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The Effects of Ultraviolet-B Radiation on Antioxidant Status and Survival in the Zebrafish

By

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Abstract

Increased exposure to solar radiation in the UV-B range (280-320 nm) is occurring globally at least partly due to anthropogenic degradation of stratospheric ozone. Evidence of UV-B radiation eliciting oxidative stress in mammalian systems coupled with the fact that UV-B readily penetrates the air-water interface led to this study. The direct impact of present ambient (1.95 W/m^2) and sub-ambient doses of UV-B radiation on muscle and skin tissue antioxidants in mature zebrafish (*Brachydanio rerio*) and on their hatching success and survival in early life stages was measured. Exposure of mature fish to sub-ambient doses of UV-B radiation both in the presence (1.28 W/m^2) and absence (1.72 W/m^2) of a cellulose acetate filter (blocks all wavelengths below 290 nm) resulted in a significant depression in muscle/skin total glutathione (TGSH) levels compared with controls and low UV-B (0.15 and 0.19 W/m^2 , respectively) treated fish after 6 and 12 h cumulative exposure, respectively. Exposure to an ambient intensity of UV-B resulted in a significant decrease in muscle/skin glutathione peroxidase (GPx) activity compared with controls and low UV-B (0.15 W/m^2) treated fish after a 6 h exposure. Activities recovered to near control levels after a 12 h cumulative exposure period. Glutathione reductase (GR) activity exhibited no significant change in activity over a 24 h cumulative exposure period. A peak in superoxide dismutase (SOD) activity was observed after a 6 h exposure period. These activities also fell to control levels after 12 h cumulative exposure. At this time a coincident significant increase in catalase activity compared with controls and low UV-B treated fish was also observed. After 24 h cumulative exposure, however, catalase activities fell to levels similar to that for low UV-B treated fish, these were significantly

higher than controls after 24 h cumulative exposure. Changes in SOD, catalase, GPx and GSH status suggests a UV-B-mediated increase in cytosolic superoxide anion radicals (O_2^-) and hydrogen peroxide (H_2O_2). Elevated muscle/skin thiobarbituric acid reactive substances (TBARS) indicate that despite fluctuations in protective antioxidants, an ambient dose of UV-B is able to elicit oxidative stress in exposed fish. Newly fertilized eggs exposed to ambient UV-B for the duration of the hatching period exhibited 1.68% hatching success, a 60- and 50-fold decrease compared with controls and eggs exposed to one third of the ambient dose, respectively. Newly hatched larvae were observed to be more sensitive to UV-B than the adults. When exposed to an ambient dose of UV-B, larvae experienced 100% mortality after a 12 h cumulative exposure period. Adults survived a minimum of 18-24 h cumulative ambient UV-B exposure.

Résumé

La dégradation de la couche d'ozone stratosphérique causé par les activités humaines a mené à une augmentation du rayonnement ultraviolet-B (280-320 nm). Des recherches antécédentes ont démontrés que le rayonnement ultraviolet-B (UV-B) peut induire un stress oxydatif chez la mammifères. Il a aussi été démontré que le rayonnement UV-B pénètre dans l'eau pouvant ainsi affecté les organismes vivant qui s'y trouvent. La présent étude vise premièrement à déterminer si une irradiation UV-B ambiante (1.95 W/m^2) ou plus faible peuvent induire des changements dans les mécanismes de protection antioxydants de la peau et des muscles chez le poisson zébré et deuxièmement, si ces traitements peuvent réduire le succès d'éclosion et la survie des juvéniles et des adultes. Comparés au groupe contrôle qui était exposé à un rayonnement UV-B bas ($0.15\text{-}0.19 \text{ W/m}^2$), une diminution de la concentration de glutathion totale (TGSH) de la peau et des muscles a été observé chez des poissons adultes exposés à un rayonnement UV-B de 1.28 W/m^2 , qui est inférieure au rayonnement environnemental naturel, pour une durée de 6 et de 12h en présence ou en absence d'un filtre de cellulose acétate (bloque l'UV-B inférieure à 290 nm). Comparé au groupe contrôle exposé à un rayonnement UV-B bas, l'activité du peroxydase glutathion (GPx) et du superoxide-dismutase (SOD) de la peau et des muscles était significativement réduite chez des adultes exposés pendant 6 h à un rayonnement UV-B de 1.95 W/m^2 . Après 12 h de traitement l'activité de ces enzyme a diminué pour atteindre un niveau comparable à celui du groupe contrôle. Par contre, l'activité de la catalase a augmenté de façon significative durant cette même période. Ces traitements n'ont eu aucun effets sur l'activité du glutathion reductase (GR). L'activité de

la catalase a diminué à un niveau semblable à celui des contrôles après une exposition continuel au rayonnement UV-B pour une durée de 24 h. Les changements d'activités des enzymes SOD, catalase, GPx ainsi que la réduction dans la concentration de GSH suggèrent que le rayonnement UV-B cause un stress oxydatif semblable à celui associé à des anions superoxydes (O_2^-) et au peroxyde d'hydrogène (H_2O_2). Le rayonnement UV-B a aussi causé une augmentation significative de *thiobarbituric acid reactive substances* (TBARS), ce qui suggère que l'épuisement des mécanismes antioxydants peut mené à un stress oxydatif chez ces poissons. Comparé au contrôle, le succès d'éclosion des oeufs exposés à un rayonnement UV-B de 1.95 W/m^2 et de 1.28 W/m^2 a respectivement diminué de 98% et 75%. Tout les juvéniles exposés à un rayonnement de 1.95 W/m^2 sont mort après 12 h de traitement. Les adultes ont survie à ce traitement au moins 18-24 h.

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List of Abbreviations

BHT	butylated hydroxytoluene
CFC	chlorofluorocarbon
Cu,Zn-SOD	cuprozinc SOD
DOC	dissolved organic carbon
DOM	dissolved organic matter
DNA	deoxyribonucleic acid
DTNB	5,5'-dithiobis-2-nitrobenzoic acid
EDTA	ethylenediaminetetraacetic acid
GPx	glutathione peroxidase
GR	glutathione reductase
GSH	reduced glutathione
GSSG	oxidized glutathione or glutathione disulfide
GST	glutathione S-transferase
H ₂ O ₂	hydrogen peroxide
KMnO ₄	potassium permanganate
MDA	malondialdehyde
MnCl ₂ ·4H ₂ O	manganese (II) chloride-tetrahydrate
Mn-SOD	mangano SOD
NADH	reduced nicotinamide adenine dinucleotide
NADPH	reduced nicotinamide adenine dinucleotide phosphate
O ₃	ozone
O ₂ ⁻	superoxide anion radical
¹ O ₂	singlet oxygen
OH [•]	hydroxyl radical
OH ₂ [•]	hydroperoxyl radical
PHGPx	phospholipid hydroperoxide glutathione peroxidase
ROS	reactive oxygen species
SOD	superoxide dismutase
TBA	thiobarbituric acid
TBARS	thiobarbituric acid reactive substances
TCDD	2,3,7,8-tetrachlorodibenzo- <i>p</i> -dioxin
TGSH	total glutathione
UV-A(B,C)	ultraviolet-A(B,C) radiation
UVR	ultraviolet radiation

1. Introduction

Recent evidence of stratospheric ozone (O_3) depletion has led to increased concern over the exposure of terrestrial and aquatic organisms to increased UV-B radiation. Ozone depletion has and is occurring as a result of the extensive release of man-made ozone depleting chemicals including chlorofluorocarbons, carbon tetrachloride, methyl bromide, methyl chloroform and various halons into the atmosphere. In the case of chlorofluorocarbons (CFCs), a chlorine atom breaks away from the CFC molecule when struck by UV-B and becomes available to react with ozone (O_3). Once in contact with an ozone molecule, the chlorine atom removes an oxygen atom forming chlorine oxide and molecular oxygen (O_2). Should the chlorine oxide molecule come in contact with another oxygen atom, O_2 is formed leaving the chlorine atom free to repeat the process. Despite international efforts to reduce the emission of these ozone depleting chemicals, because they have a lifetime of 20-100 years in the atmosphere, they will continue to remove large amounts of protective ozone from the stratosphere for many years to come. The most dramatic loss of ozone is observed over Antarctica during the austral spring, between September and November, where reductions of up to 50% are reported (Frederick and Snell, 1988). The reason is the formation of an oval-shaped polar vortex over the South Pole during the Antarctic winter. This polar vortex draws ozone rich air from above while pushing ozone poor air out away from the South pole region. With temperatures reaching -80°C in the vortex, clouds are formed which are capable of absorbing nitrogen oxide and storing it as nitric acid crystals. When solar radiation passes through these cloud

formations, ozone-depleting chlorine and chlorine oxide are formed from inactive chlorine and nitric acid. This process contributes to up to 70% of the ozone depletion over Antarctica resulting in a dramatic increase in ultraviolet radiation (UVR) penetration. In the northern hemisphere, ozone depletion is greatest during the late winter and early spring (Environment Canada, 1993). In the past, springtime losses of stratospheric ozone in the arctic have been reported between 4-10% (Environment Canada, 1993). On a regional basis, in 1993 the levels of ozone over Canada averaged 14% lower than the levels reported before 1980 during the months of January to April (Environment Canada, 1993). As a result, terrestrial organisms are at increased risk of UV-B-mediated effects including DNA damage (Davies *et al.*, 1994), melanogenesis, skin erythema, non-melanoma skin cancer (Urbach *et al.*, 1989), immunosuppression (Uřich *et al.*, 1995) and oxidative stress (Cunningham *et al.*, 1985; Pence and Naylor, 1990; Babu *et al.*, 1995; Stewart *et al.*, 1996).

1.1. Physical Properties of Ultraviolet Radiation

Ultraviolet radiation (UVR) is electromagnetic radiation that is invisible to the naked eye and cannot be felt on the skin during exposure. UVR exists in the 200-400 nm range between the shorter wavelength X-rays and the longer wavelength visible light. UVR is categorized by wavelength into three types: UV-A (320-400 nm), UV-B (280-320 nm) and UV-C (200-280 nm). The development of action spectra in the literature indicates that as wavelength decreases, energy increases (Shao *et al.*, 1993). Thus, UV-C is the most harmful followed by UV-B, and UV-A being the least harmful of the three. The

energy spectrum of incident sunlight at the earth's surface has a peak irradiance above 400 nm, with the intensity sharply falling off at the ultraviolet wavelengths to a cut off wavelength at approximately 290 nm (Davies, 1994); the cut off wavelength depends on local weather conditions as well as altitude, latitude, and ozone concentration.

Stratospheric ozone shields the earth from the most biologically injurious components of solar radiation including all of the UV-C wavelengths and UV-B wavelengths less than 290 nm. Absorption of UV-B radiation is exponential with respect to ozone concentration (Worrest *et al.*, 1976); for every 1% decrease in ozone, a 2% increase in UV-B penetration results (Scotto *et al.*, 1988). Ozone is relatively transparent to UV-A, therefore, UV-A penetration remains constant with respect to ozone concentration (Smith *et al.*, 1992; Madronich *et al.*, 1995). In addition to stratospheric ozone, UV-B penetration is also dependent on geographical and seasonal variations including earth-sun geometric factors, cloud cover, local and regional air pollutants and surface elevation reflectivity (Environment Canada, 1993; Madronich *et al.*, 1995). Between 1979 and 1992, anthropogenic declines in stratospheric ozone have resulted in increases in UV-B penetration of up to 10-20% in north-temperate regions; more dramatic increases have been reported in south-temperate regions, especially in Antarctica (Madronich, 1994).

1.2. Penetration of Ultraviolet Radiation through the Water Column

Quite evidently terrestrial organisms are at a higher risk to increased UV exposure in the face of ozone depletion. Not so obvious is the fact that aquatic organisms are also

subject to ozone-dependent increases in UV-B radiation. That is, UV-B readily penetrates the air-water interface (Frederick *et al.*, 1989). The depth of penetration of detectable UV-B, however, varies from a few centimeters in highly colored humic waters to approximately 70 meters in clear ocean waters, a depth well below that inhabited by the majority of aquatic organisms (Smith *et al.*, 1992; Kirk, 1994; Scully and Lean, 1994).

1.2.1. Dissolved organic carbon.

The principal compounds that absorb UV-B in freshwater lakes are dissolved organic carbons (DOC) (Scully and Lean, 1994; Madronich *et al.*, 1995); the absorption of UVR by DOC is due to its chemical structure and possession of UV-absorbing chromophores (Lean, 1998). DOC in most freshwater lakes contributes 40-45% of the dissolved organic matter (DOM). As a consequence of UVR absorption, DOC becomes faded, removing its ability to attenuate UVR (Miller, 1994; Zepp *et al.*, 1995). This sunlight-induced photobleaching results in greater bleaching of the UV absorbing portion of the DOM spectrum compared to the visible portion (Kouassi and Zika, 1990; Miller and Zepp, 1995) and allows deeper penetration into marine and freshwater systems. Another consequence of UV-B absorption by DOC is the liberation of a variety of transient photochemical species. These transient species include excited triplet state DOM, organic radical cations, hydrated electrons, superoxide, singlet oxygen, hydroxyl radicals and peroxy radicals with half-lives of μsec or less (Cooper, 1989; Lean, 1998). Hydrogen peroxide (H_2O_2) is a longer lived photoproduct and can build to trace concentrations over time. Reported concentrations in freshwater systems are much higher than in marine systems with levels reaching $1 \mu\text{M}$ during midday (Lean, 1998). The degradation of H_2O_2

in lakewater is believed to occur principally through biological pathways; however, in lakes with low pH and high iron, H_2O_2 may be degraded to the hydroxyl radical through the Fenton reaction (see 1.4.1.3). In general, absorption of UV-B by DOC decreases with increasing wavelength (Cooper, 1989). In parallel, as the concentration of DOC increases, absorption of UV-B also increases. DOC is reported to be more abundant in freshwater systems than in marine environments, and as a result UV-B is expected to be attenuated more rapidly in lakes than oceans (Kirk, 1994). In the clearest ocean waters, 1% UV-B attenuation depths (the depth at which 1% of surface UV-B irradiance is measured) approach 40 meters (Smith and Baker, 1979). In coastal marine systems DOC levels are higher and 1% UV-B attenuation depths are often less than 20 meters (Kirk, 1994). Therefore, it can be hypothesized that ozone-mediated increases in UV-B penetration are more important in oceans and lakes with lower levels of DOC than in marine estuaries and lakes which have higher DOC levels.

1.3. Effects of Ultraviolet Radiation in Aquatic Species

Aquatic species such as phytoplankton, that are restricted to near surface waters in order to capture sunlight, reportedly have suffered disruptions in DNA integrity, impairment of photosynthesis and nitrogen uptake, bleaching of cellular pigments and inhibition of motility and orientation (Döhler *et al.*, 1991; Häder and Worrest, 1991). Motile marine phytoplankton can ameliorate the effects of UV damage with vertical migrations in the water column (avoidance). Additionally phytoplankton can also partially escape these effects through biosynthesis or uptake of photoprotective compounds,

enzymatic repair of damaged DNA, and nocturnal cell division (Paanakker and Hallegraeff, 1978; Bidigare, 1989). Unlike phytoplankton, zooplankton do not rely directly on solar energy. Furthermore, with the presence of photoreceptors and swimming appendages, they possess a greater range of responses to minimize UV-B exposure in surface waters (Siebeck *et al.*, 1994). Because zooplankton depend on phytoplankton as a food source, population declines may be attributed either to direct exposure to increased UV-B when feeding on phytoplankton at the surface or indirectly through increases in UV-B-mediated phytoplankton mortality.

