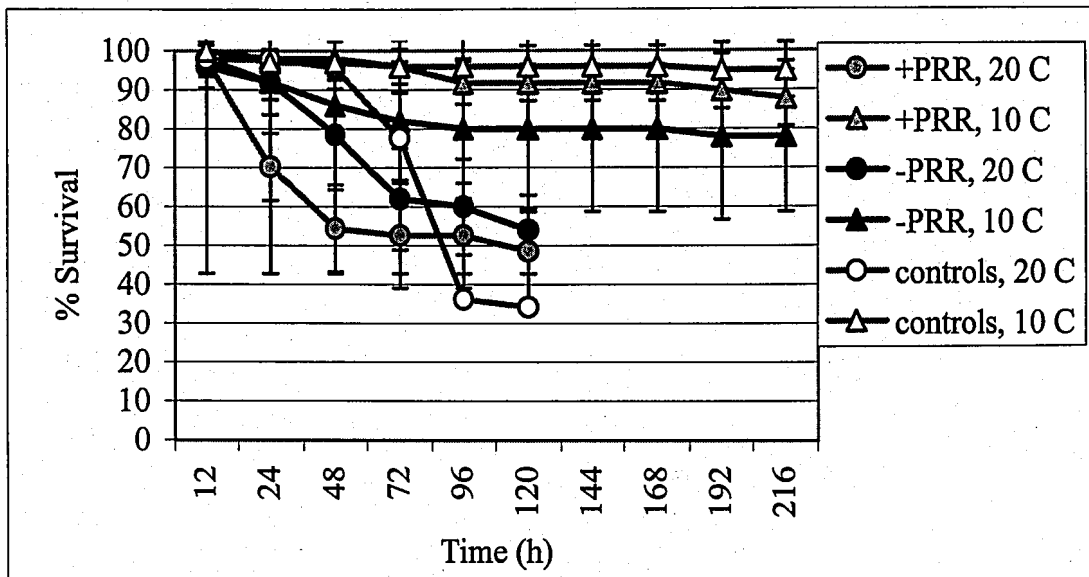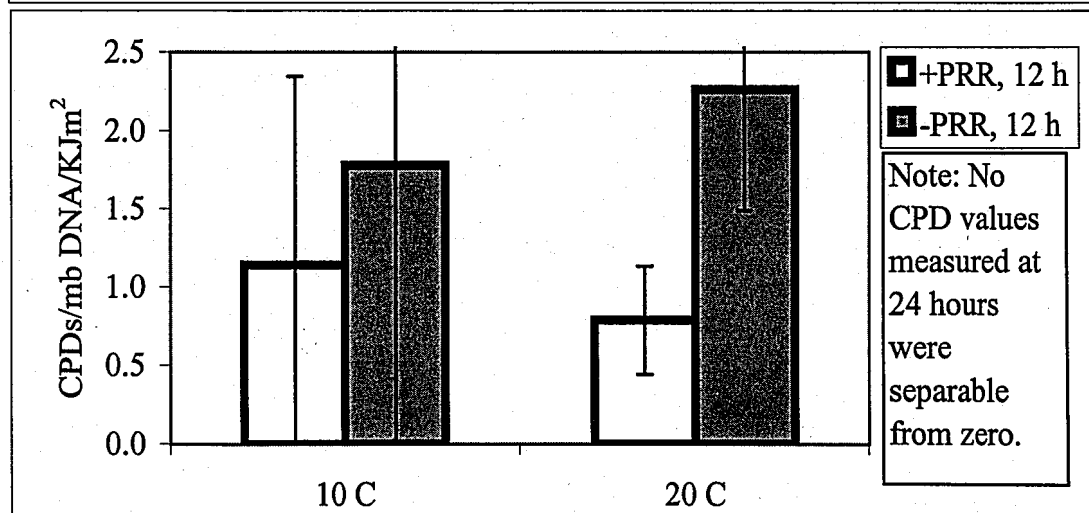

Figure 17: Exps. 16 and 17: CPDs from Daphnia at 10°C and 20°C, following 12-hour UVB exposure with and without photorepair radiation (PRR), and after an additional 12-hour dark incubation: +PRR exposed to 15 KJm2 and -PRR exposed to 7 KJm2 UVB.

