Interspecific Variation in UV Defense Mechanisms Among Temperate Freshwater Fishes

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Received 23 August 2005; accepted 16 December 2005; published online 21 December 2005 DOI: 10.1562/2005-08-23-RA-656

ABSTRACT
An important step in predicting the effects of future increases in UV radiation (UVR) is to evaluate the mechanisms that organisms use to prevent and repair DNA damage and determine how those mechanisms influence UVR sensitivity. Damage is prevented by varying degrees through photoprotection and repaired via two main pathways: nucleotide excision repair and photoenzymatic repair. At present, little is known about the generality or similarity of these defenses among temperate freshwater fishes. We used laboratory experiments to compare UVR defense mechanisms among five freshwater fish species representing four families and three orders. Purified DNA, freeze-killed larvae and live larvae were exposed to UVB radiation for 12 h in the presence or absence of photorepair radiation. After exposure, we quantified frequencies of cyclobutane pyrimidine dimers in each exposure treatment. All five species used photoprotection and proportional decreases in dimer frequency were similar for all species. Evidence of excision repair was also found for all species but proportional decreases in photoproduct frequencies varied among species. Finally, evidence of photoenzymatic repair was found for only two of the five species.

INTRODUCTION
UVB radiation (spectrum, 290–320 nm) reaching the Earth’s surface is predicted to continue increasing at midlevel and high latitudes for several decades because of the interactive effects of stratospheric ozone depletion and greenhouse gas emissions (1,2). In aquatic ecosystems the effects of increased UV radiation (UVR) exposure depend strongly on local-scale processes that determine dissolved organic matter (DOM) concentrations (3). At DOM concentrations >2 mg/L, most of the incident UVR attenuates in the top 1–2 m (4). However, losses of wetlands, acidification and drought-induced reductions in runoff can all reduce DOM inputs and thereby increase UVR penetration in individual ecosystems to depths of ≥5 m (3). Because exposure to UVR can damage DNA, these increases in UVR penetration pose a threat to aquatic organisms, ranging from bacteria to fish (5).

An important step in predicting effects of future increases in UVR is to evaluate the defense mechanisms that organisms use to minimize DNA damage. To varying degrees all eukaryotic organisms use photoprotection (PP), typically involving pigments such as melanin and carotenoids, to absorb UVR and thus prevent the formation of DNA photoproducts (6). The major DNA-damage types that do occur can be repaired through two main pathways. Nucleotide excision repair (NER) is a generalized DNA repair mechanism found in all organisms that involves multiple proteins and requires metabolic energy in the form of ATP (7). Photoenzymatic repair (PER), which is found in some organisms, is specific to UVR damage and involves a single enzyme (photolyase) that uses energy from photorepair radiation (PRR), specifically UVA (spectrum, 320–400 nm) and short-wavelength visible (blue) light (7,8).

Fish are generally thought to use both NER and PER to repair DNA photodamage (9,10). However, the extent to which this observation holds for temperate freshwater fishes is not known because it is based on relatively few species and because those that have been studied are primarily tropical (e.g. Poecilia Formosa and Carassius auratus) (11,12) or marine (e.g. Engraulis mordax and Gadus morhua) (13,14). In addition, direct comparisons among species to evaluate interspecific variation are lacking. In this study we compared UVR defense mechanisms among the larvae of five ecologically and economically important freshwater fish species representing four families and three orders. Following the approach of MacFadyen et al. (15), we used laboratory experiments to quantify the frequency of cyclobutane pyrimidine dimers (CPDs) after 12 h of UVB exposure in purified DNA, dead organisms and live organisms in the presence and absence of PRR. Pairwise comparisons among these exposure treatments allowed us to determine whether PP, NER and PER reduced CPD frequencies in each species and whether the effectiveness of each mechanism differed among species. We focused on larvae because fish are most vulnerable to UVR during early stages of life, when high surface area to volume ratios and limited avoidance capabilities can result in high exposure levels to a large proportion of cells (16,17).

MATERIALS AND METHODS
Fish. The five fish species used in this study were bluegill (Lepomis macrochirus, Perciformes, Centrarchidae), brook trout (Salvelinus fontinalis, Salmoniformes, Salmonidae) and yellow perch (Perca flavescens, Perciformes, Percidae). Because of differences in geographic range and spawning behavior, collection of experimental animals varied among species (Table 1). Brook trout and rainbow trout were obtained as eyed eggs from hatcheries and hatched in incubators before experimentation, northern pike were obtained as larvae

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from a hatchery, bluegill were collected as larvae from Lake Giles, PA, and yellow perch were collected as eyed eggs from Lake Lacawac, PA, and hatched in an incubator (18). All larvae were kept in the dark in an incubator at a temperature within the range of hatching temperatures (Table 1) in either their native water or spring water. To facilitate interspecific comparisons, all experiments were conducted on yolk sac larvae of the 5th days of hatching.

