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Induction of cytochrome P450 1 genes and stress response genes in developing zebrafish exposed to ultraviolet radiation

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Abstract

Ultraviolet (UV) radiation damages cell molecules, and has been suggested to upregulate mammalian cytochrome P4501 (*CYP1*) genes through an aryl hydrocarbon receptor (AHR) mediated mechanism. In this study, embryos and larvae of zebrafish (*Danio rerio*) were exposed to UV to determine the effects on expression of *CYP1* and stress response genes *in vivo* in these fish. Zebrafish embryos were exposed for varying times to UV on two consecutive days, with exposure beginning at 24 and 48 hours post-fertilization (hpf). Embryos exposed for 2, 4 or 6 hours twice over two days to UVB (0.62 W/m^2; 8.9–26.7 kJ/m^2) plus UVA (2.05 W/m^2; 29.5–144.6 kJ/m^2) had moderately (2.4 ± 0.8-fold) but significantly upregulated levels of *CYP1A*. UVA alone had no effect on *CYP1A* expression. Proliferating cellular nuclear antigen (*PCNA*) and Cu-Zn superoxide dismutase (*SOD1*) transcript levels were induced (2.1 ± 0.2 and 2.3 ± 0.5 fold, respectively) in embryos exposed to two 6-hour pulses of 0.62 W/m^2 UVB (26.8 kJ/m^2). *CYP1A* was induced also in embryos exposed to higher intensity UVB (0.93 W/m^2) for two 3-hour or two 4-hour pulses (20.1 or 26.8 kJ/m^2). *CYP1B1*, *SOD1* and *PCNA* expression was induced by the two 3-hour pulses of the higher intensity UVB, but not after two 4-hour pulses of the higher intensity UVB, possibly due to impaired condition of surviving embryos, reflected in a mortality of 34% at that UVB dose. A single 8-hour long exposure of zebrafish larvae (8 dpf) to UVB at 0.93 W/m^2 (26.8 kJ/m^2) significantly induced *CYP1A* and *CYP1B1* expression, but other *CYP1* genes (*CYP1C1*, *CYP1C2* and *CYP1D1*) showed no increase. The results show that UVB can induce expression of *CYP1* genes as well stress response genes in developing zebrafish, and that UVB intensity and duration influence the responses.

Introduction

Amounts of ultraviolet (UV) radiation reaching Earth's surface increased starting in the mid-20th century due to deterioration of the stratospheric ozone layer by chlorofluorocarbons (CFC) and related compounds (McKenzie *et al.* 2007; Rowland 2006). According to model calculations that include changes in CFCs use, the ozone concentrations at mid-latitudes may return to pre-1980 levels by the middle of this century (McKenzie *et al.* 2007), yet UV will continue to present challenges. UVB radiation (280–320 nm) is readily absorbed by and can cause severe damage to proteins and DNA. Other UV wavelengths reaching Earth's surface include UVA (320–400 nm) and UVC (< 280 nm), although the latter is mostly absorbed by

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stratospheric ozone. In water, UV is absorbed by dissolved organic material, but water in itself absorbs little, and UVB penetration has been observed as deep as 55 m (Tedetti *et al.* 2007).

Effects of UV radiation result from generation of reactive oxygen species (ROS) and subsequent radical formation, as well as direct damage to cellular macromolecules. The most pronounced effect of prolonged exposure to UV in humans is induction and development of skin cancer (Brash 1997; Brash *et al.* 1991; Setlow 1974). In aquatic organisms the effects of UV radiation include impairment of larval development in copepods and fish (Browman *et al.* 2003; Hakkinen *et al.* 2004; Hunter *et al.* 1979), lesions to the brain and retina of developing fish (Hunter *et al.* 1979), oxidative stress in *Daphnia* and in adult zebrafish (Vega and Pizarro 2000), and other effects as reviewed elsewhere (Dahms and Lee 2009; Häder *et al.* 2007; Zagarese and Williamson 2001).

Adverse effects of UV radiation are partially reduced by molecular defense mechanisms including glutathione and enzymes such as superoxide dismutase (SOD), catalase, and glutathione peroxidase, which are involved in quenching excessive levels of ROS and free radicals. Increased activity of enzymes protecting against oxidative stress has been reported in adult zebrafish exposed to UVB (Charron *et al.* 2000). When UV radiation damages DNA directly, the most characteristic result is the formation of cyclobutane-pyrimidine dimers and pyrimidine (6-4) photoproducts. Repairing UV-damaged DNA commonly involves two mechanisms 1) photoreactivation by photolyases and 2) excision repair. Resynthesis of DNA is facilitated by DNA polymerase δ and its processivity factor, proliferating cellular nuclear antigen (PCNA). PCNA has been found to be upregulated by exposure to UV radiation in human cell lines (Katsumi *et al.* 2001; Zeng *et al.* 1994) and mice (Lambertini *et al.* 2005).

UV radiation also has been found to induce expression of cytochrome P450 gene family 1 (*CYP1*) genes in human and animal cells in culture (Wei *et al.* 2000; Wei *et al.* 1999), and UV radiation also elicits *CYP1* induction *in vivo* in rodents and humans (Fritsche *et al.* 2007; Goerz *et al.* 1996; Katiyar *et al.* 2000). This appears to involve tryptophan, which has emerged as a prime candidate for formation of endogenous AHR agonists, *via* enzyme action or direct oxidation. Aspartate aminotransferase and D-amino acid oxidase form indole-3-pyruvate, which spontaneously oxidizes to diindole products able to activate the AHR at nanomolar concentrations (Bittinger *et al.* 2003; Chowdhury *et al.* 2009; Nguyen *et al.* 2009). Oxidation of tryptophan by UV radiation or sunlight forms multiple products that can induce *CYP1A* gene expression (Diani-Moore *et al.* 2006; Fritsche *et al.* 2007; Mukai and Tischkau 2007). In HaCaT cells exposed to UV tryptophan has been shown to be the intracellular source of highly potent AHR agonists including 6-formylindolo[3,2b]carbazole (FICZ) (Fritsche *et al.* 2007).

Recently we reported that FICZ is a potent inducer of *CYP1* genes in zebrafish embryos (Jönsson *et al.* 2009), suggesting that AHR activation as a response to UV radiation is conserved through phylogeny. Here we report on *in vivo* UV effects on expression of *CYP1* and radical defense related genes *PCNA* and *SOD* in zebrafish embryo/larval stages. Zebrafish embryos are transparent and may therefore be particularly vulnerable to the effects of UV radiation. Exposure was to different wavelengths, intensities, and duration, similar in magnitude to UVB exposure in subtropical sunlight. Zebrafish are native to eastern India, and the irradiance and total fluent energy of the current study are less than that potentially experienced by zebrafish in the wild (estimated maximum flux of 2.6 W/m^2 UVB and total daily insolation of 82.4 kJ/m^2 UVB in Western Bengal). Zebrafish tend to spawn in clear shallow waters with silt bottoms (Engeszer *et al.* 2007), and may have behavioral adaptations that minimize developmental exposure to high UV radiation similar to yellow perch in transparent lakes (Zagarese and Williamson 2001). The results indicate that UVB can have an effect on transcription of multiple *CYP1* genes as well as on *PCNA* and *SOD*, in developing zebrafish.

