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Characterization and Differential Expression of CPD and 6-4 DNA Photolyases in *Xiphophorus* Species and Interspecies Hybrids★

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Abstract

Among the many *Xiphophorus* interspecies hybrid tumor models are those that exhibit ultraviolet light (UVB) induced melanoma. In previous studies, assessment of UVB induced DNA damage and nucleotide excision DNA repair has been performed in parental lines and interspecies hybrids. Species and hybrid specific differences in the levels of DNA damage induced and the dark repair rates for cyclobutane pyrimidine dimers (CPDs) and 6-4 pyrimidine pyrimidine photoproducts (6-4PPs) have been reported. However, UVB induced DNA lesions in *Xiphophorus* fishes are thought to primarily be repaired via light dependent CPD and 6-4PP specific photolyases. Photolyases are of evolutionary interest since they are ancient and presumably function solely to ameliorate the deleterious effects of UVB exposure. Herein, we report results from detailed studies of CPD and 6-4PP photolyase gene expression within several *Xiphophorus* tissues. We determined photolyase gene expression patterns before and after exposure to fluorescent light in *X. maculatus*, *X. couchianus*, and for F₁ interspecies hybrids produced from crossing these two parental lines ($\times$. *maculatus* Jp 163 B $\times$ *X. couchianus*). We present novel results showing these two photolyase genes exhibit species, tissue, and hybrid-specific differences in basal and light induced gene expression.

Keywords

q-RT-PCR; gene expression; light induced; Photolyase; Cyclobutane pyrimidine dimer; 6-4 pyrimidine pyrimidone photoproduct; *Xiphophorus*; interspecies hybrid

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1. Introduction

The various cellular effects of ultraviolet light (UVB) exposure on vertebrate organisms have been well studied and continue to be of scientific interest (McKenzie et al, 2003). In humans, UV exposure has been linked with increased risk of skin cancers including cutaneous malignant melanoma (Armstrong et al., 1997; Jemal et al., 2006). Despite the inherent interest in induced biological effects of UVB (290–320 nm), animal models that lend themselves to experimentally controlled UVB exposure and melanoma induction are few (Walter and Kazianis, 2001). *Xiphophorus* fish interspecies hybrids have emerged as a tractable experimental model to study the genetics underlying UV induced melanoma (Nairn et al., 2001, Walter and Kazianis, 2001, Mitchell et al, 2010). Efforts to better understand UVB induced melanoma have led to many reports detailing UVB induced DNA damage and relative rates of repair of UVB induced DNA photoproducts. In particular, UVB induction and repair of cyclobutane pyrimidine dimers (CPDs) and 6-4 pyrimidine pyrimidine photoproducts (6-4PPs) has been assessed in several *Xiphophorus* species and interspecies hybrids (Mitchell et al, 2001; Meador et al, 2000, Fernandez et al, 2010). Penetration of UVB through the epidermis may cause a litany of DNA photoproducts (Rastogi et al, 2010), the most common of which are CPDs and 6-4PPs. UVB induced DNA damage may ultimately lead to activation of growth factors and is associated with an increasing incidence of human melanoma worldwide (Marks, 2000). In previous studies it has been shown that UVB induced DNA damage (CPDs and 6-4PPs) may lead to an increase in transcription of many DNA repair genes (Funayama et al, 1996), including two distinct photolyase proteins that possess specificity to repair CPDs and 6-4PPs upon exposure to visible light (Sancar, 2003; Kim et al, 1994). The repair mechanism for CPD photolyase has been defined in much greater detail than the more complex 6-4 photolyase mechanism. CPD photolyase repair occurs via photon absorption, triggering electron release into a tunneling pathway that serves to split the CPD cyclobutane ring, while the 6-4 photolyase is thought to perform repair using a mechanism involving cyclic proton transfer between the photolyase and 6-4PP substrate (Li et al, 2010).

Genes encoding CPD and 6-4 photolyases have been shown experimentally to be inducible by exposure to fluorescent light (Rastogi et al, 2010; Hirayama et al, 2009). In vertebrates these two photolyase genes are members of the cryptochrome superfamily and have only been studied in a few select organisms (opossum, amphibians, and several fishes). Research into photolyase gene level effects is confounded by an undercurrent of confusing gene nomenclature exacerbated by recent sequencing of many new genomes having disparate annotation of cryptochrome gene family members that may or may not include CPD and 6-4 photolyases. The cryptochrome gene family is comprised of a large number of genes that are known to function in maintenance of circadian rhythm, contain similar cofactors, often exhibit light induced transcription, and with the exception of the photolyases do not possess DNA repair capability. This lack of repair capability in most cryptochrome superfamily members is largely due to C-terminal extensions that may be used to distinguish cryptochromes from photolyases (Eker et al, 2009).