**Experimental design.** Fish larvae were experimentally exposed to UVB radiation in the presence and absence of PRR in a lamp phototron (19). Housed within a light- and temperature-controlled environmental chamber, the phototron had a 30 cm diameter, horizontal, opaque polyethylene methacrylate wheel that rotated at 2 rpm. Around the outside of the wheel were forty 5.1 cm diameter holes arranged in two rows of 20. During an experiment, we stocked 4–10 fish larvae (depending on the species) into 30 mL quartz dishes filled with 20 mL of source water. These dishes were then placed inside 25 mm high opaque collars and set on top of a hole in the wheel. The wheel sat on top of a light-tight Styrofoam box that was open only below the wheel. Inside the box and 32 cm below the wheel were two 40 W cool, white fluorescent lights and two 40 W Q-panel 340 bulbs (Q-Panel, Cleveland, OH). These lights supplied PRR upwards through the holes in the wheel. Depending on the species, one to three Spectronics XX15B UVB lamps (Spectronics, Westbury, NY) were suspended 24 cm above the wheel. These lamps were covered with cellulose acetate to remove the low levels (cutoff, <290 nm) of UVC that are emitted from the lamps. UVB exposure levels were manipulated by placing stainless-steel mesh screens on top of quartz dishes. Similarly, metal disks were placed over the holes in the wheel to block PRR. All experiments were conducted at the temperatures to which larvae had been acclimated in incubators (Table 1).

To determine the extent to which each species used PP, NER and PER to decrease potential DNA photodamage, we modified the experimental design of MacFadyen et al. (15). Specifically, we exposed purified DNA (derived from salmon testes and diluted to 100 µg/mL in double-distilled water), dead larvae and live larvae to UVB radiation, as well as live larvae to both UVB and PRR, for a total of 12 h. The purified DNA was contained in 10×40 mm quartz cuvettes sealed with paraffin and rubber stoppers that would lie horizontally inside a quartz dish. Dead larvae were freeze-killed immediately before the experiment to arrest metabolically driven NER. For each species, we chose UVB exposure levels that were known to cause 100% mortality within 7 days on the basis of previous experiments but had high survival at the end of the exposure period (Table 1). All exposure treatments had three to five experimental units (i.e. quartz dishes). At the end of an experiment we scored survival of larvae that were alive at the start of the exposure. Only those larvae that survived were used in the DNA-damage assay.

**DNA-damage assay.** Subsequent to UV exposure and repair, fish from each experimental unit were pooled and immediately frozen and stored at −20°C. Samples were then thawed into a 1.5 mL microcentrifuge tube (Eppendorf, Westbury, NY) containing 750 µL of lysis buffer solution (10 mM Tris–Cl [pH 8.0]; Sigma, St. Louis, MO); 1 mM EDTA [pH 8.0]; Sigma); and 0.25% sodium dodecyl sulfate (Roche, Indianapolis, IN). After incubation with 10 µg/mL heat-inactivated RNase (Sigma) at 37°C for 1 h, the lysates were treated with 30 µL Proteinase K (10 mg/mL; Roche) in a variable speed shaker (Eppendorf, Westbury, NY) overnight at 37°C with vigorous mixing. Following deproteination, samples were extracted sequentially with equal volumes of buffer-saturated phenol (Roche), phenol:Sevag (1:1) and Sevag (24:1 chloroform:isoamyl alcohol). Finally, the aqueous phase was transferred to a centrifuge tube containing one-tenth volume 3 M sodium acetate (pH 5.2). One volume ice-cold isopropanol was added and the mixture was inverted several times before overnight precipitation at −20°C. Following precipitation samples were centrifuged at 13 000 g (Beckman TJ-6; Beckman Coulter, Fullerton, CA) for 10 min at room temperature, washed with 70% ethanol, air-dried and resuspended in 1 mL 10 mM Tris and 1 mM EDTA (pH 8.0). The DNA concentration and purity were determined by absorbance.