In fish, the most sensitive tissues to UVR exposure are the dermal and epidermal tissue layers. Damage inflicted on dermal tissue by UV-B can be repaired. However, if damage is severe, programmed cell death (apoptosis) may occur resulting in a loss of dermal and epidermal tissue (Siebeck *et al.*, 1994). Eggs and larvae are especially susceptible due to inhabiting shallow waters, reduced mobility, a lack of scales or other integumental structures for protection, and the fact that sensitive processes, including organogenesis, are occurring (Hunter *et al.*, 1979). Fish eggs and larvae have been observed to show a wide range of differences in species-specific mortality rates and developmental abnormalities upon UV-B exposure. The incidence of eye and brain lesions in anchovy larvae were significantly higher when compared to mackerel larvae (Hunter *et al.*, 1979). In fact, at doses near their LD₅₀, the incidence of eye lesions was approximately 3 times higher and brain lesions 5 times higher in anchovy larvae than in mackerel larvae. Further, larvae exposed to UV-B exhibited smaller body lengths and depths, increased yolk diameter and less eye pigmentation than unexposed larvae (Hunter *et al.*, 1979). In

relation to ozone depletion, the incidence of lesions and retardation of growth in anchovy larvae could occur at doses expected to occur at the water surface in June as a result of a 25% decrease in ozone thickness; 50% mortality could be expected with decreases in ozone thickness of 25 and 50%.

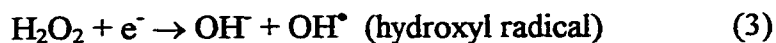
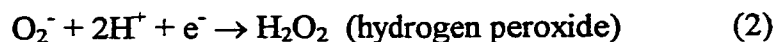
1.4. Oxidative Stress and its Consequences

When molecules in living cells absorb high energy photons of UV-B radiation they are elevated to excited electronic states. If these excited state species are unstable they can, through a photosensitization reaction (see 1.6.), transfer charge to ground state molecular oxygen forming reactive oxygen species which are detrimental to biological systems if their levels are not controlled (Cunningham *et al.*, 1983).

1.4.1. Reactive Oxygen Species (ROS)

Aerobic organisms use molecular oxygen to oxidize food substrates rich in carbon and hydrogen to obtain chemical energy which is critical for survival. In the process of oxidation, however, molecular oxygen itself is reduced through stepwise additions of electrons, resulting in the liberation of several reactive intermediates, two of which (HO_2^\bullet , OH^\bullet) are free radicals (Gutteridge, 1995). A free radical is defined as being any chemical species with one or more unpaired electrons which is capable of independent existence. The stepwise reduction of O_2 to H_2O is shown in equations 1-4 (Gutteridge, 1995).

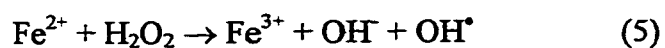




1.4.1.1. The Superoxide Anion Radical. The superoxide anion radical (O_2^-) is a one-electron reduction state of molecular oxygen. Sources include mitochondrial and microsomal respiratory chains as well as bacteriacidal activity of leukocytes and macrophages (Sies, 1986). In aqueous solution, O_2^- is a weak oxidizing agent, able to oxidize such molecules as ascorbic acid and thiols. It is, however, a much stronger reducing agent and can reduce iron complexes such as cytochrome c and ferric-EDTA. The toxicity of O_2^- depends on its rate of dismutation to H_2O_2 in aqueous solution (Gutteridge, 1995).

1.4.1.2. The Hydroperoxyl Radical. The hydroperoxyl radical (HO_2^\bullet) is a protonated form of O_2^- and is both a more powerful oxidizing and a more powerful reducing agent than O_2^- . As well it is more lipid soluble than O_2^- . But at physiological pH (7.4) HO_2^\bullet dissociates forming O_2^- and therefore is found only at low concentrations (Gutteridge, 1995).

1.4.1.3. Hydrogen Peroxide. Hydrogen peroxide (H_2O_2) is a direct two-electron reduction state of molecular oxygen formed either directly from O_2 or through the dismutation of O_2^- or HO_2^\bullet radicals (Sies, 1986). H_2O_2 is a weak oxidant and reducing agent that is relatively stable in the absence of transition metal ions (Gutteridge, 1995). It readily mixes with water and can rapidly diffuse across cell membranes (Gutteridge, 1995). In the presence of transition metal ions, H_2O_2 forms highly reactive free radicals such as the hydroxyl radical (OH^\bullet) through the Fenton reaction (Gutteridge, 1995):



1.4.1.4. The Hydroxyl Radical. The hydroxyl radical (OH^\bullet) is a three-electron reduction state of molecular oxygen formed by the Fenton reaction as shown above. The OH^\bullet radical is an aggressive oxidant attacking most biological molecules at a rate limited only by diffusion (Gutteridge, 1995).

1.4.2. Oxidative Damage and its Consequences

Reactive oxygen species can cause extensive cellular damage. To prevent this damage a balance exists between these prooxidant forces and the antioxidant forces which protect the body from oxidative damage. Oxidative stress occurs when the prooxidant forces overwhelm the protective antioxidant systems and shift the balance in favor of the prooxidants. Although oxidative damage is quite variable in terms of the different macromolecules affected, this type of damage is restricted to a small number of ROS as described above. These intermediates are reactive towards nucleic acids, amino acids and proteins, carbohydrates, and lipids (Sies, 1986) and, therefore, must be removed from biological systems.

1.4.2.1. Nucleic Acids. Oxidative damage to DNA is often complex and may be initiated by such oxidizing agents as hydroperoxides, oxygen radicals, ionizing radiation or photooxidation (Sies, 1986). Under aerobic conditions, strand scissions by peroxy radicals of the bases and sugars results in the loss of thymine and guanine groups. Strand scissions by both ionizing radiation and O_2^- are thought to be mediated by OH^\bullet (Lesko *et al.*, 1980; Von Sonntag *et al.*, 1981). These breaks lead to chromosome damage which is found to

be prevalent in diseases such as Bloom's syndrome (Emerit and Cerutti, 1981), lupus erythematosus (Emerit and Michelson, 1981) and Fanconi's anemia (Joenje et al., 1981).

1.4.2.2. Amino Acids and Proteins. The oxidation of amino acid side-chains in proteins can result in conformational changes and loss of function (Sies, 1986). This is particularly important in the case of active enzymes such as elastase. Methionine at the active site of elastase can be oxidized to sulfoxide by the hydroxyl radical, singlet oxygen or hydrogen peroxide, resulting in a loss of function (Beatty *et al.*, 1980). Loss of elastase function is reported during pulmonary emphysema (Eriksson, 1964). Histidyl residues in proteins play a similar role as methionine. The loss of one histidine residue by singlet oxygen-mediated oxidation of glutamine synthetase from *E. coli* results in inactivation and, therefore, loss of function (Levine, 1983). In the case of cysteine, disulfide bond formation is a reversible process and isn't viewed as damage; however, disulfide bridges in peptides and proteins altering thiol/disulfide status may elicit changes in the enzyme kinetic parameters K_m and V_{max} (Sies, 1986).

1.4.2.3. Carbohydrates. Deoxyribose is susceptible to oxidative attack and has been exploited for the development of the thiobarbiturate (TBARS) assay, which measures free radical damage (Halliwell and Gutteridge, 1981). Polysaccharides such as hyaluronic acid can be also be degraded by oxidative attack. The oxidative depolymerization of hyaluronic acid in synovial fluid was found to be spared addition of superoxide dismutase which removes O_2^- (McCord, 1974).

1.4.2.4. Lipids. Polyunsaturated fatty acids, largely present in the phospholipids of biological membranes, are susceptible to free-radical-mediated oxidation leading to a

number of stable degradation products dependent on the initial fatty acid involved. Attack by a reactive oxygen species (e.g. OH^\bullet) results in the abstraction of a H^\bullet from a methylene group ($-\text{CH}_2-$) with the remaining carbon radical rearranging to form a conjugated diene (Gutteridge, 1995). In the presence of molecular oxygen, the conjugated diene forms a peroxy radical which can abstract another H^\bullet from another fatty acid, initiating a chain reaction. Extensive lipid peroxidation results in losses in membrane fluidity, increased permeability to H^\bullet and other ions, decreases in membrane potential and in extreme situations, the rupturing of cell membranes and cell death (Gutteridge, 1995).

1.5. Protection from Oxidative Stress

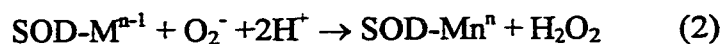
As a consequence of an aerobic lifestyle, organisms are constantly exposed to ROS. Cellular respiration involves the controlled step-wise reduction of O_2 to H_2O utilizing the electron transport chain (see 1.4.1). A second source of ROS within the cell is the activity of oxidizing enzymes such as diamine oxidase, tryptophan dioxygenase and cytochrome P450 reductase which generate O_2^\bullet and guanyl cyclase and glucose oxidase which generate H_2O_2 (Fridovich, 1974; Fridovich, 1978; Halliwell, 1978). As a result, aerobic organisms developed effective enzymatic and non-enzymatic antioxidant systems to counter these prooxidant forces. These antioxidant systems counteract toxic ROS at all levels: prevention, interception and repair. The most important non-enzymatic antioxidants are the lipid-soluble α -tocopherol (vitamin E) and ascorbic acid (vitamin C) (Burton *et al.*, 1983; Sies, 1986; Halliwell, 1990). Ascorbic acid, together with glutathione (GSH) in the aqueous phase, react with the vitamin E radical regenerating tocopherol in the membrane

(Sies, 1986; Sharma and Buettner, 1993). Essential enzymatic antioxidants are superoxide dismutase (SOD), glutathione peroxidase (GPx), catalase, as well as other hydroperoxidases and hemoprotein peroxidases (Sies, 1986). These antioxidants are characterized by the metal involved (copper, manganese, iron (heme), selenium), specific cellular content and specific organ and subcellular distribution (Sies, 1986).

1.5.1. Superoxide dismutase (SOD).

Superoxide dismutase belongs to the metalloenzyme family characterized by the use of metal groups for detoxifying activity. SOD is a dimeric enzyme existing intracellularly as cytosolic copper/zinc-SOD (Cu,Zn-SOD) and mitochondrial as mangano-SOD (Mn-SOD). Extracellular SOD also contains Cu and Zn in its active site, however, it has been shown to be distinct from the mitochondrial form (Harris, 1992). The extracellular form is secreted into the extracellular compartment binding to the plasma membrane and heparin-containing elements of the extracellular matrix (Karlsson and Marklund, 1989). In the Cu/Zn form, the zinc ion is buried in the protein with the copper ion lying at the bottom of the active site channel exposed to the medium (Deng *et al.*, 1993). The edge of the active site channel is lined with positive charged residues, forming a positive electrostatic field forcing O_2^- toward the Cu ion (Banci *et al.*, 1998). SOD functions by removing O_2^- by a very rapid dismutation process ($k > 10^9 M^{-1}sec^{-1}$) (McCord and Fridovich, 1969). It is generally accepted that in all SODs the metal ion catalyzes the dismutation of O_2^- through a cyclic oxidation-reduction (ping-pong) mechanism as illustrated below (Fridovich, 1978):





The first step involves the reduction of the metal prosthetic group by O_2^- forming O_2 . In the second step, the metal is immediately re-oxidized by another O_2^- molecule, yielding H_2O_2 (Fridovich, 1974). As a result, any system liberating O_2^- will produce H_2O_2 from the dismutation process. Because H_2O_2 can form highly ROS (ex. OH^*), unwanted H_2O_2 must be removed from cells thus eliminating its toxic effects and derived toxic radicals.

1.5.2. Catalase.

Catalase (hydrogen-peroxide:hydrogen-peroxide oxidoreductase) is a tetrameric heme protein catalyzing the reduction of H_2O_2 to O_2 and H_2O . The heme groups of catalase are found deeply buried accessible only by a narrow channel lined with hydrophobic residues (Ried et al., 1981). Catalase is found mainly in the peroxisomes, however, it has also been found in the cytosol (Eriksson *et al.*, 1992). It functions in rapidly catalyzing the reduction ($k > 10^7 \text{ M}^{-1}\text{sec}^{-1}$) of $2\text{H}_2\text{O}_2$ molecules to H_2O and O_2 (eq. 11) (Krinski, 1992; Gutteridge, 1995).



The first step in the catalase cycle involves the interaction of H_2O_2 with the active site of ferricatalase (oxidation state of Fe(III)) resulting in the formation of complex I (Fe(V)), the active form of catalase as illustrated in Figure 1.1 (Kirkman *et al.*, 1987). The interaction of compound I with another H_2O_2 molecule results in ferricatalase, O_2 and H_2O . Compound I can undergo a conversion to compound II (Fe(IV)), an inactive form of catalase, through a one-electron reduction by unknown reductants which have been

GSH synthesis occurs in several cell types, but the highest turnover occurs in the liver and kidney (DeLeve and Kaplowitz, 1991; Kelly et al., 1998). GSH synthesis is a two-step ATP-dependent process which occurs in the cytosol (DeLeve and Kaplowitz, 1991). γ -Glutamylcysteine synthetase catalyses the first step which is the formation of the dipeptide γ -glutamylcysteine. This rate limiting step is subject to feedback inhibition by GSH. In the second step, glycine is linked to the cysteine carboxyl group of γ -glutamylcysteine. Because two moles of ATP are required for each mole of GSH, GSH levels may be low when ATP levels are depleted. GSH degradation is restricted to cleavage by γ -glutamyltranspeptidase due to the linkage of N-terminal glutamate and cysteine by the γ -carboxyl group of glutamate (Fig. 1.2). In most cells, γ -glutamyltranspeptidase occurs only on the external cell surface, therefore, rendering GSH resistant to intracellular degradation. Intracellular degradation, however, can occur in certain cell types that possess γ -glutamyltranspeptidase on the cell membrane (DeLeve and Kaplowitz, 1991). GSH catabolism is further restricted by the C-terminal glycine which protects against cleavage by intracellular γ -glutamylcyclotransferase. The keystone to GSH function is cysteine which provides the reactive thiol group. GSH serves multiple roles in the cell including scavenging of reactive oxygen species, detoxification of reactive electrophiles, maintenance of essential thiol status of proteins and other molecules, and storage of cysteine for inter-organ transport (DeLeve and Kaplowitz, 1991) (Fig. 1.4).

