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Investigations into cataract formation in sciaenid fish species from the Elizabeth River, Chesapeake Bay, Virginia

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INVESTIGATIONS INTO CATARACT FORMATION IN SCIAENID FISH SPECIES FROM THE ELIZABETH RIVER, CHESAPEAKE BAY, VA.

A Dissertation

Presented to

The Faculty of the School of Marine Science

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Christopher D. Williams

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DEDICATION

I would like to dedicate this research and the completion of my Ph.D to all my family members: my sister Susan Williams and her husband Bill Wiles, their children Frankie, Alex, and Mikey; my sister Michelle and her husband John Weaver, their children Nick and Christie; my parents, especially my mom, Mrs. Blanche E. Williams and Mr. Leland C. Williams; and of course my wife, Cynthia Ann Horton-Williams and our children Samantha Marie and Mickey.

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Although no words could truly express my gratitude and feelings, I would like to give a special acknowledgement to my parents, Mrs. Blanche E. Williams and Mr. Leland C. Williams, as well as my wife, Cindi, for their undying support, confidence, and love.

Furthermore, I would like to thank God for watching

over me and blessing my family with a healthy little baby girl, Samantha Marie, that made this time in my (our) life even more special than it already is.

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ABSTRACT

Cataract formation in three important fish species (spot, *Leiostomus xanthurus;* Atlantic croaker, *Micropogonias undulatus;* and the weakfish, *Cynoscion regalis*) of the Chesapeake Bay has been reported since 1986. Development of lens opacification occurs predominantly in the Elizabeth River (ER) which is one of the most contaminated estuaries in the USA. Previous studies have concentrated on describing cataract formation as a function of exposure to polluted sediments that are heavily contaminated with polycyclic aromatic hydrocarbons (PAH). These studies, however, have not examined what processes might be involved in cataractogenesis. The overall objective of this research was to identify important processes associated with the early development of cataract in feral fish populations.

Field studies were used to systematically examine cataract development over time and their distribution. Cataract in sciaenids was not associated with previously described cataractogenic factors, but could be linked to contaminated sediments of the ER. Cataract was highly prevalent, first appeared at the visible center of the lens, varied in the degree opacification (0-100%), could be classified into stages of development (stage 1-4), and inhibited lens growth. Data showed that a positive association existed between bottom water temperatures (°C) and cataract prevalence.

Biochemical comparison of lenses with and without cataract revealed that the water content increased slightly, but significantly, in lenses that exhibited stage 1 opacification. Furthermore, lipid analysis showed that cholesterol levels and free fatty acids were significantly elevated while total esters were reduced in stage 1 lenses. Interestingly, no significant changes in the protein composition of lenses could be determined even in lenses with mature cataract (stage 4, 100% opacification). These data suggest that compositional changes may lead to modified plasma membrane permeability that could initiate lens opacification.

Investigations of cataract in fish at the cellular level have not been studied due to the absence of a reliable *in vitro* model. Protocols were established for the primary culture, successful passage (45 subcultures), and the long-term maintenance (54 months) of spot lens epithelial cells (SLEC). Cells exhibited certain morphological characteristics similar to those reported for epithelial cell differentiation *in vivo* and have maintained a normal diploid chromosome complement over time. SDS-PAGE and Western blot analysis positively identified α-, ß-, and γ-crystallins in long-term cultures. SLEC is a unique *in vitro* model as it exhibits characteristics of cellular differentiation and transformation into a cell-line.

In vitro experiments indicated that both DNA and RNA indices were positively associated with cataract development in spot lenses. Protein synthesis, on the other hand, did not appear to be influenced by cataract formation. Results suggested that high molecular weight PAH may play a role in interfering with the mitotic cycle of lens epithelial cells. These results suggest that epithelial cells are disturbed and may be involved in the initial steps of cataractogenesis.

In conclusion, results demonstrate that cataract development is associated with high levels of contamination found in the ER. Altered membrane permeability and epithelial cell function may play a key role in cataract initiation. Disturbances in the osmotic regulation of the lens could lead to cataract formation.

INVESTIGATIONS INTO CATARACT FORMATION IN SCIAENID FISH SPECIES FROM THE ELIZABETH RIVER, CHESAPEAKE BAY, VA.

CHAPTER 1

AN INTRODUCTION

Fish are by far the most numerically important class of all vertebrates accounting for over half (22,000) of all known species (Nelson, 1984). Visual capability in fish, as in most animals, is essential for predator avoidance, food acquisition, reproduction and maturation, migration, social interaction and organization, alarm signalling, and various other routine processes important to the overall success of most species.

Fish Eye Morphology

Fish eyes have evolved into various morphologies with different image reception capabilities dependent on the visual needs and environmental conditions to which particular species are exposed. Overall, the design and function of fish eyes varies greatly, from the highly limited (or even lacking) vision of cave dwelling and abyssal fish to the very sensitive perception of pelagic and piscivorous fish species.

While the basic plan of the teleost eye (Fig. 1) follows that of higher vertebrates, a few important differences do exist such as: 1) the location and the visual field of the ocular system; 2) the tissue organization of the ocular cavity; and 3) the morphology and accommodating potential of the lens. Species differences exist as well but, in general, certain modifications are routinely observed and are considered fish-associated structures.

Most fishes' eyeballs are located laterally and are separated by the snout region. This placement of the visual system gives rise to independent eye function. In some fish, a binocular field of view is possible dependent on the specific body morphometrics. Binocular vision allows for an extended field of view. Furthermore, the fish lens is positioned extremely close to the iris and actually protrudes through the pupil into the anterior chamber. The closer proximity of the lens to the cornea provides for a wider angle of vision and enhances visual coverage of the surrounding environment.

In many teleosts the ventral suture of the embryonic optic cup does not completely close. Hence, a portion of the choroid may protrude into the vitreous chamber extending toward the lens. This structure, called the falciform process, is usually highly vascularized and provides attachment for the retractor lentis muscle. Such a process is not found in the higher vertebrates.

Most finfish lenses are spherical or globular in shape whereas humans eye lenses are biconvex. The fish lens cannot change shape to accommodate and, therefore, in fish the whole lens must be moved in order to focus. In humans, on the other hand, the shape of the lens can be altered for better image detail.

The iris in most fish species cannot be varied to change pupillary diameter, as is the case in higher

vertebrates. Thus finfish cannot modify the amount of light entering the eye by this means. Hargis (1991) has described the iris in sciaenid fish species as being incomplete resulting in an open passage way between the anterior and posterior chambers. The iris does, however, contain light absorbing pigments, iridiophores, that can be manipulated to change density levels, allowing for the reduction of light entering into the vitreous chamber of the fish eye. This mechanism may be similar in function to that of pupil dilation.

In many finfish species there exists a region called the choroid space. Lying between the sclera and the choroid coat, and surrounding the retina and extending into the iris, this tissue contains many fine blood vessels and capillaries. In fish with such structures the vessels undergo extreme convolution and curving resulting in a mass of small blood vessels. The choroid gland and the persisting hyaloid blood vessels may provide a more direct pathway for plasma-borne materials to reach the lens rather than primarily via transport through the aqueous and vitreous fluids as is postulated for mammals.

In general, fish lack a Canal of Schlemm (a passage located at the junction between the cornea and the sclera that drains aqueous fluid from the anterior chamber in mammals) and substitute other mechanisms for maintenance of hydraulic balance within the eyeball. The interconnecting

chambers resulting from the incomplete iris, therefore, may be important in maintaining osmotic homeostasis.

The differences noted between teleost eyes and those of higher vertebrates do not detract significantly from the applicability of many findings on disease effects observed in eyes of mammals to those of fish and vice versa. Some distinct differences, however, must be taken into account. Fish eyes are generally unlidded, usually protrusive, and, in general, are constantly exposed to the aqueous environment making it susceptible to solubilized materials. Consequently, protection of the eye from potentially damaging external factors is much reduced. This inherent association between ambient conditions and the tissues of the fish eye allows for direct contact of water-borne material with the cornea and a direct pathway of intrusion into the internal ocular cavity.

Fish Lens Organization

The crystalline lens is a cellular structure that develops embryologically as a vesicular invagination of the surface ectoderm near the developing optic cup. Initially, the lens vesicle is a hollow sphere formed by a wall of epithelial cells. The cells of the posterior half of the sphere elongate to fill the lumen of the vesicle. Subsequent cell multiplication, both embryonic and post-embryonic, takes place around the lens equator, with new

cells elongating and compressing the old lens material towards the center. The result is the formation of a lens of variable refractive index, that of the center being higher than that of the periphery.

The fish lens (Fig. 2) is surrounded by a capsule which is composed of glucopolysaccharides which have been secreted and maintained by the underlying epithelial monolayer. The capsule is usually thickest anteriorly and thinnest posteriorly. The epithelial layer consists of the growing and dividing cells of the lens which eventually differentiate into fiber cells at the nuclear bow region. Fiber cells elongate, lose their nuclei and ability to replicate, initiate crystallin-protein production, and subsequently become almost entirely crystallin in composition. Young, recently generated fiber cells grow around the older more mature fiber cells like the rings of a tree. The youngest developing fiber cells make up the portion of the lens called the cortex while the older fiber cells become compacted and compose the central nuclear region of the lens. The process continues throughout the life of the lens but slows with advancing age similar to that reported for mammals.

Cellular Differentiation of Lens Epithelial Cells

Lens epithelial cells are characterized by a cuboidal shape and basophilic staining properties. Epithelial cells

outside of the bow region are non-mitotic and are considered to be responsible for osmotic regulation of the lens. Epithelial cells migrate into the zone of elongation (the bow region) where they become mitotically active and at some critical time initiate their transformation into fiber cells (non-replicating cells responsible for ß- and γ-crystallin protein synthesis). Upon the start of differentiation several cellular characteristics become observable, namely: 1) epithelial cells extend cytoplasmic processes anteriorly and posteriorly; 2) the nucleus and nucleoli enlarge; 3) the granular ribosomal population significantly increases in number adjacent to the enlarged nucleus; and 4) the nucleus is metabolically active and has the ability to synthesize both DNA and RNA. Upon terminal differentiation into a fiber cell several cytological and cytochemical changes have occurred that indicate that differentiation has been completed, such as: 1) the cytoplasm obtains acidophilic staining properties; 2) the nucleus and nucleoli decrease in size and the cells lose the ability to synthesize DNA and RNA; and 3) the endoplasmic reticulum becomes smooth in appearance with a subsequent reduction in population number.

Furthermore, molecular and electron microscopy studies have identified that there are major differences in macromolecular synthesis between epithelial and fiber cells. Fiber cells have a significantly greater amount of tRNA than rRNA and a much reduced amount of DNA compared to epithelial

cells. This increase in the amount of tRNA is considered to be a product of the breakdown of the ribosomal units upon terminal fiber cell formation. DNA synthesis in pre-fiber cells is considered to decline and finally cease as differentiation is completed. Thus, with the loss of nuclear activity in the fiber cell a stabilization of mRNA and a gradual breakdown of the ribosomes occurs. The synthesis of the tissue specific crystallins of the lens occurs on a relatively long-lived messenger RNA template. Stable mRNA is a common and important feature of highly differentiated cells which synthesize very specific proteins. The synthesis of crystallins in epithelial cells, that are differentiating into fiber cells, has been shown to occur on rapidly turning-over mRNA templates (Papaconstineau, 1966: Zigman, 1985).

Cataract Formation in Fish

Feral fish populations

Cataract is the opacification of the ocular lens. Light transmission into the eye is reduced and proper visualization is impaired. Cataract is one of the most recognizable and detrimental disorders of fish eyes. It is one of only a few health parameters that can be truly associated with a reduction in biological potential (Dukes, 1975). Historically, cataract formation has been considered a significant problem only in the display or culture of

certain freshwater finfish species (Hargis, 1991). With the increasing pressure of industrial and urban development in and around coastal areas, however, greater incidence of cataract formation is being reported in feral fish populations. Cataract has been reported to be caused by a number of factors, including: 1) parasitic infections (Uspenskaya 1961; Petrushevski and Shulman 1961); 2) nutritional deficiencies (Poston et al. 1977, 1978; Ketola 1979; Richardson et al. 1986; Barash 1982; Halver 1979, 1953; Hess 1935); 3) mechanical damage and trauma (Van Duijn 1967); 4) osmotic imbalance (Iwata et al. 1987 a,b); 5) biochemical disturbances (Hoffert 1966, 1970); and 6) chemical intoxication (Hargis et al 1986, 1988).

Cataract has been reported in the South American croaker (*Micropogonias furnieri*) off the coast of Brazil (Vazzoler and Phan, 1981) between Capes Frio and Torres, with the most heavily affected populations occurring in an estuarine-lagoonal system. Cataract formation in this instance was observed only in immature croakers (< 20 cm. total length) and was concluded to be connected with some physiological or metabolic factor determined by environmental conditions.

Recently, Fraser et al. (1989, 90) have described cataract formation in Atlantic salmon (*Salmo salar*), sea trout (*Salmo trutta*), saithe (*Gadus virens*), lythee (*Gadus pollachius*), and sand eel (*Ammodytes marinus*) collected from

the west coast of Scotland. Cataract formation was mainly observed in Atlantic salmon and was documented to increase from near negligible levels in 1979 to approximately 55% in 1987. This increase in cataract formation coincided with a subsequent increase in the growth of the salmon farming industry of this area and was suggested to be related to the exposure of fish to Nuvan (50% w:v Dichlorvos as the active ingredient), an organophosphate-based sea lice parasiticide. Cataracts appeared unilaterally or bilaterally, possibly developed within two months of chemical application, and ranged in morphology from a small U-shaped opacity deep in the core to an extended anterior opaque development. The authors suggested that fiber cells had become uncoupled through an increase in gap junctional resistance and that an inhibition of cholinesterase may be the mechanism by which this cataract formation occurs.

Laboratory induced cataract formation

Cataract formation has been induced in rainbow trout (*Oncorhynchus mykiss*) dietarily exposed to thioacetamide, a reported hepatic carcinogen (Von Sallmann et al., 1966). Cataract was observed in 90% of fish maintained on this diet during a 12 month trial period. Histological examination of these lenses showed a massive proliferation of the lens epithelium and its transformation into a pleomorphic cell mass which replaced a significant portion of the anterior

cortex. The relation of the epithelial proliferation to fiber damage, however, was not clear in the development of the lens pathology. Mayer et al. (1981) reported the formation of cataracts in rainbow trout fry (*Oncorhynchus mykiss*) which had been exposed to phosphate ester hydraulic fluids under laboratory controlled conditions. Furthermore, Hawkes (1977,80) and Hawkes and Stehr (1982) produced several lens alterations, as well as retinal abnormalities in surf smelt (*Osmerus mordax, Hypomesus pretiosus*) exposed to crude oil. The actual mechanism, however, by which chemical exposure initiates cataract formation and/or ocular pathogenesis is presently unknown.

Cataract formation in the Chesapeake Bay

The Elizabeth River is considered to be one of the most polluted estuaries in the world (Bieri et al., 1986). The Elizabeth River basin is a highly industrialized region of the lower James River (Hampton Roads), Chesapeake Bay, Va. that harbors various commercial enterprises including U.S Naval operations, fertilizer mills, scrap metal yards, waterside malls and marinas, hospital centers, oil storage areas, and major ship building and dry docking facilities. Furthermore, its waters are heavily utilized by international shipping, tug boats, barges, and pleasure craft.

Chemical analysis (gas chromatography and mass

spectrometry) of sediments sampled from the Elizabeth River has shown that unsubstituted PAH are the major components (Bieri et al., 1986). Concentrations of PAH were found to be highest in the Southern Branch of the Elizabeth River, may in certain areas reach in excess of 1,000 mg/kg of sediment, and can be traced to massive spills of creosote from wood preservative facilities. Over 200 individual PAH were have been determined in ER sediments, including naphthalene (precataractogenic agent) and benzo[a]pyrene (precarcinogenic agent). In general, total PAH decrease exponentially from the Virginia Power plant towards the mouth of the river. The biological effects of these chemicals on the biota of the ER are still not fully understood.

Numerous reports have described the prevalence of fish diseases within the Southern Branch of the ER (Hargis and Zwerner, 1988; Hargis et al., 1989; Thiyagarajah et al., 1989; Vogelbein et al., 1990). Sediment-borne contamination, particularly PAH and the break-down of these parent compounds into daughter metabolites, has generally been considered to be the causative factor in ER fish diseases. Direct exposure of spot to bulk sediments of the Elizabeth River produced anatomical responses very similar to field descriptions including penetrating integumental lesions, severe fin and gill erosion, and cataract formation (Hargis et al., 1984).

Cataract has been reported in fish from the Southern Branch of the Elizabeth River, Chesapeake Bay, VA (Hargis and Colvocoresses, 1985; Hargis and Zwerner, 1988). In this instance, prevalence of cataract was associated with sediments which have been heavily contaminated by the direct discharge of wastes (mostly creosote) from wood treatment plants. Hargis and co-workers (1985, 1988) conducted an extensive finfish survey of the Elizabeth River (over 74,000 fish sampled) and showed that cataract formation was the most frequently observed external lesion (3.2%), followed by ulcerations (0.8%) and integumental lesions (0.4%) from the eight species collected during 1982-85 (spot, *Leiostomus xanthurus*; Atlantic croaker, *Micropogonias undulatus*; weakfish, *Cynoscion regalis*; hogchoker, *Trinectes maculatus*; oyster toadfish, *Opsanus tau*; spotted hake, *Urophycis regia*; red hake, *Urophycis chuss*; and the gizzard shad, *Dorosoma cepedianum*). No fish sampled from the reference site (Nansemond River) were observed to have any identifiable external lesions. In addition, Huggett et al. (1987) showed that cataract prevalence in the Elizabeth River was positively associated with fish size. This response appeared to indicate that fish size may be indicative of residence time within the ER, which in turn could parallel duration of exposure. Greater exposure would result in increased cataract prevalence.

Moreover, Hargis and Zwerner (1988) showed that eye

lens opacification observed in fish sampled from the Southern Branch of the Elizabeth River was predominantly found in fish from the family Sciaenidae, namely croaker (5%), spot (3%), and weakfish (3%). Cataracts of several types (not described) and locations (not described), as well as capsular anomalies and inward-growing epithelial tumors, were reported. Furthermore, internal ocular disturbances occurred in several tissues sometimes co-existent with cataract, including: 1) hyperaemia of vitreous and aqueous chambers; 2) engorgement of choroid blood vessels extending into the iris; 3) engorgement and enlargement of the choroid rete; and 4) variation in both the texture and thickness of the retina.

Furthermore, Hargis and Zwerner (1989) described the histological effects of Elizabeth River sediments on the development and cytomorphology of feral teleost eyes. Cataract bearing fish of the Elizabeth River were described as having observable abnormal histological features, such as: 1) abnormal vertical multiplication of epithelial cells which produced an intrusive growing mass of polymorphic, anaplastic-appearing cells which caused severe inward distortion of the cortical layer; 2) compacted cortical fiber cells which took on the appearance of fibroblasts; 3) cortical fiber cell degeneration and fragmentation with a subsequent formation of densely packed eosinophilic hyaline plaque bodies and Morgagnian globules; and 4) vacuole

formation. Furthermore, degeneration of the retina and the rete mirabile were also observed.

Laboratory exposure of spot directly to Elizabeth River sediments and sediment effluents produced other effects similar to those observed in the field, i. e. penetrating integumental lesions, severe fin and gill erosion, as well as mortality (Hargis et al., 1984). Effects of exposure to effluent waters pumped through ER sediments were less marked than direct exposure (92% survivorship and mostly skin lesions observed), but did show detrimental effects similar to those observed in fish exposed directly to ER sediments. Of the ten fish that survived in the effluent exposure study, all had opaque or cloudy lenses. Control fish showed no treatment effects or mortality when exposed to bulk sediments or water effluents from York River sediments.

Relevance of Dissertation

Fish are key animals to study because they have exploited almost every conceivable niche within the aquatic environment and, therefore, have adapted to numerous conditions which vary greatly in their photic parameters and levels of illumination. Previous researchers have concluded that much can be learned about the visual system by examining aquatic vertebrates and how they have adapted and are affected by varying environmental conditions (Walls, 1942; Lythgoe, 1979). Furthermore, fish are easily

collected and maintained in high numbers, are economical specimens compared to most other test animals utilized, have a very similar ocular and lens system relative to other vertebrates which exhibit comparable disease pathologies, and have less social significance than mammalian counterparts.

As stated earlier, lens opacification in fish is a complex manifestation the mechanism of which is not easily identified. Although several studies have addressed various quantitative aspects of the toxicological effects of exposure to sediments from the Southern Branch of the ER, none have directly investigated such parameters in cataractous fish or lenses.

Therefore, the main objectives of this dissertation are to;

1) identify the extent to which the eye lesions, in general, and cataract formation, in particular, are associated with PAH-contaminated sediments;

2) investigate possible mechanisms involved in cataractogenesis in ER fish;

3) examine possible causes of cataract formation,

4) examine major biochemical changes that occur

during cataract initiation; and

5) to establish a cellular model that adequately represents *in vivo* organization so further studies can investigate the direct effects of chemical exposure on lens cell biology.

Figure 1: The structure of the fish ocular cavity (as taken from Hargis, 1991, and reprinted with the permission of Pergamon Press).

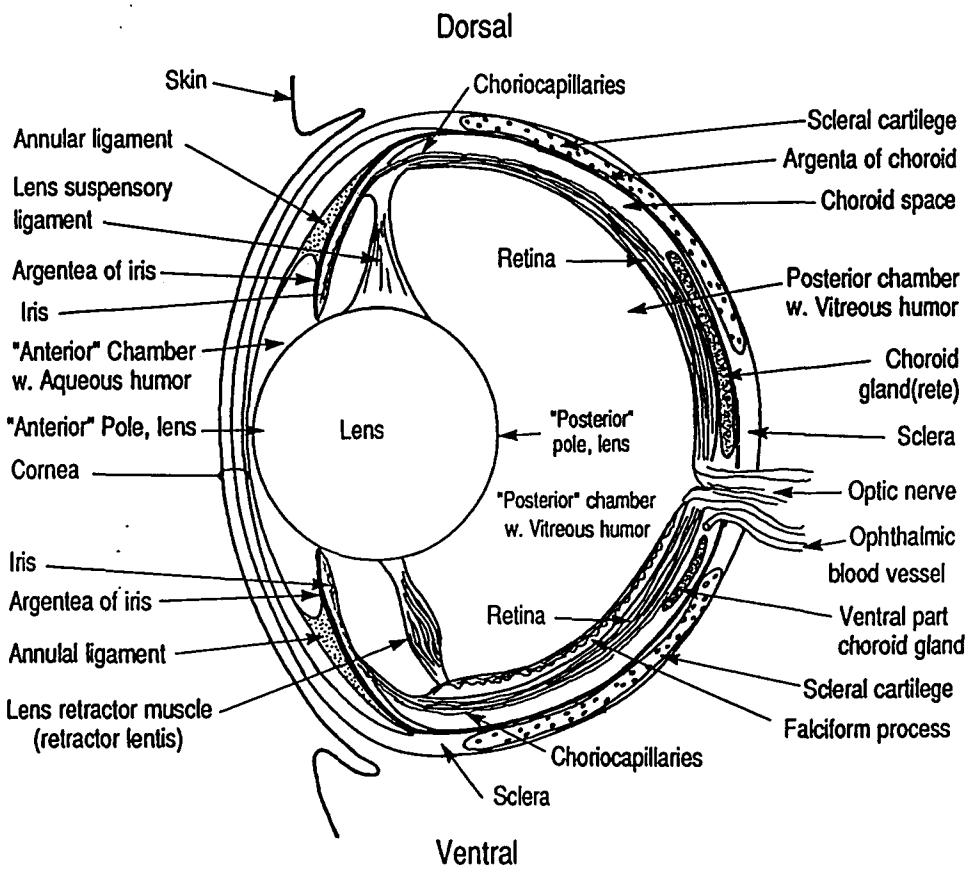
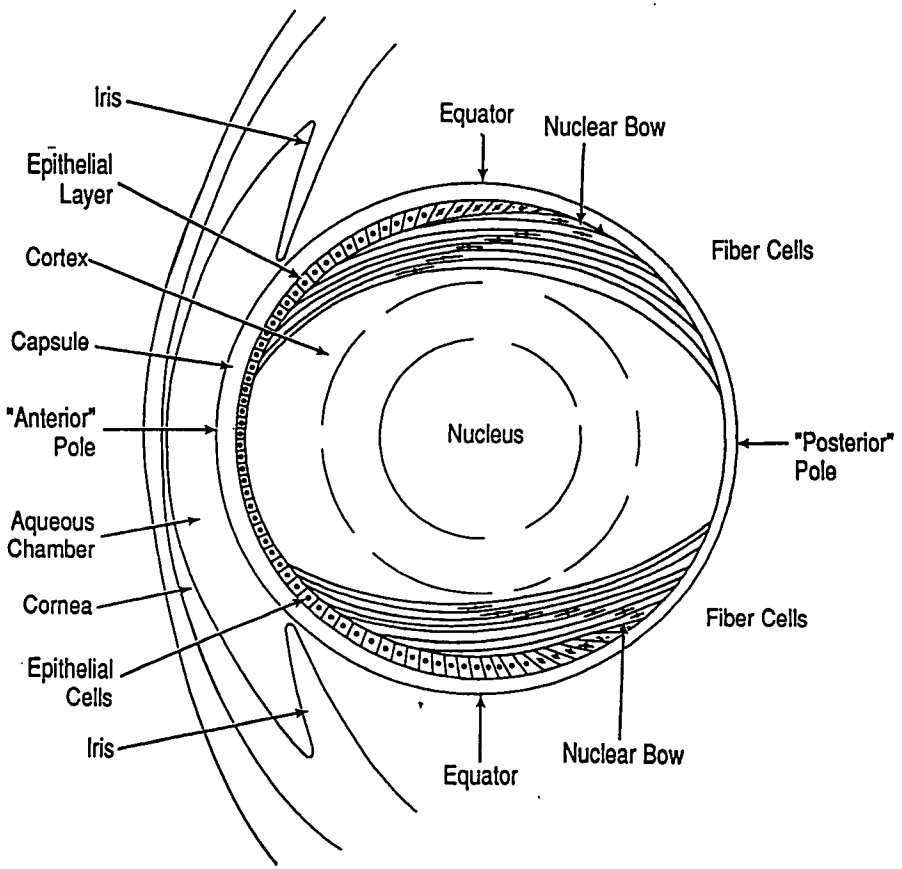

Dorsal
Skin
Choriocapillaries
Annular ligament
Scleral cartilege
Argenta of choroid
Lens suspensory ligament
Choroid space
Retina
Argentea of iris
Posterior chamber w. Vitreous humor
Iris
"Anterior" Chamber w. Aqueous humor
Choroid gland(rete)
"Anterior" Pole, lens
Lens
"Posterior" pole, lens
Sclera
Cornea
Optic nerve
"Posterior" chamber w. Vitreous humor
Ophthalmic blood vessel
Iris
Argentea of iris
Retina
Ventral part choroid gland
Annulal ligament
Scleral cartilege
Lens retractor muscle (retractor lentis)
Falciform process
Choriocapillaries
Sclera
Ventral

Figure 2: The structure of the fish lens (as taken from Hargis, 1991, and reprinted with the permission of Pergamon Press).


Iris
Epithelial Layer
Cortex
Capsule
"Anterior" Pole
Aqueous Chamber
Cornea
Epithelial Cells
Iris
Equator
Nuclear Bow
Fiber Cells
Nucleus
"Posterior" Pole
Fiber Cells
Equator
Nuclear Bow

CHAPTER 2

FISH EYE DISORDERS IN SCIAENID FISH SPECIES SAMPLED FROM SITES HEAVILY CONTAMINATED WITH POLYCYCLIC AROMATIC HYDROCARBONS IN THE CHESAPEAKE BAY

Introduction

Many types of external body lesions in fishes have been extensively studied and utilized as readily visible indicators of environmental stress, intoxication, and/or infection (Sinderman et al., 1980; Vethaak and ap Rheinallt, 1992). Eye diseases, however, which have been recognized for over a century, rarely have been investigated thoroughly (reviewed in Hargis, 1991). These diseases can be ecologically important, as reduced optical acuity may interfere with certain physiological processes associated with visual stimuli, such as predator avoidance, food gathering, schooling behavior, and possibly migratory/seasonal synchronicity. As in other vertebrates, eye disorders such as exophthalmia, endophthalmia, microphthalmia, cyclopia, keratitis, lenticulitis, and cataract have been described in the fish eye (Dukes, 1975; Hargis, 1991). Their etiologies, however, vary.

The Elizabeth River (ER), a tributary of the lower James River estuary (Chesapeake Bay, Virginia), is heavily contaminated with polycyclic aromatic hydrocarbons (PAH), which reach concentrations in excess of 400 mg/kg of dry sediment (Bieri et al. 1982), mainly due to effluents from industrial and domestic operations. Fish collected at various sites in the Southern Branch of the Elizabeth River (SBER) exhibited a wide variety of gross lesions such as

cataract, fin and integumental erosion, and body ulceration (Hargis and Colvocoresses, 1986; Hargis and Zwerner, 1988). The prevalence of these lesions was positively associated with sediment PAH contamination (Hargis and Colvocoresses, 1986). Fish from the family Sciaenidae were observed to have a greater prevalence of external body lesions than all other species sampled (Hargis and Zwerner, 1988). Croaker, spot, and the weakfish, all members of the family Sciaenidae, are major components of the ecological, commercial, and recreational fisheries of the Chesapeake Bay, as well as many other Atlantic Coast estuaries.

While fish lesions appear to be associated with sediment PAH contamination, at present it is not known if exposure to sediment-borne PAH induces disease directly or if it merely exacerbates the severity of pre-existing injuries. In addition, it is unclear if different types of lesions can be associated with sediment levels of PAH or if only certain classes of lesions are related to this type of chemical contamination. Finally, it has yet to be reported as to whether there are any discernible seasonal patterns in the occurrence of the various classes of ocular lesions.

The objective of the present investigation was to extend the earlier studies of Hargis and co-workers on the prevalence of cataract formation and to describe other ocular disorders observed in sciaenid fish collected from environments that vary in sediment levels of PAH. Young-of-

the-year sciaenids were chosen as test organisms for this study due to their known migratory patterns (Chao and Musick, 1977; Homer and Mihursky, 1991) and susceptibility to ocular disease (Hargis and Zwerner, 1988). Since temperature is known to play a role in the outbreak of diseases in poikilothermic vertebrates, an additional objective of this study was to examine the relationship between water temperature and the prevalence of ocular lesions.

Materials and Methods

Young-of-the-year fish (< 15 cm) from the family Sciaenidae were collected. Care was taken to ensure that all fish sampled during each time period were of similar size.

Trawl sampling was performed monthly from April, 1991 through June, 1992 for two stations within the Elizabeth River (ER; station 217; 96 µg/kg mean total sediment PAH concentration, latitude = 36° 50 -37° 51′, longitude 76° 17′ - 76° 18′; and station WS; 22 µg/kg mean total sediment PAH, latitude = 36° 46 - 36° 47′, longitude 76° 17′ - 76° 18′) and for one station within the York River (YR = reference site; 1 µg/kg mean total sediment PAH, latitude = 37° 15 - 37° 22′, longitude 76° 30 - 76° 40′, Fig. 3). A 14 x 30 ft. otter trawl net (1.0 inch mesh) was used for short duration

trawls (5 min). In order to attempt to sample fish migrating into and out of the rivers periodic trawl sampling was performed on a seasonal basis (March - April and September - November, 1991 and 1992) from the mouth of the Chesapeake Bay (CBM; 0.2 μg/kg mean total sediment PAH, latitude = 37° 00 -37° 20′, longitude 76° 00 - 76° 20′) and the mouth of the James River (JRM; 1.6 ng/kg mean total PAH, latitude = 37° 00 -37° 10′, longitude 76° 20 - 76° 30′, Fig. 3).

Reported sediment PAH concentrations for sampling stations were obtained from data on previously analyzed sediments archived in the Virginia Institute of Marine Science Computer Toxics Database, Division of Environmental Sciences (Craig L. Smith, VIMS, unpublished data, 1991, 1992). The figures employed herein for the sediment concentrations of total PAH at the sampling stations are the mean of all sediment grab and core samples taken at each site since 1980. Bottom water temperatures are the average of eight stations within the James River collected on a monthly basis from April, 1991 through March, 1992 (VIMS, Division of Fisheries Sciences).

Fish sampled from stations WS, 217, and YR were transferred alive to the laboratory where they were maintained in aerated holding tanks (150 gallon) and were examined within 4-12 hours after collection. Specimens collected from stations CBM and JRM were transferred in

sealed containers under ice and examined within 2-12 hours after collection. Prior to examination, all live fish were anesthetized with tricaine methanesulfonate (MS-222, 0.2g/L) (Sigma Chemical Co., St. Louis, MO). All fish were measured for total length (from the snout to the end of the caudal fin, ± 0.5 cm), weighed (± 0.1 gm), and observed for eye and body abnormalities. Smaller fish (< 8 cm) were examined using a dissection microscope (10x), while larger fish were grossly observed for body abnormalities.

The total number of fish examined by species was dependent upon trawl yield (abundant in summer months) and followed the criteria suggested by the American Fisheries Society Bluebook for detection of fish diseases (1985). During high yield months a total of at least 150 sciaenids were sampled per sampling station, while during low yield months no less than 50 specimens were obtained from each sampling site.

Condition factors were determined for individually measured fish caught in August of 1991 (maximal prevalence of eye disorders) from two PAH-contaminated sites (stations 217 and WS), as well as from the reference site (station YR). The formula utilized was $C_f = W(100)/L^3$ where W = weight (g) and L = total length (cm).

Statistical analysis was performed by using the G_H-statistic for frequency analysis (Sokal and Rohlf, 1981) to determine differences in prevalences of body lesions and eye

disorders between sample stations, as well as to indicate associations between prevalence of eye disorders and sampling seasons. In order to determine the relationship between prevalence of disease signs observed and chemical contamination of sediments, linear regression analysis was performed on the prevalence of general body disease signs (including eye abnormalities grouped) and the prevalence of individual eye abnormalities versus sediment levels of total PAH. Multiple regression and correlation analysis was performed on data obtained from station YR, WS, and 217 to determine the relationship between prevalence of total eye abnormalities with seasonality, bottom water temperature and sediment levels of PAH contamination. The MINITAB statistics package (Minitab Inc., State College, PA) was utilized for all regression and (Pearson) correlation analyses. Principal components analysis (PCA) was performed on arc-cosine transformed monthly frequencies of individual ER eye abnormalities, sediment levels of PAH, and bottom surface water temperatures. PCA was utilized as a tool to determine which variables were most significant (MINITAB statistics package). Analysis of variance (MINITAB statistics package) was utilized to determine if there were any statistical differences in condition factors between cataract and non-cataract specimens. Multiple mean comparison between ER-cataract, ER-noncataract, and YR samples was performed by utilizing a Tukeys multiple mean

comparison test ($p<0.05$, Statistica, Jandel).

Results

Lesion description

Several body disease signs were identified on a consistent basis in fish sampled from all stations. These disease signs included: fin erosion, body ulcerations, the presence of external crustacean parasites, skeletal deformities, and several eye disorders. Of the major disease signs identified, eye abnormalities were, by far, the most frequently observed classification (22.0% of all fish sampled), followed by fin erosion (7.0%), body ulcerations (3.0%), the presence of external crustacean parasites (1.0%), and skeletal deformities (0.5%), respectively. These lesions were significantly more prevalent in fish collected from the ER sampling stations than in those sampled from the CBM, JRM, and YR sampling sites (Fig. 4).

Eye disorders: Grossly visible eye abnormalities consisted of lesions associated with the cornea, lens, sclera, and the ocular cavity. Eye abnormalities could be classified into the following categories: exophthalmia, endophthalmia, corneal opacification (cloudiness), corneal ulceration (acute keratitis), haemorrhagia, scleral and pupil abnormalities, and cataract.

Exophthalmia (pop-eye, Fig. 5A) occurred primarily as a bilateral condition and appeared as enlarged and distended eye complexes which had the eyeball protruding 10-20 mm from the ocular cavity in a slightly downward direction. The cornea was normal in appearance with no apparent disruption in its integrity. The surface tension of the cornea appeared, however, to be elevated compared to normal eyes. Exophthalmia was observed in 1.8% of the total fish sampled and represented 12.4% of the recorded number of eye disorders.

Endophthalmia (sunken eye) was observed to be either bilateral or unilateral in nature with the cornea withdrawn 5-10 mm into the ocular cavity. The surface tension, the integrity, and the texture of the cornea appeared to be normal. Endophthalmia was observed in 0.2% of the total fish collected and constituted 1.8% of the observed eye disorders.

Corneal opacification (Fig. 5B) was observed unilaterally or bilaterally and varied in intensity from slight cloudiness to prominent white discoloration. Opacification was usually observed as small dots or patches of slightly cloudy areas that covered a few millimeters of surface area and were situated randomly over the eye surface. In general, the intensity of opacification increased with greater surface area affected. In rarer instances, fish exhibited severe corneal opacification with

a circular "donut-like" arrangement that incorporated up to 80% of the corneal surface area. The central region of the cornea was usually clear. Corneal opacification was most commonly observed during warmer months. It was the second most prevalent eye disorder (3.5%) in total fish sampled and constituted 22.4% of all eye abnormalities observed.

Corneal ulceration (Fig. 5C) was either unilateral or bilateral in manifestation. Ulcerations ranged from small pin-points to penetrating lesions. Affected corneas were highly opaque with the loss of structural integrity. Surface tension of the cornea was significantly reduced with the cornea abnormally soft in texture and thickened. Aphakia (lack of an eye lens) was consistently associated with corneal ulceration and was positively associated with the degree of ulceration. On three occasions, live fish (two spot and one weakfish) were collected from the ER which exhibited little or no eyeball tissue. Similar to corneal opacities, corneal ulcerations frequently appeared during warm months but were, in general, rare in occurrence (0.4% prevalence in total fish collected and 2.8% of all eye disorders).

Haemorrhagia (Fig. 5D) was observed predominantly as a bilateral condition, with visible red blood around the perimeter of the pupil and underlying the cornea. The blood appeared to originate from within the eye cavity. The amount of blood observed varied from a few small drops to

enough to completely obscure the eye face. Eyes exhibiting hemorrhagic conditions were observed in 0.8% of all fish sampled and constituted 5.4% of total eye abnormalities observed.

Scleral abnormalities (Fig. 5E) were unilateral in appearance and were observed as either irregular geometry of eye apertures, severely reduced openings or non-circular morphologies of the eye, or ulcerations/discontinuities in the scleral tissue. Since sciaenids do not have a dilating pupil abnormal apertures were designated as scleral disorders. Scleral abnormalities were infrequently noted (0.2% of all fish collected) and comprised only 1.3% of all of the eye disorders observed.

Cataract (lens opacification, Fig. 5F) was observed both as a unilateral and as a bilateral condition. The degree of opacification was, in general, strongly opaque and readily observable. Two apparent types of lens cataract were observed; opacification that appeared at the visible center of the lens (nuclear cataract) and that which appeared at the periphery of the lens (cortical cataract). Of these two types of cataract formation, "nuclear" cataract was by far the most prevalent (93%). The degree of lens impacted ranged from < 10-100%. Cataract was the most common eye disorder (7.4% of all fish sampled) and constituted 51.7% of the observed eye disorders.

Prevalence of ocular lesions

Of the major disease signs identified, eye abnormalities were the most frequently observed type (22.0% of all fish sampled), followed by fin erosion (7.0%), body ulcerations (3.0%), the presence of external crustacean parasites (1.0%), and skeletal deformities (0.5%). Fin erosion and eye abnormalities were the only disease parameters to show significant differences between sample stations.