Here we present identification of putative *Xiphophorus* CPD and 6-4 photolyase genes based on sequence similarity to *Xenopus*, zebrafish (*Danio rerio*), and medaka (*Oryzias latipes*,

Japanese rice fish) photolyases that have been functionally characterized. We show results of both phylogenetic and syntenic analyses of photolyase genes in *Xiphophorus* and other fishes that ensure identification of true platyfish CPD and 6-4 gene orthologs. We present novel quantitative real time PCR (qRT-PCR) data showing basal expression profiles for both CPD and 6-4 photolyase genes in several organs and induced expression levels after exposure to fluorescent light. We report these data for two *Xiphophorus* species (*X. maculatus* Jp 163 B and *X. couchianus*) and for F₁ interspecies hybrids produced from crossing these two species.

2. Materials & Methods

2.1 Identification of CPD and 6-4 Photolyase Orthologs in *Xiphophorus*

The medaka CPD (Funayama et al, 1996), zebrafish 6-4 (Kobayashi et al, 2000), and both CPD and 6-4 photolyases from *Xenopus* (Tanida et al, 2005; Todo et al, 1997, Kim et al, 1996) have been cloned and their respective proteins functionally verified. Therefore, we used these gene sequences as queries for BLAST searches of the platyfish genome within Geneious vR6 (<http://www.geneious.com/>). The platyfish exons were annotated by alignment with the query genes by applying their gene models.

2.2 Syntenic Analysis

Genomicus v72.01 (<http://www.genomicus.biologie.ens.fr/genomicus-72.01/cgi-bin/search.pl>) (Louis et al, 2013) was used to examine positions of the predicted platyfish CPD and 6-4 photolyase genes within conserved syntenic regions of many other fishes (Supplemental Figure 1; for supplemental materials see <http://www.xiphophorus.txstate.edu/publications-data/publishedworks-older/supplement.html>).

2.3 Phylogenetic analysis

Xiphophorus and other CPD and 6-4 photolyase sequences were identified using the Gene Tree in Ensembl vs.71 (<http://apr2013.archive.ensembl.org/index.html>); all genus and species names and Ensembl IDs for these taxa are provided in Supplemental Table 1. The gar photolyase sequences for these genes were identified by similarity- searching the Pre Ensembl genome and manually annotated the coding sequence based on similarity to other annotated photolyases. In addition to the taxa representing CPD and 6-4 photolyase in Figs. 1, several sequences that were functionally verified representatives of CRY1, and CRY4 were also included to increase the rigor of the analysis (data not shown). Protein sequences (n=46) were aligned using the Opalescent v2.1 (Wheeler and Kececioglu, 2007) module in Mesquite v2.75 (Maddison and Maddison, 2011; <http://mesquiteproject.org>). The accuracy of the alignment was assessed by ensuring that known conserved regions (e.g., conserved Trp triad residues) were generally aligned for all taxa in the dataset. Phylogenetic analysis was performed using MrBayes v3.2.1 (Ronquist and Huelsenbeck, 2003), with 2 million generations and discarding a burn-in fraction of 25%. The analysis included 2 independent runs using the WAG model for amino acid substitution (Whelan and Goldman, 2001), which had been identified as the best-fitting model in prior model-test runs. Convergence of the two runs was verified visually with Tracer v1.5 (Rambaut and Drummond, 2007; <http://tree.bio.ed.ac.uk/software/tracer/>) and using the convergence diagnostic from MrBayes, and

the trees retained after 25% burnin were used to build a consensus tree. The consensus tree was visualized and midpoint-rooted using Archaeopteryx v0.9813 A1ST (Han and Zmasek, 2009).

2.4 Fishes Utilized

All fishes utilized were supplied by the *Xiphophorus* Genetic Stock Center, Texas State University, San Marcos, TX 78666 (<http://www.Xiphophorus.txstate.edu>). All parental and F₁ hybrid fishes were mature males between 9-12 months old (Figure 2). The *X. maculatus* Jp 163 B (pedigree 100B) was in its 100th generation of sibling inbreeding, while the *X. couchianus* (pedigree 77B) was in its 77th generation of inbreeding (Walter et al, 2006; <http://www.xiphophorus.txstate.edu/stockcenter/stockcentermanual.html>). Interspecies hybrids were produced by mating the two parental lines (*X. maculatus* Jp 163 B × *X. couchianus*) in their 99th and 75th inbred generations, respectively.