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Table 13: Exps. 16 and 17 : 12 and 24 hour samples: Results of two factor with replication ANOVAs for effects of temperature (10 or 20 °C) and PER (+ or -PRR).

Source of Variation	SS	df	MS	F	P-value	F crit
12-hour samples						
Temperature	0.14	1	0.14	0.06	0.82	7.71
PRR	1.90	1	1.90	0.84	0.41	7.71
Interaction	0.50	1	0.50	0.22	0.66	7.71
Within	9.06	4	2.27			
Total	11.60	7				
24-hour samples						
Temperature	0.00	1	0.00	2.16	0.22	7.71
PRR	0.00	1	0.00	0.18	0.69	7.71
Interaction	0.00	1	0.00	0.20	0.68	7.71
Within	0.00	4	0.00			
Total	0.01	7				

Table 14: Exps. 16 and 17 : 10 and 20 °C: Results of two factor with replication ANOVAs for effect of NER (sample time: 12 or 24 hours) and PER (+ or -PRR).

Source of Variation	SS	df	MS	F	P-value	F crit
10 °C						
Sample time	6.11	1	6.11	5.03	0.06	5.32
PRR	0.29	1	0.29	0.24	0.64	5.32
Interaction	0.32	1	0.32	0.26	0.62	5.32
Within	9.71	8	1.21			
Total	16.43	11				
20 °C						
Sample time	4.48	1	4.48	24.92	0.01	7.71
PRR	1.05	1	1.05	5.81	0.07	7.71
Interaction	1.13	1	1.13	6.30	0.07	7.71
Within	0.72	4	0.18			
Total	7.38	7				

EXPERIMENT: 18 (20 °C), solar phototron (repeat of Exps 10 and 13, with modifications)

QUESTION: What is the wavelength-specific impact of UVR on damage and repair in *Daphnia*?

OBJECTIVE:

- To determine the wavelength-specific responses of DNA damage and repair due to exposure of *Daphnia* to solar radiation by developing independent biological weighting functions (BWFs) for DNA damage and repair.

- To determine if CPDs (direct damage by UVR) are an accurate indicator of total damage (direct and indirect damage by UVR) by comparing BWFs based on survival with BWFs based on DNA damage.

HYPOTHESES:

- H1 – BWFs are similar whether they are based on CPDs or survival, which suggests that measuring direct UVR-induced damage (CPDs) is a good indicator of total UVR-induced damage (direct and indirect) in *Daphnia*.

- H2 – BWFs are different whether they are based on CPDs or survival, which suggests that measuring direct UVR-induced damage (CPDs) is not a good indicator of total UVR-induced damage (direct and indirect) in *Daphnia*.

BACKGROUND:

It is known that the damage to organisms induced by ultraviolet radiation (UVR) has 2 components: (1) direct damage to DNA, as cyclobutane pyrimidine dimers (CPDs) and (6-4)photoproducts, and (2) indirect damage to cells and tissues, through photo-oxidative damage. While the indirect effects of UVR are diverse and difficult to assay, the direct

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effects of UVR are well characterized and easily quantified. This study will determine whether direct damage is a good indicator of total damage in *Daphnia*.

PRELIMINARY DATA:

A biological weighting function (BWF) for net damage has been developed for *Daphnia* using survival as the measured data endpoint (Williamson et al. in press). Independent BWFs for damage and repair are necessary to predict how *Daphnia*, an organism without dose rate – cumulative dose reciprocity, might respond to changing UVR regimes. Preliminary data suggest that direct damage might not be an indicator of total damage in *Daphnia* (i.e. low survival despite repair of damaged DNA).

METHODS:

- Organisms were collected from Dutch Springs (off steel pier to 20 meters) with a 202 um bongo net on the afternoon before the experiment.

- Collected organisms were filtered through a 363 um mesh to isolate adults. Adults were incubated overnight with *Ankistrodesmus* as food.

- Incubated overnight at 20 °C in 4 L aquaria under a 12:12 light:dark cycle

- Adults were isolated using a 363 um mesh.