1.5.3.1 Conjugation of electrophiles. In their naturally occurring form, many environmental contaminants are not toxic to organisms. However, upon metabolism by the cytochrome P450-mixed function oxygenase system, these relatively non-toxic compounds are

converted to unstable electrophilic metabolites which are both toxic and carcinogenic (Heidelberger, 1975; Miller, 1978; Gelboin, 1980; Otto and Moon, 1996). GSH functions in detoxifying these reactive metabolites either through spontaneous conjugation or by a reaction catalyzed by glutathione-S-transferase (GST). The GSH conjugate is then attacked by γ -glutamyltranspeptidase removing the γ -glutamyl moiety (DeLeve and Kaplowitz, 1991). The remaining cysteinyl-glycine conjugate is then cleaved by dipeptidase leaving a cysteinyl conjugate. Acetylation of the cysteine moiety occurs in the kidney and results in the formation of a mercapturic acid which is excreted.

1.5.3.2. Antioxidant function. In scavenging endogenously produced H_2O_2 , two GSH molecules act as substrates (provide reducing equivalents) for the selenium-dependent enzyme glutathione peroxidase (GPx). Once oxidized, the two molecules form a disulfide bridge between cysteine groups forming glutathione disulfide (GSSG) (eq. 9) (Gutteridge, 1995).



GPx is primarily a cytosolic selenoprotein, however, other forms exist in the plasma and in the lipid bilayer (Michiels *et al.*, 1991). The cytosolic form of GPx contains selenium bound to cysteine (selenocysteine) in the active site. The mechanism of action involves the oxidation and reduction of the selenocysteine group (Epp *et al.*, 1983). In the reduced state (Fig. 1.3), the selenolate anion reacts with hydrogen peroxide reducing it to H_2O . Subsequently, the selenolate anion becomes oxidized yielding selenic acid which exists only transiently due to reduction back to the selenolate anion by excess cytosolic GSH

cellular glutathione is in the form of GSH due to the high efficiency of GR and the availability of NADPH.



1.5.3.3. Thiol-disulfide exchange. Thiol-disulfide equilibrium in cells may be important in regulating certain metabolic pathways (DeLeve and Kaplowitz, 1991). Many proteins are active when key sulfhydryl groups are in the thiol form, whereas some require the oxidized, disulfide, form for activation. To prevent or reverse oxidation of essential thiols, or maintain thiol-disulfide equilibrium, the system which has evolved is thiol-disulfide exchange with GSH, catalyzed by thiol-transferase (DeLeve and Kaplowitz, 1991). With the thiol-transferase reaction being bidirectional, the redox state of the cell determines the thiol-disulfide equilibrium. Disulfides including GSSG and cystine can react with cysteine groups within enzymes to form mixed disulfides; this process would activate enzymes requiring the oxidized state (DeLeve and Kaplowitz, 1991).

1.5.3.4. Storage of cysteine. Cysteine itself cannot be stored in cells or transported in the blood because it rapidly auto-oxidizes to cystine which in turn can lead to the liberation of potentially toxic oxygen radicals (Lash and Jones, 1985). This auto-oxidation process, however, is prevented by storing non-protein cysteine in the form of GSH (DeLeve and Kaplowitz, 1991).

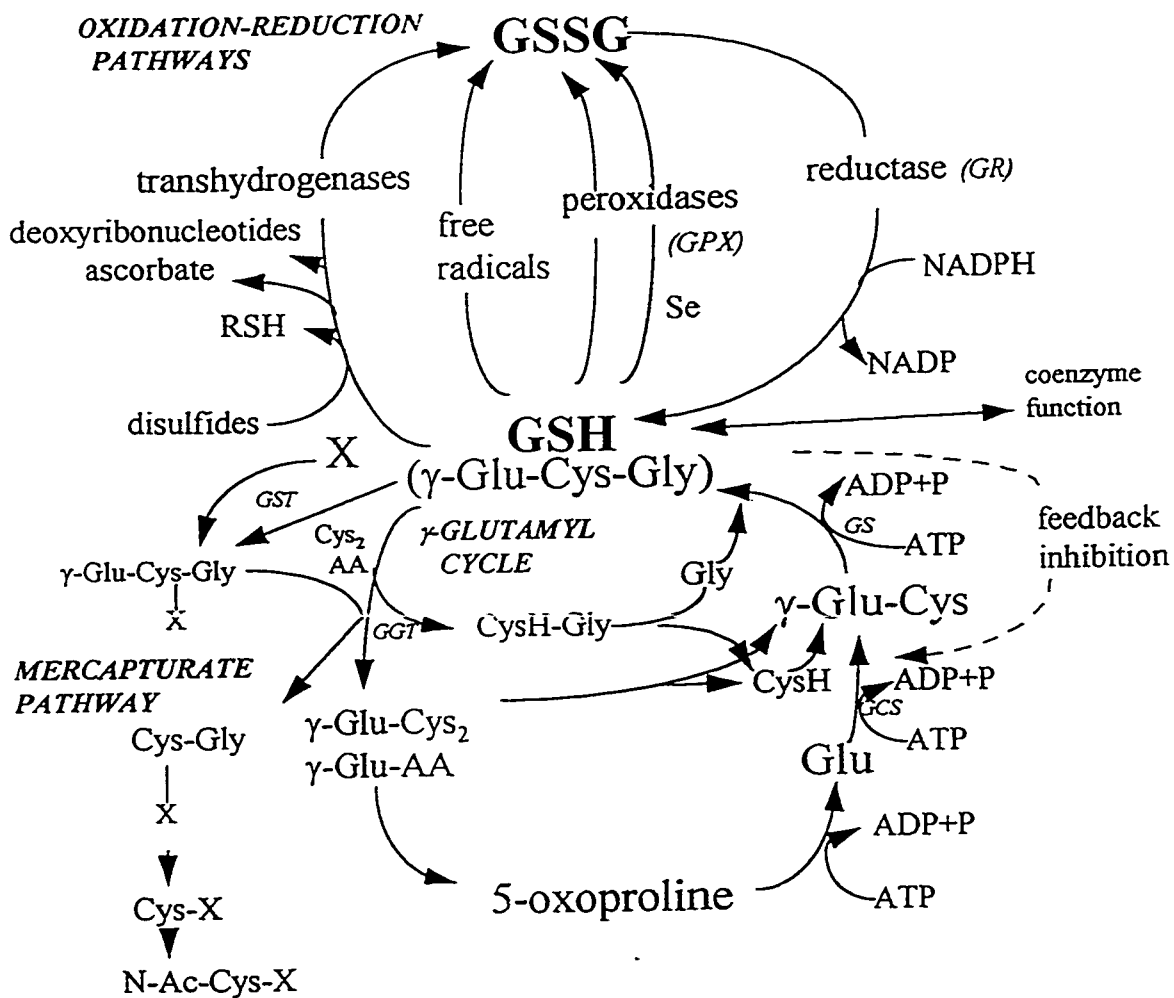


Figure 1.4. Outline of glutathione biochemistry. See list of abbreviations for GSH, GSSG, GPx, GR and GST. See text for description of main pathways. Adapted from Meister (1991).

1.6. UV-B Radiation and Oxidative Stress

In normal aerobic metabolism, ROS are continuously liberated requiring detoxification and removal by biological antioxidants. Many of these antioxidants have been measured (Cohen *et al.*, 1970; Paoletti *et al.*, 1986; Fitzgerald, 1992; Otto and Moon, 1995) and are used as indicators of oxidative stress.

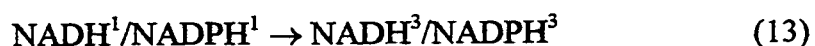
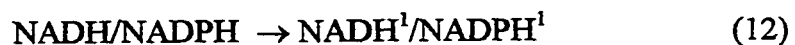
It is obvious that the integument is the tissue most readily exposed to UV-B radiation. Humans, numerous bird species, many reptiles and amphibians have a pigmented integument in which the pigment protects the epidermal and dermal tissues from UV-B-mediated damage. The principal epidermal pigment is melanin. Melanins are black, brown or yellow pigments of high molecular weight that are formed from the enzymic oxidation of phenols (Pathak and Stratton, 1968). Melanins found in mammals, birds, reptiles, amphibians and certain lower vertebrates and invertebrates are derived from tyrosine and, therefore, are termed tyrosine-melanins (Fitzpatrick, 1965). In human skin, tyrosine-melanin is produced in specialized unicellular glands called melanocytes which in turn secrete melanin-containing particles, melanosomes, into epidermal cells. The optical absorption of the melanin polymer is within the ultraviolet and visible regions of the solar spectrum (Pathak and Stratton, 1968). Through reversible oxidative-reduction reactions, melanin attenuates high energy UV-B by scattering it and dissipating the absorbed energy as heat (Fitzpatrick *et al.* 1963; Daniels *et al.* 1964). In the reduced state, melanin is less colored (tan), while the oxidized form is black. Pathak and Stratton (1968) found that skin containing melanin generated fewer radicals when exposed to UV-B compared to 'white' or unpigmented skin. The radicals generated were characterized as type I radicals

generated from epidermal proteins, like keratins, or other proteinaceous cellular constituents, and type II radicals characterized as melanin free radicals which are generated within the melanin polymer. Mason *et al.* (1960) described the role of melanin in biological systems as “melanin may act in some organisms as a biological electron exchange polymer able, by means of its capacity for oxidation and reduction, and its stable free radical state, to protect a melanin-containing tissue or associated tissues against reducing or oxidizing conditions which might otherwise set free, within living cells, reactive free radicals capable of disrupting metabolism” (p. 228). In addition to melanin, which may act in preventing the generation of ROS, epidermal antioxidant systems will also provide a crucial line of defense against UV-B-mediated damage by intercepting and removing ROS. Pence and Naylor (1990) demonstrated that exposure of hairless mice (SKH: hr-1 strain) to a single tumor promoting dose of 900 W-sec/m² of UV-B (130 sec exposure to 7.0 ± 0.2 W/m²) was sufficient to significantly depress epidermal SOD and catalase activities within 12 hours post-exposure and the depression remained for up to 72 h post-exposure. Compromises in these two antioxidants indicates that UV-B generates ROS in epidermal tissue mainly in the form of superoxide anion radicals and H₂O₂. Furthermore, Stewart *et al.* (1996) reported that the supplementation of cultured mouse keratinocytes with selenium (required for glutathione peroxidase function) increased GPx activity significantly reducing oxidative damage to DNA when exposed to 7500 W-sec/m² (see Appendix for definition of units) of UV-B.

In addition to skin, eyes are also readily exposed to increased UV-B radiation. Babu *et al.* (1995) showed that exposure of aqueous humor and lens homogenate from

albino mice to 6 W/m² of UV-B resulted in significantly higher levels of H₂O₂ in aqueous humor after 15 days and in lens tissue after 7 and 15 days. Elevated H₂O₂ levels resulted in enhanced lipid peroxidation of the eye lens and decreased levels of GSH after 15 days exposure. These data demonstrate that elevated H₂O₂, lipid peroxidation, and depressed GSH contribute to photodamage of the eye, resulting in photochemical cataractogenesis.

One of the ways in which ROS may be liberated in epidermal or eye tissue is through a photosensitization reaction in the presence of NADH or NADPH. Cunningham *et al.* (1985) reported that the reduced pyridine coenzymes NADH and NADPH, through photosensitization reactions, produced the superoxide anion radical (O₂⁻) from ground state molecular oxygen when exposed to UVR in the 290-405 nm range. The liberation of O₂⁻ was confirmed by SOD-mediated inhibition of cytochrome c reduction. UVR within the UV-B range liberated the highest quantum yield of O₂⁻ when compared with wavelengths in the UV-A range. Although these quantum yields are quite low, the yields from 334 nm radiation are sufficient to enhance DNA strand breaks (Cunningham *et al.*, 1984). Cunningham *et al.* (1985) proposed the liberation of O₂⁻ to be due to a type II photosensitization reaction (eq. 12-14). The ground state sensitizer absorbs a photon producing an unstable, excited singlet state (denoted by ¹) (eq. 12). The singlet state sensitizer can then cross over to a more stable triplet state (denoted by ³) (eq. 13) which can transfer charge to ground-state molecular O₂ forming O₂⁻ (eq. 14).



In phytoplankton damage by these photosensitization reactions can be minimized due to the possession of secondary pigments known as xanthophylls, which include diadinoxanthin, diatoxanthin, violoxanthin, zeaxanthin, alloxanthin and β -carotene. These pigments are inefficient in energy transfer and, therefore, are proposed to serve a photoprotective function (Grumbach et al., 1978; Larkum and Barrett, 1983; Paerl et al., 1983; Bidigare et al., 1987; Demmig et al., 1986). These compounds protect photosynthetic machinery and other important cellular components by quenching the triplet state of oxygen, and dissipating the energy as heat (Bidigare, 1989).

1.7. Objectives of the Present Study

The purpose of the present study is to determine the impact of varying intensities of UV-B radiation (up to present ambient levels, i.e. those levels experienced in Ottawa, Ontario (45°N) on a cloudless sunny day at noon under the present reduced ozone levels) on the antioxidant status in the zebrafish (*Brachydanio rerio*), a shallow water species native to freshwater systems in India and Pakistan proper. Although a more commercially relevant species native to North America (such as the rainbow trout, *Oncorhynchus mykiss*) was preferred for this study, the reason for using the zebrafish is that there is: 1) an abundance of information on their life cycle and embryological development; 2) they are small in size which allows for exposure of a large number of fish in a small tank at one time; 3) they are easily bred; and 4) unlike native fish eggs can be produced “on demand”. Their small size is also a disadvantage as individual tissues cannot be readily separated.

The emphasis of the study was to assess the impact of UV-B on protective endogenous antioxidants in muscle and skin tissue of adult fish. It was hypothesized that

exposure to current ambient levels of UV-B radiation would directly compromise muscle and skin antioxidant function in the zebrafish leading to oxidative stress and thus reduced survival. Preliminary studies, however, suggested that the egg and juvenile stages were the most vulnerable to UV-B, therefore, a second objective of this study was to determine the impact of UV-B on hatching success of eggs, survival of newly hatched juveniles as well as the impact on antioxidant systems in these early life stages. From the first hypothesis, it was also expected that current ambient levels of UV-B radiation will directly impair hatching success and survival in juveniles. Furthermore, this impairment was expected to be correlated to oxidative stress. This second objective raises a few questions. Can oxidative stress impair hatching success? If so, does egg mortality correlate with reduced antioxidant status? Are juveniles more sensitive to UV-B radiation than adult fish?

2. Materials and Methods

2.1. Chemicals. 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB), GR, butylated hydroxytoluene (BHT), thiobarbituric acid (TBA), diethanolamine, triethanolamine, triton X-100, hydrogen peroxide (H_2O_2), ethylenediaminetetraacetic acid (EDTA), horseradish peroxidase (HRP) and scopoletin (6-methyl-7-hydroxyl-1,2-benzopyrene) were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Manganese (II) chloride-tetrahydrate ($MnCl_2 \cdot 4H_2O$), potassium permanganate ($KMnO_4$) and butanol were obtained from VWR Canlab. 2-Mercaptoethanol, GSH and GSSG were obtained from ICN Biomedicals (Aurora, Ohio, U.S.A.). NADPH and NADH were obtained from Boehringer Mannheim (Montreal, Quebec).