Materials and Methods

Animals

Zebrafish (*Danio rerio*) embryos were raised in the Woods Hole Oceanographic Institution zebrafish facility. Animals were kept at constant temperature (~28.5°C) in aerated, filtered system water. System water was composed of Instant Ocean mixture (60 mg ml^{-1}) with addition of sodium bicarbonate (50 mg ml^{-1}), calcium sulfate (8.5 mg ml^{-1}) and Kent's freshwater essentials (53 μl L^{-1}) in distilled water. Densities were less than five fish L^{-1} and the fish were fed twice a day with decapsulated and freshly hatched brine shrimps (*Artemia salina*) and once daily with Omega One flakes. Breeding was initiated by placement of breeding traps made of plastic Tupperware covered with plastic mesh. After 1 hour the traps were removed and fertile eggs sorted into Pyrex petri dishes. Fertilized embryos were kept in 0.3 × Danieau's solution at 28.5°C until further treatment. Larvae were fed AP100 juvenile food, mixed with *Spirulina* starting at 6 days post-fertilization (dpf). Danieau's solution was exchanged on subsequent days.

Exposure to UVA and UVB radiation

Embryos were exposed to UV radiation in an enclosed incubator at constant temperature (28.5° C), UV treatments being superimposed onto the normal daily light cycle of 14 hours. White light was supplied by four Sylvania cool white 4 ft. fluorescent tubes (F340W/SS/Eco). UVA radiation was supplied by one 2 ft. Phillips Blacklight (Tl20W/08RS) with a peak irradiance at 366 nm. UVB was supplied by one or two 2 ft. UBL FS20T12/UVB tube with peak irradiance at 310 nm (National Biological Corp., Beachwood, OH, USA). UV intensities were measured using an OL 756 UV-VIS spectroradiometer (Optronic Laboratories, Orlando FL, USA). The spectral distributions of the UV radiation and white light from these sources are presented in the Supplemental Data (Fig. S1–S2).

The desired UV intensity was obtained by adjusting the distance of the samples from the sources (range of 18 to 37 cm) and by using one or two tubes. UVC (<280 nm) radiation was blocked by use of cellulose acetate filters, which were replaced with new filters before each exposure. The UV intensity was 0.62 W/m^2 (UVB) and 2.05 W/m^2 (UVA) for exposures of embryos to UVB and UVA sources simultaneously. The same intensity of UVB without supplemental UVA included UVA at irradiances of 0.5 W/m^2. We refer to this exposure as lower intensity UVB exposure. Lower intensity UVB was used to investigate the time dependent responsiveness of SOD, CYP1A and PCNA gene induction. Increased UVB irradiance (0.93 W/m^2) (which included UVA at 0.83 W/m^2) was used to investigate dose dependency and to irradiate larval zebrafish. These UV radiation doses were selected for the different exposure regimes based on previously determined mortality in zebrafish embryos (Charron *et al.* 2000; Dong *et al.* 2007). The doses of UVB we used are also in the range of UVB-irradiance in sunlight [e.g., ca. 0.96 W/m^2 in Woods Hole in August ((NCAR/ACD) 2009)]. Fertilized TL zebrafish eggs (numbers ranging from 20 to 60; see figure legends for exact numbers) were placed in 10 cm Pyrex glass petri dishes containing 25 ml of 0.3 × Danieau's solution. To prevent evaporation the dishes were covered with UV permeable plastic wrap, with small holes in the plastic to allow air exchange. Most treatments were started at 24 hours post fertilization (hpf).

Experimental exposures to UV radiation

Four different regimes of exposure were used in these studies. The duration and the timing of exposure and sampling are depicted in the figures showing the data for the various experiments.

1. Exposure of zebrafish embryos to UVA and UVB—Treatments involved 2, 4 and 6 hours of daily UVA and UVB treatments for two consecutive days, adding up to cumulative

doses of 4, 8, and 12 hours of total UV (8.9, 17.8, and 26.8 kJ/m^2 UVB). This exposure regime was chosen to account for the possibility that UV-generated compounds might increase over time, possibly reaching a higher dose after a second pulse. At 72 hpf, four separate biological replicates (each of 60 embryos) were collected from the control and each of the UVA + UVB exposure groups. The samples were immediately frozen in liquid N_2. Data from two separate experiments were pooled for statistical analysis.

As the objective of this study was to determine the effect of UVB radiation on expression of *CYP1* and a few stress response genes we did not supplement UVA in subsequent exposures although UVB bulbs emitted some UVA. For UVB+UVA treatments the ratio of UVB to UVA was ~ 1:3, for UVB "alone," the ratio was 1:0.9. The ratio in sunlight is closer to 1:10.

2. Influence of sampling time on gene expression—In this experiment the embryos were exposed to two separate pulses of UVB (with an intensity of 0.62 W/m^2) of 6 hours each on two consecutive days, adding up to a cumulative 12 hour dose of 26.8 kJ/m^2. The embryos were sampled either directly after the last UVB exposure (54 hpf) or 18 hours later at 72 hpf, and frozen in liquid N_2. Four replicates were sampled for each treatment.

3. Increased dose exposures—Two UVB bulbs were used in this experiment with a combined energy of 0.93 W/m^2 UVB. (The bulbs also emitted 0.83 W/m^2 UVA.) The embryos were exposed for either 3 or 4 hours twice over the course of two days, adding up to a total exposure time of 6 or 8 hours of UVB, equivalent to a total of 20.1 or 26.8 kJ/m^2, respectively, over the duration of treatment. All treatments were done in quadruplicate except for controls, for which five petri dishes were sampled. All samples were frozen in liquid N_2 for later analysis.

4. Exposure of larvae at eight days post fertilization—Eight-day old (8 dpf) zebrafish larvae were exposed to a single 8-hour pulse of the higher intensity radiation (0.93 W/m^2, giving 26.8 kJ/m^2 UVB, along with 23.9 kJ/m^2 UVA). We used six biological replicates with 18 fish per replicate for both the UV treatment and the controls. Fish were sampled immediately after the light exposure and the animals were frozen in liquid nitrogen for future use. We chose to use a single longer pulse of UVB at the higher intensity, as we considered that the negative effect would be less than that of two 4-hour pulses and yet have an effect strong enough to allow us to detect changes in expression of other *CYP1* genes.