Total eye abnormalities showed the most distinct pattern of occurrence (Table 1). Prevalence was not found to be significantly different at stations CBM (2.0%), JRM (6.3%), and YR (4.9%). Prevalence was, however, significantly greater at the WS station (14.1%, G_H=68.2, $p<0.001$, df=4) compared to the aforementioned sites while being significantly greatest at station 217 (24.0%, G_H=54.6, $p<0.001$, df=4).

Among individual eye abnormalities observed, lens cataract was the most prevalent (7.4%), followed by corneal opacities (3.5%), exophthalmia (1.8%), hemorrhagic eyes (0.8%), corneal ulceration (0.4%), scleral abnormalities (0.2%), and endophthalmia (0.2%, Fig. 6). Statistical analysis indicated that only the prevalences of cataract formation (G_H=354, $p<0.001$, df=4) and corneal opacities (G_H=42, $p<0.001$, df=4) differed significantly among the sampling sites. The individual contribution of individual

eye disorders to the total number of eye abnormalities observed from fish collected at stations WS and 217 is presented in Table 2.

Cataract formation was observed at three of the five sampling stations, but only at sites within the James River. Prevalence of cataract was non-existent at the CBM and YR sampling sites. In comparison to the former sampling stations, cataract was significantly elevated at stations JRM (5.8%) and WS (5.2%), although, no statistical difference was observed between these two sites. Prevalence of cataract was observed to be statistically greatest at station 217 (14.9%, G_H=91.4 , $p<0.001$, df=4).

Corneal opacification also was observed only at three of the five sampling stations, but in this case the sites were not confined to the James River system. Corneal opacities were not observed in fish sampled from stations CBM and JRM. Manifestation of this ocular abnormality was significantly higher at the YR (2.9%), WS (4.8%), and 217 (3.6%) sampling stations, although, no statistical difference was found among these sites.

Seasonal occurrence of ocular disease parameters was observed at stations YR, WS and 217. No analysis of CBM or JRM samples was performed due to incomplete sampling over the 12-month collection period. At the YR, WS, and 217 sampling sites, eye disorders were observed to be significantly associated with seasonal patterns of

occurrence, being most prevalent during summer and fall sampling (early fall, $G_{H,YR}$=5.6, $G_{H,WS}$=53.1, $G_{H,217}$=424, 0.025< p_{YR} >0.01, $p_{WS,217}$<0.001, df=3). A significant positive correlation (R=0.68, p<0.05) was observed between prevalence of eye disorders and bottom water temperature. Prevalences of total eye disorders observed, aggregated on a seasonal basis, were as follows: summer = 35.7%, n=1129; fall = 25.0%, n=588; winter = 11.5%, n=479; and spring = 3.4%, n= 1000, respectively.

Seasonal occurrence of individual eye disorders was also observed at stations YR, WS, and 217. Prevalence of cataract formation (G_H=25.2, p<0.001, df=3), haemorrhagia of the eye (G_H=8.4, p=0.05, df=3), and exophthalmia (G_H=8.6, p=0.05, df=3) were all observed to vary significantly among sample seasons. Manifestation of cataract was significantly higher in summer (20.5%) and fall collections (15.6%) than in winter (5.7%) and spring samples (1.4%). Ocular haemorrhagia, on the other hand, showed highest prevalence during summer (2.0%), fall (1.1%), and winter months (2.3%, no statistical difference in occurrence among these sample seasons) with significantly reduced occurrence in spring samples (0.1%). Fish exhibiting exophthalmic eyes were observed to have greatest prevalence during fall sampling (4.7%) with no significant differences in occurrence among winter (2.3%), spring (1.6%), and summer collections (2.1%).

Overall prevalences of ocular disorders did not vary

significantly among spot (18.7%), croaker (17.4%), and weakfish (16.9%). Cataract was the most predominant eye disorder reported in all three species ($G_H \geq 10.6$, $p=0.001$, $df=6$) and indicated that certain species differences do exist. Croaker exhibited a significantly higher overall prevalence (17.0%, $p<0.001$, $df=1$) of cataract formation compared to spot. The latter exhibited a significantly higher prevalence (12.1%, $p<0.001$, $df=1$) than weakfish (6.5%). Of the other eye abnormalities observed, only corneal opacities, exophthalmia, and hemorrhagic eyes occurred with any consistent frequency. Prevalence of fish exhibiting exophthalmic conditions, however, did not significantly differ between species collected. Of the various other classes of eye disorders (excluding cataract), only the prevalence of corneal opacification and eye haemorrhagia varied significantly among the three species. The prevalence of corneal opacification was observed to be significantly greater in spot (7.0%) and weakfish (9.2%) than in croaker specimens (1.1%; $G_H \geq 33.4$, $p<0.001$, $df=2$) and the occurrence of ocular haemorrhagia was significantly greater in croaker (2.7%) and weakfish (3.8%) than in spot samples (0.7%; $G_H \leq 18.0$, $p<0.001$). Species differences of eye disease signs for the two most often collected species, Atlantic croaker and spot, at stations WS and 217 are shown in Table 3 and 4.

Principal components analysis for all individual eye

disorders and water temperature showed, using eigenanalysis of a correlation matrix, that four principal components explained 85% of the total variance while the addition of a fifth component would explain 96%. Component 1 was comprised predominantly of the variables bottom water temperature, lenticular cataract, and corneal abnormalities (corneal opacities grouped with corneal ulceration) and explained the greatest portion of the overall variance incurred (33%). Table 5 identifies the six remaining components and their respective proportions of total variation.

Associations between sediment PAH concentrations and lesions

In general, the prevalence of all grossly observable disease signs increased at sites documented to have increased sediment PAH concentrations. A strong positive correlation was observed between total PAH levels and fish eye abnormalities (R^2=94.5, p=0.006, y=4.69+0.00031(x)). In addition, data also indicated that associations existed between prevalence of certain specific eye disorders and sediment PAH concentrations. In particular, cataract (R^2 = 84.0, p=.029, y=1.58+0.0002(x)), exophthalmia (R^2 = 83.8, p=.029, y= 0.57+ 0.000035(x)), and hemorrhagic eyes (R^2 = 99.4, p<0.000, y= -0.0048 + 0.000026(x)) all had strong positive correlations with total PAH sediment levels. The remaining individual eye abnormalities were not as strongly

associated with sediment PAH levels. Their calculated R^2-values were as follows: scleral abnormalities (59.4), corneal opacification (40.1), corneal ulceration (31.3), and endophthalmia (0.5).

Multiple regression analysis that utilized bottom water temperature (BT, °C), station sediment levels of PAH (PAH), and monthly prevalence of ocular disorders ($\log_{10}$ (%+1) transformed, all 12 months) exhibited an R^2-value of 42.0% ($y = 0.27 + 0.02BT + 0.00001PAH$, $p_{REG}=0.000$, $p_{BT}=0.01$, $p_{PAH}=0.000$, df=2). Separating the 12-month sampling procedure into blocks of time (all possible chronologically arranged combinations) in order to incorporate the seasonal component of ocular abnormality occurrence, a maximal R^2-value of 76.7% was obtained for months 6-11 (June-November, $y = 0.65 + 0.008BT + 0.000013PAH$, $p_{REG}=0.000$, $p_{BT}=0.331$, $p_{PAH}=0.000$, df=2).

Body condition index

One-way ANOVA analysis showed that significant differences in body condition factors existed between normal fish sampled at stations 217 and YR (217 fish displayed larger C_f-values, $p < .001$), as well as between fish with and without eye abnormalities collected from station 217 (fish with eye abnormalities had reduced C_f-values, $p < 0.019$). No significant difference between mean condition factors was observed between 217 fish with eye abnormalities

and fish collected within the YR sampling site. Mean condition factors for individually measured 217 fish, with and without eye abnormalities, and YR fish were 1.09 (± 0.17 SD, N = 103), 1.15 (± 0.18, N = 96), and 1.05 (± 0.16, N = 91), respectively).

Table 1: Prevalence of total eye abnormalities for all sciaenids collected from the Chesapeake Bay sampling stations over the twelve month sampling period.

	1991					Month				1992			
Sample station	4	5	6	7	8	9	10	11	12	1	2	3	Total
CBM	0.0 (51)	4.0 (49)	NS	NS	0.0 (50)	0.0 (55)	0.0 (47)	NS	NS	NS	NS	0	1.2
JRM	3.3 (31)	0.0 (30)	NS	NS	11.8 (119)	0.0 (18)	0.0 (41)	NS	NS	NS	NS	0	6.3
YR	NS	NS	5.7 (175)	1.7 (240)	4.6 (153)	11.3 (159)	5.1 (118)	4.6 (87)	0	NS	2.2 (43)	NS	4.9
WS	2.2 (178)	1.1 (285)	19.1 (310)	22.8 (175)	17.8 (73)	33.7 (101)	7.8 (64)	8.6 (70)	15.9 (88)	9.1 (33)	13.5 (57)	22.0 (50)	14.1
217	1.0 (297)	3.8 (182)	30.4 (158)	48.2 (195)	58.5 (248)	40.6 (128)	40.7 (59)	33.0 (109)	17.1 (111)	6.9 (102)	6.0 (50)	10.4 (67)	24.0
Total	1.5	2.3	16.5	22.6	28.7	23.8	11.2	17.3	16.6	5.9	7.5	11.5	14.7

NS = not sampled during this month
(#) = sample size

Table 2: Percent contribution of individual eye abnormalities (expressed as a percentage of the total number of eye disorders observed) in all sciaenid fish species collected from Stations WS and 217 in the Elizabeth River.

	1991					Month				1992		
Ocular disorder	4	5	6	7	8	9	10	11	12	1	2	3
Cataract	77.8	71.4	62.2	35.0	77.7	42.3	66.7	71.4	65.7	20.0	0.0	0.0
Corneal opacity	0.0	0.0	28.8	40.0	5.4	37.2	3.7	9.5	8.6	0.0	12.5	0.0
Exophthalmia	0.0	0.0	0.9	14.7	0.7	17.9	7.4	9.5	0.0	40.0	70.0	66.7
Endophthalmia	0.0	0.0	0.9	0.0	0.7	0.0	0.0	2.4	0.0	0.0	0.0	5.6
Hemorrhagia	0.0	14.3	3.6	4.2	9.4	0.0	14.8	0.0	11.4	40.0	20.0	0.0
Scleral disorder	0.0	14.3	0.0	0.0	0.0	1.3	0.0	4.8	0.0	0.0	10.0	0.0
Corneal ulceration	22.2	0.0	2.7	5.6	6.1	1.3	3.7	2.4	14.3	0.0	10.0	0.0
Sample size	9	7	111	143	148	78	27	42	35	10	10	18

Table 3: Prevalence of individual eye disorders for the two most commonly sampled sciaenid fish species from the Elizabeth River, Chesapeake Bay.

Sample station/ Month	Eye Disorders																											
	Cataract				Corneal opacification				Corneal ulceration				Exophthalmia				Hemorrhagic eye				Scleral abnormalities				Endophthalmia			
	Croaker		Spot		Croaker		Spot		Croaker		Spot		Croaker		Spot		Croaker		Spot		Croaker		Spot		Croaker		Spot	
	WS	217	WS	217	WS	217	WS	217	WS	217	WS	217	WS	217	WS	217	WS	217	WS	217	WS	217	WS	217	WS	217	WS	217
April	0.9	0.0	4.8	1.7	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
May	0.0	1.4	4.2	27.8	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	2.8	0.0	0.7	0.0	0.0	0.0	0.0	0.0	0.0
June	10.8	22.3	10.0	22.8	0.0	5.4	15.6	0.0	0.0	0.9	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.9	1.9	0.0	0.0	0.0	1.9	0.0	0.0	0.0	0.0	0.0
July	4.7	31.3	4.9	17.0	0.0	10.4	29.0	23.6	0.0	0.0	0.0	0.9	1.2	4.5	9.7	12.3	0.0	4.5	0.0	1.9	1.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0
August	20.0	84.0	14.3	19.0	0.0	0.9	14.3	1.6	0.0	0.0	42.8	0.8	0.0	0.0	0.0	0.8	0.0	16.0	0.0	1.6	0.0	0.0	0.0	0.0	0.0	1.9	0.0	0.0
September	0.0	40.0	16.4	15.4	0.0	0.0	20.5	14.6	0.0	0.0	1.4	1.6	0.0	0.0	6.8	11.4	0.0	0.0	0.0	0.8	0.0	20.0	0.0	0.0	0.0	0.0	0.0	0.0
October	11.1	65.2	5.4	3.7	0.0	0.0	0.0	3.7	0.0	0.0	0.0	0.0	11.1	8.7	0.0	0.0	0.0	21.7	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
November	0.0	57.5	7.8	1.8	0.0	7.5	0.0	0.0	0.0	0.0	0.0	0.0	0.0	7.5	1.6	0.0	0.0	25.0	0.0	0.0	0.0	2.5	0.0	0.0	0.0	2.5	0.0	0.0
December	0.0	0.0	0.0	17.4	0.0	0.0	0.0	2.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	2.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
January	0.0	0.0	12.5	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	5.9	2.0	0.0	0.0	0.0	4.9	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
February	0.0	0.0	0.0	0.0	2.2	0.0	0.0	0.0	2.2	0.0	0.0	0.0	11.1	4.0	0.0	0.0	2.2	10.0	0.0	0.0	0.0	10.0	0.0	0.0	0.0	0.0	0.0	0.0
March	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	24.0	7.9	0.0	25.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.6	0.0	0.0

Table 4: Sample sizes for the collection of the sciaenid fish species; Atlantic croaker (C), spot (S), and weakfish (W) from the York and Elizabeth Rivers.

	Sampling station								
	YR			WS			217		
Month/ Species	C	S	W	C	S	W	C	S	W
4	-	-	-	114	63	1	116	180	1
5	-	-	-	163	120	2	143	36	3
6	91	78	6	148	160	2	112	35	11
7	129	75	36	85	62	28	67	106	22
8	73	30	50	20	14	39	106	121	21
9	35	116	8	25	73	3	5	123	0
10	18	11	89	9	37	18	23	27	9
11	0	77	10	3	64	3	40	56	13
12	0	0	0	1	87	0	10	92	9
1	-	-	-	17	16	0	102	0	0
2	45	0	0	45	12	0	50	0	0
3	-	-	-	50	0	0	63	4	0

Table 5: Principle component analysis of individual eye abnormalities, for stations Waterside and 217 combined, using correlation matrices.

Principle component	PC1	PC2	PC3	PC4	PC5	PC6	PC7
Eigenvalue	2.3249	1.5928	1.0705	0.9742	0.7494	0.2310	0.0573
Proportion of total variation	0.332	0.228	0.153	0.139	0.107	0.033	0.008
Cumulative proportion of total variation	0.332	0.560	0.713	0.852	0.959	0.992	1.000
Variables of component	**PC1**	**PC2**	**PC3**	**PC4**	**PC5**	**PC6**	**PC7**
Cataract	0.535	0.240	-0.062	0.412	0.140	0.433	0.525
Corneal abnormalities	0.492	-0.420	0.145	0.068	0.129	-0.706	0.203
Exophthalmia	-0.192	-0.543	0.097	0.031	0.738	0.335	-0.041
Endophthalmia	-0.216	0.242	0.496	0.728	0.095	-0.185	-0.276
Hemorrhagic eyes	0.210	0.542	-0.331	-0.158	0.597	-0.246	-0.332
Scleral deformities	0.013	-0.313	-0.728	0.509	-0.161	-0.019	-0.295
Bottom water temperature (^{0}C)	-0.587	0.143	-0.283	0.109	0.169	-0.327	0.639

Figure 3: A map of the lower Chesapeake Bay showing the sampling sites. YR = York River (reference site), JRM = James River Mouth, WS = Waterside, and 217 = Station 217. INSERT shows the Chesapeake Bay Mouth (CBM) sampling site.

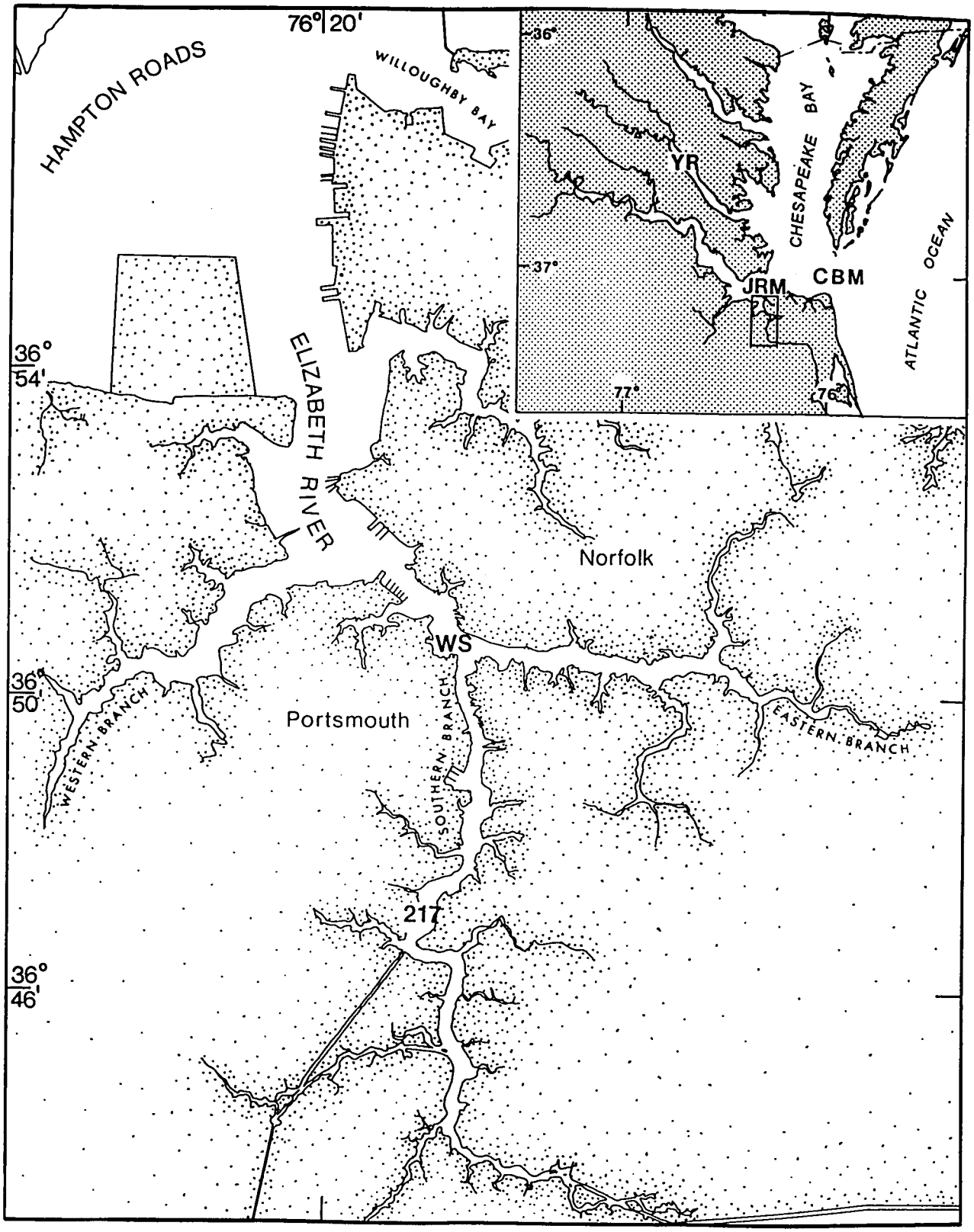

76° 20'
HAMPTON ROADS
WILLOUGHBY BAY
ELIZABETH RIVER
36° 54'
36° 50'
36° 46'
Norfolk
Portsmouth
WS
217
WESTERN BRANCH
SOUTHERN BRANCH
EASTERN BRANCH
36°
37°
77°
76°
YR
JRM
CBM
CHESAPEAKE BAY
ATLANTIC OCEAN

Figure 4: Prevalence of total externally visible disease parameters observed from the five survey stations sampled within the Lower Chesapeake Bay.

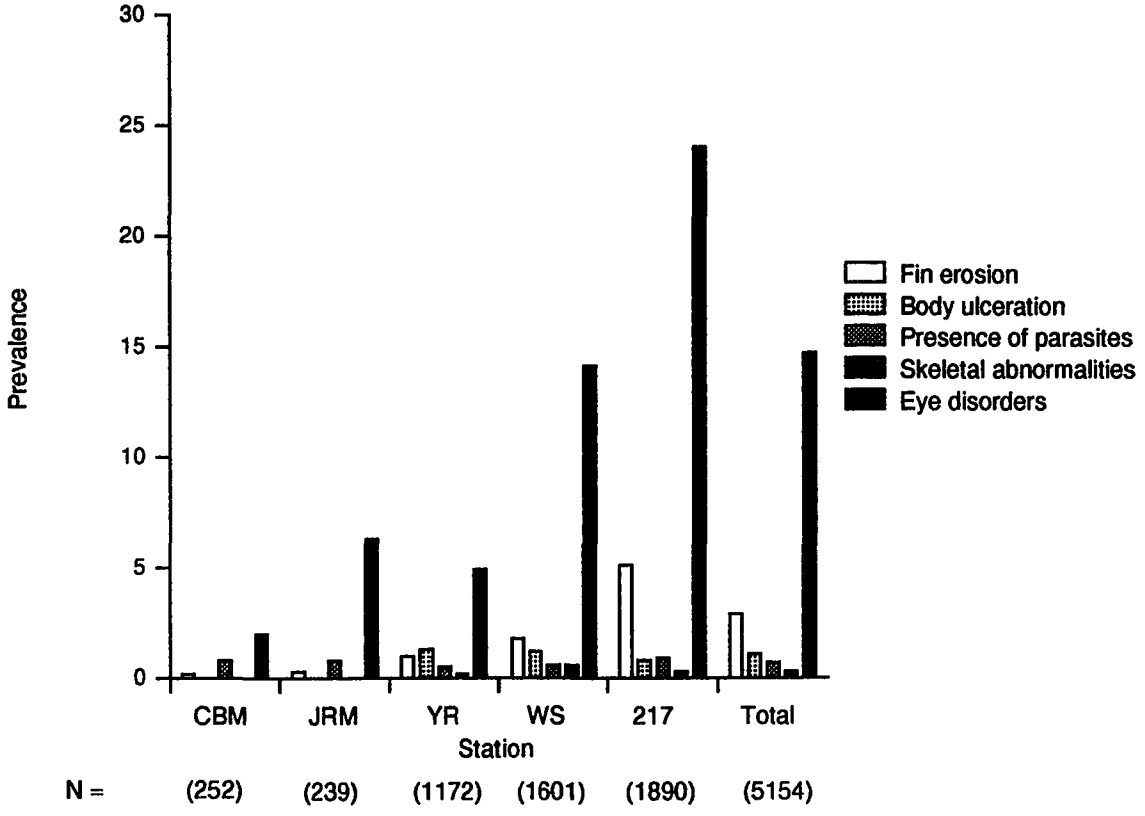

30
25
20
15
10
5
0
Prevalence
Fin erosion
Body ulceration
Presence of parasites
Skeletal abnormalities
Eye disorders
CBM
JRM
YR
WS
217
Total
Station
N =
(252)
(239)
(1172)
(1601)
(1890)
(5154)

Figure 5: Eye disorders observed in sciaenid fish: A) exophthalmia in an Atlantic croaker, B) moderate corneal opacification in an Atlantic croaker, C) ulceration of the cornea of a spot (acute keratitis), D) haemorrhagia of a spot eye completely obscured by blood, E) scleral abnormality in a spot, and F) cataract formation in an Atlantic croaker.

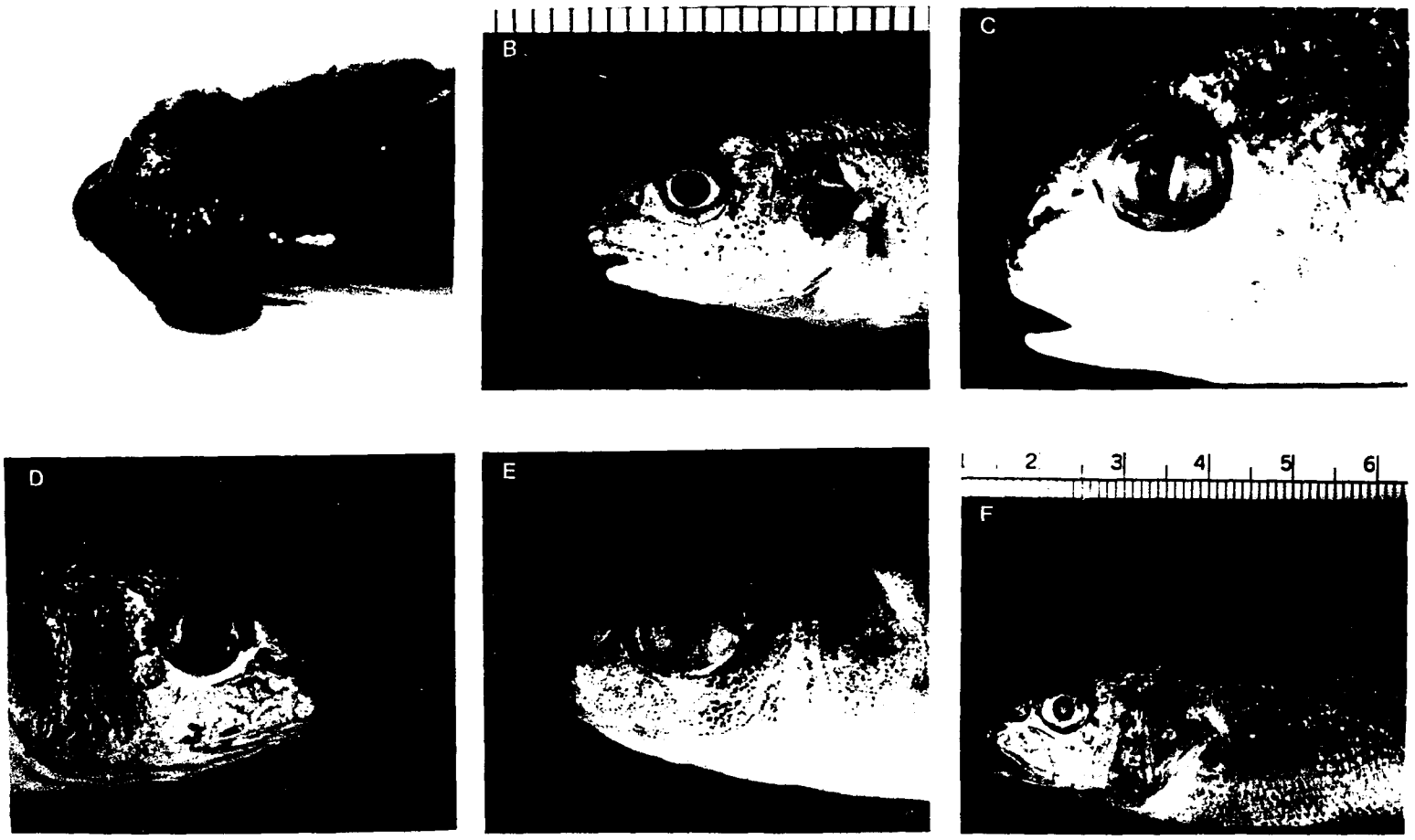
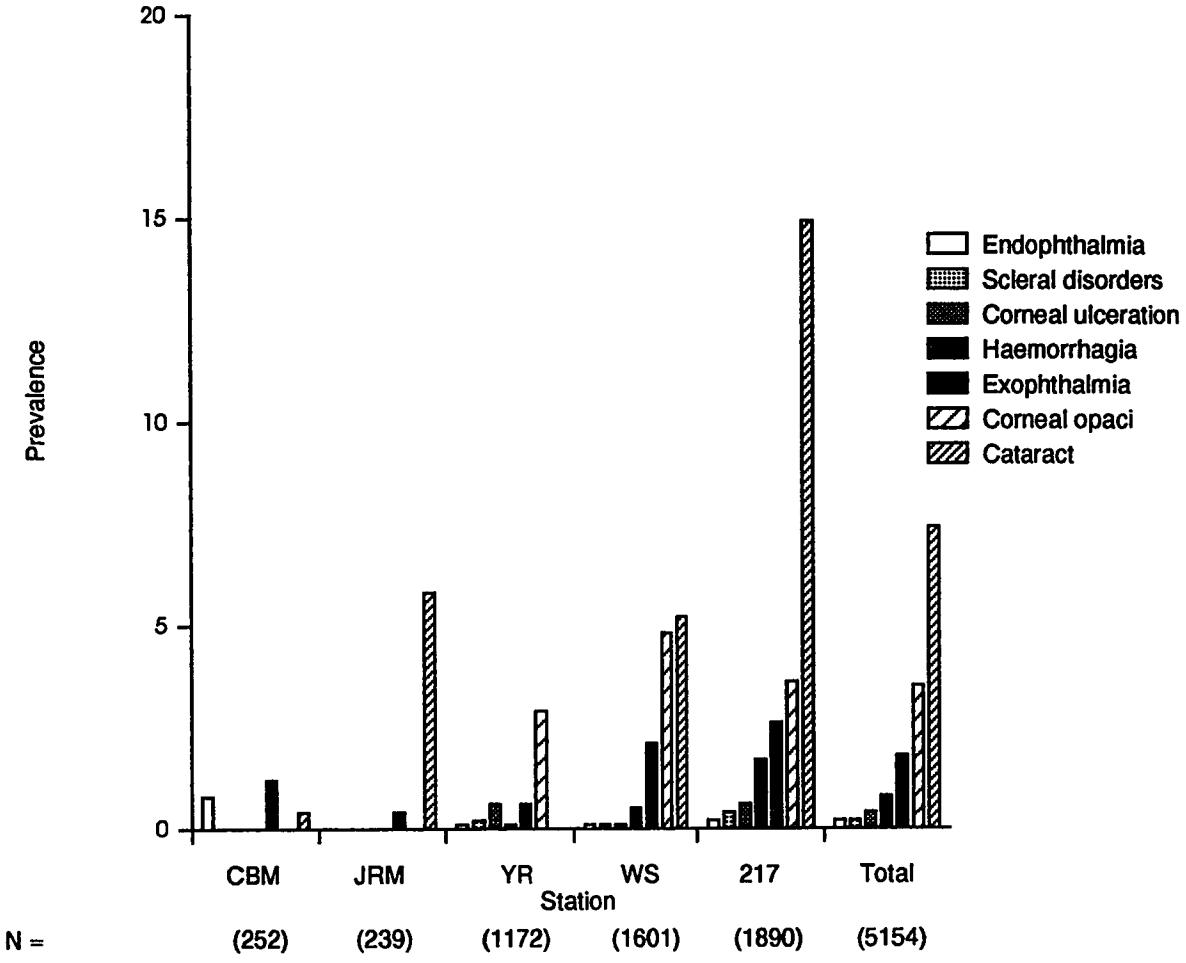

B
C
D
E
1
2
3
4
5
6
F

Figure 6: Prevalence of total individual eye abnormalities observed from the five survey stations sampled within the Chesapeake Bay.


20
15
10
5
0
Prevalence
Endophthalmia
Scleral disorders
Corneal ulceration
Haemorrhagia
Exophthalmia
Corneal opaci
Cataract
CBM
JRM
YR
WS
217
Total
Station
N =
(252)
(239)
(1172)
(1601)
(1890)
(5154)

Discussion

It has been suggested repeatedly that disease outbreaks in aquatic organisms can be linked to environmental factors such as pollution (Sindermann et al., 1980; Wedemeyer and Goodyear, 1984), but clear cause and effect relationships are difficult to establish. This lack of conclusive data can be attributed to variables in the aquatic environment, the mobility of many fish species studied, and the lack of specifically designed experiments aimed at investigating these relationships. Recent research in the North Sea, however, has associated the observance of some disease signs (fin erosion, vertebral abnormalities, liver abnormalities, ulceration, epidermal hyperplasia/papilloma) with pollution related parameters (pulp mill effluents, smeltery effluents, and oil spills; Vethaak and ap Rheinallt, 1992) and may signal the development of more precise ecotoxicological information.

In this study, as in Hargis and Colvocoresses (1986) and Hargis and Zwerner (1988), ocular disorders were observed to be prevalent at sampling sites where sediment loads of PAH are high. However, the previous studies concentrated on the presence of lens cataract in most of the species sampled, but did not describe other abnormalities associated with the ocular region. In the present study, corneal opacification, corneal ulceration, hemorrhagic eyes,

and scleral abnormalities are described for the first time from this area of the Chesapeake Bay. It should be noted, however, that ocular damage can be caused by net abrasion encountered during trawl sampling. The comparison of ER station data to a reference site (YR station) using the same material and methods hopefully has minimized any such effect.

The three sciaenid species examined in this study (spot, croaker and weakfish) appeared to be equally prone to the development of eye abnormalities as a general lesion class. These results may indicate the applicability of ocular disorders across species as an index for environmental quality (or the lack of).

The high positive correlation between sediment PAH levels and lens cataract, exophthalmia, and hemorrhagic eyes indicates that these eye abnormalities may be associated with PAH contamination. Previous studies have shown a similar relationship between cataract development and exposure to increased PAH levels (Hargis and Colvocoresses 1986, Huggett et al. 1985, Hargis and Zwerner 1988). In addition, a laboratory study by Hargis et al. (1984) demonstrated that exposure to ER sediment and effluent water caused body and ocular abnormalities in spot. Data from the present study appear to further support the hypothesis that disease conditions in fish, especially ocular disorders, occur significantly more often within the confines of the

polluted ER. For example, relatively few fish with ocular abnormalities were sampled from sites (stations CBM and JRM) and at periods of the year (early fall) where one would expect to observe such disease signs if fish were migrating in from offshore habitats with pre-existing health problems. It is noteworthy that the figures given in the present study are those of young-of-the-year and not all fish age classes as in the study of Hargis and Zwerner (1988). Thus, the fish examined were more likely to have developed their physical state solely from environmental conditions recently encountered. Atlantic croaker, spot, and weakfish become mature at the end of their first year of existence at approximately 14, 15, and 12 cm of size, respectively (Phillips et al. 1989; Mercer, 1989; Hales and Van Den Avyle, 1989) Care was taken not to include individuals which were close to but less than these lengths during early sampling periods (April-July, 1991) as larger fish may have over-wintered from the previous year.

The strong association between prevalence of eye abnormalities and season of capture indicates the increased susceptibility to or the more forceful action of causative agents in creating ocular lesions with increasing water temperatures. As indicated by regression and correlation analysis, a positive relationship was observed between bottom water temperature and the prevalence of eye abnormalities. The positive correlation could possibly be

attributed to the increased solubility of chemicals from sediments into overlying waters (Schwarz, 1977; Doucette and Andren, 1988; May et al., 1983; Friesen and Webster, 1990). It is also documented that the metabolism of high molecular weight PAH also increases as temperatures rise (Kennedy et al., 1989 and Kennedy and Walsh, 1991). Enzyme systems involved in such metabolism have been preliminarily observed in fish eyes (Hawkes, 1980). If PAH start to diffuse into the ocular cavity in conjunction with the induction of detoxifying enzymes water-soluble reactive metabolites would be produced that could interfere in the normal physiology of the eye. An interesting result of the present study was that the cornea was the second most affected ocular tissue (corneal opacification plus corneal ulceration). This finding suggests that chemical exposure, at least in part, may be directly through the corneal tissue.

On the other hand, the effects of increasing water temperature and duration of exposure are difficult to separate when the species collected are not year-round residents. Nevertheless, if duration was the key factor involved in prevalence than one would expect lesion occurrence to increase throughout the time period fish are present. Data in this study clearly show that prevalence peaks during the hottest part of the summer and declines thereafter, even when sciaenid fish are still relatively abundant. This is not to say that duration of exposure is

not important, just that water temperature appears to play a role.

The reduced C_r-values observed for fish with eye abnormalities collected from the 217 sampling site suggest a possible disturbance in the nutritional state of diseased fish. Hypothetically, if the vision of an organism is impaired, then its ability to obtain and select food items should also be reduced. Hargis et al. (1984) and Roberts et al. (1989) did observe reduced feeding in spot exposed to ER sediments under laboratory conditions. Phan and Vazzoler (1981) could show no statistical difference in condition factors for cataractous and non-cataractous South American croaker (*Micropogonias furnieri*) sampled from the coast of Brazil. The significantly reduced C_r-values observed in this study between fish which exhibited no ocular disorders collected from station 217 and YR may just be indicative of different food habitats, environmental conditions, variations between fish and river relationships, or exposure to chemical contamination.

Data from this study clearly show that ocular abnormalities are prevalent disease disorders, that certain specific ocular lesions may be indicative of fish exposed to chemically-compromised environmental habitats, that elevated water temperatures augment the manifestation of ocular disease conditions, and that ocular lesions appear to interfere in the nutritional state of fish species

inhabiting such environments. The effect of significantly increased prevalence of ocular lesions at the community/population level could be significant, due to its possible impact on other behavioral attributes such as predator avoidance, food collection, schooling-associated actions, and probably warrants greater research attention. Finally, of the ocular disease signs reported, cataract is, by far, the most prevalent as well as the most consistently observed lesion present.

CHAPTER 3

PROGRESSION OF CATARACT IN FISH COLLECTED FROM THE SOUTHERN BRANCH OF THE ELIZABETH RIVER, CHESAPEAKE BAY, VA.

Introduction

Cataract formation has long been reported to be one of the most commonly observed fish diseases (as reviewed in Hargis, 1991). Several factors are believed to induce cataract such as parasitic infestation (Petrushevski and Shulman, 1961; Uspenskaya, 1961), nutritional deficiencies (Halver, 1953; 1979; Hess, 1935; Hughes, 1985; Ketola, 1979; Poston et al.,1977; Poston et al., 1978; Richardson et al., 1986), osmotic imbalance (Iwata et al., 1987a; Iwata et al.,1987b), ultraviolet radiation (Allison, 1962;1963), and chemical intoxication (Hargis and Colvocoresses, 1986; Huggett et al., 1987; Hargis and Zwerner, 1988; Von Sallman et al., 1966).

Studies performed by Hargis and Colvocoresses (1986) and Hargis and Zwerner (1988) described the presence of cataract in fish sampled from the Elizabeth River (ER), Chesapeake Bay, VA. This phenomenon was found predominantly in fish species belonging to the family Sciaenidae, namely Atlantic croaker (*Micropogonias undulatus*), weakfish (*Cynoscion regalis*), and spot (*Leiostomus xanthurus*, Hargis and Zwerner, 1988) and was positively associated with sediment levels of polycyclic aromatic hydrocarbons (PAH, Hargis and Colvocoresses, 1986). Furthermore, Huggett et al. (1987) reported that fish size, larger fish displayed a greater prevalence, was also associated with cataract

formation. Finally, cataract lenses have been histologically characterized as having a hyperplastic epithelial layer, a compacted cortical layer, and fragmented fiber cells (Hargis and Zwerner, 1989).

The pioneer studies of Hargis and co-workers have raised several questions whose elucidation may shed light on the mechanism of cataract formation. First, it is unknown whether cataract formation is a direct result of chemical intoxication or if it is a response of fish becoming more susceptible to other cataractogenic factors because of exposure to high levels of chemical contamination. Secondly, if chemical contamination is the cause it is unclear as to whether cataract formation occurs rapidly as a result of an acute exposure or develops relatively slowly in a series of intermediate steps due to chronic/sporadic exposure.

Therefore, the primary objective of this study was to follow the formation of cataract and to describe its development. The second aim was to determine whether a relationship exists between certain environmental parameters such as salinity (osmotic imbalance), temperature (metabolism), and presence of parasites. In addition, this study further examines the relationship between cataract formation and the normal growth of fish and fish lenses.

Hence, it is the objective of the present investigation to supplement earlier studies aimed at investigating

cataract development in fish from the family Sciaenidae. This investigation describes, in detail, lens opacification with regards to initiation and progression within the Chesapeake Bay.

