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Ultraviolet-B Exposure Causes DNA Damage and Changes in Protein Expression in Northern Pike (Esox lucius) Posthatched Embryos

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ABSTRACT

The on-going anthropogenically caused ozone depletion and climate change has increased the amount of biologically harmful ultraviolet-B (UV-B) radiation, which is detrimental to fish in embryonal stages. The effects of UV-B radiation on the levels and locations of DNA damage manifested as cyclobutane pyrimidine dimers (CPDs), heat shock protein 70 (HSP70) and p53 protein in newly hatched embryos of pike were examined. Pike larvae were exposed in the laboratory to current and enhanced doses of UV-B radiation. UV-B exposure caused the formation of CPDs in a fluence rate-dependent manner, and the CPDs were found deeper in the tissues with increasing fluence rates. UV-B radiation induced HSP70 in epidermis, and caused plausible p53 activation in the brain and epidermis of some individuals. Also at a fluence rate occurring in the nature, the DNA damage in the brain and eyes of pike and changes in protein expression were followed by severe behavioural disorders, suggesting that neural molecular changes were associated with functional consequences.

INTRODUCTION

Ultraviolet-B (UV-B) radiation has been shown to be detrimental to fish, especially at embryonal stages (reviewed in 1, 2). However, there are large differences between species in sensitivity to UV-B. Post-hatched embryos of pike have been shown to be extremely sensitive to UV-B: even the low UV-B fluence rates and doses that normally occur in Northern spring cause growth retardation, severe behavioural disorders and mortality (3 - 5).

In Fennoscandia, pike spawns in April-May in shallow waters (depth less than 1 m) with vegetation as spawning base. Newly hatched embryos attach to plants and remain nonmotile for the first days of life (6). Freely swimming one-week-old fry are positively phototactic and often swim very near the lake surface thus likely being exposed to UV-B (6, 7).

The primary lesions produced by UV-B in DNA are cyclobutane pyrimidine dimers (CPDs) (8). UV-B irradiation induces CPDs in a fluence rate -dependent manner in fish species (9 - 12). Because the DNA containing CPDs cannot be effectively transcribed or replicated, these lesions either are repaired or lead to cell death by apoptosis (13 - 15). Both of these processes may be energetically costly to the animal, and excess apoptosis may even lead to death of the organism.

Heat shock protein 70 (HSP70) is a class of stress proteins with a molecular weight of 70 – 75 kDa. Cells express some proteins of this family constitutively (heat shock cognates, HSC's) and some are upregulated by a variety of environmental stressors that generate denatured proteins, including UV radiation (16 - 19). HSP70 is believed to have a protective role against

tissue damage as it binds to denatured proteins and attempts to restore their tertiary structure and function (18 - 19). It has other roles as well, and it takes part in many multi-protein complexes inside the cell. It transfers proteins into their right compartment in the cell, tags incorrectly folded proteins for ubiquitination and degradation, participates in endo- and exocytosis and protects cells from apoptosis (20 - 23). In newly hatched pike embryos UV-B, unexpectedly, has been shown to diminish whole body HSP70 (3).

The tumour suppressor protein p53 plays a major role in regulation of various cellular stress responses like hypoxia, hypoglycaemia, and DNA damage. In such conditions p53 becomes active and in turn transcriptionally activates genes involved in cell cycle control, DNA repair, and apoptosis (reviewed in 24). p53 also functions independently of transcription e.g. in apoptosis (25). Also p53 exists in up to 10 different transcripts in the cell, of which, however, the full-length classical p53 is the most important protein responsive to cellular stress (26). Many factors, including the alternative transcripts of the TP53 gene, control the activation of p53, and the downstream response varies depending on the cellular environment or other modifying factors in the cell (27). p53 protein is also involved in embryonic development: it is typically expressed at high levels in developing tissues with a high number of proliferating and differentiating cells (28). The activation of p53 occurs at the posttranslational level through various modifications of the molecule, of which phosphorylation by multiple kinases is the best characterized (29, 30).

The aim of this work was to reveal the effects and modes of action of UV-B in late embryonic stages of pike, at tissue and cellular level. Slot blot analysis of DNA damage manifested as cyclobutane pyrimidine dimers (CPDs) and immunoblots of HSP/HSC70 and p53 proteins

were used to quantify the effects of UV-B on these variables, and immunohistochemistry of the same molecules was performed to define their locations. Because UV-B irradiation of larval pike results in severe behavioural disorders (spiral swimming), followed by high mortality (3, 5), the phenomena behind this effect were of most interest and therefore the examination concentrated mainly on the brain.

MATERIALS AND METHODS

Fish and water characteristics and general living conditions of fish): The fertilized eggs of pike were obtained from Kuhmo (Kuhmon Kala hatchery, Lake Lentua, Finland, 64^{o}22' N, 29^{o}75' E) and Jämsä (Mr. Timo Paajoki, Lake Päijänne, Finland, 61^{o}24 N, 25^{o}24 E). The eggs were incubated and hatched in flow-through hatchery cones in the University of Jyväskylä at 10 ^{o}C. The light-dark cycle was 12 h: 12 h. The conductivity of the water was 24.6 µS/m, alkalinity 0.79 mmol/l, pH 7.6, chemical oxygen demand (COD_{Cr}) < 30 mg/l, Mg 7.9 mg/l and Ca 19 mg/l.

Exposure system and sampling: UV-B radiation exposure was carried out at the laboratory using a fluorescent lamp (UVB-313, Q-Panel, Cleveland, OH, USA). Ultraviolet-C (UV-C; under 290 nm) was blocked with a cellulose diacetate filter (Clarifoil, Derby, UK), which was replaced after each six hours of UV-B radiation. Visible light was used as a control treatment without UV-B (TLD 36 W/950 daylight, Philips, Eindhoven, Netherlands).