We quantified the frequencies of CPDs in each of our exposure treatments by radioimmunoassay analysis (RIA). The RIA is an assay that involves competitive binding between very small amounts of radiolabeled DNA and sample DNA for antisera raised against UV-irradiated DNA. For the RIA, 50 ng of heat-denatured sample DNA were incubated with 5–10 pg of poly(dA):poly(dT) (labeled to >5×10⁶ cpm/µg by nick translation with [3P]dTPP) in a total volume of 1 mL 10 mM Tris (pH 7.8), 150 mM NaCl, 1 mM EDTA and 0.15% gelatin (Sigma). Antiserum was added at a dilution that yielded ~35% binding to labeled ligand, and after incubation overnight at 4°C the immune complex was precipitated with goat anti-rabbit immunoglobulin (Calbiochem, San Diego, CA) and carrier serum from nonimmunized rabbits (UTMDACC, Science Park/Veterinary Division, Bastrop, TX). After centrifugation, the pellet was dissolved in tissue solubilizer (NCS, Amersham, Piscataway, NJ), mixed with ScintiSafe (Fisher, Pittsburgh, PA) containing 0.1% glacial acetic acid and the 3P quantified by liquid scintillation spectrometry. Under these conditions, antibody binding to an unlabeled competitor inhibits antibody binding to the radiolabeled ligand. These details, as well as those concerning the specificities of the RIAs and standards used for quantification, are described in Mitchell (20, 21).

**Data analysis.** We evaluated differences in the mean number of CPDs×10⁶ bases among pairs of exposure treatments to determine the extent to which a particular UVR defense mechanism was operating. Because exposure levels varied among species and among exposure treatments, all data were standardized by Setlow-weighted exposure and expressed as the frequency of CPD×10⁶ bases/weighted kJ m⁻². To test for an effect of PP, we compared purified DNA and dead larvae in the absence of PRR. To test for an effect of NER, we compared dead larvae and live larvae in the absence of PRR. To test for an effect of PER, we compared live larvae in the absence and presence of PRR. For all comparisons, we used analysis of variance (ANOVA) with exposure treatment and species as main effects. All data were log₁₀-transformed before analysis to reduce heteroscedasticity and to allow changes in CPD frequency to be interpreted as proportions. A significant interaction term indicated that the effectiveness of the defense mechanism in reducing CPD frequency varied among species. If the interaction term was significant, we then analyzed each species separately with one-way ANOVA. If the interaction term was not significant, we could interpret a significant main effect of exposure treatment to indicate a similar effectiveness of the defense mechanism across species. In the absence of an interaction, a significant main effect of species indicated that CPD frequencies averaged across exposure treatments differed among species but that all species had similar proportional decreases in CPD frequency.

**RESULTS**

For all five species, there was a decrease in the frequency of CPDs between purified DNA and freeze-killed individuals in the absence
of PRR, indicating significant photoprotection (Fig. 1a). Both the main effects of fish species and exposure treatment were significant (overall ANOVA: $F_{0.28} = 31.15, P < .0001$; species: $F_{4.28} = 8.53, P < .0001$; and exposure treatment: $F_{1.28} = 211.35, P < .0001$). However, the interaction term was not significant ($F_{4.28} = 2.15, P > .05$). Therefore, all species experienced a similar photoprotective capability; that is, a similar proportional change in CPD frequency among exposure treatments. Across species, the mean CPD frequency ($± 1$ SE) decreased by 75% ± 0.5% in freeze-killed individuals, compared with purified DNA.

A decrease in CPD frequency was also observed between freeze-killed organisms and live organisms in the absence of PRR, suggesting functional NER (Fig. 1b). Results of this overall ANOVA were significant and included significant main effects of species and exposure condition, as well as the interaction term (overall ANOVA: $F_{0.39} = 44.48, P < .0001$; species: $F_{4.39} = 20.57, P < .0001$; exposure treatment: $F_{1.39} = 503.17, P < .0001$; and interaction: $F_{4.39} = 3.87, P < .01$). Results of one-way ANOVA for each species indicated that NER was present in all five species.

**DISCUSSION**

The three main mechanisms used to reduce UV-induced DNA damage did not vary consistently across our five study species. Whereas the effect of PP was similar among species, an effect of NER was present but varied among species and evidence of PER was only observed in two species. These results suggest that generalizations about UVR defense mechanisms across fish taxa may not be valid.