2.2. Animals. Adult zebrafish were purchased from Aquarium Services (1010 Belfast Road, Ottawa, Ont. Canada). The fish were laboratory acclimated in 38 L aquaria with dechloraminated city of Ottawa tap water and kept under a 12 hour light/dark cycle. Experimental tanks were setup with a closed system of water flow. Water from a reservoir container situated below the experimental setup was pumped through a multi substrate filter into each tank with the overflow returning back to the reservoir by gravity. As necessary to compensate for evaporation, the reservoir was topped-up with dechloraminated city of Ottawa tap water. Both the holding and experimental tanks were kept in a temperature controlled room with 60% humidity to slow evaporation. The room air and water temperatures were maintained at $28.0 \pm 2.0^\circ C$ and the water was saturated with oxygen. Fish were fed once daily with a combination of Wardley Total Tropical® flake food and San Francisco Bay Brand brine shrimp which were cultured fresh each day.

2.3. Exposure to UV-B. White light was supplied by four verilux full spectrum (F40T12VLX) 4 foot fluorescent tubes purchased from Verilux Canada Corporation, Ottawa, Ontario and three 2 foot Sylvania cool white (F20T12) 20 watt tubes purchased from Buchanan Lighting, Ottawa, Ontario. UV-A was supplied by 2 foot NEC 20 watt T10 blacklight tubes with a peak irradiance of 365 nm and UV-B was supplied by 2 foot UBL F520T12/UVB tubes with a peak irradiance of 310 nm (company specified); both UV-A and UV-B tubes were purchased from Blacklock Medical Products Inc., British Columbia. The 4 foot verilux tubes were suspended over the entire experimental setup. For each experimental tank, 1 UV-A, 1 UV-B and 1 white tube were suspended independently at a distance from the water surface determined by the desired intensity. Intensities were measured using a Goldilux Oriol ultraviolet meter with Oriol UV-A and UV-B probes. Measured intensities were corrected for 10% reflection at the water surface. UV-A intensities were 2.09, 2.06 and 2.02 W/m² and UV-B intensities were 0.15, 0.99 and 1.95 W/m² for the low, medium and ambient UV-treatments, respectively. All experiments involved exposure to 6 h of UV-B daily, superimposed onto the 12 hour white light + UV-A/dark cycle. Most experiments involved the use of a cellulose acetate filter for blocking UV-B below 290 nm and all UV-C radiation (Worrest and Kimeldorf, 1976; Worrest *et al.*, 1978). Cellulose acetate sheets were placed over each tank so as not to come in contact with the water surface and were changed every three days as these sheets yellow with time which in turn impairs penetration of higher UV-B wavelengths. Adult fish were harvested after 1, 2 or 4 exposure periods (equivalent to 6, 12 and 24 h of cumulative UV exposure) by decapitation. The gut was removed and the remaining

muscle/skin tissue was frozen in liquid nitrogen. Muscle/skin tissue samples were stored at -80°C until analyzed.

For the hatching success experiments, several males and females were placed in a 55.5 cm x 22.0 cm x 20.0 cm perforated plexiglass breeding tank placed in a 38 L aquarium supplied with a false bottom made of plastic rods. Fish were placed in the breeding tank in late afternoon and remained there until the onset of the next light cycle when breeding took place. The breeding tank was exposed only to verilux full spectrum lights. Newly fertilized eggs were retrieved by siphoning and were separated into 4 replicates of n = 15 eggs for exposure to the UV regime as used for the adults until hatching (3 days). In determining survival of juveniles, eggs were hatched in the absence of UVR and newly hatched larvae were separated in the same manner as the eggs and exposed to the same UV regime as used for the adults and eggs for 5 days.

2.4. Chemical and Enzyme Analysis

2.4.1. Glutathione measurements.

Frozen muscle/skin samples were homogenized in ice cold 5% 5-sulfosalicylic acid using a Kontes microultrasonic cell disrupter (Mandel Scientific Company LTD., Saint-Laurent, Quebec) in a 1 + 8 (weight:volume) dilution and were then cleared by centrifugation at 4°C at 9000 rpm for 10 min using an eppendorf 5415C centrifuge (Brinkman Instruments Inc., Westbury, New York). The supernatant fraction was recovered and stored at -20°C for future analysis. Total glutathione (TGS) was determined according to the method by Sen *et al.* (1992) whereby the rate of reduction of 0.8 mM DTNB (final concentration) is followed at 412 nm using a Beckman DU 65

spectrophotometer. GR and NADPH were added to the reaction mixture (1 ml total volume) at final concentrations of 0.43 units and 0.23 mM, respectively, to reduce any GSSG to GSH; therefore, the rate of DTNB reduction is proportional to the sum of GSH + GSSG using a standard curve of GSH concentration versus absorbance.

2.4.2. Cytosolic enzyme assays.

All enzymes were assayed at 20-25°C (a temperature well within the range experienced by this species) using a Beckman DU 65 spectrophotometer. All homogenizations were performed using a Kontes microultrasonic cell disrupter (Mandel Scientific Company LTD., Saint-Laurent, Quebec) due to the small amount of sample obtained. Substrate concentrations were those of the original assay and were not optimized for this species.

Glutathione peroxidase (GPx) activity was determined according to the method of Tappel (1978). Muscle/skin tissue was homogenized in 50 mM tris buffer in a 1 + 8 dilution (weight:volume) containing 0.1mM β -mercaptoethanol and phenylmethanesulfonylfluoride (PMSF) (1 grain per sample) at pH 7.6. Samples were cleared by centrifugation at 10,000 rpm for 30 min using an eppendorf 5415C centrifuge (Brinkman Instruments Inc., Westbury, New York). A 20 μ l sample of the supernatant was incubated at room temperature for 5 min in a solution of 39 mM Tris buffer (final concentration: pH 7.6) containing EDTA, GSH, NADPH and GR at the final concentrations of 78 nM, 0.19 μ M, 93 nM and 0.78 units, respectively. The reaction was started by adding cumene hydroperoxide at a final concentration of 0.21 mM and the rate

of disappearance of NADPH was monitored at 340 nm. GPx activity is expressed as μmol of NADPH oxidized per min per gram of wet tissue weight ($\mu\text{mol}/\text{min}/\text{g}$ wet tissue).

Glutathione disulfide reductase (GR) activity was determined according to the method by Carlberg and Mannervik (1985) using GSSG (1 mM) and NADPH (100 μM) as substrates in 100 mM phosphate buffer containing 1 mM EDTA (final concentrations) at pH 7.0. The same enzyme preparation used for determining GPx activity was used to determine GR activity. The reaction was initiated by the addition of enzyme to the cuvette and the decrease in absorbance (oxidation) of NADPH was monitored at 340 nm. GR activity is expressed as μmol of NADPH oxidized per min per gram of wet tissue weight ($\mu\text{mol}/\text{min}/\text{g}$ wet tissue).

Superoxide dismutase (SOD) activity was measured by the method of Paoletti *et al.* (1986). Muscle/skin tissue samples were homogenized in 25 mM triethanolamine/diethanolamine buffer in a 1 + 10 (weight:volume) dilution and then were cleared by centrifugation at 25,000 rpm for 30 min using a Sorvall RC 5B Plus centrifuge (Mandel Scientific Co. Ltd., Guelph, Ontario). The method is based on the superoxide anion-mediated oxidation of NADH (0.28 mM) whereby 2-mercaptoethanol (0.94 mM) is the superoxide anion generating system and the decrease in absorbance is followed at 340 nm. The addition of SOD to the reaction mixture causes a proportionate inhibition of the rate of NADH oxidation. SOD activity is expressed as units per gram wet tissue weight (U/g wet tissue) where 1 unit of enzyme inhibits the rate of superoxide-mediated NADH oxidation by 50% as determined by linear regression of a standard curve of rate of NADH oxidation (% of control) versus the concentration of pure SOD (ng) (Figure 2.1).

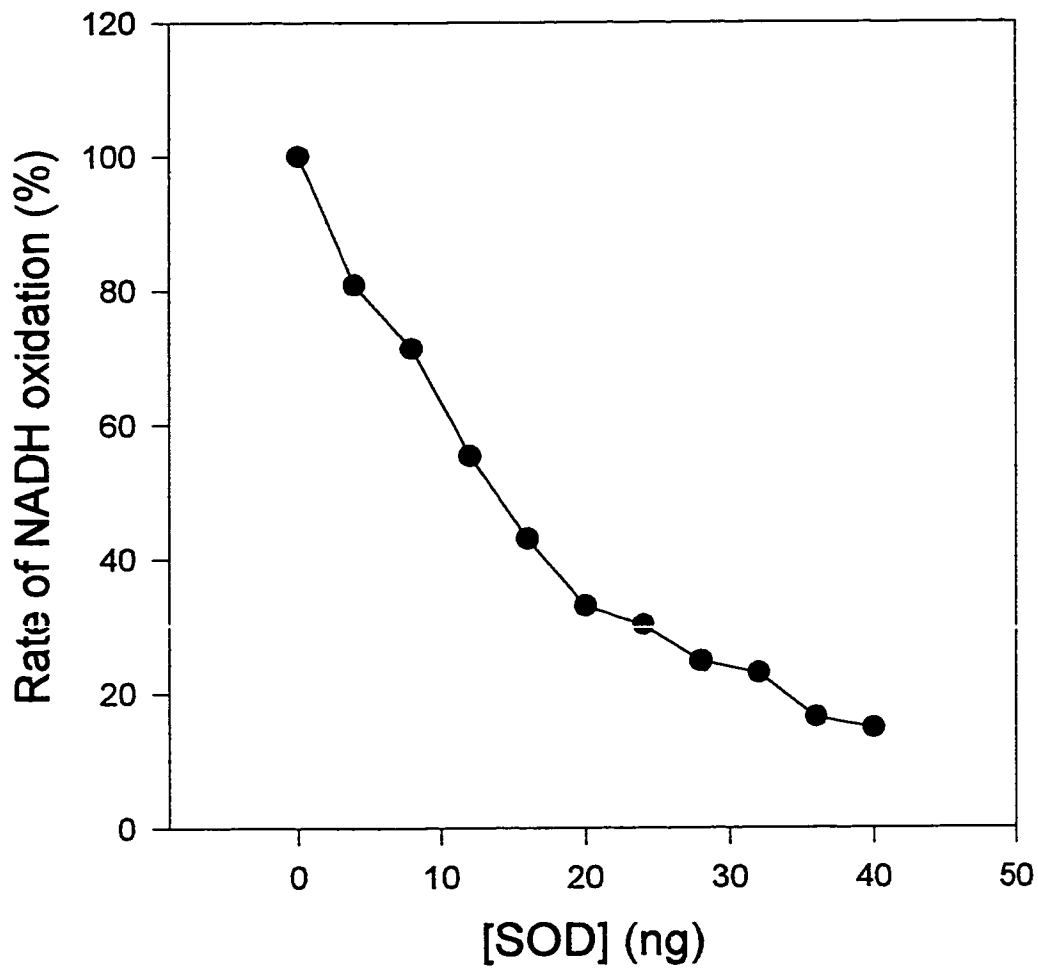


Figure 2.1. Standard curve of rate of NADH oxidation (% of control rate) versus the concentration of pure SOD.

Catalase activity was determined by the method of Cohen *et al.* (1970).

Muscle/skin samples were homogenized in isotonic medium containing 0.31 M NaCl and 0.01 M sodium phosphate buffer at pH 7.4. To increase observable catalase levels, homogenates were treated with 1% Triton-X and 0.17 M ethanol (final concentrations). Ethanol increases the observable catalase activity by degrading Complex II, an inactive complex of catalase (Chance, 1950). Triton X-100 increases the observable cytosolic catalase activity by freeing catalase from mitochondria and peroxisomes thus ensuring reproducibility from sample to sample (Adams, 1957). The catalase-mediated decomposition of H_2O_2 is determined by its reaction with 0.005 N permanganate (final concentration); residual permanganate concentrations were determined spectrophotometrically at 480 nm. Catalase activity was calculated by estimation of residual H_2O_2 by linear regression of a standard curve of H_2O_2 concentration versus residual permanganate absorbance. Catalase activity is expressed as mmoles of H_2O_2 removed per minute per gram wet tissue weight (mmol/min/g wet tissue).

2.4.3. Thiobarbituric acid reactive substances (TBARS).

The concentration of TBARS was determined according to the method of Hermes-Lima *et al.* (1993). The principle of the assay involves the measurement of malondialdehyde (MDA), a decomposition product of hydroperoxides which forms a complex with TBA that has an absorbance peak at 532-535 nm (Slater, 1984; Halliwell and Gutteridge, 1985). Frozen muscle/skin samples were homogenized in 1.1% phosphoric acid (1+8, weight:volume) using a Kontes microultrasonic cell disrupter

(Mandel Scientific Company LTD., Saint-Laurent, Quebec). and to the homogenate, 1% thiobarbituric acid (TBA), 50 mM NaOH and 99.5% butanol was added. After centrifuging for 5 min at 'level 6' using a Damon / IEC clinical benchtop centrifuge, the butanol phase was recovered and the absorbance was measured at 532-535 nm. The concentration of TBARS is expressed as nmoles per gram wet tissue weight (nmol/g wet tissue). and was calculated by dividing the observed absorbance (resultant from subtracting the difference between the absorbance of the blank at 532 nm and at 600 nm from the difference between the absorbance of the sample at 532 nm and 600 nm) by the extinction coefficient for TBARS ($156,000 \text{ cm}^2/\text{mole}/10^6$) and multiplying by the dilution factor of the analyzed butanol phase (2.5) and the sample dilution factor (9).

2.4.4. Measurement of Hydrogen Peroxide (H_2O_2). The concentration of H_2O_2 from fish tank water was determined by a modification of the method by Holm *et al.* (1987). The analysis was performed using a fluorescence decay protocol involving the oxidation of 95 nM scopoletin (6-methyl-7-hydroxyl-1,2-benzopyrene), a fluorescent chromophore, catalyzed by 99.2 nM horseradish peroxidase (HRP) (final concentrations). A Turner Designs Model 10 fluorometer equipped with filters providing excitation and emission wavelengths of 365 and 490 nm, respectively, was used for the fluorescence determinations. Twenty milliliter samples were taken from each experimental tank (in the absence of fish) at 5 intervals: before the white light/UV-A cycle, before the UV-B cycle, half way through the UV-B cycle, immediately after the UV-B cycle and immediately after the white light/UV-A cycle. The concentration of H_2O_2 was estimated by linear regression

of a standard curve of change in fluorescence (change in fluorescence before and after addition of H₂O₂) versus concentration of H₂O₂.

2.5. Statistical analysis. Significant differences between control and treatment groups over time were tested using a two-way ANOVA with pairwise comparisons. A level of significance of $P < 0.05$ was accepted as significant.