UV was quantified using Hamamatsu Photonic Multichannel Spectral analyser (model PMA-11), measuring the wavelength area 280 – 380 nm. The UV-B intensities were measured at the water surface at the beginning and end of each experiment. The daily doses were calculated as

89 unweighted, DNA-weighted and CIE-weighted (Commission Internationale de l'Eclairage;
90 International Commission on Illumination) J/m^2, i.e., the action spectrum specific for human
91 erythema (31, 32). The UV-B fluence rates and total doses are shown in Table 1. The two lowest
92 UV-B fluence rates correspond to natural fluence rates occurring in April-May, at the hatching
93 time of pike (33).

94 <Table 1>

95

96 Newly hatched (< 24 h) embryos were carefully transferred to 1-liter Pyrex glass bowls
97 filled with water from the hatchery, 40 animals in each bowl. Pike from the Lake Päijänne were
98 irradiated with UV-B at four different intensities for 3 hours on one day or for 3 hours on two
99 consecutive days (2 x 3 h). Embryos from Lake Lentua received UV-B radiation at the same
100 intensities for 3 hours on two consecutive days (2 x 3 h). Otherwise, the light-dark cycle of 12 h:
101 12 h was maintained throughout the study. Animals were sampled right after the end of irradiation
102 (fast anesthesia with 100 mg/l MS-222). Ten individuals were fixed in 10% buffered formalin for
103 24 hours, and the rest were frozen in liquid nitrogen and preserved in -80 °C until analysed
104 further.

105 **Analysis of cyclobutane pyrimidine dimers (CPDs):** UVB-radiation-induced DNA damage
106 manifested as CPDs was determined as whole body analysis, pooling five embryos. Fish frozen in
107 liquid nitrogen were homogenized quickly with plastic homogenizers in Eppendorf tubes, 500 µl
108 extraction buffer (50 mM Tris, pH 8.0; 5 mM EDTA; 1% SDS; 0.25 M NaCl; 20 µg / ml
109 proteinase-K) was added, and the tubes were placed in 55 ^{o}C heat block for a minimum of 3 hours.
110 After this, Mo Bio Microbial DNA purification kit was used (Mo Bio, Carlsbad, CA, USA). The

DNA was eluted in TE-buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). The concentration of DNA was analyzed fluorometrically using SYBR Gold dye (Molecular Probes™, Invitrogen, USA).

The CPD standards were prepared as described in (34). Briefly, larval pike genomic DNA was irradiated with UVC lamp emitting at 254 nm (G30W, Sylvania, USA) for 12 s to obtain a dose of 12 J / m^2. Molecular weight of 640 000 per kb of DNA was assumed, and the absolute number of CPDs/kb DNA was determined based on studies by Bohr and coworkers (35).

Prior to blotting, extracted DNA was denatured by adding 0.1 volume 3 M NaOH and heating at 60 °C for 1 hour. Ammonium acetate was added to obtain 1 M final concentration, and samples were vacuum blotted onto 0.45 µm cellulose acetate membranes (Protran, Schleicher & Schuell, Germany) using a 72 well slot blotter (Minifold II, Schleicher & Schuell, Germany). The membrane was baked for 1 h in 80 °C to fix the DNA onto the membrane.

Immunodetection of CPDs was carried out as described earlier (36). The membranes were blocked with 9 % non-fat dry milk in TBS-T (tris-buffered saline - Tween 20), washed with TBS-T and incubated in 1:800 dilution of antibody against CPDs (TDM-2, provided by prof. Osamu Nikaido; 37) for 1 h. After washing with TBS-T the membranes were incubated in 1:3000 peroxidase labelled anti-mouse IgG (A9044, Sigma-Aldrich Chemie, Steinheim, Germany) for 30 min, washed, and the immunodetection was performed via enhanced chemiluminescence. Scion Image 4.0.2 was used for quantification of immunoreactive bands.

Immunoblotting: Immunoblotting for p53 and HSP70 proteins was carried out as previously described (36). Briefly, embryos frozen in liquid nitrogen (5 animals, in total 25 – 40 mg) were homogenized in a glass homogenizer with potassium gluconate buffer. The homogenates were centrifuged at 1000 *g* for 5 min and total protein concentration of the supernatant was determined

by a modified Lowry method adapted for 96-well plates (BioRad DC, Bio Rad Laboratories, Hercules, CA, USA).

The p53 and HSP70 were analyzed by immunoblotting after sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) separation (100 and 20 µg protein / lane, respectively), using a Mini-Protean II apparatus. The positive controls were nuclear protein extract of MCF-7 breast carcinoma cells treated with 5 µM benzo(a)pyrene (1 µg protein/lane, 38) and bovine HSP70 (Sigma H9776, 50 ng protein/lane) for p53 and HSP70, respectively. Proteins were transferred to a nitrocellulose membrane (Protran, Schleicher & Schuell) using Bio Rad Mini-Protean II apparatus. The membrane was stained with 0.2 % Ponceau S in 3 % trichloroacetic acid for 1 min to confirm protein transfer, rinsed twice with Tris-buffered saline-Tween (TBST; 0.9 % NaCl, 10 mmol/L Tris, 0.1 % Tween-20, pH 7.4) and incubated in blocking buffer (9 % non-fat dry milk in TBST) for 14 h at 4 ^{o}C.

