

## ABSTRACT

Title of Document: EYE GROWTH AND DIFFERENTIATION IN  
THE BLIND CAVEFISH *ASTYANAX*  
*MEXICANUS*: A STUDY IN THE  
EVOLUTION OF DEVELOPMENT

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2006

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Department of Biology

“Embryology is to me by far the strongest class of facts in favor of change of forms”

-Charles Darwin, in a letter to Asa Gray, September 10, 1860.

Modern biological studies often employ a synthesis of multiple fields to accomplish a unified research goal. For instance, evolution of development (evo-devo) answers questions concerning the emergence of unique organismal phenotypes resulting from changes in evolutionary and developmental forces. I am interested in studying these forces on a microevolutionary scale. To accomplish this, I use the teleost fish *Astyanax mexicanus*. This species, indigenous to Mexico, is comprised of two forms: a surface stream dwelling form (surface fish) and a cave dwelling form (cavefish). Cavefish, which are the evolutionary descendent of surface fish, have evolved a number of constructive and regressive features as a result of being exposed to the subterranean environment, including loss of functional eyes and melanin pigment. Thus, *Astyanax* is ideal for comparative studies on a microevolutionary scale.

I am interested in studying changes in eye development between surface fish and cavefish, and how this may relate to the evolution of the two forms. I initially utilized a comparative approach, using candidate gene, cell proliferation, and cell death studies. I extended these studies to include differential gene expression analyses as a means to better understand differences between surface fish and cavefish development. To further this understanding, I ultimately performed surface fish to cavefish lens transplants and surface fish lens deletions to study the effect of the lens on eye development. Finally, I integrate these data into a theory concerning eye development in *Astyanax* and put these developmental phenomena in the context of evolution.



EYE GROWTH AND DIFFERENTIATION IN THE BLIND CAVEFISH  
*ASTYANAX MEXICANUS*: A STUDY IN THE EVOLUTION OF DEVELOPMENT

By

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Dissertation submitted to the Faculty of the Graduate School of the  
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Doctor of Philosophy  
2006

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## Dedication

*This dissertation is dedicated to the first surface fish that thought it would be a good idea to swim into a cave.*

## Acknowledgements

I would like to thank, first and foremost, my graduate advisor Bill Jeffery, who has been the best advisor a graduate student could ask for. In Bill's lab I was given the opportunity to work with proper guidance, while at the same time given the opportunity to explore my scientific interests. This aspect of Bill's lab is of utmost importance to me. Never should a scientist feel they are not able to explore their curiosities, since questioning and exploration are fundamentals of what science should be based on.

Secondly, I would like to thank my committee members for taking the time to help further my education. I am appreciative of all the support I've received during my time here. I would also like to include Catherine Carr in this acknowledgement as an important contributor to my graduate education.

I also extend a thank you to Yoshiyuki Yamamoto with whom I shared many excellent scientific conversations. He was also the individual who taught me his unique embryological techniques in addition to the help he contributed to the work of the fourth chapter of this thesis.

I would next like to thank Rose Aurigemma who taught me all I know about molecular biology. Day in and day out she was patient with my questions, ultimately resulting in me reaching a level of confidence, allowing me to be able

to perform experimental molecular biology on my own. I only hope I may pass on knowledge as she has done for me.

Next I would like to thank Ernie Hixon, Meredith Yeager, and Daphne Soares, who are all great friends and have provided hours upon hours of excellent scientific argument and discussion. Additionally, I would like to thank the Jeffery lab and anyone who has worked in the lab since my arrival in 1997, including the extended lab family.

I am indebted to my parents who have given me much support and encouragement during my educational endeavors. Finally, I thank all my family and friends who have supported me throughout my time here. Everyone I know has contributed to this work at least in some small capacity, and I am grateful for that.

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## Chapter 1: Introduction

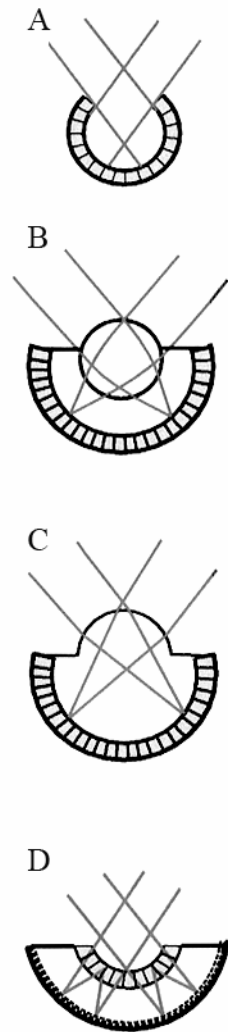
Darwin wrote in *The Origin of Species*: “The simplest organ which can be called an eye consists of an optic nerve, surrounded by pigment-cells and covered by translucent skin, but without any lens or other refractive body”. This defines the ‘prototype eye’ described by Walter Gehring. In contrast, Michael Land defines an eye as possessing multiple photoreceptor cells capable of producing an image opposed to simply detecting light intensities (Treisman, 2004).

Of the 33 extant metazoan phyla, six have evolved image forming eyes. These include *Cnidaria*, *Mollusca*, *Annelida*, *Onychophora*, *Arthropoda*, and *Chordata* (Fernald, 2004). There are eight general metazoan eye plans, which can be divided into two basic types (shown in Figure 1-1). These two types include simple eyes (also known as single chambered eyes) and compound eyes. While extensive diversity exists among different visual forms, there are features such as photoreceptor molecules common to all eye types. (Fernald, 2000). As a result, debate exists concerning the evolutionary origin(s) of eyes, similar to the aforementioned disagreement concerning the structural definition of an eye.

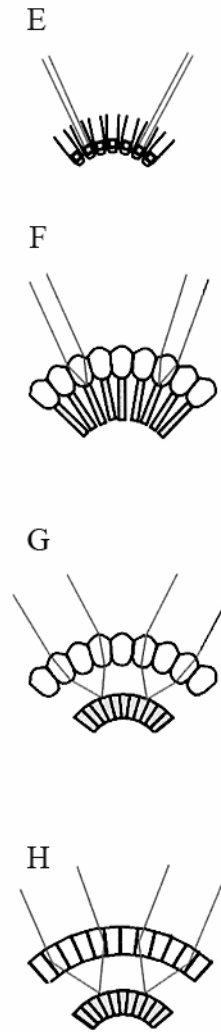
Based on several morphological characteristics, such as cell type, developmental tissue origin, and overall structure, among other things, Salvini-Plawen and Mayr concluded that eyes have independently evolved at least 40 separate times (Salvini-Plawen and Mayr, 1977). Alternatively, based on the importance of Pax6

Figure 1-1

## Single Chambered Eyes



## Compound Eyes



**Figure 1-1. Illustration of the basic types of metazoan visual systems.** A-D, Simple eye types. E-H, Compound eye types. A, simple pit eye, using only shadow for visual cues, found in platyhelminthes, annelids, and molluscs. B, Camera-type lens eye of aquatic animals such as fish and cephalopods. C, Camera-type lens eye utilizing corneal optics typical of land vertebrates. D, Concave mirror type eye typical of scallops and some crustaceans. E. Basic tube compound eye typical of ark clams, some tube worms, and starfish. F, Apposition compound eye of diurnal insects and crustaceans. G, Refracting superposition eye of nocturnal insects and some crustaceans. H, Reflecting superposition eye of decapod shrimp and lobsters. Light ray lines show how light is imaged onto the retina. (Adapted from Land, 2005).



in eye development across phyla, Walter Gehring suggests that all metazoan eyes resulted from a single prototype eye form which utilized *pax6* as a developmental master control gene (Gehring, 2004). Opposing this idea, a Pax6 homolog has been isolated from the sea urchin *Paracentrotus lividus*, which does not possess eyes (Czerny and Busslinger, 1995). As an alternative to these theories, portions of the eye such as photoreceptor cells may be monophyletic in origin, while other structures may be the result of novel evolutionary events.

The variety of eye types across phyla allows us to investigate evolution on several macroevolutionary levels, while the complexity of individual eye types provides us with opportunities to study the visual system on a microevolutionary scale. Variety and complexity, along with its uncertain evolutionary origins, make the eye an excellent model for evolution of development (evo-devo) studies.

I investigate changes in eye phenotype at the microevolutionary level by studying the teleost fish *Astyanax mexicanus* (common name, Mexican tetra). *Astyanax mexicanus* is well suited for microevolutionary studies since the species consists of two phenotypically different forms: an eyed and pigmented form which lives in surface streams (surface fish), and an eyeless, albino form which lives in caves (cavefish) (Figure 1-2). Surface fish were initially washed into caves and trapped, evolving into the cavefish descendants.

Figure 1-2



**Figure 1-2.** Adult forms of *Astyanax mexicanus*. The surface fish form is shown in the top panel of the figure, and the cavefish form is shown in the bottom panel.

Table 1-1

Feature	Change
Larval jaw	Constructive
Maxillary teeth	Constructive
Taste buds	Constructive
Cranial neuromasts	Constructive
Telencephalon	Constructive
Eyes	Regressive
Cornea	Absent
Iris	Absent
Anterior chamber	Absent
Lens	Degenerate
Posterior chamber	Absent
Neural retina	Small and distorted
RPE	Rudimentary
Sclera	Small
Optic nerve	Small degenerate
Optic Tectum	Regressive
Infraorbital Bones	Constructive
Pineal Gland	Regressive (partial)
Vertebrae	Regressive
Scales	Regressive
Pigmentation	Regressive
Egg size (Yolk)	Constructive
Fat content	Constructive
Metabolism	Regressive
Schooling behavior	Regressive
Aggressive behavior	Regressive
Circadian activity	Regressive

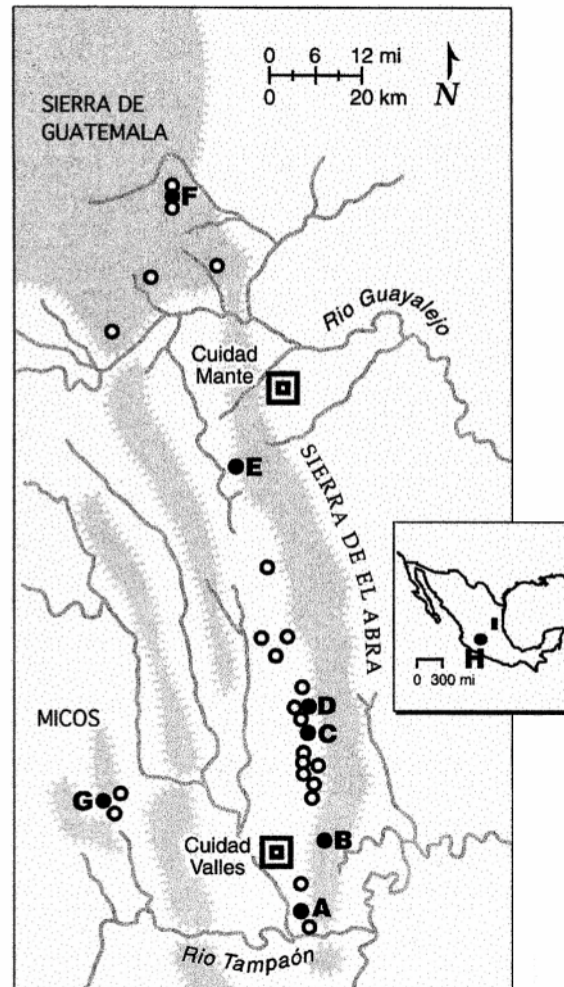
**Table 1-1. Phenotypic differences between surface fish and cavefish.** The first column lists the phenotypic change. The second column describes whether each corresponding change is constructive or regressive in cavefish when compared to surface fish. (Adapted from Jeffery, 2001).

There is debate as to when this event initially occurred, but estimates place it during the late Pleistocene (Avisé and Selander, 1972). Cavefish have subsequently evolved to possess both regressive and constructive traits (Table 1-1) (Jeffery, 2001)

Cavefish inhabit 30 separate caves in Mexico (see Figure 1-3). Of the 30 caves, 29 of the caves exist in an area surrounding the Sierra de El Abra, Sierra de Guatemala, and Micos regions of the states of Tamaulipas and San Luis Potosí in eastern central Mexico. The remaining cave, Guerrero, is in the state of Guerrero in south central Mexico (Jeffery, et. al., 2003). Images of surface fish and several cavefish are shown in Figure 1-4. La Cueva Chica, La Cueva de El Pachón, and La Cueva de Los Sabinos were the first three cavefish populations described. The fish in these caves were given the scientific names *Anoptichthys jordani*, *A. antrobius*, and *A. hubbsi* respectively (Hubbs and Innes, 1936; Alvarez, 1946; Alvarez, 1947). Currently, surface fish and cavefish are considered a single species under the name *Astyanax mexicanus*.

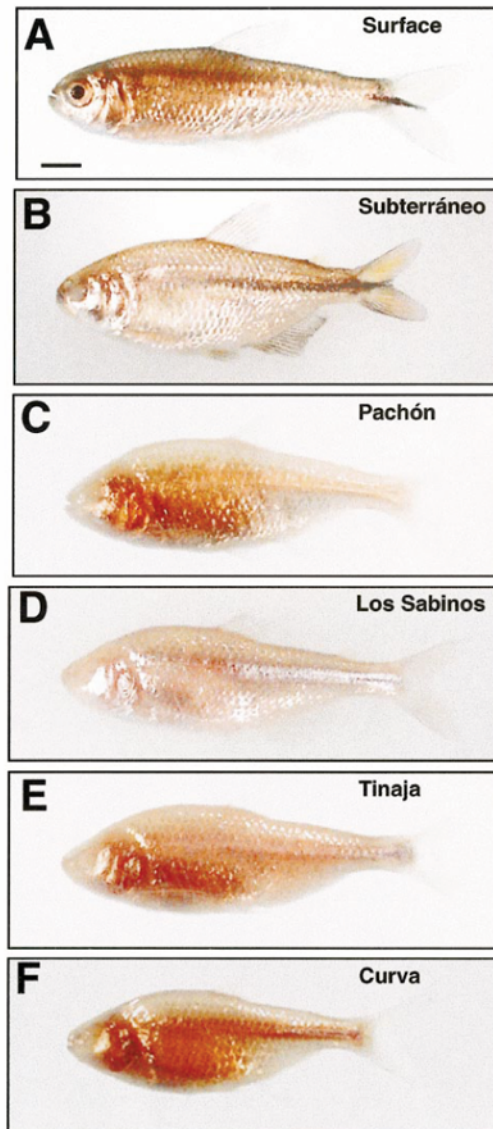
*Astyanax mexicanus* is an excellent developmental model (Jeffery, 2001). Like zebrafish, *Astyanax* is an oviparous species. Embryos can be obtained from natural spawning and in vitro fertilization. Individuals reach sexual maturity at the age of around eight months to one year. Figures 1-5 and 1-6 represent a timeline of *Astyanax* development. Surface fish and cavefish development occurs at similar rates. Adults are small, measuring approximately five to seven

Figure 1-3



**Figure 1-3. Map of caves harboring *Astyanax* cavefish populations.** A. Chica cavefish. B. Curva Cavefish. C. Tinaja Cavefish. D. Los Sabinos cavefish. E. Pachon cavefish. F. Molino cavefish. G. Subterraneo cavefish. The inset shows Mexico with the filled rectangle signifying the larger map, and H. showing the area of Guerrero cavefish. (From Jeffery, 2003).

Figure 1-4



**Figure 1-4. Surface and cave forms of *Astyanax mexicanus*.** A. Surface fish. B. Subterráneo cavefish. C. Pachon cavefish. D. Los Sabinos cavefish. E. Tinaja cavefish. F. Curva cavefish. (From Jeffery, 2003).

# Embryonic Stages of the Characin *Astyanax mexicanus*

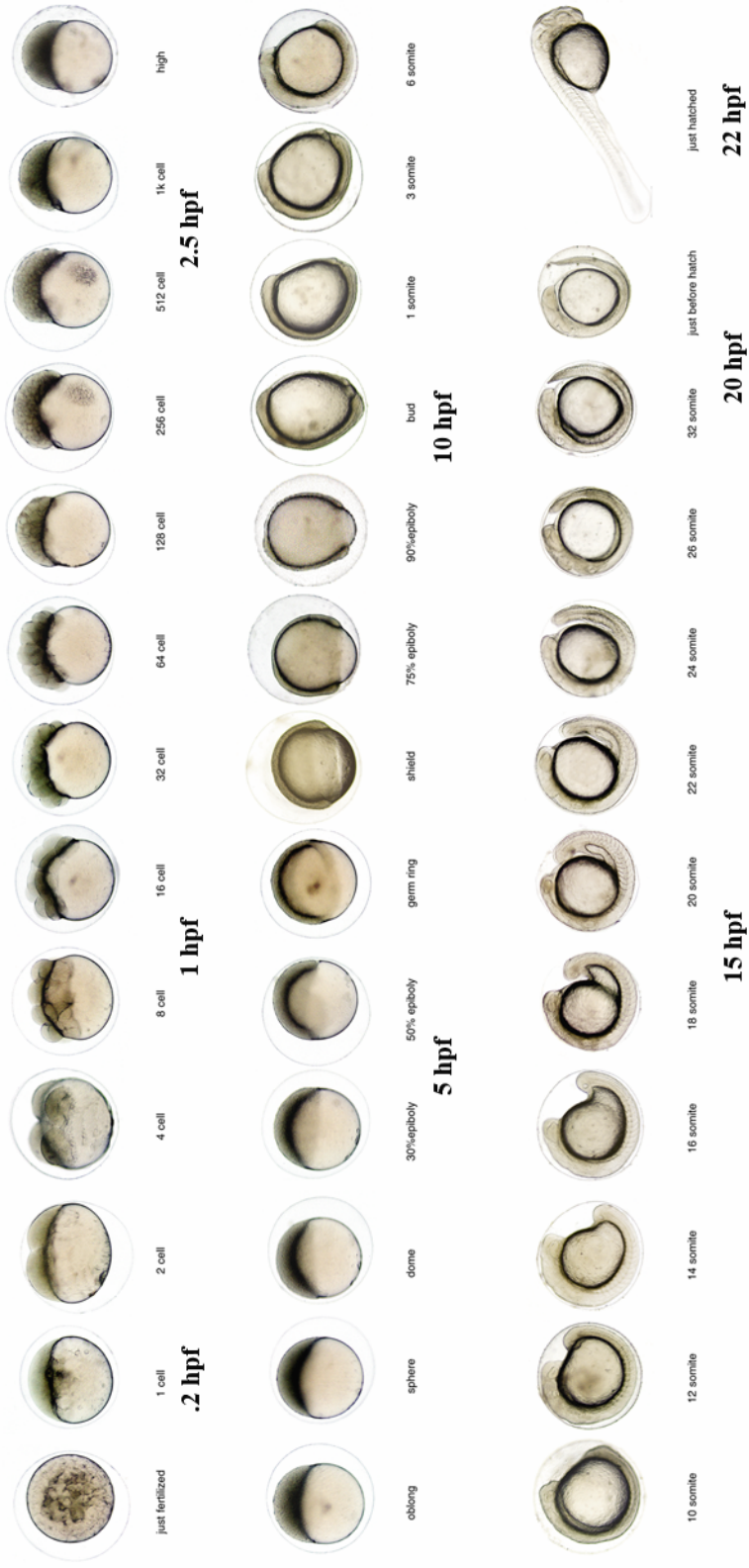


Figure 1-5



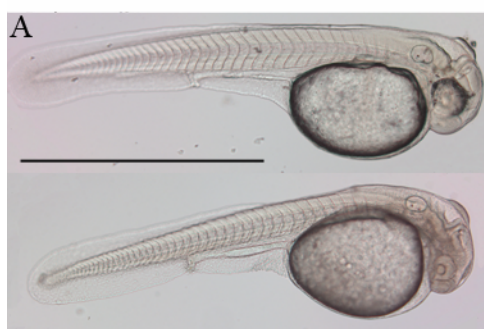
**Figure 1-5. *Astyanax* development from fertilization to hatching stage.**

Significant embryonic stages of *Astyanax* development are shown. Below each stage image is the title for that stage. A general timeline of development is indicated by the labels shown below their respective developmental stage.

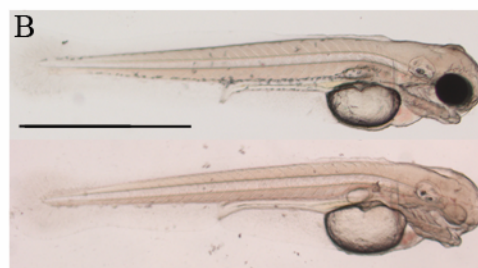
Abbreviation: hpf, hours post fertilization.