Materials and Methods

Test organisms

Young-of-the-year fish (< 15 cm) from the family Sciaenidae were collected. Care was taken to ensure that all fish sampled during each time period were of similar size.

Trawl sampling was performed monthly from April, 1991 through June, 1992 for two stations within the Elizabeth River (ER; station 217; 96 µg/kg mean total sediment PAH concentration, latitude = 36° 50 -37° 51′, longitude 76° 17′ - 76° 18′; and station WS; 22 µg/kg mean total sediment PAH, latitude = 36° 46 - 36° 47′, longitude 76° 17′ - 76° 18′) and for one station within the York River (YR = reference site; 1 µg/kg mean total sediment PAH, latitude = 37° 15 - 37° 22′, longitude 76° 30 - 76° 40′, Fig. 7). A 14 x 30 ft. otter trawl net (1.0 inch mesh) was used for short duration trawls (5 min). In order to attempt to sample fish migrating into and out of the rivers periodic trawl sampling was performed on a seasonal basis (March - April and September - November, 1991 and 1992) from the mouth of the

Chesapeake Bay (CBM; 0.2 μg/kg mean total sediment PAH, latitude = 37° 00 -37° 20′, longitude 76° 00 - 76° 20′) and the mouth of the James River (JRM; 1.6 ng/kg mean total PAH, latitude = 37° 00 -37° 10′, longitude 76° 20 - 76° 30′, Fig. 7).

Reported sediment PAH concentrations for sampling stations were obtained from the Virginia Institute of Marine Science Computer Toxics Database, Division of Environmental Sciences (Table 6, Craig L. Smith, VIMS, unpublished data, 1991, 1992). The values obtained for sediment concentrations of total PAH at the sampling stations are the mean of sediment grab and core samples taken from various locations at each site since 1984. Bottom water temperatures are the average temperatures sampled from the James River collected on a monthly basis from April, 1991 through March, 1992 (VIMS, Division of Fisheries Sciences).

Fish sampled from stations WS, 217, and YR were transferred alive to the laboratory where they were maintained in aerated holding tanks (150 gallon) and were examined within 4-12 hours after collection. Specimens collected from stations CBM and JRM were transferred in sealed containers under ice and examined within 2-12 hours after collection. Prior to examination, all live fish were anesthetized with tricaine methanesulfonate (MS-222, 0.2g/L) (Sigma Chemical Co., St. Louis, MO). All fish were measured for total length (from the snout to the end of the caudal

fin, ± 0.5 cm), weighed (± 0.1 gm), and observed for cataract formation and other abnormalities. Smaller fish (≤ 10 cm) were examined using a dissection microscope (10x) while larger fish (10-15 cm) were observed by the naked eye. Fish with cataract lenses were classified by estimating the percent opacification of the surface area of the lens for both the right and left eye. Subsamples of examined fish from stations WS and 217 were stored at -20 °C for sex determination performed at a later date.

The total number of fish examined by species was dependent upon trawl yield (abundant in summer months) and followed the criteria suggested by the American Fisheries Society Bluebook for detection of fish diseases (1985). During high yield months (June-October) a total of at least 150 sciaenids were sampled per sampling station while during low yield months no less than 50 specimens were obtained from each sampling site.

Fish condition index

In order to investigate the effect of cataract on the body condition of fish the formula $C_f = W(100)/L^3$ was utilized where W = weight (g) and L = total length (cm). The condition factor was determined on individually measured fish caught in August, 1991 (maximal prevalence of eye disorders, Chapter 2) from station 217 (ERC = fish with cataract, ERNC = fish without detectable cataract) and from

a reference site, station YR.

Depuration studies on the progression of cataract

Spot collected from the YR and ER (station 217) during August, 1993 were transferred alive to the Virginia Institute of Marine Science. Spot from the ER consisted of two groups of fish, namely stage 1 cataract (ERC) and non-cataract specimens (ERNC). The three classifications of fish (YR, ERNC, and ERC) were placed into separated flow through tanks supplied with filtered York River water (19 ppt) and adequate aeration. Fish were fed *ad-libidum* twice per day and were held under laboratory conditions for a total of 150 days. Fish were sampled at 30, 60 and 150 days, grossly observed for cataract formation under light anesthesia, and replaced back into their appropriate tank.

Lenses

Lenses were dissected from the ocular cavity and any exogenous material removed from the lens surface. Paired lenses were immediately blotted dry, placed into a petri dish, measured for diameter with an ocular micrometer (15x, mm), and weighed (mg). Lenses were classified as being cataractous or non-cataractous. Cataract lenses were further classified by the stage of cataract development visible in the whole fish.

Lens size:body size relationships

Lenses from YR, ERNC, and ERC fish (stage 1 and 2) were used for analysis. Linear regression analysis was performed on lens diameter (mm) by fish total length (cm) and lens weight (gm) by fish weight (gm). Regression analysis was used to determine differences between R^2-values (variation) and slopes (lens:body growth) for ERC (station 217, stage 1 and stage 2), ERNC, and YR lenses.

Statistics

Statistical analysis for prevalence of cataract formation was performed by using the G_H-statistic for frequency analysis (Sokal and Rohlf, 1981). Analysis of fish condition factors was performed using a one-way ANOVA model for individually measured fish using the Statistica analysis package (StatSoft, Release 3.0A, 1991-92, Tulsa, OK). Significant differences between means were determined by the use of the Scheffe multiple mean comparison test (Statistica) at $\alpha = 0.05$. Relationships between prevalence of cataract development and sediment levels of total PAH as well as water temperature (°C) were determined by linear regression and correlation analysis using the MINITAB statistics package.

Results

Description of cataract formation

Cataract was observed in all three species of sciaenids collected (Fig. 8). Opacification was observed in one (unilateral, 12.1% of total fish with cataract) or both (bilateral) lenses with no statistical difference between left or right lens occurrence. Cataractous fish displayed varying degrees of lens opacification and were classified into stages according to the degree of cataract formation (% of lens). The classification scheme utilized was as follows (Fig. 9,10): a) stage 1, 5 - 15% of the lens region showed opacification; b) stage 2, 20 - 50% of the lens region showed opacification; c) stage 3, 60 - 95% of the lens region showed opacification; and d) stage 4 (mature cataract), 100% of the lens was opaque. All stages of development were observed in each of the three species examined. Minor cataract development (<10%) predominantly manifested itself as a centrally located (within the visible center of the lens) opacification (Fig. 10). Dissection microscopy showed that non-cataract lenses were well delineated into two major sections, a large spherical central region (the nuclear region) and a circular outer band (the epithelial and cortical regions). Both of these regions were translucent when observed by dark field dissection microscopy (Fig. 10). Stage 1 cataract was

characterized by a well demarcated, centrally located opacification with varying degrees of intensity. In intermediate stage development (stages 2 and 3) opacification progressed in bands to involve the entire inner portion of the lens. In stage 4 development, opacification extended to incorporate the outer most region (the epithelial layer) of the lens and, in general, the entire lens became opaque.

Prevalence of cataract formation

Fish collected from the CBM and YR stations exhibited no signs of cataract formation throughout the entire sampling period. Fish collected at the JRM station, however, did exhibit cataract during August, 1991. The annual prevalence of lens opacification at stations 217 was significantly greater than other sampling sites ($p<0.001$). Cataract formation at station WS was not statistically different than that at JRM, but was significantly greater than those at CBM and YR ($p<0.001$). Frequency of occurrence of fish with cataract, by station, for the complete sampling period is shown in Table 7. No statistical difference in percent occurrence of cataract formation was observed between sexes.

Seasonal prevalence

An association between cataract prevalence and seasonal

occurrence was observed ($G_{H,ER}$=262.4, p<0.001, df=3; $G_{H,217}$=262.0, p<0.001, df=3). Data from Station 217 showed that the peak prevalence was during summer sampling (32.6%, n=591), followed by significantly reduced levels of occurrence during fall (20.9%, G_H=13.8, df=3, p<0.005, n=296), winter (6.1%, G_H=27.4, df=3, p<0.001, n=263), and spring sampling (1.5%, G_H=12.0, df=3, p<0.01, n=546). Samples collected at Station WS, however, showed no statistical difference in prevalences observed in summer (7.9%, n=578), fall (8.9%, n=235) or winter (5.2%, n=173) sampling periods. Although winter and spring periods (1.4%, G_H=7.2, n=509) were not statistically different, spring occurrence was significantly reduced compared to both summer and fall collections ($GH \geq 28.3$, df=3, p<0.001).

Prevalence of cataract within the ER ranged from 0% in January - March, 1992, to 47.2% in August of 1991, at station 217, and 0% in February - March, 1992, to 11.9% in September of 1991 at station WS (Table 7). Values determined for 217 showed a steady increase in cataract prevalence from the start of sampling in April through August with a subsequent reduction in prevalence from August through March. A minor peak in October (27.1%) was also observed at Station 217.

Species susceptibility (all months combined) to cataract formation indicated that significant differences did exist. Prevalence of cataract on pooled ER data

(Station 217 plus WS) was significantly higher in the Atlantic croaker (13.5%, n=1517) as compared to spot (10.4%, G_H=7.0, p<0.05, df=2, n=1483) or weakfish (6.5%, G_H=8.4, p<0.025, df=2, n=185). No significant statistical difference was observed between cataract formation in spot and weakfish. Statistical analysis for Station WS data, however, indicated that spot exhibited a significantly higher prevalence (8.1%, G_H=10.8, p<0.005, df=2, n=703) compared to either croaker (3.8%, n=680) or weakfish (2.1%, n=96), respectively. On the other hand, data collected from Station 217 were similar in nature to that determined for pooled ER data, i.e. croaker displayed a significantly elevated level of cataract formation (19.6%, G_H=18.6, p<0.001, df=2, n=903) while spot (11.9%, n=780) and weakfish (11.2%, 89) exhibited statistically similar prevalences. Species comparison by month sampled is shown in Fig. 11 and Table 8.

Cataract progression

Utilizing the previously described cataract classification scheme, prevalence of stages of cataract for samples collected at Station 217 were recorded (Fig. 12). Prevalence shows the early predominance of stage 1 development followed by (approximately 1 month) increases in both stage 2 and 3, and the final progression towards total cataract formation. Time between peak periods of stage 1

and stage 4 prevalence was approximately 1-2 months. During the two major declines in percent occurrence of stage 4 cataract fish an 80-85% reduction was observed. Differences between ER stations were minor in nature as both sites showed similar trends. Basically, stage 1 predominated early and then declined as stage 4 cataract became more prevalent. In both instances stage 2 and 3 development showed relatively low rates of occurrences, compared to stage 1 and 4, and paralleled each other with stage 2 consistently obtaining higher occurrences. As stage 4 declined, a subsequent increase in stage 1 occurrence reappeared. The major difference between the two sites was that at WS stage 1 formation never declined to below stage 4 percentages, whereas at station 217 such a phenomenon was consistently observed.

Effects of some environmental factors on cataract prevalence

Cataract prevalence was observed to increase as a function of water temperature. Pearson correlation analysis for station 217 indicated that a R-value of 0.61 ($p<0.05$) was obtained. No statistical differences were observed between YR and SBER stations or within stations when prevalence of cataract was compared with other water quality parameters, pH (7.9-8.3) and salinity (16-23 ppt).

A significant relationship between sediment PAH concentrations, water temperature, and cataract formation

appears to exist. Multiple regression analysis that utilized bottom water temperature (BT, °C), station sediment levels of PAH (PAH), and monthly prevalence of cataract formation (log_{10} (%+1) transformed, all 12 months) exhibited an R^2-value of 47.2% (y = -2.84 + 0.03 BT + 0.0001PAH, p_{REG}=0.000, p_{BT}=0.007, p_{PAH}=0.000, p_k=0.14, df=2). Separating the 12-month sampling procedure into blocks of time, in an attempt to incorporate the seasonal component of cataract occurrence, an R^2-value of 64.2% was obtained for months 6-12 (y = -0.04 + 0.01BT + 0.00002PAH, p_{REG}=0.000, p_{BT}=0.345, p_{PAH}=0.000, p_k=0.855, df=2) while months 6-10 showed an R^2 = 68.2% (y= -0.72 + 0.04BT + 0.00002PAH, pREG=0.000, pBT=0.03, pPAH=0.000, pk=0.087, df=2). The reasons why these periods of time were selected were: 1) sciaenids usually migrate offshore by the end of October; and 2) months 1-3, 1991 may have incorporated fish from more northern river systems of the Chesapeake Bay (such as the York River) which found their way into the ER.

Change in lens opacification in laboratory maintained fish

Results (Table 9) showed that over the 150 day trial period YR fish displayed no signs of cataract formation. On the other hand, ERNC fish were observed to have some progression of cataract formation (although minimal, 2.5%) between Days 30 and 60. Positive cataract development was observed in 7.3% of the fish by the end of the trial period.

As for fish with cataract, no progression in opacification had occurred after 60 days. On the other hand, two specimens (8% of the total) did show a degree of recovery by the end of the study (150 days), in that the original opacification was no longer evident.

Body condition index

One-way analysis of variance showed that significant differences existed between the average condition factor of normal fish sampled at stations 217 and YR (217 fish displayed larger C_f-values, $p<.001$, Fig. 13) as well as between cataractous and non-cataractous fish collected from station 217 ($p < 0.001$) in August, 1991. No significant difference between mean condition factors was observed between 217 fish with cataract and fish collected within the YR sampling site. Mean condition factors ($\pm$ SD) for 217 fish with and without cataract lenses and YR fish were 1.09 ($\pm$ 0.17), 1.16 ($\pm$ 0.17), and 1.05 ($\pm$ 0.16), respectively. Stratification of condition factors with regards to stage of cataract development showed that only those fish that exhibited stage 4 cataract had significantly reduced length to weight relationships. Mean values for sampled fish collected at Station 217 that exhibited an average ocular value (left eye stage value plus right eye stage value/ 2) for cataract development of 0, 0.5-1.5, 2.0-2.5, 3.0-3.5, and 4.0 were 1.16 ($\pm$ 0.18), 1.13 ($\pm$ 0.14), 1.16 ($\pm$ 0.24),

1.10 (± 0.18), and 1.03 (± 0.13), respectively.

Lens:body relationships

In general, both total length and weight of fish were good determinants of lens size (diameter or weight, Fig. 14). Simple linear regression analysis for lens diameter by fish length indicated that the R^2-values for YR ($y=0.02x+0.13$, $n=24$), ERNC ($y=0.03x+0.05$, $n=30$), ERC (stage 1, $y=0.02x+0.15$, $n=39$), and ERC (stage 2, $y=0.007x+0.28$, $n=12$) lenses were $R^2=0.84$, $R^2=0.93$, $R^2=0.94$, and $R^2=0.40$, respectively. Regression analysis for lens weight by fish weight demonstrated that the R^2-values for YR ($y=0.001x+0.010$), ERNC ($y=0.001x+0.008$), ERC (stage 1, $y=0.001x+0.015$), and ERC (stage 2, $y=0.0003x+0.022$) lenses were $R^2=0.97$, $R^2=0.94$, $R^2=0.77$, and $R^2=0.32$, respectively.

Table 6: Prevalence of cataract formation and mean chemical composition of selected polycyclic aromatic hydrocarbons (ng/g) in sediment samples as taken from the long-term Toxics Database of the Virginia Institute of Marine Science.

	Sampling station							
Prevalence/ concentration	CBM	JRM	YR	WS	217	R^2	p	regression equation
Cataract (%)	0.0	5.8	0.0	5.2	14.9	-	-	
Naphthalene*	8	37	22	432	3694	83.5	0.030	y=1.53+0.006x
LMW**	42	514	530	5187	34835	84.2	0.028	y=1.80+0.0003x
BaP	4	56	30	785	2592	85.7	0.024	y=1.89+0.0001x
BeP	6	35	30	778	2088	84.3	0.028	y=2.27+0.003x
HMW***	62	654	403	10312	39914	85.6	0.024	y=2.12+0.0004x
Total PAH	156	1637	1063	21551	96046	85.5	0.025	y=1.66+0.005x
Sample size	8	4	3	4	9	-	-	-

* does not include methyl-phenyl-naphthalene or phenyl-naphthalene
** LMW = low molecular weight PAH, includes all PAH identified with a molecular weight $\leq$ to the molecular weight of pyrene
*** HMW = high molecular weight PAH, includes all PAH identified with a molecular weight > the molecular weight of pyrene

BaP = benzo[a]pyrene, BeP = benzo[e]pyrene

Regression equations show the prevalence of cataract as a function of chemical concentration

Table 7: Prevalence of cataract in all sciaenid fish species collected within the Chesapeake Bay.

	1991		Month							1992		
Station	4	5	6	7	8	9	10	11	12	1	2	3
CBM	0.0 (51)	0.0 (49)	NS	NS	0.0 (50)	0.0 (55)	0.0 (47)	NS	NS	NS	NS	0.0 (0)
JRM	0.0 (31)	0.0 (30)	NS	NS	11.8 (119)	0.0 (18)	0.0 (41)	NS	NS	NS	NS	0.0 (0)
WS	2.2 (178)	1.8 (285)	10.3 (310)	4.6 (175)	8.2 (73)	11.9 (101)	6.2 (64)	7.1 (70)	8.0 (88)	6.1 (33)	0.0 (57)	0.0 (50)
217	1.0 (297)	6.6 (182)	22.2 (158)	21.0 (195)	47.1 (248)	16.4 (128)	27.1 (59)	22.9 (109)	14.4 (111)	0.0 (111)	0.0 (50)	0.0 (67)
YR	NS	NS	0.0 (175)	0.0 (240)	0.0 (153)	0.0 (159)	0.0 (118)	0.0 (87)	0.0 (0)	*NS	0.0 (43)	*NS

*NS = not sampled due to inclement weather conditions
NS = not sampled
(#) = sample size

Table 8: Sample sizes for the collection of the sciaenid fish species; Atlantic croaker (C), spot (S), and weakfish (W) from the York and Elizabeth Rivers.

	Sampling station								
	YR			WS			217		
Month/ Species	C	S	W	C	S	W	C	S	W
4	-	-	-	114	63	1	116	180	1
5	-	-	-	163	120	2	143	36	3
6	91	78	6	148	160	2	112	35	11
7	129	75	36	85	62	28	67	106	22
8	73	30	50	20	14	39	106	121	21
9	35	116	8	25	73	3	5	123	0
10	18	11	89	9	37	18	23	27	9
11	0	77	10	3	64	3	40	56	13
12	0	0	0	1	87	0	10	92	9
1	-	-	-	17	16	0	102	0	0
2	45	0	0	45	12	0	50	0	0
3	-	-	-	50	0	0	63	4	0

Table 9: Cataract development in spot collected from the York River and Station 217 within the Elizabeth River maintained in water from the York River.

	Period of Depuration (days)			
	0	30	60	150
Classification	C/NC	C/NC	C/NC	C/NC
ERC: stage 1	25/0	25/0	25/0	23*/2
ERNC	0/41	0/41	1/40	3/38
YR	0/30	0/30	0/30	0/30

C = cataract lens
N = non-cataract lens
* = 2 fish were classifed as having bilateral stage 2 cataract development

Figure 7: A map of the lower Chesapeake Bay showing the sampling sites. YR = York River (reference site), JRM = James River Mouth, WS = Waterside, and 217 = Station 217. INSERT shows the Chesapeake Bay Mouth (CBM) sampling site.

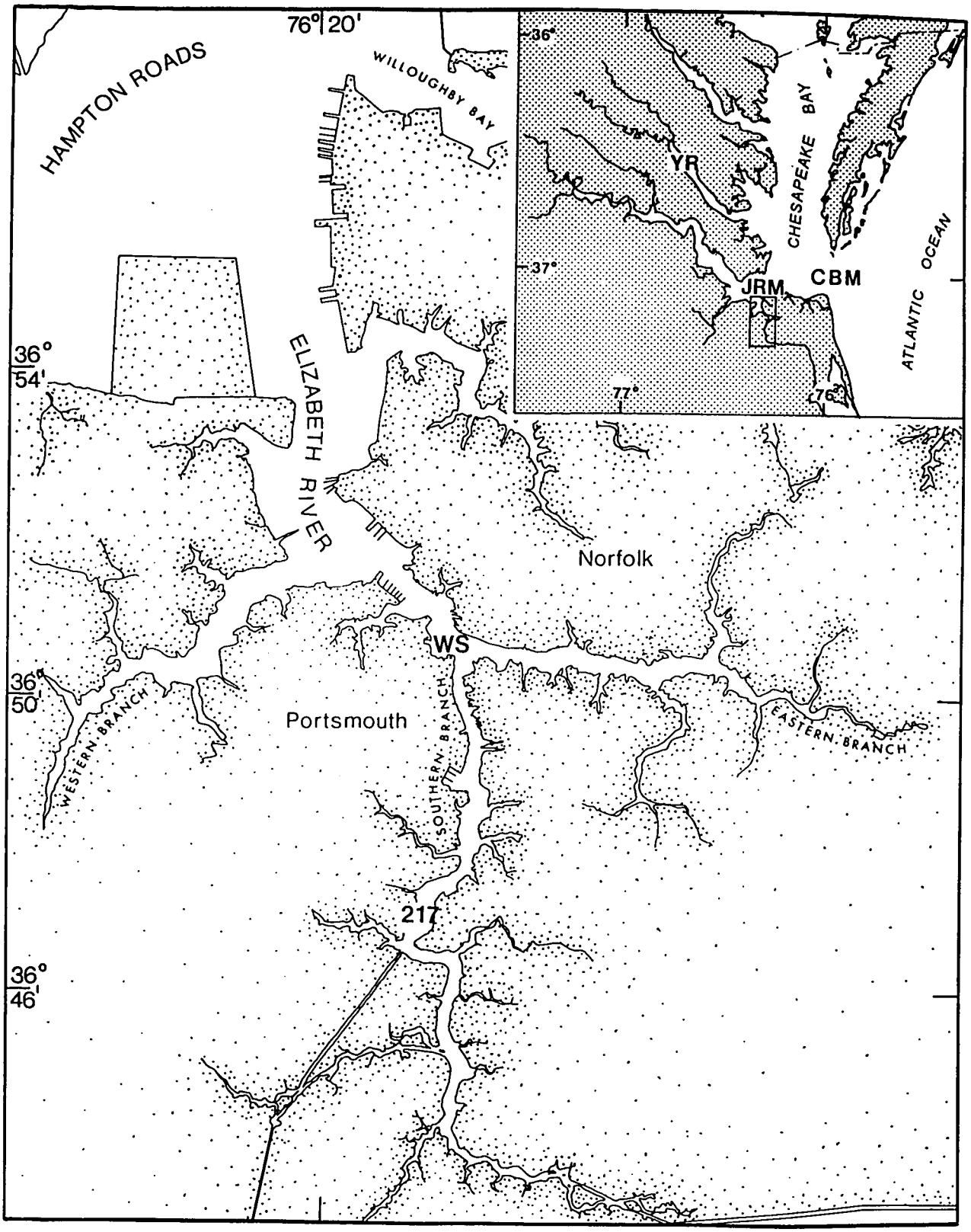

76° 20'
HAMPTON ROADS
WILLOUGHBY BAY
36°
54'
ELIZABETH RIVER
Norfolk
WS
36°
50'
Portsmouth
WESTERN BRANCH
SOUTHERN BRANCH
EASTERN BRANCH
217
36°
46'
36°
37°
YR
CHESAPEAKE BAY
JRM
CBM
ATLANTIC OCEAN
77°
76°

Figure 8: Cataract formation in sciaenids sampled from the Southern Branch of the Elizabeth River : A) spot, B) weakfish, C) Atlantic croaker.

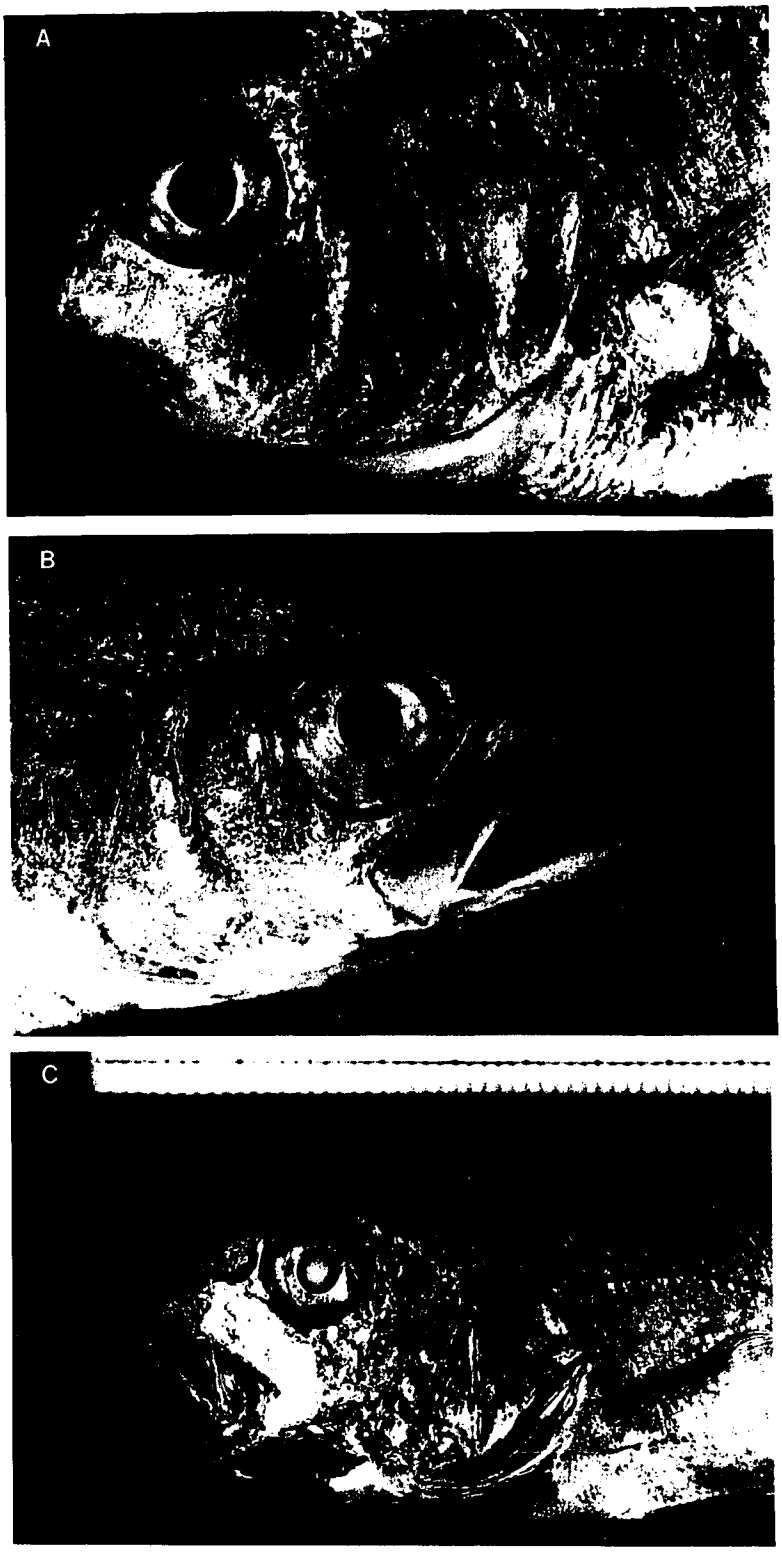

A
B
C

Figure 9: Stages of cataract development in sciaenid fish species: A) stage 1 (spot), B) stage 2 (spot), and C) stage 4 (Atlantic croaker).

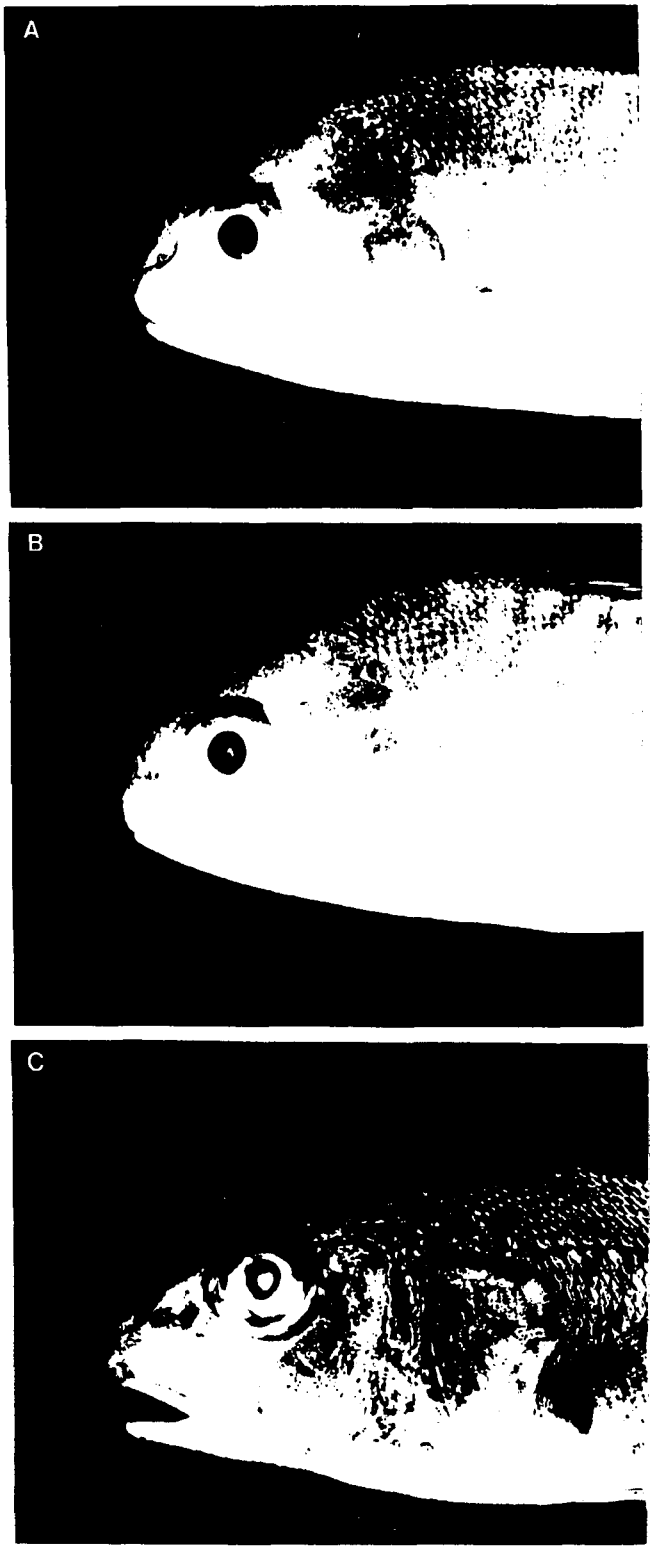

A
B
C

Figure 10: Stages of cataract formation in isolated lenses of cataractous fish: A) normal eye lens, B) stage 1, and C) stage 4.

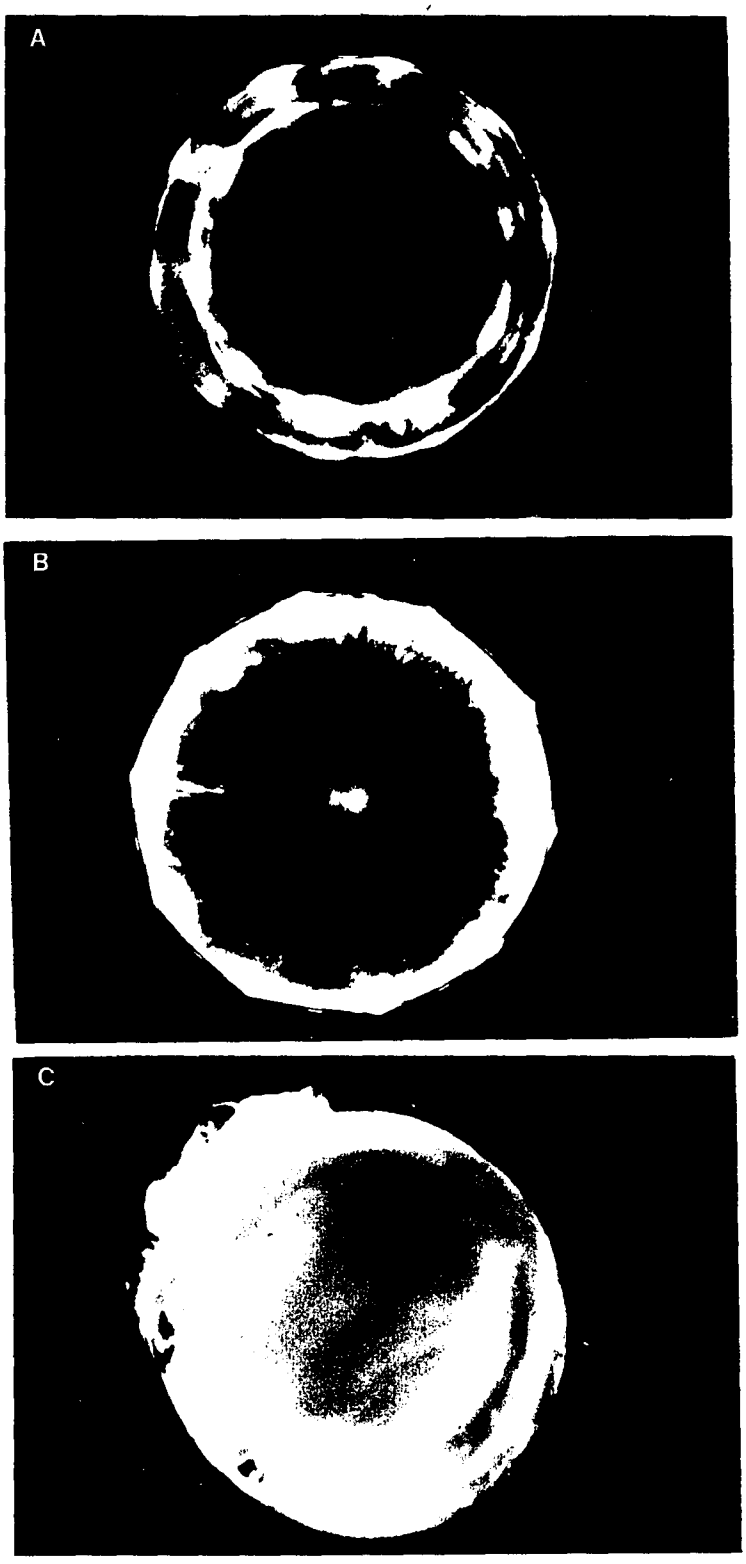

A
B
C

Figure 11: Prevalence of cataract formation (all stages combined) within the ER by species.

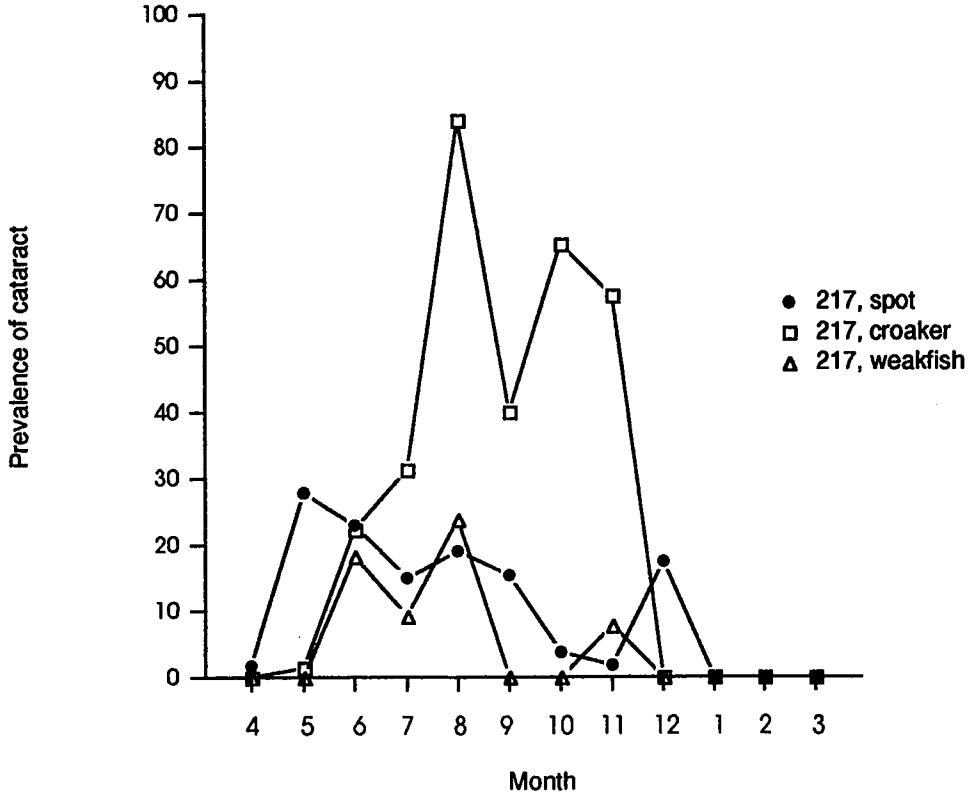

Prevalence of cataract
100
90
80
70
60
50
40
30
20
10
0
4
5
6
7
8
9
10
11
12
1
2
3
Month
217, spot
217, croaker
217, weakfish

Figure 12: Prevalence of cataract formation by stages of development for all species collected within the ER.

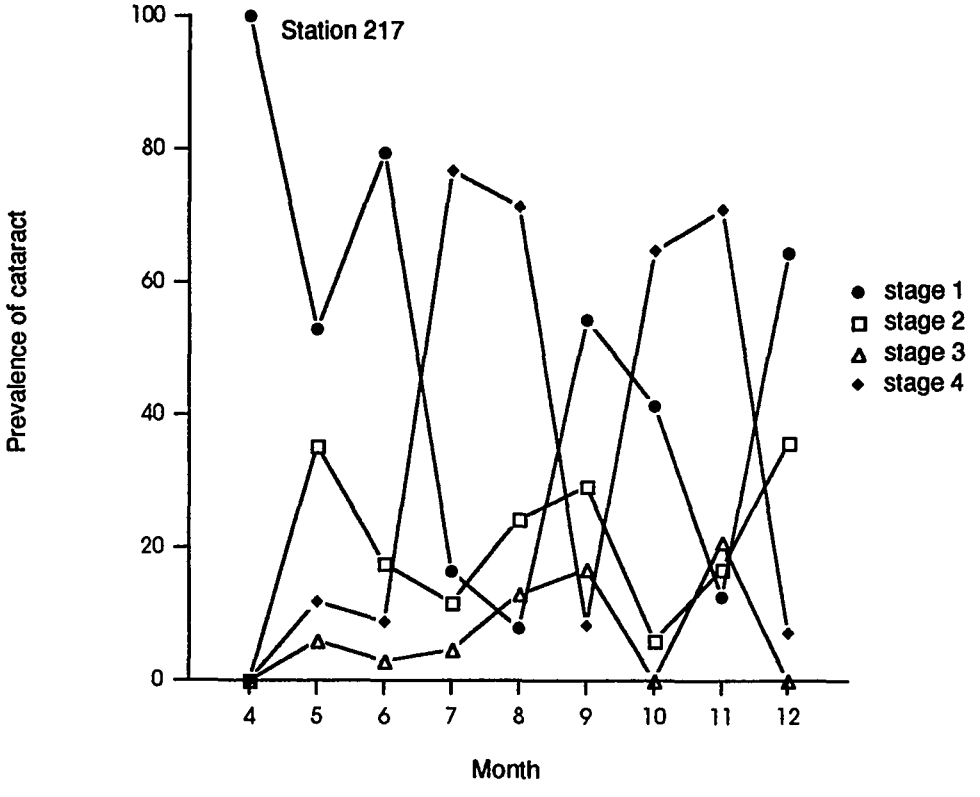

Station 217
Prevalence of cataract
100
80
60
40
20
0
4
5
6
7
8
9
10
11
12
Month
stage 1
stage 2
stage 3
stage 4

Figure 13: Mean condition factors of cataractous and non-cataractous fish sampled from station 217 and the York River during August of 1991 (the month of peak cataract prevalence).