The blot was probed with 1:500 anti-p53 (NCL-p53-CM1, Novocastra) or 1:5000 anti-HSP70 (MA3-006, Affinity BioReagents) in blocking buffer for 2 h. These antibodies recognize p53 and HSC70/HSP70, respectively, of various species including fish species, amphibians, and mammals (39; Murphy SP, Fox S, Myers MP, Morimoto RI. Unpublished data, Affinity Bioreagents). After washing (TBST, 1x15 min, 2x5 min), the blot was probed with secondary antibody, 1:10000 peroxidase labeled anti-rabbit IgG (81-6120, Zymed) for p53-stained and 1:3000 peroxidase labeled anti-mouse IgG (A9044, Sigma) for HSP70-stained blots in TBST for 1 hour. After washing (TBST, 1x15 min, 2x5 min) the immunodetection was performed via enhanced chemiluminescence using Star-Glo chemiluminescent substrate (ICN Biomedicals, Irvine, CA, USA). Hyperfilm ECL® high performance chemiluminescence film (Amersham Pharmacia, Uppsala, Sweden) was used for visualization.

The exposed films were scanned and the pictures analyzed using Scion Image 4.0.2. Different blots were made comparable to each other by calibrating them with the positive controls, fixing each positive control at a value of 1.

Immunohistochemistry of CPDs, HSP70 and p53: The samples were dehydrated through a graded series of ethanol solutions up to 100 %, followed by xylene before embedding in paraffin. Animals were sectioned longitudinally along the vertical axis at 5 µm using a Leica microtome. The sections were placed on Superfrost Plus slides to avoid floating of the sections in later incubations. Fresh sections (< 48 h) were always used for the analysis.

Sections were deparaffinized with xylene followed by rehydration with a graded series of ethanol. Antigen unmasking was done by cooking the slides in 10 mM sodium citrate, pH 6.0, in a water-bath in microwave oven (40). Slides were then washed three times in water and thereafter endogenous peroxidase was quenched with 3 % hydrogen peroxide, 1 % sodium azide in phosphate salt buffer. Slides were further washed three times with PBS and blocked with 5 % non-fat dry milk in TBS-Tween. After three washes, the samples were incubated with primary antibody (1:1000 TDM-2 against CPDs, 1:1000 NCL-p53-CM1 against p53 from Novocastra Laboratories Ltd, Newcastle upon Tyne, United Kingdom, or 1:1000 MA3-006 against HSP-70 from Affinity BioReagents, Golden, CO, USA) in +4 ^{o}C overnight. The next day the slides were washed with PBS and incubated with the secondary antibody (1:500 anti-rabbit-HR, Zymed Laboratories, San Fransisco, CA, USA for p53 slides and 1:400 anti-mouse-HRP, A9044, Sigma-Aldrich Chemie, Steinheim, Germany for others) for 30 min at room temperature. After washing, the specimens were stained with 3,3'-diaminobenzidine tetrahydrochloride (DAB), counterstained with Mayer's hematoxylin, dehydrated with a graded series of ethanol solutions up to 100 %, followed by xylene and mounted in DePeX (Electron Microscopy Sciences, USA). Negative

controls consisted of sections in which all the reagents were added with the exception of the primary antibody. Positive control for p53 was Zymed positive control for p53 (09-0025, Zymed, USA), sections of colon cancer tissue with a nuclear staining pattern of p53.

Five to nine slides per exposure per each probe were randomly examined with microscope. Sections were examined for presence of the probe in tissues. For p53, all specimen were first viewed to assess average staining intensity. After that, p53 antibody staining intensity was scored in each individual as 0 = no staining, 1 = diminished staining, 2 = average staining, 3 = strong staining, and the mean of the scores was calculated and designated as mean antibody staining intensity.

Statistics: Probit analysis was used to calculate fluence rates leading to response in 50 % of the animals (effective fluence rate 50 %, EFR_{50}) in immunohistochemical analysis. Difference in the concentration of HSP/HSC70 was tested by the ANOVA and Tukey's test. PASW Statistics 18 was used to perform the statistical analyses.

RESULTS

DNA damage

DNA damage measured as cyclobutane pyrimidine dimers (CPD) from pooled five animals was fluence rate dependent (Fig. 1). In immunohistochemical analysis, CPDs were completely absent from the controls, but their amount increased with increasing fluence rate (Fig. 2, Table 2). Also the depth from the integument where CPDs were found in the tissues depended on the fluence rate; the highest rates (300 and 540 mW/m^2 CIE) caused CPD formation not only in the epidermis but also in the subepidermal tissues such as brain, eye and striated muscle (Fig.

2, Table 2). The EFR_{50} values grew in the order epidermis < muscle < brain < eye, and duration of exposure (3 h vs. 2 x 3 h) did not have a marked effect on them (Table 3). The fluence rate needed to produce CPDs in the epidermis, eye, and muscle was higher for Lake Lentua than Lake Päijänne embryos (Table 2).

<Figure 1>

<Figure 2>

<Table 2>

<Table 3>

HSP70

The immunoblots revealed an increase in HSP/HSC70 levels in pike embryos irradiated for 3 hours with the two lowest UV-B fluence rates (80 and 190 mW/m^2 CIE, Fig. 3), whereas irradiation with two highest fluence rates diminished HSP/HSC70 levels (Fig. 3). The embryos irradiated for 2 x 3 hours were not analysed for HSP/HSC70 by immunoblots.

<Figure 3>

The immunohistochemical staining showed that UV-B induced HSP70 in the epidermis of pike from both populations (Table 4). The immunohistochemical samples showed very dark HSP/HSC70 staining in the controls, especially in the brain, but even a complete loss of staining in the brain with the highest fluence rates (300 and 540 mW/m^2 CIE) in some larvae (Table 4, Fig. 4). However, there were large individual differences.