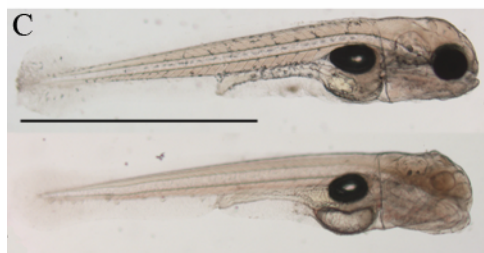
Figure 1-6



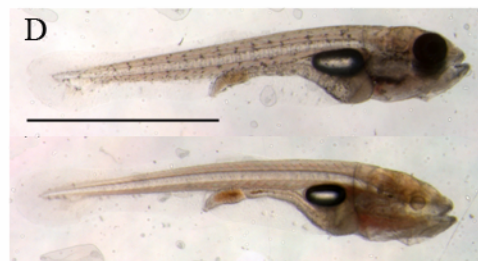
24 hpf



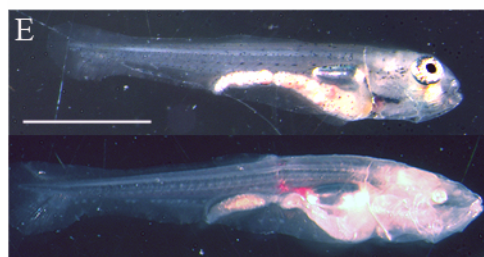
72 hpf



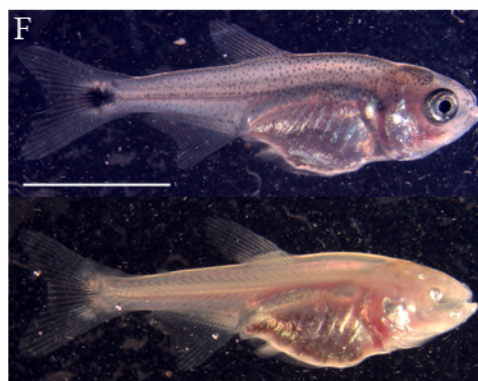
one wfp



two wfp



three wfp



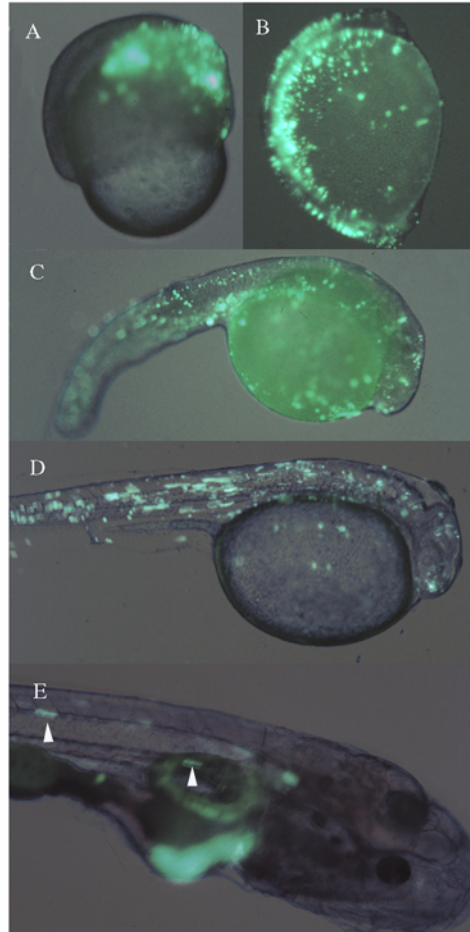
one mpf

**Figure 1-6. Older developmental stages of *Astyanax*.** A. 24 hours post fertilization larva. B. 72 hours post fertilization larva. C. One week post fertilization larva. D. Two week post fertilization larva. E. Three week post fertilization larva. F. One month post fertilization juvenile. Developmental stages post hatching are shown. For each time point, surface fish appear above, with cavefish below. Caudal is to the left and rostral is to the right. Abbreviations: hpf, hours post fertilization; wpf, weeks post fertilization; mpf, months post fertilization. Scale bar in A, 62.5mm; B, 125mm; C, 250mm; D, 250mm; E, 250mm; F, 500mm.

centimeters in length, and can be fed a commercial fish food diet. *Astyanax* embryos are transparent and develop rapidly in a manner similar to zebrafish. This allows the application of zebrafish experimental procedures to *Astyanax* with relative ease. For example, RNA, DNA, and morpholino microinjection procedures used in zebrafish are easily adapted to *Astyanax* developmental studies (see Figure 1-7; Yamamoto, et. al., 2004). Furthermore, genes cloned from zebrafish facilitate the design of degenerate oligonucleotides for use in *Astyanax* gene cloning. *Astyanax* genes cloned in the lab of William Jeffery are shown in Table 1-2.

Similar to debate surrounding visual system evolution, it is still unclear whether cavefish resulted from a single evolutionary event, or from multiple episodes resulting in evolutionary convergence. However, current data supports the second scenario. Initially, Avise and Selander (1972), using 17 allozyme loci, concluded that all cavefish descended from a common origin. Since this time, several researchers have suggested that cavefish resulted from multiple evolutionary events (Mitchell, et. al., 1977; Espinasa and Borowsky, 2001). Most recently, Dowling et al. (2002) and Strecker et al. (2003), using the mitochondrial genes NAD<sup>+</sup> dehydrogenase-2 (ND-2) and cytochrome b respectively, concluded that there are at least two separate cavefish origins.

Figure 1-7



**Figure 1-7. GFP DNA injections performed in Chica cavefish.** A. 70% epiboly. B. Tailbud stage. C. 20 hpf larva. D. 30 hpf larva. E. one week old larva. Approximately 300pl of 0.01pg/pl plasmid containing GFP linked to a CMZ promoter was injected into 1 to 8 cell stage embryos. These developing embryos and larvae were viewed using fluorescence microscopy to visualize cells expressing GFP. Arrowheads in E show these cells in the one week old larva.

Table 1-2

***Astyanax* Genes (136)****Structural Molecules (24)**

alpha-Actin	Type I Keratin, Type II Keratin
Vimentin	Major (lens) Intrinsic Protein (MIP)
Alpha1Type I Collagen	Cofilin, Profilin II
alphaA-Crystallin, alphaB-Crystallin	Semaphorin
beta-A1/A3-Crystallin	beta-B1-Crystallin
gamma S-Crystallin, gamma M-Crystallin	Lens intrinsic membrane protein (MP-19)
Rhodopsin, Green Opsin	Zinc transporter-like protein-2
Fibronectin	18S and 28S rRNA
Red opsin, blue opsin	

**Signaling Components (40)**

Nodal (Cyclops)	Sonic hedgehog (Shh)
Tiggy winkle hedgehog (Twhh)	Echidna hedgehog (Ehh)
Patched1, Patched 2	Frizzled b1
Wnt 8, Wnt 8.2, Wnt 8b, Wnt 2b,	Hsp 90 alpha, Hsp 90 beta, Hsp70
BMP2a, BMP2b, BMP 4, BMP 6, BMP7	FGF4, FGF8, FGF17, FGF 18, FGF10, FGF20
IGF1, IGF2	LEDGF, HDGF
Cmaf, Maf B, Maf G	Cyclin dependent kinase 8
Ryanodine receptor 1	Serotonin receptor 3
PDGF1	EAR-2 nuclear receptor subfamily 2
Rho8	RACK1
Chordin	

**Transcription Factors (55)**

Msx d, Msx e	Vsx2	GA TA3, GA TA5
Rx1	Otx1, Otx2, Otx3	Vax1
Optx2	Prox1	Foxa2
Pax2.1, Pax6.1, Pax9	Pitx2	TAF2b
Eya 1, Eya2, Eya3, Eya4	TFE3	Barx1, Barx2
Sox1, Sox 3, Sox 19, Sox 21, Sox 31	Eng-2	MyoD
Six 1, Six 2, Six 3, Six 4.1	Six4.3, Six 6, Six7	hx6, lhx7, HuG
Nkx2.1a, Nkx2.1b, Nkx2.2	Hoxa3, Hox3, Hoxd3, Hoxb1b	Krox2e
Goosecoid, Dkk1	Dlx1, Dlx2, Dlx3, Dlx4	Max4

**Others (18)**

Vasa	NADP7	HAX-1
Bax	Adenyl CAP	C3VS
C3VS protein (uveal autoantigen)	eF3-alpha, eF3-delta	CAD1
Prostaglandin endoperoxide synthetase-1	CSA-19	AANA T
NAD dehydrogenase 2	Carbonic anhydrase	cyclin 1
Complement Protein C3-H1	Tyrosinase	cytochrome B

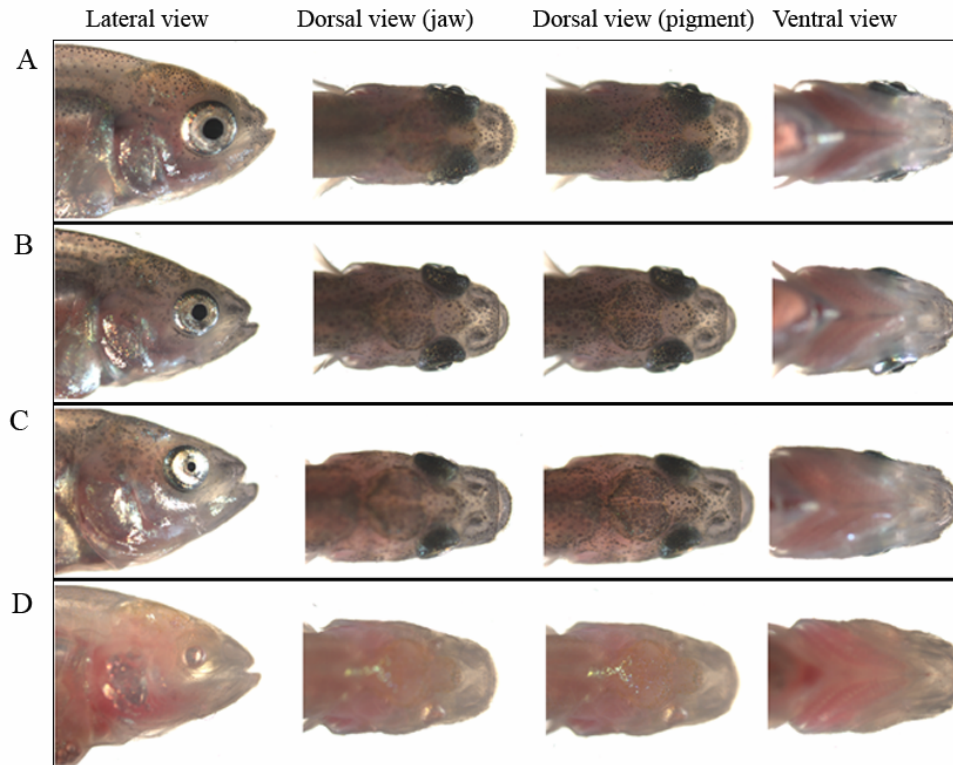
**Table 1-2. Genes that have been cloned from *Astyanax mexicanus*.** Genes are listed by family (Structural molecules, Signaling components, Transcription Factors, and Others). The number in parentheses beside each gene family represents the number of *Astyanax* genes cloned from that family, with a total of 136.

Surface and cave *Astyanax* are interfertile and crosses can be performed in both surface female  $\times$  cave male, and cave female  $\times$  surface male directions (Figure 1-9). This, along with synteny with the zebrafish genomic map, facilitates genetic experiments such as quantitative trait loci (QTL) (Borowsky and Wilkens, 2002; Protas, et. al., 2006). Furthermore, crosses between various cave populations can be performed. Some crosses result in F1 individuals with a degree of eye development greater than either parent cave population (Wilkens, 1971). This indicates that different eye genes are mutated in individual cavefish populations, further supporting that cavefish have evolved multiple times.

The cavefish eye provides a unique model for the study of regressive evolution. There are numerous examples of cave adapted organisms lacking sight and pigment (Deamer, 1964). It is commonly assumed these organisms are descendents of surface dwelling animals which may either be extant or extinct. Animals adapted to the lightless cave environment present an interesting evolutionary problem, since it is unclear whether their regressive traits are a result of neutral evolution or natural selection.

The neutral mutation theory (Kimura and Ohta, 1971) assumes selective pressure is relaxed on the cave animal eye phenotype. Thus, mutations are allowed to accumulate in genes controlling this character, eventually causing

Figure 1-8



**Figure 1-8. One month old surface fish, cavefish, and F1 crosses.** Row A, Surface fish. Row B, F1 from a female surface fish and male cavefish cross. Row C, F1 from a female cavefish and male surface fish cross. Row D, Cavefish. The columns represent a general lateral view, a dorsal view focusing on the jaw, a dorsal view focusing on the pigment of the head, and a ventral view.

degeneration or loss. In contrast, the theory of natural selection states that eye loss is advantageous (Poulson, 1963). There are several possible ways to explain eye degeneration under the natural selection theory. One theory states that eye formation is not needed by the animal because it is not energetically feasible (Poulson and White, 1969). Another argument assumes that the eye may serve as an entryway for parasites, subjecting it to negative selection pressure. Indirect selection through pleiotropy has also been proposed as a mechanism of regressive evolution (Barr, 1968). *Astyanax* is an excellent model species to investigate these evolutionary hypotheses since the derived cavefish form and ancestral surface fish form are both available for study.

I am interested in studying how developmental changes of the eye can provide us with information about *Astyanax* evolution. Unique ecology, evolutionary history, and developmental processes make *Astyanax* an excellent evo-devo model species. It is interesting from both a divergent evolution (surface fish versus cavefish) and a convergent evolution standpoint (cave versus cave). Not often are we offered an opportunity to study drastically different phenotypes on a microevolutionary scale, which *Astyanax* provides. By studying differences such as eye development between the two forms, we are able to answer questions about evolution in the context of development and phylogenetics. In the future, this information can be integrated with data from other organisms so we may better understand the processes of speciation and evolution relating not only to the evolution of eye development, but to the organism as a whole.



## Chapter 2: Larval Eye Growth and Development in the Blind Cavefish *Astyanax mexicanus*: a Comparative Study

### Abstract

Proper eye development is crucial for allowing proper visual perception. I am interested in normal and aberrant modes of eye development in the context of natural evolution and development. To study this, I work with the teleost *Astyanax mexicanus*. This species consists of an eyed (surface fish) and eyeless (cavefish) form. Both forms develop an embryonic eye. The surface fish eye continues to grow normally through larval and juvenile stages while the cavefish eye arrests in development and degenerates. I studied several molecular and cellular processes in an attempt to elucidate the causes of altered cavefish eye development. While expression of retinal genes such as *pax6*, *rx1*, and *vsx2* appeared to be relatively similar between surface fish and cavefish, lens genes encoding *MIP*, *MP19*, and the *crystallins* had altered expression patterns. Cell proliferation is preserved in the appropriate zones of the cavefish eye. However, I discovered that cells of the cavefish lens and retina undergo programmed cell death (PCD). I believe programmed cell death is responsible for altered gene expression in the lens of cavefish. Moreover, programmed cell death may be a major factor in the overall degeneration and lack of growth in the cavefish eye.

### **Introduction**

Eye development is an important process that involves the complex interaction of several cell and tissue types. The eyes of cold-blooded animals such as teleost fish and amphibians continue to grow throughout the life of the organism (Kubota, et al., 2002). This makes them especially interesting candidates to study ocular growth and development. Teleost eye development begins during neurulation as optic pits form from the anterior neural tube at the area of the presumptive forebrain (Sivak and Sivak, 2000). These pits continue to outpocket from the neural tube forming the optic vesicles. These structures remain attached to the presumptive brain via the optic stalk. As development progresses, the lateral wall of the optic vesicle invaginates in a medial direction forming the optic cup. As the optic cup is forming, the overlying surface ectoderm thickens to form a lens placode. These cells invaginate to form a lens vesicle. Eventually, this vesicle pinches off from the surface ectoderm to form the lens. At this point, the developing embryo possesses a rudimentary retina and lens.

During late embryonic and larval stages, the retina and lens begin to undergo cellular differentiation. The posterior cells of the lens elongate forming primary lens fibers, while the anterior cells form a proliferative epithelium. This epithelium will continue to form cells that will move to the back of the lens and differentiate into secondary lens fibers. During early larval stages, retinal cells begin to differentiate into their presumptive cell types. The outer layer of the retina will form the retinal pigment epithelium (RPE) and the inner layer will

form the neural retina (Graw, 1996). Parts of the optic cup will also form the iris and ciliary body. Additionally, neurons will begin to grow out of the retina forming the optic nerve. The neural retina will first form an inner neuroblastic layer and an outer neuroblastic layer (Tripathi, et al., 1991). These two layers will later become the differentiated layers of the retina. The lateral anterior edge of the retina most proximal to the lens will form the ciliary marginal zone (CMZ), which is comprised of cells that stay in a proliferative state and contribute to future growth of the retina. Eventually, the cornea forms from the overlying surface ectoderm. Additionally, mesoderm and neural crest cells contribute to the sclera, ocular muscles, and other supporting tissues of the eye (Tripathi, et al., 1991).

Several important processes that contribute to the development and growth of the eye have been discovered. Many important eye development genes have been cloned and described (Malicki, 2000; Ogino and Yasuda, 2000; Tripathi, et al., 1991; Jean, et al., 1998). These represent several gene families, including genes encoding transcription factors, signaling molecules, structural proteins, and proteins involved with cell physiology processes. Some of these genes are expressed solely in ocular structures and have unique temporal expression patterns.

Many genes specifically involved in lens development have been characterized, including transcription factors, signaling molecules and structural genes (Zygar, et

al., 1998; Lovicu and McAvoy, 2005). Of the structural genes identified, some of the most important are the crystallins. These genes are responsible for producing up to 90% of the protein that is present in the lens. Crystallin protein products are necessary for creating the specific refractive index of the lens responsible for promoting proper sight (Posner, et al., 1999; Andley, et al., 2000).

In addition to gene expression in the eye, cellular processes such as proliferation and programmed cell death (PCD) have been documented as important regulators of eye growth and development (Wilson, 1999; Jimeno, et al., 2003). These molecular and cellular process all work in concert to form the complicated structures of the eye, helping to enable the organism to perceive its environment via visual information.

I study eye development using *Astyanax mexicanus* as a comparative developmental model. *Astyanax mexicanus* is a teleost fish consisting of an eyed hypogean form (surface fish) and a blind epigean form (cavefish). During larval development the cavefish forms a morphologically normal lens and optic cup, albeit smaller than the surface fish. The surface fish eye continues to develop normally. In contrast, the cavefish eye appears to grow only slightly, eventually undergoing developmental arrest. In late larval and adult cavefish stages, only a small regressed eye vestige remains that is sunk into the orbit below a layer of fat and surface epithelium (Jeffery, 2001).

In this study, I was specifically interested in characterizing changes in eye development between the two forms of *Astyanax* to provide an initial understanding of how developmental alterations may affect the adult cavefish eye phenotype. I chose to focus on larval stage development since this is when the most severe arrests in eye development seem to occur in cavefish. I found that when comparing retinal genes between the two forms, all genes examined had similar patterns of expression. However, I found several differences in genes expressed in the lens. Using PCNA and BrdU experiments, I determined that the proliferative zones of the cavefish eye appear to be intact. However, I determined that there is widespread PCD in the cavefish lens and retina compared to surface fish. This PCD appears to be an important factor in the alteration of cavefish eye development.

## **Materials and Methods**

### **Biological Materials**

Surface fish were originally collected at Balmorhea State Park, TX, USA.

Cavefish were collected from Cueva de El Pachón, Tamaulipas, Mexico. Fish were kept in the laboratory on a 14 hour light and 10 hours of darkness photoperiod at 25°C and were allowed to spawn naturally. Embryos were collected and raised at 25°C.

### **Gene Cloning**

Briefly, genes were cloned by isolating RNA from developing *Astyanax mexicanus* surface fish using the RNeasy Maxi Kit (Qiagen Inc., Valencia, CA, USA) or the Oligotex Direct mRNA Midi/Maxi Kit (Qiagen Inc., Valencia, CA, USA). RNA was made into cDNA using the First Strand cDNA Synthesis Kit for RT-PCR (AMV) (Roche Applied Science, Indianapolis, IN, USA). PCR using degenerate primers was performed using the PCR Master Kit (Roche Applied Science, Indianapolis, IN, USA) under the following cycling conditions: one cycle for 2 min at 94°C, five cycles for 1 min at 94°C, 2 min at 35°C, and 3 min at 72°C, five cycles for 1 min at 94°C, 2 min at 45°C, and 3 min at 72°C, 20 cycles for 1 min at 94°C, 2 min at 55°C, and 3 min at 72°C, and one cycle for 10 min at 72°C.

*pax6* DNA was cloned according to Strickler et al. (2001). *Rx1* and *vsx2* were cloned according to Strickler et al. (2002). The sequences for *pax6*, *rx1*, and *vsx2* used in this study match previously cloned sequences. *MIP* DNA was cloned using MIP1 (5'-CCAGTRTAATACAWCCCAAAGAGGTG-3') and MIP2 (5'-AACCCWGCRGTCACCTTTTGCCTTCC-3') degenerate primers. *MP19* was cloned using LIM2 (5'-CCCGTGGAGATACTGCACGCC-3') and LIM2 (5'-CTGCGGGCTGTCCATGCATCC-3') degenerate primers. The primers used to clone  *$\alpha A$ -crystallin* were Alpha1 (5'-ATGGATATTGCCATCCAGCACC-3') and Alpha2 (5'-CTAACCGCCAAACCTACTGGTG-3'). The  *$\beta B$ -crystallin* and  *$\gamma M$ -crystallin* sequences were cloned according to Jeffery et al. (2000).

PCR products of the expected size were gel extracted and ligated into either the pPCR-Script AMP SK(+) vector (Stratagene, La Jolla, CA, USA) or the pSTBlue-1 vector (Novagen, Madison, WI, USA). Ligated vectors were transformed and putative positive colonies were grown in liquid culture. Vector DNA was extracted from these cultures using the QIAprep Spin Miniprep Kit (Qiagen Inc., Valencia, CA, USA) and sequenced at the DNA sequencing centers on the campuses of the Pennsylvania State University or the University of Maryland. The sequences were subjected to BLAST analysis to confirm the proper DNA fragments had been cloned. Relevant GenBank accession numbers are as follows: *pax6* AY651762, *rx1* AF264703, *vsx2* AY986759,  *$\beta B$ -crystallin* AF195949,  *$\gamma M$ -crystallin* AF195948.

## **Phylogenetic Analysis**

Sequence alignment was performed using ClustalX (Thompson et al., 1997).

Phylogenetic trees were constructed using the MEGA2 version 2.1 software (Kumar et al., 2001). Trees were constructed based on nucleotide sequence with distances calculated using the p-distance method. Trees were constructed using the neighbor joining method, with 1000 bootstrap replications used to support internal nodes.

## ***In situ* Hybridization**

Antisense and sense riboprobes were generated from cloned DNA sequences using the DIG RNA Labeling Kit (SP6/T7) (Roche Applied Science, Indianapolis, IN, USA). *In situ* hybridization was performed on samples fixed overnight in 4% paraformaldehyde in PBS according to the procedure described by Puschel et al. (1992). Samples were viewed as whole mounts or subsequently embedded in paraplast, cross sectioned at 8µm, mounted on subbed slides, and viewed using light microscopy.



### **PNCA Immunohistochemistry**

Samples were fixed, embedded in paraplast, and cross sectioned at 8µm. Sections were incubated with a polyclonal antibody to PCNA (Santa Cruz Biotechnology, Santa Cruz, CA, USA) according to the procedure used by Yamamoto and Jeffery (2000). Staining was visualized using DAB substrate. The sections were counterstained with hematoxylin, mounted, and viewed by light microscopy.