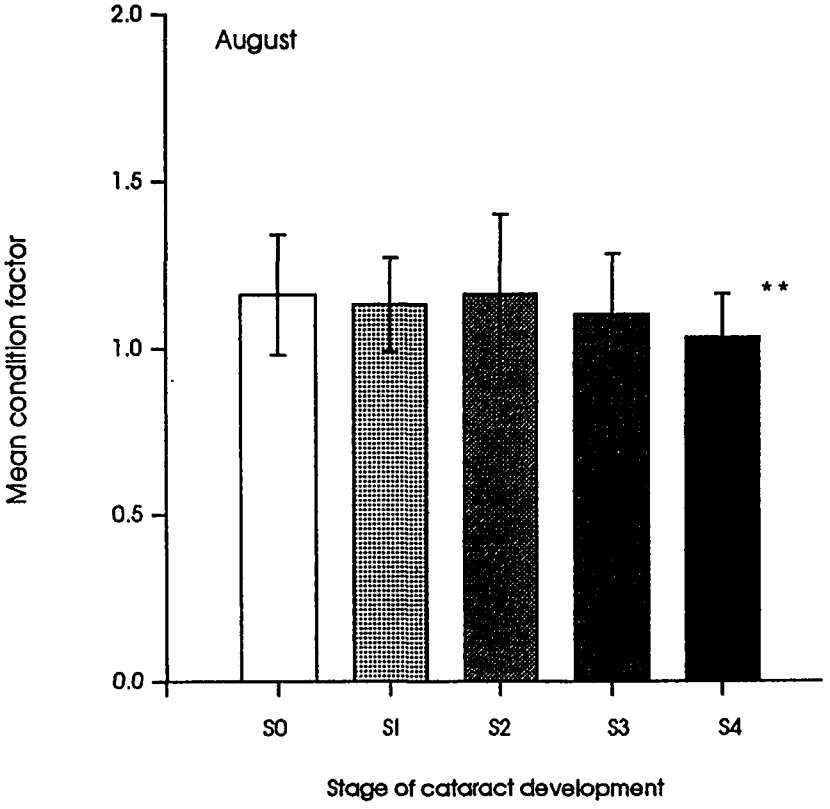
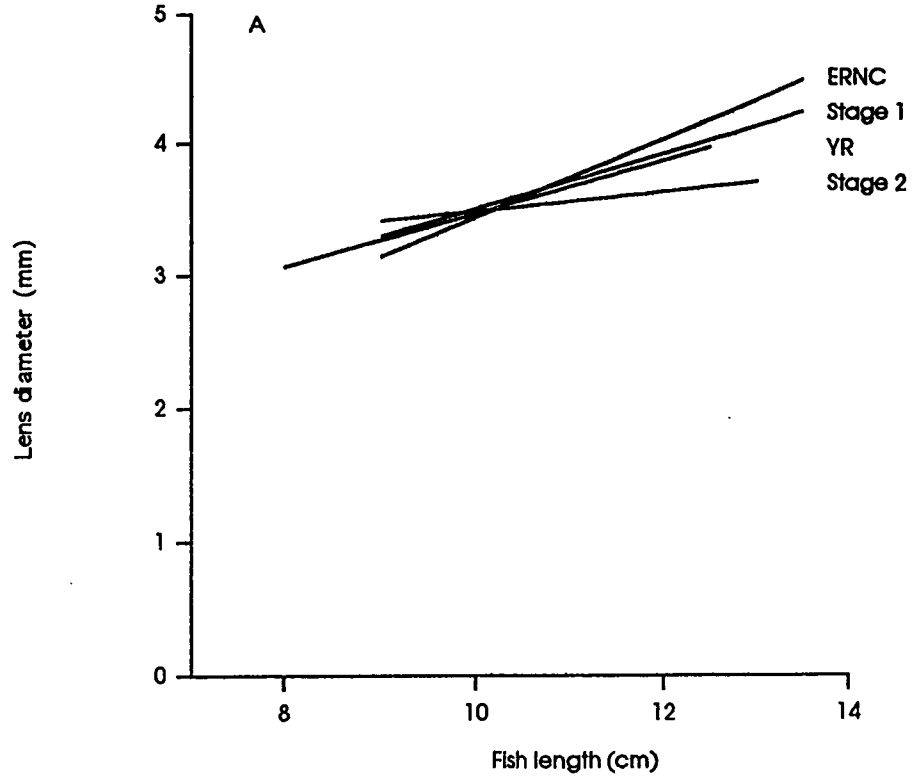
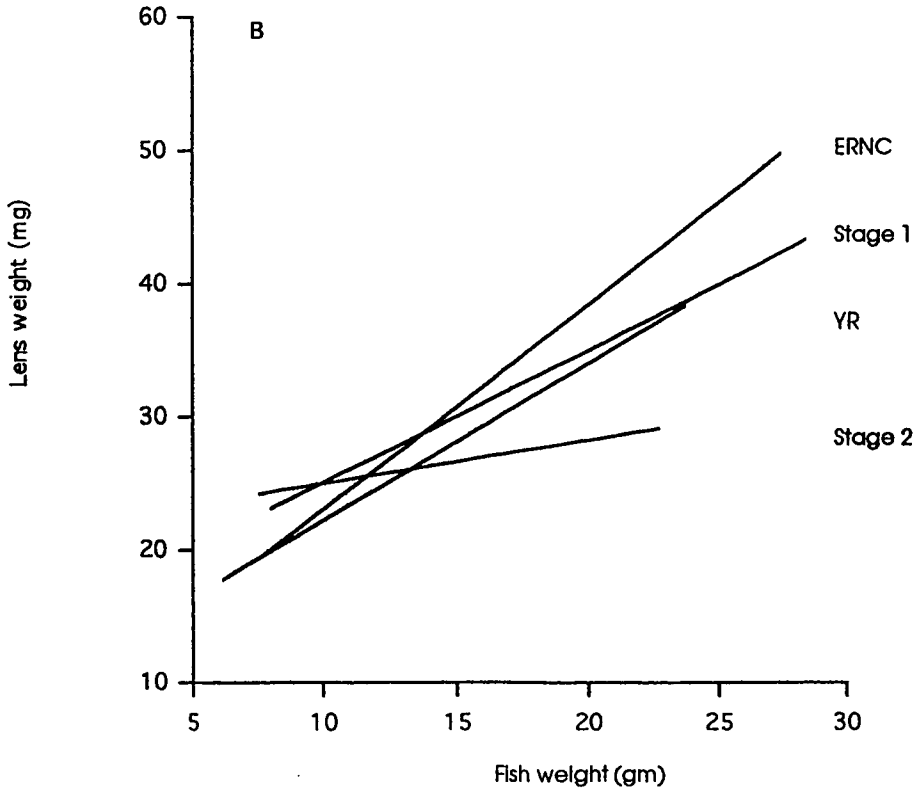

August
2.0
1.5
1.0
0.5
0.0
Mean condition factor
**
S0
SI
S2
S3
S4
Stage of cataract development

Figure 14: Linear regression analysis for the relationship of lens size to fish size for cataract and non-cataract specimens.


A
Lens diameter (mm)
0
1
2
3
4
5
8
10
12
14
Fish length (cm)
ERNC
Stage 1
YR
Stage 2


B
Lens weight (mg)
10
20
30
40
50
60
5
10
15
20
25
30
Fish weight (gm)
ERNC
Stage 1
YR
Stage 2

Discussion

The results of our data suggest that cataract is induced within the ER. This conclusion is based on 1) the lack of cataractous specimens collected from the YR, JRM, and CBM stations between January-May (entrance of sciaenids into the CB from offshore), 2) the absence of cataract in young-of-the-year fish sampled from within the ER during the same time period, 3) the high prevalence of cataract formation stations within the ER, and 4) the distributional pattern in stages of cataract development in fish of the ER. Although a collection of cataract specimens (n=11, stage 1) did occur at the JRM station this was a singular event and was considered to be a school of fish that had migrated outside of the ER. These findings support the conclusions made by Hargis and coworkers (1986, 1988) and Huggett et al. (1987) that cataract formation could be attributed to exposure to sediments heavily contaminated with PAH. Transport of specimens under ice from Stations CBM and JRM appeared to have no effect on the observation of cataract in fish collected from these sites.

As previously mentioned, cataract formation in fish can be caused by a number of factors. Based on our findings, several known inducers of cataract can be excluded, or at least minimized, such as infection with parasites (since parasites of the lens were extremely rare, N>500, n=1) and

UV radiation (due to the limited ability of UV to significantly penetrate turbid estuarine waters). Nutritional deficiencies can be excluded due to the fact that reduced conditioned factors were not associated with any of the intermediate stages of cataract formation and rarely do nutritional deficiencies occur in feral fish populations. Osmotic imbalances seem improbable because 1) sciaenids are euryhaline in nature (Phillips et. al, 1989; Hales and Van Den Avyle, 1989; Mercer, 1989) and are known to tolerate wide ranges of salinities, and 2) osmotic cataract formation is usually temporary while sciaenid cataract is basically irreversible (Table 9). It appears, therefore, that the most likely causative factor most associated with cataract formation is the aquatic environment of the ER. Recently, cataract formation in fish was reported in which feral Atlantic salmon (*Salmo salar*) were observed to develop cataract in the North Sea (Fraser et al., 1989). Chemical induction was reported to be associated with exposure to dichlorvos (Nuvan), an organophosphate-based parasiticide.

In the ER, PAH levels increase from the mouth downriver to station 217 where the highest prevalence of cataract was observed (Bieri, et al. 1986). The significant association between increased cataract prevalence with greater sediment PAH concentrations denotes that a possible cause and effect relationship as previously hypothesized (Hargis and

Colvocoresses, 1986; Huggett et al., 1987; Hargis and Zwerner, 1988) might exist. Furthermore, croaker enter the ER earlier in the year than either spot or weakfish. This earlier presence in the ER would increase their duration of exposure which could in turn contribute to their increased prevalence of cataract formation. A common component of sediments collected from Station 217 as well as other sites within the ER is naphthalene (Table 6), a low molecular weight PAH, and benzo[a]pyrene (BaP, Table 6), a high molecular weight PAH. Previous work in mammalian research has shown naphthalene to be a cataractogenic agent (van Heyningen, 1967) while BaP has been reported to be a precarcinogen that when metabolized can interfere directly with DNA synthesis (Smolerak, 1988). Enzyme systems involved in the oxidative metabolism of naphthalene have been preliminarily observed in the eyes of fish (Hawkes, 1980). The sediments of the ER, however, are laden with a number of chemical substances (heavy metals, polychlorinated biphenyls, tributyltin, etc...) other than PAH that makes the identification of a causative factor extremely difficult.

Temperature may play an important part in cataract formation as shown by the seasonality of occurrence and the positive correlation with bottom water temperature.. Prevalence was low in late fall and early spring, rose rapidly from spring--early summer, peaked in August, and

declined thereafter. Young-of-the-year fish sampled during the months of January-March (the colder water months) were not observed to exhibit any form of cataract development. The positive association between cataract formation and water temperature, as previously stated in Chapter 2 for ocular lesions, in general, may be attributed to: 1) the increased solubility of chemicals from sediments into overlying waters (Schwarz, 1977; Doucette and Andren, 1988; May et al., 1983; Friesen and Webster, 1990); and/or 2) the increased metabolism of high molecular weight PAH (Kennedy et al., 1989; Kennedy and Walsh, 1991).

It should be noted, however, that duration of exposure closely parallels increasing water temperatures and the separation of the individual influences of these two parameters is difficult, especially in fish that are not year-round residents. Nevertheless, if duration was the key factor involved in prevalence than one would expect cataract to increase throughout the time period fish are present. Data in this study, similar to that observed for eye abnormalities as a whole (Chapter 2), clearly show that prevalence peaks during the hottest part of the summer and declines thereafter, even when sciaenid fish are still relatively abundant. This is not to say that duration of exposure is not important, just that water temperature appears to play a role. Further research aimed at isolating the influences of these two parameters is necessary before

any conclusions can be obtained.

Whether or not cataract formation in ER sciaenids is induced by the direct exposure of lenses to PAH remains to be confirmed. Hargis et al (1989) has reported cataract formation in spot exposed to ER-sediment effluents. Balk et al. (1984) showed that water and diets contaminated with ^{3}H-benzo[a]pyrene rapidly distributed into the ocular region. Furthermore, Xu et al. (1992) reported naphthalene metabolites (naphthalene dihydrodiol) in the aqueous humor and the lens of naphthalene fed rats. If PAH are carried to or diffuse into the ocular cavity in conjunction with the activation of detoxifying enzymes, then it is likely that such contamination could interfere with a number of ocular tissues.

The increased prevalence of cataract formation in Atlantic croaker indicates that genetic susceptibility may play a role, at least to some degree, in cataract formation. Previous work by Hargis and Zwerner (1988) indicated that fish from the family Sciaenidae showed increased prevalence of cataract compared to other species collected. The coincidental report of cataract formation in feral South American croaker (Vazzoler and Phan, 1981) and Atlantic croaker of the Chesapeake Bay is likely an indication of some inherent susceptibility. It is possible, therefore, that a genetic predisposition may exist that increases the probability of lens opacification.

Progression of cataract formation in fish from the ER was predominantly observed as an opacity at the visible center of the lens. Nuclear cataract formation can be indicative of physical-chemical modifications of the lens structural proteins, especially the γ-crystallins which are, in general, more concentrated in the nuclear portion of the lens (Simirskii et al., 1983). γ-crystallins are highly susceptible to oxidative modifications due to their high cysteine and methionine content (Zigler, 1994; Datiles and Kinoshita, 1991). PAH induce the mixed function oxidation system which produces reactive oxidative products such as H_2O_2. Such an activation has been suggested to be a mechanism by which oxidative-cataract formation could occur (Datiles and Kinoshita, 1991). Previous work by Van Veld et al (1988,90,91) and Roberts et al (1987) have indicated that enzymes associated with PAH metabolism and conjugation are elevated in non-ocular tissues of fish collected from the ER. Interesting to note is that exposure to naphthalene is also characterized by nuclear opacification. Xu et al. (1992) described the development of cataract formation in naphthalene fed rats. After one week of feeding water-clefts and spoke-like opacities appeared, by week three clefts and opacities had merged and formed an opaque shell deep in the cortex region, by 4-6 weeks the opacification became denser and deeper and appeared yellow to light brown upon excision from the ocular cavity.

Data from the depuration experiment indicated that, in general, cataract formation in spot is irreversible and that progression is dependent upon environmental conditions specific to the ER. Although a few instances of both cataract reversal and cataract progression (7.6% of ER spot) were observed, the general trend was for lens classification to remain unchanged.

Classification of fish cataract formation into stages of development, especially early stages in development, allows for the investigation into the mechanism of cataractogenesis. Comparison of non-cataract and stage 1 lenses enables research to be designed aimed at revealing processes involved in lens opacification. The lack of human specimens severely inhibits the ability of researchers in understanding how and why cataract formation occurs (Zigler, 1994). Cataract formation in sciaenid fish species occurs in a distinct pattern. The synchronicity between the prevalence of stage 1 cataract formation and the more advanced stages of cataract development indicates a progression in development. Development of stage 1 to stage 4 appears to take 30-60 days under spring and summer conditions with the intermediate steps (stages 2 and 3) relatively short in duration. Furthermore, the absence of a consistent increase in stage 4 fish throughout the summer seems to indicate that the ultimate fate of these fish may be death, presumably by either predation or fatal chemical

intoxication (skeletal remains at times have been trawled from the bottom of station 217). The reduced condition factors of fish with stage 4 cataract indicate the detrimental effects on the biological welfare of impacted individuals. Vazzoler and Phan (1981) indicated that fish with cataract had a significantly higher rate of mortality than did non-cataract specimens. They suggested that fish exhibiting bilateral fully developed cataract had only a 15% survival rate.

The greater variability in the relationship between lens weight and fish weight in fish that exhibited stage 1 and stage 2 cataract formation compared to ERNC lenses suggests that the normal growth of the lens is disturbed. The relationship between lens diameter and fish size was less affected, but still showed a significant reduction in stage 2 cataract lenses. These results indicate that the growth of the lens is interfered with during the early stages of cataract formation. Except during embryogenesis of the lens, the lens grows slowly but continuously throughout life (Jaffe and Horwitz, 1991) in mammals, the same would be expected for fish. Previous research had indicated that exposure to crude oil significantly increases lens diameter, probably due to osmotic imbalance and lens swelling. Hawkes (1977) reported an increase in lens diameter in trout fed a diet containing 1% crude oil for several months while Payne et al. (1978) reported a similar

response in cunners (*Tautogolabrus adspersus*) exposed to Venezuelan crude oil (0.6ml/L of water). The difference between these studies and the present is hard to interpret and may be due to a number of different parameters such as different types of contamination, different concentration levels, different exposure conditions, or species differences.

In summary, it would appear that cataract formation and progression in our fish samples (family Sciaenidae) is restricted to the ER and is most associated with levels of PAH, duration of exposure, and/or rising water temperatures. Opacification appears to initiate predominantly at the center of the lens. This may suggest that certain specific processes usually associated with this type of cataract formation (e.g. oxidation of γ-crystallins) might have occurred. Although the conditional index (i.e., the nutritional status) of affected fish is reduced, it would appear to be a secondary response to cataract formation and not the initiating factor. The alterations to the growth relationship that exists between lenses and fish size may indicate that a compositional change has occurred.

CHAPTER 4

ALTERATIONS TO THE BIOCHEMICAL COMPOSITION OF THE SPOT, *LEIOSTOMUS XANTHURUS*,, EYE LENS DURING CATARACT FORMATION

Introduction

Cataract formation has been reported in fish collected from sites in the Elizabeth River (ER), Chesapeake Bay, VA. (Hargis and Colvocoresses, 1986; Huggett et al., 1987; Hargis and Zwerner, 1988; Williams, Chapter 2). Cataract formation was described as a small spherical opacification at the (visible) center of the lens (stage 1) that appears to progress in stages until the entire lens is opacified (stage 4, Williams, Chapter 3). Cataracts were observed in species belonging to the family Sciaenidae (Hargis and Zwerner, 1988) and have been shown to be positively correlated with both sediment levels of polycyclic aromatic hydrocarbons (PAH; Hargis and Colvocoresses, 1986; Huggett et al., 1987, Hargis and Zwerner, 1988; Williams et al., Chapter 3) and bottom water temperature (Williams et al., Chapter 3). Previous work (Williams et al., Chapter 3) has shown that the lens weights (normalized for fish size) of stage 1 cataract lenses (<15% opacification) were significantly reduced compared to non-cataract lenses, while lens diameters were unaltered. This finding indicated that cataract formation may be associated with compositional changes in the lens.

Cataract formation, in general, is a complicated process and hence the mechanism(s) of induction has been difficult to elucidate, however certain major compositional

changes have been associated with different types of cataracts. For instance, changes in the protein composition of the lens, such as protein aggregation (Benedek, 1971), precipitation (Garner and Spector, 1979), or proteolysis (Van Heyningen and Waley, 1962; Blow et al., 1975) have been shown to be involved in cataract formation in mammals (Rathbun, 1989; Xu et al., 1992). As reviewed by Zigler (1992), crystallins are the major structural proteins of the lens (90-95% of all lens proteins). The water-soluble crystallins are highly stable, are vital to the maintenance of lens transparency, and can have intimate interactions with cellular membranes and intrinsic membrane proteins. The three major crystallins found in all vertebrate lenses are α-, ß-, and γ-crystallins. Therefore, the first objective of this study was to analyze if changes in the crystallin composition (qualitative and quantitative) of spot lenses are associated with cataract formation.

Disruption of normal membrane function has been suggested to be an early event in the initiation of lens opacification in humans (Kinoshita, 1974; Kern, 1979; Garner and Spector, 1980). Hargis and Zwerner (1989) showed that fiber cells were fragmented in cataractous lenses of spot. Phospholipids are the major structural components of cellular membranes and aid in maintaining the fluidity and flexible nature of membranes (Gurr and Harwood, 1991). Cholesterol also plays a significant role in cell membrane

permeability and equilibrium (Deull, 1957). Cholesterol has been reported to account for almost 50% of the total lipid complement of human lenses (Feldman, 1967,68: Broekhuyse, 1973; Andrews, 1979), while phospholipids contribute approximately 45% (Cotlier, 1987). Sphingomyelin is the major component of the phospholipid fraction (Broekhuyse, 1973). To date, little information exists as to the lipid composition of the fish lens. Broekhuyse (1970) did show that the major components of the phospholipid fraction in lenses of codfish were phosphotidylcholine and phosphotidylethanolamine, while phosphotidylserine, phosphotidylinositol, and sphingomyelin were minor constituents. Modifications to the lipid composition of lens membranes could lead to altered cell permeability and osmotic imbalance. The lens is a dehydrated tissue that is dependent on the strict regulation of its internal environment (especially Na^{+}, K^{+}, Ca^{2-}, and H_2O) to maintain lens transparency (Cotlier, 1987). Hence, the second objective of this study was to evaluate if changes in the lipid composition and water content of spot lenses were associated with cataract development.

Naphthalene, a low molecular weight PAH and a common component of sediments of the ER, has been documented to induce nuclear cataract in mammalian systems (van Heyningen and Pirie, 1967; Koch et al., 1976). Xu et al. (1992) showed that glutathione (GSH) levels were diminished in cataractous

rat lenses that were induced by the feeding of naphthalene. These data indicated that oxidative processes were important in naphthalene induced cataract. Therefore, the third aim of this study was to investigate if reduction in GSH levels can be associated with cataract formation.

Therefore, it is the objective of this study to investigate the compositional changes, if any, that are associated with the early stages of cataract formation (stage 1 lenses). Such information should help clarify what processes might be important in sciaenid cataractogenesis.

Materials and Methods

Fish

Young-of-the-year spot (< 15 cm, Pisces:Sciaenidae)) were utilized as experimental organisms. Specimens were sampled from the ER and the York River (YR, reference site) during the summers of 1992 and 1993. Upon collection, specimens were immediately transferred to aerated water and transported alive to the Virginia Institute of Marine Science.

Prior to dissection, fish were anesthetized with methanesulfonate (MS-222, 0.2g/L) (Sigma Chemical Co., St. Louis, MO), total length measured (± 0.5 cm), weighed (± 0.1 gm), and examined for cataract development. Examined fish sampled were classified into two categories, namely:

cataract (C) and non-cataract (NC). Cataract-bearing samples were further classified into four stages (S1-4) of cataract development, as previously described in Chapter 3. In order to determine the initial processes that may be involved in the induction of fish cataractogenesis, only lenses from fish that exhibited stage 1 cataract formation were analyzed.

Lenses were dissected from the fish, any extraneous non-lenticular material removed, weighed (+0.0000 gm), measured for diameter (+ 0.1mm), and placed into 3 ml of chloroform:methanol (1:2 v/v) for storage at -20° C.

On occasion, lenses were quick frozen in cryogenic vials by the immediate placement of individual lens samples (single lenses, not paired lenses) into a bath of liquid nitrogen. Upon completion of the dissection procedures samples were subsequently stored at -70° C.

Lens water composition

Dry weight analysis was performed on lenses dissected from spot collected in April - October, 1992 at Stations 217 and YR. Lenses were placed into a petri dish filled with 5 ml of ambient seawater. Lens diameters were immediately measured utilizing a dissection microscope (10x) and a calibrated ocular micrometer. After diameter estimation, lenses were blotted dry, placed into tared aluminum pans and weighed ($Wt._{1}$, $\pm$ 0.0000 g), and then transferred to a

drying oven for 96 hours. Oven temperature was maintained at 60 °C. Upon completion of the drying period, lenses were allowed to cool for 20 minutes inside an activated desiccator and measured for final weight ($Wt._2$).

$$\text{Final dry weight (\%)} = \frac{Wt._1 - Wt._2}{Wt._1} \text{ (x 100)}$$

Protein separation

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described by Laemmli (45) using a Hoefer apparatus. Gels contained 15% acrylamide. Cell samples were heat-denatured, 20 μl aliquots were mixed with bromophenol blue tracking dye, and loaded onto gels. Molecular weight protein standards (Pharmacia-LKB, Biotechnology) and positive control rat lens fractions (water-soluble and urea-soluble) were used as positive detection markers. Electrophoresis was carried out at 130 V for ~1.66 hours. Gels were stained with Coomassie Blue R-250 for 30 minutes and destained overnight at 50° C.

Crystallin bands (α, β, and γ-crystallins) were identified by Western Blot analysis using bovine polyclonal antibodies raised against the various crystallin proteins (antibodies were kindly provided by Dr. J. Samuel Zigler, National Eye Institute at the National Institutes of Health, Bethesda, MD). Immunoblots were developed according to the Bio-Rad protocol. Transfer of proteins from SDS-PAGE gels

to nitrocellulose paper was accomplished using a Bio-Rad transfer unit and protein bands visualized via the peroxidase reaction. Peroxidase labelled goat anti-rabbit IgG and the detection reagent (4-chloro-1-naphthol) were from Kirkegaard and Perry (Gaithersburg, Md.).

Gel filtration chromatography of the water-soluble and urea-soluble proteins of the lens was performed by High Performance Liquid Chromatography (HPLC) using a Superose 12 column (Pharmacia-LKB, Piscataway, NJ). The column was equilibrated with 0.1*M* phosphate buffer with 0.1*M* NaCl at a pH of 7.4 for water soluble fractions. The same column was equilibrated with 3.5*M* urea (0.1*M* NaCl, and 50*mM* Tris) solution for the urea soluble fractions. All protein fractions were eluted at a flow rate of 0.30 ml $minute^{-1}$ and 0.6 ml fractions collected with continuous monitoring of absorbance at 280 nm. A total of 26 fractions were collected per trial run with a 15 minute delay period. Fractions were tested for the presence of α, β, and γ-crystallins by SDS-PAGE and Western blotting as described above.

Glutathione assays

GSH was measured in whole lenses from YR, ERNC, and ERC spot by the modified method of Ellman (1959). Whole lenses were homogenized at a ratio of 0.4 ml of 5% trichloracetic acid (TCA) per 23 mg of lens wet weight, and centrifuged at

1500 *g* for 20 min. Aliquots of 50 and 150 µl of the supernatant were then mixed with 150 and 100 µl of 20 *mM* EDTA, respectively, and 400 ul of 0.4 *M* Tris (pH 8.9). Aliquots (200 µl) of the sample solutions were placed into microplates, all samples augmented with 10 µl of Ellman's solution (99 mg DTNB in 25 ml methanol), and incubated for 10 minutes. Absorbance of the solution was then measured at 410 nm. Standards of purified GSH (Sigma Chemical Co.) were run in parallel with sample solutions. Total GSH was determined by the comparison of the absorbance of sample solutions to the linear regression relationship between concentration levels and the purified GSH standards. Total GSH was determined as the mean of the two concentration levels used (in triplicates) and was expressed as ug GSH/mg of lens wet weight.

Lipid analysis

Total lipid was extracted from paired-lenses (i.e., two lenses/sample) of cataractous and non-cataractous spot via chloroform-methanol-water (1:1:0.8 v:v:v) extraction as described by Bligh and Dyer (1959). Upon separation, the chloroform layer was isolated and evaporated under N_2 to complete dryness. The dried fraction was then redissolved in 1.0 ml of chloroform, layered with N_2, and stored at -20° C.

Total lipid was determined using a modification of the

method of that previously reported by Holland and Gabbitt (1971). Briefly, 50 ul aliquots (2 replicates/lens-pair) were dried at 90 °C, 250 ul of H_2SO_4 added, charred at 190° C, supplemented with 5 ml of distilled water, and read for absorbance on a spectrophotometer at 375 nm. Sample absorbances were transformed into µg of lipid extracted by using a linear regression equation generated from a standard curve of 0, 5, 10, 20, and 40 ul aliquots of Atlantic menhaden oil standard lipid mixture (1 ug/ul). Mean absorbance was the result of duplicate samples. Results were expressed as a percentage of the total lens wet weight.

Iatroscan analysis

Classes of lipids were analyzed using an Iatroscan TH-10 lipid analyzer (Mark IV, Iatroscan Laboratories, Tokyo, Japan) according to the procedure reported by Cosper and Ackman (1983). Briefly, each lipid sample (1.0 ml) was concentrated to 10 µg/ul, spotted (2 µl) on silica gel chromarods (S-3 chromarods, 10 rods per set, ??) and focused (2x) in a 1:1 chloroform:methanol solution. Migration of lipid classes was performed by incubation in an hexane:diethylether:formic acid solution (85:15:0.1) for 45 min. Upon termination of development, chromarods were analyzed by an Iatroscan flame ionization detector (scan speed = 30, zero = 10, attenuation = 6, chart speed = 10, peak width = 0.04 and area reject = 0).

Standards (Sigma Chemical Co.) for phospholipids (lecithin), cholesterol, free fatty acids (stearic acid), triacylglycerides (menhaden oil), and wax/cholesterol esters (oleic acid) were analyzed in parallel with experimental samples. Regression analysis was determined between peak areas observed by the total amount spotted (0.5-3.0 ul, 5 μg/ml) in order to identify individual standard response factors and peak retention times.

Phospholipid classes

Phospholipid classes were quantified using multi-scan chromatography as described by Jackson (Lipid Analysis by TLC-FID, 1993, RSS, Inc. Bemis, TN). In general, samples were spotted and developed as previously described for lipid class separation using a solvent mixture of hexane:diethylether:formic acid (50:20:0.1). Developed chromarods were then placed through the TLC/FID system for a partial run (80% scan, that did not include the polar lipid fraction, i.e., phospholipids). After scan 1 was performed, the chromarods were then redeveloped in a polar solvent mixture of chloroform:methanol:water at a ratio of 50:20:1 for 45 min. Upon final development, rods were again processed as previously described, but for 100% of the chromarod lengths.

A suite of phospholipid standards were (phosphotidyl-serine, -inositol, -choline, -ethanolamine, and

sphingomyelin, Sigma Chemical Corp.,) run in parallel on the same chromarods as experimental samples, in order to construct standard curves, determine specific peak retention times, and to obtain response factors. As for lipid classes, 0.5-3 μl per standard (10μg/μl) were spotted and analyzed.

High performance thin layer chromatography

High Performance Thin Layer Chromatography (HPTLC) was performed according to the method described by Olsen and Henderson (1989). In general, activated HPTLC plates (Whatman) were spotted with 10 μl of total lipid (10μg/μl in chloroform), and developed in a hexane:ethyl ether:acetic acid solution (80:20:1) for approximately 45 min. Plates were then charred with 3% cupric acid in 8% aqueous phosphoric acid at 180° for 20 min. (Fewster et al., 1969) and quantified utilizing a GS-300 transmittance/reflectance scanning densitometer (Hoefer Scientific Instruments, San Francisco, CA. USA). Purified standards, as previously described for Iatroscan analysis, were run in parallel on the same plate and the response factor of each standard was calculated. Data were corrected with calculated response factors and were expressed as a percentage of total lipid weight.

Analysis of fatty acid composition

The total lipid extract was transesterified with methanol and boron trifluoride according to the procedure described by Cosper and Ackman (1983). Separation of the fatty acid methyl esters (FAME) was carried out on a gas liquid chromatograph (GLC Varian 3300) equipped with a flame ionization detector, using a fused silica capillary column coated with SP-2330 (30m x 0.25mm i.d., Supelco, Bellefronte, PA). The column was temperature programmed from 120-180 °C at 120 °C/min. and from 180-220° C at 60 C/min. The injector and detector temperatures were 220 and 240 °C, respectively. The flow rates of compressed air and hydrogen were 300 ml/min. and 30 ml/min., respectively. Helium was used as the carrier gas at a flow rate of 1.5 ml/min. Individual fatty acids were identified based on the comparison of sample retention times to those of known standards (Sigma Chemical Co.). Results were corrected with the response factors of external standards and were expressed as a percentage of the total fatty acid methyl esters observed.

Statistical analysis

All statistical analyses for percent composition were performed on $\log_{10}$-transformed values and compared for significant treatment effect at $p < 0.05$ via a one-way ANOVA analysis (Minitab statistical package). Differences between

sample means were determined by utilizing the Scheffe multiple means comparison test (Statistica, Jandel, 1993). Once again, differences between means were considered to be significant if p-values were ≤ 0.05.

Results

Proteins

Lenses from YR, ERNC, and ERC spot were used for protein analysis. Protein analysis was performed on lenses from both spot and Atlantic croaker (*Micropogonias undulatus*). Lenses with varying stages of cataract development (S1-3) were also used for compositional determinations.

SDS-PAGE analysis

Preliminary analysis of the protein composition of whole lens homogenates indicated that no differences could be observed between cataract and non-cataract samples. Therefore, in an attempt to enhance any significant compositional alterations, the epithelial/cortical portion of the lens was removed and the nuclear core used as the test region. Furthermore, lenses differing in stages of cataract development were also employed in order to heighten differences between cataract and non-cataract samples.

Intensity of protein staining was inconsistent.

Several SDS-PAGE gels indicated that the total protein content was reduced in cataract samples (Fig. 15), while in other samples no differences could be observed. On occasion, a band representative of high molecular weight proteins was observed in certain cataract samples that was not observed in reference lenses. Protein staining patterns, however, were inconsistent and although specific gels suggest that protein aggregation may be taking place during cataract formation, no clear protein response was evident. No significant differences could be observed in either staining properties or migration patterns of any of the crystallin-proteins when cataract and non-cataract lenses were compared.

Western Blot analysis

Polyclonal antibodies to bovine α-, ß-, and γ-crystallins were used to detect the presence of these crystallins in lens samples. ß-crystallin polypeptides were observed to have a molecular weight range of approximately 25-30 kDa, while α- and γ-crystallin polypeptides exhibited a molecular weight close to, but slightly higher than, 20 kDa. When solubilized in HPLC-grade water, all three crystallins were positively identified in both the water-soluble and the urea soluble fractions.

HPLC analysis

The water soluble fraction was found to contain eight peaks that appeared in both cataract and non-cataract samples. Comparison of the water-soluble fractions of whole spot lenses and nuclear regions of spot lenses are depicted in Fig. 16. Comparison of fish lens profiles (nuclear region) to that of a rat lens (positive control) indicated that five types of proteins were present, namely fraction 1 (peak 1) that contained a high molecular weight aggregate, fraction 2 (peaks 2 and 3) that contained α-crystallin, fraction 3 (peaks 4 and 5) that contained ß-crystallin, fraction 4 (peak 6) that contained γ-crystallin, and fraction 5 (peaks 7 and 8) that contained low molecular weight proteins. SDS-PAGE in conjunction with Western Blot analysis positively identified that α, ß, and γ-crystallins were present. Fraction 4 (γ-crystallin) was by far the most abundant of the protein classifications observed and constituted between 55-62% while fraction 2 (α-crystallin) and 3 (ß-crystallin) comprised 13-15% and 7-8% of the total water soluble fraction, respectively. The percent composition for all fractions for the three types of lenses is reported in Table 10.

Statistical analysis showed that no significant differences in the mean percent composition of individual peaks (fractions) of proteins or the mean total area calculated (total protein content) could be determined

between ERC, ERNC, and YR lens classifications. Stratification of data by species or varying stages of cataract development also showed no significant differences.

Urea-soluble proteins

HPLC analysis identified six peaks in the urea-soluble fraction that appeared in both cataract and non-cataract samples. Data indicated that the two major components of the urea-soluble fraction for all samples were fraction 1 (46-53%) and fraction 4 (~30%).

Statistical analysis, once again, indicated that no significant differences could be determined in any of the protein fractions when cataract and non-cataract lenses were compared. Table 11 shows the mean composition of the urea-soluble fraction as determined by HPLC. Separation of data by species and cataract development was similar to that determined for the water-soluble fraction in that no significant differences could be determined.

Glutathione

No significant differences in total GSH concentrations could be determined between ERC (n=15) and ERNC (n=15) or YR spot lenses (n=14). ERNC lenses, on the other hand, were shown to have significantly decreased levels ($p<0.01$) of GSH compared to YR lenses. The average GSH concentrations in ERC, ERNC, and YR spot lenses were 3.9 ($\pm$0.8 SD), 3.1

(±1.5), and 4.4 ug/mg lens wet weight (±0.7), respectively.

Lipid analysis

Iatroscan analysis was performed on samples collected during August-October, 1992. Iatroscan data provided estimates for individual lipid classes, as well as individual phospholipid compositions. HPTLC analyses was utilized to reproduce the effects observed in Iatroscan data and was performed on samples collected during September and October, 1993. HPTLC results was used only to estimate the composition of individual lipid classes. During the 1992 sampling period the distribution and abundance of spot were severely reduced compared to previous years and hence cataract samples were also limited. In fact, spot with stage 1, bilateral cataract formation were only collected in the months of September and October. Statistical analysis, therefore, was performed only on samples collected during this time period for all lens classifications.

Sample sizes for the Iatroscan analysis incorporated (N_{YR}=46, N_{ERNC}=41, N_{ERC}=16) a total of 103 specimens. Sample sizes for statistical comparison (i.e., September-October, 1992; N_{YR}=33, N_{ERNC}=28, N_{ERC}=16) included 76 organisms. Sample sizes used for HPTLC analysis, on the other hand, consisted of 17, 20, and 20 fish lens pairs, respectively for YRNC, ERNC, and ERC classifications. All spot samples were analyzed for all lipid classifications, however, not all

lipid classifications (lipid classes or phospholipids) were observed in all samples. Therefore, the mean concentration reported represents the average concentration level when that lipid designation was observed (n=total number of samples lipid class identified in).

Total lipid

The total lipid contents of lenses sampled from YR, ERNC, and ERC (S1) spot were determined to have mean values of 0.62% (± 0.17SD, N=33), 0.62% (± 0.25%SD), 0.69% (± 0.33%) of the total lens wet weight, respectively. No statistical differences in mean lipid concentrations could be determined between any of the three lens classifications.

Lipid classes

Iatroscan analysis for lipid classes of spot lenses revealed the following lipids: phospholipids (PLs), cholesterol (C), wax/cholesterol esters (WCE), free fatty acids (FFAs), and triacylglycerides (TAGs). Table 12 shows the results of lipid class analysis as determined by Iatroscan flame ionization detection. Comparison of Iatroscan data with that obtained from HPTLC analysis showed slight differences in percent compositions but, in general, produced the same relative tissue concentrations. It should be noted, that Iatroscan analyses performed as described under the Materials and Methods were unable to separate wax

and cholesterol esters and were therefore identified jointly as a total ester composite (TE). HPTLC analysis, on the other hand, did not exhibit a peak equivalent to the wax/cholesterol ester designation and therefore no estimation of this classification could be attempted.

Phospholipids

Phospholipids (PL) constituted the major lipid class present in all lens classifications. Statistical analysis indicated that phospholipids from ERNC samples did not vary significantly from YR reference lenses. Analysis of PL via HPTLC was similar to that of Iatroscan and indicated that the mean concentration was 42.9% (n=20). Statistical analysis for HPTLC data, however, showed that PL in ERNC lenses were significantly lower than mean PL concentrations in YR lenses ($p<0.002$). No difference in mean concentration levels could be determined between stage 1 cataract and either ERNC or YR lenses. Data from HPTLC analysis indicated that the mean concentration level of PL in ERC samples was 40.2% ($\pm$6.2%). Using HPTLC results, statistical analysis showed that no significant difference could be determined between ERC and ERNC lenses, but did indicate that ERC lenses exhibited significantly lower mean values than YR reference samples ($p=0.001$).