2.5 Fluorescent Light Exposures

Fluorescent light (FL) exposures were carried out in a specially designed wooden box (77 cm in length, 41 cm in height, and 36 cm in depth), with a hinged wooden lid capable of sealing the interior of the box from external light. On the bottom of each of the two sides (41 cm × 36 cm) were 15.5 cm diameter high-speed fans that maintained interior temperatures of the closed box at less than 24 °C. For FL exposures single fish were placed into UV transparent (UVT) plastic cuvettes (9 cm × 7.5 cm × 1.5 cm) in about 95 mL water and the cuvettes were suspended in a rack centered between and about 10 cm from the FL bulbs inside the exposure chamber. All animals were kept in the dark 14 h prior to exposure. FL exposures were 2 h with a bank of “cool white” fluorescent lamps (Philips F20T-12/D, 4100K “cool white” lamps) filtered through Mylar 500D to exclude any wavelengths <320 nm.

After FL exposure, fish were maintained in the dark for appropriate times (see results) to allow for gene expression prior to sacrifice and tissue dissection. At dissection fish were sacrificed using a lethal dose of anesthesia (MS-222) and skin, as well as other organs, dissected directly into RNAlater (Ambion Inc., Austin, TX, USA), frozen, and stored at –80 °C.

2.6 RNA Isolation

Total RNA was isolated after maceration of liquid nitrogen-frozen whole tissues using a pestle followed by resuspension in TRizol (Invitrogen, Carlsbad, CA, USA). RNA was further purified using 5Prime phase lock gel tubes (ThermoFisher Scientific, Waltham, MA, USA). Any residual DNA was eliminated by performing column DNase I digestion at 37 °C (30 min). The integrity of RNA was determined by gel electrophoresis (2% agarose in TAE running buffer) and concentration was determined using a spectrophotometer (Nano Drop Technologies, Wilmington, DE, USA).

2.7 Quantitative Real Time PCR

Total RNA isolated from tissues of two independent biological replicates of highly inbred parental and hybrid fishes. Isolated RNA was used in cDNA synthesis employing a High

Capacity cDNA reverse transcription kit (Applied Bioscience, Carlsbad, CA, USA). The reaction was performed in two steps, a DNase I treatment was performed for 15 min at room temperature and deactivated by the addition of 25 mM EDTA at 65 °C for 10 min. Following DNase treatment, reverse transcription was performed by adding 100 mM dNTPs, 10× RT random primers, RNase inhibitor, and MultScribe Reverse Transcriptase in a 20 µL reaction employing standard thermocycler conditions (25 °C for 10 min, 37 °C for 120 min, and 85 °C for 5 min). Each sample was diluted to a final volume of 500 µL and analyzed for CPD and 6-4 photolyase expression by qRT-PCR utilizing SYBR Green-based detection on an Applied Biosystems 7500Fast system (Applied Bioscience). PCR primers were designed using Geneious (Biomatters Ltd, Auckland, New Zealand) bioinformatic software. All primers were designed with T_m between 60 and 62°C in 3.0 mM K, 50 mM Na, and 0.8 mM dNTPs.

Primer lengths were limited to 18-25 bp long with 55 – 60% GC content, and not more than 1°C between T_{ms} for each primer in a set. In addition, all amplicons were limited to 100-150 bp in length and were designed to cross at least one exon junction. Primers meeting these criteria and specific for the *Xiphophorus* CPD and 6-4 photolyases are given in Table 1. Initially, the efficiency of each set of designed primers was tested in triplicate in a 20 µL reaction consisting of a standard serial dilution series of 100, 10, 1, 0.1 ng cDNA, 0.5 µM of each primer, and 10 µL SYBR Green ready mix (Applied Bioscience). Each reaction was subjected to 40 cycles each at 95 °C for 20 s, 95 °C for 15 s, and 60 °C for 30 s, before being subjected to melting curve analysis. Amplified products were also analyzed for size by agarose gel electrophoresis (2% agarose in TAE running buffer). The 18S rRNA was selected as the transcript for normalization of all samples. Only primers with efficiencies between 70 and 120%, with not more than ±10% differences between *Xiphophorus* species, were selected for further use. Once the efficiency and specificity were established, the primers were used to test relative expression of each gene from cDNAs (10 ng) produced from each organ sample from *X. maculatus*, *X. couchianus* and the F₁ interspecies hybrid for two independent biological replicates and four technical replicates. The threshold cycle (C_T) for each test target and 18S rRNA control was determined by the 7500 Fast System SDS 2.0.6. The average 18S control C_T was subtracted from the average target C_T for each biological replicate independently over 4 technical replicates to give a C_T value. Photolyase expression was then determined relative to the 18S rRNA endogenous control by applying 2^{-C_T} . This represents the mRNA expression level for each target tested relative to the 18S rRNA control. Fold changes for samples exposed to FL were determined relative to each respective basal level tissue (C_T) (Schmittgen and Livak, 2008) and standard deviations were calculated for the average of 3 technical replicates from each biological replicate. Hybrid basal liver samples were included on every plate to ensure that data was not skewed to differences in protocol or detection. An unpaired Student's *t*-test was performed on all fold change differences to determine statistical significance; *p*-values were recorded (Supplementary Table 5).