3. Results

UV-B Exposure and H₂O₂ Liberation in Experimental Water

The penetration of UV-B radiation into the water column produces numerous transient photochemical species due to absorption by DOC (see 1.2.1). One of the longer lived transient species is H₂O₂. Water from the experimental tanks was assayed for H₂O₂ to verify that any change in oxidative stress in the fish was a consequence of UV-B exposure of the animal rather than a H₂O₂ build-up in the water. Water samples were taken at 5 time intervals: before the white light cycle, before the UV-B cycle, half way through the UV-B cycle, immediately after the UV-B cycle and immediately after the white light cycle. H₂O₂ levels over the complete light cycle are plotted in Figure 3.1. Peak levels were observed at 6.74 ± 2.53 nmoles/L in the high UV-B treatment at the end of the UV-B cycle.

UV-B Exposure and Behavioral Responses

Fish exposed to different intensities of UV-B were observed to display different behavior patterns. On the first day, fish displayed similar behaviors. On day 2, that is after 2 exposure periods of 6 h (or a cumulative dose of 12 h), those exposed to the ambient UV-B dose remained at the bottom of the experimental tank when the lights were on while those in the low UV-B treatment exploited all levels of the water column. In addition, fish exposed to the ambient UV-B dose did not feed or swim as actively as those in the low UV-B treatment. Fish that survived 24 h cumulative exposure (4 exposure periods of 6 h) to the ambient UV-B dose had lesions around the dorsal fin and extending to the caudal fin, lost parts of their caudal fin and displayed reduced movement.

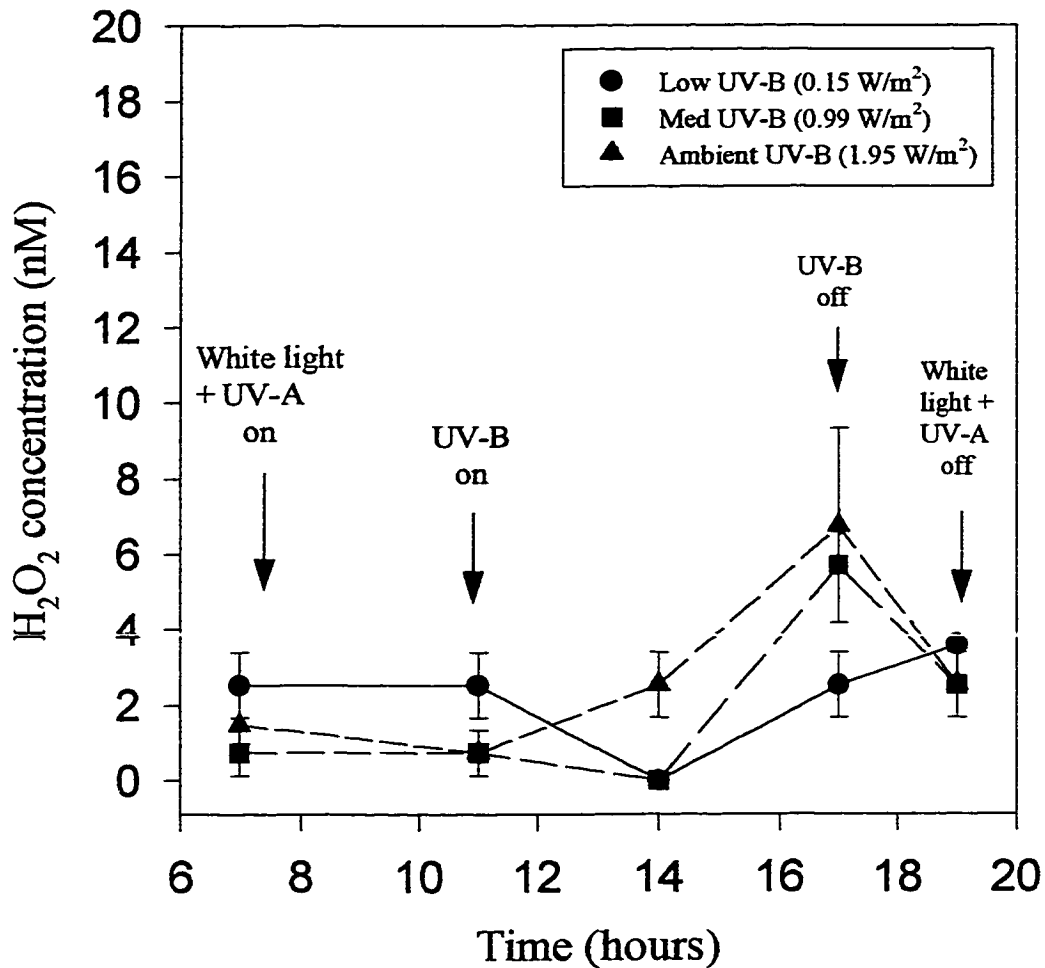


Figure 3.1. Concentration of hydrogen peroxide (H₂O₂) in the water of three UV-B treatment tanks over the course of the light cycle. Samples were taken at the start of the white light cycle (7 h), the start of the UV-B cycle (11 h), mid-way through the UV-B cycle (14 h), the end of the UV-B cycle (17 h) and the end of the white light cycle (19 h). Data points represent means of n = 3 samples taken on 3 different days using the same recycled water; error bars represent SEM. Fish were absent from the tanks.

UV-B Exposure and Impact on Muscle/Skin Antioxidants

To test the effects of UV-B exposure on muscle/skin TGSH levels, fish were exposed to a low (0.15 W/m^2) and a sub-ambient (1.28 W/m^2) dose of UV-B radiation in the presence of a cellulose acetate filter (Fig. 3.2). After one 6 h exposure period, levels of muscle/skin total glutathione (TGSH) were significantly lower (1.4 times) in fish exposed to both the sub-ambient and low UV-B doses compared with controls (fish unexposed to UV-B or time 0 fish). After 2 exposure periods (or 12 h cumulative exposure), however, muscle/skin TGSH levels in fish exposed to the low UV-B dose recovered back to control levels; muscle/skin TGSH levels in sub-ambient UV-B exposed fish were further reduced to 1.7 times that of controls and remained relatively unchanged for the remainder of the experimental period. The same experiment was repeated with the cellulose acetate filters removed allowing exposure to the full UV-B spectrum emitted from the UV-B tubes (Fig. 3.3). After 18 h cumulative exposure, fish treated with 1.72 W/m^2 of UV-B had half of the levels of muscle/skin TGSH compared with controls and low UV-B-treated fish. In comparing the data from Figures 3.2 and 3.3, no significant difference in TGSH was observed between high UV-B-treated fish, however, the slope of the line Figure 3.3, is much steeper compared to the line in Figure 3.2. In addition, fish subjected to the sub-ambient UV-B dose in the absence of the cellulose acetate filter survived only 18 h of cumulative exposure. Given that the cellulose acetate sheets block UV-B below 290 nm and all UV-C wavelengths, this result implies a greater impact of the lower UV-B wavelengths on GSH levels.

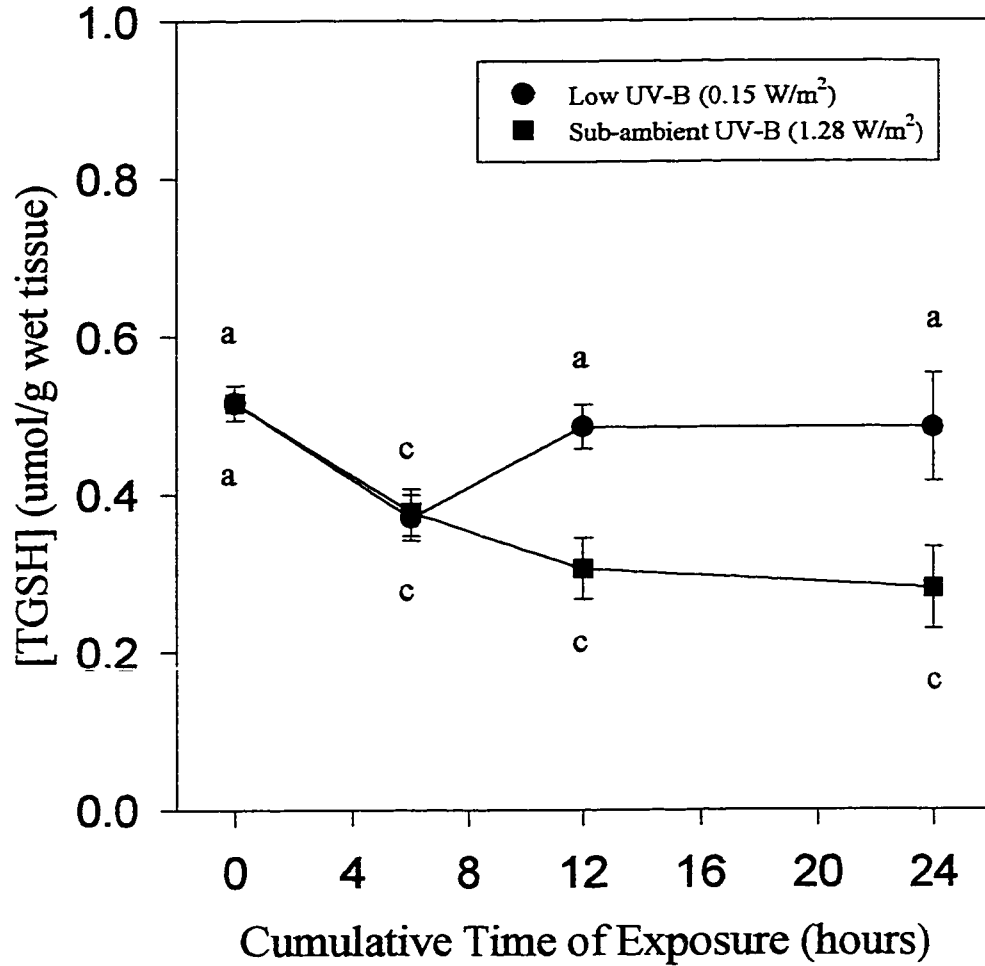


Figure 3.2. Concentration of total glutathione (TGSH) in zebrafish muscle/skin samples after exposure to two different UV-B treatments in the presence of a cellulose acetate filter. Data points represent mean concentrations from $n = 3-5$ fish; error bars represent SEM. Data points at $T = 0$ represent control levels. Different letters are significantly different ($P < 0.001$).

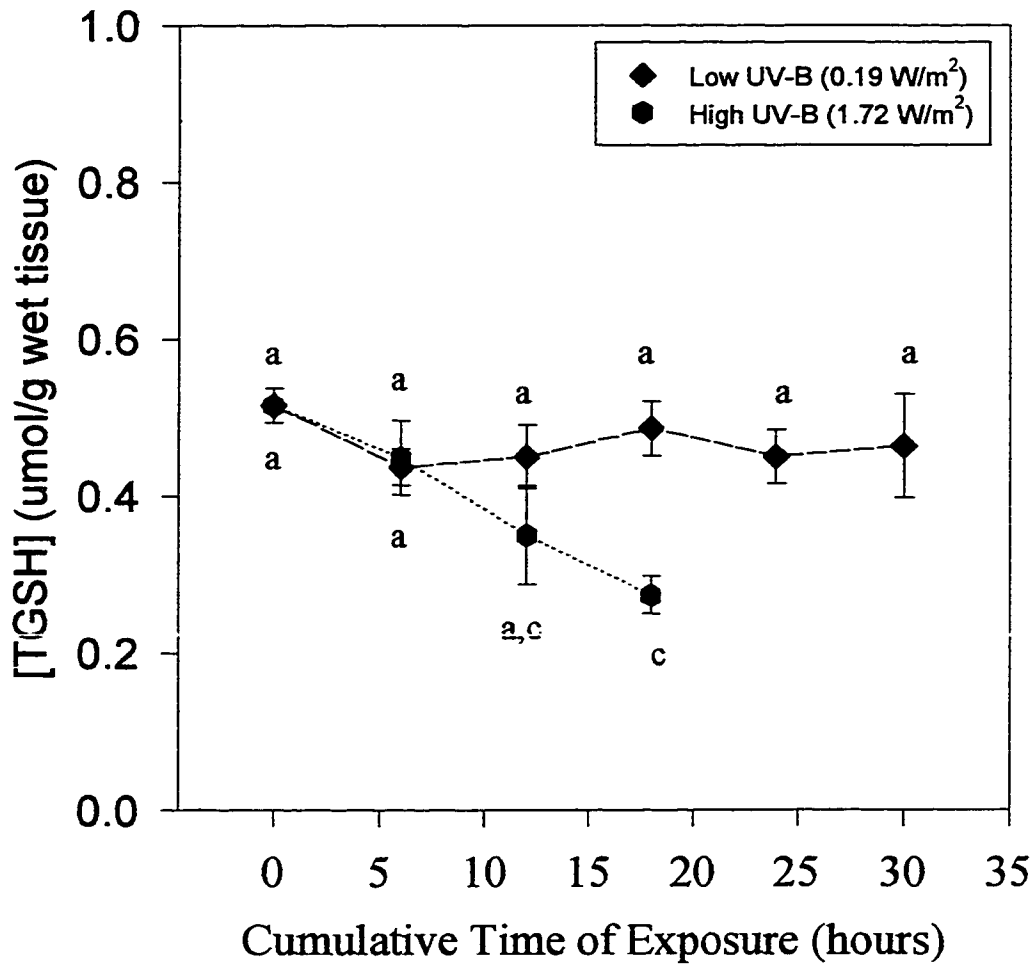


Figure 3.3. Concentration of total glutathione (TGSH) in zebra fish muscle/skin samples after exposure to two UV-B treatments in the absence of a cellulose acetate filter. Data points represent mean concentrations from ($n = 3-5$ fish); error bars represent SEM. Data points at T = 0 represent control levels. Different letters are significantly different ($P < 0.001$).

The impact of UV-B on muscle/skin glutathione peroxidase (GPx) and glutathione disulfide reductase (GR) is shown in Figures 3.4 and 3.5. Exposure to the ambient dose of UV-B (1.95 W/m^2) for a 6 h exposure period depressed GPx activity to 1.6 times that of the controls (time 0) and low UV-B-exposed fish with a recovery to near control activities after 12 h exposure (Figure 3.4). GR activities were not significantly different between UV-B doses or compared with controls (Fig. 3.5).

The impact of UV-B exposure on SOD activity is shown in Figure 3.6. After a 6 h exposure period, SOD activity was significantly higher ($P = 0.006$) in fish exposed to both the low and ambient UV-B doses compared with controls (time 0). These elevated activities after 12 h cumulative exposure declined back to near control values. After 24 h cumulative exposure, SOD activity in ambient UV-B-treated fish continued on a downward trend, whereas SOD activity began to climb in fish exposed to the low UV-B dose.

Catalase activity (Fig. 3.7) was also induced by UV-B exposure. After 12 h cumulative exposure, fish exposed to the ambient and low UV-B dose exhibited catalase activities 6.7 and 4 times higher, respectively, than controls (time 0) and 6 h exposed fish. With 24 h cumulative exposure, however, catalase activity fell to a level comparable with that observed in low UV-B-treated fish after 12 and 24 h cumulative exposure.