Cholesterol

Cholesterol (C) was observed to be the second most abundant class of lipid present in all lens classifications. Variability between samples collected from September (11.1%, ± 4.4%, n=18) and October (28.6%, ±4.6%, n=15) of 1992, however, was considerable. Mean C-composition from lenses collected during these two sampling periods was observed to be significantly different ($p < 0.001$). HPTLC analysis (avg.= 21.6%) was similar to that for Iatroscan, but did not exhibit the same monthly variability. Statistical analysis showed that no significant difference could be observed between the mean percent cholesterol in ERNC and YR samples. Once again, however, variability between sample months (September, 11.8%, ±4.4%, n=15; October, 25.1%, ±3.8%, n=13) for 1992 was considerable. No statistical differences were observed between ERNC and YR data for September or October. HPTLC analysis indicated that the mean concentration level for cholesterol was 23.5%. Statistical analysis on data obtained from HPTLC showed that ERNC lenses had significantly elevated mean concentration compared to YR reference samples ($p=0.047$). No significant variability in C-concentrations for ERNC lenses between the collection months was observed in the 1993 data.

No significant difference between lens C-content in ERC lenses was observed between sampling periods. Statistical analysis indicated that cholesterol levels in stage 1

cataract lenses were not significantly different (p=0.121) compared to both ERNC and YR pooled lens compositions due to the significant monthly variability found in both YR and ERNC samples. Stratification of data by month sampled and lens type indicated that only cataract lenses collected in September of 1992 had significantly elevated C-content compared to both YR ($p<0.001$) and ERNC samples (p=0.002). HPTLC analysis, on the other hand, showed ERC lenses possessed a mean C-concentration level of 25.5% (±2.0%). Statistical analysis for this set of data indicated that ERC lenses exhibited significantly elevated C-concentrations compared to both ERNC (p=0.004) and YR samples ($p<0.001$).

Total esters

Total esters were considered to be almost totally composed of cholesterol esters due to the relatively high C-content and the lack of information suggesting wax is a significant contributor to the lens lipid composition. TE was the third largest fraction observed in all lens classifications. The mean composition of ERNC lenses was not found to be significantly different from that of YR lenses. Comparison of mean concentrations indicated that lenses from ERC spot had significantly decreased levels of TE compared to YR reference lenses (p=0.003) but not significantly decreased levels (although close) compared to ERNC lenses (p=0.063).

Free fatty acids

Free fatty acid content of ERNC lenses was not found to be statistically different from YR samples. Cataract lenses demonstrated statistically greater concentrations of free fatty acids than did YR lenses ($p=0.027$). Statistical analysis indicated that no significant differences existed between ERC and ERNC lenses, while YR lenses were significantly lower ($p=0.002$) than either of these classifications.

HPTLC analysis for YR lenses indicated a higher mean percent composition for FFA, 23.9%, but exhibited considerable variability (SD=$\pm$10.7%). Similar to YR-FFA, HPTLC analysis indicated a significantly higher mean concentration for FFA in ERNC lenses than did Iatroscan, 32.1% ($\pm$5.7%, n=20). HPTLC analysis indicated that the mean concentration level of the FFA-fraction was 3.5% ($\pm$0.2%). Using the results obtained from HPTLC analysis, ERNC samples exhibited significantly higher levels of FFAs than did YR lenses ($p=0.001$).

Triacylglycerides

TAG was the only lipid class that was not consistently observed in all samples. TAG was detected in only 21% (n=7) of YR lenses, 21% (n=6) of ERNC lenses, and 19% (n=3) of ERC lenses respectively. The TAG fraction in general was the smallest fraction observed in all lens classifications. TAG

were also not consistently detected in HPTLC analyses. Statistical analysis for the TAG fraction was not performed (see Materials and Methods).

Phospholipid classes

Iatroscan analysis indicated that phophotidylcholine (PC, ~40%) and phosphotidylethanalamine (~40%) were by far the dominant phospholipids found in the spot lens followed by phosphotidylinositol (PI, ~9%), sphingomyelin (S, ~6%), and phosphotidylserine (PS, ~4%). Composition of individual phospholipids are reported in Table 13. The relative composition of all individual phospholipids was similar amongst all lens classifications. Statistical analysis indicated that no significant differences in any of the individual phospholipid compositions could be observed between YR, ERNC, and ERC samples.

Individual fatty acid composition

Preliminary analysis of the fatty acid composition of YR (N=2), ERNC (N=2), and ERC lenses (N=2) indicated that no statistical differences could be observed (Table 14). Therefore, due to the small sample sizes between lens classifications the results reported here are a mean composition of pooled data from all lens samples analyzed (N=6).

The major components identified were palmitic (16:0,

30.4%) and oleic acids (18:1w9, 27.8%). Other less concentrated fatty acids that were present in significant amounts were myristic acid (14:0, 8.3%), stearic acid (18:0, 7.7%), docosahexaenoic acid (22:6w3, 7.0%), and palmitoleic acid (16:1w7, 7.0). An average total of 88.2% of the total area of the peaks produced were identified.

Stratification of fatty acids based on the position and number of double bonds indicated the following: saturated (46.4%), unsaturated (49.2%), monoenoic (35.6%), polyunsaturated (11.5%), w3 (8.6%), w6 (4.8%), w7 (7.0%), and w9 (27.8%).

Lens moisture composition

Spot lenses with stage 1 development showed a significant reduction in percent dry weight ($\log_{10}$ transformed) compared to non-cataract lenses (Fig. 17). Cataractous lenses demonstrated a significant decrease in dry matter content (increase in water content) compared to non-cataractous and reference YR samples ($p<0.003$) during months (August and September, 1992) when all three types of samples were collected. Mean values for percent dry weight ($\pm$ SD) for cataractous, non-cataractous, and YR reference samples were 51.1% ($\pm$ 3.8%, N=20), 53.2% ($\pm$ 1.7%, N=35), and 53.5% ($\pm$ 0.7%, N=45), respectively.

Table 10: Mean percent composition for the water-soluble protein fraction of sciaenid eye lenses as determined by HPLC analysis.

	Lens classification		
HPLC peak	YR	ERNC	*ERC
1	1.3 (2.0)	1.0 (1.0)	1.8 (2.0)
2/3	13.3 (4.6)	13.0 (7.0)	15.3 (6.6)
4/5	7.5 (1.9)	8.3 (2.4)	8.6 (2.9)
6	62.1 (8.8	62.0 (14.6)	56.3 (14.9)
7/8	2.6 (0.6)	2.7 (0.5)	2.6 (1.0)
Total	86.7	87.0	84.7
N	4	5	7

*ERC: includes different stages of cataract formation

Table 11: Mean percent composition for the urea-soluble protein fraction of sciaenid eye lenses as determined by HPLC analysis.

	Lens classification		
HPLC peak	YR	ERNC	*ERC
1	50.0 (7.6)	52.8 (8.2)	46.1 (15.7)
2	35.1 (4.6)	9.4 (3.0)	16.8 (9.6)
3	5.2 (6.5)	4.7 (2.8)	7.0 (4.6)
4	31.2 (2.6)	31.4 (3.0)	30.5 (15.0)
5/6	1.6 (0.4)	1.6 (0.3)	3.8 (0.9)
Total	122.9	99.9	104.2
N	4	4	4

*ERC: includes different stages of cataract formation

Table 12: Percent lipid class composition of eye lenses dissected from the marine teleost, spot (*Leiostomus xanthurus*: Pisces, Sciaenidae)

	Lens classification		
Lipid class	Non-cataract: York River (n=33)	Non-cataract: Elizabeth River (n=28)	Cataract: Elizabeth River (n=16)
Total esters	18.1 (±12.8)	15.3 (±14.9)	5.2** (±3.8)
Triacylglycerides	1.8 (±1.4)	9.7 (±0.6)	12.5* (±8.6)
Free fatty acids	11.4 (±5.1)	12.7 (±5.9)	21.5** (±7.4)
Cholesterol	19.1 (±9.8)	18.7 (±7.8)	24.8** (±5.8)
Phospholipids	47.6 (±10.5)	49.8 (±11.5)	45.1 (±6.4)

* = significantly different from YR lens classification ($p<0.05$)
** = significantly different from both YR ad ERNC lens classifications ($p<0.05$)

Table 13: Percent phospholipid composition of eye lenses dissected from the marine teleost, spot (*Leiostomus xanthurus*: Pisces, Sciaenidae)

	Lens classification		
Class of phospholipid	Non-cataract: York River (n=33)	Non-cataract: Elizabeth River (n=28)	Cataract: Elizabeth River (n=16)
P-ethanalomine	46.8 (±12.5)	38.1 (±10.1)	39.8 (±8.6)
P-inositol	7.7 (±2.4)	9.2 (±3.0)	10.0 (±2.0)
P-serine	2.9 (±3.2)	4.2 (±3.9)	4.7 (±4.7)
P-choline	37.4 (±10.9)	41.0 (±10.7	41.0 (±10.1)
Sphingomyelin	6.9 (±4.0)	5.7 (±2.6)	5.5 (±1.3)
Total	101.7	98.2	101.0

Table 14: Percent fatty acid compositon of spot eye lenses.

	Lens classification			
Fatty acid	York River: Non-cataract (n=2)	Elizabeth River: Non-cataract (n=2)	Elizabeth River: Cataract (n=2)	Name
14:0	9.2 (+0.6)	8.2 (+0.6)	7.5 (+0.1)	Myristic acid
16:0	30.0 (+2.4)	28.2 (+0.5)	33.0 (+3.3)	Palmitic acid
16:1 (w7)	7.0 (+2.0)	8.8 (+0.6)	5.2 (+0.5)	Palmitoleic acid
16:2 (w6)	5.3	ND	ND	Hexadecadienoic acid
16:3 (w6)	ND	ND	ND	Hexadecatrienoic acid
18:0	7.7 (+0.7)	7.4 (+0.1)	8.0 (+0.7)	Stearic acid
18:1 (w9)	26.1 (+1.1)	28.7 (+0.1)	28.6 (+1.5)	Oleic acid
18:2 (w6)	0.57	ND	ND	Octadecaenoic acid
20:1 (w11)	1.2 (+0.1)	1.1 (+0.1)	1.3 (+0.3)	Eicosenoic acid
20:4 (w6)	2.6 (+0.3)	3.2 (+0.005)	2.8 (+0.3)	Arachidonic acid
20:5 (w3)	1.0 (+0.02)	1.4 (+0.1)	1.1 (+0.1)	Eicosapentaenoic acid
22:5 (w3)	ND	0.7 (+0.1)	0.8 (+0.2)	Docosapentaenoic acid
22:6 (w3)	7.7 (+1.5)	7.3 (+1.4)	5.9 (+1.2)	Docosahexaenoic acid
Total	98.4	95.0	94.2	
w3:w6	1.0	2.9	2.8	
Sat:Unsat	0.9	0.8	1.1	
Monoenes	34.3%	38.6%	35.1%	
Unidentified	1.6	5.0	5.8	

ND = not detected

Figure 15: SDS-PAGE protein separation for spot lenses. A. An example of the reduction in water-soluble proteins in stage 1 cataractous lenses from spot (Lane 1 = positive control, rat lens water-soluble homogenate; Lanes 2-7, water-soluble fraction of spot lenses; Lanes 2-3, cataract stage 1; Lanes 4-5, spot from the ER, non-cataract; Lanes 6-7, spot from the YR, non-cataract; Lane 8, molecular weight ladder; Lanes 9-14, urea-soluble (7*M*) fraction homogenate; Lanes 9-10, cataract stage 1; Lanes 11-12, spot from the ER, non-cataract; Lanes 13-14, spot from the YR, non-cataract; Lane 15, positive control, rat lens water-soluble homogenate). B. An example of the formation of high molecular weight aggregates (arrows) in stage 1 cataractous lenses from spot (Lane 1 = positive control, rat lens water-soluble homogenate; Lanes 2-7, water-soluble fraction of spot lenses; Lanes 2-3, cataract stage 1; Lanes 4-5, spot from the ER, non-cataract; Lanes 6-7, spot from the YR, non-cataract; Lane 8, molecular weight ladder; Lanes 9-14, urea-soluble (7*M*) fraction homogenate; Lanes 9-10, cataract stage 1; Lanes 11-12, spot from the ER, non-cataract; Lanes 13-14, spot from the YR, non-cataract; Lane 15, positive control, rat lens water-soluble homogenate)

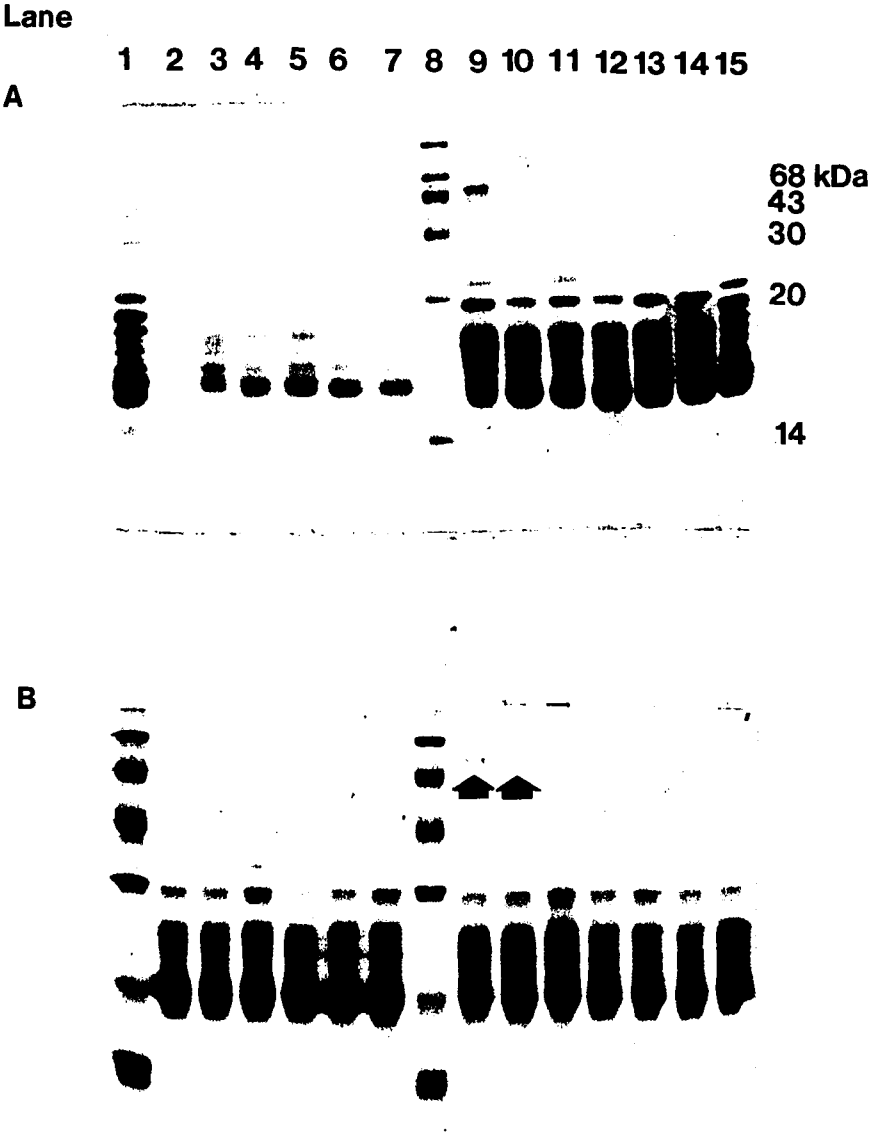

Lane
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
A
68 kDa
43
30
20
14
B

Figure 16: HPLC analysis for the quantification of protein fractions (mean composition) in spot lenses.

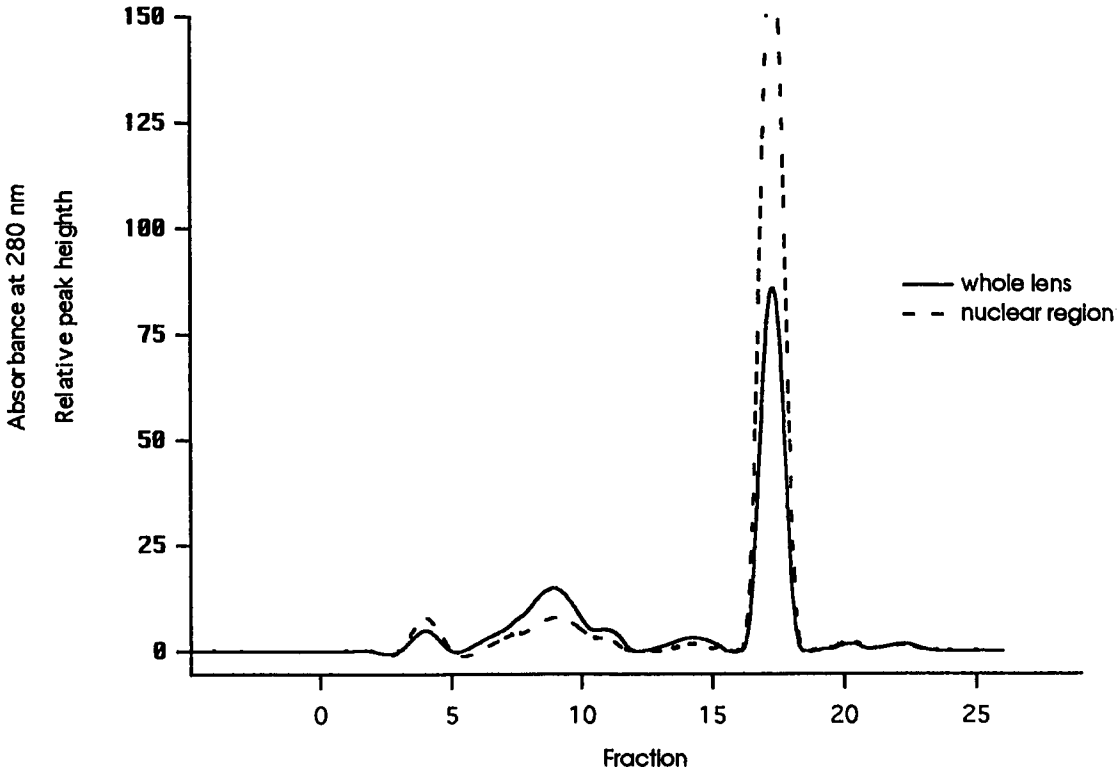
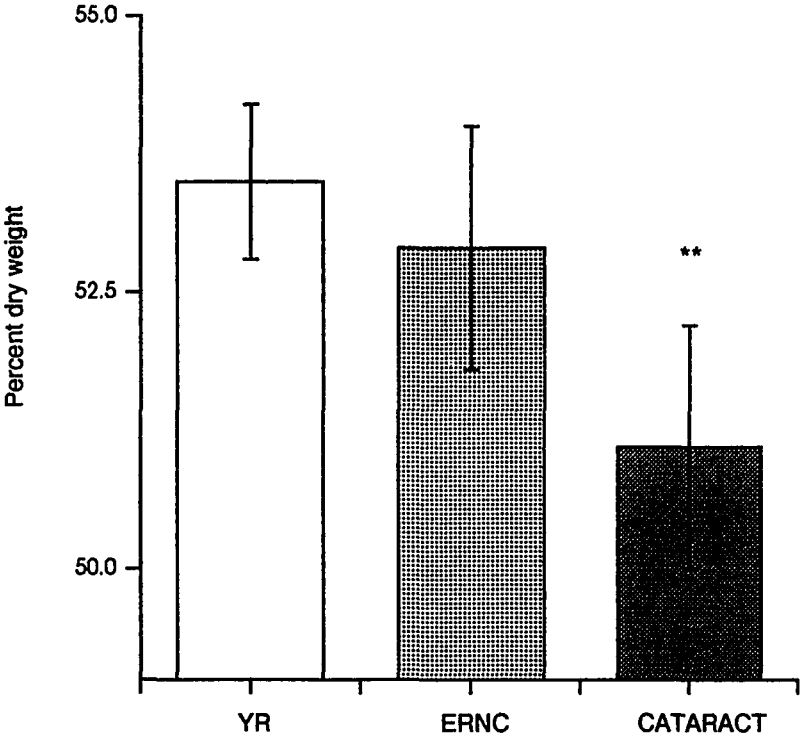

Absorbance at 280 nm
Relative peak heighth
150
125
100
75
50
25
0
0
5
10
15
20
25
Fraction
whole lens
nuclear region

Figure 17: Mean dry matter composition of spot lenses. YR = lenses from spot collected from the York River, ERNC = non-cataract lenses from spot collected from the Elizabeth River, ERC = stage 1 cataract lenses from spot collected from the Elizabeth River. ** = significantly lower than YR and ERNC at $\alpha = 0.05$.


55.0
52.5
50.0
Percent dry weight
**
YR
ERNC
CATARACT

Discussion

Alterations to the lipid composition and the water content of spot lenses during early stages of cataract formation indicate that disturbances to cell permeability and/or osmotic regulation of lens cells may play a significant role in fish cataractogenesis.

The stability of the percent total lipid composition between cataract and non-cataract samples indicates the lack of interference in the overall lipid synthesis of the lens. This response, however, does not exclude the possibility of structural alterations to lens cell membranes via PAH partitioning into the non-polar fraction. Previous work by Broekhuyse et al. (1973), investigating the effects of cataract formation on lipid metabolism in human lenses, also indicated that the total lipid content was unchanged.

The augmentation of cholesterol concentrations in lenses with stage 1 cataract development indicates that such an elevation may play a role in the mechanism of sciaenid cataractogenesis. Furthermore, the inverse relationship between cholesterol and total esters (cholesterol esters) may be indicative of a disruption in the normal processing of these compounds. Cholesterol has been shown to assist in maintaining the balance between cell permeability and the membrane equilibrium of living cells. The actual effect of increased cholesterol on the fluidity of lens membranes

would be to lower the critical transitional temperature (T_c) between the highly ordered gel-phase to the less ordered liquid-crystalline phase. Lens cells exposed to temperatures above T_c would become less permeable to solutes, while cells exposed to temperatures below T_c would become more permeable. Considering that the prevalence of cataract formation predominantly occurs during the hotter months of the summer (maximum in August, avg temp.= 29 °C) the problem in membrane function may be one of less permeability. Furthermore, increased levels of cholesterol in membranes would make lens cells more susceptible to osmotic shock (Gurr and Harwood, 1991).

As previously mentioned, prevalence of cataract formation has been positively correlated with sediment levels of PAH. Previous research has shown cholesterol levels to be elevated in the serum and the liver upon exposure to petroleum products containing PAH. For example, Thomas et al. (1980) showed that striped mullet (*Mugil cephalus*) exposed to the water-soluble fraction of No. 2 crude oil displayed increased plasma cholesterol levels after 3-4 hours. On the other hand, Eurell and Haensley (1981) showed that liver sections of the Atlantic croaker had increased cholesterol levels after exposure to 5% and 10% dilutions of the water-soluble fraction of southern Louisiana crude oil. Therefore, exposure to PAH may have caused cholesterol augmentation in spot lenses.

Lipids are, in general, not found as free fatty acids. This structural component of all lipids usually comprises a minor portion of the overall content. Data indicated increased levels of FFA in lenses with cataract formation. Although data from 1993 showed a significantly higher mean concentration than did 1992, overall variability was high and no statistical differences could be determined between the two sets of results. Samples from 1993 were processed, stored (-70 °C), and analyzed in a timely fashion (3 months). Therefore, elevated values were not considered to be a result of systematic error. The significant increase in free fatty acid concentrations found in cataract lenses, was thus considered to be a sign of certain degradation processes, possibly involving lipid oxidation. Microscopic studies have shown severe membrane damage in mature cataracts (Matsuto 1973; Hazlett and Bradley 1978; Harding et al., 1980; Farnsworth et al., 1981). In general, reactive oxidative compounds, such as H_2O_2, singlet oxygen, and oxygen free radicals, are major causes of cytological damage and death. Furthermore, oxidation is considered to be a key factor in the process of cataract formation in other vertebrate systems (rats, rabbits, humans) including that involved in the chemical induction of cataract via naphthalene exposure (Xu et al., 1992). PAH have been shown to elicit lipid peroxidation in fish (Wofford and Thomas, 1988).

Although phospholipid data were inconclusive, results obtained from the 1993 sampling indicated a significant reduction in the mean phospholipid composition of cataract lenses compared to reference lenses. Degradation of phospholipids into FFA would exacerbate problems with membrane function and osmoregulatory ability. Unfortunately, individual phospholipid determinations were not performed on 1993 data. It is known, however, that phosphotidylinositol plays a specific role in membrane protein anchoring and acts as a secondary messenger involved in intercellular communication (Gurr and Harwood, 1991). Future research may wish to pursue this avenue.

Dry weight analysis indicated that an increase in the water composition of cataractous lenses versus non-cataractous lenses had occurred. This data suggests that certain osmoregulatory functions of the lens may have been compromised and also suggests that membrane structure or function may have been modified. The lens is a highly dehydrated tissue that strongly regulates Na^{+}, K^{+}, Ca^{2-}, and water content (Cotlier, 1987). This regulation is vital to the maintenance of lens transparency. Previous research in mammals showed that the reduction in Na^{+}-K^{+} ATPase activity, the major membrane bound osmoregulatory enzyme, was responsible for cataract formation in the Nakano mouse (Iwata and Kinoshita, 1971; Kinoshita, 1974). Studies aimed at determining the levels of Na^{+}, K^{+}, and Ca^{2-} in cataract

(S1) and non-cataract lenses need to be performed.

The apparent lack of involvement of crystallin-proteins in sciaenid cataractogenesis further indicates the importance of lipids to the sciaenid lens. It should be noted, however, that a significant reduction in the mean total amount of fraction 4, which contains the γ-crystallins, in the water-soluble fraction was observed in cataract lenses compared to either YR ($p=0.01$) or ERNC lenses ($p=0.05$), as well as in the urea-soluble fraction between cataract and ERNC lenses ($p=0.05$). Furthermore, when cataract lenses were stratified by stage of development (data not shown) the greatest percent decrease in γ-crystallins (~12%, although not statistically significant) was observed between reference samples and lenses that exhibited stage 1 formation (< 10% opacification). γ-crystallin has been shown to be extremely susceptible to oxidative modification due to its a high cysteine and methionine content (Zigler, 1994). A greater sample size of lenses evaluated and/or reducing the nuclear lens core to a smaller unit may reduce data variability or increase sensitivity to where a clearer picture can be depicted. Previous work by Vazzoler and Phan (1990) looking at cataract formation in the South American croaker (*Micropogonias furnieri*, Pisces:Sciaenidae) also could not find significant changes to proteins isolated via electrophoresis. These authors also noted marked

variability between samples.

The lack of change in the total GSH concentrations between ERC-stage 1 and ERNC lenses suggests that oxidation (at least that associated with GSH reduction) may not play a significant role in the initiation of cataract in spot. Previous work by Xu et al. (1992) had shown that rats fed naphthalene developed nuclear opacification which was associated with a subsequent reduction in total lens GSH.

In conclusion, it appears that alterations to the lipid composition of sciaenid lenses (possibly membrane structure or function) may be associated with the initiation of lens opacification. Changes to the lenticular water content and not to GSH levels suggests that osmotic, as opposed to oxidative, problems may play a role in the formation of spot cataract and further supports the hypothesis that plasma membrane disturbances may be important. Such a disturbance could destabilize the physicochemical balance necessary for maintaining lens transparency.

CHAPTER 5

LONG-TERM CULTURES OF LENS EPITHELIUM FROM SPOT (*LEIOSTOMUS XANTHURUS*) PRODUCE α, ß, and τ-CRYSTALLINS

Introduction

Cataract has been reported in fish from the Elizabeth River (ER). The Southern Branch of the ER are characterized by sediments that are heavily contaminated with polycyclic aromatic hydrocarbons (PAH, Bieri et al., 1986). A positive correlation has been suggested between the prevalence of cataract and PAH levels (Hargis and Colvocoresses, 1986; Huggett et al., 1987; Hargis and Zwerner, 1988; Williams, Chapters 2,3). Histological examination of cataractous lenses have shown hyperplastic changes in epithelial cells and fragmentation of fiber cells within the cortical layer (Hargis and Zwerner, 1989). Investigations into the mechanism(s) leading to this type of cataract and its relationship to PAH exposure were hampered by the inability to culture lens cells of fish.

Several protocols for the primary culture of mammalian and avian lens epithelium have been developed (Okada et al., 1971; Hamada and Okada, 1977; Russell et al., 1977; Yasuda et al., 1978; Van Venrooij et al., 1974) and were successfully applied in various fields of vision research (Yasuda et al., 1978; Van Venrooij et al., 1974; Muggleton-Harris and Wang, 1989; Nagineni and Bhat, 1989; Nagineni and Bhat, 1988; Tomarev et al., 1994) including cataractogenesis (Hamada and Okada, 1978; Duncan et al., 1994; Eguchi and Kodama, 1979; Reddan and Dziedzic, 1994; Lipman and

Muggleton-Harris, 1982; Muggleton-Harris et al., 1981). Despite the relative ease in obtaining confluent monolayers of cultured lens epithelium, the success in developing cell lines of these cultures has been limited. The failure to establish long-term cultures of cells exhibiting characteristics of lens cells *in vivo* could be attributed to limited growth potential (Reddan et al.,1981; Ringens et al., 1982; Reddan et al., 1983; Vermorken et al., 1977), the loss of the ability to synthesize lens specific proteins over time (Iwig et al., 1978; Simonneau et al., 1983; Ramaekers et al., 1984), and the inability of lens epithelium to differentiate *in vitro* (Vermorken et al., 1977; Hamada et al., 1979; Simonneau et al., 1983; Ramaekers and Bloemendal, 1981).

To date, there are a few established cell lines that are derived from lens epithelium of mammalian tissues. Two of these cell lines were established in 1978 by Russell et al. from normal mouse tissues and from the Na+,K+-ATPase inhibited-Nakano mouse model. These cell lines had population doubling times (PDT) of approximately 31 hours, were easily subcultured, produced small amounts of γ-crystallin, and exhibited cell morphologies indicative of moderate cellular differentiation (Russell et al., 1977; Russell et al., 1978). In 1980, Reddan et al. established numerous cell lines from normal rabbit lens epithelium, one of which (designated N/N1003A, PDT=24 hr) exhibited an

epithelial morphology, contained the normal diploid complement of chromosomes (Reddan et al., 1986), and produced α-crystallin (Reddan et al., 1986; Yamada et al., 1990). An additional cell line (designated αTN4) from lens epithelium of transgenic mice was developed by Mahon et al. (1987). This α-crystallin producing cell line was developed from an animal that had the T-antigen of the SV40 virus linked to the αA-crystallin promoter. Most recently, Andley et al. (1994) reported the extended lifespan of human lens epithelium via infecting cells with the adenovirus-12 SV40. One of these virally-infected human cell lines (B3) was reported to produce ß (subculture 17) and another γ-crystallins (subculture 4).

It is well documented that production and localization of crystallin-proteins during ontogeny differ markedly among organisms from different classes. Simirskii et al. (1991) showed that in frog lenses α-crystallins were confined to the epithelial and cortical regions, ß-crystallins in the epithelial, cortical, and nuclear region (trace amounts), and γ-crystallins to the cortical and nuclear regions (trace amounts). On the other hand, in the young mammal α-crystallin is present in epithelial and fiber cells, whereas ß and γ-crystallins are found in fiber cells (Lovicu and McAvoy, 1989; Van Leen et al., 1987). γ-crystallin is found exclusively in the fiber cell and therefore, the presence of γ-crystallin can serve as a marker for the differentiation

of lens epithelium (Papaconstantinou, 1967; Zigman, 1985).

The aim of the present study was to develop a reliable procedure to produce long-term cultures of fish lens epithelium and to develop a cell line, capable of synthesizing lens specific crystallin-proteins.

Materials and Methods

Fish

Healthy spot (10-20 cm) were trawled from the York River, Virginia, transferred immediately into the laboratory, maintained in 500-gallon flow-through tanks supplied continuously with filtered York River water (salinity approximately 20 ppt), provided proper aeration, and fed *ad libidum* until sacrifice. Fish were maintained and sacrificed in accordance with the principles of the Declaration of Helsinki and The Guiding Principles in the Care and Use of Animals at the College of William and Mary. Length and weight of donors were recorded immediately prior to dissection.

Preparation of lens epithelium

Fish were anesthetized with tricaine methanesulfonate (MS-222, Sigma Chemical Co., St. Louis, MO). Eye regions were disinfected and lenses were dissected from the ocular cavity. Lenses were transferred to sterile petri dishes

containing tissue culture media adjusted to an osmolality of 335 mOsm/kg (approximately equivalent to the osmolality of this species serum) and supplemented with 100 IU/ml penicillin, 100 mg/ml streptomycin, 0.3 mg/ml L(+) glutamine, 20 *mM* Hepes buffer solution, and 10% Fetal Bovine Serum (FBS, Sigma Chemical Co.).

Four procedures were compared for the initiation of primary cultures: 1) lenses were cultured intact (organ culture); 2) lenses were fragmented and all pieces from the two lenses were cultured (explant culture), 3) the epithelial layer was stripped from the lens and cultured along with the remaining lens material (cell culture); and 4) similar to procedure 3, except the epithelial layer was minced before culture (minced culture). For procedures 3 and 4, the lens epithelial layer was treated with 10 ml of 0.25% trypsin solution (cell culture, Sigma Chemical Co.), and then stirred at low speed for 10 minutes at room temperature. Cells and lens nuclei were then centrifuged, washed twice with 2% FBS medium, resuspended in 10% FBS medium, and the total number of viable cells estimated by means of trypan blue exclusion staining (Gibco, Grand Island, NY).

Cell morphology

Cell morphologies and growth patterns were determined by observation of cultures with an inverted phase contrast

microscope, cytospin smears, and coverslip cultures stained with Wright's stain (Camco Quick, Cambridge Chemical Products, Fort Lauderdale, FL).

Optimization of the culture environment

Successful cultures of epithelial cells were seeded at 1 x 10^5/ml in media containing between 2.5 and 25% FBS (either Leibovitz-15 or RPMI-1640, Sigma Chemical Co.). Cells were incubated at 15°, 21°, 27°, and 37° C in both the presence and absence of 5% CO_2 in 95% air. Cultures were maintained with and without the lens nucleus. Cultures were periodically observed for adherence, growth, and replication using inverted phase contrast microscopy. The medium was changed every 14 days and subcultures were performed upon confluency. Subcultures were performed by dissociation of the monolayer with 0.25% trypsin, rinsing the single cell suspension twice with the appropriate growth media (2% FBS), centrifuging cells at 400 *g* for 5 minutes, and reseeding cells at one-half the confluency density in 6 ml of growth media.

Proliferation of primary cultures

Growth curves were produced by performing proliferation assays on pooled fish lens epithelial cells to determine the increase in numbers over time. Cell numbers were adjusted to obtain three concentration levels: 5 x 10^4, 1 x 10^5, and

1.5 x 10^5/ml in L-15 media, supplemented with 10% FBS. Four replicates per concentration were used. Cultures were incubated at 27 °C in the presence of 5% CO_2. Medium (100%) was replenished on day 14. Cells were harvested as previously described and the total number of viable cells estimated via the trypan blue exclusion method. Two experiments were conducted with average values calculated to determine general growth patterns. Linear regression analysis of cell estimates was used to describe differences in mitotic rates.

Growth curves were produced by performing proliferation assays on pooled fish lens epithelial cells to determine the increase in uptake of ^{3}H-thymidine over time. Cell numbers were adjusted and cultured as previously described. Twelve replicates per concentration level were used. 96-well culture plates were incubated at 27° C in the presence of 5% CO_2. Wells were inoculated 72 hours after initial seeding with 1 μCi of ^{3}H-methylthymidine (specific activity, 6.7 μCi/*mM*, Dupont, Wilmington, DE) in 25 μl of 10% FBS media. Plates were incubated for an additional 24 hours and then cells harvested with 0.25% trypsin and deionized water onto glass-fiber filters with the aid of an automatic cell harvester (Cambridge Tech., Baltimore, MD). The radioactivity, which represents uptake of ^{3}H-methylthymidine by the proliferating cells, was assessed in a Beckman liquid scintillation counter using Beckman Ready-Safe cocktail

mixture (Beckman, Fullerton, CA) as scintillant. Number of studies, cell densities, and growth media used was as previously described for the proliferation assays. The data were expressed as disintegrations per minute (dpm) ± standard deviations and simple linear regression analysis was performed to determine differences in rates of cellular proliferation.

Characteristics of long-term cultures

Since it was possible to passage one of the Spot Lens Epithelial Cell cultures 41 times within 49 months (designated as SLEC), the following experiments were performed:

Seeding density In order to determine the effects of cell concentration on growth and proliferation potential, SLEC-40 cells were grown at three density levels (1.2×10^6, 0.6×10^6, and 0.2×10^6) for 28-35 days. Cultures were placed into 25 cm^2 flask in 6 ml of L-15 growth media (10% FBS) as previously described. Eight identical cultures per concentration level were initially seeded. Cells were harvested and counted at various stages of culture development via the trypan blue exclusion method. Cultures that were allowed to grow past 21 days received a 100% media change on day 18.

Temperature In order to determine the effects of incubation temperature on growth and proliferation potential, SLEC-40 cells were grown at 10°, 17°, 27°, and 37° C. Temperature studies were similar in nature to the density trials, but upon seeding were maintained at room temperature for 2 hours in order to establish an adherent cell population. A cell density of 0.6 x 10^6 cells per 25 cm^2 flask in 6 ml of L-15 growth media was used as previously described. Cultures that were allowed to grow past 21 days received a 100% media change on day 18.

Karyotyping Chromosome analysis for possible alteration in number and morphology was performed on SLEC-6 and SLEC-41 cultures that had been maintained under *in vitro* conditions for eight and 49 months, respectively.

Chromosomes were isolated and fixed via the method by Rothfels and Siminovitch (1958) and Worton and Duff (1979) as described in Freshney (1987).

Identification of crystallins

Confluent cultures of SLEC (37th passage, 45 months past original seeding) were pelletted, rinsed twice with phosphate buffered saline solution (PBS, osmotic concentration = 325 mOsm), and resuspended in 125 μl of HPLC grade water and sonicated to disrupt cell membranes. Sonicated cell suspensions were centrifuged at 14,000 *g* for

15 minutes and supernates mixed with 25 μl of 2% sodium dodecyl sulfate (at 4° C). The remaining cell pellet was then rinsed twice with 500 μl of HPLC grade water, pelletted using centrifugation as previously described, and supernates discarded. The water-insoluble (urea-soluble) fraction was extracted by resuspending the previous cell pellet in 100 μl of 7 *M* urea (0.1*M* NaCl, 0.05*M* Tris) and incubated for 12 hr. at -20° C. Final preparation of the urea fraction consisted of solution resuspension, the addition of an equivalent amount (100 μl) of HPLC grade water, and the solution pelletted as previously described. After centrifugation, the supernate was isolated.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described by Laemmli (1970) using a Hoefer apparatus. Gels contained 15% acrylamide. Cell samples were heat-denatured, 20 μl aliquots were mixed with bromophenol blue tracking dye, and loaded onto gels. Molecular weight protein standards (Pharmacia-LKB, Biotechnology) and positive control rat lens fractions (water-soluble and urea-soluble) were used as positive detection markers. Electrophoresis was carried out at a 130 V for ~1.66 hours. Gels were stained with Coomassie Blue R-250 for 30 minutes and destained overnight at 50° C.

Crystallin bands (α, ß, and γ) were identified by Western blotting using bovine polyclonal antibodies raised

against the various purified crystallins (antibodies were the kind gift of Dr. J. Samuel Zigler, National Eye Institute at the National Institutes of Health, Bethesda, MD). Immunoblots were developed according to the Bio-Rad protocol. Transfer of proteins from SDS-PAGE gels to nitrocellulose paper was accomplished using a Bio-Rad transfer unit and protein bands visualized via the peroxidase reaction. Peroxidase labelled goat anti-rabbit IgG and the detection reagent (4-chloro-1-naphthol) were from Kirkegaard and Perry (Gaithersburg, Md.).