3. Results

3.1 Identification of *Xiphophorus* CPD and 6-4 Photolyase Orthologs

We identified putative *Xiphophorus* CPD and 6-4 photolyase genes through similarity to *Xenopus*, medaka, and zebrafish CPD and 6-4 photolyases, several of which had previously been functionally verified as coding for proteins that possess photolyase activity. Both *Xiphophorus* photolyase genes chosen reside within syntenic regions that are very well conserved in the context of other teleost genomes (Supplemental Figure 1). Phylogenetic analysis presented in Figure 1 show it is highly probable all vertebrate CPD (Figure 1A) and all 6-4 photolyase proteins (Figure 1B) cluster monophyletically relative to each other and to other groups of functional vertebrate cryptochrome genes (not shown). The putative *X. maculatus* photolyase sequences cluster within the appropriate clade with strong support (Figure 1). The estimated probability the identified putative *Xiphophorus* CPD groups with functionally tested CPDs is 100% (Figure 1A, bolded probabilities). The estimated probability that the putative *Xiphophorus* 6-4 photolyase groups with zebrafish 6-4 is also 100%, and it is ~90% likely that it groups with the *Xenopus* 6-4 photolyase given the alignment and model (Figure 1B, bolded probabilities).

3.2 Basal Expression Levels of *Xiphophorus* CPD and 6-4 photolyases

Both CPD and 6-4 photolyases have been extensively studied as DNA repair proteins based on their ability to reverse UV induced DNA photoproducts upon absorption of visible light (Li et al, 2010; Rastogi et al, 2010; Hirayama et al, 2009; Eker et al, 2009). In addition, for several experimental aquatic models (frogs and fishes) it has been shown that CPD photolyase transcription is induced upon exposure to visible and blue light wavelengths (Hirayama et al, 2009; Funayama et al 1996). Also, photolyase gene expression in cultured cells, and in the skin for various organisms, has been documented (Rastogi et al, 2010; Hirayama et al, 2009; Hiroaki et al, 2005; Todo et al, 1996; Yasuhira and Yasui, 1992). Given the high conservation of these two genes among vertebrates (Figure 1) and reports indicating photolyases may be expressed in organs other than skin (Hiroaki et al, 2005), we first assessed the general basal expression patterns for the *Xiphophorus* CPD and 6-4 photolyases in several light responsive external (skin, eyes) and internal (brain, heart, gill, testes, liver) organs.

Figure 3 and Supplemental Table 2 show basal transcription levels assessed by qRT-PCR for CPD (Figure 3 panel A) and 6-4 photolyase genes (Figure 3 panel B) in two *Xiphophorus* species (*X. maculatus* Jp 163 B and *X. couchianus*) and in F₁ interspecies hybrids made by crossing these two parental lines (i.e., *X. maculatus* Jp 163 B × *X. couchianus*; Figure 3). For these studies the fish were dark adapted (~14 h in dark) prior to immediate sacrifice, dissection, and RNA isolation. As shown in Figure 3 panel A and Supplemental Table 2, the *X. maculatus* parental line shows the highest basal expression of CPD photolyase in the brain with the lowest, of organs tested, in the liver. *X. couchianus* expresses the CPD photolyase gene at lower basal levels than either *X. maculatus* or the F₁ interspecies hybrid in all organs tested except the heart and testes. Interestingly, the F₁ interspecies hybrid exhibits much higher basal transcription levels of CPD photolyase than either parental line in light responsive organs (~30 fold in skin and ~4 fold in eyes). For other organs, the F₁

interspecies hybrid exhibits higher basal expression of CPD photolyase than either parental line in gill and heart, while showing basal levels similar to the *X. maculatus* in brain (≈ 2.5 fold above *X. couchianus*) and basal levels similar to *X. couchianus* for testes and liver (9.4 and 7.7 fold above *X. maculatus*, respectively).

A pattern of “hybrid” vigor, where the F_1 interspecies hybrid exhibits upregulated basal expression of CPD photolyase in light responsive organs (skin and eyes) and higher basal expression than at least one parental line in other organs (gill, brain, heart, and testes) is also observed for 6-4 photolyase expression (Figure 3, panel B, Supplemental Table 2). The expression of 6-4 photolyase in F_1 interspecies hybrids appears to be ≈ 3 to 4 fold above the parental line expression in the skin and eyes. In the brain and gill, the F_1 interspecies hybrid 6-4 photolyase expression is similar to that of *X. couchianus*, and both the F_1 and *X. couchianus* exhibit a greater than 5 fold higher basal expression in the heart compared to *X. maculatus*. The 6-4 photolyase becomes dramatically dysregulated in testes compared to either parent exhibiting substantial upregulated basal expression (>30 fold compared to *X. couchianus* and >50 fold for *X. maculatus*). Liver shows much reduced 6-4 basal expression and this is consistent among all three fish types.