### **BrdU Pulse Chase Experiments**

*Astyanax mexicanus* larvae were incubated in a solution of 100µM BrdU from day 8 to day 10. A portion of the larvae were removed and fixed overnight in 4% paraformaldehyde in PBS (PFA in PBS). These samples comprised the BrdU pulse portion of the experiment. The remaining larvae were removed from the BrdU solution and put into fresh tank water containing no BrdU to develop further. These samples comprised the BrdU chase portion of the experiment. Some of these samples were allowed to develop from day 10 to 12 and fixed in PFA in PBS (pulse with chase 1) while the rest were allowed to develop from day 10 to 14 and fixed in PFA in PBS (pulse with chase 2).

BrdU incorporation was detected using a fluorescein linked antibody to BrdU (Roche Applied Science, Indianapolis, IN, USA). After BrdU incubation

experiments and fixation, samples were embedded in paraffin and cross sectioned at 10µm. These sections were dewaxed, rehydrated, and subjected to the BrdU detection procedure.

Slide glasses with sections were incubated twice for five minutes in PBSAT (PBS with 0.5% BSA and 0.1% Tween20). They were then treated with Trypsin solution (0.05% trypsin and 0.05% Calcium Chloride in PBS) for three min at room temperature. This was followed by a 15 min incubation in trypsin inhibitor (Roche Applied Science, Indianapolis, IN, USA). Samples were washed twice for five minutes in PBSAT followed by a 15 min treatment in 4M HCl at room temperature. This was followed by three washes in PBSAT, assuring that the pH of the final wash solution was 6.5 or higher after incubation. The sections were then covered with BrdU antibody solution (diluted to 50ug/ml in PBSAT) for one hour at 37°C in a humid chamber. After BrdU antibody incubation, sections were washed three times for five min in PBSAT and cover-slipped. Finally, samples were viewed and photographed using fluorescence microscopy.

### **Detection of PCD by TUNEL**

Larvae were raised until the appropriate time and fixed overnight in 4% paraformaldehyde in PBS. Samples after 10 dpf were embedded, cross sectioned at 8µm, and processed following the protocol included with the *In Situ* Cell Death

Detection, POD Kit (Roche Applied Science, Indianapolis, IN, USA). Samples to be viewed by fluorescence were only taken to the TUNEL labeling step, mounted, then viewed by fluorescence microscopy. Some samples were taken past this step and further processed using converter solution and DAB substrate to visualize PCD. These samples were subsequently mounted and viewed by light microscopy. Samples 10 dpf and younger were subjected to the TUNEL assay as whole mounts using TUNEL reaction mixture and converter solution from the In Situ Cell Death Detection, POD Kit (Roche Applied Science, Indianapolis, IN, USA). Samples were brought from 100% methanol to 100% PBS through graded washes, washed twice in PBS for 5 min, digested in 10 $\mu$ g/ml proteinase K in PBS for 2 to 20 minutes depending on age, washed in PBS, fixed in 4% paraformaldehyde in PBS for 20 min, washed twice in PBS for 5 min, incubated in a solution of 0.1% Triton X-100, 0.1% sodium citrate in PBS for 3 minutes on ice, washed three times in PBS for 5 min, incubated in TUNEL reaction mixture for 1 hr at 37°C, washed four times for 5 min in PBS, incubated in converter solution for 30 min at 37°C, washed four times for 5 min in PBS, incubated in DAB solution to visualize labeling for 2 to 5 minutes, and finally washed with PBS and fixed in 4% paraformaldehyde in PBS overnight. All steps were performed at room temperature unless indicated. After these samples were viewed by light microscopy, they were embedded, cross sectioned at 8 $\mu$ m, mounted, and viewed again in section by light microscopy.

**Immunohistochemistry using an antibody to  $\gamma$ -crystallin**

Samples were fixed overnight in 4% paraformaldehyde in PBS. After being subjected to the whole mount TUNEL assay previously described, samples were embedded, cross sectioned at 8 $\mu$ m, and subjected to immunohistochemistry using an antibody to  $\gamma$ -crystallin (gift of Dr. Robert Grainger, University of Virginia). The immunochemistry protocol used was a modified antibody staining procedure also provided by the laboratory of Dr. Robert Grainger. Briefly, sectioned samples were washed in PBS two times for 5 min, subjected to antigen denaturation for 15 min in 5M urea, blocked in 5% non-fat dry milk for 30 min, rinsed twice in PBS for 10 min, incubated in primary antibody (diluted 1:100 in 5% non-fat dry milk in PBS) for 45 min, rinsed in PBS for 10 min 2 times, incubated in secondary antibody (biotin-conjugated goat anti-rabbit diluted 1:60 in 5% non-fat dry milk in PBS) for 60 min, rinsed twice for 10 min in PBS, incubated in Avidin-TRITC (diluted 1:50 in 5% non-fat dry milk in PBS) for 60 min, and rinsed twice for 10 min in PBS. All procedures were performed at room temperature. Finally, samples were mounted and viewed using fluorescence microscopy.

## **Results**

### **Gene Expression in the Retina**

#### *Pax6*

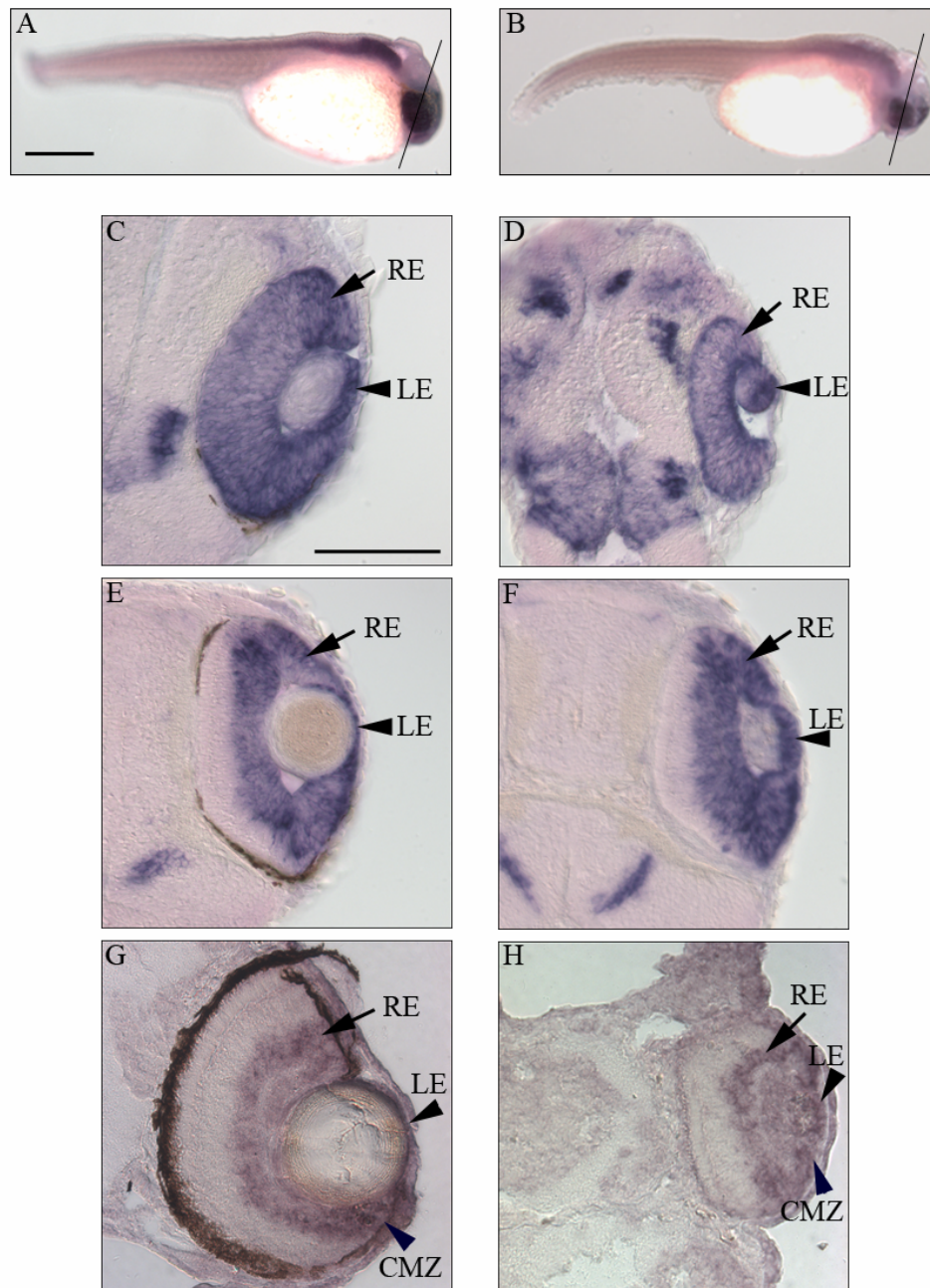
I investigated molecular differences that may be involved in *Astyanax* eye development using the homeobox gene *pax6*. I studied expression of *pax6* by *in situ hybridization* in early larval stages since this is when crucial eye developmental processes occur. *Pax6* is expressed at the 24 hour stage in both the developing eye and neural tube (Figure 2-1, A and B). I sectioned samples subjected to whole mount *in situ* hybridization to find out specifically where *pax6* transcripts are located in the retina. At the 24 hpf stage, *pax6* is expressed throughout the retina of both surface fish and cavefish (Figure 2-1, C and D). By 48 hpf, expression is restricted to the inner layer of the retina as well as the CMZ in both surface fish and cavefish (Figure 2-1, E and F). By 72 hpf, *pax6* transcripts still exist in the CMZ (Figure 2-1, G and H). However, the inner layer expression is now split into two distinct domains. The inner most layer closest to the lens represents developing ganglion cells. The layer outer to this represents expression in developing amacrine cells. This expression pattern is also similar between surface fish and cavefish. In addition to this *pax6* expression, there is documentation of *pax6* mRNA and protein expression in *Astyanax* at various other stages (Strickler, et al., 2001).

### *Rx1 and Vsx2*

I investigated gene expression in the CMZ by studying the expression patterns of the homeobox genes *rx1* and *vsx2*. *Rx1* transcripts are first detected in the optic vesicles of developing *Astyanax mexicanus* (Strickler, et al., 2002). I again used *in situ* hybridization to determine if *rx1* expression differences exist in surface fish and cavefish larval stages, when the CMZ becomes active. At 24 hpf, expression is seen throughout the retina of both surface fish and cavefish (Figure 2-2, A and B). By 48 hpf, the major area of *rx1* transcripts exists in the CMZ (Figure 2-2, C and D). By 72 hpf, expression continues to be seen in the CMZ of both surface fish and cavefish (Figure 2-2, E and F). Later in development, *rx1*, in addition to being expressed in the CMZ, is seen in the outer layer of the retina (Chuang, et al., 1999). This expression domain is seen in surface fish, but appears fragmented in cavefish (see Strickler, et al., 2002).

Like other vertebrates, *vsx2* transcripts are first detected in the developing optical vesicles of both surface fish and cavefish (Chen and Cepko, 2000; Strickler, et al., 2002). At 24 hpf, expression is seen throughout the retina (Figure 2-3, A and B). By 48 and 72 hpf, expression is restricted to the CMZ of both surface fish and cavefish (Figure 2-3, C-F). *Vsx* genes are reported to be expressed in the inner nuclear layer, presumably in bipolar cells (Liu, et al., 1994). Low levels of expression in the zone containing bipolar cells can sometimes be seen up to 96 hpf in both surface and cave *Astyanax* (Strickler, et al., 2002).

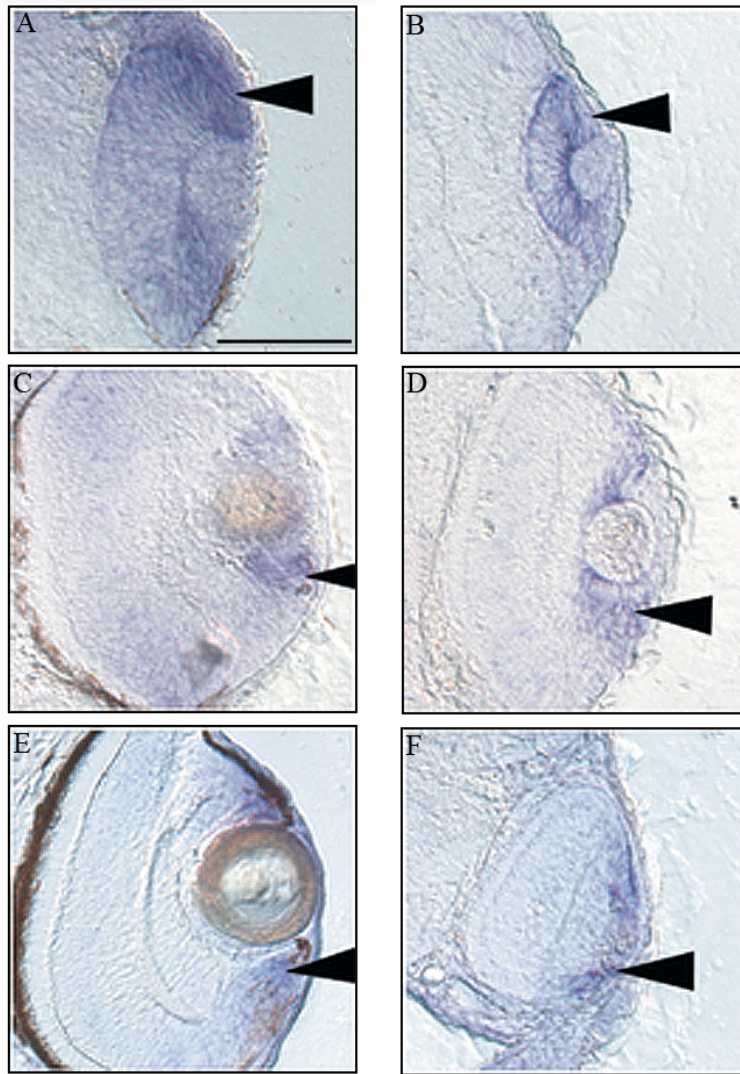
Figure 2-1



**Figure 2-1. Expression of *pax6* in early larvae of *Astyanax mexicanus*.** A, C, E G. Surface fish. B, D, F, H Cavefish. Larva were subjected to whole mount *in situ* hybridization at 24 hpf (A,B), in addition to the 48 hpf and 72 hpf stages. Larva subjected to whole mount *in situ* hybridization were subsequently cross sectioned at the 24 hpf (C,D), 48 hpf (E,F), and 72 hpf (G,H) stages. Lines in A and B through the eye of the whole mount larva indicate the plane of section. Abbreviations: LE, lens epithelium; RE, retina; CMZ, ciliary marginal zone. Scale bar in A, 200um; magnification is the same in A,B. Scale bar in C, 50um; magnification is the same in C-H.

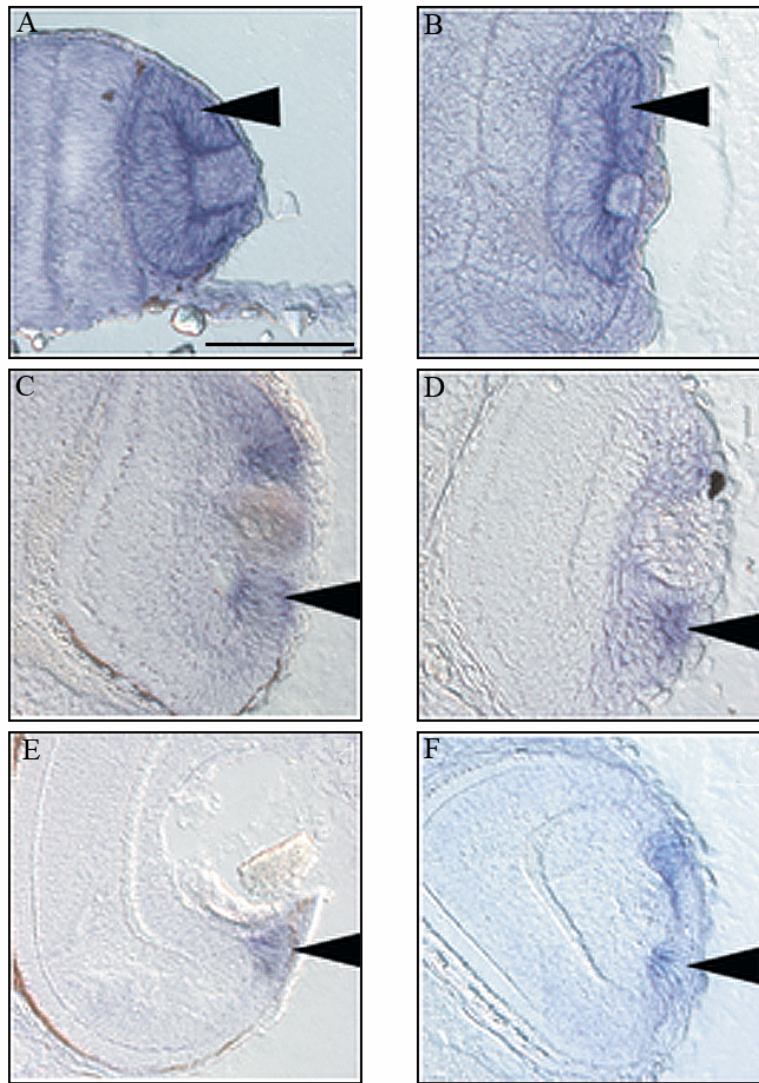


Figure 2-2



**Figure 2-2. Expression of *rx1* in early stage larvae of *Astyanax mexicanus*.** A, C, E Surface fish. B, D, F Cavefish. Larva were subjected to whole mount *in situ* hybridization and subsequently embedded and cross sectioned at the 24 hpf (A,B), 48 hpf (C,D), and 72 hpf (E,F) stages. Arrowheads indicate staining in the ciliary marginal zone of the retina. Scale bar in A, 50um; magnification is the same in A-F.

Figure 2-3



**Figure 2-3. Expression of *vsx2* in early stage larvae of *Astyanax mexicanus*.**

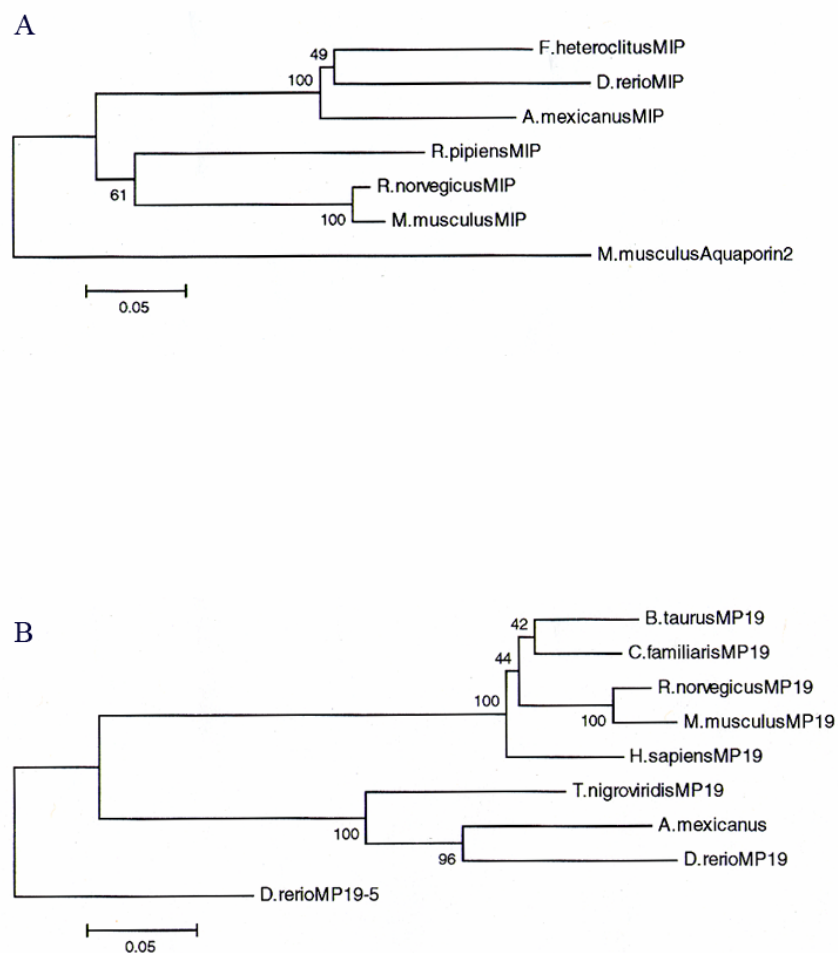
A, C, E Surface fish. B, D, F Cavefish. Larva were subjected to whole mount *in situ* hybridization and subsequently embedded and cross sectioned at the 24 hpf (A,B), 48 hpf (C,D), and 72 hpf (E,F) stages. Arrowheads indicate staining in the ciliary marginal zone of the retina. Scale bar in A, 50um; magnification is the same in A-F.