Mortality of embryos exposed to UV was assessed in these and additional experiments by examining embryos for beating heart. Only embryos with beating heart were included in gene expression analyses.

Quantification of mRNA with quantitative PCR

Frozen embryos or larvae were homogenized and RNA was extracted using RNA Stat-60 (Tel. Test Inc. Friendswood, TX, USA). Isolated RNA was DNase treated using the TURBO DNA free kit (Ambion). Synthesis of cDNA was performed using the Omniscript Reverse Transcriptase kit (Qiagen, Inc. Valencia, CA, USA), random hexamer primers (Operon Biotechnologies Inc.) and the RNasin RNase Inhibitor (Promega, Madison, WI, USA). The RNA was then quantified spectrophotometrically (NanoDrop, ND-1000, Nanodrop Technologies, Wilmington, DE, USA). *CYP1A*, *CYP1B1*, *CYP1C1*, *CYP1C2*, *CYP1D1*, *ARNT2* and *PCNA* real time PCR primer were taken from (Jönsson *et al.* 2009) (see Table 1) and were synthesized by Operon. The sequences for copper-zinc *SOD* (*SOD1*) and *β-actin* real time PCR primers generously were provided by Dr. Mark Hahn (Woods Hole, MA, USA). Real time quantitative PCR was performed using the IQ SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA, USA), forward and reverse primers (5 pmol each per reaction) and cDNA (derived from 0.1 μg total RNA per reaction). In each sample genes were analyzed

in technical triplicates in a 96 well plate on the iCycler IQ Real Time PCR detection System (Bio-Rad Laboratories, Hercules, CA, USA). Amplification conditions for all templates were, 95 °C for 4.5 min followed by 40 cycles of 95 ° C for 15 s, 62 ° C for 1 min, and 72 ° C for 30 s. Melt curve analysis was performed at the end of each PCR run to ensure that single products only were amplified

Calculations and Statistics

Relative mRNA expression was calculated using the $2^{\Delta\Delta Ct}$ method (Livak and Schmittgen 2001), with aryl hydrocarbon receptor nuclear translocator (*ARNT2*) was used as a reference gene in all UV- experiments. There was no significant difference in ARNT2 expression between control and UVB-treated groups in the experiment with 8 dpf larvae, or in most experiments with embryos. There was a modest increase in ARNT transcript levels in the 54 hpf UVB group in experiment 2, although there still was a detectable increase in SOD and PCNA expression at that exposure (Fig. 2C–D). We also measured β-actin expression, which was notably less stable than the expression of ARNT, consistent with the observations of McCurley and Callard (McCurley and Callard 2008). qPCR efficiency was assumed to be 2 throughout all calculations. Grubbs test (Grubbs 1969) was performed on all data sets to remove possible outliers. One way ANOVA and Dunnett's post-hoc test was performed on data from experiments number 1 (UVA+UVB exposure) and 3 (increased UVB dose). Student's t-test was performed on data from experiments number 2 (influence of sampling time) and 4 (8dpf larval exposure). Statistical analysis was done using Prism 4.03 (GraphPad Software Inc., San Diego, CA, USA).

Results

Exposure of zebrafish embryos to UVA and UVB

In order to determine whether the combination of UVA and UVB has an effect on *CYP1A* expression, we exposed zebrafish embryos to both UVB (0.62 W/m^2) and UVA (2.05 W/m^2) for 2, 4, or 6 hours per day on two consecutive days (i.e., in total 4, 8, or 12 h; Fig. 1A). Embryos irradiated with the two 6-hour pulses of UVA plus UVB exhibited a modest yet significant induction of *CYP1A* transcripts (2.4-fold control, ± 0.8; $p < 0.01$; Fig. 1B). Exposure to UVA plus UVB for shorter periods had either less strong or no significant effect on *CYP1A* expression in embryonic zebrafish (Fig. 1B).

Another set of experiments (data not shown) demonstrated that radiation from a UVA source alone at 2.8 W/m^2 (123.1 kJ/m^2) had no effect on *CYP1A* transcript levels in zebrafish embryos, and therefore UVA was not supplemented in subsequent exposures.

Influence of sampling time on gene expression

As CYP1 induction by tryptophan photoproducts can be transient (Wei *et al.* 2000; Wincent *et al.* 2009), another experiment was conducted to determine whether UV-induced changes in *CYP1A* expression in zebrafish embryos are potentially stronger immediately post-exposure and might diminish with time after exposure. We included measurements of *SOD1* and *PCNA* to examine other effects than those on *CYP1* gene expression, and as the response of these genes also could have a temporal aspect. Treatments involved two 6-hour pulses of UVB with exposure beginning at 24 and 48 hpf. Sampling occurred either immediately after the second pulse (at 54 hpf) or 18 hours later, i.e., at 72 hpf (Fig. 2A). In this experiment there was a trend toward increased *CYP1A* expression at 54 hpf and at 72 hpf, although the increases were not significant (Fig. 2B). In the same samples, significant induction of *SOD1* (2.3-fold, $p< 0.01$) was observed at both 54 and 72 hpf (Fig. 2C). *PCNA* transcript levels were significantly elevated in samples taken after the second UVB-pulse (at 54 hpf; $p< 0.05$); the mean value for *PCNA* levels was greater at 72 hpf, though the difference from control values

was not significant (Fig 2D). The samples taken at 18 hours after the second UVB pulse showed a greater variation in mRNA levels for all three genes (*CYP1A*, *SOD*, *PCNA*) when compared to the samples taken immediately after that second pulse (Fig. 2B–D).

Exposure to increased UVB doses

In the previous experiments, *CYP1A*, *SOD1* and *PCNA* expression levels were examined after UVB exposure at 0.62 W/m^2. To assess the response of zebrafish embryos to a greater dose of UVB, the irradiance was increased to 0.93 W/m^2, with exposure in two daily pulses of either 3 or 4 hours each (20.1 and 26.8 kJ/m^2 total UVB, respectively), and sampling was done directly after the second pulse, i.e., at 54 or 56 hpf (Fig. 3A). In addition to *SOD1*, *PCNA* and *CYP1A*, we also examined expression of *CYP1B1* in this set of experiments. The observed expression patterns can be sorted into two distinct groups. The embryos exposed to two 3-hour pulses of UVB, showed significant up-regulation of *SOD1* and *PCNA* ($p< 0.001$ and $p< 0.01$, respectively) (Fig 3D–E). When embryos were exposed for two additional hours (i.e. two pulses of 4 hours each) the levels of *SOD1* and *PCNA* transcript expression were only at about the levels in control embryos.