It is interesting that testes exhibit very high basal expression values in the F_1 interspecies hybrid for both photolyases (CPD ≈ 9.4 fold and 6-4 photolyase >100 fold higher than liver; Figure 3 and Supplemental Table 2). Also, both CPD and 6-4 photolyase exhibit higher expression in *X. couchianus* testes than in *X. maculatus* (≈ 9 and 5.8 fold, respectively; Figure 3 and Supplemental Table 2).

3.3 Fluorescent Light Induction of CPD and 6-4 Photolyases in *X. maculatus* Skin

The induction of CPD photolyase in cultured fish cell lines and in skin or eyes from *Xenopus* has been reported (Hirayama et al, 2009; Hiroaki et al, 2005; Yasuhira & Yasui, 1992). In plants the induction of cytochrome genes, including photolyase, has been shown after exposure to blue light wavelengths (Rastogi et al, 2010). However, determination of light induced photolyase gene expression in varied organs, including non-light responsive organs, has not been extensively investigated within intact vertebrate animals. To study this we first sought to characterize the gene expression kinetics for the *Xiphophorus* CPD and 6-4 photolyases in skin after exposure to FL. Dark adapted (14 h incubation in the dark) *X. maculatus* Jp 163 B were exposed to FL followed by incubation in the dark to allow time for expression (2, 4, 6, 8 and 14 h post exposure dark incubation; Figure 4). Following each post exposure dark period fish skin was dissected, utilized for RNA isolation and qRT-PCR based assessment of CPD and 6-4 photolyase gene expression performed (Figure 4). The qRT-PCR analyses indicate both the CPD and 6-4 photolyases are similarly induced to about $4.5\times$ basal levels by 6 h post FL exposure. In skin, the CPD photolyase appears to begin a trend back toward basal levels by 14 h post exposure; while the 6-4 photolyase shows a continuing induced transcriptional response even after 14h incubation on the dark.

3.4 Fluorescent Light Induction of CPD and 6-4 Photolyases in Various Organs

Expression of DNA photolyases in several different experimental systems has been shown inducible by exposure to visible light (Eker et al, 2009; Yasuhira & Yasui, 1992). However,

most of these reports assessed inducible gene expression only in skin or cultured cells of various types (e.g., goldfish and medaka; Funayama et al, 1996; Yasuhira & Yasui, 1992). We employed qRT-PCR to assess FL induction of CPD and 6-4 photolyase gene expression in a varied set of organs for *X. maculatus*, *X. couchianus*, and F₁ interspecies hybrids after 4 or 6 h post exposure dark incubation.

3.4a CPD Photolyase Gene Expression

Figure 5 and Supplemental Table 3 present a comparative display of qRT-PCR transcriptional expression data for skin, eyes, brain, gill, and liver derived from independent FL exposure experiments of *X. maculatus*, *X. couchianus*, and F₁ interspecies hybrids. As shown, CPD photolyase transcription exhibits a robust induction in *X. maculatus* by 4 h post exposure in most organs (4.5× skin, 16× eyes, 8.4× gill, and 15× liver; Figure 5 and Supplemental Table 3) except brain, where expression is nearly the same as the basal levels. Also, note that except for the skin, the *X. maculatus* CPD response for eyes, brain, gill and liver appear to trend toward return to basal levels by 6 h post exposure. Light induction of CPD photolyase in *X. couchianus* also exhibits a robust induction response, but it is less rapid and results in peak values shifted toward the 6 h time point when compared to *X. maculatus*. The *X. couchianus* data indicate very strong FL induction of CPD photolyase 6 h post exposure (33× skin, 5.5× eyes, 2.8× brain, 4.8× gill, and 37× liver). The F₁ interspecies hybrid shows a CPD photolyase response to light exposure that is much different than either parental line. For all organs except the liver, the F₁ hybrid appears either to not induce, or perhaps suppress, CPD photolyase transcription after light exposure (6 h expression down regulated (-)3.0× skin, (-)1.3× eyes, (-)1.5× brain, 1.5× gill). However, liver in the F₁ interspecies hybrid does exhibit a strong CPD induction of >50× at 6 h post exposure, similar to the liver expression response in *X. couchianus*.