Gel filtration chromatography of the water-soluble and urea-soluble proteins of the lens was performed by High Performance Liquid Chromatography (HPLC) using a Superose 12 column (Pharmacia-LKB, Piscataway, NJ). The column was equilibrated with 0.1*M* phosphate buffer with 0.1*M* NaCl at a pH of 7.4. for water soluble fractions. The same column was equilibrated with 3.5*M* urea (0.1*M* NaCl, and 50*mM* Tris) solution for the urea soluble fractions. All protein fractions were eluted at a flow rate of 0.30 ml $minute^{-1}$ and fractions of 0.6 ml were collected with continuous monitoring of absorbance at 280 nm. A total of 26 fractions were collected per trial run with a 15 minute delay period. Fractions were tested for the presence of α, β, and γ-crystallins by SDS-PAGE and Western blotting using polyclonal antibodies raised against the appropriate crystallins as described above.

Results

Culture characteristics

Cultures obtained using each of the four techniques exhibited varying degrees of cell growth and displayed similar growth patterns during culture progression. Confluent monolayers of spot lens epithelium, however, were consistently established only from trypsinized epithelial/cortical layers (Table 15).

Enzymatic digestion of lens epithelial cells Trypsinization (T) and trypsinization/mincing (MT) of the eye lens epithelial/cortical layers produced the highest occurrence of successful cultures (> 70%). Growth of cells during culture was similar in progression to that previously described for mechanical separation. Trypsinization of particulate lens material resulted in cellular yields (Table 15) which were greater than yields from other methods attempted and proximity of cellular clusters was enhanced. Cell adherence was evident by day 3 and time to confluency averaged approximately 30 days. The presence of the lens nucleus did not have a significant effect on the success or the morphology of cells. Cultures did become confluent in the absence of the lens nucleus. Cells obtained via mincing and trypsinization (MT) showed a significantly greater plating efficiency within the first 24-48 hours and were

found more uniformly distributed throughout the culture container. Cellular yields from the MT method were the highest of all procedures attempted and viability remained consistently > 80%. A period of rapid cellular proliferation was observed early and the initial development of a monolayer was apparent within 72 hours after seeding. Time to confluency, however, was dependent upon number of cells inoculated and averaged approximately 24 days. Cells from confluent cultures did appear to arrange in a parallel fashion with adjacent cells.

Subsequent passage of confluent primary, secondary, tertiary, (quartenary, etc...) cultures of fish lens cells showed a positive association between cell size and culture number. Time to confluency (approx. 30 days) was consistent and demonstrated both epithelioid and fibroblastic morphologies. In general, a fibroblastic appearance has predominated throughout the 45 subcultures that have been completed. Lentoid bodies were observed in a random fashion in subcultures 1-20. Trypsinization methods produced 45 and 13 subcultures, respectively, with approximately equal rates of success for passage of primary confluent cultures (72%, % = number of cultures passaged/total number of cultures prepared).

Culture progression and cell morphology

Seeded primary cells were small (2-3 μm), rounded, and

contained spherical nuclei. Cells which became adherent under *in vitro* conditions were heteromorphic and exhibited triangular, stellate, polygonal, round, and rectangular morphologies. Adherent cells (triangular, stellate, and polygonal, Fig. 18A) would then elongate and spread, becoming thin and transparent. Successful cultures developed numerous clusters of adherent cells (30-50, Fig. 18B) which would develop into large, long, and attached assemblages several thousands of microns^2 in area. Cellular division was frequently observed in the early stages of culture growth. Through growth and spreading (Fig. 18C), assemblages would migrate to other cellular clusters and develop into a confluent monolayer, usually within 30 days. In general, a parallel array of adjacent elongated cells was observed (Fig. 18D) after the development of the confluent monolayer was complete. Rate of spread would increase upon cell cluster formation but decrease in the later stages of monolayer development. Within 24 hours of culture, spherical bodies with no observable internal structures were observed. Over time, these structures would fuse and create large, transparent bodies. Evacuation of the intracellular constituents directly from cells was frequently observed.

In stained cytospin smears (Fig. 19A-D) and coverslip cultures (Fig. 20A-H) a number of cellular types were recognized. Cells ranged in size from approximately 5 um up to about 100 μm and varied significantly in nuclear to

cytoplasmic ratio. Small cells (5 μm) exhibited a well delineated nucleus with a basophilic staining cytoplasm. Nucleus:cytoplasm ratios decreased proportional to cell size and exhibited nuclear compaction. Nuclear compaction became more advanced in larger cells until completely devoid of all visible internal structures. Structureless bodies that were translucent in appearance, but which varied in size, were observed in both procedures.

Optimal *in vitro* environment

Observation of the growth patterns of lens epithelium incubated in L-15 and RPMI-1640 media indicated that L-15 better supported the growth and proliferation of cells as indicated by reduced time to confluency and increased percentage of subcultures produced (Table 15). Cells grew well in both CO_2 and non-CO_2 enriched environments. Cells have been subcultured through 45 passages while being maintained in a non-CO_2 environment, have grown well between 23° through 27° C at a pH between 7.2-7.8, and have been maintained under culture conditions for 48 months. Elevation of FBS levels up to 25% and reduced down to 2.5% had no apparent effect on the time needed for early cultures (SLEC-10 through SLEC-15, 12-17 months) to obtain confluency.

Growth rates

Primary cultures Enumeration of lens epithelial cells via the direct estimation of the total number of viable cells (Fig. 21) shows the long-term replicative production (log-transformed) of four cell densities (5 x 10^4, 1 x 10^5, 1.5 x 10^5, and 2 x 10^5 cells/ ml) observed over a 29 day trial period. All density levels exhibited positive rates of growth except for the lowest seeded density. Increased density concentrations (up to 2 x 10^5/ml) demonstrated increased cellular production and greater success of culture.

Estimation of the mitotic rates of lens epithelial cells via the uptake of ^{3}H-thymidine indicated that 5 x 10^4 cells/ml displayed low uptake after day 3 of the trial period and exhibited negative slopes during both studies performed. On the other hand, 10 x 10^4 cells/ml (concentration 2) peaked by day 12 (experiment 1) at a average level of 1273 dpm, whereas 1.5 x 10^5/ml (concentration 3) peaked at day nine at an average level of 1471 dpm. In experiment 2, concentration two was observed to increase throughout the 15 day study period, reaching a level of 3050 dpm. Concentration three peaked, again, at day nine at 3349 dpm. Figure 22 displays the linear regressions for the average ^{3}H-thymidine uptake for both experimental trials. Both concentration levels exhibited positive slopes in both studies.

Long-term cultures Studies on the normal passage kinetics (1:2 split) of SLEC-39 showed a 24-hour plating efficiency of 108% ($1.3x10^6$ cells adhered/$1.2x\ 10^6$ seeded). The most rapid accumulation of cells was in the first 48-96 hours of incubation with a gradual but steady increase in cell number thereafter. Cell cultures became confluent within 10-14 days. 25 cm^2 culture flasks produced an average of 2.8×10^6 ($\pm 0.4 \times 10^6$SD) cells per flask over a 28-35 day incubation period. Large cell-like bodies without an apparent nucleus were observed between 23-27 days in 27 °C cultures (Fig. 23).

Density studies performed on SLEC-40 cells (Fig. 24) indicated that the number of population doublings per unit time were 0.50, 0.13, and 0.03 for the low (0.2×10^6), medium (0.6×10^6), and high density (1.2×10^6) concentrations, respectively. Confluency, on the other hand, was observed to occur most rapidly as concentration levels were increased. High, medium, and low cell concentrations progressed to confluency by Day 8 ($\pm$1.0 SD), 12 ($\pm$1.5 SD), and 17 ($\pm$1.5 SD), respectively.

Temperature studies performed on SLEC-40 cells showed that cells incubated at 27° C displayed the fastest proliferation potential. Cells grown at all temperature regimes showed positive growth and limited mortality (< 10%) except for the 37° C treatment (Fig. 25). Cells exposed to temperatures of 37° C maintained viability for 24 hours (>

85%) but exhibited reduced cell numbers (5 x 10^4) and significant cell mortality (54%) by Day 3. All cells were judged to be dead by Day 7. In the 17° C cultures a curious observation was exhibited on Day 16 (Fig. 26), namely almost all cells were observed to lack any visible internal organelles. In fact, throughout the 28 day trial period the 17° C treatment showed a reduced mitotic rate as compared to either the 10° or 27° C incubated cells. By Day 24, both the 10° (96 x 10^4, $\pm$ 11.9) and 17° C (91.5 x 10^4, $\pm$13.4) treatments had equivalent numbers of cells. Final examination of cultures indicated that the total number of viable cells per 25 cm^2 flask for the 10°, 17°, and 27 °C treatments were 1.2 x 10^6, 0.9 x 10^6, and 3.1 x 10^6, respectively. All treatments maintained > 95% viability.

Chromosomal counts from SLEC-6 and SLEC-40 were determined and a modal distribution of 48 telocentric chromosomes was observed (Fig. 27). Chromosome counts were equally distributed around the mode in SLEC-6, while SLEC-40 was hypermodal with an increased frequency of counts around the 51-53 range. Two separate spreads of 105 chromosomes were observed during observation of the SLEC-40 culture. Chromatid lengths ranged from 2.5-5.0 μm and averaged approximately 4 μm in length for both cultures. Chromosome morphology was completely telocentric in SLEC-6, while some morphological variation occurred in SLEC-41 in the form of metacentric chromosomes.

Protein analysis of SLEC

A total of four composite samples of SLEC (37 passages, 45 months after original seeding) were analyzed for crystallin protein composition. Average number of cells per sample was 7.1×10^6 cells.

SDS-PAGE indicated that strong protein bands existed in the water-soluble fractions at molecular weights similar to those of crystallin-proteins (Fig. 28). Significant protein bands appeared in the 20 and 30 kDa range, characteristic of lens α-, ß-, and γ-crystallins.

Western blot analysis utilizing polyclonal antibodies specific for bovine lens α, ß, and γ crystallins (Fig. 29) confirmed the presence of these in SLEC-37. SLEC-37 cultures displayed mild to strong reactions for all three polyclonal antibodies. All three major crystallins were positively identified in the water-soluble fractions (lanes 1 and 2) of the cell culture preparations, while only γ-crystallins (lanes 3 and 4) were observed in the urea-soluble fraction. For all three crystallin antibodies there were also positive reactions observed at molecular weights higher than expected for the native crystallin. The strongest such bands were in the area where dimers of crystallin polypeptides would be expected (Fig. 29). It is likely that these bands represent crosslinked crystallins as have been shown to occur commonly in the lenses of other species.

HPLC analysis (Fig. 30) of the water soluble fraction of SLEC-37 cultures showed three major areas of absorbance. The major peak eluted near the void volume of the column (fractions 3-5) indicating proteins with relatively high molecular weights. A second region of significant absorbance includes three distinct peaks in fractions 12-18. As indicated in Fig. 30, this region encompasses the area where the ß- and γ-crystallins elute, i.e. a molecular weight range of ca 20-180 kDa. The final peak (fractions 20-22) elutes near the column volume and represents proteins smaller than γ-crystallin or, more probably, non-protein components including peptides and nucleotides.

Table 15: Comparison of different procedures for the primary culture of spot lens epithelium.

Method	Average time to confluency (days) (±SD)	Average time to adherence (days) (±SD)	Maximum number of subcultures obtained	Percent of cultures passaged	Maximum duration of cell growth (months)	Average cellular yield/lens (±SD)
IL	NA	10 (3)	0	0	2	NA
MS	40 (5)	6 (2)	2	30	3	2.0×10^6 (0.8×10^5)
T	30 (3)	3 (1)	45*	70	52*	2.5×10^6 (0.8×10^6)
M/T	24 (3)	2 (1)	13	75	16	3.5×10^6 (1.0×10^6)
Media Used						
L-15	30 (4)	10 (3)	45	60	23	NA
RPMI	45 (5)	10 (3)	6	75	4	NA

IL = intact lens, MS = mechanical separation, T = trypsinization, M/T = mincing and trypsinization.
NA = not applicable
* = still in progress

Figure 18 (A-D): Progression of fish lens cells towards confluency under *in vitro* conditions. A. Individually settled cell from the epithelial/cortex layer of the fish lens (5-7 days). B. Aggregation of lens epithelial cells actively spreading and contacting surrounding lens cells (7-14 days). C. Leading edge of an actively spreading cluster of cells (15-25 days). D. Confluent monolayer displaying parallel organization of adjacent cells (30-40 days). Phase-contrast microscopy, 40x.

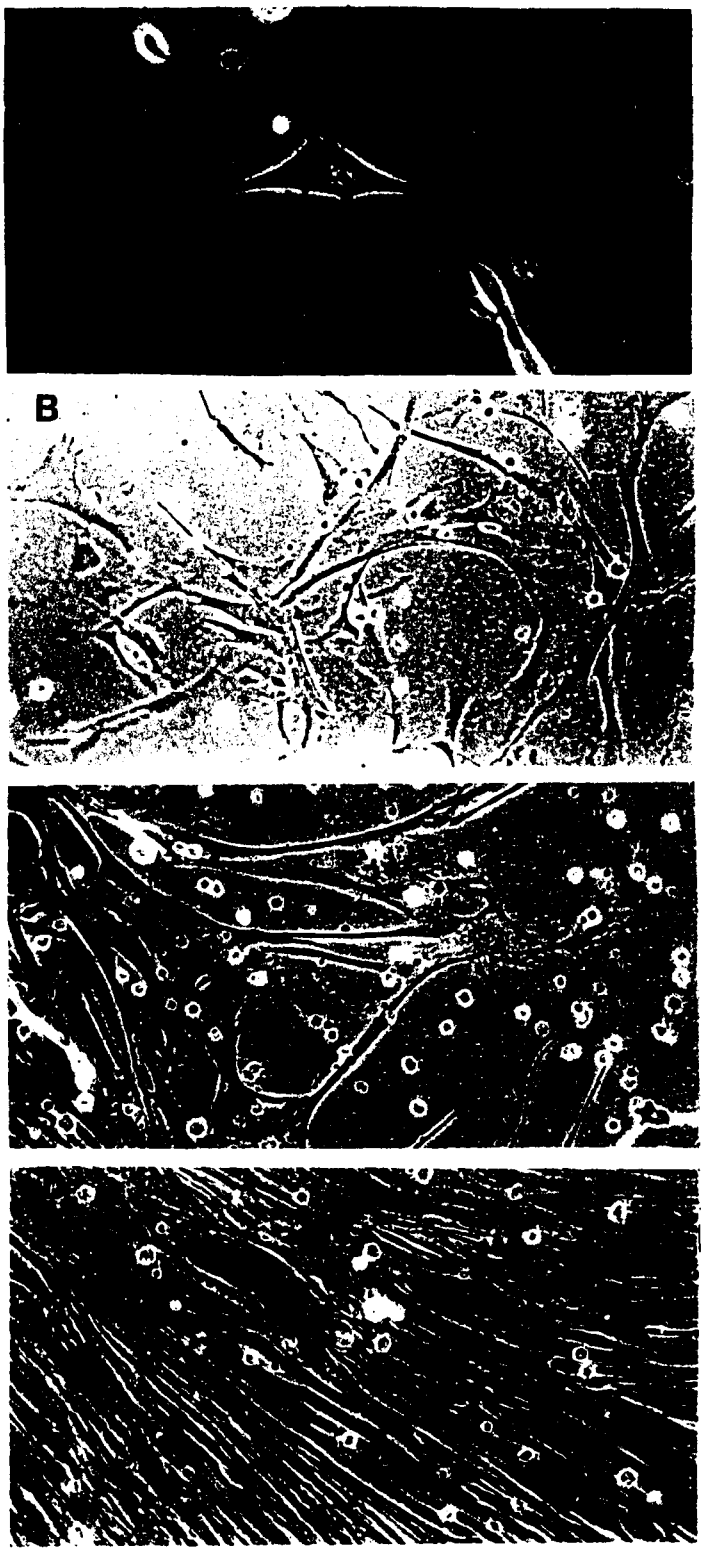

B

Figure 19 (A-D): Cytospin preparations displaying series of cellular progressions involved in the production of a cell void of internal structures. A. Spot lens cell starting to exhibit early stage of reduction in nucleus:cytoplasm ratio. B and C. Lens cell demonstrating loss of internal organization and reduction in the nucleus:cytoplasm ratio. D. Lens cells which have lost almost all of their nuclear material. n = nucleus sb = structureless body. Light microscopy at 140x under oil emersion.

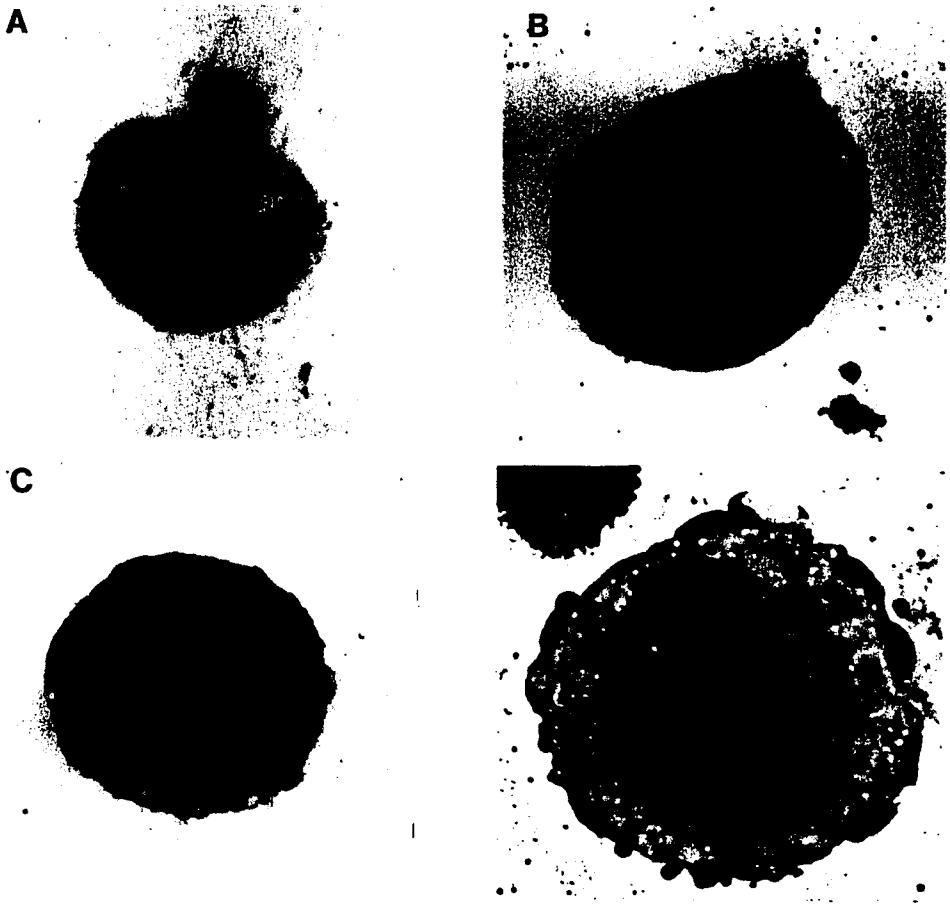
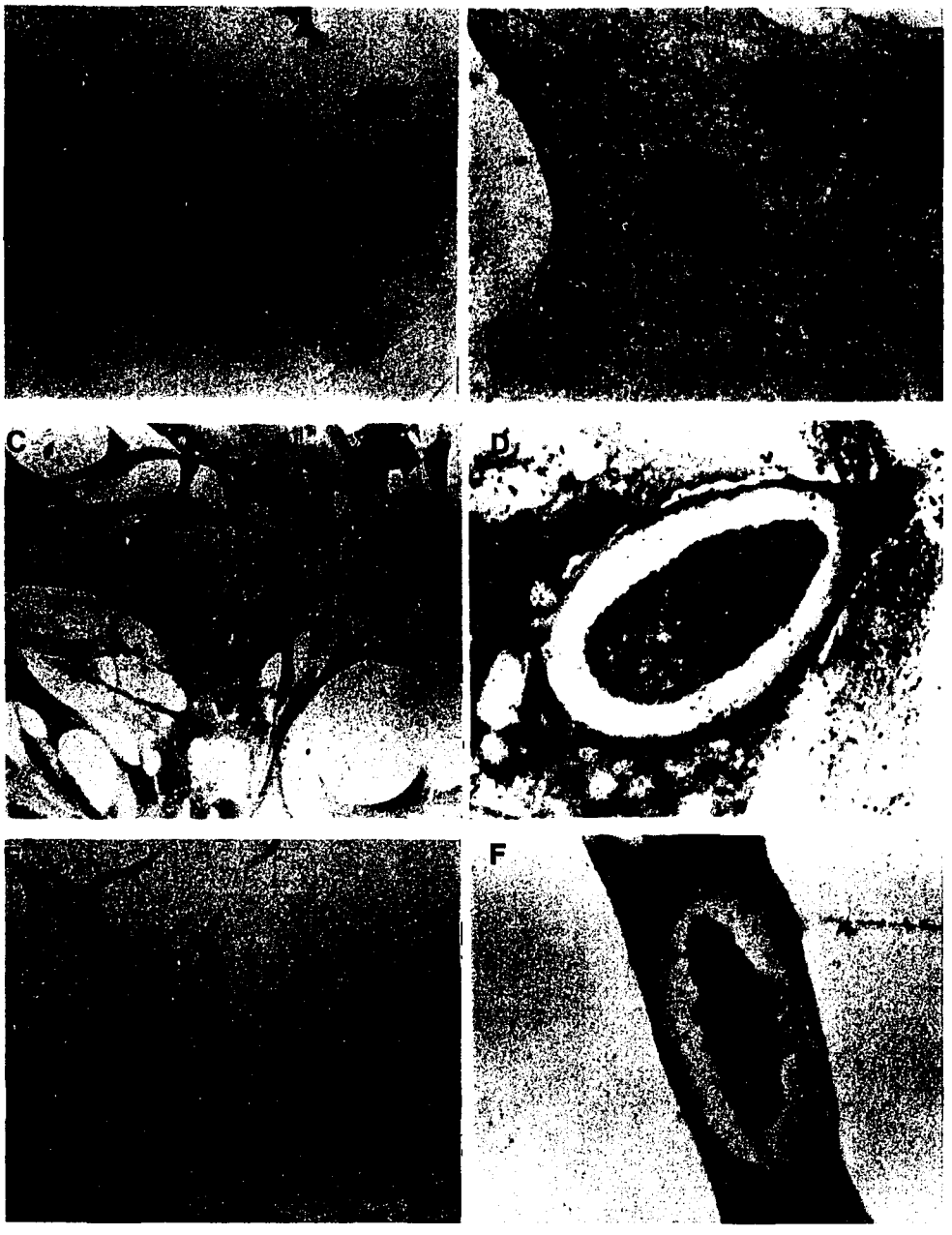

A
B
C

Figure 20 (A-H): Coverslip slide preparations of spot lens cells displaying differential morphology. A. Spot lens cell with intact nucleus and cytoplasm complement (10x). B. same as A but at 140x under oil emersion. C. Spot lens cell exhibiting reduction of the nucleus:cytoplasm ratio. D. same as C but at 140x under oil emersion. E. Spot lens cell with significant loss in nucleus:cytoplasm ratio. F. same as E but at 140x under oil emersion. G. Individual spot lens cell showing total reduction in nucleus:cytoplasm ration, i.e. formation of a "fiber cell". H. same as G but at 140x under oil emersion. Arrows indicate specific cellular example of intracellular changes.


C
D
F


H

Figure 21: Proliferation assays of primary lens epithelial cell cultures. Plotted values are the average log-transformed estimates using the trypan blue exclusion method.

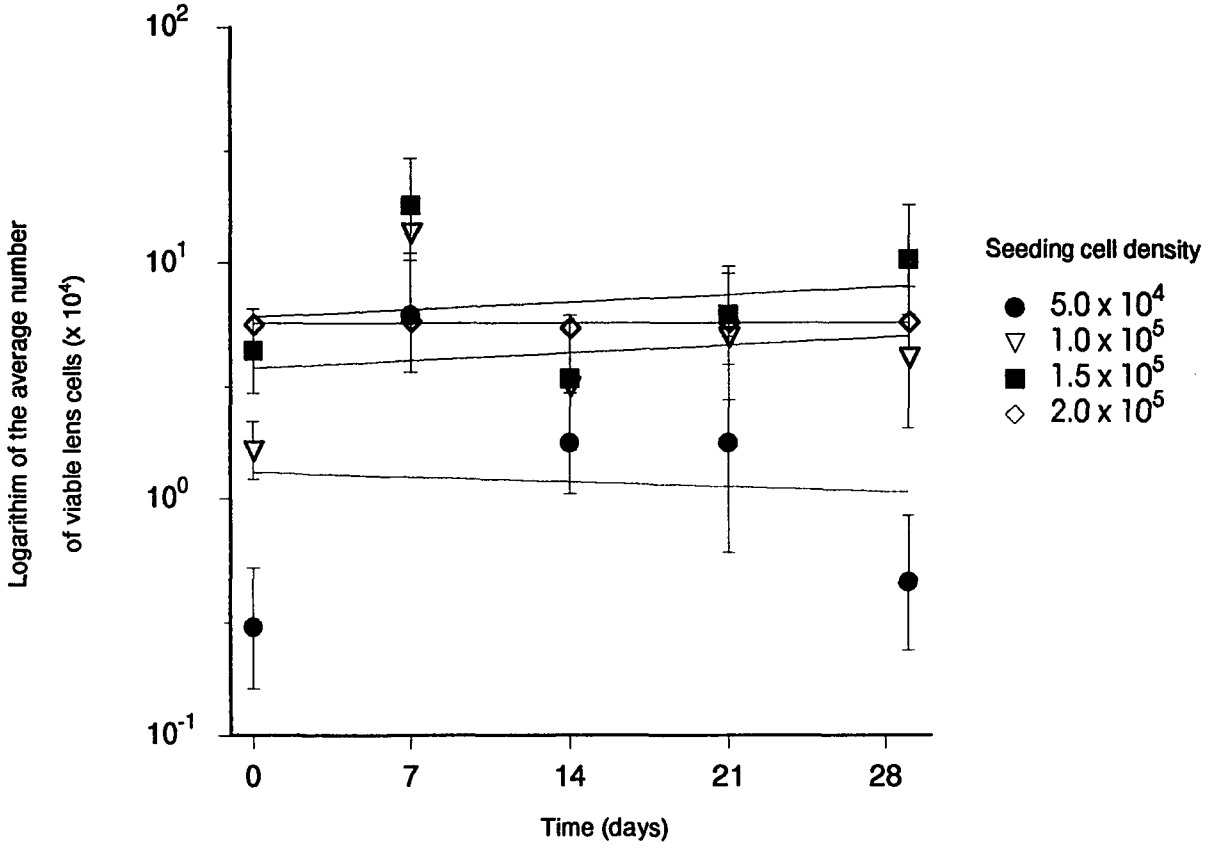

Logarithim of the average number
of viable lens cells (x 10⁴)
10²
10¹
10⁰
10⁻¹
0
7
14
21
28
Time (days)
Seeding cell density
5.0 x 10⁴
1.0 x 10⁵
1.5 x 10⁵
2.0 x 10⁵

Figure 22: Thymidine uptake showing the replication potential of primary epithelial cell cultures as measured by the uptake of ^{3}H-thymidine for three different concentration levels of freshly harvested spot lens cells.

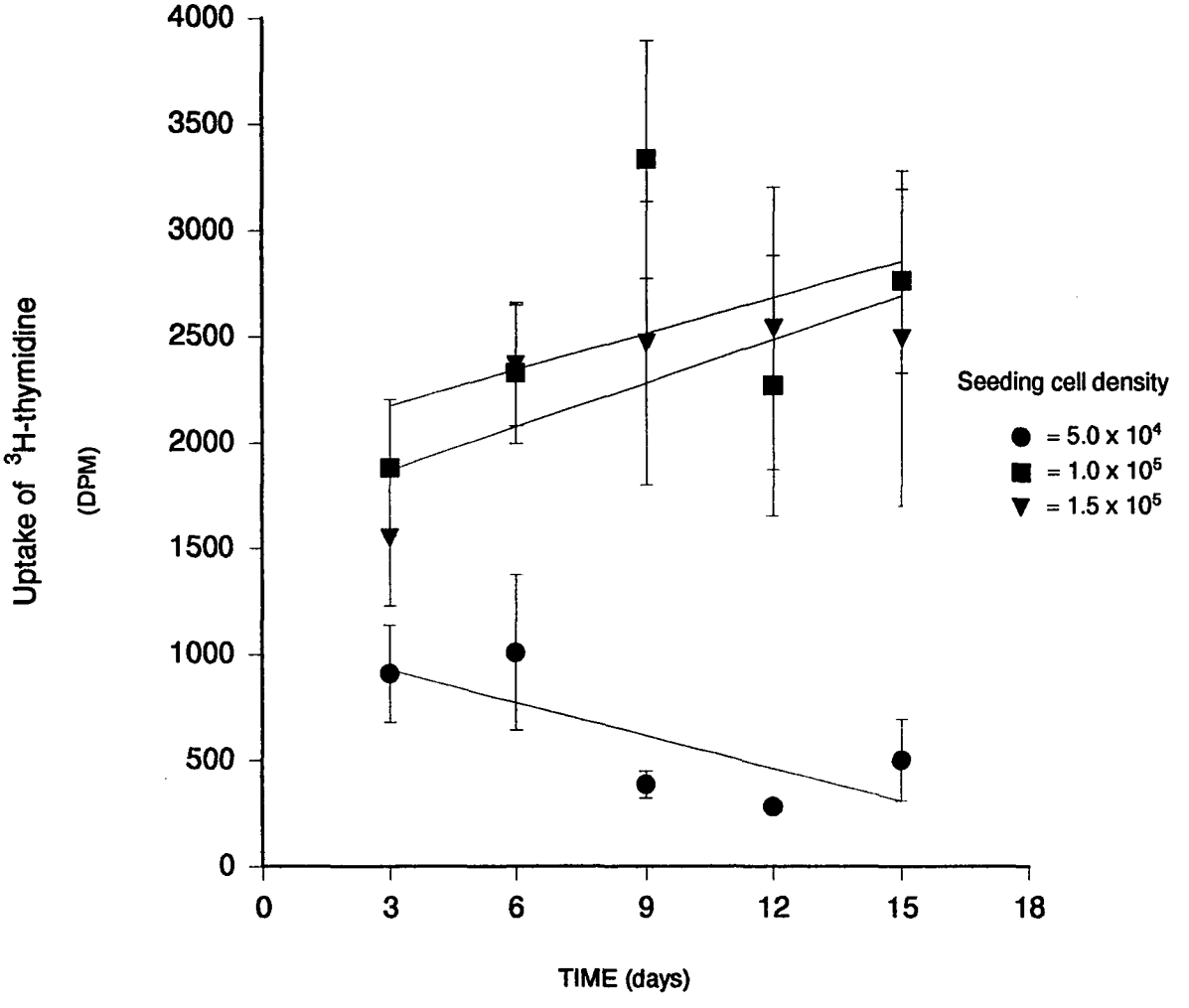
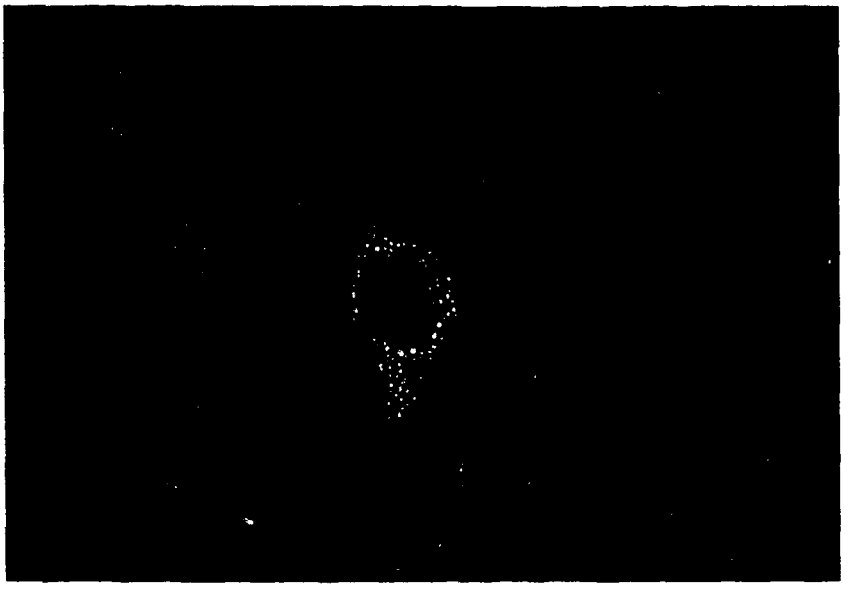

Uptake of ^{3}H-thymidine
(DPM)
4000
3500
3000
2500
2000
1500
1000
500
0
0
3
6
9
12
15
18
TIME (days)
Seeding cell density
= 5.0 x 10^{4}
= 1.0 x 10^{5}
= 1.5 x 10^{5}

Figure 23: Cell-like bodies without an apparent nucleus. A. SLEC-40 grown at 27 °C for 25 days, B. SLEC-40 grown at 27 °C for 34 days.

A



B

Figure 24: Proliferation assays of SLEC-40 cultures (48 months under *in vitro* culture) performed at varying cell density levels (100% media change at day 20). Estimates are replicate samples of a single flask of cells.

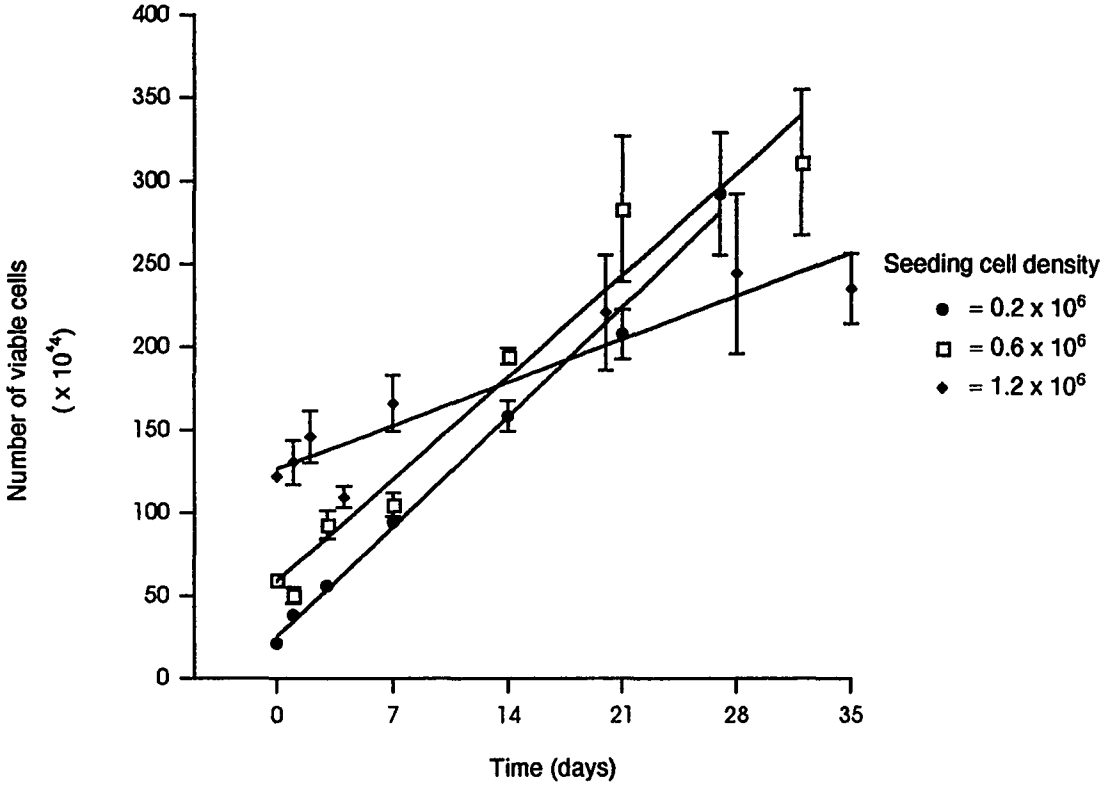

Number of viable cells
(x 10⁴)
400
350
300
250
200
150
100
50
0
0
7
14
21
28
35
Time (days)
Seeding cell density
● = 0.2 x 10⁶
□ = 0.6 x 10⁶
◆ = 1.2 x 10⁶

Figure 25: Proliferation assays of SLEC-40 cultures (49 months under *in vitro* culture) performed at varying exposure temperatures (100% media change at day 18).

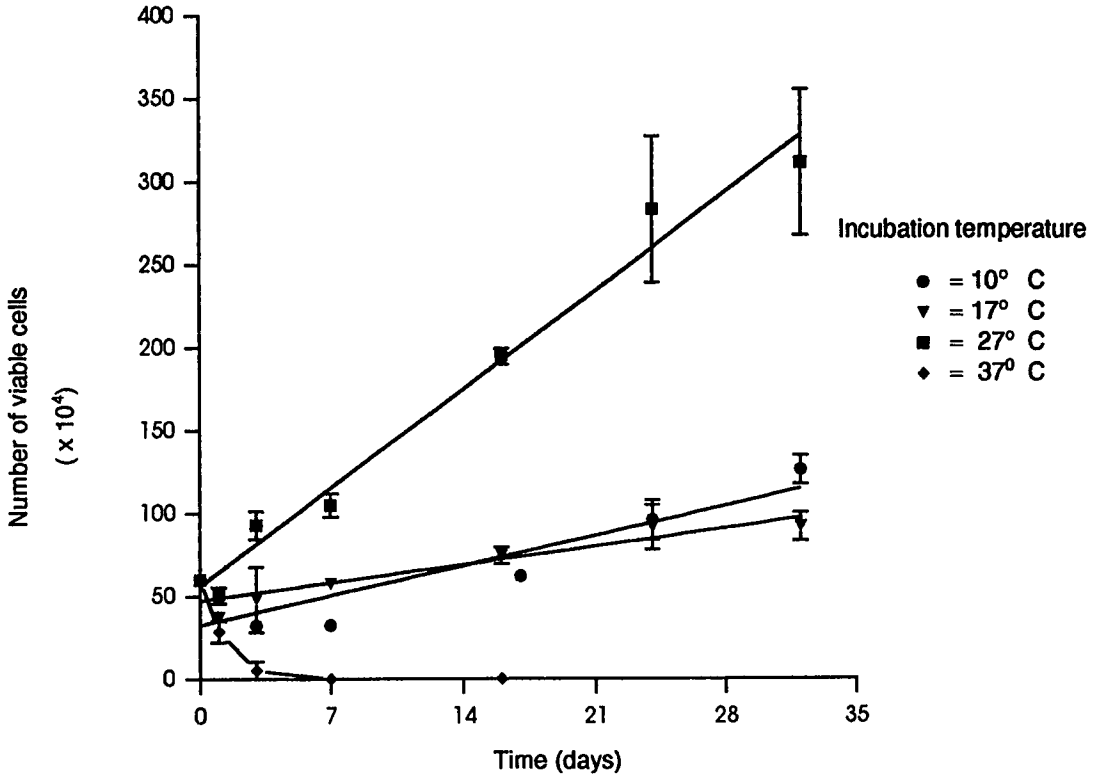

400
350
300
250
200
150
100
50
0
0
7
14
21
28
35
Time (days)
Number of viable cells
(x 10⁴)
Incubation temperature
● = 10° C
▼ = 17° C
■ = 27° C
◆ = 37° C

Figure 26: SLEC-40 grown at 17 °C for 16 days.