As with *SOD1* and *PCNA*, *CYP1B1* mRNA levels were significantly induced in embryos that were exposed to two 3-hour pulses, and declined with the additional exposure (i.e. after two 4-hour pulses; Fig. 3C). The effects of UVB at 0.93 W/m^2 on *CYP1A* gene expression differed somewhat from the effects observed in *SOD1*, *PCNA* and *CYP1B1* genes. Thus, *CYP1A* expression levels appeared to increase after two 3-hour pulses of UVB, and were significantly elevated after the two 4-hour pulses ($p< 0.05$; Fig. 3B), while expression of the other genes declined after the two 4-hour pulses.

Expression of additional *CYP1* genes

We used larval zebrafish to determine the response of the full complement of *CYP1* genes to UVB exposure at another stage of development. Zebrafish larvae at 8 dpf were exposed to a single 8-hour long pulse of UVB, at 0.93 W/m^2, with sampling immediately after exposure. Both *CYP1A* and *CYP1B1* were up-regulated significantly in comparison with controls (Fig. 4), *CYP1C1* showed a trend to increase, and *CYP1C2* and *CYP1D1* expression did not change in response to this UVB exposure in the 8-dpf zebrafish.

Mortality

The condition of the embryos exposed to UVB could be a factor in the observed levels of gene expression. Embryo condition may be reflected by mortality in groups exposed to a particular UV dose. Mortality was monitored in the above experiments on a daily basis, by examining embryos for beating heart at the end of the UV exposures. UVA was found not to cause mortality in embryonic zebrafish, even at high irradiances (> 2.8 W/m^2) and high doses (808.4 kJ/m^2). The lower UVB irradiance (0.62 W/m^2) used in these experiments only elicited minor mortality in the exposed embryos, ranging from 3 to 4 % (Table 2). This was confirmed in separate experiments (data not shown). Increasing the UVB irradiance to 0.93 W/m^2 caused much greater mortality, to 27% in those groups of embryos exposed to two 3-hour pulses (20.1 kJ/m^2), and to 34% in those exposed to two 4-hour pulses (26.8 kJ/m^2; Table 2). In a separate experiment to examine mortality further, we observed that mortality was only slightly above background 24 hours after the beginning of the first pulse of either 3 or 4 hours exposure to the higher dose (data not shown). However, the second pulse of either 3 or 4 hours exposure to the higher dose of UVB resulted in a dramatic and immediate increase in mortality.

Discussion

The results here show that exposure of zebrafish embryos or larvae to varied intensities or durations of UV can induce the expression of *CYP1A* and *CYP1B1*, as well as affect the expression of genes involved in protection from radicals (*SOD1*) and in DNA repair (*PCNA*). Our results are similar to results with mammalian cells and tissues showing that UV radiation causes an increase in *CYP1A* and *CYP1B1* expression (Katiyar *et al.* 2000; Wei *et al.* 1999). As well, our results (Fig. 3) agree with reports that UV induces the levels of *SOD* and *PCNA* transcripts (Hall *et al.* 1993; Zeng *et al.* 1994). The effects in zebrafish also were observed at irradiances similar to those causing effects in mammalian systems. Changes in levels of expression of all four genes were modest, and varied with duration and intensity of UVB exposure. Similarly variable responses to UV have been recorded also in transcriptional effects in mammalian systems.

Zebrafish embryos exposed to UVB plus UVA over two days had significantly up-regulated levels of *CYP1A* mRNA (Fig. 1). The order of magnitude of response in experiments (1.5- to 2.4-fold over the control), is comparable to the magnitude of increase in *CYP1A1* observed in HaCaT cells exposed to UVB (Fritsche *et al.* 2007), in which an approximate twofold increase in *CYP1A1* mRNA transcripts was observed. The same study also found that UVA did not affect the AHR translocation into the nucleus, suggesting that UVA is not likely to be involved in the induction of *CYP1A* gene expression (Fritsche *et al.* 2007). In keeping with these findings we found no effect of UVA alone on *CYP1A* gene expression in zebrafish embryos.

The induction of *CYP1* genes in vertebrates occurs through an AHR-mediated pathway. Mammalian, fish and frog AHRs can be activated by FICZ, a product of tryptophan photooxidation and one of the most potent inducers of mammalian *CYP1A1* known (Fritsche *et al.* 2007; Jönsson *et al.* 2009; Laub *et al.*). It is thus plausible that tryptophan derivatives are involved in induction of *CYP1A* and *CYP1B1* in the UV-treated zebrafish embryos and larvae. While the identity of the *CYP1*-inducing compounds formed in zebrafish embryos or larvae exposed to UV has not been determined, the potency of FICZ in various species suggests it as one candidate for the UV effects on *CYP1* gene induction in these fish

The modest induction of *CYP1A* transcript in zebrafish could be related to the levels, persistence, and/or the efficacy and potency of inducing compounds formed by UV. The amounts of AHR agonists that might be formed at any one time in embryos *in vivo* are presumed to be much less than the amounts of known photoproducts of tryptophan added experimentally to zebrafish embryos (Jönsson *et al.* 2009). Many potent inducers formed by UV are transiently active as well; for example, *CYP1A* induction by FICZ is transient in rat hepatocytes and human keratinocytes, with induction no longer detected after 24 hours (Kocarek *et al.* 1993; Oberg *et al.* 2005; Wei *et al.* 1999). Jönsson et al. found that induction of *CYP1A* by FICZ is also transient in zebrafish embryos; *CYP1A* was more highly induced after 6 hours of exposure than 12 hours of exposure (Jönsson *et al.* 2009). Such results could result from a rapid metabolism of inducer (Wincent *et al.* 2009).

Whether CYP1A protein is detectably induced was not determined. Hakkinen et al (2004 AqTox) did not see induction of CYP1A protein by western blot analysis of pike larvae exposed to UVB. This is not necessarily inconsistent with our results. Western blotting is less sensitive than qPCR, and Hakkinen et al did not measure transcript levels. A very slight induction of CYP1A protein was detected in whitefish larvae exposed to a low dose of UVB but not a higher UVB dose (Vehniäinen et al., 2003), a dose response pattern not unlike that which we observed. (The doses are approximately equivalent to our low and high doses respectively; see supplemental data.) However, given the slight induction of mRNA in zebrafish larvae, and the limits of detection, we would not expect to detect CYP1A protein by western blot.