## Gene Expression in the Lens

### *MIP and MP19*

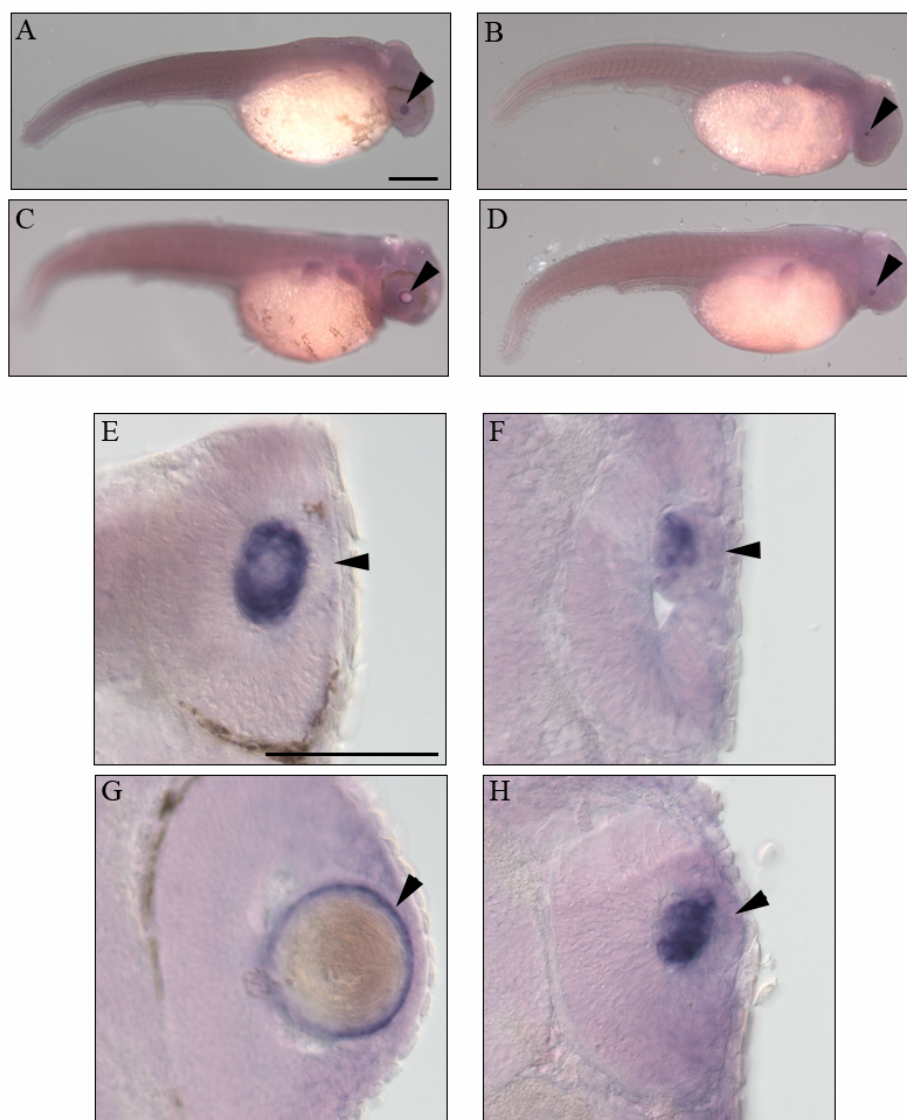
In order to elucidate the role of the lens in cavefish eye degeneration, I checked the expression pattern of several key lens genes. I first looked at the expression of the *MIP* and *MP19* genes. *MIP* and *MP19* are the first and second most abundant intermembrane lens proteins respectively and are expressed exclusively in the lens (Chepelinsky, et al., 1991; Church and Wang, et al., 1993). After cloning putative gene fragments, I performed a phylogenetic analysis on the resulting sequences to verify that I had cloned the true *Astyanax* *MIP* and *MP19* sequences. As seen in A of Figure 2-4, *Astyanax* *MIP* lies within a clade with zebrafish (*D.rerio*) and killifish (*F. heteroclitus*) *MIP* sequences. The *MP19* tree is shown in B of Figure 2-4. The *Astyanax* (*A.mexicanus*) sequence clusters with zebrafish (*D.rerio*) and pufferfish (*T. nigroviridis*). The other clade in the tree represents mammalian *MP19* sequences. For the *MIP* sequence, I obtained a bootstrap value of 100 supporting that the *Astyanax* sequence clusters with other fish *MIP* sequences. For *MP19*, I obtained a bootstrap value of 96, supporting that *Astyanax* sequence clusters with the zebrafish *MP19* sequence. These results support that I cloned the true *Astyanax* *MIP* and *MP19* genes.

Figure 2-4



**Figure 2-4. Phylogenetic trees of *MIP* and *MP19* nucleotide sequences constructed by the NJ method.** A, *MIP* sequences. B, *MP19* sequences. The branch lengths are proportional to phylogenetic distance. The scale bar represents and evolutionary distance of 0.05 nucleotide substitutions. Percentage values appearing at the nodes indicate bootstrap values for 1000 replicates.

Figure 2-5

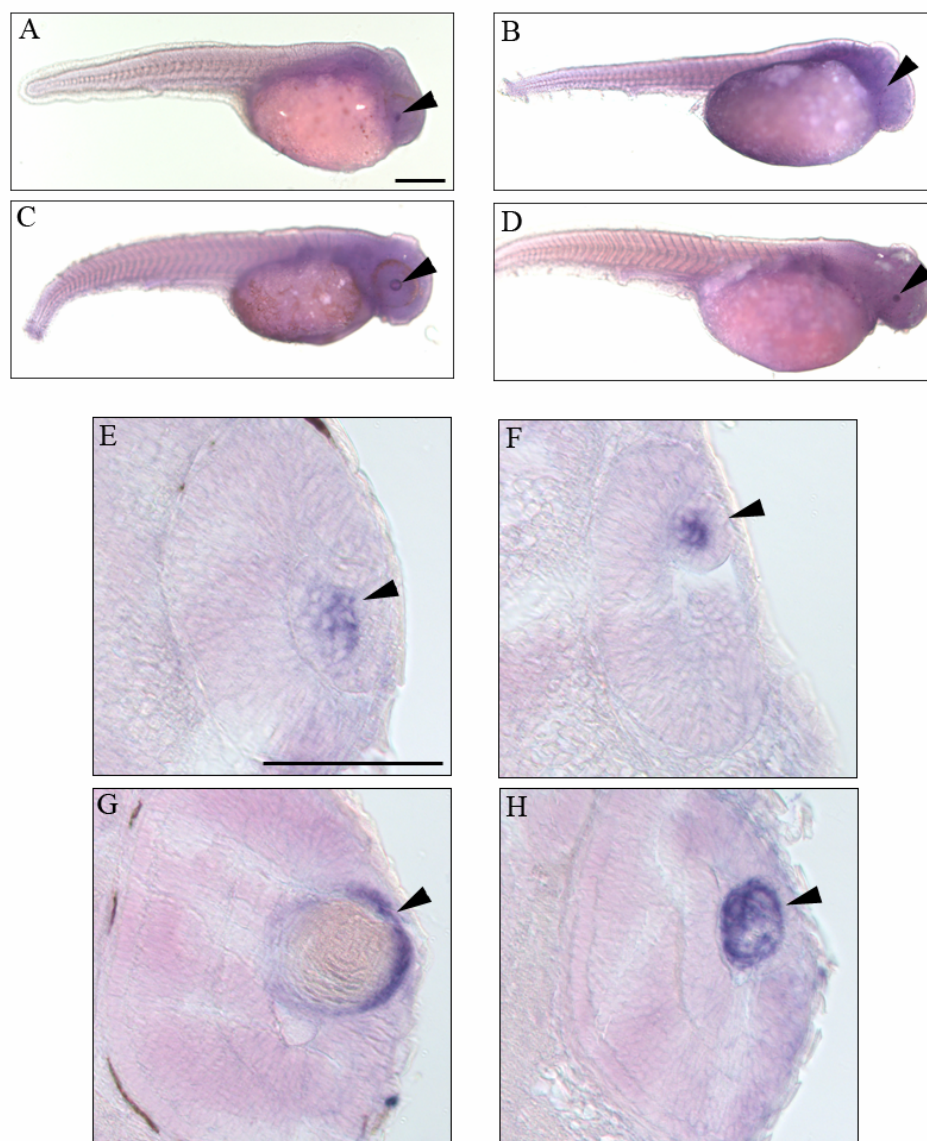


**Figure 2-5. Expression of *MIP* in early stage larvae of *Astyanax mexicanus*.**

A, C, E, G Surface fish. B, D, F, H Cavefish. Larva were subjected to whole mount *in situ* hybridization at 24 hpf (A,B) and 48 hpf (C,D) stages. Some of these samples were subsequently embedded and cross sectioned at 24 hpf (E,F) and 48 hpf (E,G) stages. Arrowheads indicate the lens. Scale bar in A, 200um; magnification is the same in A-D. Scale bar in E, 100um; magnification is the same in E-H.



Figure 2-6



**Figure 2-6. Expression of *MP19* in early stage larvae of *Astyanax mexicanus*.**

A, C, E, G Surface fish. B, D, F, H Cavefish. Larva were subjected to whole mount *in situ* hybridization at 24 hpf (A,B) and 48 hpf (C,D) stages. Some of these samples were subsequently embedded and cross sectioned at 24 hpf (E,F) and 48 hpf (E,G) stages. Arrowheads indicate the lens. Scale bar in A, 200um; magnification is the same in A-D. Scale bar in E, 100um; magnification is the same in E-H.



Transcripts of the *MIP* and *MP19* genes are seen in the surface fish and cavefish lens at 24 hpf and 48 hpf stages (Figure 2-5, A-D; Figure 2-6, A-D). I cross sectioned whole mount samples to determine more specific expression patterns. Both genes are found in the presumptive fiber cells of 24 hpf lenses (Figure 2-5, E and F; Figure 2-6, E and F). At this stage, there appears to be fewer *MIP* transcripts in the cavefish lens. However, since I used a probe produced from a surface fish *MIP* sequence, there may be a lower hybridization signal due to possible differences in sequence between surface fish and cavefish. Additionally, the area of staining is smaller in the cavefish lens for both genes. By 48 hpf, I see dramatic changes in the expression pattern of both genes when comparing surface fish to cavefish. At this stage, both *MIP* and *MP19* transcripts are found in a ring of cells surrounding already differentiated lens fibers in the surface fish (Figure 2-5, G; Figure 2-6 G). In cavefish, the expression pattern appears to be similar to the 24 hpf stage. However, the field of expression appears larger for both genes (Figure 2-5, H; Figure 2-6, H).

### *Pax6 and the Crystallins*

Pax6 is an important regulator of eye development. Therefore, I was interested in checking *pax6* expression in developing lens of *Astyanax mexicanus*. At the 24 hpf stage, *pax6* expression appears in the lens epithelium (Figure 2-1, C and D), similar to previously published studies in zebrafish (Macdonald and Wilson, 1997). This expression pattern continues throughout the 48 hpf and 72 hpf stages (Figure 2-1, E-H). The noticeable difference between surface fish and cavefish appears to be a response to lens morphology. In the surface fish, the lens epithelium becomes thin with squamous or cuboidal shaped cells, while cells of the cavefish lens epithelium retain a thicker columnar shape.

In addition to the *pax6* transcription factor and lens membrane proteins, I was interested in checking the expression patterns of crystallins, which make up the major water soluble protein component of the lens (Van Leen, 1987). The three basic groups of crystallins are  $\alpha$ ,  $\beta$ , and  $\gamma$ , with  $\beta$  and  $\gamma$  being more closely related than either are to  $\alpha$  (Wang, et al., 2004). I cloned putative crystallin sequences and verified that they corresponded to the proper gene of interest. *Astyanax*  $\alpha A$ -crystallin has been cloned previously, and my cloned sequence matched this sequence (Behrens, et al., 1998). I subjected the  $\beta$ - and  $\gamma$ -crystallin sequences to phylogenetic analysis as a verification technique (see Figure 2-7). The  $\gamma$ -crystallin sequence I used in these studies appears in the tree as *A.mexicanusG* and lies in a clade with other  $\gamma M$ -crystallin sequences. I cloned a second

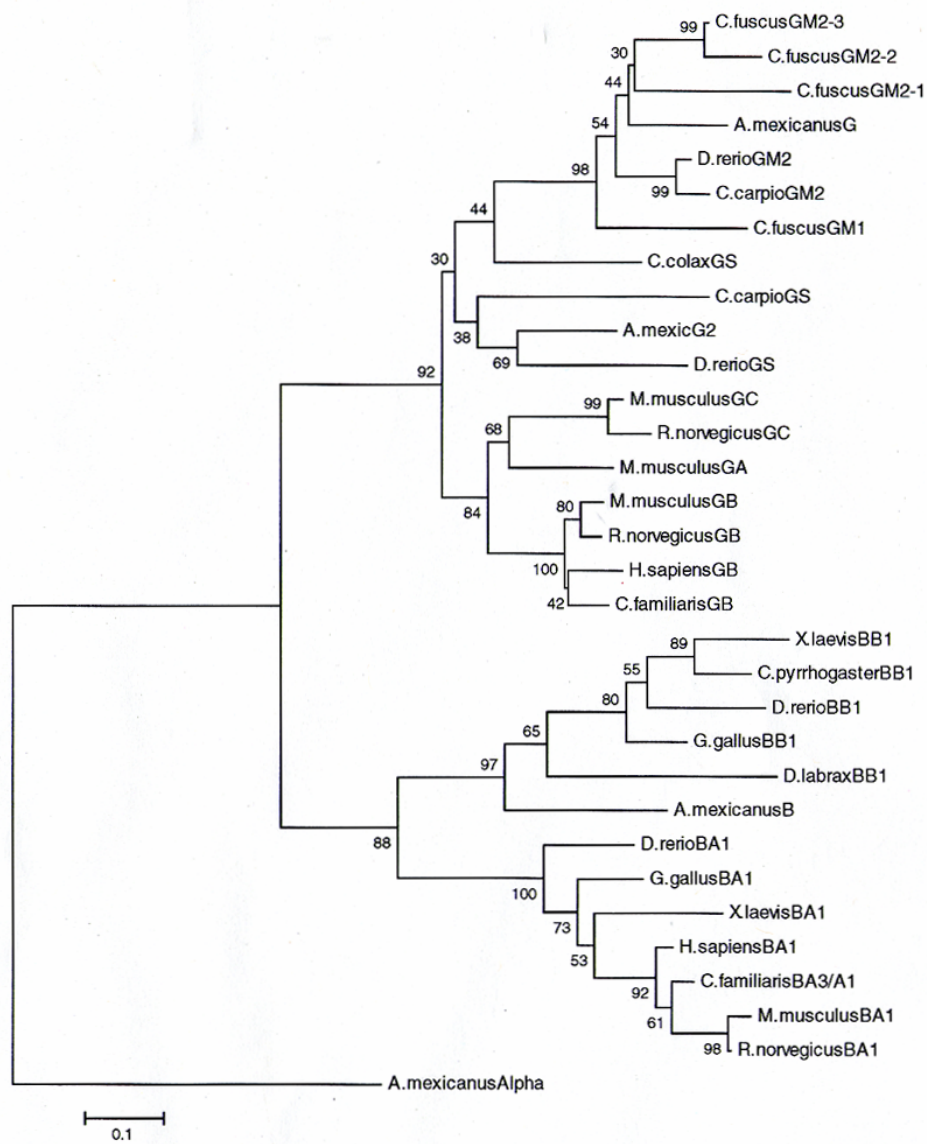
*Astyanax*  $\gamma$ -crystallin sequence not used in these studies, which lies in a clade with  $\gamma S$ -crystallins from other fish species. The *Astyanax*  $\beta$ -crystallin sequence (*A. mexicanus*B) lies in a clade with other  $\beta B$ -crystallin sequences, which group together separately from the  $\beta A$ -crystallin sequences. The  $\gamma M$ -crystallin lies within a clade of fish  $\gamma M$ -crystallin sequences supported by a bootstrap value of 98. The  $\beta B$ -crystallin sequence lies within a clade of other  $\beta B$ -crystallin sequences supported by a bootstrap value of 97. From this information, I conclude that I cloned *Astyanax*  $\gamma M$ -crystallin and  $\beta B$ -crystallin.

I first checked the expression pattern of  $\alpha A$ -crystallin in lens of developing larval stages of *Astyanax mexicanus*. Both  $\alpha A$ - and  $\alpha B$ -crystallin have been cloned in zebrafish and are expressed primarily in the lens (Posner, et al., 1999; Runkle, et al., 2002). It is thought that  $\alpha$ -crystallins serve both a structural and protective purpose, helping to produce a transparent lens and serving to prevent the accumulation of stress proteins, respectively (Dahlman, et al., 2005). At 24 hpf, there was no accumulation of transcripts in surface fish or cavefish (Figure 2-8, A and B). By 36 hpf, there is a ring of expression surrounding differentiated lens fibers in the surface fish (Figure 2-8, C). In some cavefish samples, there is an area of weak expression detectable in the central portion of the lens (Figure 2-8, D). This weak expression is seen in approximately one out of four 36 hpf samples (unpublished data). The ring of  $\alpha A$ -crystallin expression surrounding differentiated lens fibers continues in surface fish throughout the 48 hpf, 60 hpf,

and 72 hpf stages (Figure 2-8, E, G, I). In contrast, expression is absent in the cavefish lens after 36 hpf (Figure 2-8, F, H, J).

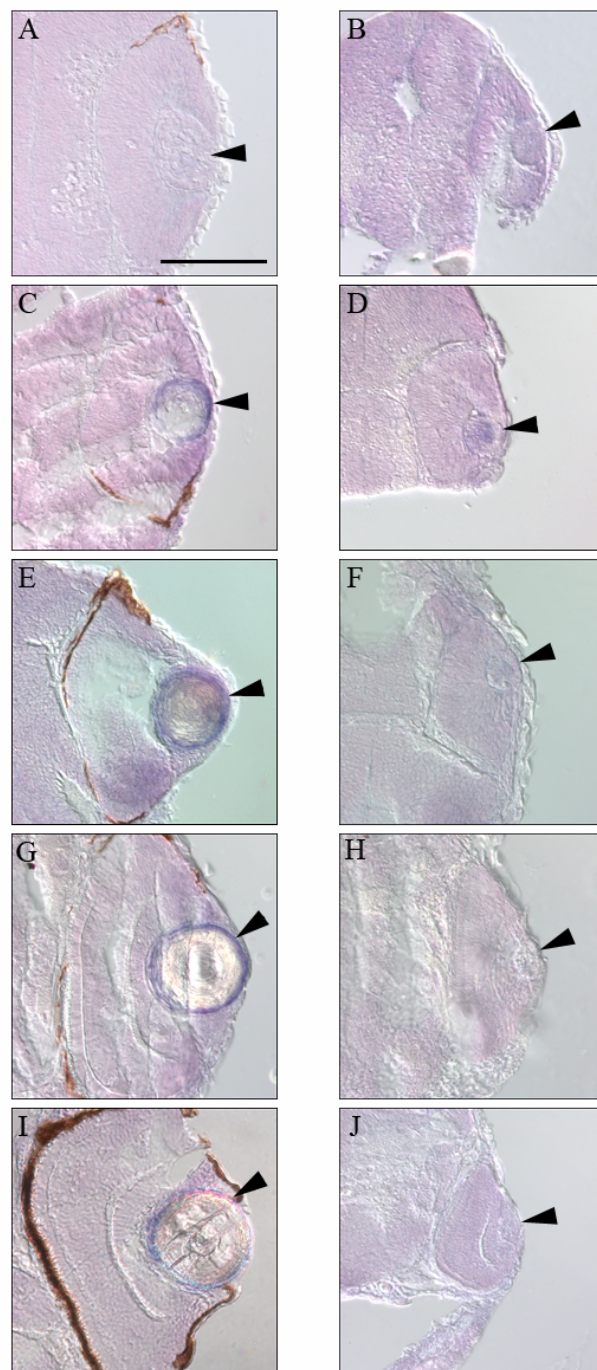
To further my studies on crystallin expression in *Astyanax mexicanus*, I checked the accumulation of  $\beta B$ - and  $\gamma M$ -crystallin transcripts in the lens. At the 24 hpf stage, both  $\beta B$ - and  $\gamma M$ -crystallins are expressed in the central and posterior regions of the lens (Figure 2-9, A and B; Figure 2-10, A and B). In the 36 hpf, 48 hpf, 60 hpf and 72 hpf surface fish lens, both  $\beta B$ - and  $\gamma M$ -crystallin are expressed in a ring of cells surrounding already differentiated lens fiber cells in a similar manner to  $\alpha A$ -crystallin (Figure 2-9, C, E, G, I; Figure 2-10, C, E, G, I). In the cavefish lens,  $\beta B$ - and  $\gamma M$ -crystallin expression persists throughout the 36 hpf, 48 hpf, 60 hpf, and 72 hpf stages. However, the expression pattern does not take on the ring shape typical of the surface fish lens. Instead, expression is seen in the central and posterior region of the lens similar to the 24 hpf stage (Figure 2-9, D, F, H, J; Figure 2-10, D, F, H, J). Furthermore, presumably in response to the changing lens size in cavefish, the area of expression of both  $\beta B$ - and  $\gamma M$ -crystallin increases from the 24 hpf to the 48 hpf stage, but subsequently decreases from the 48 hpf to the 72 hpf stage.

Figure 2-7



**Figure 2-7. Phylogenetic tree of crystallin nucleotide sequences constructed by the NJ method.** The branch lengths are proportional to phylogenetic distance. The scale bar represents an evolutionary distance of 0.05 nucleotide substitutions. Values appearing at the nodes indicate the percentage of times that particular internal branch was obtained out of 1000 bootstrap replicates. *Astyanax*  $\gamma M$ -crystallin groups with other  $\gamma M$ -crystallin sequences, while *Astyanax*  $\beta B$ -crystallin groups with other  $\beta B$ -crystallin sequences.

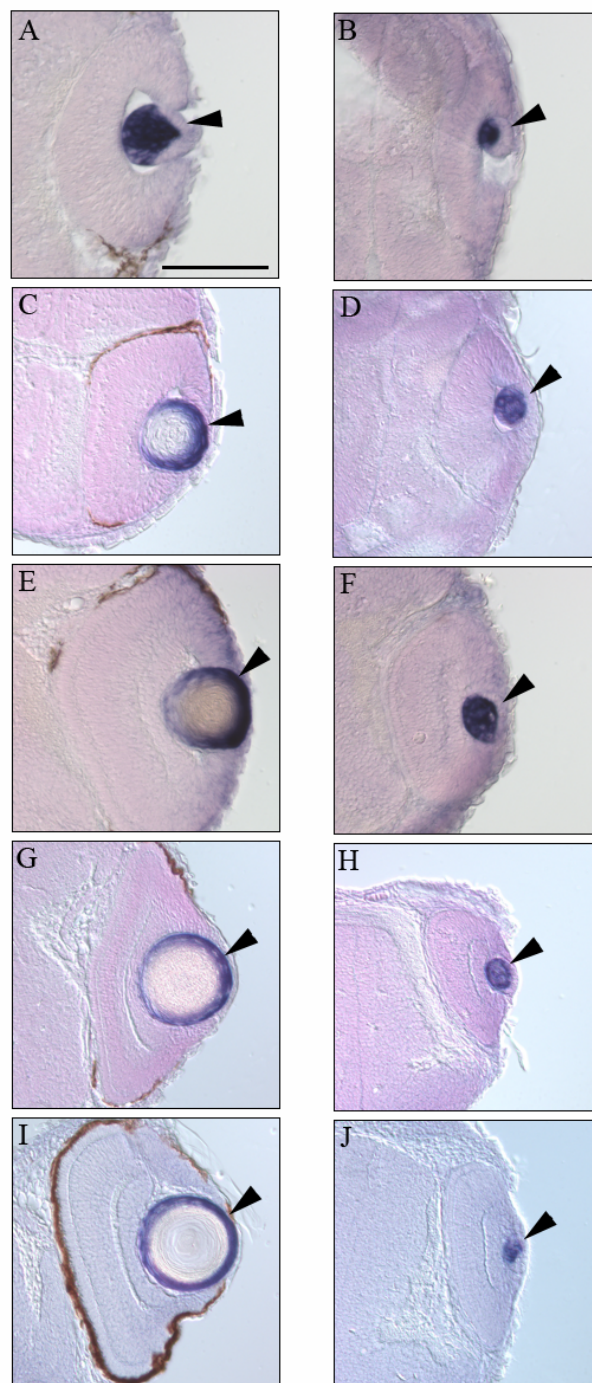
Figure 2-8



**Figure 2-8. Expression of  $\alpha A$ -crystallin in early stage larvae of *Astyanax mexicanus*.** A, C, E, G, I Surface fish. B, D, F, H, J Cavefish. Larva were subjected to whole mount *in situ* hybridization and subsequently embedded and cross sectioned at 24 hpf (A,B), 36 hpf (C,D), 48 hpf (E,F), 60 hpf (G,H), and 72 hpf (I,J) stages. Arrowheads indicate the lens. Scale bar in A, 100um; magnification is the same in A-J.