- Healthy-looking medium-large individuals were placed into black petri dishes (50/dish: 40 to be sampled for dimers and 10 to be followed survival).

- A DNA dosimeter was used for each treatment (DNA in buffered solution, salmon testes DNA from Carolina Biological Supply).

- Solar phototron was used to isolate wavelength-specific effects of damage and repair processes (PER and NER) on CPD accumulation and survival.

- All dishes, with the exception of dark controls (black-plastic-covered), were exposed to full solar radiation (under quartz lid) for 5 hours (from the start of the experiment at 8:40 am to 1:40 pm). At 4.5 hours, filters were placed above the quartz lids to cut out solar radiation below specific wavelengths. Filters with cutoffs of 305, 326, 352, 370, 404, 423, 433 and 450 nm, as well as a dark treatment, were applied to dishes of dosimeter and *Daphnia*.

- *Daphnia* and dosimeter samples for CPD analysis were taken at 0 hours, 4.5 hours (at the time of filter application), 8 hours (at the end of solar exposure) and at 24 hours (following 16 hours of dark incubation). Only live individuals were included in *Daphnia* samples. Samples were frozen immediately after collection to prevent repair of CPDs or degradation of DNA.

- Samples were sent to Dr. David Mitchell (M.D. Anderson Cancer Center, Smithville, TX) for CPD analysis.

- Survival data were recorded for 5 days following solar exposure.

PROBLEMS AND OTHER COMMENTS:

- Dimer data have not been analyzed

RESULTS:

Dimer data have not been analyzed. Survival data are presented in Figure 18.

DISCUSSION:

After Exp 18 was conducted, data from Exp 10 were received. Results from Exp 10 suggested that dimer levels would be too low to distinguish among treatments in samples from Exp 18.

Figure 18: Exp. 18: Survival of Daphnia at 20°C, following exposure in solar phototron, 08/02/01.
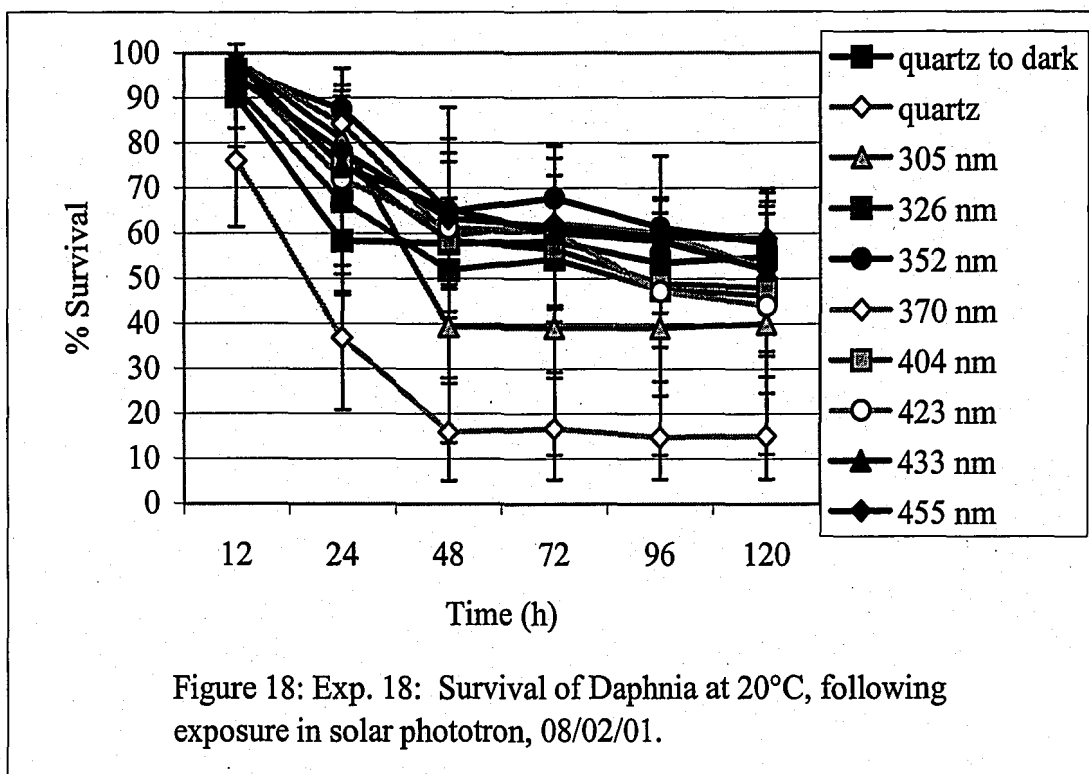
EXPERIMENTS: 19 (20 °C) and 20 (5 °C)

