THE EFFECTS OF ACUTE ULTRAVIOLET IRRADIATION ON ZEBRAFISH (*DANIO RERIO)* EYE STRUCTURE AND LENS PROTEIN COMPOSITION

A Report of a Senior Study

by

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ABSTRACT

For terrestrial vertebrates, refraction of light entering the eye is accomplished by both the cornea and lens, while in many aquatic vertebrates the lens is mainly responsible for focusing incoming light. Ultraviolet-B (UV-B) radiation has been implicated in many ocular disorders, and this study investigated the impact of UV exposure on the cornea and lens of the vertebrate model species *Danio rerio*. It was hypothesized that lens and cornea damages would be apparent through histological and SDS-PAGE analysis. Three individual 15-minute UV-B (301nm) irradiation periods were completed on 14 fish, and 6 control fish were left unexposed. To quantitatively determine if the UV radiation (UVR) affected the structure of the *Danio rerio* eye, measurements were taken of the corneal thickness, lens fiber thickness, and lens capsule diameter of the sagittally sectioned slides created through histology. The mean for the corneal thickness was significantly reduced from 49.2µm in control group to 17.9µm in animals exposed to UVR (p-value = 0.018). The mean values for the lens capsule diameter (p-value = 0.214) and lens fiber thickness (p-value = 0.855) showed no significant difference between exposed and unexposed eyes. The lens proteins were analyzed quantitatively by electrophoresis on the SDS-PAGE gel showing a loss of proteins at 22.3 and 45.1kDa in the exposed group. These sizes correspond to zebrafish α-crystallin and cytoskeleton proteins respectively, suggesting a loss of these proteins. These results support the hypothesis that UV-B radiation would affect vertebrate eye structure. Future studies should examine the implications of such UV exposure, and continue to utilize zebrafish as a model for human ocular disturbances.

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CHAPTER I

INTRODUCTION

Structure of Vertebrate Lenses

The vertebrate ocular lens is suspended in the eyeball, situated behind the cornea and iris, and is supported by the vitreous body (Figure 1). The slightly convex anterior surface of the lens is in contact with the pupillary margins of the iris. In most vertebrates, the primary support of the lens is provided by many suspended ligaments, commonly known as the zonular fibers, which extend from the equatorial rim of the lens to the surrounding muscle of the ciliary body. With relaxation of the ciliary muscle, the lens flattens, losing some of its convexity (Robinson, et al., 2004). This variation of lens convexity is what allows a clear image of an object to be focused on the retina.

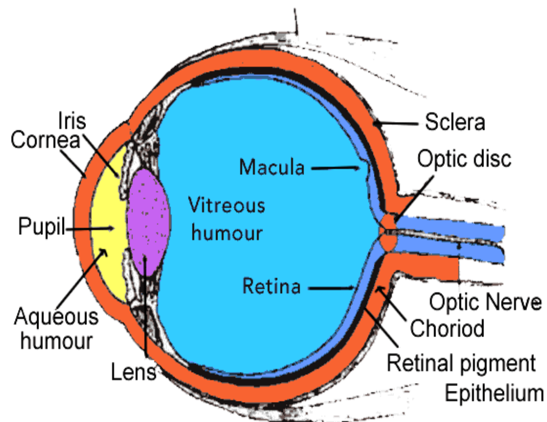


Figure 1: Human Adult Eye Anatomy (Haines 2005).

The anterior segment of the vertebrate eye is comprised of the cornea, lens, iris, ciliary body, and highly specialized tissue at the iridocorneal angle. Two main functions are ascribed to the ocular anterior segment: (1) to focus incoming light onto the neural retina and (2) to regulate intraocular pressure. For mammals and other terrestrial vertebrates, refraction of light entering the eye is accomplished by both the transparent cornea and lens. In many aquatic vertebrates, including fish, the lens is mainly responsible for focusing incoming light (Soules et al., 2005).

Development and Function of Vertebrate Lenses

The vertebrate eye is derived from three types of embryonic tissue. From the neural tube, known as the neuroectoderm, the retina proper and its associated pigment cell layer is developed. The mesoderm of the head region produces the corneoscleral and uveal coats, while the lens is fabricated from the surface ectoderm (Caceci, 2001). This eye ontogeny has been most thoroughly examined in chickens and mice (Evans et al., 2005). A group of surface ectodermal cells immediately overlying the developing optic vesicle are induced to thicken and form the lens placode (Kuszak et al., 2004). In humans, this process begins around day 22 of development (Caceci, 2001). The lens placode is a region that is visible on the surface of the embryo, once its formation has begun, the expanding optic vesicle begins to enclose to form a cup-shaped structure, (Caceci, 2001) known as the optic cup. Figure 2 below presents a light microscopic image of the eye of a 41-day-old human embryo.

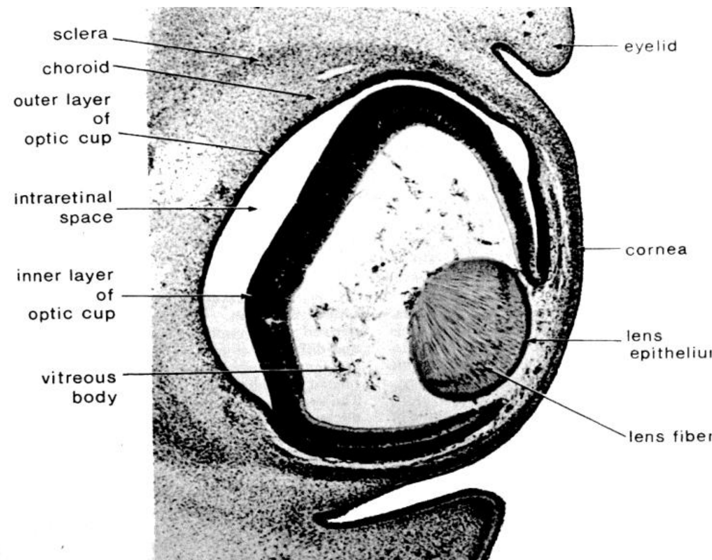


Figure 2: Light microscopic image of the eye of a 41-day-old human embryo. The retina has begun to develop; the intraretinal space will eventually be obliterated. The lens has detached from the surface and the cornea is forming from the thickened ectoderm (Caceci, 2001).

Zebrafish eye formation progresses in a similar manner to mammalian and avian development, with lens formation beginning at 16–20 hours post-fertilization (hpf) when the optic vesicle contacting the overlying head ectoderm. By 24–26 hpf, the lens has detached from the surface ectoderm and invaginates into the optic cup, forming the presumptive lens (Evans et al., 2005). As the lens develops, the central core appears significantly different from the cuboidal epithelium lining the lens periphery. The concentric organization of lens fiber cells becomes apparent by 36 hpf, and by 48 hpf differentiated primary fibers that have lost their nuclei and organelles have begun to appear at the lens core. Subsequent cell differentiation of secondary fibers derived from the lateral lens epithelium eventually leads to the formation of a mature lens that allows for functional vision by 3 days post-fertilization (dpf), as shown in Figure 3 (Evans et al., 2005).

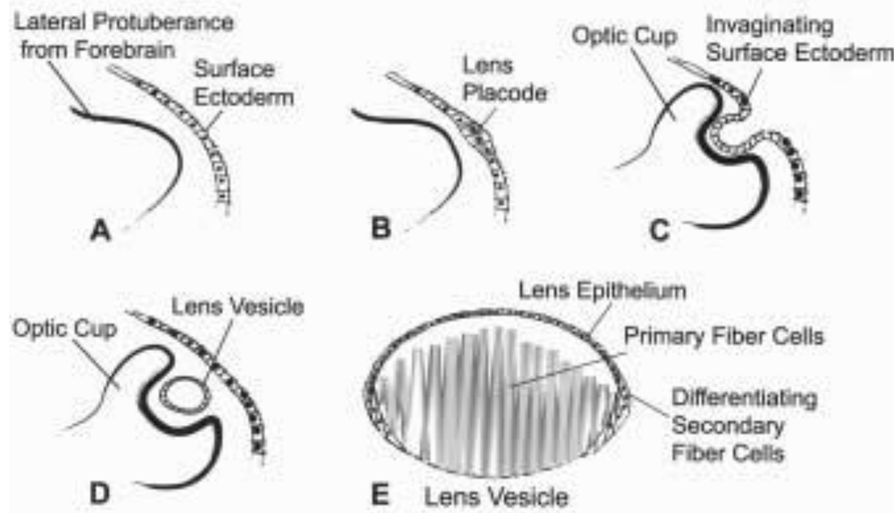


Figure 3: Early stages in the formation of the lens vesicle (A–D) and the lens (Bhat, 2001, pp. 541).