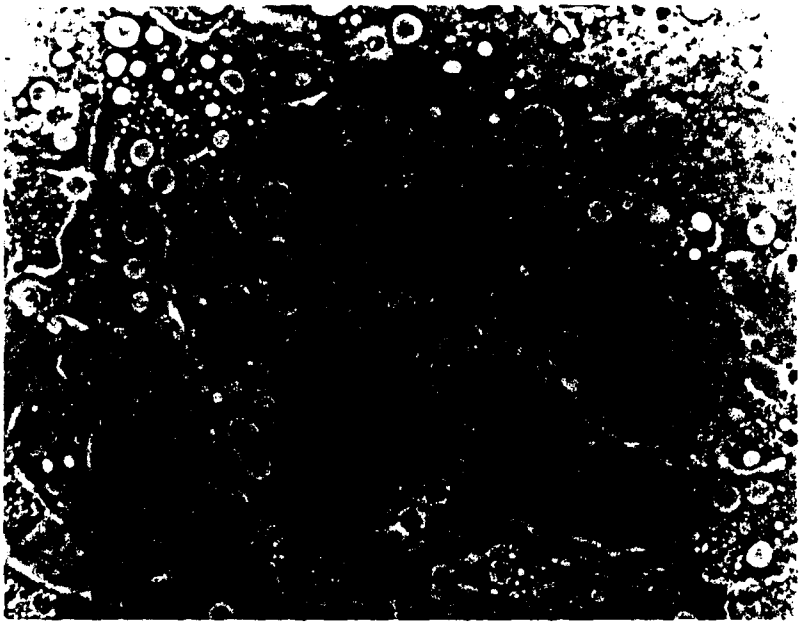


Figure 27: Karyotyping exhibiting the modal distribution of the number of chromosomes present in SLEC-6 (8 months after initial seeding) and in SLEC-40 (49 months after initial seeding). Note the presence of two spreads with 105 chromosomes in SLEC-40 that may be indicative of cellular transformation.

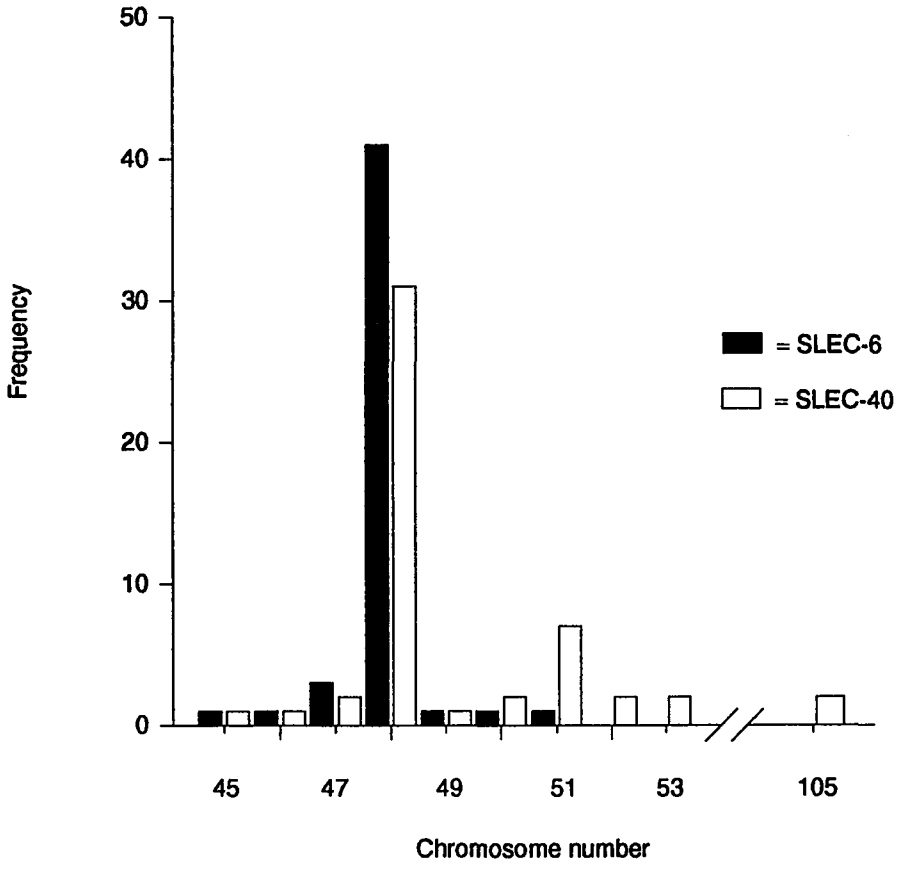

50
40
30
20
10
0
Frequency
= SLEC-6
= SLEC-40
45
47
49
51
53
105
Chromosome number

Figure 28: SDS-PAGE analysis. A. Long-term cultures of spot lens epithelium (SC37, 45 months under *in vitro* conditions). Lane 1 = molecular weight ladder (C), Lanes 2-3 = urea soluble fraction (US), Lanes 4-5 = water soluble fraction (WS). B. Water-soluble homogenates of lens tissue. Lane 1 = rat lens positive control, Lane 2-6 = spot lens replicates, Lane 7 = molecular weight standard.

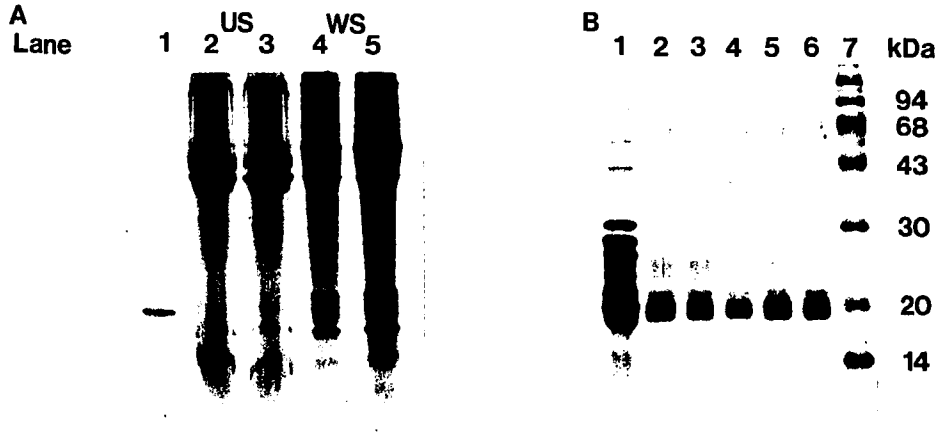

A
Lane
1
2
US
3
4
WS
5
B
1 2 3 4 5 6 7 kDa
94
68
43
30
20
14

Figure 29: Western blot analysis of long-term cultures of spot lens epithelium (SC37, 45 months under *in vitro* conditions). A. α-crystallins. B. ß-crystallins. C. γ-crystallins. Lanes 1-2 = water soluble fraction (WS), Lanes 3-4 = urea soluble fraction (US), C = positive control (rat lens).

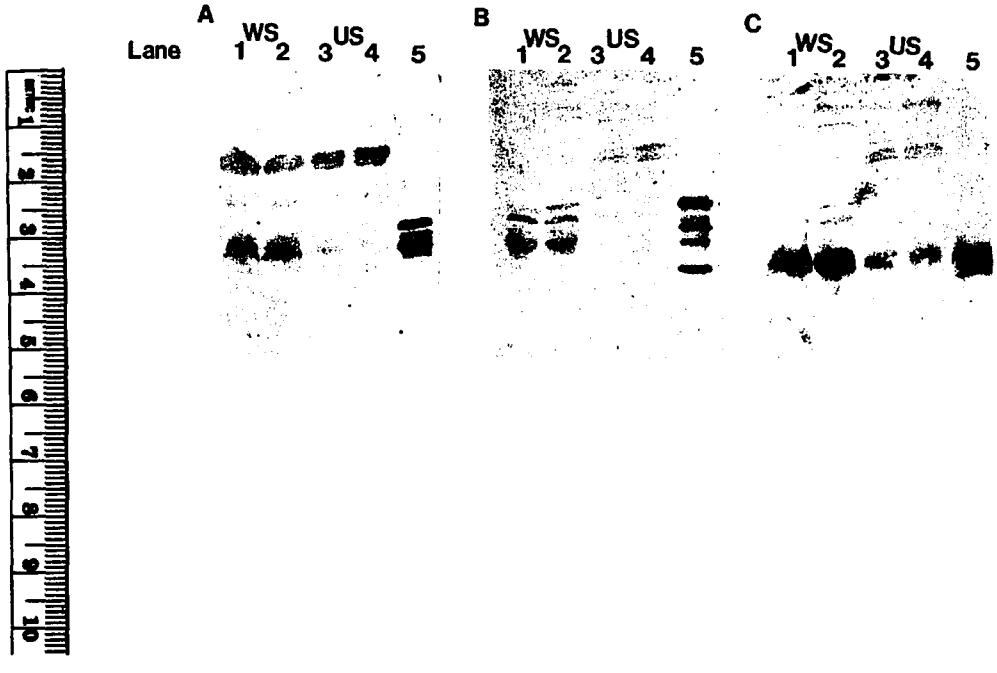
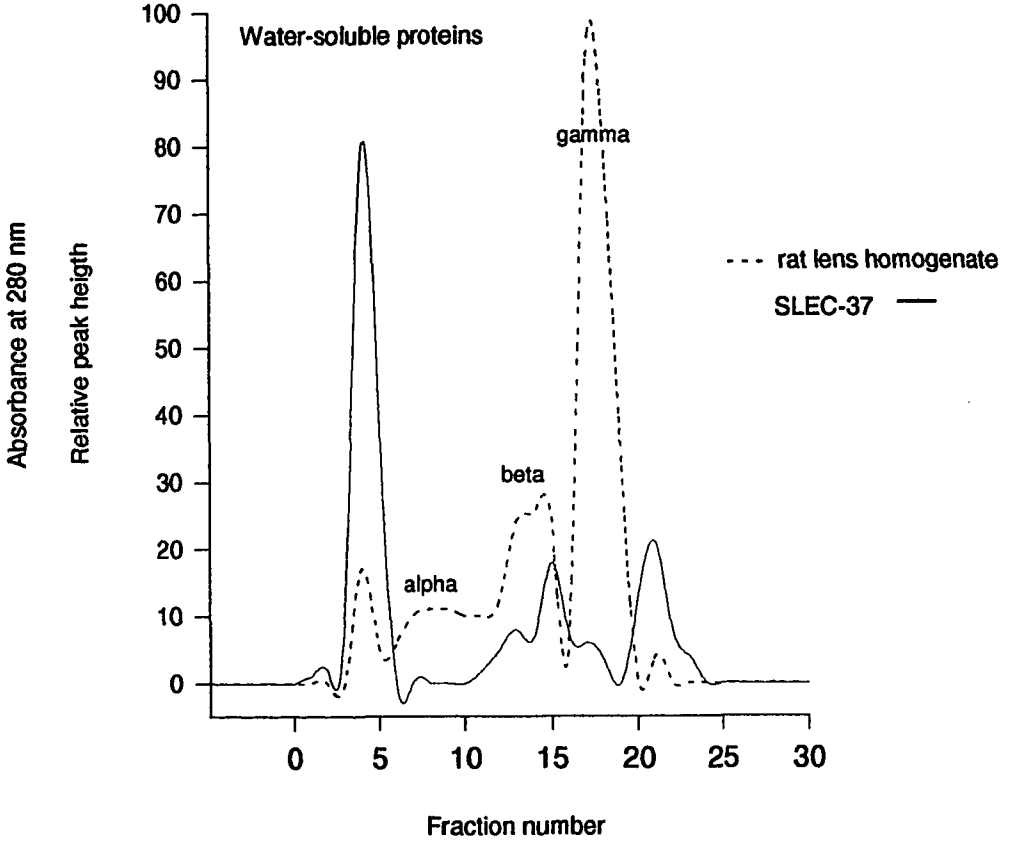

A
B
C
Lane
1 WS 2 3 US 4 5
1 WS 2 3 US 4 5
1 WS 2 3 US 4 5

Figure 30: HPLC elution patterns of soluble proteins from long-term cultures of spot lens epithelium (SC37, 45 months under *in vitro* conditions). Values are the average of two composite samples made up of four separate 25 cm^2 confluent cultures (30 days post initial seeding).


Water-soluble proteins
gamma
beta
alpha
rat lens homogenate
SLEC-37
Absorbance at 280 nm
Relative peak heigth
100
90
80
70
60
50
40
30
20
10
0
0
5
10
15
20
25
30
Fraction number

Discussion

Successful culture of fish lens epithelium has been established with a high potential for secondary and tertiary subculture and culture conditions have been developed which support long-term maintenance of cells and provide an environment in which cell differentiation and crystallin-protein synthesis appear to occur.

Primary cultures and early passages exhibited greatest growth and obtained confluency most consistently when lenses were stripped of the epithelial and cortical layers, minced into small pieces, and the remaining suspension enzymatically digested. This technique provided greatest success most likely because of the higher surface area to enzyme ratio, allowing for greater dissociation of cells (larger number of cells allowed to adhere to the culture flask). Furthermore, this technique led to a more homogeneous distribution of cells (cell colonies in close proximity) throughout the culture container, quicker cell to cell contact, and therefore faster development of a monolayer assemblage.

Culture conditions that proved to be most optimal were an incubation temperature range between 23-27 °C, L-15 growth media with 10% FBS supplemented with Hepes buffer solution, a non-CO_2 enriched atmosphere, and a high seeding density ($\leq$ 1 x 106 cells/25 cm^3 flask). Cultures became

confluent within 25-35 days and exhibited structures similar in appearance to those previously described as "lentoid bodies". Von Sallman et al (1969), who had previously cultured fish lens epithelium, also reported the lack of CO_2 dependence for rainbow trout cultures. Many researchers have described the presence of "lentoid bodies" during the culture of chick, mice, rat, and human lens epithelium (Tomarev et al., 1994; Vermorken et al., 1977; Iwig et al., 1978), however, the formation of such a structure is not a specific marker for the differentiation of lens epithelium.

Cellular composition of early cultures indicated the presence of certain sub-populations of cells that could be distinguished by a reduction in their nucleus:cytoplasmic ratios. These cell types were observed upon confluency during the passage of cells into new subculture generations and could be identified using both cytospin and cell slide preparations. The morphological attributes of these cells appeared to be similar in description to previous reports of the cytostructural changes that occur when epithelial cells differentiate into fiber cells (Papaconstantinou, 1967; Zigman, 1985). These unique morphologies of viable lens cells in subcultures 1-15 suggested that differentiation may be taking place.

Kinetic studies, utilizing ^{3}H-thymidine uptake as an index of cell mitosis, indicated that density levels of 1 x 10^5 and 5.0 x 10^5 cells/ml exhibited positive but slow

replicative potential. No difference in rates of uptake between these two levels was discernable. For both concentrations the population doubling time (PDT) for primary cell cultures was between 9-12 days (210-290 hours). Direct observation of cells under *in vitro* conditions produced similar PDT estimations with a continuous increase over the trial period. Uptake of radiolabelled thymidine does not indicate incorporation into macromolecules, but can be used as an index to support, quantitatively, the mitotic rate (TCA precipitation was not performed). Direct observation of increasing cell numbers, however, clearly identifies cellular replication and therefore supports the thymidine uptake studies.

Chromosome analysis indicated that cultures originated from spot, were still diploid, and had not altered either numerically or morphologically, as of the 6th passage (eight months past initial seeding) when compared with the typical chromosome numbers (hypodistribution of modal 48 chromosome complement) and morphology (telocentric) previously reported for spot (LeGrande and Fitzsimons, 1988). On the other hand, cultures into their 40th subculture (48 months past initial seeding) showed slight modifications (hyperdistribution of a 48 chromosome complement, metacentric shaped chromosomes, and chromosome clusters of 105) indicative of transformation.

Despite the similarity in morphological and growth

characteristics between early and long-term cultures several striking differences exist. For instance, long-term cultures obtain confluency in 7-10 days, whereas earlier cultures took 21-30 days, lower cell densities (1:10 splits) continue to confluency while earlier cultures were dependent on high cell concentrations (1:2 splits), and finally long-term cultures exhibit the formation of large well circumscribed bodies lacking nuclei within the culture monolayer. These bodies can be single isolated structures or can consist of multiple bodies, can be spherical in shape or irregular, can be refractile or non-refractile under phase contrast microscopy, and are visible within the monolayer using an inverted microscope (40x). To the author's knowledge, no other published data of similar types of structures have been reported. Whether these specific structures are specifically associated with crystallin-protein production has yet to be determined.

Several attributes of the long-term culture of spot lens epithelium indicate that these cultures may have progressed into a cell line. The increased mitotic potential of the cells, as evidenced by the decrease in time to confluency, the subtle changes observed in the chromosome complement, and the growth stability of cells after 50 months in culture are characteristics of an established cell line. SLEC cultures were purposely not cloned in an attempt to maintain cell heterogeneity. In the future, however,

trials to clone certain types of cells may be investigated.

The relatively slow growth of the SLEC cell line may in fact be a significant advantage compared to other long-term cultures, as it may allow for certain necessary interactions and processes to occur. Furthermore, although the lens grows throughout life epithelial cells must be relatively slow in their replicative rate *in vivo* (post-embryogenesis) in order to limit the potential size of the lens that is confined to a finite cavity.

Identification of all three major crystallin-proteins in SLEC indicates the continuity in synthesis of lenticular components. To the authors knowledge, no reports exist that identify the presence of a cell line that produces all three major crystallin-proteins. The positive reaction to bovine polyclonal antibodies indicates the existence of significant homology between fish and bovine crystallins. The presence of these three types is an indication of an ongoing process of cellular differentiation that coincides with our morphological observations. Fish lenses, in general, have a higher relative composition of γ-crystallins compared to mammalian species (Zigler, 1994). This may suggest that fish lens epithelium has an increased potential for γ-crystallin production. In general, crystallin production, if present at all, is reduced upon the continued culture of lens epithelium. In bovine and rat lens epithelial cultures a gradual loss of α-crystallin (α_B) content was reported

(Simonneau et al., 1983; Ramaekers, et al., 1984; Hamada, et al., 1979). Several researchers have also documented the reduction in synthesis of ß-crystallin ($ß_{B2}$) *in vitro* (Simonneau et al., 1983; Ramaekers et al., 1984; Simonneau et al., 1983).

Although earlier cultures of the SLEC cell line were not analyzed for crystallin composition, the presence of crystallin-proteins in spot lens cultures after 4-years of culture indicates long-term synthesis. This may be the result of allowing cultures to be maintained for significant periods of time past confluency since specialized biosynthesis in cultures usually occurs after confluency is obtained (Freshney, 1987). The relatively unique ability of spot epithelium to re-enter a growth phase after significant periods of time in a resting phase allows for both *in vitro* crystallin production and long-term culture maintenance. Nagineni and Bhat (1988) reported the progressive expression of ß-crystallin in human fetal lens epithelial cell cultures but these cells were only maintained under *in vitro* conditions for 5-6 months (passaged 2-3 times). The cultures described in this dissertation, however, differ from previously reported long-term cultures in that they are unaltered cells from normal, healthy tissue, have been maintained in culture for over 55 months, and continue to exhibit morphological changes and crystallin production characteristic of cellular differentiation. These

characteristics of spot epithelium indicate an elevated capacity for *in vitro* potential.

In summary, studies described herein indicate that the culture of spot lens epithelium is feasible, cultures can be established, long-term cultures developed, and the synthesis of all three major crystallin-proteins occurs. It is our hope that the SLEC cell line will provide an *in vitro* model to investigate the mechanism(s) of cataractogenesis.

CHAPTER 6

AUGMENTATION OF MACROMOLECULAR SYNTHESIS IN CULTURED LENS EPITHELIUM FROM FERAL FISH EXHIBITING CATARACT

Introduction

Cataract has been observed in several fish species from the Elizabeth River, Virginia (Huggett et al., 1987; Hargis and Colvocoresses, 1986). Spot (*Leiostomus xanthurus*), Atlantic croaker (*Micropogonias undulatus*), and the weakfish (*Cynoscion regalis*, all members of the family Sciaenidae), however, are reported to be preferentially susceptible (Hargis and Zwerner, 1988). It was shown that cataract prevalence increased in fish sampled from sites of the river where sediments are highly contaminated with creosote-associated polycyclic aromatic hydrocarbons (PAH, Huggett et al.,1987; Hargis and Colvocoresses, 1986; Hargis and Zwerner, 1988; Williams, et al., 1992). Despite the wealth of knowledge on the carcinogenic and hepatotoxic effects of PAH (Gelboin, 1980; Baumann, 1989; Varanasi et al., 1986; Dunn et al., 1987) little is known about the mechanism(s) that leads to cataractogenesis in PAH-exposed fish. Several PAH-compounds are metabolized by drug mixed function oxygenases into reactive metabolites that may bind to cellular macromolecules and thus may impair vital functions of the cells (Varanasi et al., 1986; Dunn et al., 1987; Smolarek et al.,1988; Smolerak et al., 1987). The liver is the main site for PAH biotransformation and detoxification, however, some extrahepatic metabolism has also been reported (Van Veld et al., 1990; Stegeman et al., 1984; Stegeman and

Kloepper-Sams, 1987; Stegeman, 1989; Goddard et al., 1987; Miller et al., 1988; Miller et al., 1989). The bottom feeding nature of some sciaenid fish such as spot and croaker, in which the highest prevalence of cataract occurs, raises suspicion that cataract may have resulted from the direct exposure to high concentrations of PAH compounds. Most recently, Williams et al. (Chapter 5) developed a protocol that allowed for the isolation and long-term culture of lens epithelium of the sciaenid fish, spot. *In vitro*, spot lens cells multiplied, elongated, differentiated, and produced α, β, and γ-crystallins.

The purpose of this study was to investigate the synthesis of the major cellular macromolecules, DNA, RNA, and protein, in cultured lens epithelium. Additional goals were to evaluate if the synthesis of these macromolecules is altered in fish collected from sites highly contaminated with PAH within the ER or that exhibit cataract formation.

Materials and Methods

Fish

Young-of-the-year spot were trawled monthly from June-September, 1991 from two stations (217 and Waterside) in the Elizabeth River, Virginia. The mean PAH concentrations in sediment samples taken over the past 15 years at Station 217 (latitude = 36° 50 -37° 51′, longitude 76° 17′ - 76° 18′) are

considered to be one of the highest ever reported and average approximately 96 µg/kg (n=4, SD=25,000). Sediments from Station Waterside (WS, latitude = 36° 46 - 36° 47′, longitude 76° 17′ - 76° 18′) have less total PAH (approximately 22 µg/kg, SD=6500, n=3, VIMS, unpublished data). Moreover, spot were sampled concurrently from the York River, Gloucester Pt., VA. (YR, latitude = 37° 15 - 37° 22′, longitude 76° 30 - 76° 40′) whose sediments contain lower levels of PAH (approximately 1.2 µg/kg, SD=350, n=10) and were considered as reference samples.

Fish sampled from Stations WS, 217, and YR were transferred to the laboratory, maintained in well-aerated holding tanks, and processed within 4 hours after collection. Prior to dissection, all fish were anesthetized with methanesulfonate (MS-222, 0.2g/L) (Sigma Chemical Co., St. Louis, MO), total length measured (from the snout to the end of the caudal fin, ± 0.5 cm), weighed (± 0.1 gm), and observed for cataract development, as well as any other body abnormalities.

Culture procedure

In vitro preparations were performed according to the enzymatic digestion protocol previously established by this author (Chapter 5). In general, lenses were aseptically removed from the ocular cavity and transferred to a sterile petri dish containing tissue culture media (Leibovitz-15,

Sigma Chemical Co.) adjusted to an osmolality of 365 mOsm/kg and supplemented with antibiotics and fetal bovine serum (FBS, Sigma Chemical Co.). Cells were harvested by stripping the epithelial layer from the lens and mincing, trypsinizing and stirring the epithelial fragments. Cells were then centrifuged, washed twice with 2% FBS culture media, and resuspended in 10% FBS L-15 media. The total number of viable cells harvested was estimated by means of trypan blue exclusion staining (Gibco, Grand Island, NY).

Macromolecular synthesis

The uptake of radiolabelled isotopes was performed as described by Williams et al. (1992). Briefly, the uptake of ^{3}H-thymidine, ^{3}H-uridine, and ^{3}H-leucine was determined on cells pooled from 5-10 fish (10-20 lenses). Lens cells were resuspended in 96-well microtiter plates at a density of 1 x 10^{5} cells/ml in 200 ul of 10%-FBS growth media. Six replicates per treatment level were utilized. Following 72 hr of incubation, microtiter plates were pulsed with radiolabelled precursors, further incubated for 24 hours, and harvested with an automatic cell harvester (Cambridge Tech, Baltimore, MD.) onto glass-fiber filters. The radioactivity, which represents the uptake of the radiolabelled precursors, was then assessed in a Beckman Liquid Scintillation counter using Beckman Ready-Safe (Beckman, Fullerton, CA.) as scintillant. All values

measured were expressed in disintegrations per minute (dpm).

Statistical analysis

Data were analyzed by a one-way ANOVA model to determine if any significant differences among treatment levels existed (Minitab statistical package). Comparison between YR and ER data was performed using the Student's t-test at alpha = 0.05 (Sigmaplot 4.1, Jandel Corporation, 1991). Analysis of mean treatment levels between different ER classifications was performed utilizing a Duncan's multiple mean comparison test (Statistica, Jandel Corporation, 1992).

Results

Macromolecular synthesis by cultured lens cells

York River fish

The uptake of radiolabelled precursors by cultured YR reference lens cells indicated that cells were synthesizing all three macromolecular compounds (DNA, RNA, and protein, as measured by the uptake of ^{3}H-thymidine, Fig. 31, ^{3}H-uridine, Fig. 32, and ^{3}H-leucine, Fig. 33, respectively) and that cells were viable and in a positive growth phase. At Day 3, ^{3}H-thymidine uptake was observed to be significantly greater than either that of ^{3}H-uridine or ^{3}H-leucine ($p<0.001$). The mean uptake was 1945 dpm (SE = $\pm$ 178 dpm)

for ^{3}H-thymidine, 1012 dpm (± 181 dpm) for ^{3}H-uridine, and 1122 dpm,(± 288 dpm) for ^{3}H-leucine. The lack of significant differences in the rates of uptake between ^{3}H-leucine and uridine indicated that the synthesis of proteins was comparable to RNA synthesis.

Elizabeth River fish

Classified collectively, lens cells harvested from ER spot (Station 217, Station WS, cataract, and non-cataract fish combined) exhibited significantly higher ^{3}H-thymidine, 4007 dpm (± 499 dpm, $p<0.0034$), and ^{3}H-uridine uptake responses, 3172 dpm (± 1380 dpm, $p<0.001$), compared to cells taken from YR fish. On the other hand, ER lens cells had an average uptake rate for ^{3}H-leucine of 1185 dpm (± 582 dpm). This was not observed to be significantly different from YR samples.

Classification of ER data by individual sampling stations and collected fish specimens into cataract and non-cataract samples indicated that differences did exist. Lens cell uptake of ^{3}H-thymidine was significantly higher ($p<.002$) at sampling stations where sediments have been documented to have increased PAH content (Stations WS and 217). The mean value for samples taken from Station WS was 1534 dpm (± 31 dpm), while the mean response of assays harvested from fish collected at Station 217 was 3057 dpm (± 237 dpm).

Contrary to the data obtained from the thymidine uptake assays, ^{3}H-uridine uptake in cells harvested from fish sampled from station WS (884 ± 108 dpm) and 217 (1388 ± 512) showed no significant statistical difference in uptake responses.

Similar in response to the uridine assays, no significant difference could be determined between mean ^{3}H-leucine uptake responses between ER sampling stations. Lens cell uptake rates for ^{3}H-leucine for WS and 217 specimens displayed mean responses of 653 dpm (± 57 dpm) and 1185 dpm (± 582 dpm).

Fish exhibiting lens cataract

Lens cells harvested from fish exhibiting cataract showed the greatest ^{3}H-thymidine uptake of all classification treatments. Cells taken from cataract lenses displayed mean uptake value of 7050 dpm (± 667 dpm). A t-test comparison indicated that cataractous cells demonstrated significantly higher uptake values than cells taken from non-cataractous fish collected from either Station WS or 217 ($p<0.001$).

Similar in nature to the thymidine assays, the uptake of ^{3}H-uridine by lens cells harvested from cataract lenses showed significant augmentation ($p<0.001$) compared to both station WS and 217 samples. The mean uptake response for cells harvested from cataract lenses was 6992 (± 244 dpm).

On the other hand, as was observed in the station data, no significant difference was observed in protein synthesis by cataract specimens to either the WS or 217 data. The average uptake rate for all cataract lens cell assays was 1263 (± 310 dpm).

Stages of cataract development

Significant differences in uptake of ^{3}H-thymidine were observed between differing stages of cataract development, i.e. 100% mature cataracts versus < 50% opacified lenses. Cells isolated from partial cataract lenses had significantly ($p<0.0001$) lower mean uptake, 5679 dpm (± 299 dpm), compared to those harvested from mature cataract tissues, 8878 dpm (± 100 dpm).

Differences in RNA synthesis (^{3}H-uridine uptake) between stages of cataract development also showed significant differences in uptake response. Cells from 100% mature cataract lenses showed a significantly greater mean uptake response, 9396 dpm (± 939 dpm, $p<0.0001$), compared to cells isolated from partial cataract lenses (< 50% opacified) that demonstrated a mean uptake response of 4588 dpm (± 112 dpm).

The only significant difference observed in any of the protein assays performed was observed in the comparison between cells isolated from partial and mature cataract lenses. Cells harvested from lenses with only partial

cataract development had significantly higher mean uptake values, 1573 ± 81, than those cells taken from 100% mature cataract lenses, 952 ± 61 ($p < .0001$).

Figure 31: DNA synthesis, as measured by the uptake of ^{3}H-thymidine, in single cell suspensions of spot (*Leiostomus xanthurus*) lens cells. YR = epithelium harvested from lenses excised from spot collected from the York River, WS = epithelium harvested from lenses excised from spot collected from Station Waterside, 217 = epithelium harvested from non-cataractous lenses excised from spot collected from Station 217, PC = epithelium harvested from stage 1 and stage 2 cataractous lenses excised from spot collected from Station 217, MC = epithelium harvested from stage 4 cataractous lenses excised from spot collected from Station 217.

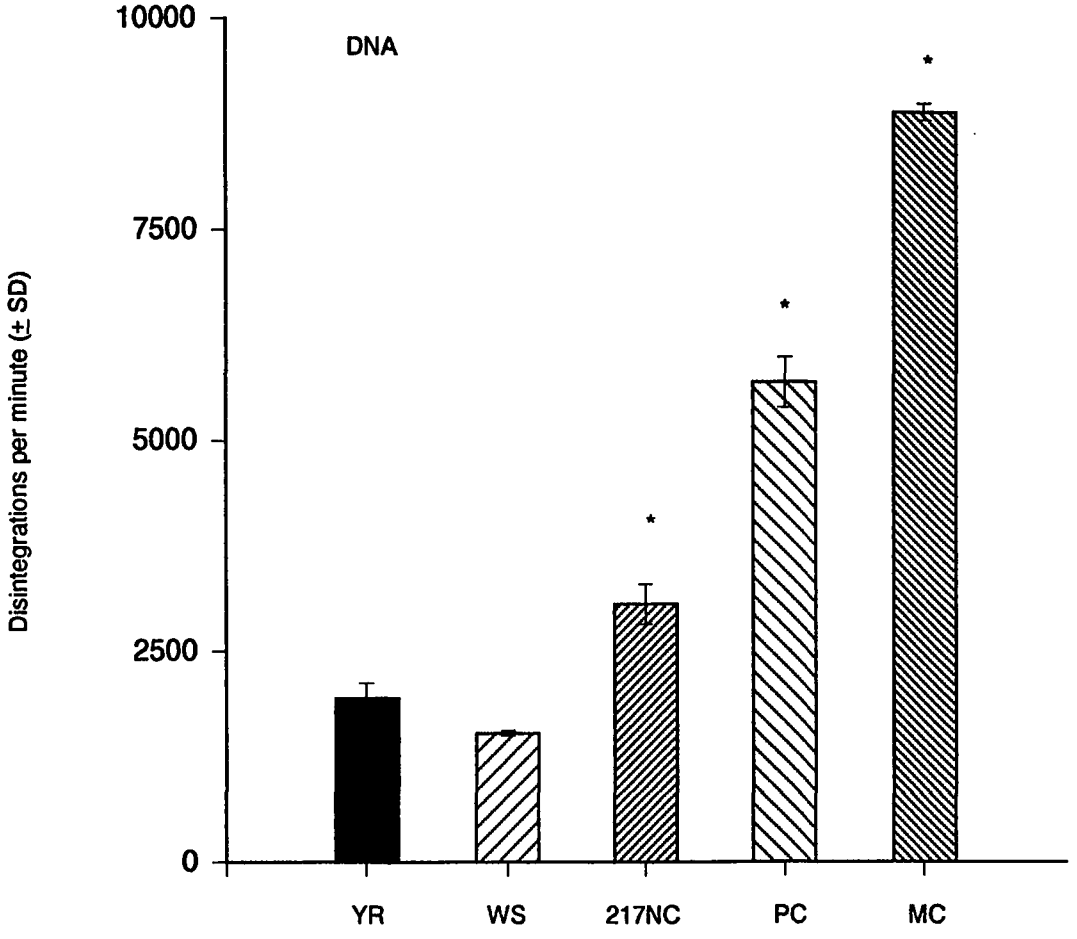

DNA
Disintegrations per minute (± SD)
10000
7500
5000
2500
0
YR
WS
217NC
PC
MC
*
*
*
*

Figure 32: RNA synthesis, as measured by the uptake of ^{3}H-uridine, in single cell suspensions of spot (*Leiostomus xanthurus*) lens cells. YR = epithelium harvested from lenses excised from spot collected from the York River, WS = epithelium harvested from lenses excised from spot collected from Station Waterside, 217 = epithelium harvested from non-cataractous lenses excised from spot collected from Station 217, PC = epithelium harvested from stage 1 and stage 2 cataractous lenses excised from spot collected from Station 217, MC = epithelium harvested from stage 4 cataractous lenses excised from spot collected from Station 217.

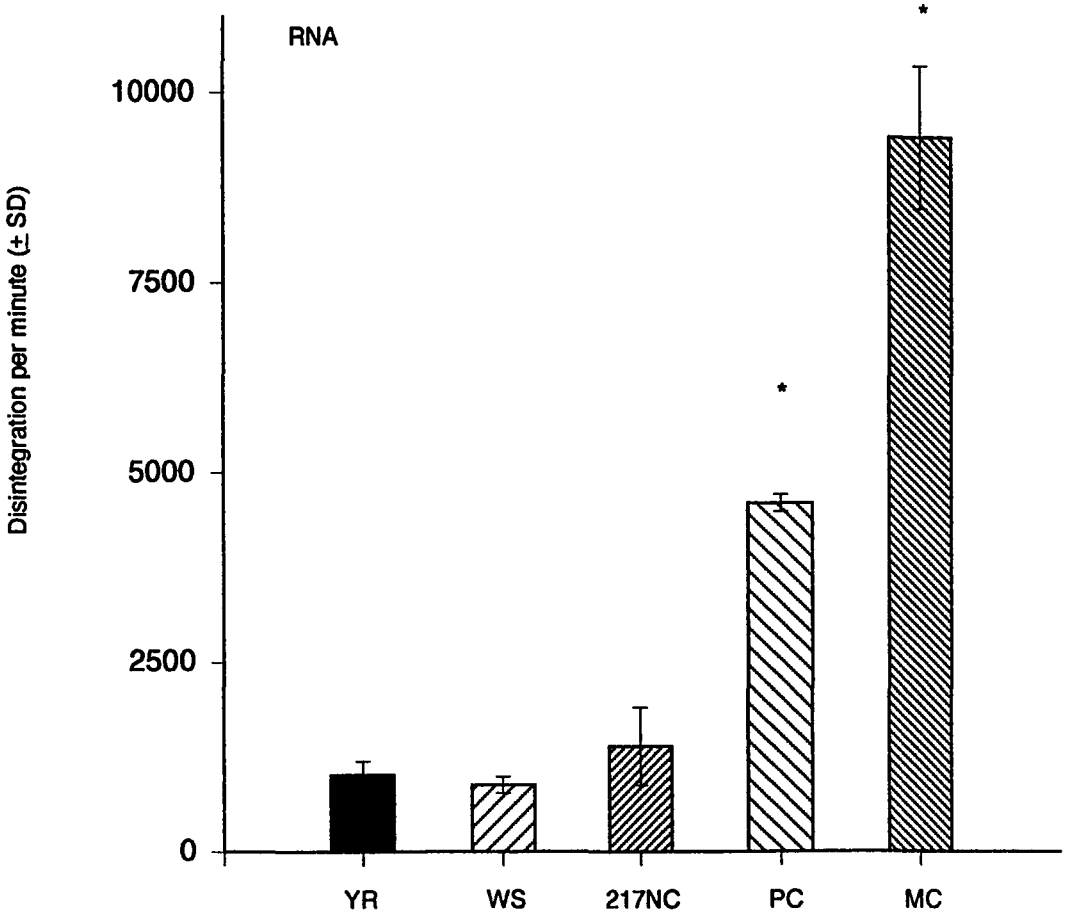
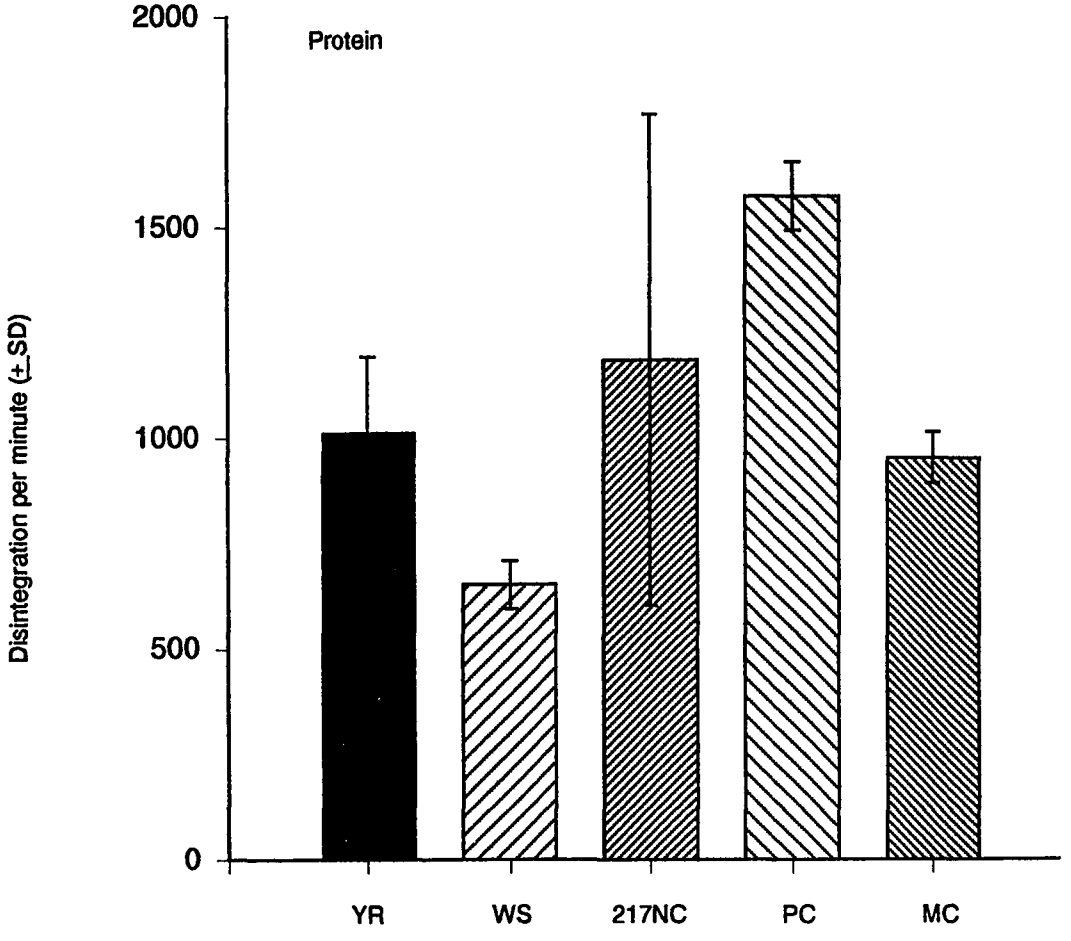

RNA
Disintegration per minute (± SD)
10000
7500
5000
2500
0
*
*
YR
WS
217NC
PC
MC

Figure 33: Protein synthesis, as measured by the uptake of ^{3}H-leucine, in single cell suspensions of spot (*Leiostomus xanthurus*) lens cells. YR = cells harvested from lenses excised from spot collected from the York River, WS = cells harvested from lenses excised from spot collected from Station Waterside, 217 = cells harvested from non-cataractous lenses excised from spot collected from Station 217, PC = cells harvested from stage 1 and stage 2 cataractous lenses excised from spot collected from Station 217, MC = cells harvested from stage 4 cataractous lenses excised from spot collected from Station 217.