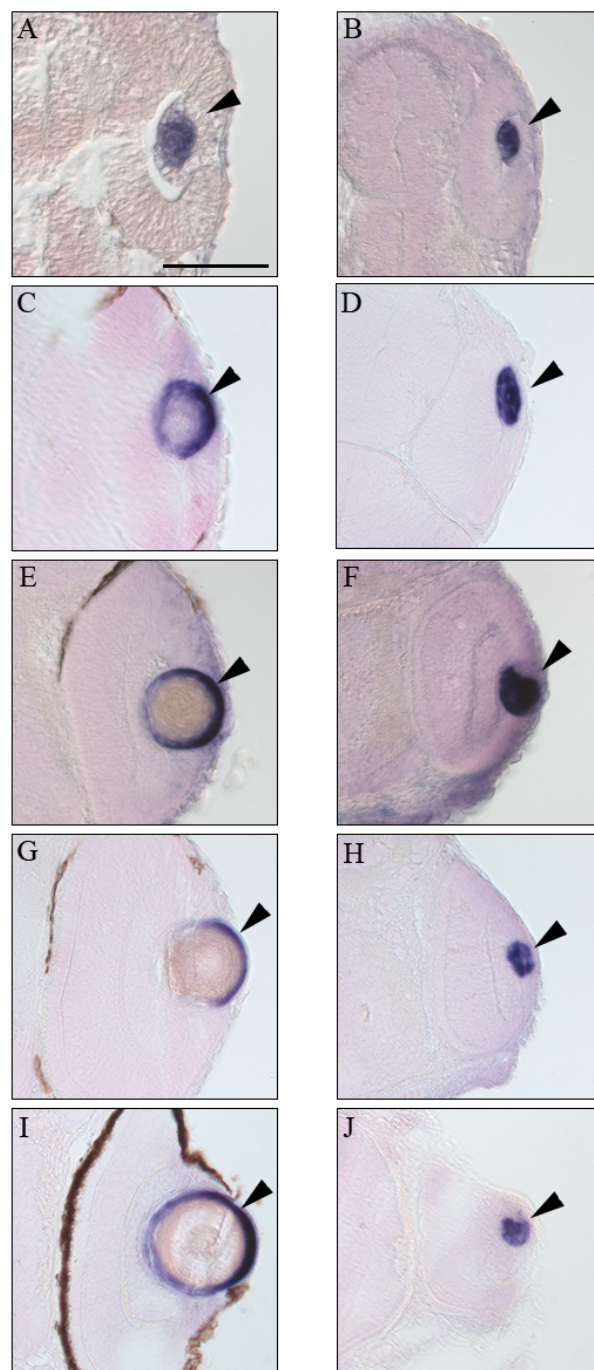


Figure 2-9



**Figure 2-9. Expression of  $\beta B$ -crystallin in early stage larvae of *Astyanax mexicanus*.** A, C, E, G, I Surface fish. B, D, F, H, J Cavefish. Larva were subjected to whole mount *in situ* hybridization and subsequently embedded and cross sectioned at 24 hpf (A,B), 36 hpf (C,D), 48 hpf (E,F), 60 hpf (G,H), and 72 hpf (I,J) stages. Arrowheads indicate the lens. Scale bar in A, 100um; magnification is the same in A-J.

Figure 2-10



**Figure 2-10. Expression of  $\gamma M$ -crystallin in early stage larvae of *Astyanax mexicanus*.** A, C, E, G, I Surface fish. B, D, F, H, J Cavefish. Larva were subjected to whole mount *in situ* hybridization and subsequently embedded and cross sectioned at 24 hpf (A,B), 36 hpf (C,D), 48 hpf (E,F), 60 hpf (G,H), and 72 hpf (I,J) stages. Arrowheads indicate the lens. Scale bar in A, 100um; magnification is the same in A-J.

## Cell Proliferation in the Eye

### *PCNA Labeling*

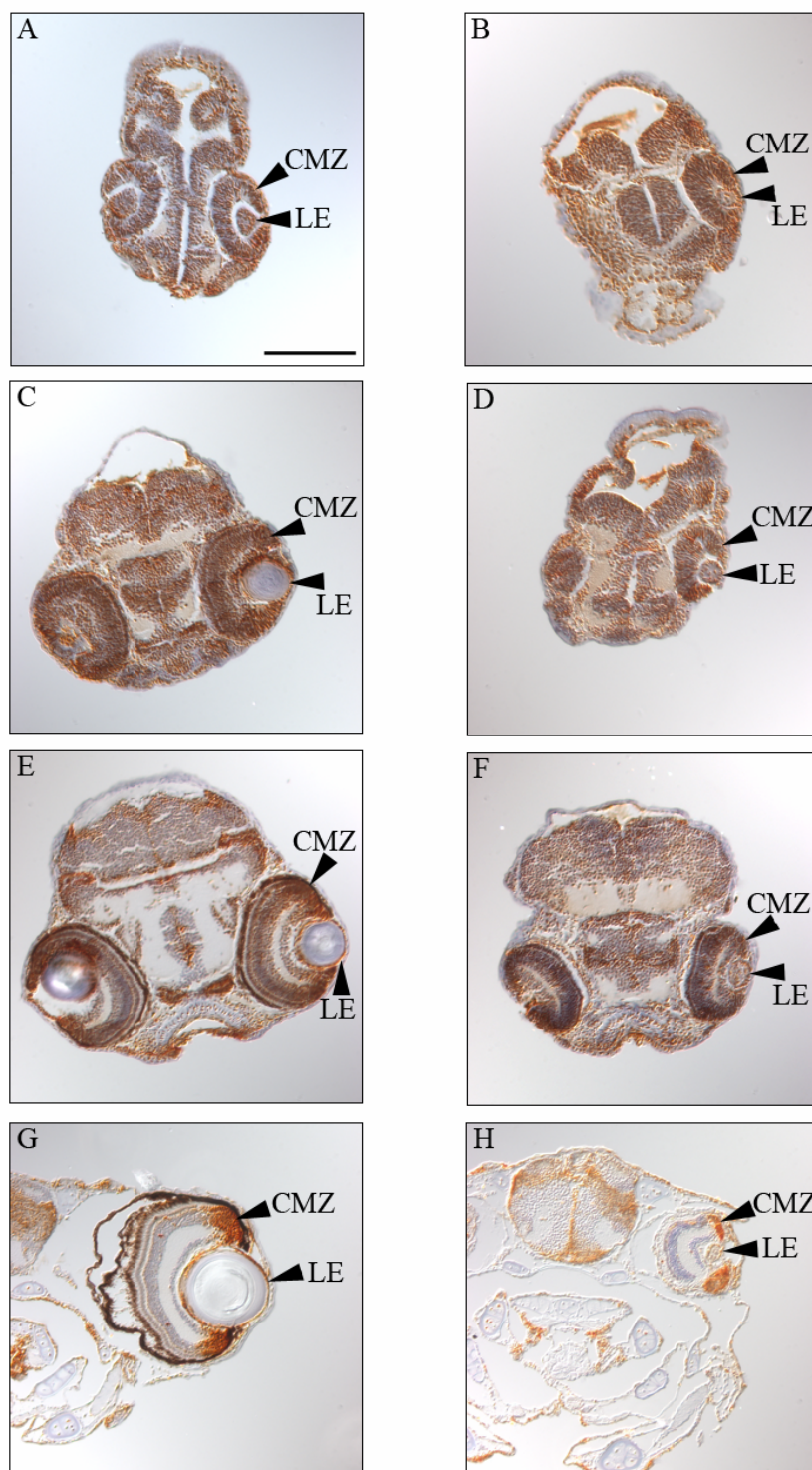
To study cell proliferation in the eye, I used an antibody to PCNA to detect mitotically active cells (Strickler, et al., 2002). PCNA expression was ubiquitous at the 24 hpf and 48 hpf stages in both surface fish and cave fish (Figure 2-11, A-D). By 72 hpf, PCNA begins to be restricted to the major proliferative zones of the eye. Labeling is seen in the CMZ and the lens epithelium. This labeling is even more defined at the 5 dpf stage (Figure 2-11, E-H). As development progresses, PCNA labeling is present in both the CMZ and the lens epithelium of both surface fish and cavefish at the 17 dpf, 1 mpf, 2 mpf, and 3 mpf stages (Figure 2-12, A-H). Additionally, labeling is seen in the outer layer of the retina in surface fish (arrowhead in Figure 2-12, G) and in cells throughout the retina in cavefish (arrowhead in Figure 2-12, H).

### *BrdU Labeling*

To further my studies of cell proliferation in the eye of *Astyanax mexicanus*, I used BrdU to label and track dividing cells in the retina (Strickler, et al., 2002). In samples that were labeled with BrdU from day 8 to 10 (pulse), proliferating cells are seen in the CMZ of the retina in both surface fish and cavefish (Figure 2-13, A

and B). Samples that were incubated in BrdU from day 8 to 10 and subsequently left to develop in water with no BrdU from day 10 to 12 (pulse with chase 1) showed labeled cells that had moved back into the retina (arrows in Figure 2-13, C and D). This area of labeled cells continues to progress further into the retina. This is evident in samples that were labeled with BrdU from day 8 to 10, but subsequently left in water with no BrdU from day 10 to 14 (pulse with chase 2) (arrows in Figure 2-13, E and F). In samples that were allowed to develop further in fresh water after the BrdU pulse, it appears that the area of labeled cells that moves back into the retina is more disorganized and made up of fewer cells in cavefish when compared to surface fish (arrows in Figure 2-13, C-F). Also, BrdU labeling is seen in the outer layer of the retina in surface fish in the pulse with chase 1 sample, while this cell labeling is absent in the corresponding cavefish sample (arrowhead in Figure 2-13, C). This pattern of loss will be further investigated in Chapter 4.

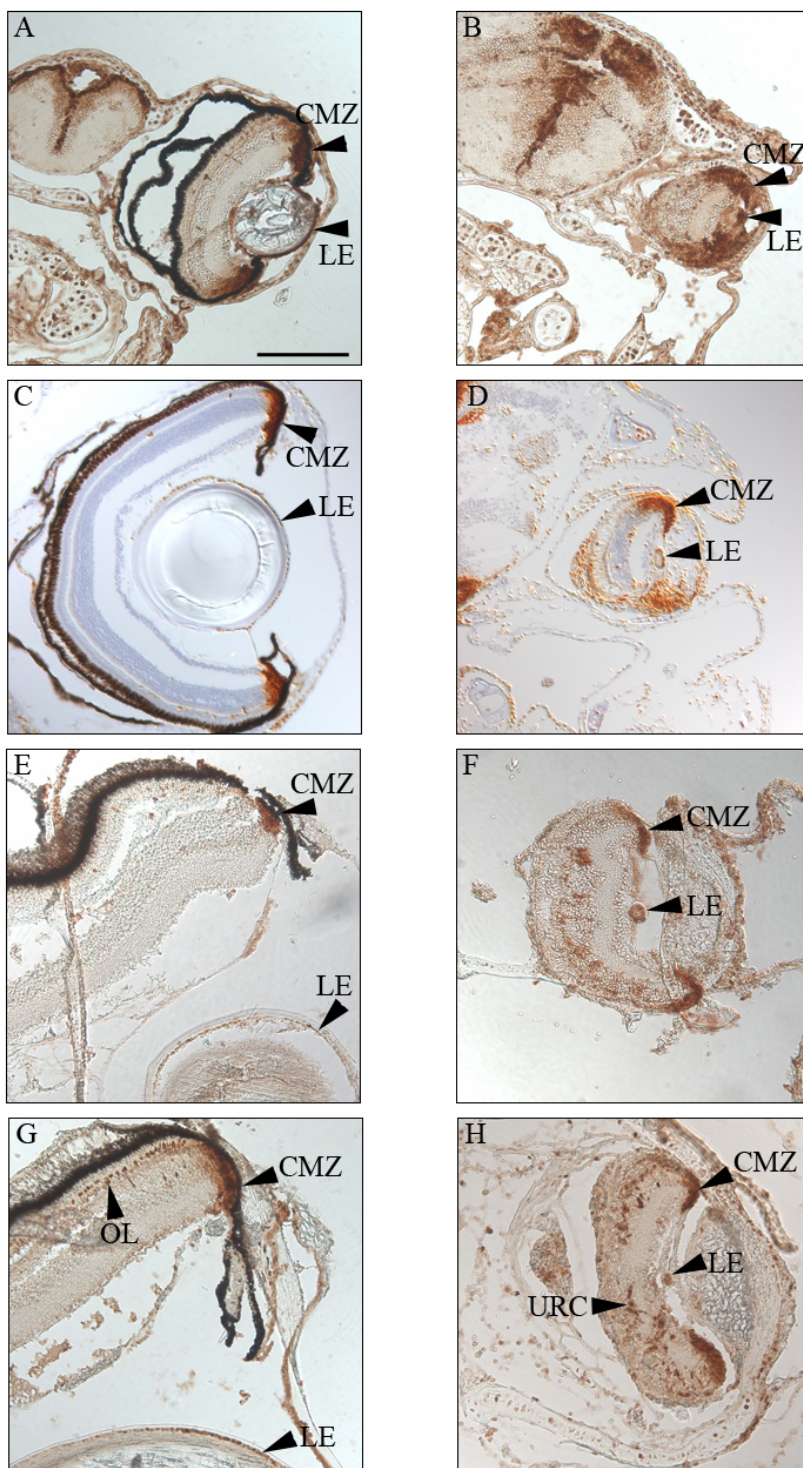
Figure 2-11



**Figure 2-11. PCNA expression in the eye of early stages of development of *Astyanax mexicanus*.** A, C, E, G Surface fish. B, D, F, H Cavefish. Developing larva of *Astyanax mexicanus* were fixed, embedded, and cross sectioned. These samples were then subjected to immunohistochemistry using an antibody to PCNA at 24 hpf (A,B), 48 hpf (C,D), 72 hpf (E, F) and 5 dpf (G,H) stages. Brown staining represents areas of PCNA expression. Abbreviations: CMZ, ciliary marginal zone; LE, lens. Scale bar in A, 100um; magnification is the same in A-H.

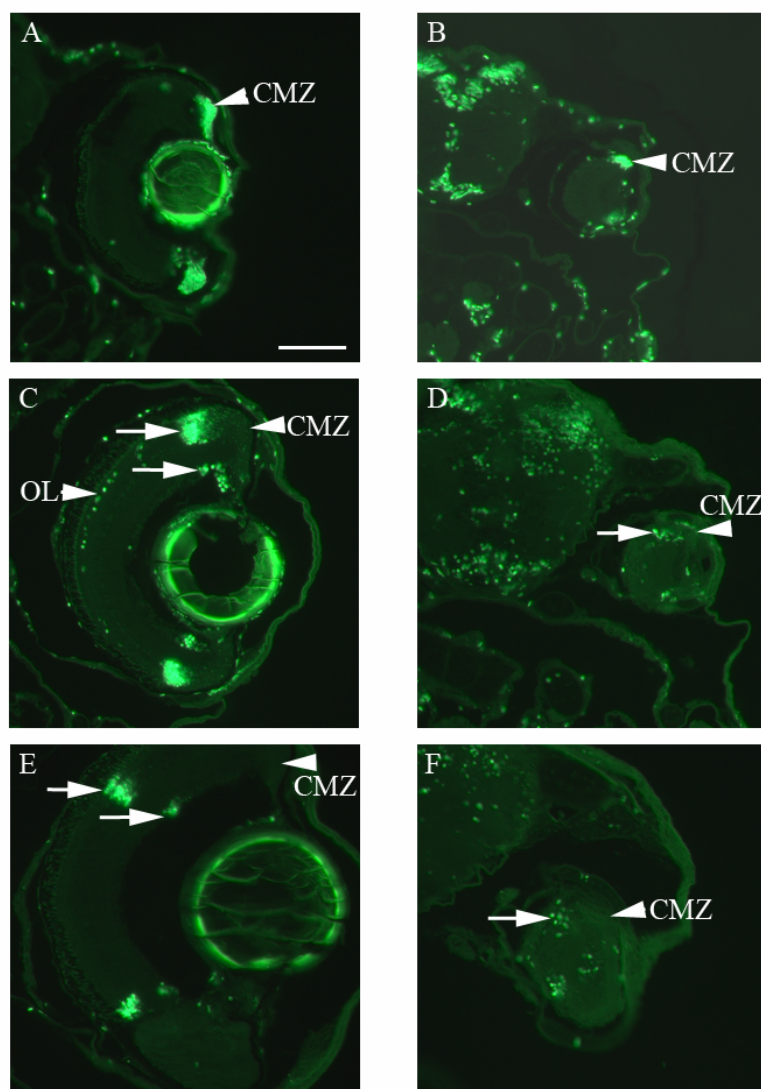


Figure 2-12



**Figure 2-12. PCNA expression in the eye of later stages of *Astyanax mexicanus* development.** A, C, E, G Surface fish. B, D, F, H Cavefish. Developing juveniles of *Astyanax mexicanus* were fixed, embedded, and cross sectioned. These samples were then subjected to immunohistochemistry using an antibody to PCNA at 17 dpf (A,B), 1 mpf (C,D), 2 mpf (E, F) and 3 mpf (G,H) stages. Abbreviations: CMZ, ciliary marginal zone; LE, lens; OL, outer layer; URC, unidentified retinal cells. Scale bar in A, 100um; magnification is the same in A-H.

Figure 2-13



**Figure 2-13. BrdU pulse-chase experiments in the developing eye of *Astyanax mexicanus*.** A, C, E Surface fish. B, D, F Cavefish. Developing *Astyanax mexicanus* were incubated in BrdU from 8-10 dpf. Some samples were immediately fixed (pulse with no chase) (A,B). Some samples were left to develop from 10-12 dpf in water without BrdU and subsequently fixed (pulse with chase 1) (C,D). Finally, some samples were left to develop from 10-14 dpf in water without BrdU and subsequently fixed (pulse with chase 2) (E,F). After the samples were embedded and cross sectioned, they were subjected to immunohistochemistry using an antibody to BrdU. Abbreviations: CMZ, ciliary marginal zone; OL, outer nuclear layer. Scale bar in A, 100  $\mu$ m; magnification is the same in A-F.

## PCD in the Eye

I was interested in studying PCD in the eye of *Astyanax mexicanus*, using the TUNEL method to label dying cells. I labeled whole mount samples to see if there were any differences between surface fish and cavefish. At the 24 hpf stage, I saw labeling around the lens area in both surface fish and cavefish (Fig 2-14, A and B). After 24 hpf, no labeling is seen in surface fish, although at 72 hpf eye pigment development makes it impossible to tell if labeling is present (Figure 2-14, C, E, G, I, J, M). In cavefish, TUNEL labeled cells are present in the lens and retina at the 36 hpf, 48 hpf, 60 hpf, 72 hpf, 5 dpf, and 10 dpf stages (Figure 2-14, D, F, H, J, L, N).

To more fully understand the dynamics of PCD in the eye, I studied TUNEL labeled sections of *Astyanax mexicanus*. The labeled cells at the 24 hpf stage seen in the whole mount samples appear to be cells dying as the lens vesicle pinches off from the surface ectoderm (Figure 2-15, A and B). At 36 hpf, there appear to be remnants of cells dying from the lens pinching off from the surface ectoderm. However, I also see labeled cells beginning to appear in the cavefish lens (Figure 2-15, C and D). As development progresses, TUNEL labeled cells are seldom, if ever, seen in the developing lens and retina of surface fish (Figure 2-15, E, G, I, K, M; Figure 2-16, A, C, E, G). Beginning at 48 hpf, widespread PCD is present in the lens of the cavefish and continues throughout development (Figure 2-15, F, H, J, L, N). Beginning at 48 hpf, TUNEL labeled cells are present in the retina of

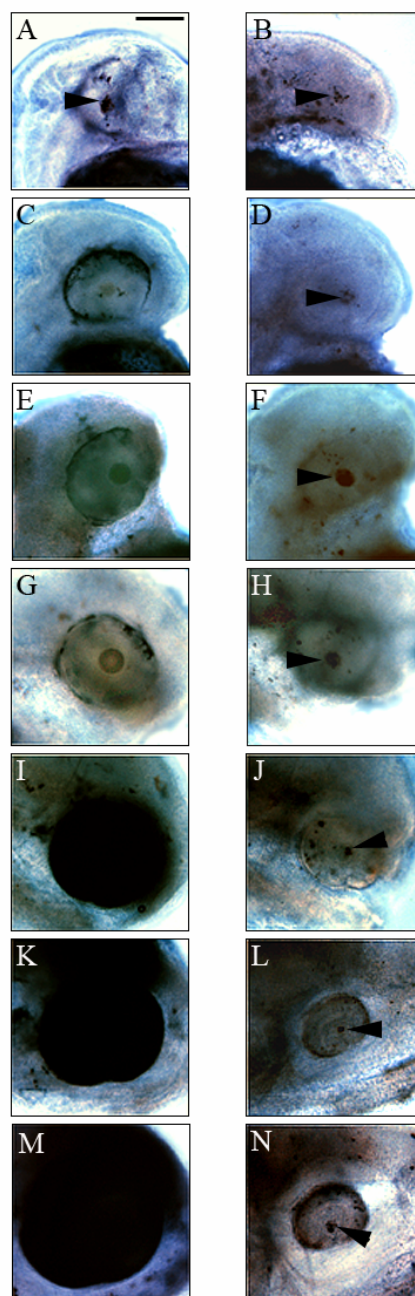
the cavefish (Figure 2-15, F). Similar to the lens, I see TUNEL labeled cells in every stage studied after 48 hpf, although this labeling is more restricted at later stages (Figure 2-15, H, J, L, N; Figure 2-16, B, D, F, H). Most notably, large patches of PCD appear in the retina of the cavefish at 72 hpf stage (Figure 2-15, J). Additionally, TUNEL labeling was detected in the area of the retinal pigmented epithelium at the 5 dpf and 10 dpf stages in cavefish (Figure 2-15, F, L and N).