Cells of the lens vesicle are induced to differentiate in an inside-to-outside fashion. A significant structural consequence of the terminal differentiation is the original lens vesicle cells are transformed into elongated, primary fiber cells (Kuszak et al., 2004). These fibers are classified as either cortical or nuclear fibers. The lens produces fibers of defined shape and size and arranges these cells into radial cell columns and growth shells throughout a lifetime (Kuszak et al., 2004). As the lens fiber cells are retained, the entire life history of the lens is conserved in the tissue (Robinson et al., 2004). Cells in the anterior segment of the lens vesicle differentiate into epithelial cells, which continue to proliferate, allowing the lens to grow (Lang et al., 2004).

The divergent fates of an embryonic lens cells give the lens its distinctive polarity (Lang et al., 2004). The polarity is very important in maintaining the ordered cellular structure that contributes to the transparency and optical properties of the lens (Lang et al., 2004). From this stage on, the lens grows by continued proliferation of epithelial cells and differentiation of fiber cells. The growth patterns of the lens ensure that polarity is

maintained as new fibers continue to differentiate and are added to the fiber mass throughout life (Lang et al., 2004).

Since these cells accumulate over time, the transparency of the lens is closely linked to the structure and function of its cell membranes. To minimize light scattering, lens fiber cells are packed together in a tightly ordered array so that the space between the cells is smaller than the wavelength of light (Kistler et al., 2004). Age-related changes in lens crystallins, cytoskeleton, and cell membranes can make the lens incapable of preserving its necessary structure-function routine indefinitely, which can lead to common lens pathologies, such as cataract and presbyopia, a condition in which the lens loses its ability to focus. (Kuszak et al., 2004).

Transcription Factors and Genes Involved in Lens Formation

Additional insight into the development of the ocular lens can be gained by looking at the molecular events involved in this process. Genes from different classes of transcriptional factors are involved in early vertebrate lens formation. Table 1 displays specific transcriptional factors involved in lens development and their function. As the field of developmental eye research continues to advance in years to come, additional transcription factors will likely be found to be involved in eye and lens formation.

Table 1: Transcription factors involved in lens development (based on Goudreau et al, 2004).

Gene Family	Subfamily	Transcription Factor	Species	Loss-of-Function Phenotype	Gain-of-Function Phenotype	Assumed Function/ Target Genes
Homeobox (DNA-binding)	Pax	Pax6	M, Hu, Xe	Heterozygous: small eye Homozygous: arrest at optic vesicle stage, no lens	Ectopic eye structures, ectopic lenses	Lens placode formation/ + crystallins, + Pax6, Sox2, Six3, Prox1
	Six	Six3	M, Hu	Not determined	Ectopic eyes, including ectopic lenses	+Pax6
	Prox	Prox1	Xe, Ze, M, Hu	Defects in lens fiber formation	Not determined	Cell cycle arrest, cell elongation/ +γD- crystallin
	Pitx	Pitx3	Xe, M, Hu	Aphakia (absence of crystalline lens)	Not determined	Lens differentiation
	Otx	Otx1	Xe, Ch, M, Hu	Eye defects, no lens defect	Not determined	
		Otx2	Xe, Ch, M, Hu	Lethal at high levels	Not determined	Specification of eye field
		Otx1/Otx2	Xe, Ch, M, Hu	No lens or impaired lens differentiation	Not determined	Control of morphogenetic movements
	LIM	Lhx2	M, R, Hu	No eye differentiation beyond optical vesicle stage; no lens placode formation	Not determined	+ in surface ectoderm Pax6 via secreted factor
	Rx	Rx	Xe, Ch, M, R, Hu	No optic vesicle	Hyperproliferation of NR and RPE repressing lens tissue	
	Sox	Sox1	Ze, Ch, M, Hu	No lens fiber elongation	+ on all δ-/γ-crystallins	Cell cycle arrest
		Sox2	Ze, Ch, M, Hu	Preimplantation lethality		
		Sox3	Ze, Xe, Ch, M, Hu	Not determined		
bZIP	Maf	c-Maf	Ze, Ch, M, R, Hu	No lens fiber elongation due to less γF-/β-crystallins	Not determined	+γF-/β-crystallins
HSH	AP	AP2α	M, Hu	Anophthalmia (absence of one or both eyes), or lens defects	Not determined	Cell adhesion

Key: Ch=Chicken, Hu=Human, M=Mouse, R=Rat, Xe=Xenopus, Ze=Zebrafish

Comparative Lens Structures of Amphibian, Fish, Reptiles, Birds and Mammals

The simplest vertebrate eyes belong to cyclostomes, a class of primitive, jawless vertebrates including hagfishes and lampreys. These simple eyes contain a large primitive lens not attached to the walls of the eyeball but fixed in place by the cornea and the vitreous humour (Robinson et al., 2004). In terrestrial vertebrates, the cornea contributes to the focusing and protection, strongly refracting most of the incident light rays. The corneal epithelium is responsible for protecting deeper corneal structures in the eye from damage, like ultraviolet exposure (Podskochy, 2004). However, in aquatic vertebrates, such as fishes, most of the focusing is performed by the ocular lens, and, in most cases, the cornea is not relied on for refraction (Schwab, 2007). As a result, the lenses of fish are very large, almost spherical, and have a higher refractive index than that of any other vertebrate lens (Robinson et al., 2004). The cornea of the fish is flattened, used mostly for protection, and because of immersion, nearly ineffective for focusing, leaving the lens to focus light on the retina (Schwab, 2007). Since the lens is serving dual purposes, it needs to be situated far forward in the ocular globe, bulging through the pupil and dwelling close to the cornea. In contrast, land vertebrates have a less rounded lens, relatively flat in higher vertebrates, that is situated farther back in the eyeball. The spherical shape of the fish lens and its close proximity to the cornea is also observed in amphibians in their larval stages. As amphibians began to exploit terrestrial niches, the burden of focusing light was partially accomplished by the cornea, and the lens became situated posteriorly, behind the iris, and became somewhat flattened (Robinson et al., 2004). The lens of reptiles is also flattened anteroposteriorly (front-to-back) and has a greater convexity posteriorly. Reptiles are able to accommodate for near and far vision by making the lens

rounder (near sight) or flatter (far sight) through utilization of muscles that do not exist in amphibians or fish (Robinson et al., 2004).

Birds, throughout their evolution have attained the highest degree of vertebrate eye specialization, shared only with mammals. The eyes of birds are designed in the same general way of reptile development. Though, one of the most significant improvements in the eyes of birds (as well as mammals) is the positioning of the lens, which is brought forward toward the cornea, allowing for an increased image size on the retina. Because of its flattened shape, the avian eye is capable of maintaining an entire visual field in focus at any one time, giving it a major advantage over the rounder mammalian eye where their point of focus is limited to a small area of the visual field (Robinson et al., 2004). The mechanism of accommodation is essentially similar in reptiles, birds, and mammals. Reptiles and birds have a well-developed ciliary body containing a ring of pad-like processes which make contact with the lens periphery. With contraction of the ciliary muscle, these processes push on the lens, forcing it to take on a rounded shape suitable for close-up vision. In more advanced land mammals, the lens is suspended by the zonular fibers that attach to the ciliary muscles, so the ciliary body is not in direct contact with the lens. Contraction and relaxation of the ciliary muscles alter the tension on the zonular fibers which allow the lens to alter its shape for near and far vision (Robinson et al., 2004).

One thing that remains constant among vertebrates is the manner in which the different parts of the eye, especially the lens, continue to develop, differentiate, and grow throughout the entire life of an organism. The lens' rate of development is rapid during the embryonic stage and early development, but gradually slows down as the growth of

the whole organism slows. Very few mitotic divisions are seen in epithelial cells of an adult, but these cells do divide rapidly when the lens is damaged and attempts to regenerate (Bloemendal, 1977).