In our study species, PP decreased CPD frequency by approximately 75%. Because this finding is based on comparisons of freeze-killed individuals and purified DNA, we cannot attribute this effect solely to the presence of photoprotective pigments or mycosporine-like amino acids. Other chromophoric biomolecules, such as lipids and proteins, may also absorb UVR and reduce CPD frequency (15, 23). Consequently, DNA located in the interior of fish larvae would receive less UVR and less photodamage, thus resulting in diluted CPD frequencies when DNA from throughout the larvae was analyzed (24). Nevertheless, our results indicate that species did not differ in their ability to absorb UVR and prevent DNA damage. One caveat to this finding is that our results may be influenced by the sources of experimental animals. Specifically, three species were obtained from hatcheries and two species were collected from wild populations. In other fishes, melanin concent-

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**Table 2.** Decreases in CPD frequency (CPDs × 10⁻⁶ bases/weighted kJ m⁻²) due to nucleotide excision repair (NER) and photoenzymatic repair (PER).

<table>
<thead>
<tr>
<th>Species</th>
<th>NER Decrease (int, df)</th>
<th>NER $P$</th>
<th>PER Decrease (int, df)</th>
<th>PER $P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Northern Pike</td>
<td>95.8 (287.17, 8)</td>
<td>0.0001</td>
<td>9.8 (4.23, 1)</td>
<td>0.0001</td>
</tr>
<tr>
<td>Brook Trout</td>
<td>95.2 (343.89, 8)</td>
<td>0.0001</td>
<td>17.6 (2.01, 8)</td>
<td>0.01</td>
</tr>
<tr>
<td>Rainbow Trout</td>
<td>94.3 (77.60, 8)</td>
<td>0.0001</td>
<td>10.9 (3.58, 8)</td>
<td>0.02</td>
</tr>
<tr>
<td>Bluegill</td>
<td>78.9 (12.24, 8)</td>
<td>0.0001</td>
<td>90.1 (8.98, 6)</td>
<td>0.002</td>
</tr>
<tr>
<td>Yellow perch</td>
<td>92.7 (80.88, 7)</td>
<td>0.0001</td>
<td>55.4 (14.55, 7)</td>
<td>0.007</td>
</tr>
</tbody>
</table>

*Change in mean CPD frequencies for each exposure treatment.
†Results of one-way ANOVA tests for each species. Nonsignificant results are denoted by “ns.”
§Reported for the denominator. All tests had 1 df in the numerator.
§Increase in CPD frequency.

(Table 2). Therefore, decreases in CPD frequency were observed in all species but the magnitude of these proportions differed (Fig. 1b). The lowest CPD reduction was observed in bluegill and the highest was observed in northern pike (Table 2).

We tested for the presence of PER by analyzing differences in CPD frequency between live individuals in the presence and absence of PRR (Fig. 1c). Results of the overall ANOVA were significant and included significant main effects of species and exposure treatment, as well as the interaction (overall ANOVA: $F_{0.35} = 11.23, P < .0001$; species: $F_{4.35} = 11.63, P < .0001$; exposure treatment: $F_{1.35} = 5.48, P < .05$; and interaction: $F_{4.35} = 9.04, P < .0001$). The frequency of CPDs decreased slightly for brook trout and actually increased for northern pike and rainbow trout (Fig. 1c), although results of one-way ANOVA analyses indicated that differences were not significant for these three species (Table 2). In contrast, CPD frequency decreased significantly for bluegill and yellow perch (Table 2).
trations are known to increase following UVR exposure (25,26). A similar response may have occurred in the species that we collected from the wild but would be less likely to have occurred in the species that we obtained from hatcheries. Had all species experienced similar exposure conditions before experimentation, we might have observed interspecific variation in the effect of PP.

The variation in NER that we observed among species does not appear to simply reflect temperature-dependent enzyme kinetics. The two smallest decreases in CPD frequency occurred in bluegill and brook trout, which had the highest and lowest experimental temperatures, respectively. Furthermore, the correlation coefficient for temperature and percent decreases in CPD frequency across our five species was only $-0.18$. It is important to note that temperature likely affects NER rates within a species (27) and that the pattern of variation we observed among species would probably be different if all species were compared at a common temperature. From an ecological perspective, however, NER rates at hatching temperatures are most relevant for understanding how UV defense mechanisms vary among species. Phylogenetic differences also do not appear to play a role in explaining variation in NER. Reductions in CPD frequency for rainbow trout were more similar to those for northern pike and yellow perch than to those for brook trout, despite the fact that rainbow trout and brook trout were the most closely related species we studied (28). Other studies have also found significant differences in DNA repair between closely related species and even between strains within a species (10,29).