## **PCD and Other Molecular Markers in the Eye**

### *PCD and $\gamma$ -crystallin Antibody Labeling*

To study  $\gamma$ -crystallin protein accumulation in relation to PCD, I dual labeled sections of the eye with TUNEL and an antibody to  $\gamma$ -crystallin. In the 48 hpf surface fish, the lens is developing normally (Figure 2-17, A) and shows accumulation of  $\gamma$ -crystallin protein, but no TUNEL labeling (Figure 2-17, B, and C). The cavefish lens also shows  $\gamma$ -crystallin protein accumulation. However, TUNEL labeling indicates that cells within this area are also undergoing PCD (Figure 2-17, D-F).

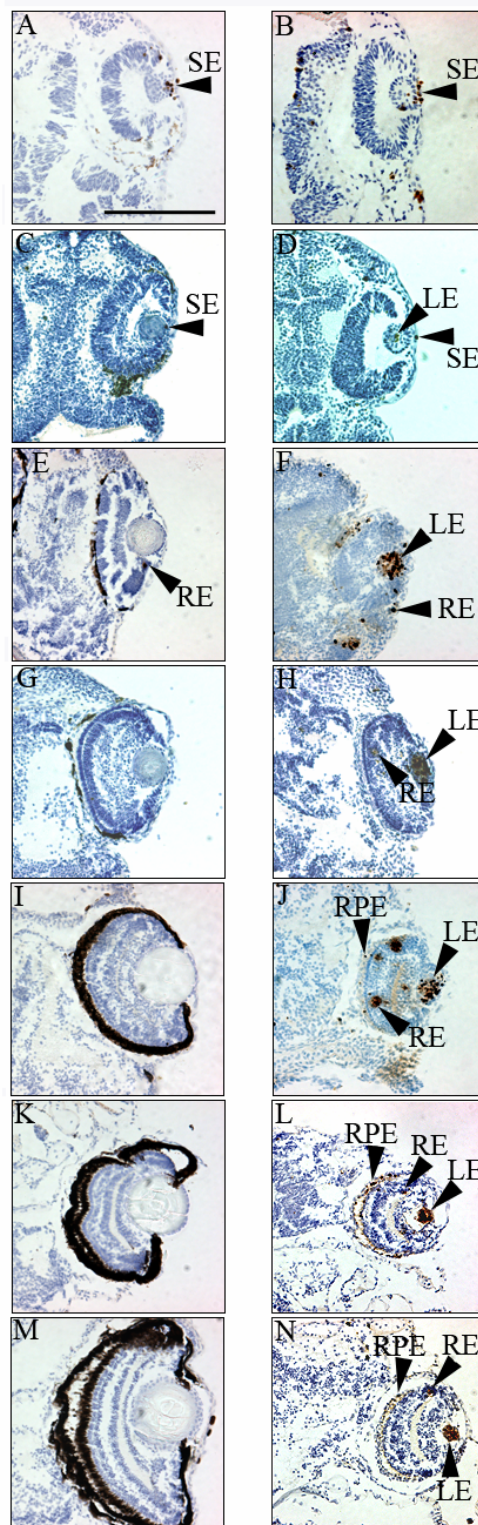
Figure 2-14



**Figure 2-14. Whole mount programmed cell death in the developing eye of *Astyanax mexicanus*.** A, C, E, G, I, K, M Surface fish. B, D, F, H, J, L, N Cavefish. Programmed cell death was detected in *Astyanax mexicanus* by subjecting whole mount samples to the TUNEL assay at the following stages: 24 hpf (A,B), 36 hpf (C,D), 48 hpf (E,F), 60 hpf (G,H), 72 hpf (I,J), 5 dpf (K,L), and 10 dpf (M,N). Arrowheads indicate programmed cell death in the lens area. Scale bar in A, 100um; magnification is the same in A-N.

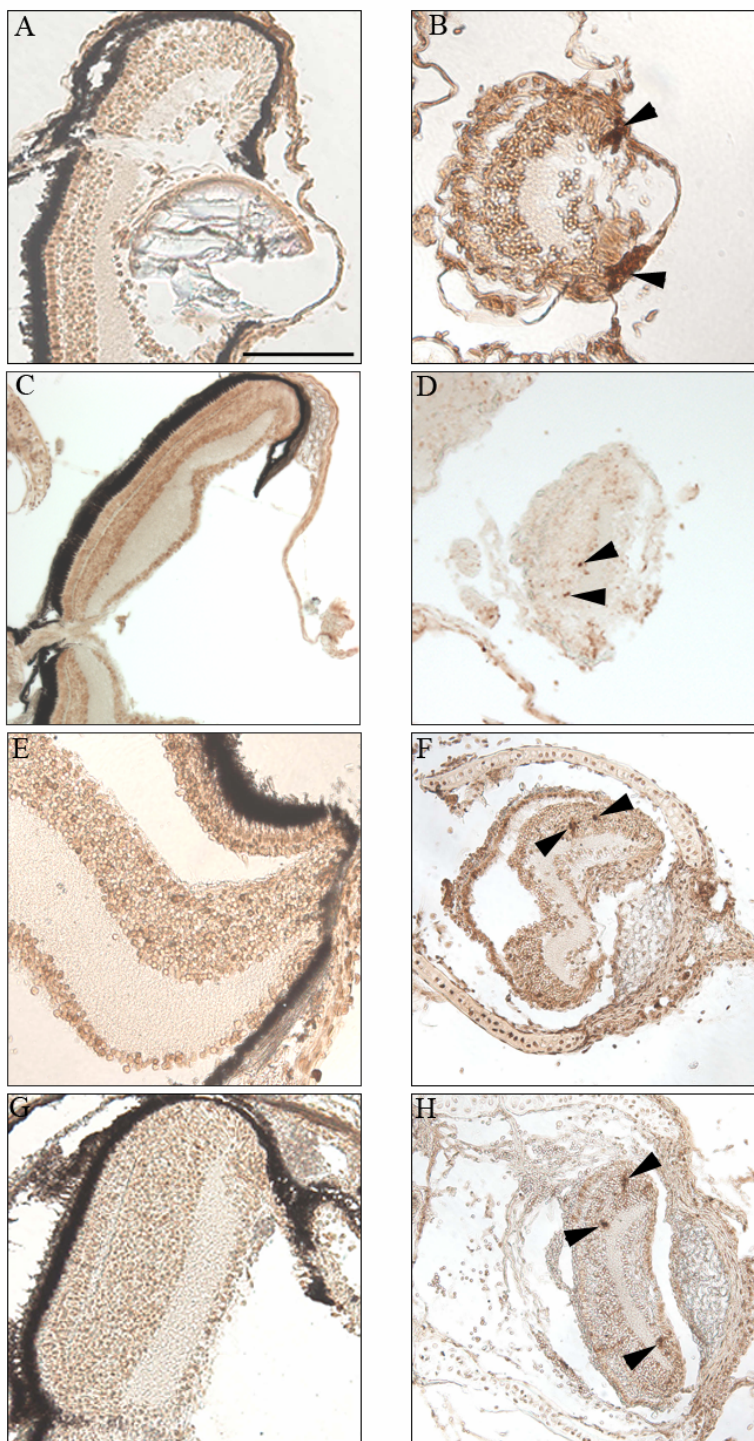


Figure 2-15



**Figure 2-15. Programmed cell death in the developing eye of early stage *Astyanax mexicanus*.** A, C, E, G, I, K, M Surface fish. B, D, F, H, J, L, N Cavefish. Programmed cell death was detected in *Astyanax mexicanus* by subjecting whole mount samples to the TUNEL assay. These samples were subsequently embedded and cross sectioned at the following stages: 24 hpf (A,B), 36 hpf (C,D), 48 hpf (E,F), 60 hpf (G,H), 72 hpf (I,J), 5 dpf (K,L), and 10 dpf (M,N). Abbreviations: SE, surface ectoderm; LE, lens; RE, retina; RPE, retinal pigmented epithelium. Scale bar in A, 200um; magnification is the same in A-N.

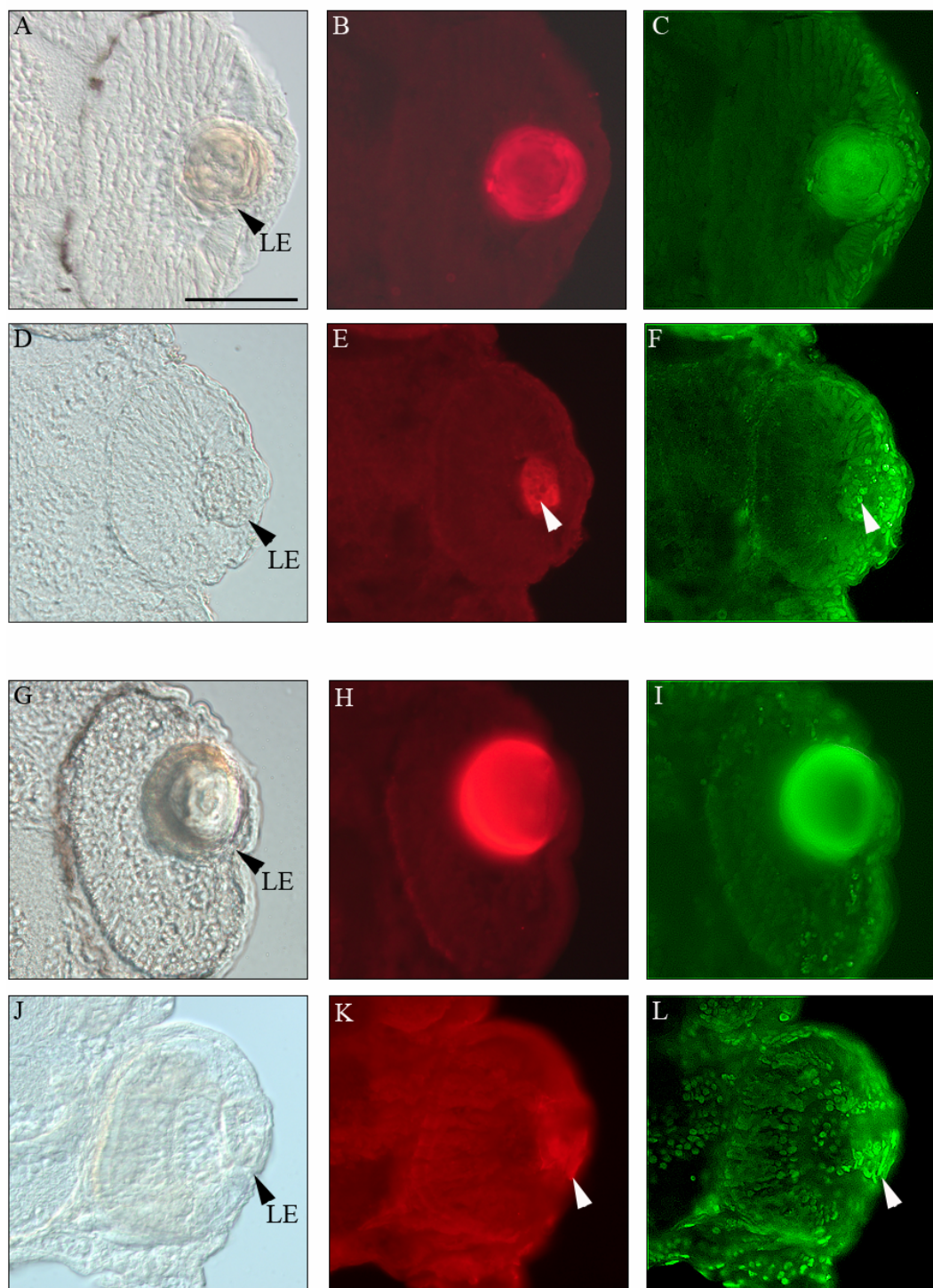
Figure 2-16



**Figure 2-16. Programmed cell death in the developing eye of later stage *Astyanax mexicanus*.** A, C, E, G Surface fish. B, D, F, H Cavefish. Developing juveniles were fixed, embedded and cross sectioned at 17 dpf (A,B), 1 mpf (C,D), 2 mpf (E,F), and 3 mpf (G,H) stages. The sectioned material was subjected to the TUNEL assay to detect cells undergoing programmed cell death. Arrowheads indicate cells or areas of programmed cell death. Scale bar in A, 200um; magnification is the same in A-H.

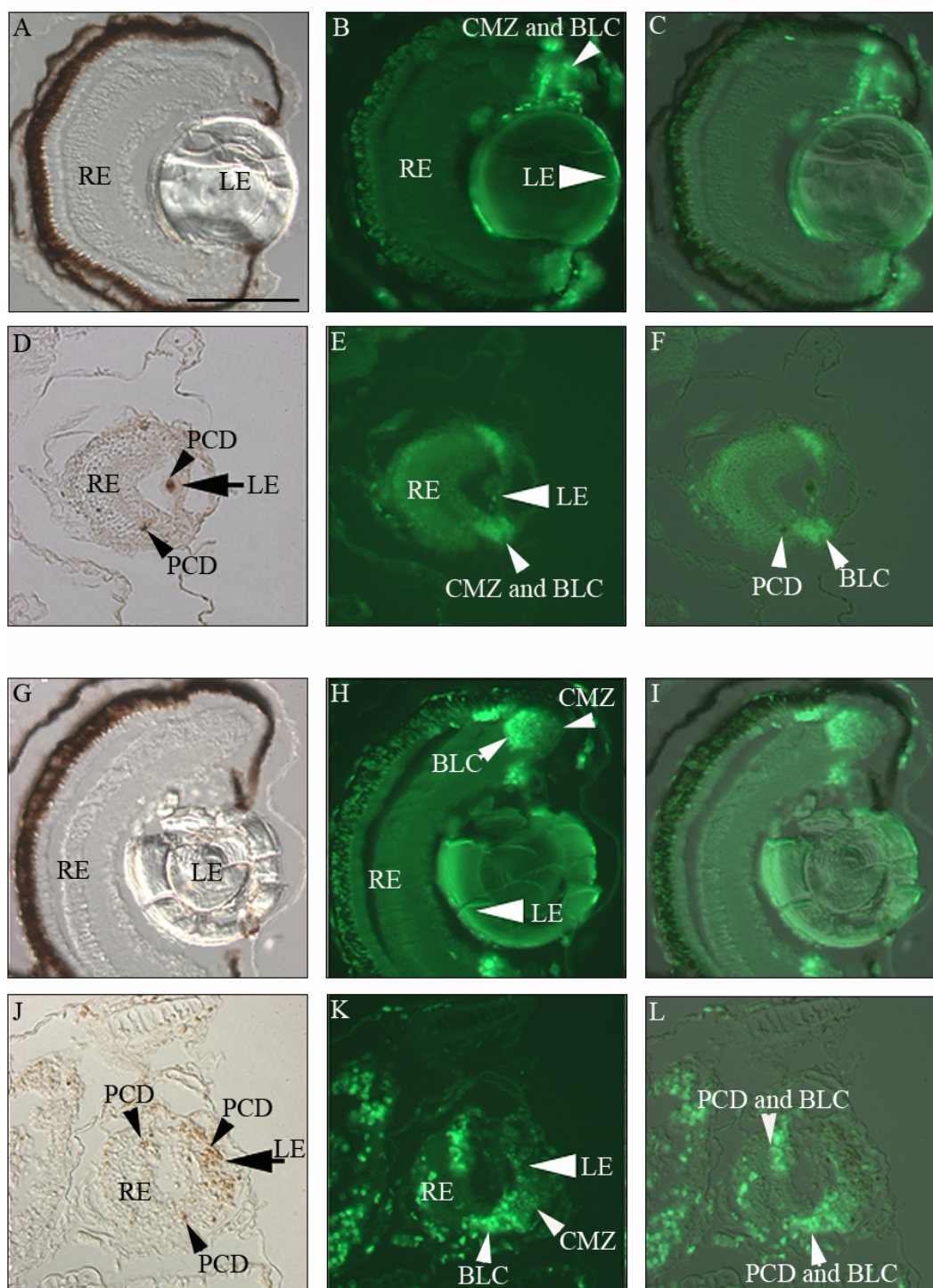


Figure 2-17



**Figure 2-17. Gamma-crystallin protein expression and programmed cell death in *Astyanax mexicanus*.** A, B, C, G, H, I Surface fish. D, E, F, J, K, L Cavefish. Samples were fixed, embedded and cross sectioned at 48 hpf (A-F) and 72 hpf (G-L). Samples were subjected to immunohistochemistry with an antibody to Gamma-crystallin (B,E,H,K). These same samples were also tested for programmed cell death using the TUNEL assay (C,F,I,L). Corresponding light micrographs are shown in A,D,G,J. Abbreviation: LE, lens. Arrowheads in E and K indicate the area of Gamma-crystallin protein expression in the cavefish lens. Arrowheads in F and L indicate areas of programmed cell death in the cavefish lens. Scale bar in A, 100um; magnification is the same in A-L.

Figure 2-18



**Figure 2-18. Programmed cell death and BrdU labeling in the eye of *Astyanax mexicanus*.** A, B, C, G, H, I Surface fish. D, E, F, J, K, L Cavefish. *Astyanax mexicanus* were incubated in BrdU from 8 to 10 dpf. Some samples were fixed and analyzed immediately (pulse) (A-F), while others were put back into fresh water and allowed to develop further from 10 to 12 dpf (chase) (G-L). Samples were subjected to the TUNEL assay to detect programmed cell death (A,D,G,J). These same samples were also tested for BrdU incorporation by immunohistochemistry using an antibody to BrdU (B,E,H,K). Merged images of programmed cell death light micrographs and BrdU fluorescent immunohistochemistry are shown in C, F, I, and L. Abbreviations: RE, retina; LE, lens; CMZ, ciliary marginal zone; PCD, programmed cell death; BLC, BrdU labeled cells. Scalebar in A, 200  $\mu$ m; magnification is the same in A-L.



At the 72 hpf stage,  $\gamma$ -crystallin protein continues to accumulate in the surface fish lens without any PCD (Figure 2-17, G-I). At this stage, the cavefish lens appears to have lower amounts of  $\gamma$ -crystallin accumulation. Furthermore, TUNEL labeled cells continue to correspond to this area of labeling (Figure 2-17, J-L).

### *PCD and BrdU Labeling*

To study the dynamics of cell proliferation versus PCD, I dual-labeled sections of the eye of *Astyanax mexicanus* with BrdU and TUNEL. Surface fish incubated with BrdU from day 8 to 10 and immediately fixed and analyzed (pulse) possess labeled cells in the CMZ and lens epithelium but no TUNEL labeled cells (Figure 2-18, A-C). Cavefish labeled with BrdU in the same manner (pulse) also possess labeled cells in the CMZ (Figure 2-18, D-F). There are also a few labeled cells in the reduced lens of the cavefish. In contrast to surface fish, I also see TUNEL labeled cells in the cavefish retina. This area of PCD appears outside the CMZ and does not correspond to BrdU labeling.

Samples were also incubated in BrdU from day 8 to 10 and subsequently allowed to develop in fresh water from day 10 to 12 before they were fixed and analyzed (pulse with chase 1). The area of BrdU labeling in the lens and retina has moved away from the areas of proliferation in surface fish, and no cells undergoing PCD are present (Figure 2-18, G-I). In cavefish, PCD occurs in the retina (arrowheads

labeled PCD in Figure 2-18, J). Furthermore, there are disorganized areas of BrdU labeled cells in the retina and lens (arrowhead labeled BLC in Figure 2-18, K). Unlike the original 8 to 10 day pulse samples, TUNEL labeled cells now correspond to areas of BrdU labeling (arrowheads in Figure 2-18, L).

### **Discussion**

The purpose of this study was to determine factors that may be responsible for the process of eye degeneration in *Astyanax* cavefish. I began by assessing the expression patterns of several key genes in the retina and lens. I found expression pattern differences in certain lens genes. However, I found that most genes retain their expression integrity in cavefish. My PCNA and BrdU studies indicated that cavefish eye proliferative zones remain relatively intact. Finally, I found that PCD may be an important factor in cavefish eye degeneration throughout development.

My first approach to elucidating cavefish eye degeneration mechanisms was to study genes expressed in the retina. I initially chose to study the transcription factor Pax6. The Pax6 protein contains an N-terminal paired domain characteristic of all Pax genes in addition to a homeodomain located near the central portion of the protein. Both of these regions act as DNA binding domains (Wawersik, et al., 2000). In vertebrates, Pax6 is important for development of the nervous system, pituitary, and pancreas (Callaerts, et al., 1997). In *Astyanax*, Pax6 is first expressed during late epiboly and continues to be expressed through gastrulation and neurulation around the tailbud stage. At this time, Pax6 transcripts are seen most notably in two domains in the anterior region of the developing embryo corresponding to presumptive forebrain and optic primordia. Pax6 continues to be expressed in the optic vesicles as they outpocket from the diencephalon at approximately the five somite stage. As eye development

continues, Pax6 expression becomes more restricted to certain areas of the eye (Strickler, et al., 2001). Although there are important changes in expression between surface fish and cavefish at both the tailbud stages and later stages, I did not see significant *pax6* expression differences in the larval stage retina.