Lens Proteins

The lens must be extremely dense to refract light in the vitreous humor in which is it suspended. The necessary density is achieved by the presence of the crystallin proteins (Duncan et al., 2004). Crystallin proteins are a large family of proteins that constitute 90% of the soluble protein in lens fiber cells. Crystallin is a protein that is essential for maintaining the transparency of the eye's lens by forming a crystallin array (Mostafapour, 1978). Lens crystallins are expressed at high concentrations in lens cells to achieve the high index for refraction required for normal optical function. Since most proteins would aggregate and strongly scatter light long before accumulating such high concentrations, the crystallins are believed to have a number of special properties that allow for the lens to be transparent (Duncan et al., 2004). Crystallin proteins are arranged in short-range, glass-like order in the cytoplasm and are vital for the development and maintenance of lens transparency (Greiling et al., 2009). Figure 4 below shows the crystal structure of αA and αB-crystallin polymerized in their ordered assembly to maintain transparency.

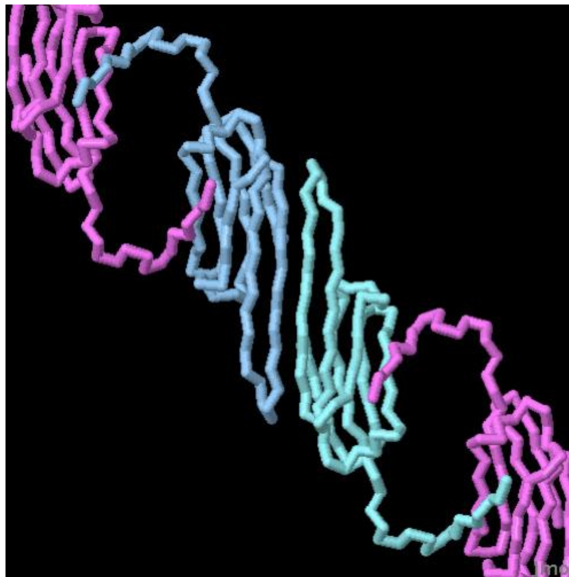


Figure 4: Polymerized structure of αA-crystallin revealing the short-ordered, glass-like structure needed for lens transparency (Goodsell, 2010).

The α-, β-, and γ-crystallins are the major protein components of the vertebrate lens. The predominant type is α-crystallin, which accounts for up to 40% of the protein content of the mammalian lens, contributes structurally to the transparency and refractive index of the lens necessary for clear vision, but also has molecular chaperone-like activity (Bloemendal et al., 2004). Alpha-crystallin is found as a heteroaggregate of two proteins in the lens, αA-crystallin and αB-crystallin. Both proteins are members of a small heat shock protein family, which are typically stress inducible and act as molecular chaperone-like proteins by binding to and preventing the aggregation of denaturing proteins. Unlike true molecular chaperones, α-crystallins are not able to refold non-native proteins. The α-crystallins' chaperone-like activity also prevents the aggregation of aging and stressed proteins that would otherwise lead to lens opacity, known commonly as cataract (Dahlman, 2005). Beta- and γ-crystallins serve only as structural proteins in the lens. Both β- and γ-crystallins are comprised of several proteins related by sequence and structure (Sharma et al., 2009). Unlike α-crystallins, no nonrefractive physiological

functions have been directly ascribed to the β- and γ-crystallins. However, it is likely that nonrefractive functions do exist (Duncan et al., 2004).

Since a primary function of crystallins is to focus light on the retina by maintaining the necessary refractive characteristics of the lens, modifications of the crystallins can have adverse affects on the clarity of vision. Changes in β- or γ-crystallin size and composition are precursors for cataract formation. These normally soluble structural proteins change composition and become insoluble to make the lens cloudy and aid in the formation of a cataract as shown in Figure 5 (Mostafapour, 1978).

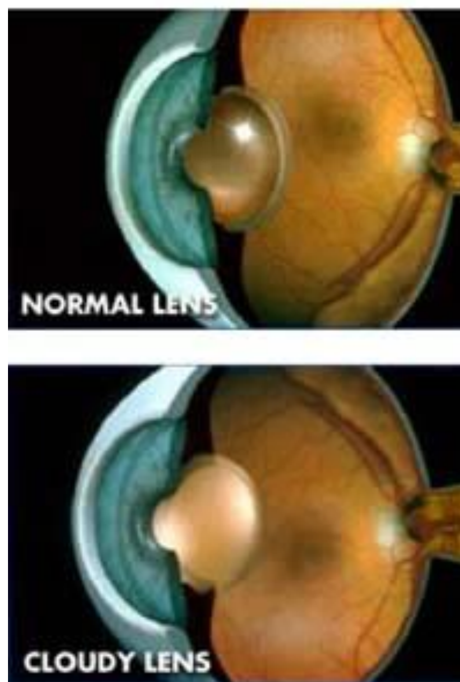


Figure 5: Comparison of a normal human lens and a lens clouded with aggregated β-crystallin proteins (Foley, 2008)

Lens proteins undergo very little turnover, but they do undergo various changes during aging and cataractogenesis, including increased crystallin proteolysis, fragmentation, and aggregation (Sharma et al., 2009). For the lens to be able to maintain life-long transparency in the absence of protein turnover, the crystallins must meet not

only the requirement of solubility associated with high cellular concentration, but that of longevity as well. For proteins, longevity is commonly assumed to be correlated with long-term retention of native structure. This retention can be due to inherent thermodynamic stability, efficient capture and refolding of non-native proteins by chaperones or a combination of both (Bloemendal et al., 2004).

There are many similarities in the optical and biophysical properties of zebrafish and mammalian lenses including the expression of many of the same crystallins. Both zebrafish and mammalian lenses contain αA- and αB- crystallins. The β-crystallin proteins are also similar between zebrafish and mammals, and it has been proposed that six β-crystallin genes are found in all vertebrates. The γ-crystallins are more evolutionarily divergent. Both zebrafish and mammals express γN- and γS-crystallins, and zebrafish additionally have multiple members of the γM-crystallin family of the aquatic crystallin in the lens (Greiling et al., 2009).

External Factors that Perturb the Lens

Several factors can perturb the development or overall function of the ocular lens. Table 2 lists numerous factors that have been found to effect the organizational or activational development of the crystallin lens.

Table 2: Factors Found to Perturb the Crystallin Lens.

Factor	Comment	Citation
Age-Related	Age-related changes in lens crystallins, cytoskeleton, and membrane render the lens incapable of preserving the necessary structure-function regimen.	Kuszak et al., 2004
Ultra-Violet Radiation	Exposure to ultraviolet light is an important factor through thermal or photochemical effects.	Cruickshanks et al., 1992
Eye Injury	Foreign objects can damage the crystallin lens.	Duncan et al., 2004
Family History and Congenital Disorders	Congenital cataracts can be the result of genetic abnormalities, the intrauterine environment, errors of morphogenesis, or a chromosomal abnormality. Diseases such as muscular dystrophy can also result in such perturbation.	Menko et al., 2004
Loss of Corneal Epithelium	Loss of the corneal epithelium causes damage to deeper underlying ocular structures such as the lens	Podskochy, 2004
Steroid Use	Both systemic and topical steroids are significant risk factors in the formation of posterior subcapsular cataracts.	Robman et al., 2005
Diet	Unhealthy diet, especially to the point of Type II diabetes, increases the risk of cortical cataracts. In addition, lack of antioxidants, vitamins A,C, & E, folic acid, selenium and zinc in the diet can affect the lens.	Robman et al., 2005
Smoking	Smoking increases the risk of nuclear cataract.	Robman et al, 2005
Gene and Protein Mutations	Lenses with mutations in the genes and proteins needed for proper development effect the development of the crystallin lens.	Duncan et al., 2004
Lack of Necessary Genes and Proteins	Lenses lacking the genes or proteins needed for proper development will be affected.	Duncan et al., 2004
Lens Depolarization	Depolarization of fiber cells causes an influx of ions and water, ultimately causing cell swelling	Kistler et al., 2004

Ultraviolet Radiation

Ultraviolet light (220-380 nm) is part of the electromagnetic spectrum and beyond the visible spectrum (Foley, 2008). Ultraviolet (UV) radiation is the portion of the electromagnetic spectrum between x rays (40-220 nm) and visible light (380-760 nm). UV light is absorbed by molecules known as chromophores, which are present in the eye cells and tissues. Chromophores absorb light energy from the various wavelengths at different rates - a pattern known as the absorption spectrum. If too much UV light is absorbed, eye structures such as the cornea, the lens, and the retina can be damaged (Di Girolamo et al., 2005). Unique hazards apply to the different sources depending on the wavelength range of the emitted ultraviolet radiation. The ultraviolet spectrum is further divided into UV-A, UV-B, and UV-C radiation.