<Table 4>

<Figure 4>

p53 protein

Immunoblots of p53 protein showed very faint staining signal at around 40 kDa in all embryos. It was not possible to quantify the amount due to the weak signal. The amount of protein per lane (100 µg) should have been enough for the signal detection, as the HSP70 antibody gave a signal with a substantially smaller amount of protein (20 µg / lane). The positive control lanes showed only one band at 53 kD (the size of human p53), proving that the antibody worked. Immunohistochemical sections showed very slight overall cytoplasmic staining in all animals (Fig. 5a). In some irradiated embryos an additional deep staining in the epidermis or uppermost part of the brain could be seen (Figs. 5b and c, “strongly stained areas” in Table 5). However, there were strongly stained areas also in two control embryos (Table 5).

In pike irradiated for 2 x 3 hours, there seemed to be a shift towards stronger p53 staining in post-hatched embryos irradiated with the two highest UV-B fluence rates, but this was not statistically significant (Table 5). In pike irradiated for 3 hours, there was more staining in the nucleus with increasing fluence rate (Table 5).

< Figure 5>

< Table 5 >

DISCUSSION

DNA damage

UV-B radiation caused DNA damage (CPDs) in pike in a fluence rate –dependent manner, as reported in the literature for other fishes (8 - 12). This is the first study locating CPDs in boreal fish. Interestingly, the two highest fluence rates cause damage that reaches not only epidermal cells but also tissues underneath: brain, muscle tissue, and eyes. These fluence rates cause severe behavioural disorders that can be seen already 24 hours after irradiation in pike embryos (3, 5, 41). These behavioural changes are followed by significant or even total mortality (5, 41). It can be assumed that there was severe damage in multiple tissues, resulting in behavioural disorders and ultimately death. The behavioural changes could result from damage to the brain, or they could be associated with eye damage, or damage to the striated muscle.

It has been shown before that with similar fluence rates as the ones in this study reciprocity does not hold in post-hatched embryos of pike; that is, the UV-B-induced mortality is not only dose-dependent but also fluence rate plays a role (5). The findings of this study further emphasize the importance of the fluence rate: The target tissue of UV-B-induced DNA damage clearly changes with the fluence rate, and the EFR_{50} values are not markedly different between animals irradiated at the same fluence rates for 3 h and 2 x 3 h. Regarding to environmental realism, this again shows how important it is to use relevant fluence rates when assessing the UV-B-associated risk to fishes.

The two highest fluence rates in this study were higher than those occurring in the nature nowadays or predicted for the future. However, even the two lowest fluence rates that were environmentally relevant, caused DNA damage in brain, eyes, and striated muscle of Lake Päijänne larvae. However, the spectrum of UVR and especially the amount of UVA and visible light in the current experiments differed from the natural spectrum, and thus, based on this experiment, no definite conclusions can be made about the risk UVR poses to pike embryos in the nature.

There were differences between the populations in CPD formation. Higher fluence rates were needed to produce CPDs in all tissues in pike from Lake Lentua than in pike from Lake Päijänne. This is in accordance with our earlier finding that the pike embryos from Lake Lentua are more tolerant to UV-B than those from Lake Päijänne (41). Lake Lentua animals may possess more UV-screening pigments or have more efficient repair of UV-induced DNA-damage than those from Lake Päijänne. There are differences between fish species and populations both in pigments sheltering from UV-B and in efficacy of DNA-damage repair systems (11, 42 - 44).

Changes in protein expression

Immunohistochemical staining revealed that UV-B increased the amount of HSP/HSC70 in the epidermis at all fluence rates in pike embryos irradiated for 3 hours on two consecutive days. HSP/HSC70 induction could be seen in immunoblots in larvae irradiated for 3 hours at the two lowest fluence rates. This finding is not contradictory to the earlier findings where UV-B diminished HSP/HSC70 levels (3), because these two lowest fluence rates were lower than those used before.

The immunoblots revealed decreased levels of HSP/HSC70 in pike treated with the two highest fluence rates of UV-B. This decrease is probably due to cell damage, and related to protein degradation or ceasing of protein synthesis. High sublethal doses of toxic compounds evoke a similar response in fish: stress protein synthesis slows down or ceases at the mRNA level, while the synthesis of house-keeping proteins is induced (45). In immunohistochemical examination, diminished HSP/HSC70 staining could be seen in some individuals irradiated with the two highest fluence rates. As the samples in the immunoblots were pooled from five animals, it is a possibility that the decrease in HSP/HSC70 staining may be caused by a decrease in only some of the fish in the sample.

Both constitutive (HSC70) and inducible (HSP70) heat shock proteins are continuously expressed in brains of rabbit, rat and western painted turtle (46 - 49). In mammalian nerve cells, HSC70 has a role in regulating neurotransmitter endo- and exocytosis, and HSPs protect nerve cells from damage induced by ischemia and neurogenerative diseases (21, 22, 50, 51). The diminishing and even vanishing of HSP/HSC70 staining after irradiation with UV-B in some animals was followed by severe behavioural disorders (spiral swimming) in our study. This may suggest an important role for HSP/HSC70 also in the brain of post-hatched embryos of pike.

HSP70 may work to protect cells from apoptosis - the level of inducible HSP70 has been shown to be lower in apoptotic than non-apoptotic cells (20, 52). In larval pike, the possible apoptosis of cells damaged by UV-B is an issue of further investigation, but the low level of HSP/HSC70 in brain cells in some individuals suggests that these cells may be more prone to die by apoptosis than cells containing more HSP/HSC70.

In cod embryos UV-B irradiation increases the amount of p53 (10). We could not detect any clear induction in pike embryos by UV-B by immunoblotting. The rather faint signal, and very small differences between irradiated and unirradiated larvae in the amount of p53 in immunoblotting may rise from the fact that whole body analysis of embryos was used. Post-hatched embryos of pike still possess much yolk, the protein of which may have diluted the signal. On the other hand, more nuclear p53 staining was detected in the UV-B-irradiated pike than controls in the 3 h exposure group. This, and the strongly stained regions in the brain and epidermis of some animals, could point to an activation of p53, but as the immunoblotting results did not show a similar trend, this cannot be verified.