To verify that retinal gene expression, especially in the CMZ, persisted in the cavefish retina, I studied the *rx1* and *vsx2* genes. Like *pax6*, these genes are homeodomain proteins that act as transcription factors in retinal development (Mathers, et al., 1997; Levine, et al., 1997). More specifically, both genes are expressed in proliferative cells of the CMZ (Mathers and Jamrich, 2000; Passini, et al., 1997). Like *pax6*, I found that the expression patterns of *rx1* and *vsx2* were similar between surface fish and cavefish. It is unclear if these genes are directly involved with cell proliferation in the retina, but their continued expression in the cavefish indicates that cells of the CMZ are still (Strickler, et al., 2002)

In contrast to the retina, I found significant differences in lens gene expression. I initially checked the expression patterns of the intramembrane proteins *MIP* and *MP19*. These genes are expressed specifically in the lens (Shiels and Bassnett, 1996; Kumar, et al., 1993) and are the two most abundant intramembrane proteins of the lens respectively (Kumar, et al., 1993). It is unclear whether these proteins form junctions to maintain fluid balance in the lens or if they serve some other function (Kumar, et al., 1993; Gorin, et al., 1984). Transcripts of both genes initially accumulate in the presumptive primary lens fiber cells. Later in surface

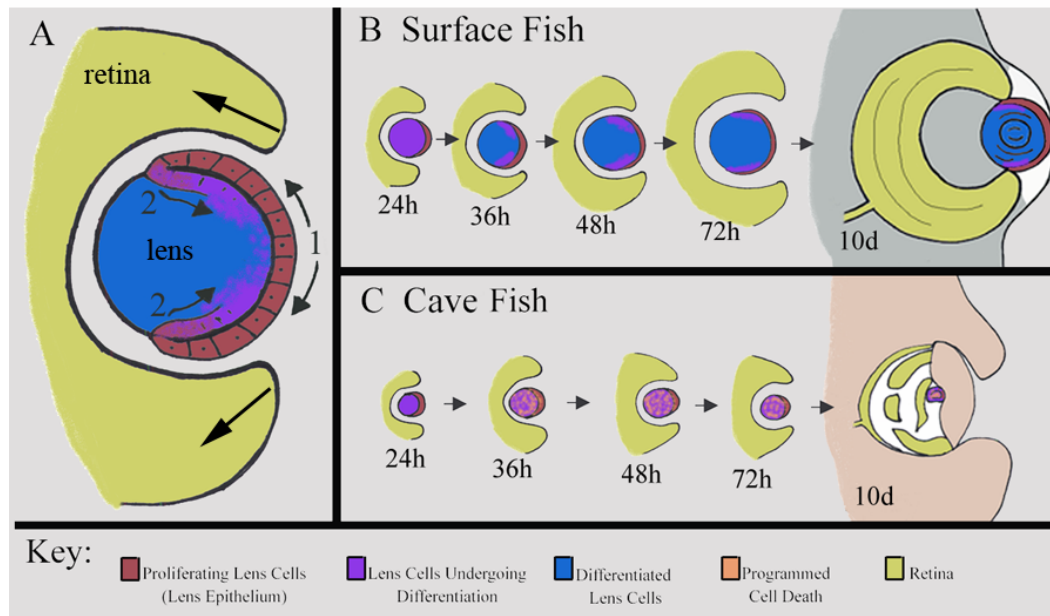
fish development, *MIP* and *MP19* transcripts accumulate in cells before they form terminally differentiated lens fiber cells. In cavefish, it appears that terminally differentiated lens cells fail to develop. As a consequence, the expression of *MIP* and *MP19* remains the same throughout most of development in the cavefish lens instead of eventually taking on the characteristic ring of expression seen in surface fish.

In response to altered *MIP* and *MP19* expression patterns, I was interested in studying the expression of crystallins in the lens of *Astyanax*. There are three main families of vertebrate lens crystallins,  $\alpha$ -,  $\beta$ -, and  $\gamma$ -crystallins (Graw, 1997). Additionally, taxon specific crystallins exist (Graw, 1997; Tomarev, et al., 1984). The origin of  $\alpha$ -crystallins can be traced to the HSP26 superfamily. The  $\beta$ - and  $\gamma$ -crystallins are more closely related to one another than either are to the  $\alpha$ -crystallins, but their molecular origins are currently unclear (Clout, et al., 1997). At the onset of expression,  $\beta B$ - and  $\gamma M$ -crystallins transcripts are found in presumptive primary lens cells in both surface fish and cavefish. The expression of  $\beta B$ - and  $\gamma M$ -crystallins in the lens of surface fish takes on a characteristic ring shape. Presumably, this expression is indicative of differentiating secondary lens fiber cells. In contrast, transcripts appear as a solid mass in the cavefish lens throughout all stages studied. Like *MIP* and *MP19*, this is most likely due to a lack of distinguishable terminally differentiated lens fibers in cavefish.

A  $\gamma S$ -crystallin sequence was previously isolated from *Astyanax*. The transcript is transiently expressed in the surface fish lens from about 35 to 45 hpf, and is totally absent from the cavefish lens (Langecker, et al., 1995). Similar to  $\gamma S$ -crystallin, the onset of  $\alpha A$ -crystallin expression appears at 36 hpf which is approximately 12 hours after  $\beta B$ - and  $\gamma M$ -crystallin transcripts appear in *Astyanax*. Unlike  $\beta B$ - and  $\gamma M$ -crystallin, expression does not occur in the presumptive primary lens fiber cells. Instead, it appears as a ring of expression surrounding already differentiated lens fibers in surface fish. This expression pattern continues as the lens develops. The delayed onset of  $\alpha A$ -crystallin expression compared to  $\beta B$ - and  $\gamma M$ -crystallin is documented in frog (Mikhailov, et al., 1997). In contrast,  $\alpha A$ -crystallin transcripts are absent from the cavefish lens other than a small amount of expression found in some samples at the 36 hpf stage. It is evident that the lens epithelium remains intact in the cavefish, as does the competency to begin to produce primary and secondary lens fiber cells. However, it appears something happens to these cells between the onset of  $\beta B$ -/ $\gamma M$ -crystallin expression and  $\alpha A$ -crystallin. It is also possible that there is a mutation in the cavefish  $\alpha A$ -crystallin gene, or that the mechanisms controlling the expression of  $\alpha A$ -crystallin are altered in cavefish.

I studied cell proliferation to further investigate changes in cavefish eye development. As shown in figure 2-19(A), cells of the retina normally undergo proliferation at the CMZ and move posteriorly. In the lens, cells proliferate in the lens ectoderm and typically move posteriorly to the lens equator. At this point,

Figure 2-19



**Figure 2-19. Schematic summary of basic eye growth and development in *Astyanax mexicanus*.** The schematic in A shows cell movements post proliferation of both the lens and retina. The images in B and C represent growth and development of surface fish and cavefish respectively.

they differentiate and are added to the centrally located lens fiber cells. My *Astyanax* gene expression data indicated that proliferative zones of the cavefish eye likely remain intact. To confirm this, I investigated PCNA expression to obtain a preliminary view of which cells are proliferating in the eye at a specific moment in time (Takasaki, et al., 1981). I found that as PCNA expression moves from a ubiquitous state to a more discrete pattern, there is no significant difference between surface fish and cavefish. At the latest time point studied, the 3 month old juvenile, PCNA expression remains in the CMZ and the lens epithelium of the cavefish. At this stage, staining is also present in the outer layer of the surface fish retina. Staining also exists in the cavefish retina. However, it is unclear which non-CMZ cells are expressing PCNA in the cavefish. Based on these data, I conclude that the lens epithelium and the CMZ remain intact in the cavefish and cell proliferation continues in these areas.

To further investigate cell proliferation, I used BrdU labeling. In addition to labeling proliferating cells, BrdU can also be used to track subsequent cell movements (Li, et al., 2000). I found far fewer labeled cells in the cavefish lens when compared to the surface fish at the early larval stage. Furthermore, I found that cells move away from the CMZ through the retina normally in both surface fish and cavefish. However, as time progresses from an initial BrdU labeling pulse, fewer labeled cells remain in the cavefish retina, despite the fact that the initial pulse data is comparable between surface fish and cavefish. Also, this



group of labeled cells is more disorganized in the cavefish in contrast to the continuous group of labeled cells of the surface fish retina.

The combined results of PCNA and BrdU proliferation data suggest loss of cells by a mechanism such as PCD in the cavefish eye. I used TUNEL labeling to test this hypothesis. I initially found labeling around the surface ectoderm covering the lens at early stages of both surface fish and cavefish. Presumably, these are cells that are dying in response to the lens vesicle pinching off from the surface ectoderm (Mohamed and Amemiya, 2003). The continuation of PCD in the cavefish appears to be a main factor in the severely reduced size of the lens over time. As seen in figure 2-19, B and C, I show general eye development of surface fish and cavefish respectively. The surface fish lens develops normally with a large accumulation of differentiated lens fiber cells while the cavefish lens undergoes PCD and does not accumulate differentiated fiber cells.

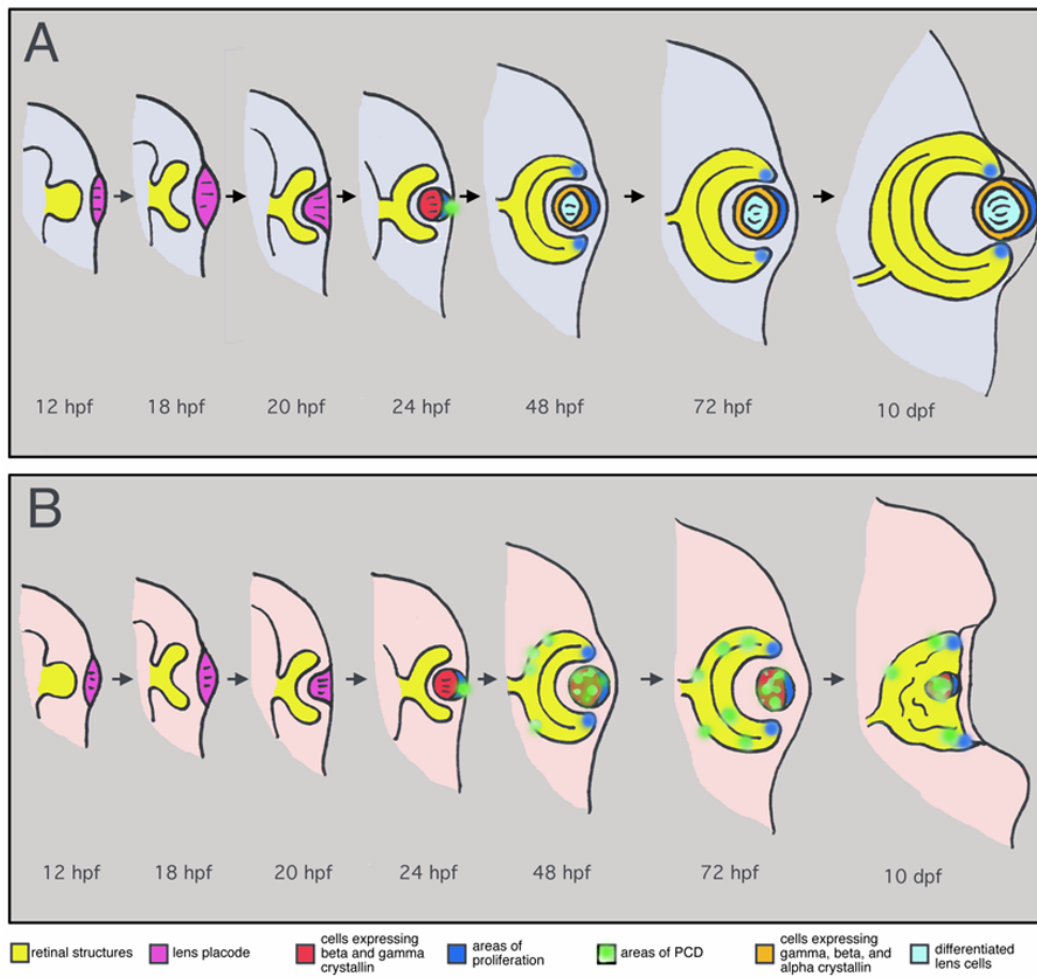
Like the lens, cells of the cavefish retina undergo PCD. I found large areas of TUNEL labeling during the 60 to 72 hpf stages. At the 5 to 10 dpf stages, I found TUNEL labeling in the area of the RPE. Additionally, there is PCD occurring in the neural retina of the cavefish at every stage I looked at starting at approximately 48 hpf. I propose this PCD in the retina is also partly responsible for the reduced size of the cavefish eye over time.

I found that PCD and  $\gamma$ -crystallin protein accumulation in the cavefish lens correspond to the same cells. This explains why  $\gamma$ -crystallin transcripts are detected in the cavefish lens despite the fact that a morphologically mature lens with terminally differentiated lens fibers fails to develop. Furthermore, when I repeated BrdU pulse and chase experiments with PCD assessment, I initially found that dying cells of the cavefish eye did not correspond to cells that were undergoing proliferation. However, after the labeled cavefish were allowed to develop further, I found that areas of PCD did correspond to the BrdU labeled cells. This suggests that cells of the cavefish retina proliferate normally, but these cells later die by PCD as they move away from the CMZ. The size of the cavefish retina may be a result of this mechanism. This will be investigated further in Chapter 4.

### **Characteristics of Cavefish Eye Development**

A summary of early *Astyanax* eye development is shown in Figure 2-20. During embryonic stages (up to the 24 hpf hatching stage), the optic cup and lens placode develop into the retina and lens respectively. During early larval stages (post 24 hpf) cavefish eye structures are significantly smaller than surface fish (Jeffery 2001). By the second day post fertilization, the cavefish lens and retina begin to undergo PCD. Several genes including those expressed in the CMZ retain their

Figure 2-20



**Figure 2-20. Schematic summary of processes involved in eye development of *Astyanax mexicanus*.** A, Surface fish. B, Cavefish. Colored blocks indicated at the bottom correspond to colored areas in the eye schematics where that process is involved both spatially and temporally.

expression integrity in the cavefish retina. Consistent with these data, I found that based on my cell proliferation studies, the cavefish CMZ remains functional.

Despite the drastic changes in gene expression patterns, I believe that lens developmental changes are most likely due to the onset of PCD, and not the molecular nature of the genes themselves. The fact that  $\beta B$ - and  $\gamma M$ -crystallin are present in the cavefish lens, coupled with the observed weak  $\alpha A$ -crystallin expression in the lens of some 36 hpf cavefish, but not in later samples, supports this assertion. It has been shown previously that lack of  $\alpha$ -crystallin expression can cause PCD (Ray, et al., 2001). Thus, lack of  $\alpha A$ -crystallin expression may cause cavefish lens PCD. However, based on the temporal nature of PCD and the onset of  $\alpha A$ -crystallin expression in the lens,  $\alpha A$ -crystallin transcripts would not be expected to accumulate if this were true.

I propose a model of cavefish eye development resembling the normal mode of teleost eye development. Initially, the cavefish optic bulb outpockets from the neural tube in the area of the presumptive diencephalon. The lens ectoderm thickens, presumably in response to signals from the optic bulb (Jean, et al., 1998). The optic cup continues to form from the optic bulb as the lens placode thickens and begins to invaginate inward toward the optic cup, forming the lens vesicle. The lens continues to develop, eventually pinching off from the surface ectoderm forming the lens proper. At this stage, all cells of the eye are proliferating. In general, the eye primordium appears similar to the surface fish

counterpart, except for the smaller overall size. At approximately 36 hpf, the lens begins to undergo widespread PCD which appears to be abnormal when compared to typical eye development. At about 48 hpf, parts of the retina begin to undergo PCD, with large areas of dying cells occurring at 60 to 72 hpf. At 5 to 10 dpf, I see the area of the retinal pigmented epithelium undergoing PCD. At about this time cells of the CMZ and lens epithelium label for proliferation markers. The cells continue to proliferate in the cavefish. However, PCD continues to occur throughout development. Presumably, an increased rate of PCD counteracts the normal rate of proliferation, contributing to cavefish eye degeneration

### **The Effects of PCD in the Eye of *Astyanax***

PCD has been documented as a normal process in eye development. In the zebrafish, PCD occurs in both the developing retina and lens (Cole and Ross, 2001). In the zebrafish, PCD occurs in the lens and the surface ectoderm during a discrete time period between 20 to 48 hpf. *Astyanax* has a faster rate of development in the laboratory, and the PCD found in the zebrafish is most likely similar to that seen in both forms of *Astyanax* at the 24 hpf stage when the lens vesicle is pinching off from the surface ectoderm.

As lens cells differentiate into lens fibers, they lose their nuclei and organelles in a process that has been compared to classic PCD. However, the cell is not totally removed during lens development as it is in PCD (Dahm, 1999). I believe that PCD in the cavefish lens is a deviation from the normal developmental program opposed to a process involved in typical lens fiber cell formation. I hypothesize that instead of the attenuated form of lens PCD, something causes cavefish lens cells to undergo complete PCD. For instance there may be a molecular factor(s) that prevents cells from completing the process of PCD during normal development. This factor may be mutated or absent in the cavefish. It is unclear why normally differentiating lens cells of certain other organisms label for PCD while the lens of the surface form of *Astyanax* does not. However, it is evident that PCD is directly involved in the degeneration of the cavefish lens.

I found PCD occurring in the cavefish retina, but TUNEL staining was absent from the surface fish retina. There may be several reasons why I do not detect PCD in the surface fish retina. The mode of *Astyanax* eye development may have evolved so that the retina does not undergo drastic neural remodeling events. Alternatively, discrete episodes of PCD may not be occurring in the retina at the time points studied. The most likely reason may involve cell cycling and cell turnover. PCD may be occurring in both the surface fish and cavefish retina at a normal basal development rate. This rate may be low enough so that it was not detected in the samples studied. Furthermore, this basal retinal PCD may occur at a faster rate from onset to final cell elimination, making it harder to detect. Thus,

PCD may occur in the cavefish retina above that of the basal PCD rate. This PCD may involve a slower cellular physiological process making it easier to detect. Moreover, the sheer amount of PCD occurring in the cavefish retina may be statistically harder to detect.

I found differences in expression patterns in some developmental genes expressed in the eye. I also determined that proliferative zones of the cavefish eye are intact. Finally, I discovered that the cavefish eye undergoes a significantly greater amount of PCD when compared to the surface fish. It appears that PCD is the probable cause of altered gene expression patterns in the cavefish eye.

Furthermore, PCD in the lens and retina is most likely responsible for lack of overall eye growth. There is evidence that the lens and retina interact to affect the development of one another (Jean, et al., 1998). There is evidence that the faulty cavefish lens causes several eye abnormalities during development and a normal surface fish lens transplanted into a cavefish retina can prevent the development of these abnormalities (Yamamoto and Jeffery, 2000). It is likely that PCD in the cavefish lens affects developmental processes of the retina. It is still unclear what initially causes the onset of PCD in the cavefish eye.

It has been suggested that a threshold number of lens cells may have to be present to promote normal lens development (Wride, 1996). It is possible the smaller cavefish lens (and presumably fewer number of initial lens cells) promotes lens PCD. As a result, differentiating lens cells die and are removed at a critical time

in their development when they would normally secrete factors important for promoting development of the retina and other ocular structures. Without proper lens signaling processes, PCD may be triggered in the retina further propagating faulty eye development. If this process occurs in the cavefish eye, faulty lens-to-retina signaling does not have a significant effect on proliferation of retinal cells in the CMZ. Instead, cells of the retina may lose some type of ‘competency for survival’ as a result of the lack of proper lens signaling.

Further studies involving the eye of *Astyanax* will be required to determine the exact role the lens and retina play in cavefish eye degeneration. In addition, it will hopefully become clear how the different parts of the developing eye interact with one another to promote normal eye development versus the faulty eye development of cavefish. It will also be important to determine what other factors may be affecting eye development. For example, there may be events occurring in the embryo prior to eye formation which have profound effects on eye development. Early developmental events may have a direct impact on events occurring later in development, or these events may be independent of one another. Moreover, additional studies should determine how excess PCD is triggered in the eye of the cavefish and how it more specifically affects eye development and growth in *Astyanax*. As a result of these studies, we will have a better understanding of how evolutionary changes can result in phenotypically different organisms.



### Chapter 3: Using Differential Expression Techniques to Uncover Trends in Evolution and Development of the Blind Cavefish *Astyanax mexicanus*.

#### **Abstract**

The study of evolution and development gives insights into how selective forces cause phenotypic change through altered ontogeny. I approach this concept by looking at micro-evolutionary shifts in closely related organisms. I specifically study the teleost fish *Astyanax mexicanus*, which consists of an eyed surface stream dwelling form, and an eyeless cave dwelling form. This species is interesting because the two forms are closely related, yet have obvious phenotypic alterations. In addition to differences in eyes, cavefish lack body melanin pigment and develop differences in neuroanatomy, behavior, jaw size, other sensory input structures, and metabolism. I used differential hybridization and microarray technology to uncover specific genes that may be involved in the development of these phenotypic differences. Furthermore, I used these candidate genes to infer larger genetic trends that may be occurring in the evolution of *Astyanax* development. I conclude that differential gene analyses are important in exploring the mechanisms of evolution and development. It is also evident that research involving the biology of model organisms can successfully be applied to non-model organism systems. Finally, the information provided by these studies will be used to answer questions about evolution and development by integrating what we learn from these studies with other information, such as molecular, cellular, and genetic data.

### **Introduction**

Molecular genetics has become an important component of developmental and evolutionary biological research. Knowledge in this field is rapidly expanding with the characterization of specific genes, their expression patterns, and functions. As we collect information pertaining to individual genes and genomes as a whole, we come closer to answering important questions regarding development, speciation, and evolution.