UV-A radiation has a wavelength falling between 320-380 nm. It is the most commonly encountered type of UV light because atmospheric ozone absorbs very little of this part of the UV spectrum from the sun (Zeman, 2009). UV-A light is often called “black” light. Most phototherapy and tanning booths use UV-A lamps. UV-A exposure has an initial pigment-darkening effect (tanning) followed by erythema (sunburn) if the exposure is excessive. UV-A is needed by humans for synthesis of vitamin D; however, overexposure to UV-A has been associated with toughening of the skin, suppression of the immune system, and cataract formation (Zeman, 2009).

UV-B radiation has a wavelength between 290-320 nm. UV-B is typically the most destructive form of UV radiation to human and animal because it has enough energy to cause photochemical damage to cellular DNA, and yet is not completely absorbed by

the atmosphere. UV-B is needed by humans for synthesis of vitamin D; however, harmful effects can include erythema, cataracts, and development of skin cancer. It is well known that UV-B not only damages the skin, but also causes many ocular disorders—lid cancer, pterygia (external ocular mass), keratitis (inflammation of the cornea), photokeratitis , and cortical cataracts. UV-B also accelerates the inflammatory pathway (Holladay, 2005). Individuals working outdoors are at the greatest risk of UV-B effects. Most solar UV-B is blocked by ozone in the atmosphere, and there is concern that reductions in atmospheric ozone could increase the prevalence of skin cancer (Zeman, 2009).

UV-C radiation has a wavelength between 220-290 nm. UV-C is almost never observed nature because these wavelengths are absorbed completely by the atmosphere. Germicidal lamps are designed to emit UV-C radiation because of its ability to kill bacteria. In humans, UV-C is absorbed in the outer dead layers of the epidermis (Zeman, 2009). Accidental overexposure to UV-C can cause corneal burns, also known as “welders' flash”, and severe sunburn to the face and eyes, “snow blindness”. While a UV-C injury can be relieved in a day or two, it can be extremely painful (Zeman, 2009).

Ultraviolet Attenuation in Aquatic Environments

Aquatic environments vary tremendously in their UV attenuation. The proportion of transmitted sunlight in water depends on water depth; turbidity caused by organic and inorganic particles in suspension; optical properties as modified by the presence in solution of light-absorbing substances such as coloring materials and mineral salts; and wavelength of the incident radiation (Acra et al., 1990). Coastal areas and shallow continental shelf waters have a lower transparency than open ocean waters due to the runoff of silt and dissolved organic carbon (DOC) from shores. Due to the high input of

inorganic and decaying organic material, freshwater ecosystems usually have a high UV absorption (Hader, 2007). Besides inorganic particulate matter, dissolved and particulate organic carbon (DOC and POC) are the main attenuating substances in freshwater and coastal marine waters. Recent models analyzing the absorption of the components show that DOC mainly attenuates UV-B radiation while POC mainly decreases the UV-A radiation in the water column (Hader, 2007). The normal aqueous environment attenuates the majority ultraviolet rays after only a few millimeters, though health of marine plankton in the ocean as deep as 2 meters has been compromised by UV-B radiation (Sparling, 2001). The exponential attenuation values of UV radiation (200-400 nm) in distilled water are less than in seawater and range from 10 m at 200 nm to a minimum of 0.05 m at 375 nm. Values rise sharply in the visible and infrared regions of the spectrum, showing that solar UV-A has a greater penetration power in water than UV-B or visible light. However, the dangers of UV-B are of much higher concern and up to 10% of the solar UV-B intensity at the surface of clear seawater may penetrate to a depth of 15 m (Acra et al., 1990).

In the terrestrial eye, as previously stated, most of the refraction occurs at the air-cornea interface where the refractive index of air is approximately 1.0 and cornea is approximately 1.3. In a fish eye, refraction of light is minimal at the water – cornea interface where the refractive index of water is approximately 1.3 and nearly identical to the refractive index of the cornea. As a result, much more refraction occurs in a fish lens where the index of refraction is greater than in a terrestrial lens and it is anticipated that the refractive index of the zebra fish lens may be even higher (Greiling, 2008).

Zebrafish as a Model Visual System

The zebrafish (*Danio rerio)* has become an important vertebrate model in developmental neuroscience because it is a useful model for embryology, developmental biology, and genetic analysis. Given the extensive homology of its genome with that of other vertebrate species, including humans, and given the available genetic tools, zebrafish have become popular model organisms (Gerlai, 2003). Zebrafish produce a large number of offspring that can be kept in a small area and are fairly inexpensive to maintain. The similarities of its visual system to that of other vertebrates also make this animal a valuable model in vision science.

The anatomical, physiological, and behavioral components of zebrafish visual processing have been studied in adult and in developing zebrafish. The retinal anatomy of *Danio rerio* continues to develop following hatching, providing an opportunity to correlate the development of retinal structure with visual physiology and behavior. In addition, a number of genetic mutations have been developed which are used to examine the contributions of genetics to visual development and function making them a preferred subject of genetic analysis (Bilotta and Saszik, 2001). Because zebrafish have an ocular structure and organization resembling those of human, pathways underlying ocular development and physiological processes are also conserved between zebrafish and human. In particular, zebrafish have been used to investigate eye development, glaucoma, retinal degeneration, and cataracts. Also, the genes for a large number of lens crystallins, the major structural and protective protein of the lens, have been cloned for zebrafish (Posner et al., 2008). Zebrafish provide a useful model for identifying and characterizing

genes relevant to ocular development, formation, and human diseases (Wang et al., 2008) they have been chosen as the focus of this senior study.

Purpose

This study examines the effects of UV-B radiation on eye structure and lens composition in *Danio rerio*. After controlled UV irradiation periods, it is hypothesized that damages will be seen to the histological structure, particularly the cornea and lens, and changes will be apparent in the protein composition.

CHAPTER II

MATERIALS AND METHODS

Animal Husbandry

Twenty *Danio rerio* adult fish were purchased from Aquarium (Knoxville, TN; 865-588-2073), and their exact ages were not known. Two clean 5-gallon tanks were filled with water dechlorinated with Start Right Complete Water Conditioner by Jungle (1/2 cap/ 5-gal). Two heaters (Tetra Whisper Heater Calentador) were inserted into each full tank to maintain a 28.5°C water temperature. In order to prevent temperature shock, the fish were acclimated slowly (2 hours) before they were released into the breeding net in the 5-gallon control tank. Fourteen *D. rerio* were randomly selected and moved to the experimental tank.

Fish were fed twice daily with brine shrimp hatched from frozen eggs (Brine Shrimp Direct) and Tropical Fish Flakes (Wardley). To obtain brine shrimp from frozen eggs, 1.0 g of frozen brine shrimp eggs was introduced into a 1.0 L evacuation flask filled with water and 25.0 g of Instant Ocean. An air stone was inserted into the evacuation beaker and a lamp was set up nearby for 24-hour illumination for optimal hatching. Tanks were cleaned twice a week using a vacuum method, and soiled water was replaced with fresh, dechlorinated water. No more than half of the tanks' total volume was

siphoned out at one time. The room maintained a 14:10 photoperiod throughout the experiment.

Exposure

Three randomly selected experimental fish were transferred into a large watch glass containing 45-mL of clean tank water. A 301 nm ultraviolet lamp was situated 7.5cm from the top of the work bench and 5.5cm from the top of the water level in the watch glass. Fish were irradiated for a total of 15 minutes. The experiment was repeated with all 14 subjects in the experimental tank. After exposure, fish were returned to their designated 5-gallon tank. The exposures were completed 2 more times with a 24-hour wait period between exposures for a total of 45 minutes of UV irradiation.