3.4b 6-4 Photolyase Gene Expression

Figure 6 and Supplemental Table 4 show that exposure to fluorescent light induces gene expression of 6-4 photolyase in both *X. maculatus* and *X. couchianus* in all organs tested (i.e., skin, eyes, brain, gill, and liver). The amplitude of the induction response relative to the basal expression in *X. maculatus* occurred within the first 4 h post exposure (2.7× in skin, 24× eyes, 13× brain, 2× gill, and 13.5× in liver). *X. couchianus* showed robust light induction of 6-4 photolyase in skin (38× above the *X. maculatus* response) while presenting a more subdued response than *X. maculatus* for several other organs (10× eyes, 5.4× brain, 4.5× gill, and 1.4× liver). As observed for the CPD photolyase, the F₁ interspecies hybrid appeared to not induce, and perhaps even suppress, 6-4 photolyase transcription in all organs except liver (6 h time point: (-)1.4× skin, (-) 2.6× eyes, 2.6× brain, (-) 2.9× gill). For liver, the F₁ interspecies hybrid appears to upregulate the 6-4 photolyase about 8.4 fold in the first 4 h post exposure time point and 4.7 fold at 6 h post exposure. Also, as observed for CPD photolyase induction, most organs in *X. maculatus* appeared to return 6-4 photolyase levels nearly to basal expression by 6 h post exposure while *X. couchianus* 6-4 photolyase transcription continued to exhibit the induced response at 6 h post exposure.

4. Discussion

Herein we present identification of *Xiphophorus* CPD and 6-4 photolyase genes employing both syntenic and phylogenetic analyses. We observed extensive shared synteny in genome regions harboring these photolyase genes between *X. maculatus* and other teleosts (Supplemental Figure 1) and show phylogenetic analysis of vertebrate protein sequences for CPD photolyase and 6-4 photolyase each clustering monophyletically and with high confidence (Figure 2). These *Xiphophorus* photolyase genes grouped with other known vertebrate photolyase sequences with strong support. Together these data strongly indicate the putative *Xiphophorus* CPD and 6-4 photolyase genes studied herein are true photolyase orthologs.

4.1 Basal Comparison among Parental Lines and Hybrids

Figure 3 and Supplemental Table 2 present novel data regarding basal transcription of these two presumed DNA repair genes in organs of two parental *Xiphophorus* species and F₁ interspecies hybrids produced from crossing them. The species utilized are highly inbred, and as expected, were found to exhibit fairly consistent basal transcription values that we suspect represent actual transcription states for dark-adapted animals. These comparisons, in many cases, support previously published reports while in other cases the data presented suggest these genes have presently unknown functions.

Comparison of the basal CPD gene expression between *X. maculatus* and *X. couchianus* indicate these two parental fish lines regulate the CPD photolyase similarly in eyes, gill, heart, and liver, but show some species specific differences in basal expression within skin, brain (*X. maculatus* expresses higher) and testes (*X. couchianus* shows higher expression). Likewise, the 6-4 photolyase shows consistent basal expression levels in these two species for eyes, gill, brain, and liver. However, the 6-4 photolyase expression patterns in skin (*X. maculatus* higher), heart and testes (*X. couchianus* expresses higher) were different for the two species. That skin and testes show expression differences in the same directions for both CPD and 6-4 photolyase genes supports the contention these are species-specific differences.

The consistency and similarity of basal photolyase expression between *X. maculatus* and *X. couchianus* is not reflected in the expression patterns of the F₁ interspecies hybrid. In these data there are many cases where novel expression patterns (i.e., not like either parental line) are presented by the hybrid. For example, compared to the parental lines, the F₁ hybrid exhibited very high (over 30 fold higher) basal expression of these two photolyases in some organs (e.g., CPD skin, 6-4 heart and testes), compared to the higher expressing parent. In addition, the F₁ hybrid showed higher basal expression than the highest expressing parent (CPD eyes, gill, heart; 6-4 skin, eyes, heart) or showed expression at levels about equal to the one of the two parents (CPD brain, testes, liver; 6-4 gill, brain, liver). In a previous study of base excision repair it was shown that different organs of *Xiphophorus* F₁ interspecies hybrids tended to acquire repair activities about equal to one or the other parent, or in some organs show a brand new phenotype that did not match either parent (Walter et al, 2001). It has been documented for these same fish lines that expression in several base excision repair genes becomes dysregulated in hybrids and shows expression patterns that are quite different than either parent (Heater et al, 2007). Further, using these same parental lines and

F₁ interspecies hybrid fishes, UVB exposed skin was shown to possess very different half-lives for nucleotide excision repair (i.e., dark repair) of both CPD and 6-4PPs in their DNA (Mitchell et al, 2001; David et al, 2004). In these studies the skin of the F₁ interspecies hybrids lost almost all ability to perform dark repair of 6-4 photoproducts. Thus, the observation of differential basal expression patterns of these two photolyases for the parents and F₁ hybrids is consistent with previous findings and underscores the extent of genetic dysregulation that occurs in the interspecies hybrid genetic background.