UV-B Exposure and Lipid Peroxidation

One way in which oxidative damage may be assessed is through the measurement of the end products of free radical-mediated lipid membrane peroxidation. These end

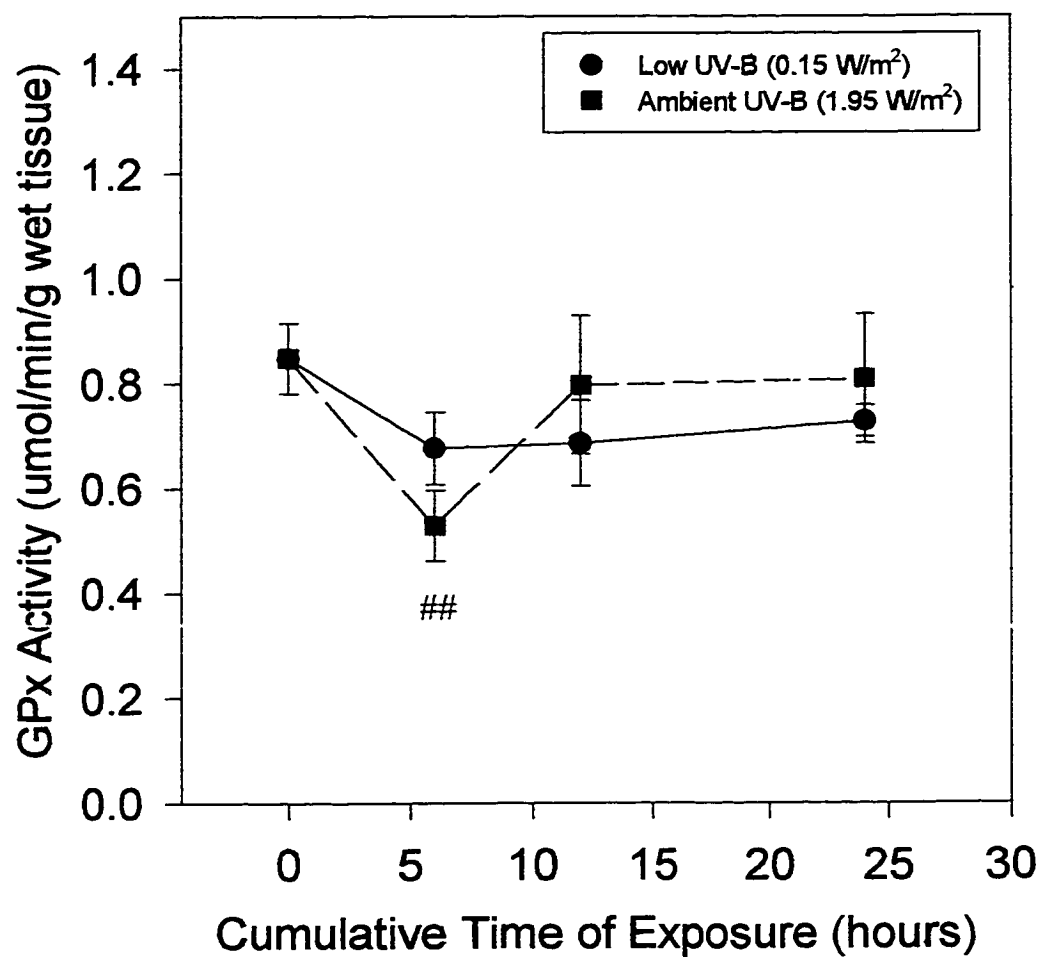


Figure 3.4. Glutathione peroxidase (GPx) activity in zebrafish muscle/skin samples after exposure to two different UV-B treatments. Data points represent mean activities from $n = 5$ fish; error bars represent SEM. Data points at $T = 0$ represent control activities. ##: significantly different ($P < 0.01$) from all other points.

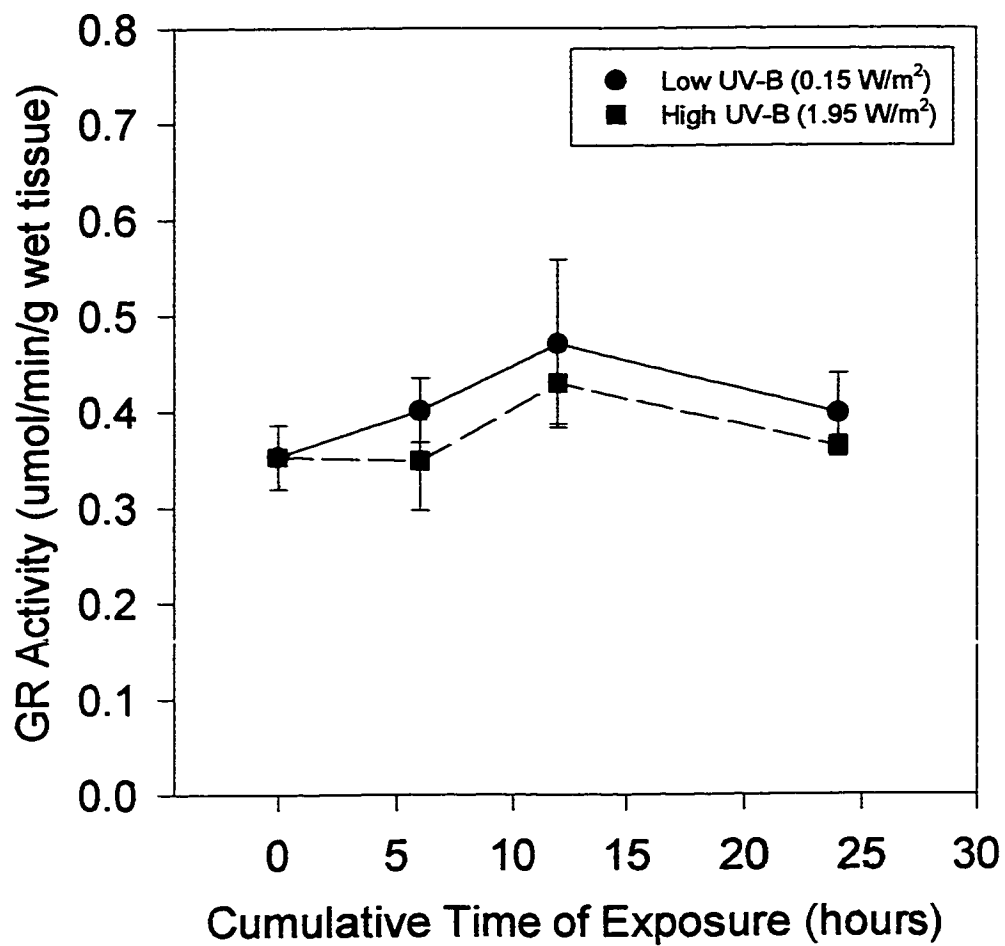


Figure 3.5. Glutathione disulfide reductase (GR) activity in zebrafish muscle/skin samples after exposure to two different UV-B treatments. Data points represent mean activities from ($n = 5$) fish; error bars represent SEM. Data points at $T = 0$ represent control activities.

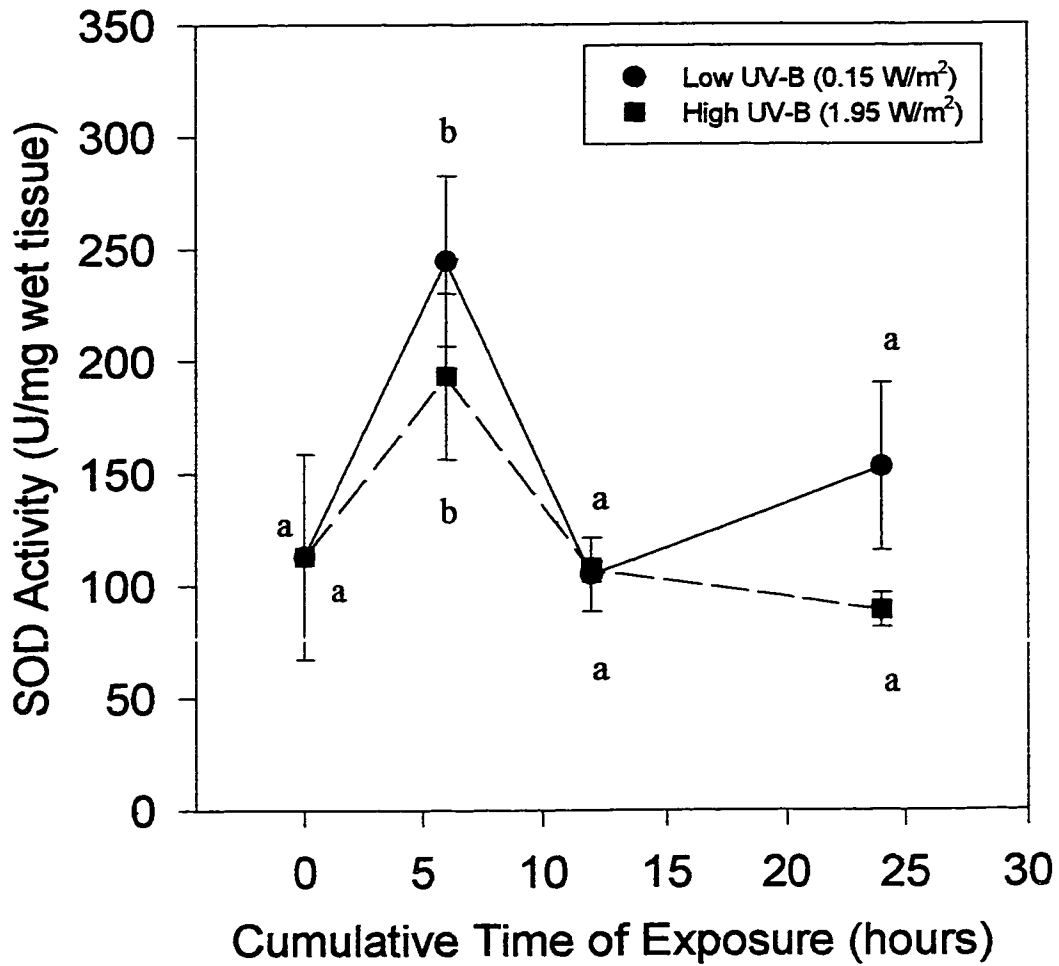


Figure 3.6. Superoxide dismutase (SOD) activity in zebrafish muscle/skin samples after exposure to two different UV-B treatments. Data points represent mean concentrations of (n = 5) fish; error bars represent SEM. Different letters are significantly different (P = 0.006).

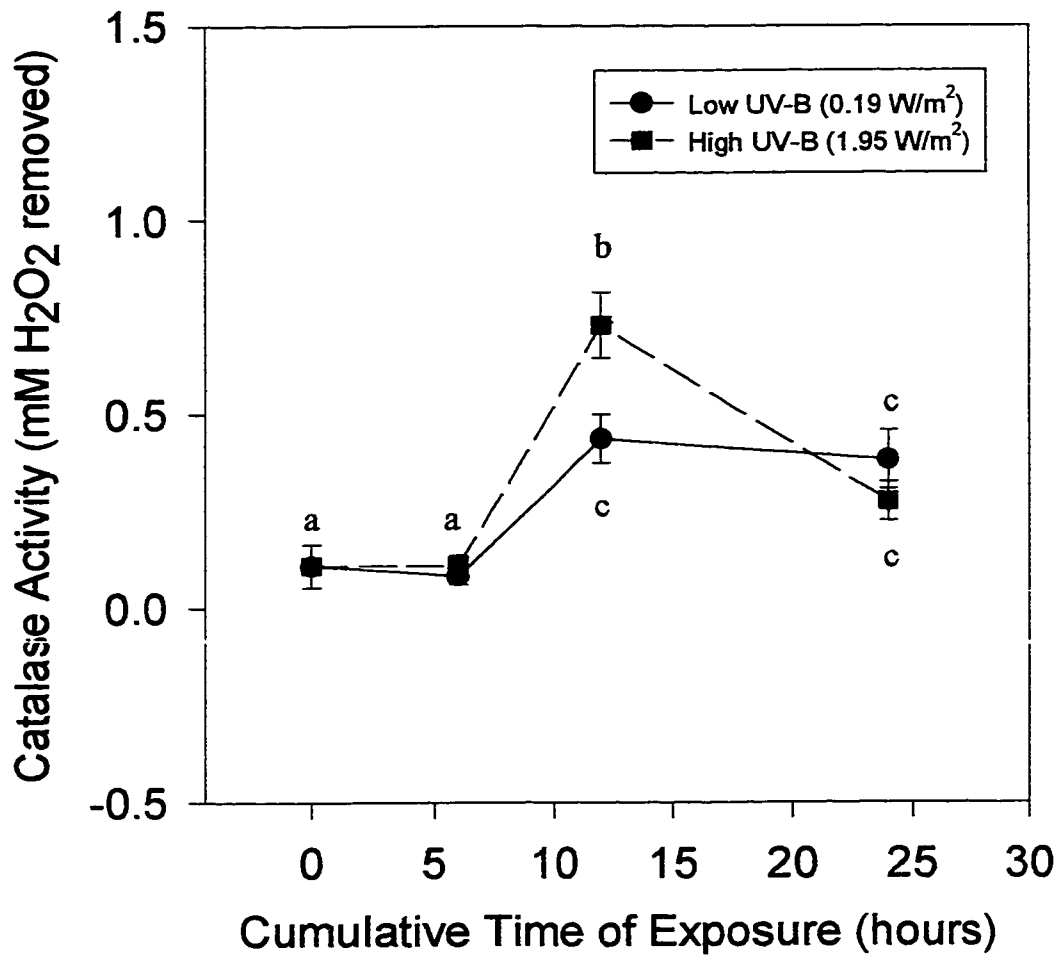


Figure 3.7. Catalase activity in zebrafish muscle/skin samples after exposure to two different UV-B treatments. Data points represent mean activities from (n = 5) fish; error bars represent SEM. Data points at T = 0 represent control activities. Different letters are significantly different (P < 0.001).

products are known as hydroperoxides which in turn decompose forming malondialdehyde (MDA) (see 1.4.2.4). MDA in addition to other aldehydes resulting from lipid peroxidation readily react with thiobarbituric acid (TBA) yielding identical fluorometric fingerprints and hence are called thiobarbituric acid reactive substances (TBARS). Figure 3.8 shows the concentration of TBARS in fish exposed to a low and ambient dose of UV-B radiation. After a 6 h exposure period, and for the remainder of the experimental period, fish exposed to the ambient dose of UV-B exhibited TBARS levels 1.3-1.5 times higher than control levels. Fish exposed to the low UV-B dose, on the other hand, exhibited a 23% decrease in TBARS levels after 6 h when compared with control levels. This general decline was observed up to 24 h cumulative exposure.