Three control fish were also transferred into a large watch glass containing 45-mL of clean tank water. They were placed on the work bench, for a total of 15 minutes without UV illumination before returning to their designated 5-gallon tank. The procedure was repeated with all six control fish, and conducted 2 more times for a total of 45 minutes with a 24-hour wait period in between procedures.

Tissue Preparation

After three individual 15-minute exposures under the ultraviolet light, the *D. rerio* were euthanized using 400-g/mL ethyl 3-aminobenzoate methanesulfonate salt, also known as MS 222 (Sigma). Immediately after euthanization, a dissection microscope and small forceps, were used to remove eyes from fish and eyes were placed into Bouin's fixative compound (Labchem Inc, Oct 2005) in a 1.0-mL centrifuge tube. After 1 week fixation, eyes were cleared with 70 % ethanol and prepped for histology by embedding

them into wax blocks with the procedure outlined in Table 3. One eye was embedded to produce sagittal sections, whereas the other was embedded to produce transverse sections of the eye.

Table 3: Prep method followed for wax embedding of the dissected, exposed eyes.

Solution	Time (min)
80% EtOH	50 mins
95% EtOH	60 mins
100% EtOH I	45 mins
100% EtOH II	45 mins
Citrisolv I	45 mins
Citrisolv II	45 mins
Wax I	45 mins
Wax II	45 mins
Wax III	45 mins
Wax IV	45 mins

The wax-embedded eyes were allowed to harden overnight and were removed from the metal mold. A Shandon Finesse 325 microtome (Thermo Electron Corp) was used to section the wax block into 12 micrometer thick ribbons. A warm water bath was prepared in the Fisher Tissue Prep™ Water Bath 134 with a pinch of unflavored gelatin (Knox). After sectioning the entire eye, slide length sections of ribbon were cut and floated in the water bath. Ribbons were situated onto Fisherbrand® Superfrost® pre-cleaned microscope slides and placed in the slide warmer (Fisher) at 32°C and remained on the warmer overnight.

Staining

Slides were placed into slide caddies, and the hematoxylin and eosin staining method was utilized for the tissue staining in this experiment (Presnell, 1997). This procedure is outlined in a flowchart in Figure 6. Upon completing the procedure, slides

were removed from the caddy and Permount (Fisher Scientific) was applied to the slides. Slide covers (Fisher Scientific) were set in place on the Permount and allowed to set overnight.

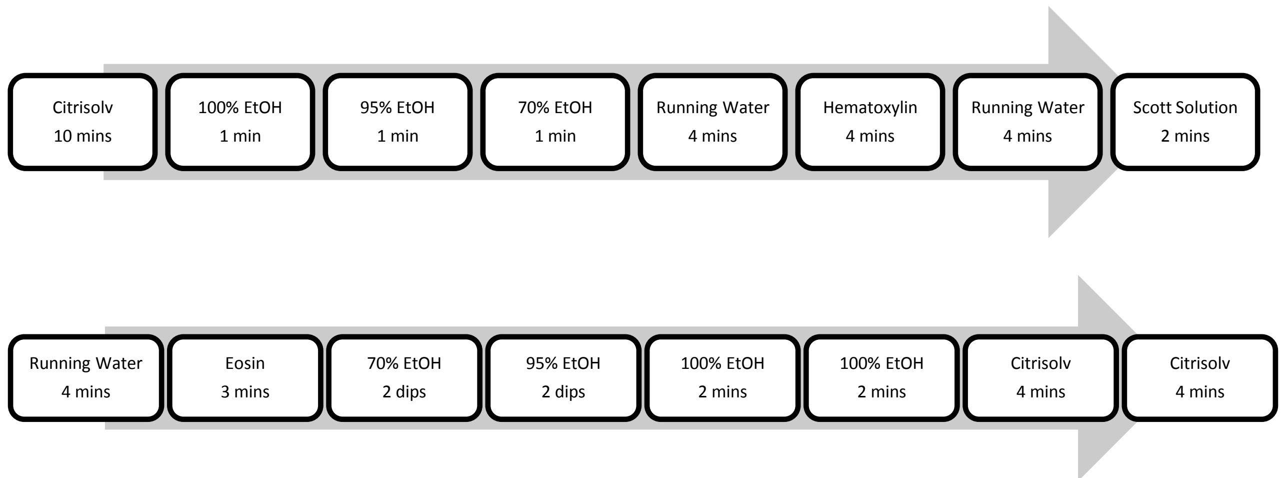


Figure 6: Flow chart outlining the Hematoxylin and Eosin tissue staining procedure.

SDS-PAGE Procedure

Using micro-dissection tools, 11 exposed lenses and 6 unexposed, control lenses were extracted after euthanization. Freshly extracted experimental and control lenses were suspended in 100µL of phosphate buffered saline (PBS; 10mM, 0.9% NaCl, pH 7.4). The lenses were vortexed vigorously and pulled through a 20 gauge needle for homogenization and then centrifuged (Eppendorf Centrifuge 5415-D) for 10 minutes at 14,000rpm. This procedure did not sufficiently homogenize the lenses, and experimental tube cracked during centrifugation. The supernatant was lost. However, whole lenses were visible still intact and unhomogenized at the bottom of the cracked tube. These lenses were resuspended in 100µL of PBS in a new tube and centrifuged again for 10 minutes at 14,000rpm. Lenses and supernatants (S1) of each group were then put into separate microfuge tubes and frozen overnight. After 24-hours, lens protein pellets were

thawed and resuspended in 150µL of PBS, vortexed vigorously, and transferred into 1.5mL microfuge tubes for homogenization through sonication. These lens solutions were insulated with ice and sonicated with 5 10-second pulses (Natural Scientific UltraSonics, Inc Model W-375 Sonicator), with 30-second cool-down periods between pulses. Ten microliters of water was added to 2 protein standards (Thermo Scientific Pierce ® Blue Protein Molecular Weight Marker Mix). All samples were centrifuged (Thermo Electron Corp Centrifuge) for 10 minutes at 14,000rpm after sonication. Supernatants from this round of centrifugation (S2) were transferred into a new 1.5mL tube and pellets were resuspended in 50 µL of Laemmli SDS-PAGE (250 mM Tris, 10% SDS, 1 mg/mL bromophenol blue, 50% glycerol, 5% β-mercaptoethanol, pH 6.8) loading dye. Fifty microliters of experimental and control lens supernatants (S1 and S2) were combined with 50µL of loading dye in four new centrifuge tubes. All protein-dye samples were incubated in a beaker of boiling water bath for 10 minutes. Fifty microliters of each of the experimental and control lens resuspended pellets, 50µL of supernatant (S1 and S2) samples, and 10µL of the standards were loaded into the prepared sodium dodecyl sulfate (SDS) polyacrylamide gel (Thermo Scientific 12% Precise Protein Gel) wells. The gel was run for 30 minutes at 180 volts in a BupH Tris-HEPES-SDS running buffer (Thermo Scientific). The gel was then stained with 0.25% Coomassie Protein Stain (0.25% Coomassie Dye, 40% methanol, 10% acetic acid) for 20 minutes. The gel was destained with Coomassie Destain (30% methanol, 10% mL acetic acid) and left overnight.

Data Analysis

To quantitatively determine if the ultraviolet radiation affected the structure of the *Danio rerio* eye, the slides created through histology were examined. The center of each

sagittally sectioned eye was selected for both experimental and control measurements. The ocular micrometer on the microscope was calibrated for conversion to micrometers, and the coronal thickness, lens capsule diameter, and lens fiber thickness was measured at 40x magnification for each selected center. Measurements were taken on the center of each sagittally sectioned eye. The corneal width was measured at the centermost point of the cornea. The lens capsule diameter was measured in the center of the lens sphere and parallel to the cornea, and covered the entire distance of the lens. The lens fiber thickness was measured at the same beginning location as the capsule diameter (centered and parallel to the cornea), but only covered the distance of the lens fibers. Figure 7 below outlines exactly what was measured for each sagittally sectioned slide. For each measurement, data for experimental and control animals were compared using a two-tailed t-test assuming equal variances ($\alpha<0.05$). Graphs were constructed to provide

visual representation of the results.