QUESTION: What is the temperature dependence of PER in *Daphnia*?

OBJECTIVE:

To determine how temperature affects DNA damage, nucleotide excision repair (NER) and photoenzymatic repair (PER) by quantifying CPDs (cyclobutane pyrimidine dimers) and (6-4)photoproducts ((6-4)s) in *Daphnia* at 5 and 20 °C (+PRR exposed to 52 KJm^2 UVB and –PRR exposed to 25 KJm^2 UVB).

HYPOTHESES:

- Damage is not temperature sensitive: Net CPD and (6-4) accumulation in the absence of repair radiation (–PRR) is not affected by temperature (the same at 5 °C and 20 °C).

- PER is temperature sensitive: Net CPD and (6-4) accumulation in the presence of repair radiation (+PRR) increases with decreases in temperature from 5 to 20 °C.

LITERATURE REVIEW:

Malloy et al. (1997) found that rate of PER increased as a linear function with temperature (6-25 °C) in Antarctic zooplankton (predominantly ichthyoplankton).

PRELIMINARY DATA:

Preliminary experiments were conducted at 10 °C and 20 °C with field-collected *Daphnia*, incubated overnight at the experimental temperature. Data from these experiments show no significant difference in CPD accumulation between 10 °C and 20 °C.

METHODS:

- *Daphnia* were collected from Dutch Springs, Bethlehem, PA. Adults were isolated in the lab using a 363 um mesh.
  - Incubated for 2 days (20 °C) or 3 days (5 °C) in 4 L aquaria at experimental temperature under a 12:12 light:dark cycle
  - Fed *Ankistrodesmus* (ANK) as food
- Lamp phototron was used to separate the effects of damage and repair processes (PER and NER) on CPD and 6-4 accumulation and survival.
  - PRR+ treatment was exposed to 52 KJm^2 of UVB lamp
  - PRR− treatment was exposed to 25 KJm^2 of UVB lamp
- A DNA dosimeter was used for each treatment (DNA in buffered solution, salmon testes DNA from Carolina Biological Supply).
- *Daphnia* and dosimeter samples for dimer analysis were taken at 12 hours. Only live individuals were included in *Daphnia* samples. Samples were frozen immediately after collection to prevent repair of CPDs or degradation of DNA. Samples were sent to Dr. David Mitchell (M.D. Anderson Cancer Center, Smithville, TX) for CPD and 6-4 analyses.
- Survival was scored for 5 days (20 °C) or 10 days (5 °C) following the exposure.

PROBLEMS AND OTHER COMMENTS:

- Experiment 19 (20 °C): none
- Experiment 20 (5 °C): none

RESULTS:

At 20 °C, accumulation of both CPDs and (6-4)s was significantly greater in the absence

of PRR than in its presence (Figure 3). The concentration of CPD damage sustained by

Daphnia exposed to UV-B without PRR was 3.69 times higher (at 20 °C) than that of

Daphnia exposed to UV-B with PRR. The response was even more pronounced in the

(6-4)s, where concentration of damage was 5.82 times higher (at 20 °C) in the treatment

without PRR than in the treatment with PRR. This pattern was not seen at 5 °C,

however, where there was no significant difference between the treatments with and

without PRR in either the CPDs or the (6-4)s.

As expected, net CPD and (6-4) accumulation in the presence of PRR increased

with decreased temperature from 20 to 5 °C. There were approximately two times as

many CPDs accumulated at 5 °C, than as at 20 °C. This response was even more

pronounced in the (6-4)s, which exhibited approximately a 3-fold increase from 20 °C to

5 °C. Results from the treatments without PRR, however, did not exhibit the anticipated

results. Instead of being higher at 5 °C– or the same at 5 and 20 °C – the CPD and (6-4)

concentrations were lower by 29% and 46%, respectively, at 5 °C than at 20 °C.