UV-B Exposure and Impact on Early Life Stages

In assessing the impact of UV exposure on early life stages, newly fertilized eggs were exposed to three different intensities of UV-B for the duration of the embryonic and hatching period (Fig. 3.9). Eggs exposed to an ambient dose of UV-B experienced a 98% decrease in hatching success (units on right y-axis) compared with controls and those exposed to 0.55 W/m² UV-B. Exposure to half of the ambient dose resulted in a 75% decrease in hatching success compared with controls and a 70% decrease compared with eggs exposed to 0.55 W/m² UV-B.

Survival of newly hatched juveniles exposed to various UV-B intensities is shown in Figure 3.10. Exposure to an ambient UV-B dose resulted in 100% mortality after 12 h cumulative exposure; after a 6 h dose, % survival was reduced by 38% compared with all other treatment groups. Juveniles exposed to half of the ambient dose survived an 18 h cumulative exposure period unlike those exposed to the low dose of UV-B which survived

the entire exposure period.

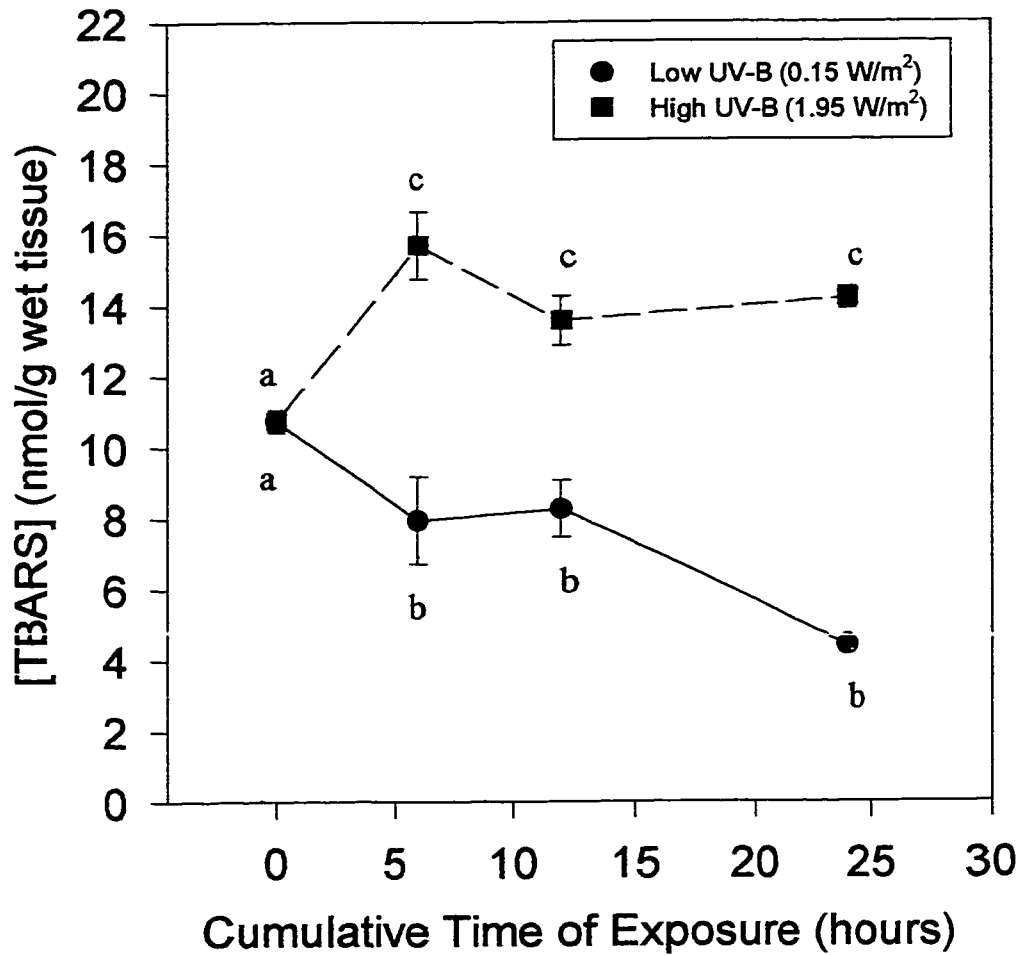


Figure 3.8. Concentration of thiobarbituric acid reactive substances (TBARS) in zebrafish muscle/skin samples exposed to two different UV-B treatments. Data points represent mean concentrations of (n = 4-5 fish); error bars represent SEM. Data points at T = 0 represent control levels. Different letters are significantly different (P < 0.001).

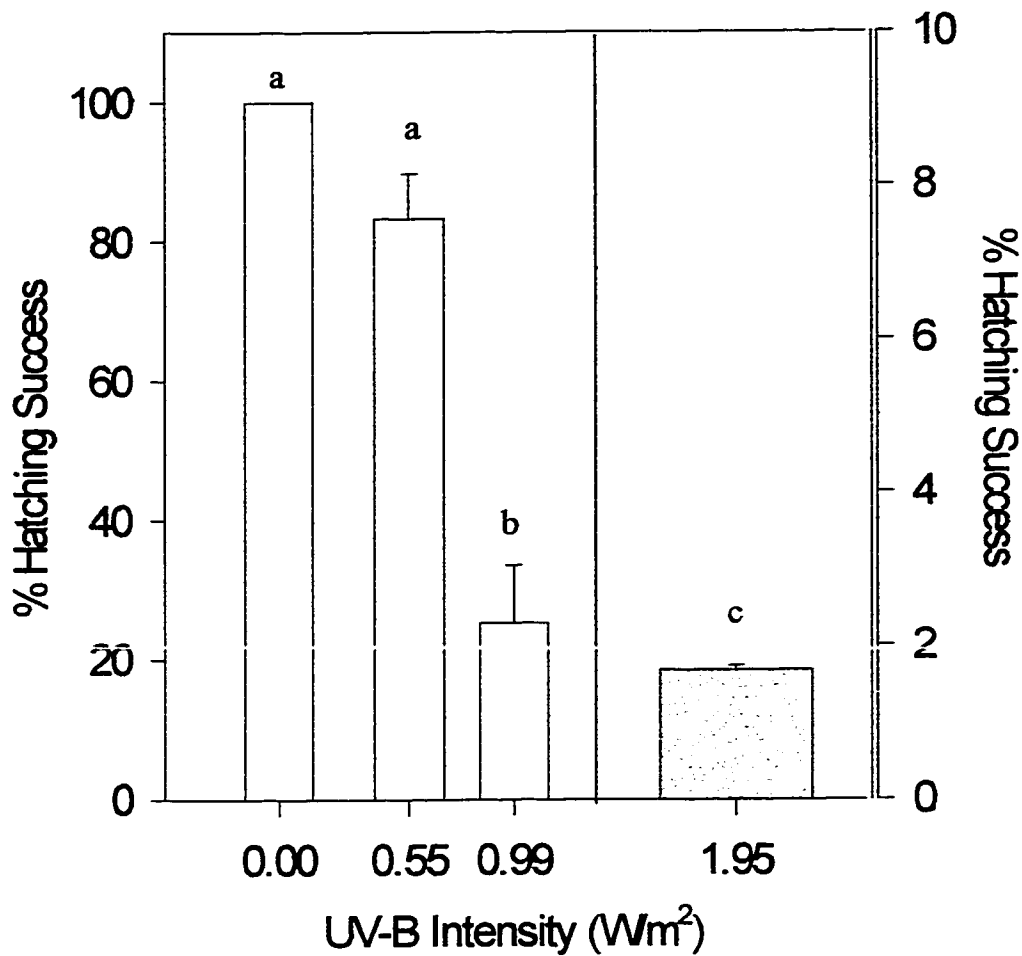


Figure 3.9. Hatching success (as a %) of newly fertilized eggs exposed to various UV-B treatments for 3 days. Data represents means of 4 replicates per treatment of (n = 15) eggs; error bars represent SEM. 0 treatment represents controls. Different letters are significantly different (P < 0.001).

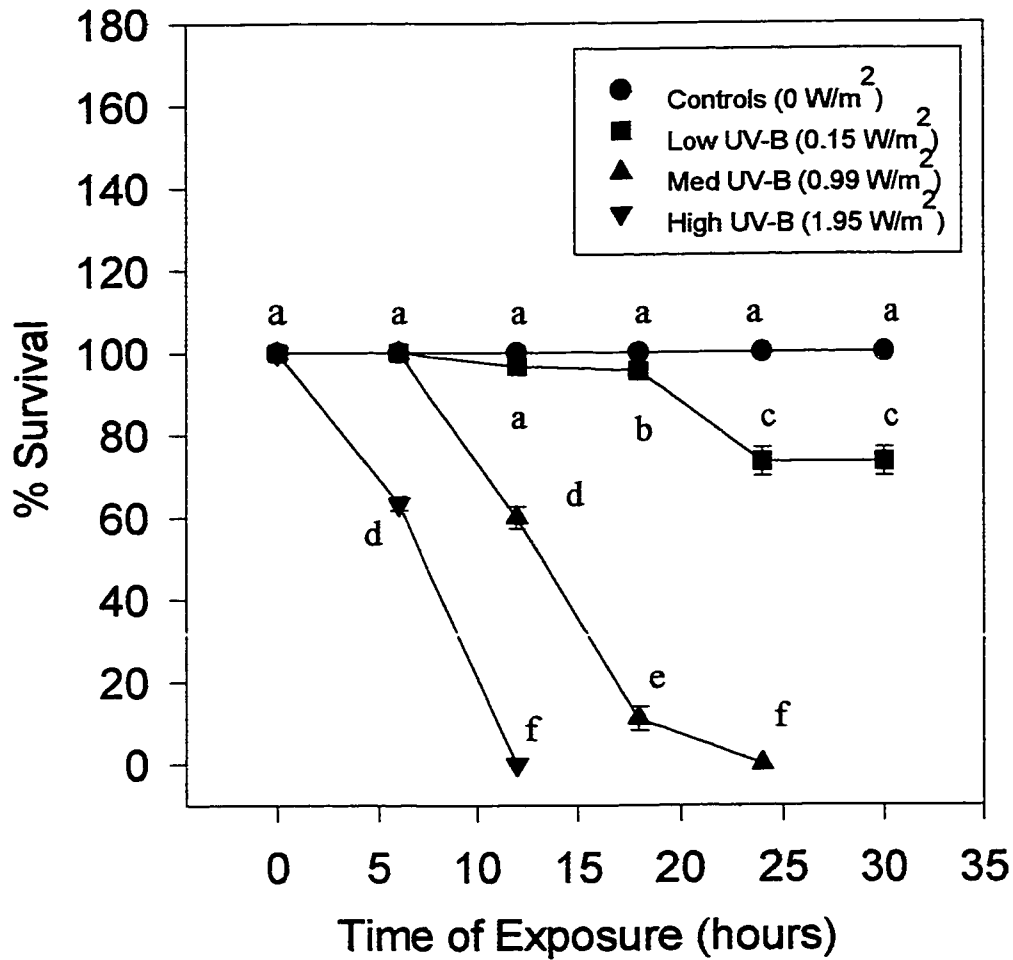


Figure 3.10. Survival (as a %) of newly hatched larvae exposed to various UV-B treatments. Data points represent means of 4 replicates per treatment of ($n = 15$) larvae; error bars represent SEM. Different letters are significantly different ($P < 0.001$).

Discussion

The penetration of UV-B radiation into freshwater systems causes the liberation of several transient photochemical species due to absorption by DOC (see 1.2.1). One of the more persistent photochemical species which can build to trace amounts is H₂O₂. With the evidence of elevated cellular H₂O₂ leading to oxidative stress (Fridovich, 1978; Sies *et al.*, 1986; Babu *et al.*, 1993; Kelly *et al.*, 1998), and the focus of this study being on the direct impact of UV-B radiation on muscle/skin antioxidants, water samples from each experimental treatment were analyzed for H₂O₂ over the course of the UV-B and white light/UV-A cycles to confirm that any change in oxidative stress in the exposed fish was a consequence of UV-B exposure and not due to H₂O₂ build-up in the water (Fig. 3.1). The levels of H₂O₂ recorded in this study were 10-50 times lower than those reported by Cooper and Zika (1983) in natural freshwater systems of the southeastern U.S.. Therefore, I conclude that the observed impacts on tissue antioxidant status were more likely the effect of UV-B exposure, than from changes in water H₂O₂ levels.

The tissue most readily exposed to high energy UVR is the integument. In fish, the outer dermal and epidermal tissue layers are among the most sensitive tissues to UVR exposure (Siebeck *et al.*, 1994). For many aquatic and terrestrial species, the recruitment of integumental pigments like melanin for the absorption and dissipation of high energy UV wavelengths provides the first line of defense against UV-mediated damage. In a study with juvenile scalloped hammerhead sharks (*Sphyrna lewini*), Lowe and Lowe (1996) reported that pups exposed to natural solar irradiation in 1m deep holding ponds experienced a $14 \pm 6\%$ and $28 \pm 6\%$ increase in integumental melanin after 21 and 215

days exposure, respectively. Spectral transmittance through solutions of melanin extracted from skin of tanned pups showed the greatest reduction within the UV-B range indicating an adaptive role for melanin in protection against UV-B-mediated damage. Instead of, or in addition to, possessing integumental pigments, some organisms possess feathers, hair, shells or scales which may act as "shields" against UVR. In the case where integumental pigments and/or "shields" are insufficient in protecting the organism from UV-mediated effects, epidermal antioxidants may play a vital role in protecting cellular components from UV-mediated damage by ROS. In a study with hairless mice, Pence and Naylor (1990) demonstrated that exposure to a single tumor promoting dose of 900 W-sec/m^2 of UV-B (or $7.0 \pm 0.2 \text{ W/m}^2$ for 130 sec, see Appendix) significantly depressed epidermal SOD and catalase activities compared with controls and remained so for up to 72 h post-exposure. Compromises in these two antioxidant enzymes indicate that UV-B is able to generate reactive oxygen species (ROS) in mammalian epidermal tissues in the form of superoxide anion radicals (O_2^-) and hydrogen peroxide (H_2O_2).