Immunohistochemistry of zebrafish embryos could reveal whether selected cells express higher levels of CYP1A protein; such studies are planned

The induction of *CYP1B1* by UV was similar in magnitude to the induction of *CYP1B1* by exposure directly to FICZ. The magnitude of *CYP1B1* response seen here (Fig. 3 and 4) also was similar to that of *CYP1A*, while in direct FICZ exposures *CYP1B1* was induced much less strongly than *CYP1A* (Jönsson *et al.* 2009). However, the EC_{50} for induction by FICZ was similar for the two genes. There also was no evident induction of *CYP1C1* by UVB, while *CYP1C1* was induced, albeit weakly, by direct FICZ exposure (Jönsson *et al.* 2009). The difference between FICZ results and UVB results suggest that the induction by UV could involve compound(s) other than FICZ. Multiple AHR agonists are formed by exposure of tryptophan to sunlight, and some of these apparently can elicit a more sustained induction of *CYP1A* than FICZ (Diani-Moore *et al.* 2006). Fully understanding the kinetics of response of *CYP1* genes to UV radiation will depend on knowing the identity and amounts of AHR agonists formed *in vivo*, as well as issues of gene specific responses as previously outlined (Jönsson *et al.* 2009).

SOD and PCNA expression

SOD1 transcript levels were significantly induced when embryos exposed to lower dose UVB were sampled at 72 hpf, i.e.,18 hours after a second pulse of UV exposure, while embryos that were sampled immediately after that second UV pulse showed no increase in *SOD1* expression (see Fig. 3B). At a higher dose *SOD1* was induced by shorter but not longer exposures. Others also have reported varying effects of UV on SOD expression and enzyme activity levels. Charron *et al*. (2000) observed maximum SOD enzyme activities in muscle/skin of adult zebrafish after exposure to UVB for 6 hours at either 0.15 or 1.95 W/m^2 while longer exposures (>6 h) resulted in less SOD enzyme activity. Northern pike larvae irradiated with UVB exhibited no significant increase in SOD activity (Hakkinen *et al.* 2004), whereas in mice and HeLa cells a continual decline in epidermal SOD activity was observed after a single pulse of UVB, up to 72 hours after exposure (Isoherranen *et al.* 1997;Pence and Naylor 1990). SOD enzymes are known to be damaged through excess levels of H_2O_2 (Salo *et al.* 1990).

In contrast, Choung et al (2004) found that UVB induced *SOD1 (Cu-Zn SOD)*, *SOD2 (Mn SOD)*, and *SOD3* (*ecSOD)* transcript levels 2–3 fold in mouse skin, although with differing UVB sensitivity and post-irradiation time-dependences (Choung *et al.* 2004). Similarly, Black et al (2008) found different expression patterns for *SOD1* and *SOD2* in mouse keratinocytes: *SOD2* expression increased 1.5-fold after 24 hours at low UVB but decreased at higher exposure levels, while *SOD1* expression increased ~2 fold at the higher but not at the lower doses (Black *et al.* 2008). Clearly, different SOD isoforms exhibit differential responses to UV irradiation, and these responses can vary with exposure conditions. As in those studies, we also observed that *SOD1* transcript expression varied with UVB irradiance and duration of exposure. The reasons for such variation are not known, but could involve a balance between enzyme damage and transcriptional activation mediated by ROS.

At lower intensities of UVB, significant induction of *PCNA* was observed in zebrafish embryos when sampling occurred at the end of the second pulse (see Fig. 2D). While the mean value was greater at the longer time after the second pulse, the difference was not significant, although the samples taken at 72 hpf (18 hr post-exposure) exhibited much greater variability in *PCNA* expression. At higher irradiance there was induction at a shorter exposure but not at a longer exposure. Maeda et. al. (2001) reported a 2–3-fold induction of PCNA transcript levels in human keratinocytes at low doses of UVB (0.1–0.3 kJ/m^2), but down-regulation with higher doses (0.6 kJ/m^2) (Maeda *et al.* 2001). Many studies of UV effects on PCNA in mammalian systems often consider protein levels or activity, rather than gene expression, making direct comparisons with our results difficult. However, Moore et al (2004) concluded that PCNA

could serve as a marker of DNA repair and indirectly as an indicator of UVB-induced damage, and found that expression varied with exposure time and dose (Moore *et al.* 2004). Thus, the changes in PCNA transcript expression that we found in zebrafish embryos, characterized by modest induction and responses that varied with exposure conditions, are not unlike the variation in PCNA protein levels in the studies with mammalian systems.

The variation observed in gene expression suggests that there are complex relationships between UV exposure and specific responses to that exposure, which must be overlain on the variable detrimental effects of UV. We observed an average mortality of about 2% in embryos exposed to 13.4 kJ/m^2 (over one 6 hour period of 0.62 W/m^2; data not shown), and ca. 34% in embryos exposed to a total of 26.8 kJ/m^2 of UVB. Dong et al (2007) found about 30% mortality in zebrafish embryos exposed to 15.5 kJ/m^2 and more than 70% mortality at 31 kJ/m^2, although their irradiation intensity was 5.2 W/m^2, more than five times the irradiance used here. Dong et al (2007) also reported a high incidence of malformations in embryos exposed to 9.3 kJ/m^2, although the mortality at that dose was low (Dong *et al.* 2007). Our results and those of others thus indicate that UVB can elicit substantial mortality in zebrafish embryos. However, the levels of mortality observed may be artificially high; Hakkinen et al. (2004) observed that the mortality in pike larvae tested in the field was significantly less than seen in experimental settings, attributed to the modifying effects of organic material in the water. It remains to be seen if this would affect the mortality in developing zebrafish. In any case, the possible effects of UV on gene expression, in experimental or natural settings, must be viewed in a context of toxicity.

When embryos were exposed to shorter durations of higher intensity UVB (0.93 W/m^2), *CYP1B1*, *SOD1* and *PCNA* all were induced significantly, while additional exposure to this higher intensity caused a decline in expression of these three genes (Fig. 3). The decline after two hours longer exposure at the higher doses of UVB could reflect the condition of the animals; mortality rose significantly from 3–4 % in the low dose experiments, to 27–34% in the high dose exposure of embryos. *CYP1A* was the only gene significantly upregulated after two 4 hour pulses of higher intensity UVB (Fig. 3). The induction of *CYP1A* therefore appears to be on the sharp part of a dose response curve, with a steep increase being limited by increasing mortality.

Conceivably, UV-generated ROS and direct damage could swamp the protective apparatus and overwhelm the physiological capability of the embryos, resulting in conditions that not only would increase mortality but also decrease transcriptional activity. Our studies also included doses of UV that did not elicit substantial mortality. Importantly, we observed that the first pulse of higher intensity UVB did not result in substantial mortality, even 24 hours after that exposure (Table 2). The immediate jump in mortality with a second UVB pulse of 3 or 4 hours strongly suggests that the ability of the embryos to protect against UV had diminished greatly, and more in those groups exposed for 4 hours than for 3 hours. The mechanism is not known, yet this suggests that the gene expression results with the two 3-hour exposures (20.1 kJ/m^2) may be less affected by deteriorating condition than with the two 4-hour exposures (26.8 kJ/m^2).