DISCUSSION:

Results from this experiment provide the first evidence that *Daphnia* utilizes

light-dependent enzymes to repair both CPD and (6-4) lesions in its DNA. The results

support the hypothesis that DNA repair of UV-induced lesions is temperature mediated.

The results from the treatments without PRR (CPD and (6-4) damage was higher at 5 °C

than at 20 °C) were not consistent with the hypothesis that damage is temperature independent.

The mechanism responsible for the observed difference in CPDs and (6-4)s between 5 °C and 20 °C in the treatments without PRR is not known; however, there are several potential explanations. One potential explanation for this finding is that dark repair processes may differ from PER in their temperature sensitivity; NER may have a temperature optimum that is closer to 5 °C than 20 °C. Also, there is no consensus in the literature about whether PER inhibits or facilitates NER. If NER is, in fact, inhibited by PER, then a suppression of PER at 5 °C could potentially release NER from inhibition.

Another potential explanation for this finding is that DNA is more sensitive to damage at the higher temperature. The mechanism for such a response could likely be explained by considering the relationship between DNA damage and DNA repair in faster versus slower growing cells. At a higher temperature, the physical protection of the DNA molecule from UVR damage may be compromised when the DNA is unwound during DNA replication, transcription, and cell division. Meanwhile, repair processes would likely not be enhanced during rapid cell growth and division. In such a situation, faster growing cells may be vulnerable to UVR damage. A large proportion of *Daphnia*'s UVR tolerance can be attributed to PER (based on past studies of differential survival in +PRR and −PRR treatments). Therefore, it may be reasonable to assume that rapid cell division could compromise the protection of DNA.

When the results obtained from the two temperatures are compared, results suggest that (6-4) photolyase may be more sensitive to variations in temperature between 5 and 20 °C than CPD photolyase.

SIGNIFICANCE:

This study represents the first quantitative study of repair of UV-induced DNA damage in zooplankton, and is one of few on a multicellular organism.

The success of this set of experiments justifies conducting a follow-up study using the same experimental design, and conducted over a wider range of temperatures, at higher resolution intervals, such as 5, 10, 15, 20, 25 and 30 °C.

<center>CURRICULUM VITAE</center>

Emily J. MacFadyen

Place of birth: Oakland, California
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Professional and Teaching Experience:
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         University, January 2001 – December 2001
      Teaching assistant for Introduction to Environmental Biology (EES 031), Lehigh
         University, September 2000 – December 2001
      Biologist, URS Corporation, Blue Bell, PA 19422, August 1998 – February 2000
      NSF REU Intern, Lehigh University, May 1997 – August 1997

Professional Activities (Memberships)
      Ecological Society of American, Student Member, March 2002 – present
      Sigma Xi, Scientific Research Society, Associate Member, May 1998 – present

Honors
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      Mary Lyon Scholar. Mount Holyoke College, May 1998, awarded for exceptional
         Honors Paper
      Upperclass Academic Advisor, Mount Holyoke College, September 1996-May
         1998, awarded by Department Chairpersons and Dean of First-Year Studies

Awards:
      Lehigh University Earth and Environmental Sciences Department Travel Grants:
         March 2001 and August 2002 (funds awarded for past and future travel)

Publications
      Manuscript of M.S. research in preparation
      Manuscript of undergraduate honors thesis in preparation

Presentations
      AIBS Annual Meeting, March 2001, Poster: "Photoenzymatic repair in the Water
         flea, *Daphnia* pulicaria."

Mount Holyoke College Student Research Symposium, April 1998, talk:
   "Ammonium inhibition of root growth and the prevention of this effect by
   potassium in small-seeded species."

Pocono Comparative Lakes Program (PCLP) Annual Meeting, October 1997,
   2000 and 2001, talks
Mount Holyoke College Biological Sciences Department, September 1997, talk:
   Results of NSF REU research

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TITLE