Protein
2000
1500
1000
500
0
Disintegration per minute (±SD)
YR
WS
217NC
PC
MC

Discussion

Augmentation of macromolecular synthesis in fish lenses that exhibit cataract formation indicates that cataract and alteration in the cellular cycle of lens epithelial cells are intimately associated. Bow region epithelial cells are the only actively mitotic cells of the vertebrate lens and therefore must be the affected cell type (Papaconstantinou, 1967; Zigman, 1985).

The positive association between stimulation of DNA and RNA indices and stages of cataract formation suggest that interference in the mitotic cell cycle may be one of the initial steps in chemically induced cataract of fish. Hargis and Zwerner (1989) described massive hyperplasia of epithelial cells in histologically examined ER cataractous spot lenses. Indeed, stimulation of cells to divide by exposure to very low concentrations of certain chemicals, chemical hormesis, has been previously reported in mice (Schumann et al., 1982).

Two general mechanisms of toxic action that could lead to increased DNA synthesis were postulated by Schumann and co-workers (1982). The first, the somatic mutation theory, would occur as a result of electrophilic xenobiotics preferentially binding to nucleophilic receptor sites on the surface of DNA strands causing adduct formation. Such a lesion, if not properly repaired, would be fixed into the

genetic composition upon replication, the cell would become transformed, and growth might go unregulated [21]. The second process was an epigenetic mechanism, non-preferential binding of xenobiotics to DNA, that would result in increased DNA synthesis caused by activated cellular repair mechanisms stimulated by damaged tissue. In general, unscheduled repair mechanisms only account for a minor increase in DNA synthesis, therefore, it is unlikely that such action could account for the dramatic increases observed in this study. It would appear than that the former theory is more likely.

Recent work in this laboratory (Williams, Chapter 4) has indicated that cell membrane permeability may be altered in cataractous lenses due to certain compositional changes in lipids. If such changes were to increase permeability than it is possible that mitogenic substances such as growth factors could leak from lens cells, thereby stimulating replication and DNA synthesis. Growth factors have been shown to be present in lens cells (McAvoy and Chamberlain, 1991).

The differential response of DNA (R=70.0) and RNA synthesis (R=60.0), relative to environmental levels of PAH, suggests that RNA synthesis may be a secondary response related to increased cell mitosis. Uridine uptake is a receptor-mediated process that coincides with the growth status of cells. Uptake rates have been shown to greatly

increase in cell cultures that are actively stimulated with mitogenic treatments (Kocan et al., 1985). On the other hand, RNA synthesis showed the greatest level of augmentation between cataract and reference specimens and may be directly related to disease processes involved in cataract formation. RNA is vital to protein synthesis, cell and gene regulation, as well as cell differentiation (Alberts et al., 1983) and therefore could, if chemical interference occurs, create severe biochemical ramifications to the proper functioning of exposed cells.

The lens is a highly proteinaceous (80-90% by dry weight, Zigler, 1994) tissue. In general, protein synthesis in mammals is disturbed with the onset of cataract formation. The lack of response in the ^{3}H-leucine uptake assays would appear to indicate that protein synthesis may not be affected by cataract development in these fish. The lens is composed of crystallin proteins that may represent up to 90-95% of all proteins present (Zigler, 1994) and which have been shown to be modified by precipitation, aggregation, or lysis upon cataract formation [reviewed by Maisal, 1990]. While leucine is a common amino acid that is incorporated into almost all proteins, it may not adequately represent crystallin-protein synthesis. Crystallins, especially γ-crystallin, contain a high number of sulfhydryl bonds. Quite possibly, the utilization of radiolabelled sulfur-containing amino acids, such as methionine or

cysteine, would lead to more specific insight to the effects of PAH exposure on lenticular protein metabolism and cataract development.

The role of PAH in fish cataractogenesis is still far from clear and the data from this study are suggestive, at best, due in great part to the inexact nature of field studies, the patchiness of PAH distributions, and the mobility of sciaenid fish species. Furthermore, sediments of the ER undoubtedly contain numerous other xenobiotics such as tributyltin, polychlorinated biphenyls, polychlorinated triphenyls, and heavy metals. No matter what the cause the ER environment has been associated with numerous detrimental effects on fish health (Faisal et al., 1991; Hargis and Zwerner, 1988; Hargis et al., 1989; Seeley and Weeks-Perkins, 1991; Thiyagarajah et al., 1989; Vogelbein et al., 1990). Hargis et al. (1984) reported cataract formation in spot exposed to ER sediment effluent. Spot are bottom feeders that root through muds for food, resuspend sediments while searching for food, and ingest a high load of sediments during feeding. All these characteristics would greatly increase exposure of spot to sediment-borne PAH.

Whether or not xenobiotics, PAH in particular, directly come into contact with the lens has yet to be shown. Naphthalene, a low molecular weight PAH, and benzo[a]pyrene, a high molecular weight PAH that is reported to be a

precarcinogen, are common contaminants of the Elizabeth River (Bieri et al., 1986). Naphthalene is a relatively water-soluble PAH that has been shown to be cataractogenic in mammals (Van Heyningen and Pirie, 1966; Wells et al., 1989). Therefore, the probability of naphthalene being able to enter the ocular cavity, either by blood circulation or diffusion through the cornea, is feasible. Xu et al. (1992) showed that rats dietarily exposed to naphthalene exhibited naphthalene metabolites in the ocular cavity and the lens. BaP, on the other hand, is a relatively hydrophobic compound. Nevertheless, investigations done by Balk et al. (1984) have shown that radiolabelled benzo(a)pyrene rapidly distributed to the eye region (lenses) of the northern pike (*Esox lucius*), following exposure to a contaminated diet or water.

Previous research has indicated that exposure to PAH can cause increased cell division. Kocan and coworkers (1985) reported that the organic extract of some PAH-contaminated sediments of the Duwamisch River augmented mitosis in the fish-derived cell line, RTG-2. Previous work by Simakov (1982) further supports these findings. Data showed that cells derived from the rainbow trout, *Salmo gairdneri*, lens exhibited increased cell mitosis when directly exposed to the benzene derivatives, trichloro-benzene, nitrobenzene, and 2-naphthol (a naphthalene metabolite). The actual mechanism, unfortunately, by which

stimulation occurred was not investigated or discussed. Therefore, the increased uptake of ^{3}H-thymidine and ^{3}H-uridine could have resulted from the direct exposure of the fish lens to PAH.

In conclusion, results indicate that the normal lens cell cycle of fish collected from areas whose sediments are heavily contaminated with PAH and cataractous fish are significantly altered. The actual mechanism by which these changes occur is presently unclear and is under further investigation in this laboratory. Furthermore, the more direct measurement of PAH-exposure (P-450 induction or elevated EROD activity in the liver or the bile) may be more conclusive of the role PAH play in the interference in lens cell regulation as well as formation of cataract.

CHAPTER 7

POLYNUCLEAR AROMATIC HYDROCARBONS AND FISH CATARACT: EFFECTS OF BENZO[A]PYRENE AND NAPHTHALENE ON THE DNA SYNTHESIS OF CULTURED LENS CELLS OF SPOT (*LEIOSTOMUS XANTHURUS*: PISCES, SCIAENIDAE)

Introduction

Cataracts have been reported in fish species of the family Sciaenidae from waters whose underlying sediments are heavily contaminated by polycyclic aromatic hydrocarbons (PAH, Bieri et al., 1986; VIMS, 1992). Presently, however, it is unknown whether cataracts in fish from chemically-contaminated sites are the result of a direct effect of one or several PAH or other chemical species on the lens, or, if it is a manifestation of a more general reaction at the organismal level. This lack of information is hindered by the absence of an *in vitro* system which would allow for the investigation of the direct effects of chemicals on lens cell metabolism. For this reason, a protocol for the *in vitro* growth and differentiation of lens epithelium (Chapter 5) from the marine teleost spot (*Leiostomus xanthurus*; Pisces:Sciaenidae) was established.

The direct exposure of lens epithelium to xenobiotics, such as benzene derivatives, is capable of interfering with the mitotic activity of undifferentiated fish lens epithelium as shown in rainbow trout (*Oncorhynchus mykiss*; Simakhov, 1982). PAH, on the other hand, such as benzo[a]pyrene (BaP), have been shown to be rapidly incorporated into the eyes of the northern pike (*Esox lucius*) via both dietary and water exposure (Balk et al., 1984).

On the other hand, naphthalene (N), a low molecular weight aromatic hydrocarbon that is also a common contaminant of ER sediments, is relatively water soluble and has been reported to be cataractogenic in rats, rabbits, and humans (Xu et al., 1992; Van Heyningen and Pirie, 1967; Koch et al., 1976; Hockwin et al., 1984).

The aim of the present study was to investigate the effects of the direct exposure of naphthalene and benzo[a]pyrene on the DNA synthesis of normal cells isolated from the lens of the marine teleost spot.

Materials and Methods

Spot lens cells were isolated via trypsin dissociation and cultured at 27 °C (5% CO_2). Uptake of ^{3}H-thymidine was determined on pooled cells at a density of 1 x 10^5 cells/ml. Medium controls contained 200 µl of Leibovitz-15 growth media, whereas BaP and N treatments contained 180 µl of lens cell suspensions mixed with 20 µl of BaP or N at stock concentrations of 1 x 10^{-4}*M* (a final concentration of 1 x 10^{-5} *M*). Serial dilutions (1 ml/ 9 ml of L-15) were subsequently performed to produce concentration levels used. Solvent controls were formulated for all BaP-treatments and in the same manner by serial dilutions of a stock solution. Medium control, PAH treatment, and solvent control values were the average of eight-ten replicates per test.

Microtitre plates containing all treatment levels were individually pulsed with radiolabelled precursors every 48-72 hours during the 15 day trial period (depending on the test), incubated for an additional 24 hours, and then harvested with an automatic cell harvester (Cambridge Tech, Baltimore, MD.). The radioactivity, which represents the uptake of the radiolabelled precursors, was then assessed in a Beckman Liquid Scintillation counter using Beckman Ready-Safe (Beckman, Fullerton, CA.) as scintillant and measured as disintegrations per minute (DPM).

Statistical analysis was performed on mean treatment DPM-values for a specific day and compared against media and solvent controls using analysis of variance (ANOVA) and Tukey's multiple mean comparison test at $\alpha = 0.05$.

Results

Naphthalene exposure

Data showed that the average ^{3}H-thymidine uptake, indicative of DNA synthesis, for the medium control of cultured lens cells increased by 291% over the incubation period. Due to the relative solubility of naphthalene (30mg/l; Eisler, 1987) no carrier solvent was necessary. Exposure of lens cells to naphthalene showed no significant differences between any of the treatment concentrations between days 2-10 (Fig. 34A). However, on the final day of

exposure (Day 14) significant differences did become apparent. On Day 14, media control cells were determined to have a significantly higher thymidine uptake compared to all naphthalene treatment levels (Fig. 34B, $p<0.01$). A slight dose response was observed as the highest concentration of naphthalene (10^{-5} *M*) was shown to have a significantly reduced uptake response ($p<0.05$) compared to all other concentration levels. Concentrations of naphthalene between 10^{-7} and 10^{-13}*M* showed no significant differences among these treatment levels.

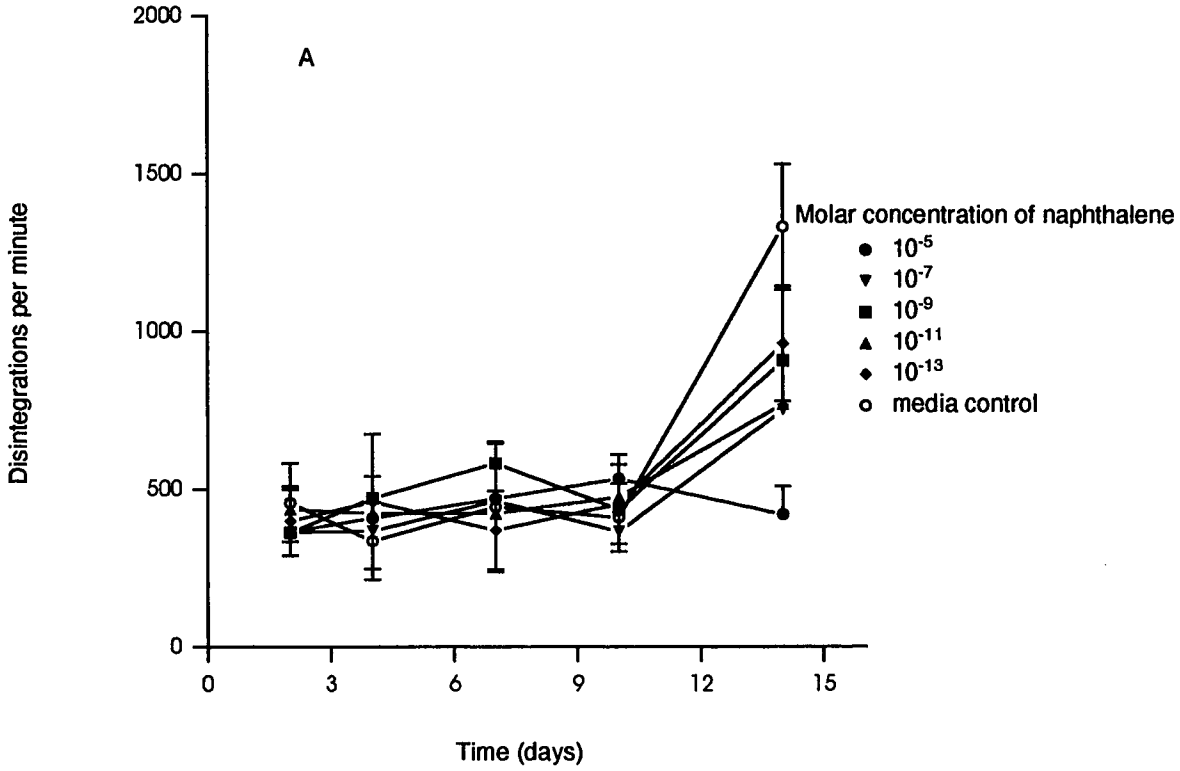
Benzo[a]pyrene exposure

Data showed that the average ^{3}H-thymidine uptake for the medium control of cultured lens cells did not exhibit positive growth. Solvent controls were utilized due to the relative insolubility of BaP (0.0038 mg/l, Eisler, 1987). In general, all treatment and control groups were observed to only maintain initial uptake responses (Fig. 35A). The one treatment level that did exhibit positive growth (155%) was the lowest concentration of the solvent control (10^{-13} *M*). Overall, solvent controls consistently displayed the highest uptake responses and were therefore used as reference samples. Although no effects were found to be statistically significant, the expression in cells exposed to BaP as a percentage of the solvent control (Fig. 35B) indicated that both the low and high concentration levels of

BaP inhibited growth, while the moderate level showed a slight stimulation.

Time series analysis of exposure of lens cells to moderate levels of BaP (10^{-8} *M*, Fig. 36) showed that during early exposure (Days 3 and 6) a slight inhibition in ^{3}H-thymidine uptake was observed. On the other hand, after 9-12 days of exposure significant stimulation was apparent. Once again, solvent controls were observed to have higher uptake responses than media controls and were, therefore, used as reference samples.

Figure 34: A. DNA synthesis, as measured by the uptake of ^{3}H-thymidine in disintegrations per minute (DPM), in fish lens epithelium exposed to varying levels of naphthalene, B. DNA synthesis on day 14 indicating the suppression of ^{3}H-thymidine uptake in spot lens epithelium by varying levels of naphthalene exposure. (* significant difference from solvent control, ** significant differences between solvent control and all other concentration levels).


A
2000
1500
1000
500
0
Disintegrations per minute
0
3
6
9
12
15
Time (days)
Molar concentration of naphthalene
10-5
10-7
10-9
10-11
10-13
media control

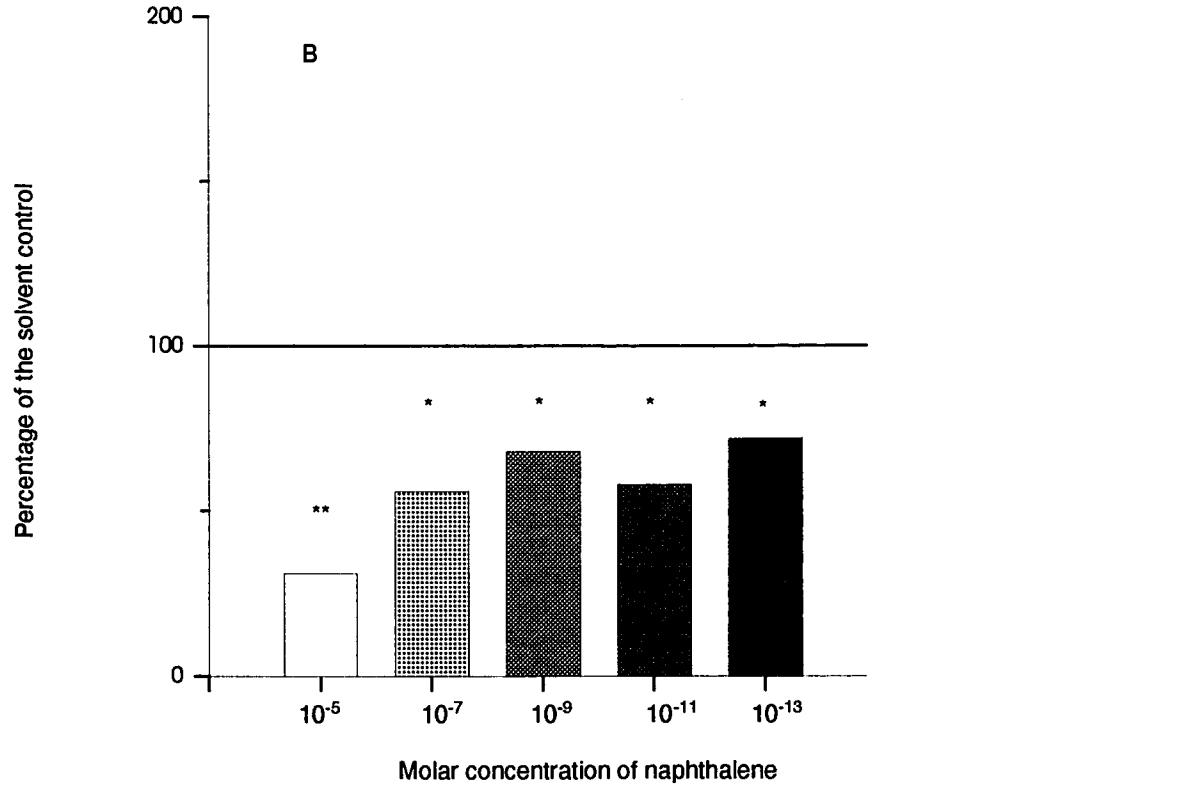
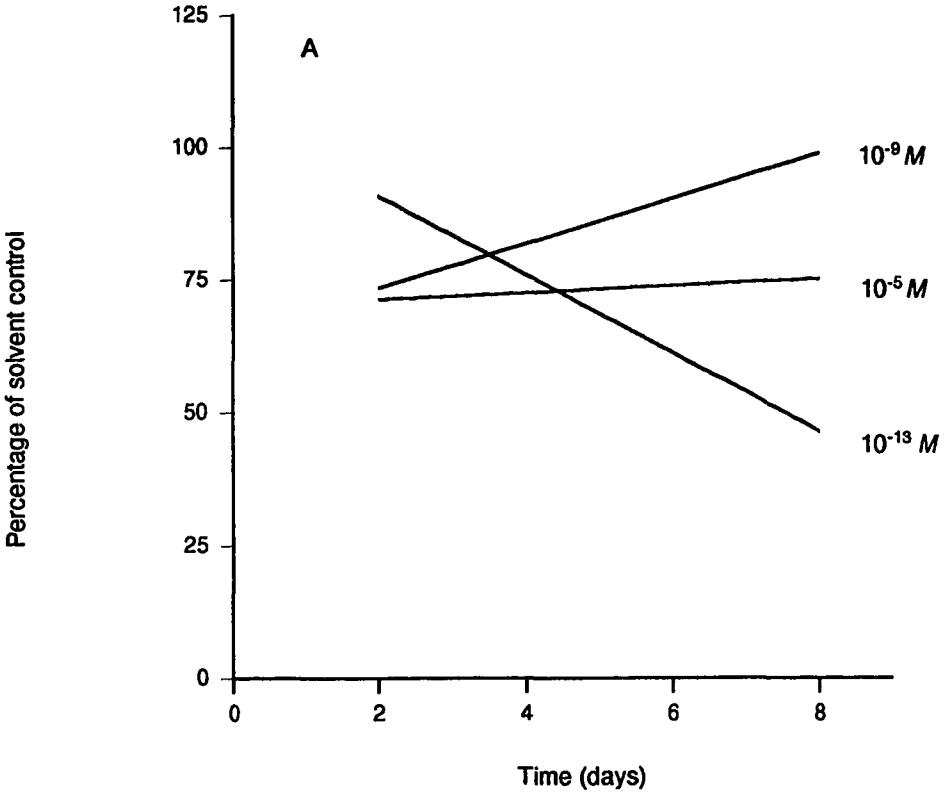

B
200
100
0
Percentage of the solvent control
**
*
*
*
*
10-5
10-7
10-9
10-11
10-13
Molar concentration of naphthalene

Figure 35: A. Regression analysis of DNA synthesis, as measured by the uptake of ^{3}H-thymidine in disintegrations per minute (DPM), in fish lens epithelium exposed to varying levels of benzo[a]pyrene, B. DNA synthesis on day 8 indicating the suppression/stimulation of ^{3}H-thymidine uptake in spot lens epithelium by varying levels of benzo[a]pyrene exposure.


A
125
100
75
50
25
0
0
2
4
6
8
Percentage of solvent control
Time (days)
10^{-9} M
10^{-5} M
10^{-13} M

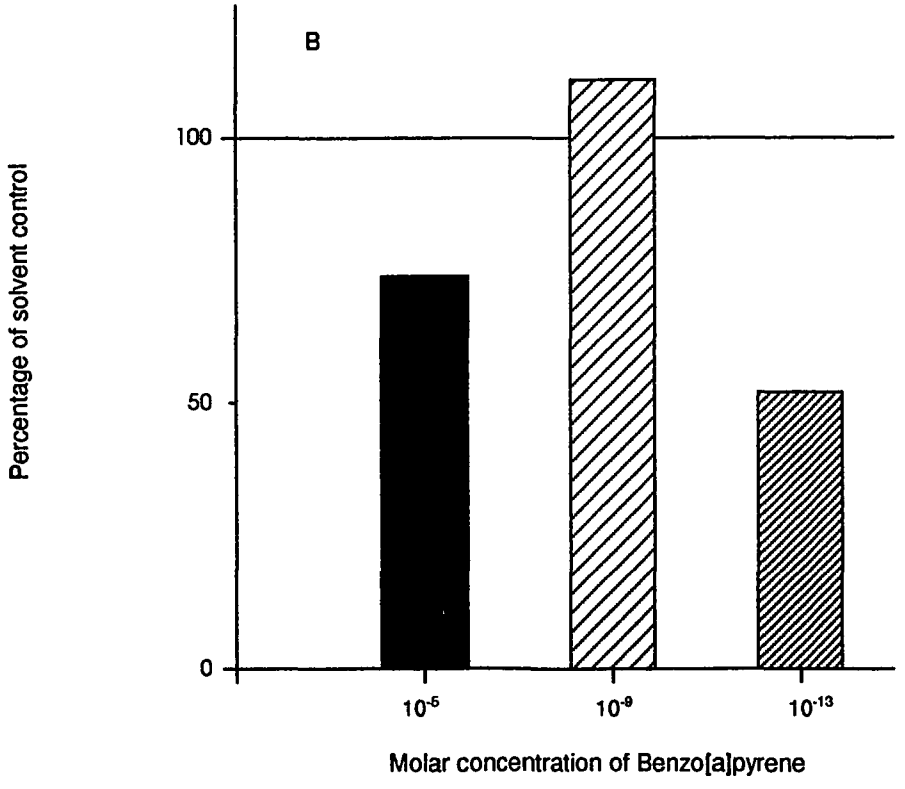
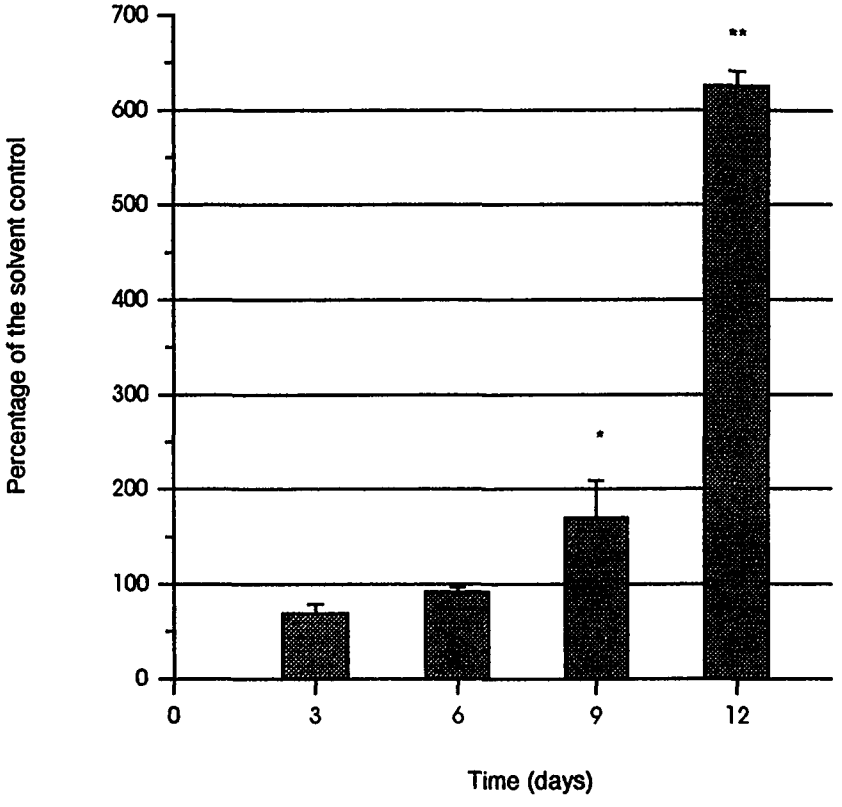

B
100
50
0
10^{-5}
10^{-9}
10^{-13}
Percentage of solvent control
Molar concentration of Benzo[a]pyrene

Figure 36: DNA synthesis over time indicating the suppression/stimulation of ^{3}H-thymidine uptake in spot lens epithelium by exposure to benzo[a]pyrene at a concentration level of 1×10^{-8} *M*. (* significant difference at $\alpha = 0.05$, ** significant difference at $\alpha = 0.01$).


700
600
500
400
300
200
100
0
Percentage of the solvent control
**
*
0
3
6
9
12
Time (days)

Discussion

Techniques employing radiolabelled precursors have been widely utilized to study the *in vitro* synthesis of cellular macromolecules such as DNA, RNA, and protein to determine cell replication and cellular growth potential (Babich et al., 1986; Seglen, 1976; Shopsis, 1984; Zakharova and Wallace, 1986; Marion and Denizeau, 1983a; Marion and Denizeau, 1983b).

The reduction in uptake of ^{3}H-thymidine during exposure to a reported cataractogenic PAH, indicates the possible inhibitory potential of naphthalene and its daughter compounds on the cellular replicative cycle of lens epithelium. Xu et al. (1992) exposing rat lenses to different metabolites of naphthalene, suggested that naphthalene dihydrodiol may be the actual cataractogenic agent as it was the only compound that adequately simulated *in vivo* cataract formation. A previous report by Hawkes (1977) indicated that the fish lens possesses enzymes capable of metabolizing naphthalene into daughter compounds. Previous work by Hargis et al. (1984) indicated that the water-soluble fraction of ER sediments could cause cataract formation in exposed spot. Whether or not naphthalene or its metabolites are involved in cataractogenesis in spot is still unknown.

The stimulation of ^{3}H-thymidine uptake by exposure to

moderate levels of BaP may indicate the importance of these compounds, or high molecular weight PAH in general, in the cell cycle of lens epithelial cells. The stimulatory response observed here is interesting as it parallels that observed in lens cells harvested from cataract lenses. It is well known that high molecular weight PAH, such as BaP that is a common component of ER sediments, are metabolized by the monooxygenase system to yield water soluble products, some of which are considered to be potential carcinogens (Varanasi et al., 1986; Babich et al., 1987, 1988; Dunn et al., 1987). Such chemicals play an important role in the deregulation of cell growth characteristic of carcinogenesis and therefore may assist in heightened DNA synthesis during cataract development as well.

This study investigated two representative xenobiotics that are commonly found in relatively high concentrations in ER sediments and attempted to analyze their influence on the replicative potential of lens epithelium under *in vitro* conditions. The degree to which the results of this study correlate with the *in vivo* toxicity of PAH on the spot lens as well as cataract induction needs to be further evaluated.

Chapter 8

A SUMMARY AND SYNTHESIS

Major Findings have indicated the following:

1. Eye disorders, as a general disease classification, occurred frequently and significantly more often at stations in the ER than at reference stations;

2. Of the eye disorders reported, cataract was by far the most predominant, was observed to be highly prevalent, did not appear to be associated with the presence of lens parasites, changes in environmental salinity or mean condition factors of sampled fish but was positively associated with sediment PAH levels. Cataract formation was observed to occur preferentially in summer months, and was associated with changes in the normal growth rate of lenses;

3. In general, lenses with cataract formation exhibited various degrees (0-100% of the lens) and intensities of opacification. Minor lens opacification (< 20%, stage 1 and 2) usually was observed at the visible center of the lens, while the last region to become opacified appeared to be the outer most section of the lens (epithelial layer);

4. Lenses that exhibited stage 1 cataract development (< 10%) displayed significantly elevated water, cholesterol, and free fatty acid levels (as compared to normal lenses), significantly reduced total ester (assumed to be cholesterol esters) concentrations, while the protein composition was apparently not significantly altered (although certain indications suggested levels of γ-crystallin may be reduced);

5. The *in vitro* growth of spot lens epithelium was possible and the culture of such cells could be maintained for long periods of time (> 55 months). These cultures produced α-, ß-, and γ-crystallins (indicative of lens epithelial cell differentiation);

6. The uptake of ^{3}H-thymidine (DNA synthesis) and -uridine (RNA synthesis) by lens cells was positively associated with stage of cataract development while the uptake of ^{3}H-leucine (protein synthesis) was unaffected.

Implications for the Mechanism Involved in Cataract Formation

Determination of the mechanism involved in cataract formation is complicated and hard to elucidate. Numerous types of cataract formation in mammals has been investigated and yet the mechanism involved in most is still unclear. One of the few known mechanisms is the induction of cataract in the Nakano mouse which is caused by a genetic inhibition of the Na^+,K^+-ATPase regulatory enzyme. The objective of this dissertation was to initiate a research direction in which quantitative data could be used to investigate what processes may be important during cataractogenesis. Actual determination of the cellular, biochemical, or molecular events that lead to fish cataractogenesis will require continued research and the induction of cataract *in vivo* under control conditions.

Data collected from sciaenid fish species that exhibited cataract formation suggested that cataract occurs and is initiated at sites specific to the ER environment. This would indicate that certain environmental parameters specific to this area are probably the major inducers of cataract. Previous research (Hargis and Colvocoresses, 1986; Huggett et al, 1987) centered around cataract formation in the ER, as compared to a single near-by reference station, but did not address the question as to

whether or not fish might have entered the ER with cataract formation. In the present study, the incorporation of several widely distributed sampling stations at points along the migration pathway of incoming sciaenids, in conjunction with year-round sampling, provided sufficient data to suggest that such an occurrence is unlikely.

Upon entering the ER environment, sciaenid fish become exposed to environmental contamination. Although the ER system is undoubtedly contaminated with a number of chemicals that have the potential to be detrimental to the well-being of fish (petroleum products, polychlorinated biphenyls, tributyltin, and heavy metals; VIMS, 1992), the sediments of the ER are characterized by a high degree of PAH contamination. The lack of association in the prevalence of cataract formation with other environmental parameters previously reported to induce cataract formation in fish, coupled with a positive relationship between sediment levels of PAH and cataract formation, supports the hypothesis that PAH may be a key component involved in cataract induction. Furthermore, the reported PAH composition in sediments from the ER includes a previously documented cataractogenic substance, namely naphthalene.

Naphthalene induced cataract in rats has been promoted via forced dietary intake, is initiated within the nuclear region of the lens, and has been strongly linked to oxidative mechanisms. GSH levels were observed to be

decreased as cataract formation occurred, while the activity of Na^+,K^+-ATPase was unaffected (Xu et al., 1992). In general, GSH levels are consistently found to be decreased in lenses with opacification (Rathbun et al., 1993). Minor cataract development (stage 1) in spot lenses, however, was not observed to have significantly altered GSH levels. This information may indicate the lack of involvement of GSH-associated oxidative mechanisms.

The effects of exposure to the ER environment appears to be positively associated with an increase in water temperatures. This relationship is not surprising as the metabolism of PAH, the solubilities of PAH, and the overall metabolism of fish are all significantly influenced by ambient temperatures. Furthermore, the general susceptibility, especially corneal lesions, of the ocular region to environmental influence suggest that transport of sediment-borne or water-soluble chemicals directly through the cornea may be an important route of uptake. Even in a healthy state the cornea, an extremely hydrated tissue, is probably not much of a barrier to the diffusion of water-soluble chemicals. This is not to say that dietary intake and uptake via the gills are not the major routes of exposure. It just suggests that exposure via corneal passage may significantly augment, especially at certain times of the year, exposure conditions.

Lens opacification first appears at or near the center

of the lens and progresses peripherally. As previously mentioned, cataract induction in rats via naphthalene exposure also occurs as a nuclear opacification (Xu et al., 1992). Initiation of opacification, however, does not necessarily mean that the critical event(s) leading to cataract formation occurs in this region. For instance, if the epithelial layer or the cortical layers of the lens were to become disturbed this may lead to opacification in the nuclear region. The nuclear region depends on the epithelial layer for osmotic regulation. In fact, the percent composition of γ-crystallins, the dominant structural protein in the central region of the lens, was not observed to be significantly altered. Fraser et al. (1989) suggested that cataract formation in salmon (Salmo salar) from the West Coast of Scotland, associated with the exposure of fish to an organophosphate-based pesticide, might be linked to the uncoupling of lens fiber cells through an increase in gap junctional resistance and altered membrane characteristics.

On the other hand, cholesterol levels as well as the water content in the lens, were observed to be elevated in spot with minor cataract development (stage 1). These data suggest that changes in the composition of membranes may lead to the increased permeability of lens cells. Increased permeability could create problems in osmotic regulation and could lead to leaky membranes. Hargis and Zwerner (1988)

indicated through histological examination that cataract lenses of spot had fragmented fiber cells which probably involved disrupted membranes. Furthermore, Cotlier (1987) in his review on the lens and cataract formation in mammals clearly showed the strict relationship between lens transparency and proper osmotic regulation.

The epithelial layer is the only mitotically active layer within the lens. *In vitro* investigations indicated that the epithelial layer shows significantly elevated levels of mitotic indices during cataract formation. Previous work by Hargis and Zwerner (1988) showed that hyperplasia of the epithelial layer was associated with cataract formation. Unfortunately, the reason for such a stimulation is presently unknown. However, if osmotic regulation of epithelial cells were to become compromised, cells could become more fragile and the potential for lysis would be increased. The lens has been shown to contain certain growth factors, for example fibroblast growth factors, that are mitogenic to lens epithelial cells (McAvoy and Chamberlain, 1990). If cells were to lyse or become susceptible to leakage these factors would be released and would induce increased cellular division. Furthermore, the neural retina also contains significant concentrations of growth factors. Previous research (Hargis and Zwerner, 1988) has indicated that the retina is disrupted in fish with cataract lenses. Presumably, cellular mortality and

lysis occurs. This retinal break-down would flood the vitreous with numerous products, including growth factors, and might create an environment suitable for increased cell mitosis. Whether or not mitogenic epithelial cells are stimulated to divide at a greater rate or whether previously mitotically passive epithelial cells are activated to divide is presently unclear. Non-mitotic epithelial cells are responsible for the osmotic regulation of internal fiber cells. If these cells were stimulated to divide than they might subsequently lose their ability to transport compounds to and from the nuclear region. This uncoupling would make it very hard for the internal region to maintain its proper physicochemical balance and such a disturbance might lead to nuclear opacification.

The successful culture of spot lens epithelium, both primary and long-term, has indicated that *in vitro* investigations are feasible and that cells adequately parallel *in vivo* development. The establishment of such a model, used in conjunction with *in vivo* exposure studies, allows the mechanism of cataract formation to be studied at the cellular and molecular level.

In conclusion, although many important processes have yet to be determined, certain important parameters have been associated with cataract formation, as well as initiation, in sciaenid fish species sampled from the ER. In general, the proposed mechanism is that juvenile sciaenids enter the

Chesapeake Bay and make their way into the James River and ultimately into the Elizabeth River system. Fish are exposed to environmental conditions specific to this region. As the water temperature rises, lenses begin to exhibit altered membrane compositions that may cause an interference in the ability of cells to osmotically regulate their intracellular environment. Cells may become more rigid and susceptible to osmotic shock. Damaged (possibly peroxidation of membrane lipids) or lysed cells from either the lens or the neural retina then release growth factors that stimulate mitosis of the lens epithelium. Once membrane composition is compromised, intercellular communication is inhibited. Epithelial cells responsible for the osmotic regulation of the nuclear positioned fiber cells break down and opacification occurs as a result of osmotic stress. Cells at the interior of the lens (nuclear region) are harder to regulate than those that are positioned closer to the epithelial layer. Over time, osmotic stress builds and cataract formation progresses outwardly. As the degree of opacification increases, the lens becomes more hydrated as a consequence of its reduced ability to maintain osmotic control. Cataract eventually progresses to a stage where fish can become totally blind.

The ecological importance of cataract formation has yet to be studied. The estuarine environment is one that is characterized by high turbidity and therefore the reduction

in visual potential may be of little immediate consequence, especially during early development. As cataract formation becomes more extensive, however, vision is undoubtedly impaired and may lead to a competitive disadvantage when and if fish enter a more visual-associated environment. Reduction in body weight to length relationships indicates that complete opacification has some effect on growth (whether it be reduced foraging ability, increased metabolism, or some other unidentified interference). Data suggest that fish with stage 4 cataract development are lost (probably via death) from the system. The complete lack of sight must affect schooling and migratory behavior and more than likely negatively influences predator avoidance.

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