Previously, ecologically realistic intensities of UV radiation have been shown to cause epidermal, eye and brain damage in fishes (11, 53, 54). Our results suggest DNA damage in epidermis, eye and brain, and changes in protein expression in pike embryos. These events most probably lie behind the behavioural disorders and mortality (3, 5, 41). The possible role of apoptosis remains to be investigated in the future.

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459

460 TABLES

461 Table 1. The experimental design showing the UV-B fluence rates and the total doses in the
462 experimental treatments. Newly hatched pike embryos were exposed to UV in Pyrex glass
463 bowls, 40 animals in each bowl. Embryos from Lake Päijänne were irradiated once at four
464 fluence rates for 3 hours or for 3 hours on two consecutive days (2 x 3 h), and embryos from
465 Lake Lentua for 2 x 3 hours.

Treatment		Fluence rate, mW/m^2	Dose for 3 h, kJ/m^2	Dose for 2 x 3 h, kJ/m^2
0	control	0	0	0
1	unweighted	240	2.6	5.2
	CIE	**80**	**0.8**	**1.7**
	DNA	220	2.4	4.7
2	unweighted	640	6.9	13.8
	CIE	**190**	**2.0**	**4.1**
	DNA	430	4.7	9.3
3	unweighted	940	10.2	20.3
	CIE	**300**	**3.3**	**6.5**
	DNA	660	7.1	14.2
4	unweighted	1700	18.4	36.7
	CIE	**540**	**5.9**	**11.7**
	DNA	1080	11.7	23.3

466

467 Table 2. Cyclobutane pyrimidine dimer (CPD) immunohistochemical staining in Lake
468 Päijänne pike embryos irradiated for 3 h (3 h) and Lake Päijänne (P) and Lake Lentua (L) pike
469 embryos irradiated for 2 x 3 h. Numbers show positive staining / all samples examined.

UVB fluence rate (mW/m^2 CIE)	epidermis			brain			eye			muscle		
	3 h	P	L	3 h	P	L	3 h	P	L	3 h	P	L
0	0/5	0/7	0/5	0/5	0/7	0/4	0/5	0/6	0/2	0/4	0/6	0/5
80	2/5	5/7	0/5	0/5	0/6	0/5	0/5	0/5	0/3	1/4	1/5	0/5
190	4/5	5/7	4/5	1/4	0/7	0/5	1/3	2/5	0/3	4/5	2/5	0/5
300	3/5	3/5	5/5	2/5	1/5	2/5	1/2	3/4	1/4	3/4	2/3	2/5
540	5/5	5/5	4/5	3/5	4/4	3/5	0/3	3/4	1/4	2/3	3/3	2/3

Table 3. Effective fluence rate at which 50% of animals show CPD staining (EFR_{50}) for different tissues. P 3 h = embryos from Lake Päijänne irradiated for 3 hours, P 2x3 h = embryos from Lake Päijänne irradiated for 3 hours on two consecutive days, and L 2x3 h = embryos from Lake Lentua irradiated for 3 hours on two consecutive days.

	EFR_{50} epidermis (mW/m^2 CIE)	EFR_{50} brain (mW/m^2 CIE)	EFR_{50} eye (mW/m^2 CIE)	EFR_{50} muscle (mW/m^2 CIE)
P 3 h	169	398	578	206
P 2x3 h	132	416	342	250
L 2x3 h	207	449	649	439

Table 4. HSP70 immunohistochemical staining in Lake Päijänne pike embryos irradiated for 3 h (3 h), and Lake Päijänne (P) and Lake Lentua (L) pike embryos irradiated for 2 x 3 h. Numbers show positive staining / all samples examined.

UVB fluence rate (mW/m^2 CIE)	epidermis	brain

	3 h	P	L	3 h	P	L
0	0/5	0/9	1/5	3/3	6/8	4/5
80	1/5	3/8	5/5	3/4	5/7	4/4
190	0/6	3/9	4/5	5/5	7/8	4/4
300	1/6	2/4	5/5	3/5	4/4	5/5
540	1/6	4/4	3/5	3/6	3/3	3/5

479

480 Table 5. p53 immunohistochemical staining in Lake Päijänne embryos irradiated for 3 h (3 h)
481 and in Lake Päijänne (P) and Lake Lentua (L) pike embryos irradiated for 2 x 3 h. Numbers
482 show positive staining / all samples examined, except mean ab staining intensity = mean
483 antibody staining intensity; see materials and method for description. Strongly stained areas,
484 see Fig. 5.

UVB fluence rate (mW/m^2 CIE)	nucleus			mean ab staining intensity			strongly stained areas		
	3 h	P	L	3 h	P	L	3 h	P	L
0	0/6	3/9	0/5	1.2	1.1	1	0/6	1/9	1/5
80	1/6	2/9	2/5	1	1.1	1.6	0/6	1/9	0/5
190	1/6	2/9	0/5	1	1.1	1.4	1/6	0/9	2/5
300	2/5	3/5	2/5	1.2	1.8	1.8	0/5	0/5	3/5
540	2/5	3/5	1/5	1.2	2.0	2.0	1/5	2/5	2/5