4.2 Fluorescent Light Induced Photolyase Expression

For the parental species, *X. maculatus* and *X. couchianus*, all assayed tissues exhibited some level of light induced gene expression for both CPD and 6-4 photolyases. These levels ranged from negligible increase in expression (i.e., CPD brain from 1.16 to 2.8 fold) to very large changes in expression (CPD liver from 15 to 88 fold). It is worth noting that basal levels of gene expression are sometimes difficult to assess since a small amount of variability among individual animals is amplified due to the very low basal transcript expression levels. In fact, we often observe that variable basal expression values become very tightly clustered upon experimental treatment and/or after an organismal response to a stimulus is elicited. We recognize the effect that increased basal value variability can have on determination of induced expression (i.e., the ratio of basal to induced values). However, even with this in mind we expect the larger differences reported here between the parental line fishes (e.g., CPD skin, brain, and testes; and 6-4 heart and testes basal levels) are likely due to fixed species specific differences. Overall, *X. maculatus* exhibited a faster (4 h post exposure) induction of the photolyase gene expression with a more rapid return to near basal expression levels at 6 h post exposure compared to *X. couchianus*, which seemed to show continued transcriptional induction at 6 h post exposure. However, gene induction kinetics of both parental lines are highly contrasted by a lack of FL induced gene expression in the F₁ interspecies hybrid. This observation is surprising and unexpected. Although, as stated above, declination of dark repair capability for both base excision and nucleotide excision repair have been reported in F₁ interspecies hybrids, a loss of repair potential due to loss of FL induction of a single protein(s) that presumably exists solely to repair UV induced DNA damage was considered rather unlikely. However, this is the case as all organs except liver in the interspecies hybrid exhibited either lack of induced expression, or depletion, and loss of repair potential for CPD and 6-4 photoproducts in the DNA. It is possible that expression of CPD and 6-4 photolyases is not so much due to their direct repair potential but rather to some poorly understood recruitment function related to dark repair pathways which becomes compromised in hybrids. In such a scenario, the loss of light driven induction of the photolyases is an unexpected consequence of the general loss in repair capability previously reported for *Xiphophorus* interspecies hybrids. It is worth noting that F₁ hybrid liver, a metabolically active but non-light responsive organ, appears to exhibit normal FL induced expression of CPD (over 80 fold) and 6-4 (8.4 fold) photolyases when compared with the parental species. Thus, in addition to suggesting alternative roles may exist for these light responsive and presumed repair proteins, the data suggest there may be regulatory circuitry producing induction of CPD and 6-4 photolyases in liver that are distinct from the “normal” light induction circuitry utilized by most other organs. This may also indicate molecular

crosstalk between light and dark repair in light responsive and unresponsive organs. Further investigation may establish new roles for these photo-responsive proteins.

Xiphophorus species are thought to have diverged ≈ 4 -6 million years ago (Mateos et al 2002; Kallman and Kazianis, 2006) and the two species used in the studies reported, *X. maculatus* and *X. couchianus*, segregate into divergent clades in most recent phylogenetic reconstructions of this genus (Cui et al, 2013; Kang et al, 2013). In addition, sites where these two species were collected ranged from Southern (*X. maculatus*, Rio Jamapa, Veracruz) and Northern (*X. couchianus*, Huasteca Canyon, Nuevo Leon) regions of Mexico over a distance of over 850 kilometers and with very different climates and conditions. Also, these lines have been maintained by brother-sister matings as inbred lines for well over 60 years (Walter et al, 2006) and as such possess fixed genetic characters. Thus, large differences in gene expression patterns due to fixed alleles in each species may be somewhat expected for complex biochemical pathways involving many genes. Indeed, the F₁ hybrid studied herein, when backcrossed to *X. couchianus*, produce subsets of backcross hybrids progeny that are susceptible to induced melanoma development upon exposure to UVB or direct acting alkylating agents (Nairn et al, 2001; Kazianis et al, 2001; Walter and Kazianis, 2001). However, given the results of this study, it will be interesting to assess the possibility of association between photolyase gene expression and DNA repair declination in tumor susceptible and tumor resistant backcross hybrids.

The basal expression patterns and light induction within organs that are unlikely to experience DNA damage, and the evolutionary similarity of CPD and 6-4 photolyases with members of the cryptochrome gene superfamily, all suggest these proteins have far more extensive functions within the intact organism than the direct repair of UVB induced DNA damage. It will be interesting from many different points of view to further delineate the roles photolyases may play in organismal development and homeostasis.