This hypothesis would be consistent with the observed pattern of significant up-regulation of *SOD1* and *PCNA* followed by decline with additional exposure. The pathway for *CYP1A* and *CYP1B1* induction in zebrafish is probably dependent on the generation of an AHR ligand, whereas *SOD1* and *PCNA* are induced by ROS and DNA damage, respectively (Essers *et al.* 2005; Punnonen *et al.* 1995). Regardless of the mechanism of induction, if the physiological capability of embryos to withstand additional UV exposure were overwhelmed, this could adversely affect the capacity to respond, seen in all four genes examined.

Conclusion

In vivo studies on UV induced *CYP1A* gene expression in non-mammalian organisms are rare. This study demonstrates up-regulation of *CYP1A* and *CYP1B1 in vivo* in response to UV radiation in the widely used model organism *Danio rerio*. Levels of *SOD1* and *PCNA* expression also were up-regulated in response to UV radiation. *SOD1* and *PCNA* induction likely reflect ROS formation and DNA damage, respectively, whereas the mechanism for induction of *CYP1A* and *CYP1B1* presumably involves photochemical formation of some AHR agonists in zebrafish. Although the effect on *CYP1* gene expression in this study was variable and slight, it was in the range of *CYP1* responses to UV seen in mammals. While induction of *CYP1A* and *CYP1B1* appear to be involved in the response of developing zebrafish to UV radiation, the significance of this induction, or the AHR activation it implies, to the physiology of the organism remains to be determined. The observation that UV increases sensitivity of keratinocytes to DNA damage by PAH that are substrates of CYP1 enzymes (Nair *et al.* 2009) suggests synergistic effects are possible.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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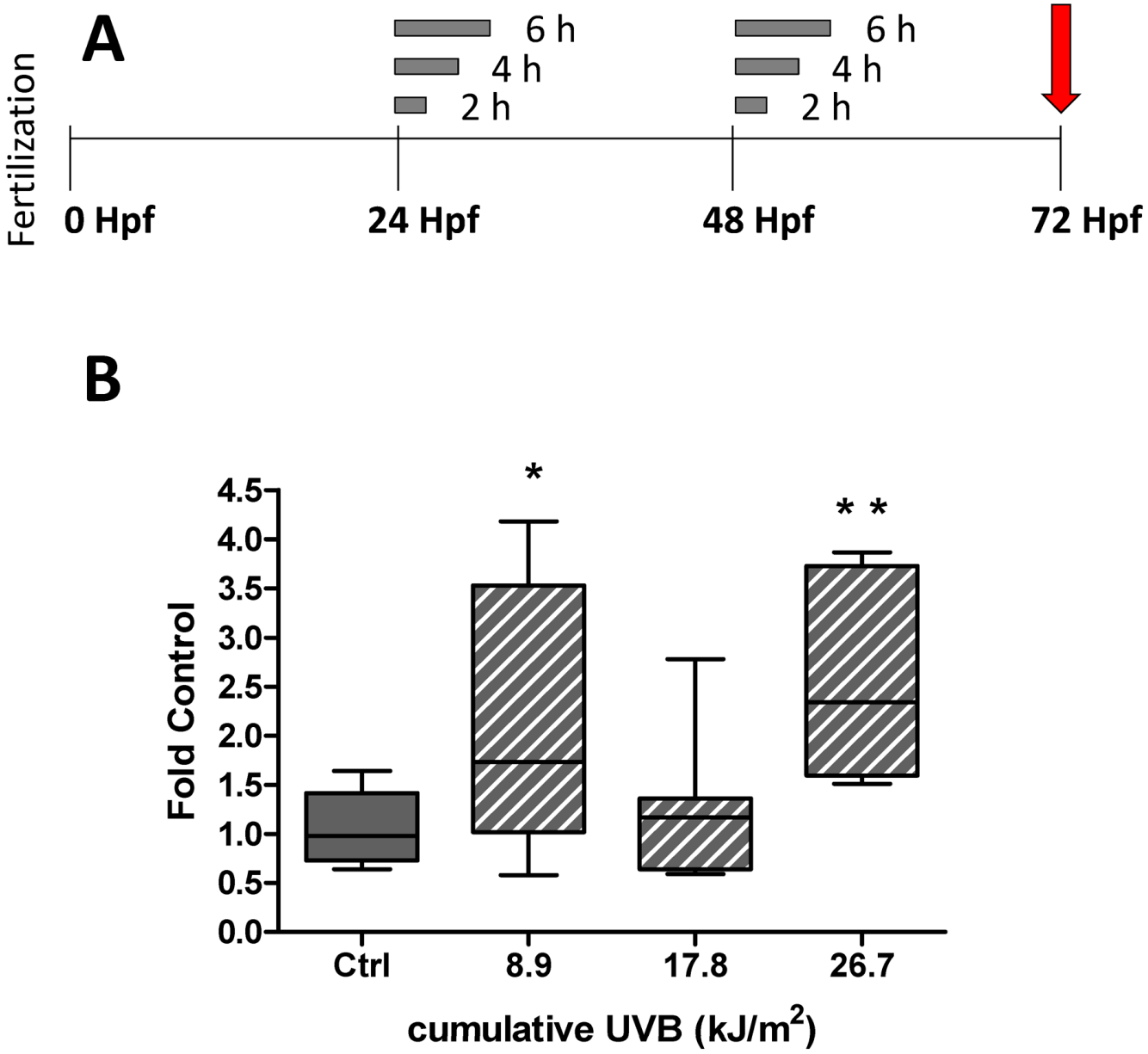

Figure 1.

Levels of *CYP1A* gene expression after 4, 8 and 12 hours of cumulative UVA+ UVB radiation exposure over two days. (A) Embryos were exposed to 0.62 W/m^2 UVB and 2.05 W/m^2 UVA. Embryos in each biological replicate (n=4) were pooled and sampled at 72 hours post fertilization (arrows). (B) *CYP1A* gene expression. Error bars represent one standard deviation. Differences from control values were determined by one-way ANOVA followed by Dunnett's-Post-hoc test (* $p < 0.05$, ** $p < 0.01$).