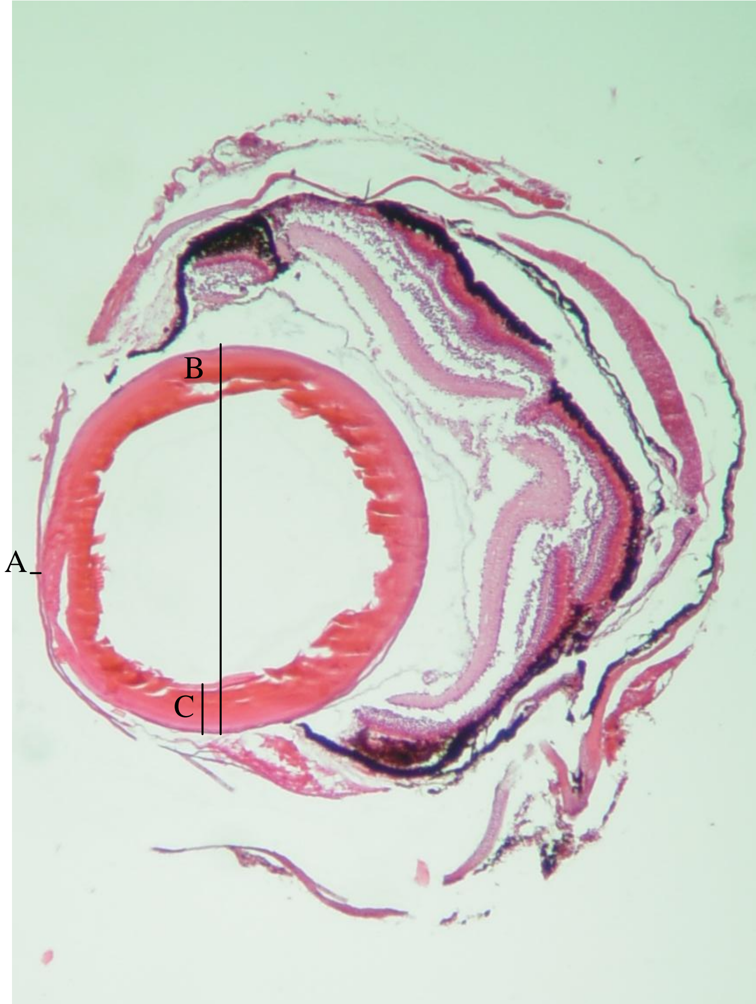


Figure 7: Example of measurement technique on center of sagittally sectioned *Danio rerio* eye. Measurements were recorded at 40x magnification of coronal thickness (A), lens diameter (B), and lens fiber thickness (C).

The lens proteins were analyzed quantitatively by electrophoresis on the SDS-PAGE gel along with a protein molecular weight marker. The migration distance of the lens protein bands were compared with the standard ladder. The marker contained the proteins in Table 4 with the listed known sizes.

Table 4: Proteins and their known sizes (kilodaltons) in Thermo Scientific Pierce ® Blue Protein Molecular Weight Marker used in SDS-PAGE for the electrophoresis of lens proteins.

Protein	Size (kilodaltons)
Myosin	208
Phosphorylase B	114
BSA	81.2
Ovalbumin	47.9
Carbonic Anhydrase	31.5
Trypsin Inhibitor	24.8
Lysozyme	16.6

CHAPTER III

RESULTS

The results of three individual15-minute exposures of ultraviolet irradiation on the zebrafish eye structure were examined by comparing the mean values of the experimental and control coronal thickness, lens capsule diameter, and lens fiber thickness. The results were also quantified by comparing the migration of the experimental and control lens protein bands to the known bands on the standard marker.

Three of the 14 experimental zebrafish died after 30 minutes of exposure to ultraviolet light. Prior to death, fish experienced retarded swimming rates and remained near the bottom of the tank at all times during the day, even for feeding.

Tissue Analysis

Histological analysis was performed on the cornea and lens of 6 treated and 4 untreated *Danio rerio.* Table 5 presents the mean and standard error of the corneal thickness, lens capsule diameter, and lens fiber thickness. The mean for the corneal thickness was significantly reduced from 49.2µm to 17.9µm in animals exposed to ultraviolet radiation (Figure 8; p-value = 0.018). The mean values found for the lens capsule diameter (p-value = 0.214) and lens fiber thickness (p-value = 0.855), Figures 9 and 10 respectively, showed no significant difference between exposed and unexposed eyes.

Table 5: Data collected from measurements taken from slides at 40x magnification from six *Danio rerio* eyes exposed to 45 mins of ultraviolet radiation and four control, unexposed eyes.

Specimen	Corneal Thickness (µm)	Lens Capsule Diameter (µm)	Lens Fiber Thickness (µm)
Exposed 1	25.0	825.0	75.0
Exposed 2	12.5	825.0	75.0
Exposed 3	25.0	970.0	100.0
Exposed 4	25.0	772.5	100.0
Exposed 5	7.5	780.0	82.5
Exposed 6	12.5	850.0	92.5
Mean of Exposed	**17.9**	**837.1**	**87.5**
Control 1	50.0	750.0	100.0
Control 2	72.5	397.5	62.5
Control 3	25.0	825.0	75.0
Control 4	n/a	875.0	105.0
Mean of Control	**49.2**	**711.9**	**85.6**

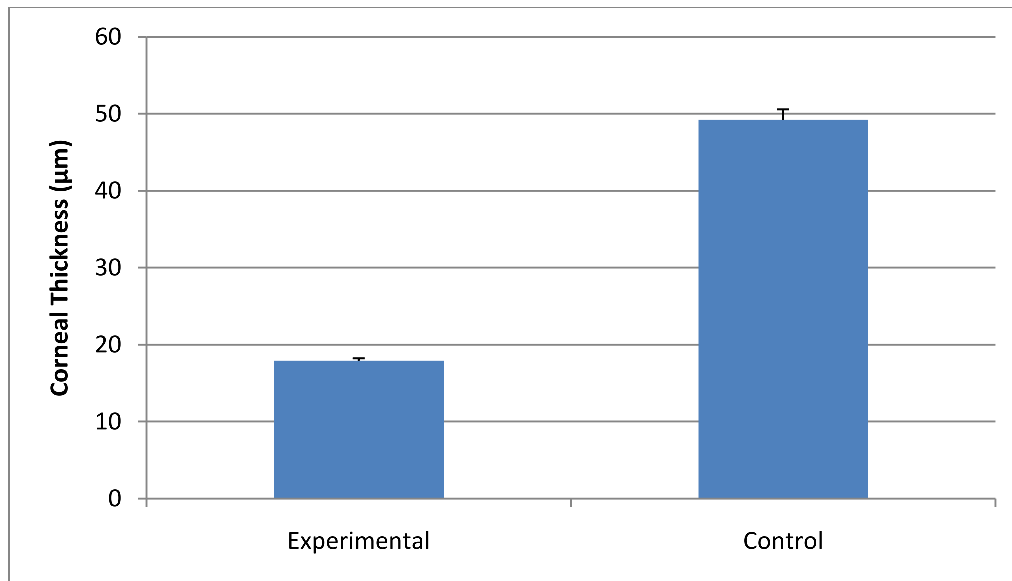


Figure 8: Average corneal thickness (+1SE) of control and ultraviolet radiation exposed *Danio rerio* eyes at 40x magnification.