485

486 FIGURE CAPTIONS

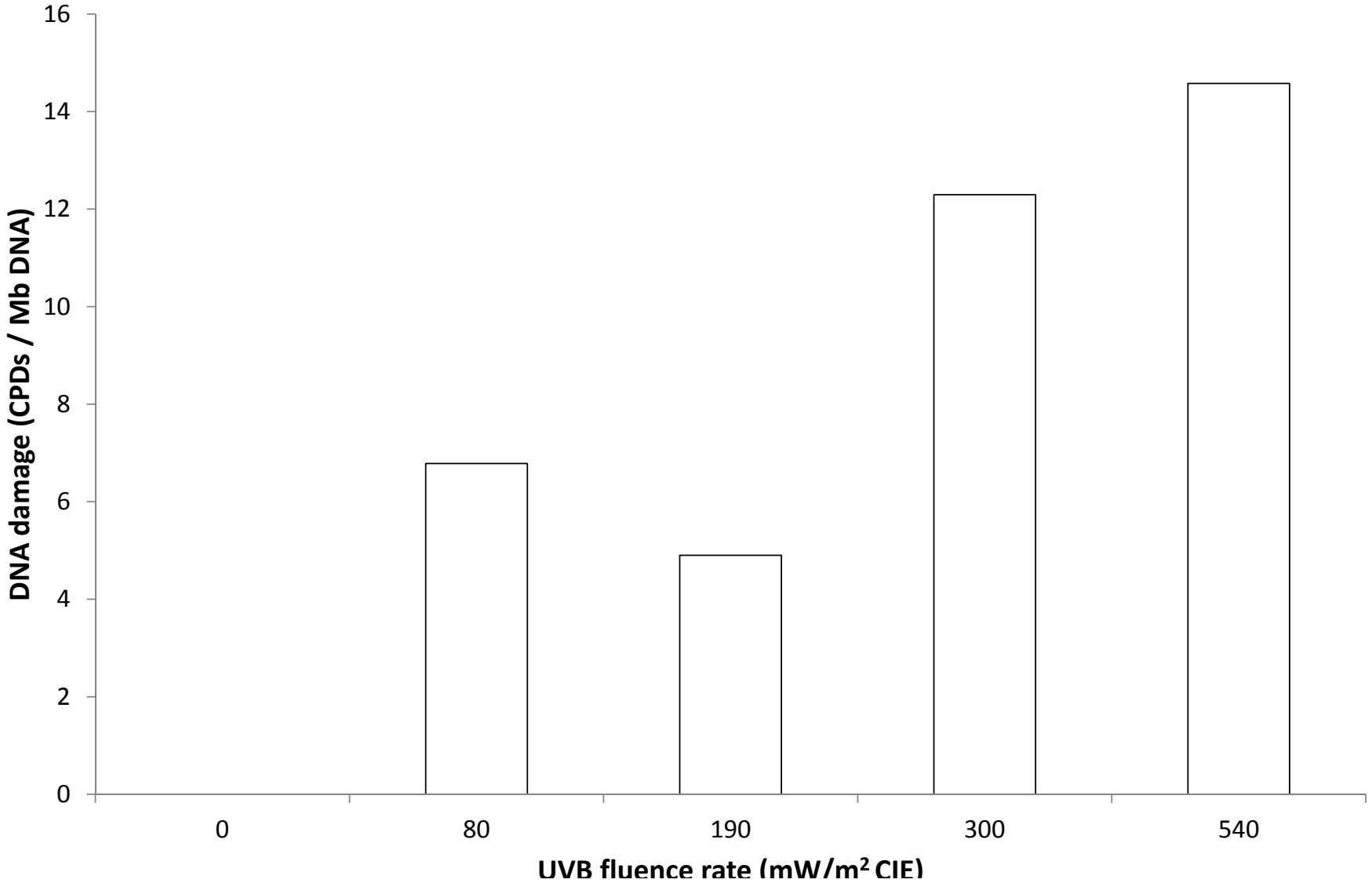
Figure 1. UV-specific DNA damage manifested as cyclobutane pyrimidine dimers (CPDs). Pike embryos form Lake Lentua irradiated for 3 hours on two consecutive days, n=5 animals in one sample analyzed.

Figure 2. Immunohistochemical analysis of CPDs in pike embryos. a) control brain, b) brain of an embryo irradiated with 2.0 kJ/m^2 CIE (3h, 190 mW/ m^2) c) brain of an embryo irradiated with 5.9 kJ/m^2 CIE (3 h, 540 mW/ m^2). Arrows point to CPDs in the epidermis and brain. E = epidermis, ME = mesencephalon, MO= medulla oblongata, SM = striated muscle.

Figure 3. Amount of HSP70 in pike embryos irradiated with UV-B for 3 hours. Fluence rates as in Fig.1. Data shows mean of 3 - 8 analyses, each pooled of 5 animals, ±SD. Letters denote groups not differing from each other significantly ($P > 0.05$, Tukey).

Figure 4. Immunohistochemical analysis of HSP70 in pike embryos. a) control head, b) head of embryo irradiated with 6.5 kJ/m^2 CIE (2x3 h, 300 mW/ m^2), c) head of an embryo irradiated with 11.7 kJ/m^2 CIE (2x3 h, 540 mW/ m^2), d) brain of an embryo irradiated with 5.9 kJ/m^2 CIE (3h, 540 mW/ m^2). CV = cerebral ventricle, E = epidermis, ME = mesencephalon.

Figure 5. Immunohistochemical analysis of p53 in pike embryos. a) Diffuse cytoplasmic staining in brain seen in all animals. b) “Strongly staining region” in an embryo irradiated with 190 mW/m^2 CIE for 2x3 hours. c) “Strongly staining region” in the epidermis and mesencephalon of an embryo irradiated with 300 mW/m^2 CIE for 2x3 hours. CV = cerebral ventricle, E = epidermis, ME = mesencephalon.


16
14
12
10
8
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DNA damage (CPDs / Mb DNA)
0
80
190
300
540
UVB fluence rate (mW/m² CIE)

Figure 1.