It was hypothesized that exposure of zebrafish to the current ambient dose of UV-B radiation would compromise muscle/skin antioxidant function leading to oxidative stress; and if sufficiently severe, this could lead to reduced survival. Exposure to a sub-ambient dose of UV-B radiation significantly depressed ($P < 0.001$) muscle/skin TGSH compared with both controls and fish exposed to low UV-B (Figs. 3.2, 3.3). Control levels were found to be 2 times higher than the TGSH levels in white muscle of mature rainbow trout (*Oncorhynchus mykiss*) and black bullhead (*Ameiurus melas*) as reported by Otto and Moon (1995; 1996). As a consequence of their small size, tissue harvested from

experimental fish included white muscle, skin tissue and perhaps small amounts of red muscle which may have contributed to the higher TGSH content compared with rainbow trout. Otto and Moon (1995) observed control red muscle TGSH content to be 2.3 times higher than white muscle. Changes in TGSH were shown to be a reliable indicator of exposure to ROS (Sen *et al.*, 1992). In addition to GSH directly reducing ROS including singlet oxygen ($^1\text{O}_2$), hydroxyl radicals (HO^\bullet) and superoxide anion radicals (O_2^-), it also acts as a substrate for GPx in the scavenging of H_2O_2 (DeLeve and Koplowitz, 1991; Kelly *et al.*, 1998). Many authors have shown that under severe oxidative stress, GSSG is actively removed from the cell when the ability of the cell to reduce GSSG by GR has been overcome by high levels of cytosolic ROS (Srivistava and Beutler, 1969; Sies *et al.*, 1972, 1978; Kondo *et al.*, 1980). With no significant increase observed in GR activity throughout the UV-B exposure period (Fig. 3.5), it is possible that excess cytosolic GSSG was transported to locations other than muscle and skin tissue thus leading to the observed significant reduction in muscle/skin TGSH. Depletion of cytosolic TGSH may not only compromise antioxidant poise through a diminished ability to directly reduce ROS, but also by providing insufficient levels of substrate for the removal of H_2O_2 by GPx. In nature, it is conceivable that a compromise in GSH antioxidant capacity may render fish more vulnerable to the effects of environmental toxicants given that GSH also serves as a substrate for the glutathione S-transferase (GST)-mediated detoxication of environmental pollutants (DeLeve and Kaplowitz, 1991; Otto and Moon, 1995; 1996). GSH is also known to be involved in DNA and protein synthesis, coenzyme function and detoxication processes (Meister and Anderson, 1983). A depletion in muscle/skin GSH, therefore, may

also impair other metabolic processes consequently disturbing the physiological state of the organism. A perturbed physiological state in addition to an increase in oxidative stress, as indicated by significantly elevated TBARS concentrations compared with controls (Fig. 3.8), are possible factors contributing to reduced health and survival of fish exposed to the high UV-B treatment. As noted in the Results, long term exposed zebrafish developed body lesions on the dorsal surface.

In response to exposure to an ambient dose (1.95 W/m^2) of UV-B radiation, GPx activity dropped significantly ($P < 0.001$) after 6 h exposure but recovered to near control levels after 12 h cumulative exposure (Fig. 3.4). Otto and Moon (1996) reported white muscle GPx activities in mature rainbow trout and black bullheads as 4.3 and 3.8 nmol/min/mg protein, respectively. For the purpose of comparison with other studies, the units of enzyme activity reported in my study have been converted from activity per g wet tissue weight to activity per mg protein. According to Moon (1983), American eel (*Anguilla rostrata*) white muscle contains 50 mg protein/g wet tissue weight. As a result, all enzyme activities reported here were divided by 50 to yield activity units per mg protein. The estimation of enzyme activity per mg protein using eel white muscle protein content, however may overestimate zebrafish muscle/skin activities given that the protein content of skin was not taken into consideration due to the lack of information pertaining to skin protein content. Accepting these assumptions, control muscle/skin GPx activity in mature zebrafish, in $\mu\text{mol/min/mg protein}$, was found to be 4.5 and 4 times higher than the activities reported in mature rainbow trout and black bullheads, respectively (Otto and Moon, 1996). As with TGSH, the additional presence of small amounts of red muscle and

skin with white muscle tissue may have contributed to the observed higher activity since GPx activity was observed to be higher in red muscle compared with white muscle (Otto and Moon, 1996). In contrast, control muscle/skin GPx activity reported here was observed to be 3.2 times lower than whole body GPx activity observed in larval turbot (*Scophthalmus maximus*) (Peters and Livingstone, 1996). During development, processes such as cell division and tissue formation require increased metabolic rates which increase the demand for oxygen uptake in both fish embryos (Rombough, 1988) and larvae (Walsh *et al.*, 1989). Elevated oxygen consumption leading to higher ROS activities in larval fish perhaps explains the elevated enzyme activity observed by Peters and Livingstone (1996). Although traditionally known for catalyzing the reduction of H₂O₂ to H₂O, GPx has also been shown to sequentially act with phospholipase A₂ in repairing peroxidized phospholipids resulting from lipid peroxidation (Tappel, 1980). Another enzyme involved in the repair of lipid peroxidation is phospholipid hydroperoxide glutathione peroxidase (PHGPx) (Ursini *et al.*, 1985). PHGPx, like GPx, contains selenium, however, it possesses a greater affinity for phospholipid peroxides that do not serve as substrates for GPx and a lower affinity for H₂O₂ (Grossmann and Wendel, 1983) suggesting that GPx and PHGPx act as a multilevel enzyme system functioning in the reduction of various hydroperoxide substrates generated by lipid peroxidation. The observed decrease in GPx activity in ambient UV-B exposed fish was found to parallel the highest measured level of TBARS (Fig. 3.8). The observed drop in GPx activity could perhaps be a result of increased degradation of the enzyme in order to recruit selenium for increased PHGPx synthesis and repair of peroxidized lipid membranes. Although PHGPx

activity was not measured in this study, determining the activity of PHGPx would test the above prediction. Another prediction is the ROS-mediated oxidation of amino acid side-chains which could cause a conformational change in GPx leading to loss of function due to a temporary overwhelming of muscle/skin cells with ROS. Beatty *et al.* (1980) reported that an observed loss of function of elastase, detected during pulmonary emphysema, was attributed to the ROS-mediated oxidation of methionine at the active site. In *E. coli*, singlet oxygen-mediated oxidation of one histidine residue in glutamine synthetase resulted in inactivation of the enzyme and, therefore, loss of function (Levine, 1983).

Despite the observed UV-B-mediated fluctuation in GPx activity and the fall in TGSH, muscle/skin GR activity remained unchanged. Control muscle/skin GR activity was 2.3 and 11.4 times higher than control activities observed in mature rainbow trout and black bullhead white muscle tissue, respectively, as reported by Otto and Moon (1996). Like GPx, whole body GR activity in larval turbot was higher (2.3 times) than that observed in this study for mature zebrafish muscle/skin tissue once again probably due to a higher metabolic rate at the larval life stage.

SOD activity exhibited an increased activity in response to UV-B exposure. A significant increase ($P < 0.001$) in SOD activity was observed after only 6 h exposure to both an ambient and low UV-B dose (Fig. 3.6), suggesting a UV-B-mediated increase in muscle/skin O_2^- levels. After 12 h cumulative exposure, however, SOD activity declined back to near control activities indicating either a rapid removal of O_2^- from the cytosol or perhaps a modification of SOD tertiary structure through an ROS-mediated oxidation of amino acid side-chains resulting in a loss of function (see above). The continued

downward trend in SOD activity following 24 h cumulative exposure in the ambient UV-B-treated group supports the concept of loss of function as observed in other non-antioxidant enzymes (Beatty *et al.*, 1980; Levine, 1983). In contrast to the results observed in the present study, Pence and Naylor (1990) observed a continual significant decrease in epidermal SOD activity up to 72 h following a single dose of UV-B to Skh:HR-1 mice comparable with approximately 8 h cumulative exposure to the ambient UV-B dose used in this study. Control SOD activity in zebrafish muscle/skin tissue was comparable with whole body SOD activity reported in larval turbot (Peters and Livingstone, 1996), but was observed to be 4.5-7.6 times lower than control SOD activity observed by Pence and Naylor (1990) in Skh:HR-1 mice. The lowest activity observed by Pence and Naylor (1990) at 72 h post exposure to 900 Wsec/m^2 (130 sec of 7.0 W/m^2) of UV-B was found to be comparable with peak SOD activity observed after exposure to 702 Wsec/m^2 of UV-B (6 h exposure to the ambient dose). With a SOD-mediated removal of O_2^- comes a subsequent increase in cytosolic H_2O_2 (see 1.5.1). This was confirmed by a peak in catalase activity following 12 h cumulative exposure (Fig. 3.7), the time at which SOD activity dropped to near control levels. Control muscle/skin catalase activity was observed to be much higher than white muscle activities in mature rainbow trout and black bullhead (3617 and 128 times higher) reported by Otto and Moon (1996) and whole body activity in larval turbot (5.9 times higher) reported by Peters and Livingstone (1996). Otto and Moon (1996) reported catalase activities in both mature rainbow trout and black bullheads to be highest in liver tissue; however, these activities were still found to be lower (4.6 and 7 times lower) than control activities observed in zebrafish muscle/skin tissue.

Despite the observed fluctuations in antioxidant defences, the degree of lipid peroxidation in ambient UV-B exposed fish, indicated by the concentration of TBARS, was significantly higher ($P < 0.001$) compared with controls and low UV-B treated fish; these levels remained consistently elevated for the duration of the exposure period (Fig. 3.8). Even with 24 h cumulative exposure to an ambient UV-B dose, zebrafish muscle/skin TBARS levels were 3 and 2.2 times lower than control levels observed in mature rainbow trout and black bullhead white muscle observed by Otto and Moon (1996). In contrast, fish exposed to the low UV-B dose exhibited significantly lower ($P < 0.001$) TBARS levels compared with controls. This observed decline in TBARS suggests that the ratio of UV-B:white light/UV-A in the low UV-B treatment beneficially affected exposed fish resulting in a reduction in the amount of lipid peroxidation compared with controls. Some authors reported that UV-A and visible light wavelengths are used by DNA photolyase, an enzyme involved in the photorepair of UV-induced DNA damage (pyrimidine dimers) (Friedberg, 1984; Shima and Setlow, 1984). Shima and Setlow (1984) reported that cultured goldfish (*Carasius auratus*) RBCF-1 cells (a fibroblastic cell line) exhibited photoreactivation (photorepair) of UV-B-mediated pyrimidine dimers following exposure to both UV-A and visible light wavelengths. Pyrimidine dimers have been associated with mutational events, carcinogenesis and cell death (Sage *et al.*, 1993). Medaka (*Oryzias latipes*) tail fin epithelial cells were also shown to possess photorepair ability when exposed to visible white light; after one hour treatment with fluorescent light, approximately 90% of UV-induced pyrimidine dimers in epithelial cells were repaired.

Many toxicant exposure studies show that the embryonic stage of development is the most sensitive life stage to toxicant-mediated effects (Wisk and Cooper, 1990; Spitsbergen *et al.*, 1991; Peterson *et al.*, 1993; Zabel *et al.*, 1995). Cantrell *et al.* (1998) reported that cytotoxicity in medaka (*Orizias latipes*) embryos exposed to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) was mediated by a shift in cellular redox balance toward oxidative stress due to the liberation of ROS by an upregulation of cytochrome P450 1A activity. Oxidative stress was shown to perturb cellular signaling pathways controlling cell proliferation and differentiation. More specifically, oxidative stress activates an intrinsic cytosolic signaling pathway which mediate apoptotic cell death (Hockenberry *et al.*, 1993; Kane *et al.*, 1993). With the observed fluctuations in protective antioxidant enzymes and elevated TBARS indicating the liberation of ROS by UV-B radiation, perturbations in critical developmental processes such as those listed above, serve as plausible mechanisms leading to the observed decreases in hatching success of fertilized eggs exposed to ambient and sub-ambient doses of UV-B radiation (Fig. 3.9). Newly hatched larvae were more sensitive to UV-B exposure and larvae exposed to an ambient dose of UV-B experienced 100% mortality after only 12 h of cumulative exposure to 1,404 W-sec/m² (see Appendix for definition of units). LD₅₀ values for larval Northern anchovy (*Engraulis mordax*) and Pacific mackerel (*Scomber japonicus*) exposed to UV-B (285-320 nm) were reported as 91,200 and 125,000 W-sec/m², respectively (Hunter *et al.*, 1979). Although the LD₅₀ values for anchovy and mackerel larvae suggest a much lower sensitivity to UV-B radiation compared with zebrafish larvae, the UV-B dose was administered at a lower intensity but for a longer duration and this probably contributed to

the higher LD₅₀ values. In addition to the embryo stage, larvae were also more sensitive to UV-B compared with mature fish which were observed to withstand at least 18-24 h cumulative exposure to ambient UV-B. Both the egg and juvenile stages are transparent in color, with integumental pigmentation becoming apparent much later in development. Certainly the lack of protective integumental melanin for the absorption and dissipation of high energy UV-B renders these earlier life stages more vulnerable to UV-B-mediated damage (Hunter *et al.*, 1979; Siebeck *et al.*, 1994).

Although these results confirm that UV-B radiation elicits oxidative stress in an aquatic species in the laboratory, it is difficult to extrapolate these results to other aquatic species in nature. One problem is species-dependent sensitivity to UV-B radiation. Hunter *et al.* (1979) reported that northern anchovy and Pacific mackerel displayed different sensitivities to UV-B; the LD₅₀ dose for northern anchovy was found to be 1.4 times lower than that for mackerel. In addition, at doses near the LD₅₀ values for each species, the incidence of eye lesions was approximately 3 times higher and brain lesions 5 times higher in anchovy compared with mackerel larvae indicating that anchovy larvae are more sensitive to UV-B radiation. Species-dependent sensitivities may be explained in part by the variety of strategies employed by different organisms to minimize the detrimental effects of increased UVR irradiance. These strategies include avoidance by vertical migration in the water column, photoprotection through increased recruitment of integumental pigments and/or antioxidants, photorepair and adaptation (Paanakker and Hallegraeff, 1978; Bidigare, 1989; Vincent and Roy, 1993; Siebeck *et al.*, 1994; Lean, 1998). Some organisms may employ only one of these strategies, whereas the response of

other species may involve a combination of the above strategies providing a multilevel response to UV-B exposure. It is clear, however, that UV-B radiation may be an important determinant in the fitness of fish species in their environment.

In conclusion, the observed changes in critical antioxidant enzymes and GSH in response to UV-B exposure supports the liberation of ROS in muscle/skin tissue of mature zebrafish by ambient UV-B radiation. Furthermore, an elevation in TBARS levels indicates that existing antioxidant defences were inadequate to remove ROS leading to a shift in cellular redox balance toward oxidative stress. In nature, with declines in DOC occurring due to increased photobleaching lake acidification, ozone-dependent increases in UV-B may not only provoke declines in zebrafish populations due to reductions in hatching success and survival of larvae, but may also render all life stages more vulnerable to environmental contaminants whose effects are mediated through ROS. It is evident that further studies need to be under taken on more commercially relevant species to establish the general nature of UV-B-mediated changes in muscle and skin tissue antioxidants.

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Appendix

Various units exist in the literature for defining UV intensities.

(W/m²): Is becoming the universal unit of measure for UV intensity. Variations may include W/cm² and W/mm².

(Wsec/m²): A cumulative measure of UV intensity. The units W/m² are multiplied by time of exposure in seconds.

(eff_{DNA} W/m²): Represents a measure of UV effect in damaging DNA, based on the DNA action spectrum by Setlow (1974).

(erythema equivalents): A dose effective in eliciting erythema (sunburn), calculated by multiplying the absolute irradiation energy by the relative energy effectiveness as determined by the erythema action spectrum by Green et al. (1974), as cited in Damkaer et al. (1980).

(J/m²): The old units of measure of UV intensity: 1 (J/m²) is equal to 1 (Ws/m²).

(ESS/Kb): The number of endonuclease sensitive sites per kilobase of DNA. 1 Kb of DNA is approximately 326 daltons in length.