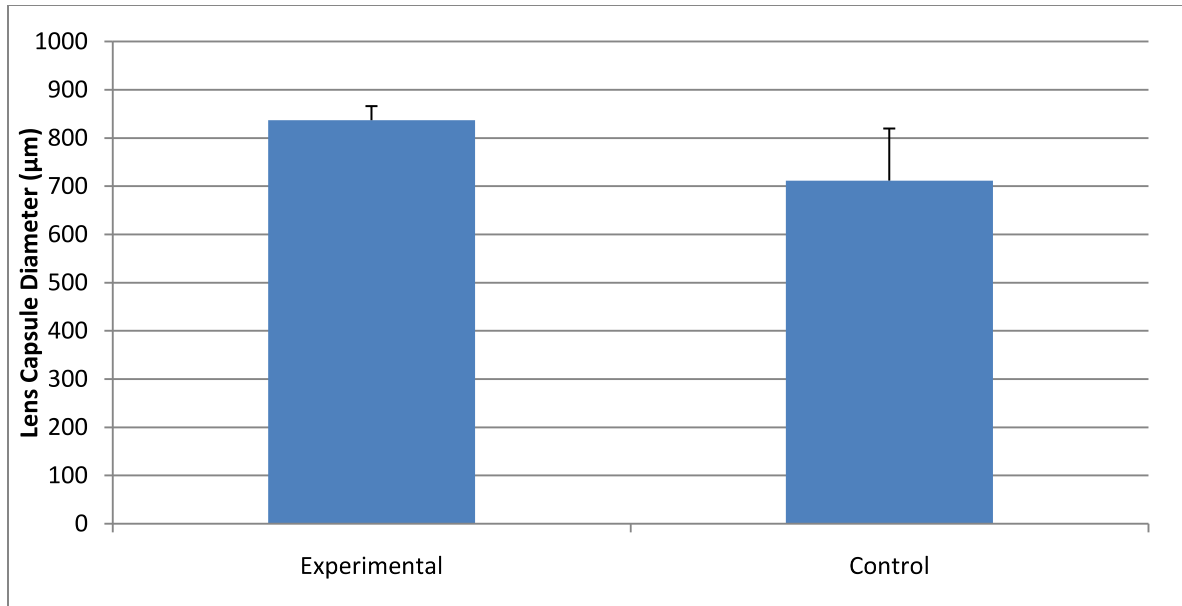


Figure 9: Average of lens capsule diameter (+1 SE) of control and ultraviolet radiation exposed *Danio rerio* eyes at 40x magnification.

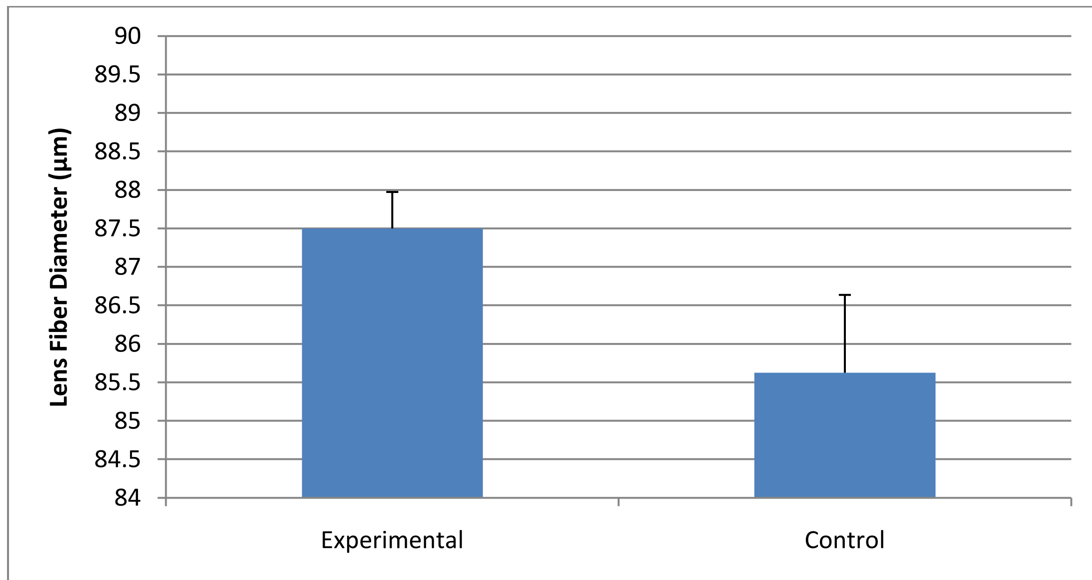


Figure 10: Average lens fiber thickness (+1SE) of control and ultraviolet radiation exposed *Danio rerio* eyes at 40x magnification.

Protein Analysis

To determine if the ultra violet exposure yielded an effect on the lens crystallin proteins, the SDS-PAGE procedure was utilized. A photograph of the gel obtained from this process is displayed in Figure 11.

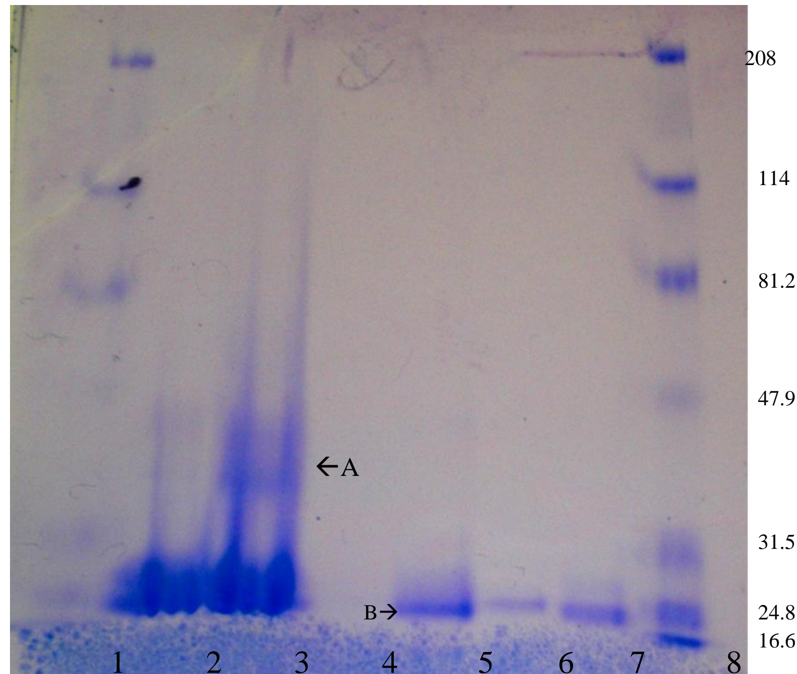


Figure 11: SDS-PAGE Analysis of Zebrafish Lens Proteins. Coomassie blue stained 12% SDS-PAGE gel of 10µL of each standard marker and 50µL of each ultraviolet radiation (45min) exposed and control sample of zebrafish lens proteins. The lanes labeled 1-8 contain the following: Lane 1: standard protein marker, Lane 2: exposed protein pellet, Lane 3: control protein resuspended pellet, Lane 4: exposed protein supernatant 1, Lane 5: control protein supernatant 1, Lane 6: exposed protein supernatant 2, Lane 7: control protein supernatant 2, Lane 8: standard protein marker. Arrow A indicates a presence of proteins in the control protein pellet that is not present in the exposed lane 2. Arrow B indicates the presence of proteins in the control supernatant 1 that are absent in the exposed supernatant 1 in Lane 3. The standard bands are labeled to the right of the figure with their molecular size in kilodaltons.

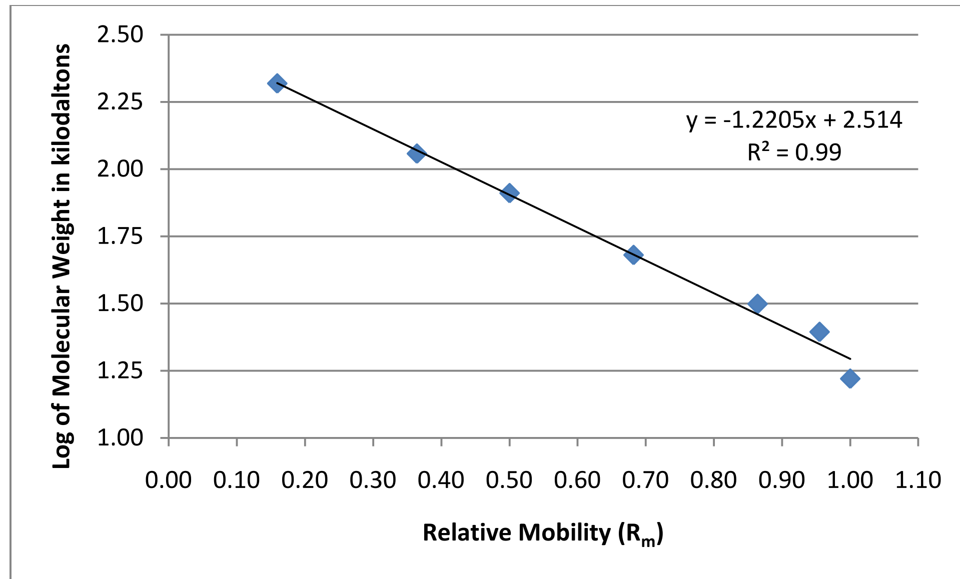


Figure 12: Standard curve of detectable zebrafish lens proteins using the log of the Thermo Scientific Pierce ® Blue Protein Molecular Weight Marker and the relative mobility of each distinguishable band of the standard ladder after 30 minutes of SDS-PAGE at 180 volts.