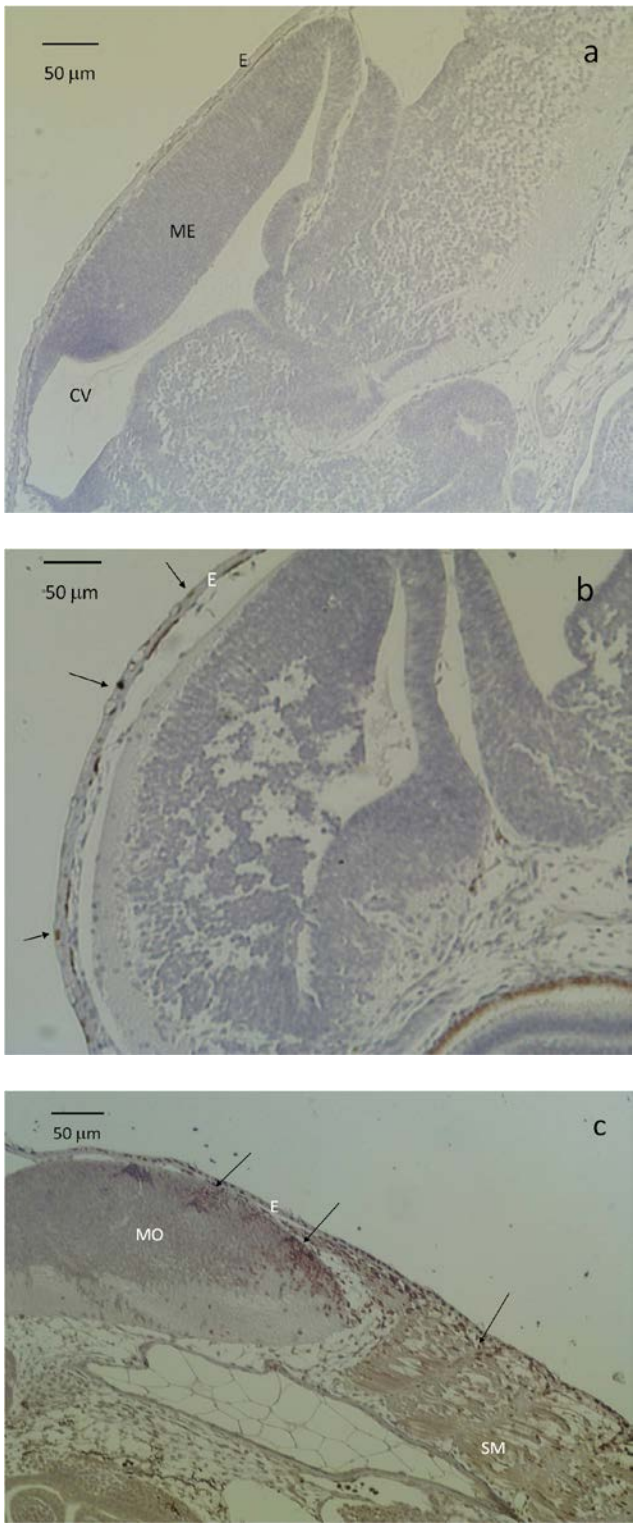


Figure 2.

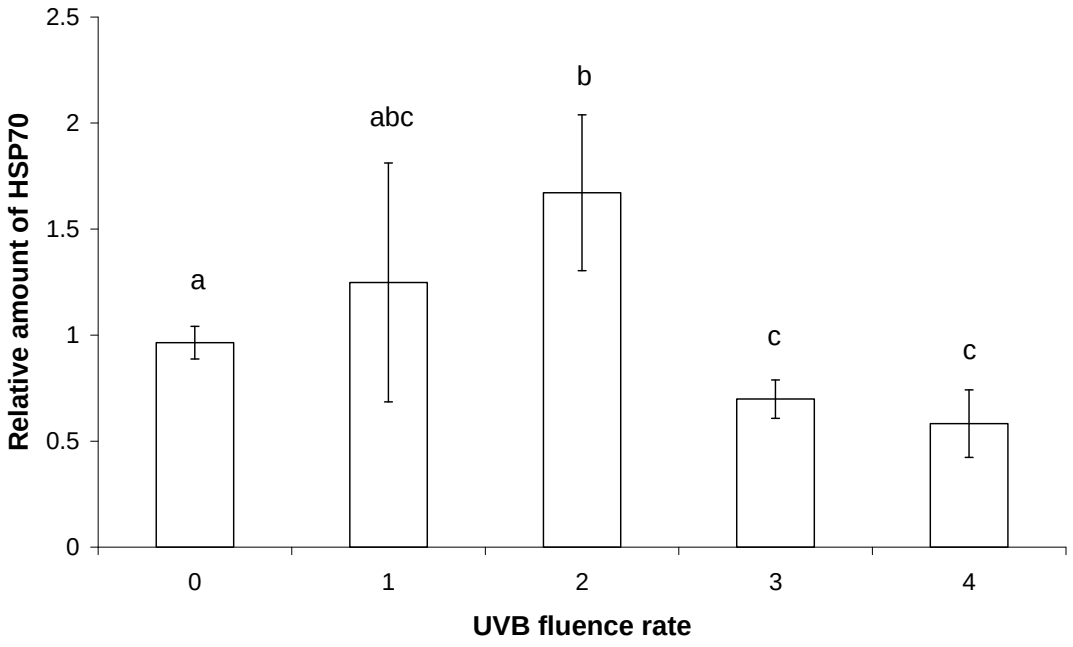

2.5
2
1.5
1
0.5
0
Relative amount of HSP70
a
abc
b
c
c
0
1
2
3
4
UVB fluence rate

Figure 3.