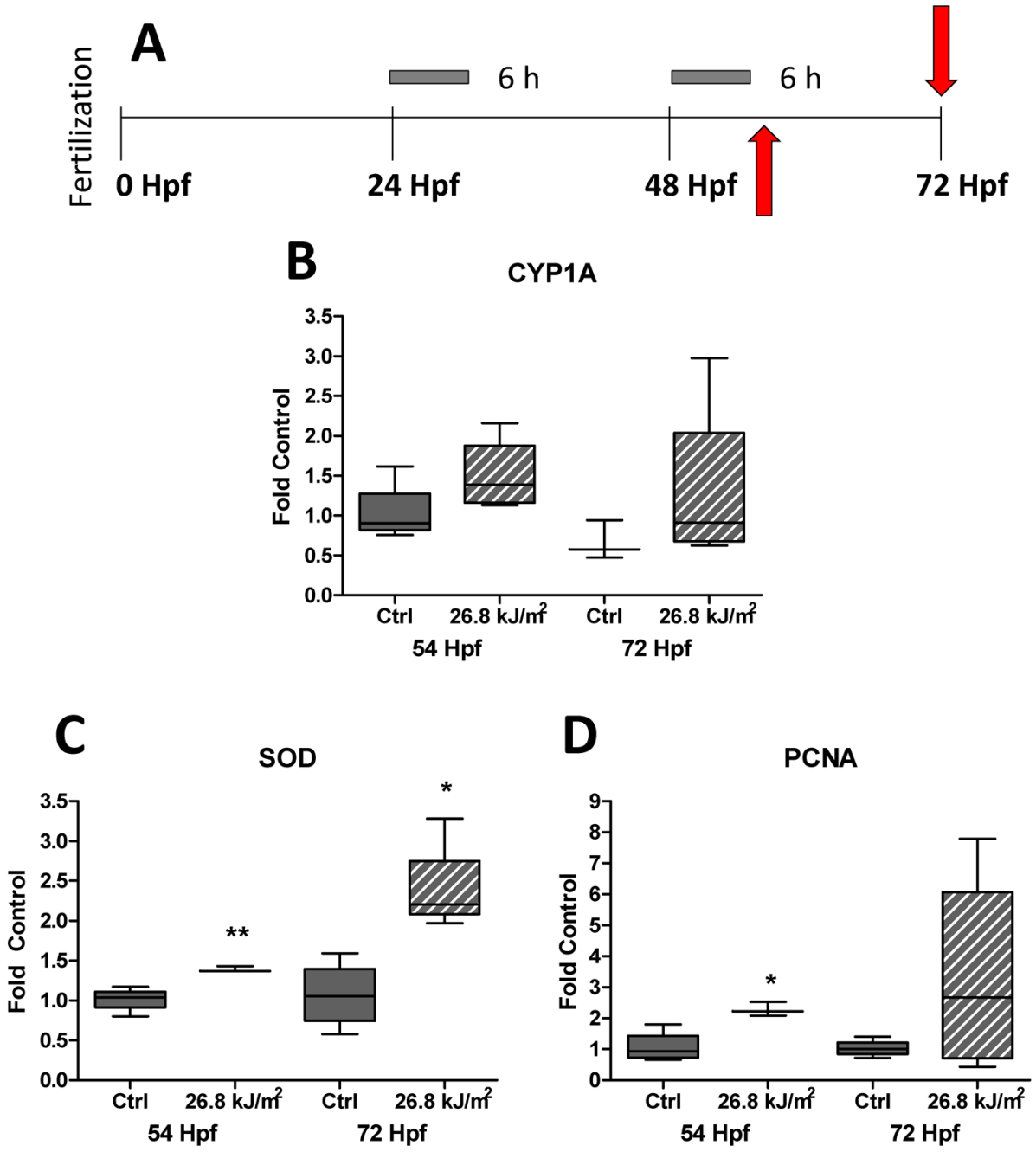

Figure 2.

Levels of *CYP1A*, *SOD1* and *PCNA* gene expression after 12h of cumulative UVB exposure. (A) Embryos were exposed to UVB at 0.62 W/m^2 and were sampled from each biological replicate immediately after a second pulse of UVB and 18 hours later, i.e., at 54 and 72 hours post fertilization (arrows), respectively (n=4). (B) Levels of *CYP1A* expression, (C) *SOD1* expression, and (C) *PCNA* expression. Error bars represent one standard deviation. Differences from control values were determined by unpaired t-tests. (*p< 0.05, ** p< 0.01).