An important assumption in our experimental design is that freezing arrested metabolically-driven NER (15). By freezing larvae before exposure, we aimed to disrupt the membrane potentials necessary for ATP synthesis and, therefore, sustained NER (30). However, any ATP present before freezing may have been available for NER, provided that the multiple proteins involved in NER were also present and functional. Consequently, our estimates of the percentage decrease in CPD frequency reported in Table 2 may be underestimated (because we quantified NER by difference between living and dead larvae during the 12 h exposure). Nevertheless, our conclusions that NER is present in all species but that its effectiveness varies interspecifically would not be affected by this underestimate.

Although PER has been found in many fish species (11–14, 31–34), we failed to detect evidence of PER in northern pike, brook trout and rainbow trout larvae. Because we only examined a single early life stage, we cannot rule out the possibility that other life stages of these species may be capable of PER, as has been demonstrated in other species (35). Brook trout and rainbow trout both occupy hyporheic zones of streams as larvae (36,37) and may therefore experience little to no UV exposure in the first few weeks after hatching. Consequently, there would be no benefit to the immediate development of PER for these species. However, the lack of PER in larval northern pike is surprising because both eggs and larvae adhere to aquatic vegetation (28) and are therefore exposed to UV.

Alternatively, the differences in PER we observed among species could reflect phylogenetic processes. Specifically, the three species that lacked PER are all from the superorder Protacanthopterygii (38), whereas the two species that had PER were both from the order Perciformes (in the superorder Acanthopterigii). PER may have been lost secondarily in the protacanthopterygians, because it is present in species that are considered more ancestral, such as goldfish and anchovy (12,13). Mechanistically, the loss of PER in northern pike, brook trout and rainbow trout may reflect evolutionary changes in species that spawn and live in low water temper-atures. As an enzymatic process, the efficiency of PER is strongly temperature dependent (15,39). Assuming there is a cost associated with the synthesis of photolyase, natural selection may have favored individuals in colder environments that did not produce the enzyme. As with PP, we must recognize a possible role of the sources of our experimental animals. The three species that lack PER were all obtained from hatcheries, although the broodstock of northern pike and brook trout were wild. However, our hatchery fish were exposed to light before our experiments, and visible light is known to increase efficiency of PER (40,41).

Our experimental design does not allow for a direct comparison of the effectiveness of the different repair mechanisms within a single species. Rather than isolating individual mechanisms, we sequentially inhibited specific mechanisms in our pairwise comparisons by using viability and light conditions. Consequently, we could only examine PER in the presence of NER. Because both mechanisms can repair the same types of photodamage (42), we cannot evaluate the relative contributions of each repair mechanism to overall repair rates when both are operating (i.e. in the presence of PRR). PER operates much more quickly than NER and can repair damage on a scale of minutes, compared with hours or days for NER (9,33). Therefore, PER may be the predominant repair mechanism under natural conditions for those species that possess it, although our results suggest NER repairs more damage.

Fish species that lack PER must rely on NER to repair UV-induced DNA damage. NER is slower than PER in fish and much slower in fish than in other vertebrates, particularly mammals (8). In addition, the efficiency of NER is nonrandom in genomic DNA displaying significantly higher rates of repair in actively-transcribing (“open”) regions of DNA and, presumably, reduced repair in more inaccessible heterochromatic regions (26). Accumulation of UV damage in such regions may ultimately prolong the pathogenic effects of UVR on species that lack PER and may help explain decreases in marine survival rates among anadromous salmonids, such as seagoing rainbow trout (i.e. steelhead trout), as hypothesized by Walters and Ward (43). Finally, PER is an energetically expensive process (7) that can potentially increase metabolic rates and thus reduce growth. Because growth is critical to survival in early life stages of fish (44), UVR may have a strong indirect effect on survival in fish that lack PER.

Acknowledgements—We thank Victoria Mead for assistance with photon experiments, and Lakshmi Paniker for assistance with DNA-damage assays. Wade Jeffrey and Craig Williamson provided helpful comments on an earlier version of this manuscript. The Blooming Grove Hunting and Fishing Club and the Lacawac Sanctuary provided access to Lake Giles and Lake Lacawac, respectively. This research was supported by NSF grant DEC-IRCEB-0210972 (to M.H.O. and D.L.M.).

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