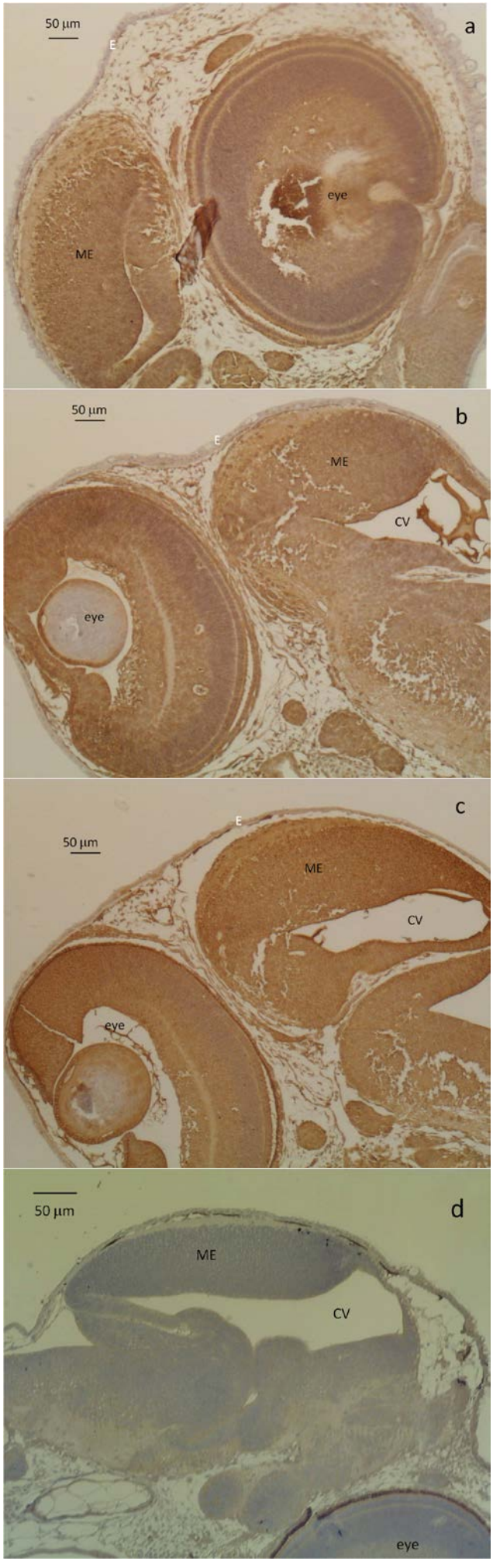
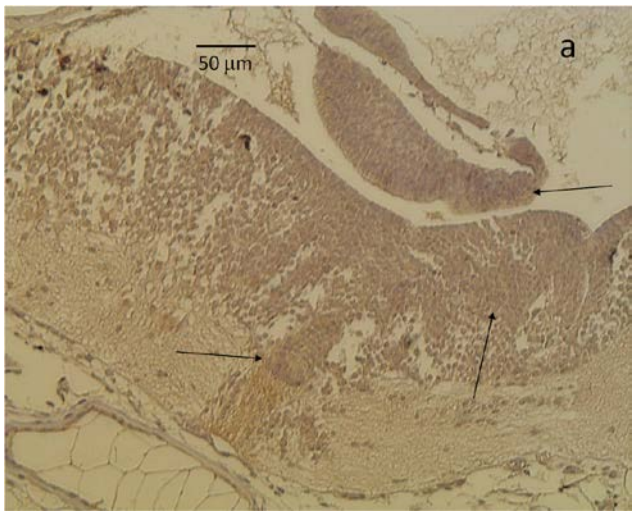
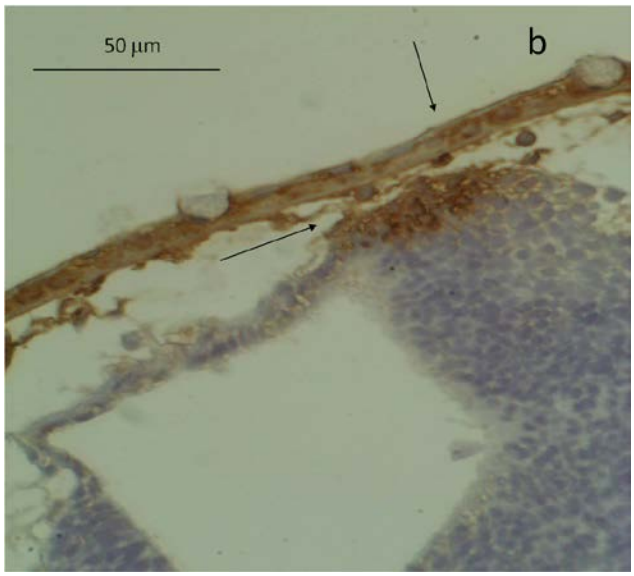


Figure 4.





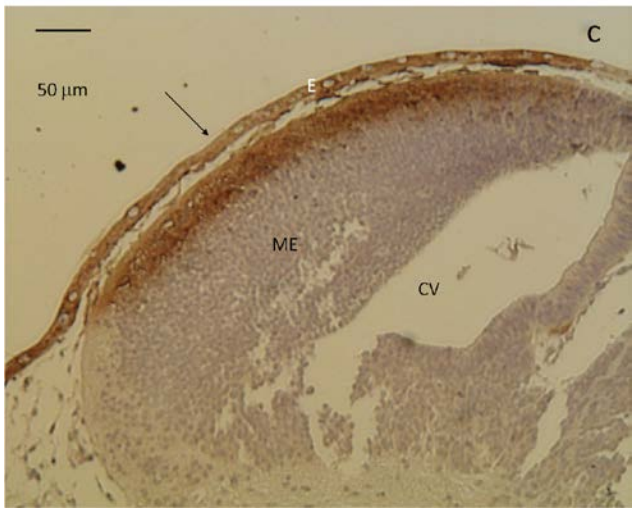


Figure 5.