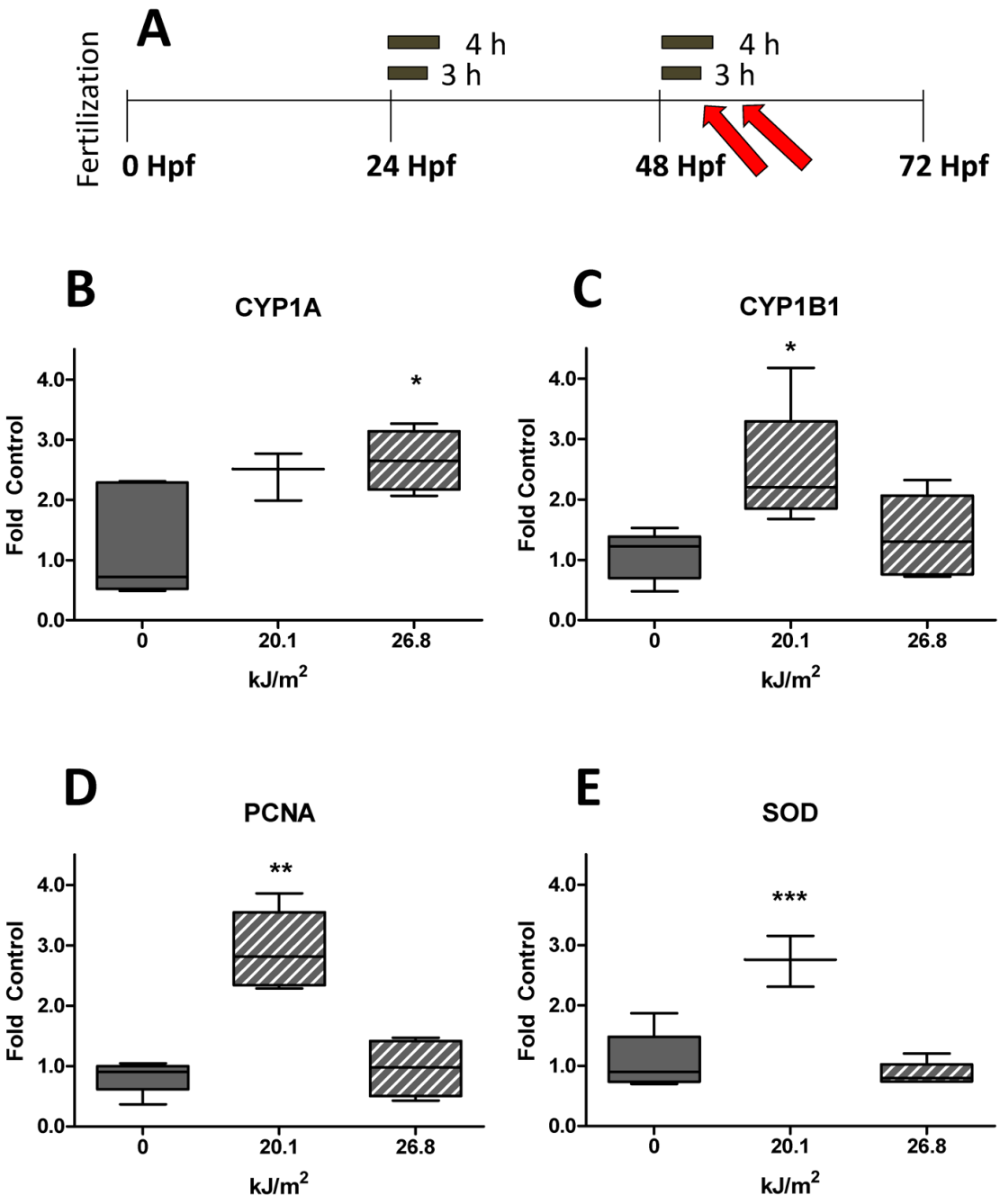

Figure 3.

Levels of *CYP1A*, *CYP1B1*, *PCNA*, and *SOD1* gene expression after UVB exposure. (A) Embryos were exposed to 0.93 W/m^2 for two periods during development. Embryos treated with 6 hours of UVB were sampled at 51 hours post fertilization and 8 hour treatments were sampled at 52 hpf (arrows). The embryos from each biological treatment were pooled (n=4). (B) Levels of *CYP1A* expression, (C) *CYP1B1* expression, (C) *PCNA* expression, and (D) *SOD1* expression. Error bars represent one standard deviation. Significant differences from control values were determined by one-way ANOVA followed by Dunnett's- Post-hoc test (* $p< 0.05$, ** $p< 0.01$, *** $p<0.001$).

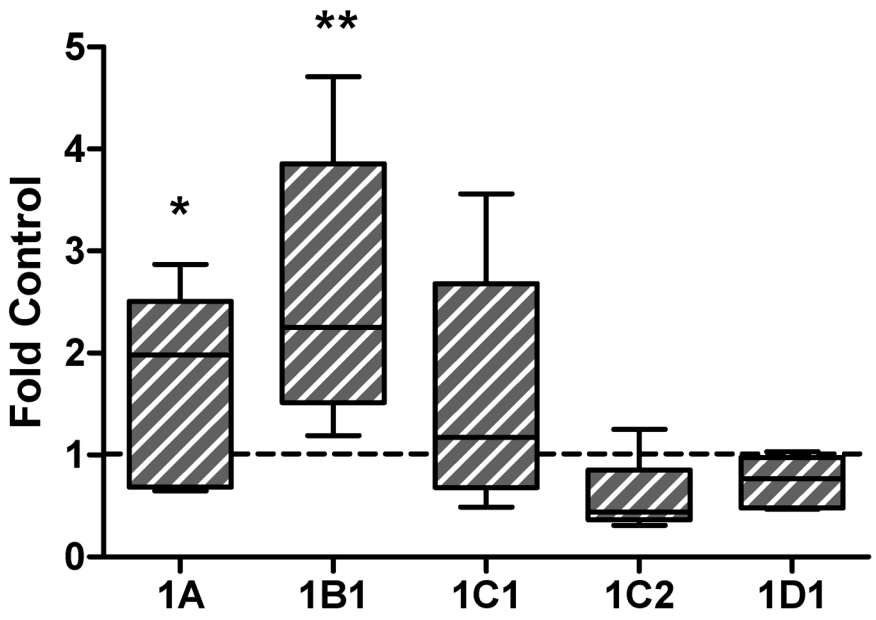

Figure 4.

Levels of *CYP1* gene expression in 8 day old zebrafish larvae after UVB exposure. Larvae were irradiated for 8 hours with 0.93 W/m^2 UVB, resulting in a cumulative dose of 26.8 kJ/m2 UVB. Sampling occurred directly after irradiation and each biological replicate contained eighteen larvae (n=6). Error bars represent one standard deviation. Significant differences from control values were determined by unpaired t-test (* $p < 0.05$, ** $p < 0.01$).

Table 1

Primer sequences used for the quantification of CYP1, SOD, PCNA, ARNT and β-Actin gene expression by real-time PCR.

Real- time PCR primers	Sequence
ZF-CYP1A F′	5′-GCATTACGATACGTTCGATAAGGAC
ZF-CYP1A R′	5′-GCTCCGAATAGGTCATTGACGAT
ZF-CYP1B1 F′	5′-GCTCAGCTGGTCCATTGATACC
ZF-CYP1B1 R′	5′-CATCAGCGACAGCAACACAC
ZF-CYP1C1 F′	5′-AGTGGCACAGTCTACTTTGAGAG
ZF-CYP1C1 R′	5′-TCGTCCATCAGCACTCAG
ZF-CYP1C2 F′	5′-GTGGTGGAGCACAGACTAAG
ZF-CYP1C2 R′	5′-TTCAGTATGAGCCTCAGTCAAAC
ZF-CYP1D1 F′	5′-ATCGTCCAAGAGATAGATAACCAAG
ZF-CYP1D1 R′	5′-TGGTGAATGGCATGTAGGAC
ZF-PCNA F′	5′-GCTGTACGACGAGTCTAAC
ZF-PCNA R′	5′-CTCTTTCACAGGCTGATACTCTAC
ZF-SOD1 F′	5′-CGTCTATTTCAATCAAGAGGGTG
ZF-SOD1 R′	5′-GATGCAGCCGTTTGTGTTGTC
ZF-β-Actin F′	5′-CAACAGAGAGAAGATGACACAGATCA
ZF-β-Actin R′	5′-GTCACACCATCACCAGAGTCCATCAC
ZF-ARNT F′	5′-CACCTTTGGATCACATCTCATTG
ZF-ARNT R′	5′-TCACCCTCCTTAGACGGACC

Table 2

Mortality due to UV irradiation.

Irradiance (W/m^2)	Number of pulses	Duration of pulse (hr)	Total UVB energy (kJ/m^2)	Age at sampling (hpf)	% Mortality after final dose
0	-	-	0	54	0
0.61	2[a]	6	26.8	54	4
0.93	2	3	20.1	51	27
0.93	2	4	26.8	52	36

[a]Mortality was also assessed after the first pulse (e.g. after 13.4 kJ/m^2) and was 2%.