5. Conclusions

We employed quantitative real time PCR to assess CPD and 6-4 photolyase gene expression in two *Xiphophorus* species (*X. maculatus* and *X. couchianus*) and F₁ interspecies hybrids produced from these two lines. We report basal expression profiles for seven organs (skin, eyes, brain, gill, heart, testes and liver) and FL induced expression in five organs representing light (skin, eyes) or non-light responsive organs (brain, gill, liver). The two *Xiphophorus* species differed in basal expression in heart and testes, but showed similar expression values for other organs. FL induction of photolyase gene expression was time shifted with *X. maculatus* showing a more rapid response that returned to nearly basal levels in 6 h while *X. couchianus* showed a sustained induced response of at least 6 h. Both *Xiphophorus* species exhibited robust expression of both CPD and 6-4 photolyase genes in most organs testes, including non-light responsive organs, perhaps indicating alternative functions for the encoded proteins. The interspecies hybrid showed higher basal expression values for both photolyases but this was surprisingly concomitant with loss of ability of the FL to induce CPD or 6-4 photolyase gene expression in all organs except liver. Loss of ability to induce gene expression in all organs but liver with a very robust inducible response in liver suggests at least two alternative regulatory circuits for these two photolyase genes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Baysian phylogenetic analysis of select vertebrate photolyase genes. *Xiphophorus* photolyases (A: CPD photolyase; B: 6-4 photolyase) are indicated with arrows; examples of functionally verified photolyases are marked with stars. Nodes with strong support (>0.8 posterior probability) are labeled with the posterior probability. Scale bars are in the lower left corner. Species key and Ensembl IDs for common names in figure provided in Supplemental Table 1.

Figure 2.

X. maculatus Jp 163 B (top left) and *X. couchianus* (top right) and an F₁ interspecies hybrid produced from crossing these two parental lines (bottom).

Figure 3.

Basal expression patterns of CPD (panel A) and 6-4 (panel B) photolyase in *Xiphophorus* fish. CPD and 6-4 photolyase basal expression levels in dark adapted *X. maculatus* (blue), *X. couchianus* (red) and the F₁ hybrid progeny (green) in skin, eyes, gill, brain, testes, and liver as determined by qRT-PCR. Data are presented as relative mRNA levels from three technical replicates normalized using 18S rRNA as the internal standard $\pm$ standard deviation from 2 independently tested biological replicates.

***X. maculatus* CPD and 6-4 Photolyase Expression following FL Exposure determined by qRT-PCR**

Figure 4.

CPD (blue) and 6-4 (red) photolyase expression following FL exposure in *X. maculatus* was determined following 2 h of FL exposure. Relative trends of expression were observed by isolating RNA before and after FL exposure and comparing the relative mRNA concentrations to a sham treatment equivalent to the 6 h time point. qRT-PCR analysis was conducted using 3 technical replicates at each time point tested and C_T values were calculated relative to the 18S rRNA internal standard. Standard curves were fit to the data to indicate expression trends.

Figure 5.

CPD Photolyase relative expression levels (panel A) and fold change (panel B) in *Xiphophorus* fish. CPD Photolyase was measured using qRT-PCR in basal, dark adapted fish (light bars) and fish exposed to 2h of FL then allowed to recover in the dark for 4 (primary colored bars) or 6h (dark bars) in *X. maculatus* (blue), *X. couchianus* (red) and the F_1 interspecies hybrid. In addition, fold change was calculated for each tissue relative to basal level for the same samples. qRT-PCR data was tabulated from 2 biological replicates and 3 technical replicates for each species and all time points. All samples were normalized using the 18S rRNA as an internal standard and C_T values were calculated relative to basal level tissues for fold change determination. A *t*-test was performed for all fold differences and confidence scores are indicated above each bar in panel B by a 1 for $p < 0.01$, a 5 for

$p < 0.05$ or an N for non-significant differences. Exact confidence scores are reported in Supplementary Table 5.

Figure 6.

6-4 Photolyase relative expression levels (panel A) and fold change (panel B) in *Xiphophorus* fish. 6-4 Photolyase was measured using qRT-PCR in basal, dark adapted fish (light bars) and fish exposed to 2h of FL then allowed to recover in the dark for 4 (primary colored bars) or 6h (dark bars) in *X. maculatus* (blue), *X. couchianus* (red) and the F₁ interspecies hybrid. In addition, fold change was calculated for each tissue relative to basal level for the same samples. qRT-PCR data was tabulated from 2 biological replicates and 3 technical replicates for each species and all time points. All samples were normalized using the 18S rRNA as an internal standard and C_T values were calculated relative to basal level tissues for fold change determination. A *t*-test was performed for all fold differences and confidence scores are indicated above each bar in panel B by a 1 for *p*<0.01, a 5 for *p*<0.05 or an N for non-significant differences. Exact confidence scores are reported in Supplementary Table 5.

Table 1

Primers Utilized for qRT-PCR

Target Transcript	Forward Primer	Reverse Primer	Amplicon length (bp)	Exons Amplified
CPD Photolyase	CTCTCAGCCAGCTCTCCC	ACCAGCTCCTCGATGAAGG	124	5 & 6
6-4 Photolyase	TTGGGGCACTCAAAGACCTG	GAACACATCTTCTGGTTTCCCC	86	2 & 3