Using the line equation found from the standard curve, the molecular weight of the unknown band A of the control protein pellet was found to be 45.1 kilodaltons (kDa) using a migration distance of 3.10 cm of the protein band and a migration distance of the solvent front of 4.40 cm. The molecular weight was also determined to be 22.3 kDa for unknown band B using a migration distance of 4.20 cm.

CHAPTER IV

DISCUSSION

Summary

The data presented in this study show that zebrafish eye structure and lens proteins are in fact affected by ultraviolet radiation. Whereas cornea structure was affected by the acute irradiation period of 45 minutes, lens structure was not significantly damaged by the short exposures utilized in this experiment. The lens proteins, however, did show different banding patterns in the exposed lenses compared to the control, unexposed lenses. These findings support the hypothesis and further support the zebrafish as a model organism for ocular research.

Histological Lens Data

The data for lens capsule diameter and lens fiber thickness showed no significant change between the exposed zebrafish and the control group. Analyzing the measurement data taken from the slides produced from the sagittally embedded eyes, the two-tailed t-test ($\alpha<0.05$) revealed no considerable difference between the two groups of *D. rerio* for both lens capsule diameter and lens fiber thickness. The mean value for the lens capsule diameter for the irradiated eyes was 837.1µm and for the control the mean was 711.9µm. Statistically, this does not provide strong enough evidence to prove that the UV-B radiation altered the lens capsule structure, and any difference could most likely be

attributed to different sized eyes from different subjects in the control and exposed groups. Even though they were all mature adult zebrafish, their eyes were not identically sized. The mean of the lens fiber thickness changed from 85.6µm in the control eyes to 87.5µm in the experimental eyes. This suggests that the short irradiation period of UV-B radiation did not have substantial damaging affect on the lens fiber structure either. In a study of cataract from ultraviolet radiation conducted by Stefan Lofgren, M.D., it was found that the 300nm of UV-B radiation only penetrated 0.36mm into a human lens (Lofgren, 2001). With the measurement from the cornea to the zebrafish lens being approximately 0.13mm and the entire distance from the cornea to the back of the lens of a sagittally sectioned zebrafish eye being approximately 0.70mm (Fadool & Dowling, 2008), the light should have penetrated half of the lens depth. However, the attenuation of ultraviolet light by water would minimize this penetration of the UV-B into the zebrafish lens in the experiment. This helps to explain the lack of supporting evidence that the acute irradiation affected the lens structures. The aforementioned study also suggested because of the relatively shallow penetration of the lens that a focus on the aqueous humor or lens epithelium would be more efficient than whole-lens measurements (Lofgren, 2001).

Histological Cornea Data

The corneal thickness measurements are where the substantial difference was detected between the exposed zebrafish and the controls. With a difference of 0.031µm (Figure 7) between the mean values of the experimental and control, the data shows that the UV-B radiation damaged the exposed eyes and had an effect on the corneal thickness by reducing the measurement. These results seem logical, especially regarding the

Lofgren study (Lofgren, 2001). If the 300nm UV-B radiation can penetrate even partially into the lens, then it has fully penetrated the cornea and would have stronger, more damaging affects, even in an acute exposure period.

Lens Protein Data

The lens protein SDS-PAGE analysis resulted in two significant bands (A and B) represented in the control lanes that were absent in their corresponding exposed lanes (Figure 10). The crystallin subunits (α, β, and γ) have a molecular weight in the range of 18-32 kDa (Mathew et al., 2003), with zebrafish α-crystallin producing bands between 20 and 28 kDa (Dahlman et al., 2005). The molecular weight of band B was found to be 22.3 kDa (Lane 5), corresponding to the size of zebrafish α-crystallin. Its absence in the exposed lane 4 is evidence of some alteration, perhaps degradation, occurring to the crystallin as a result of the UV-B exposure. Band A corresponds to proteins with a molecular weight of 45.1 kDa. According to a study conducted by J.P. Mathew et al., a major decrease or loss of protein band above 45 kDa among lens proteins represent the loss of the high molecular weight cytoskeletal proteins of the lens. This loss of cytoskeletal proteins contributes to membrane damage and loss of fiber structure (Mathew et al., 2003).

The Lofgren study supports the idea that cytoskeletal proteins could have been lost. If the UV-B radiation penetrated shallowly into the lens, it would be reasonable to believe that the outer structure would be more affected than the inner structure. The change in these lens membrane proteins would also support the differences found in the

histological measurements, even though the t-test could not validate the certainty. Future studies could further examine these ideas and verify the differences found in this study.

Future Research

Future studies of zebrafish eye structure and protein composition are warranted. It would be interesting to see the effects on the eye with a longer exposure time. Given more exposure time, it would be advantageous to compare the refractive indexes of the exposed and unexposed lenses, expecting to see fluctuation in these values, as in the case of aggregate formation and cataract. The strength, shape, and rigidity of the lenses could also be assessed in future studies to analyze the effects of the UV-B radiation on these components of lens structure. Since this study had limited exposure time because of the mortality of the fish, future studies should explore the effects the ultraviolet radiation has on well-being of fish as a whole so longer, or more, exposure periods can be employed. Regarding the lens protein data, utilizing high performance liquid chromatography (HPLC), mass spectrometry, 2D-electrophoresis, or immunoblotting techniques would have further validated the findings of this experiment by confirming the identity of proteins in the lens extracts and adding supplemental information to the data already collected.

Conclusion

Using histological and SDS-PAGE techniques, the effects of UV-B radiation on zebrafish (*Danio rerio*) eye structure and protein composition were examined to further support its use as a model organism for the visual system. Overall, the results suggest that the UV-B had damaging effects on the eye structure after 45 minutes of exposure. This was present in the data for corneal thickness and lens proteins. The results for lens

capsule diameter and lens fiber thickness did not fully demonstrate damages relating to the exposure. Further research should be conducted to further elucidate the mechanisms through which ultraviolet light alters ocular function.

APPENDIX

MARYVILLE COLLEGE
Institutional Animal Care & Use Committee (IACUC)
Student Animal Research Form

Provide information after each bold item
Student Name: Hope Marlow

Student Email Address: hope.marlow@my.maryvillecollege.edu

Date: 2/5/2010

Senior Study Advisor: Drew Crain, PhD and Angelia D. Gibson, PhD

Species to be used: *Danio rerio* (commonly referred to as zebrafish)

Age of animals: embryos-1year

Number of animals in study: up to 30 adult fish; embryos up to 100 per day

Duration of study: 1 year

Location of animals during the study (building and room): Sutton Science Center Room 114

List personnel to call if problems with animals develop:

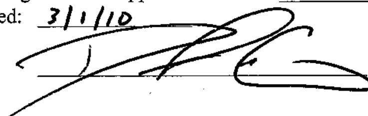
Name	Daytime Phone	Nighttime Phone	Emergency No.
Andrew Crain, PhD	865-981-8238	865-379-1706	865-850-5709
Angelia D. Gibson, PhD	865-273-8892	865-980-3572	865-414-5071
Hope Marlow	423-536-8556	423-536-8556	423-536-8556

Husbandry Requirements: Is anything other than routine care and equipment required?
YES___ No X If "YES", please list below.

What will happen to the animals at the end of the study? If euthanasia is required, state the methods.
Fish will be euthanized in a beaker containing 0.5% tricaine in water.

(Do not write below line: For MC IACUC Use)

Maryville College IACUC Approval Number: 201003
Date Approved: 3/1/10
Signed:



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