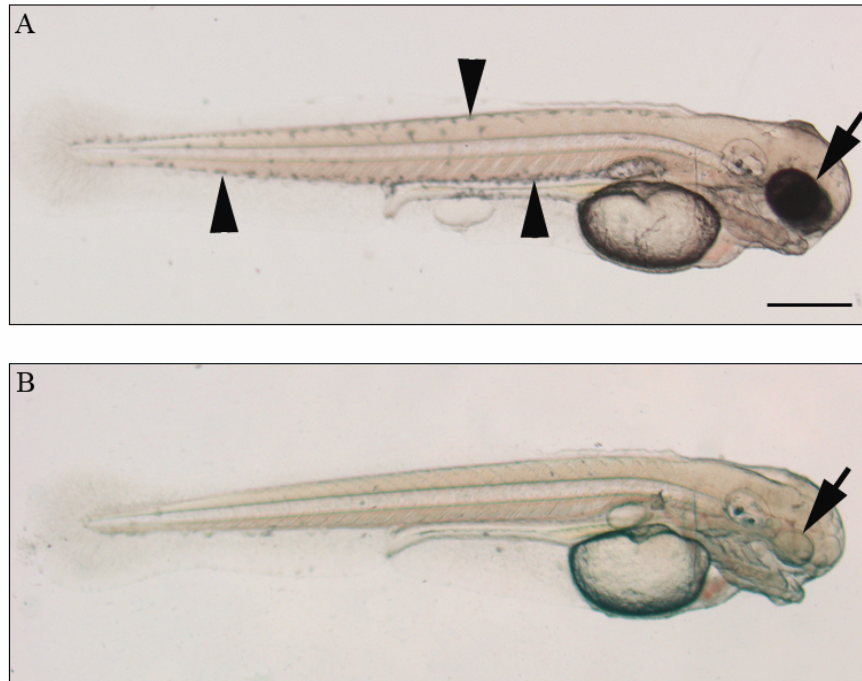
Differential gene expression techniques have been used by researchers to solve numerous types of these important biological questions. Differential display, differential hybridization libraries, and microarray analysis are the most common techniques used for this purpose (Gonzalez et al., 2006; Mathavan et al., 2005; Namkoong et al., 2006). Differential hybridization libraries are advantageous because they are species specific and can give immediate sequence information. However, it is difficult to elucidate large scale or genomic trends from this type of analysis.

The use of microarrays is becoming more common as researchers are interested in studying organisms at the genome level. In the past, microarray experiments have been accused of giving false data that is not repeatable. The technology has advanced to the point where these problems are being overcome (Sherlock, 2005). Microarray technology can be used to solve problems such as the effects a gene mutation has on numerous transcribed sequences of an organism both temporally

and spatially (Chauhan et al., 2002; Michaut et al., 2003). Microarrays are applied to all areas of science including neurobiology and human disease research (Dougherty and Geschwind, 2005; Flechner et al., 2004). Historically, the use of microarray technology has been restricted to model organisms. Producing useful chips requires a significant number of known transcribed sequences. This wealth of information is typically restricted to human, yeast, mouse, zebrafish, *Drosophila*, and other common model research organisms. However, there are documented cases of microarray studies where the research organism did not match the organism used to produce the microarray chip. For example, human microarray chips have been used to analyze data from rhesus monkeys (Kayo et al., 2001).

I am interested in using differential gene expression technologies to study evolution and development of the blind cavefish, *Astyanax mexicanus*. This teleost fish species consists of two extant forms: a surface stream dwelling form (surface fish) and a cave dwelling form (cavefish). Surface fish have well developed eyes as adults. Cavefish develop eyes as embryos, but these eye structures begin to degenerate during larval stages. Eventually, only very small remnants of eye structures remain beneath a layer of epidermis and connective tissue in the adult. Surface fish also have normal pigmentation as adults while cavefish do not develop any significant amount of body melanin pigmentation. For the differential gene expression studies, I chose the 72 hour post-fertilization (hpf) larval stage for analysis because at this time point there are

Figure 3-1



**Figure 3-1. 72 hpf surface fish and cavefish.** A, Surface fish. B, Cavefish. Arrows indicate developing eyes of surface fish and cavefish. Arrowheads indicate areas of melanin pigment formation of the torso of the surface fish. Scale bar in A, 1 mm; magnification is the same in A,B.

significant differences in eye size and body pigmentation between surface fish and cavefish (Figure 3-1). Furthermore, programmed cell death is present in the cavefish eye, yet absent in the surface fish eye at this stage. These trends continue throughout development. In addition to these more obvious phenotypic differences, cavefish develop larger jaws, smaller optic tecta, and more tastebuds, among numerous other regressive and constructive traits (Jeffery, 2001).

In the present study, I used differential hybridization and microarray technology to discover candidate genes and genetic pathways that may be important in cavefish evolution. Borrowing from advancements in model organism research, I used zebrafish microarray chips. *Astyanax* sequences share considerable homology to zebrafish sequences, and of fish species commonly used in biological research, only goldfish are more closely related to zebrafish (Jeffery, 2001). Therefore, I expected enough cross hybridization of sequences to provide us with relevant genetic information. After I obtained specific information from my analyses, I provide data confirming trends and expression patterns relating to several genes. This information helps answer several questions concerning the evolution and development of *Astyanax*, while providing insights into future directions of research.

## **Materials and Methods**

### **Biological Materials**

Animal collection and care are as stated in part A of Materials and Methods in Chapter 1.

### **Differential Hybridization Experiments**

Surface fish and cavefish RNA was isolated from 72 hpf larva using the RiboPure RNA Isolation kit (Ambion, Austin, TX, USA). RNA was converted into double stranded cDNA using the cDNA Synthesis System kit (Roche Applied Science, Indianapolis, IN, USA). The differentially selected libraries were constructed according to the Clontech PCR-Select cDNA Subtraction Kit User Manual (BD Biosciences, Palo Alto, CA, USA). Surface fish and cavefish double stranded cDNA was digested using Rsa I, and adapters were ligated onto the digested products. These surface fish and cavefish tester cDNA's (the designated experimental samples) were then subjected to two rounds of differential selection using the opposing driver cDNA. For example, a putative up regulated surface fish gene library was eventually made using surface fish tester cDNA differentially selected by using cavefish driver cDNA, and vice versa. After selection, PCR amplification was performed, and the products were cloned into the pCRII-TOPO vector (Invitrogen, Carlsbad, CA, USA). Finally, these vectors

were transformed into Electro Ten-Blue Electroporation-Competent Cells (Stratagene, La Jolla, CA, USA). Bacterial colonies were chosen representing individual library clones. These colonies were grown in liquid culture in 96 well plates and stored as glycerol stocks at -80°C.

In an initial run of the experiment, I obtained clones that consisted almost entirely of  $\gamma$ -crystallin sequence of the same fragment length. To create libraries representing different types and sizes of cDNA I used two alternate techniques in addition to the original experimental procedure.

My first alternate library production technique included a gel extraction step. After the PCR amplification step, aliquots were run on an agarose gel. The portion of the gel above 1 kilobases was excised and gel extracted. These sample pools were cloned to produce surface fish and cavefish gel extracted/size selected differential cDNA libraries. To help obtain different clones I used Pml I, an enzyme that cuts a site within the  $\gamma$ -crystallin sequence, as another alternate library production technique. I subjected ligated library samples to restriction enzyme digestion and subsequently transformed the sample pools in the hopes that the blunt digested vectors would not transform with as much efficiency. These samples were also cloned to produce restriction digest selected libraries.

At this point I had six differentially selected libraries consisting of surface fish and cavefish original tester/driver libraries, surface fish and cavefish gel

extracted/size selected libraries, and surface fish and cavefish restriction digest selected libraries. Each library was represented by individual clones in a 96 well plate in glycerol stock form.

Each 96 well plate library was replica spotted onto nitrocellulose filters in quadruplicate and screened based on procedures outlined in the PCR-Select Differential Screening Kit User Manual (BD Biosciences, Palo Alto, CA, USA). For each of the six libraries, four different probe mixtures were used to screen each of the four individual replicate filters per library. Probes 1 and 2 consisted of surface fish and cavefish non-selected cDNA respectively. Probe 3 was made up of cavefish experimental tester cDNA differently selected using surface fish driver cDNA. Probe 4 was surface fish experimental tester cDNA differentially selected using cavefish driver cDNA. Probes were made using DIG DNA labeling mix (Roche Applied Science, Indianapolis, IN, USA) and the Random Primers DNA Labeling System (Invitrogen, Carlsbad, CA, USA). Spotted filters were hybridized with probe overnight at 72°C. These were subsequently processed and visualized using colorimetric methods according to the Roche Molecular Biochemicals DIG Application Manual (Roche Applied Science, Indianapolis, IN, USA). For each of the libraries, a clone was chosen to be analyzed based on quality of signal and/or frequency of appearance on the four filters. Chosen clones were grown up from their corresponding glycerol stock, minipreped, and sequenced. Sequences were analyzed by BlastX to determine gene homology or similarity.



## Microarray Procedures

Total RNA was extracted from 72 hpf surface fish and cavefish larva using the RiboPure RNA Isolation kit (Ambion, Austin, TX, USA). RNA was converted into double stranded cDNA. Biotin labeled antisense cRNA was produced from this cDNA template to use as microarray probes. Surface fish and cavefish labeled cRNA was hybridized to Affymetrix Zebrafish Genome Array chips with 16 oligonucleotide pairs representing each of over 14,900 transcripts (Affymetrix, Inc., Santa Clara, CA, USA). In total, five separate pairwise hybridizations were performed. Replications consisted of probes made from both surface fish and cavefish RNA extracted from three different pools of 72 hpf embryos obtained from separate spawning events. Each of these three RNA extractions was used for a separate hybridization experiment. Additionally, equal amounts of each of the three RNA samples were combined for the fourth sample run. Finally, in a separate event from the fourth sample run, I again combined equal amounts of each of the three original samples for the fifth sample run. Probes were hybridized to the arrays overnight at 45°C and subsequently washed, stained, and scanned based on procedures documented in the Affymetrix GeneChip Expression Analysis Technical Manual. Array chip scans were stored as digital CEL images for analysis purposes.

## Microarray Analysis

Microarray chip analysis was performed using the DNA-Chip Analyzer ([www.dchip.org](http://www.dchip.org); Li and Hung Wong, 2001a). Surface fish and cavefish CEL images from each of the five replications were loaded into dChip. CEL files were read and normalized using the invariant set normalization method (Li and Wong, 2001). Samples were then analyzed using the model-based expression method using the PM/MM difference model (Li and Wong, 2001b). A comparison analysis was performed using the five cavefish chip arrays as the baseline and the five surface fish arrays as the experimental samples. To perform the analysis, group means of intensity with standard error for each probe set were determined for both baseline (cavefish) and experimental (surface fish) samples. Comparison criteria of at least a two fold change between the means using the lower 90% confidence bound of fold change were used. During the analysis, a random permutation comparison was run 50 times to determine the false discovery rate of putative significant genes. This value was given as a median percent value. This 50 permutation analysis was repeated 20 times to determine a mean of the given percent values.

The main analysis results were further interpreted by entering the probe set identification numbers into an expression batch query to determine known gene homologies (<http://www.affymetrix.com/index.affx>). This analysis also provided known gene ontology (GO) information including descriptions of biological

processes, molecular functions, and cellular components. Probe sets not directly identified by the affymetrix expression batch query were subjected to a further analysis. Similarities or possible homologies for these probe sets were determined by performing a BlastX analysis on genome and probe sequence information included in the details for each probe set on the Affymetrix website.

### ***Rhodopsin and $\gamma$ M-Crystallin Gene Cloning***

RNA extraction, cDNA synthesis, and PCR amplification, and  *$\gamma$ M-crystallin* isolation techniques were performed according to the procedures outlined in section B of Chapter 2. The oligos used to amplify *rhodopsin* sequence were Rho1 (5'-ATGAACGGGACAGAGGGTCCATAC-3') and Rho2 (5'-TTATGCCGGGGACACGGAGGAGAC-3'). The PCR product was run on an agarose gel and bands of the expected size were gel extracted and cloned into the pPCR-Script AMP SK(+) vector (Stratagene, La Jolla, CA, USA). The  *$\gamma$ M-crystallin* sequence was cloned as described in section B of Chapter 2.

### ***In situ* Hybridization**

In situ analysis was performed based on procedures outlined in section C of Materials and Methods in Chapter 2.

### **Rhodopsin Immunohistochemistry**

Samples at the 10 days post-fertilization (dpf) age were fixed, embedded in paraplast, and cross sectioned at 8 $\mu$ m. Sections were incubated with a polyclonal antibody to rhodopsin (Leinco Technologies, Inc. St. Louis, MO, USA) according to a procedure used previously (Yamamoto and Jeffery, 2000). Staining was visualized using DAB substrate. The sections were counterstained with hematoxylin, mounted, and viewed by light microscopy.

### **Detection of PCD by TUNEL**

PCD was detected in 72 hpf larva based on the whole mount TUNEL procedure outlined in section E of Materials and Methods in Chapter 2.

## **Results**

### **Differential Hybridization Analysis**

I constructed surface fish and cavefish differentially selected libraries and screened them to select for genes overexpressed in one of the two forms at the 72 hpf stage. I chose this stage since obvious gross phenotypic differences between the larval forms are seen at this time. Initially, I obtained 24 putative upregulated surface fish clones. Seventeen of these clones consisted of nucleotide sequence identical to *γM-crystallin* (data not shown). I repeated this experiment with additional analyses to indentify genes other than *γM-crystallin*. In addition to repeating the initial experimental procedures (tester/driver experiment, Figure 3-2 and 3-3), I performed a gel selected experiment to exclude gene fragments smaller than 1kb since the *γM-crystallin* fragment was significantly smaller than 1kb (see size selected experiment, Figure 3-4 and 3-5). I also performed a restriction digest experiment to cut and exclude *γM-crystallin* fragments (see restriction digest experiment, Figure 3-5 and 3-6).

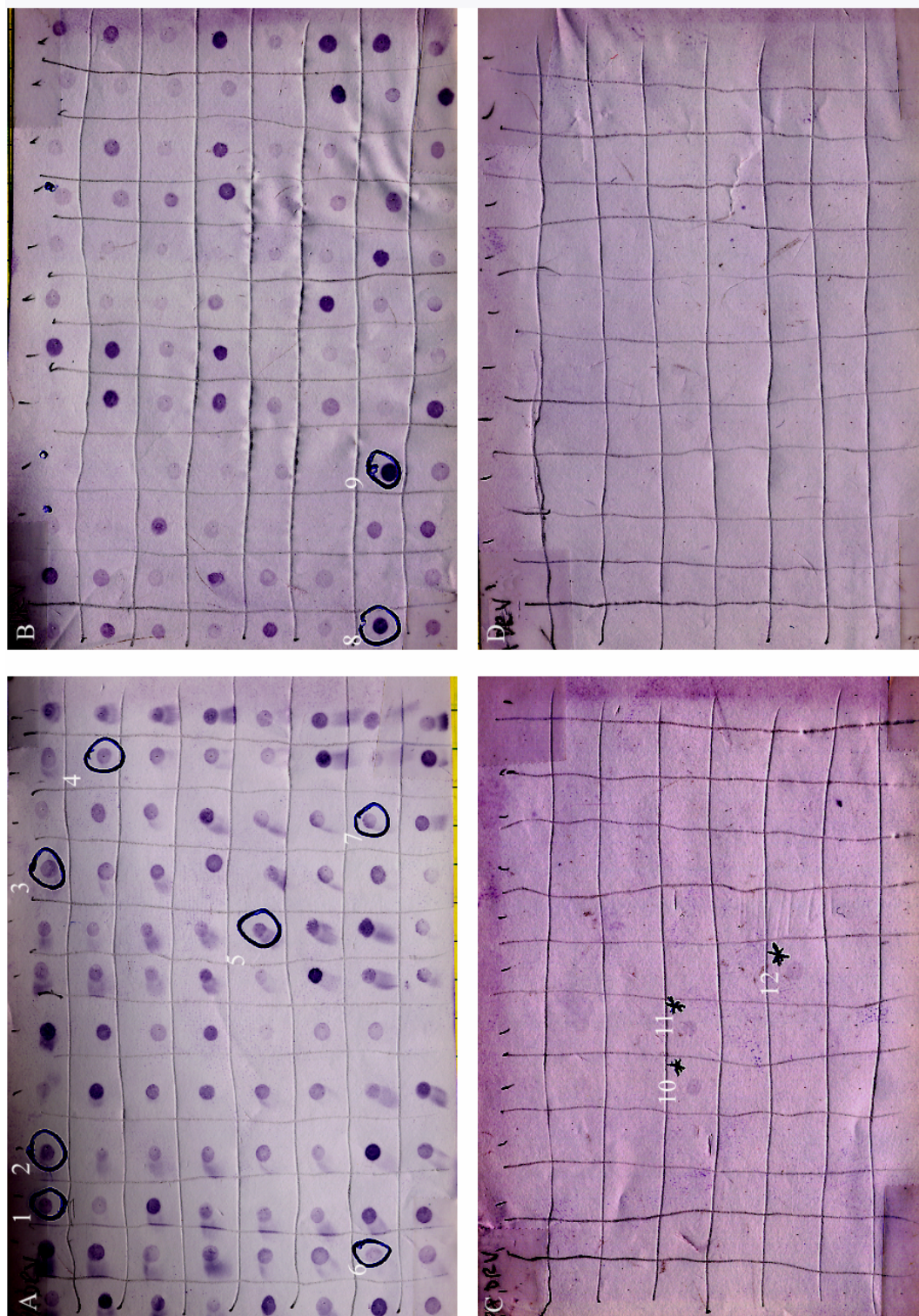
In the repeat analysis of the original tester/driver experiment, I found 13 putative significant clones upregulated in surface fish, and seven in cavefish (Table 3-1). The size selected experiment resulted in seven surface fish upregulated clones and seven cavefish upregulated clones (Table 3-2). I obtained eight upregulated surface fish clones, and 16 cavefish clones from the restriction digest experiment (Table 3-3).

## Microarray Analysis

To further analyze differential gene expression in *Astyanax*, I hybridized 72 hpf surface fish and cavefish RNA to Affymetrix Zebrafish Genome Array chips. My analysis revealed 67 putative significant genes, 61 of which were upregulated in surface fish, and six upregulated in cavefish (Table 3-4). These 67 genes are listed in Table 3-4 and are ordered based on absolute value of fold change. I assigned each probe set an arbitrary ID number for cross referencing purposes. The experimental surface fish samples are listed first as positive values, meaning upregulation in surface fish (ID numbers 1-61). These are followed by the baseline cavefish negative (absolute) values, indicating upregulation in cavefish (ID numbers 62-67).

During the dChip analysis process, a 50 replication permutation analysis was performed 20 times to determine a mean false discovery rate value of 13.4%. This translates into nine of the 67 putative genes being possible false discoveries. It is possible that probe sets with lower fold changes may have a higher chance of representing a false positive. However, without further analysis such as *in situ* hybridizations or real time PCR, it is impossible to identify which probe sets may represent actual false discoveries.

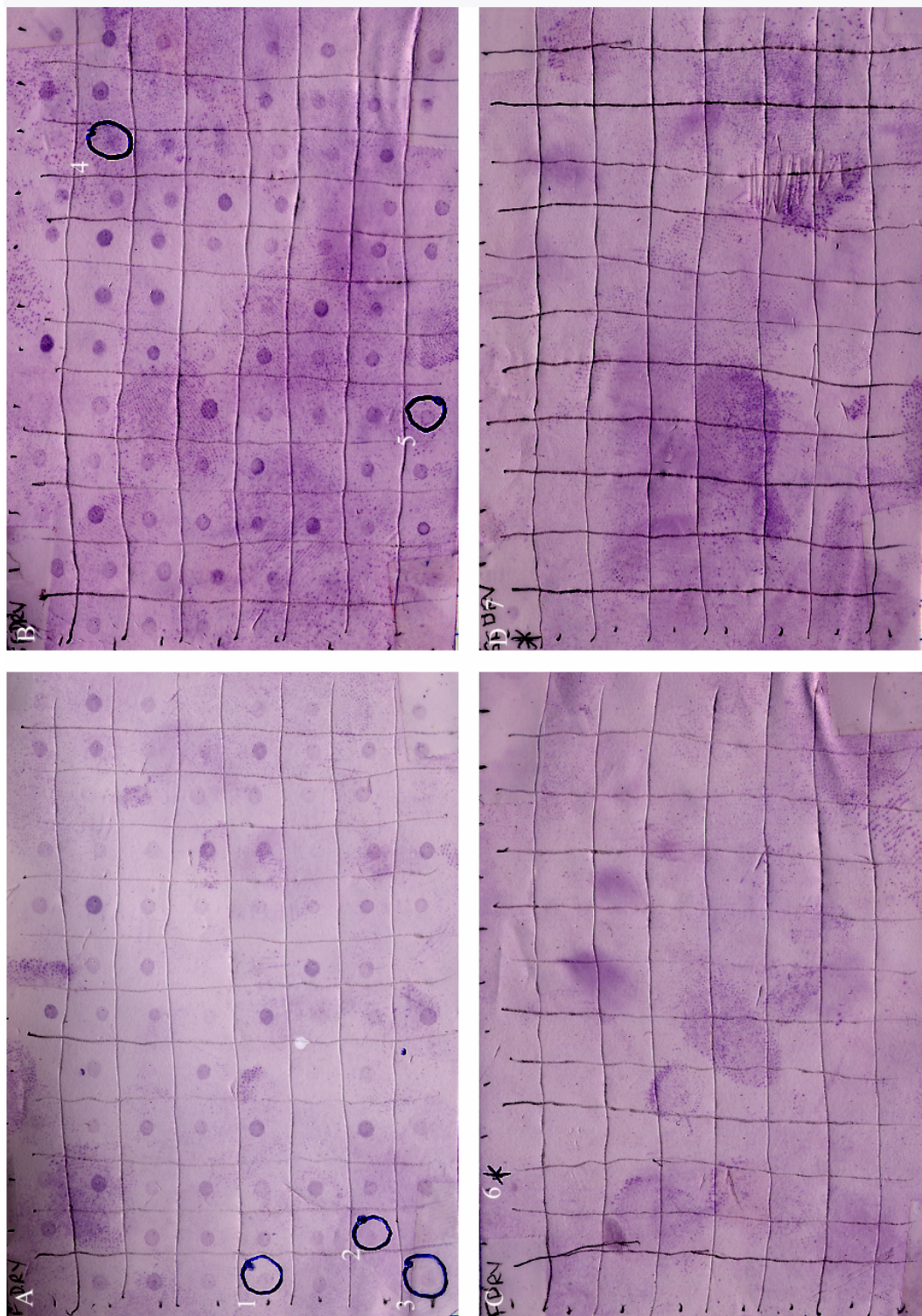
Figure 3-2



**Figure 3-2. Filter hybridizations for the original surface fish tester and cavefish driver experiment.** Each filter represents a replica spotting of the surface fish tester differentially subtracted library. Filter A was hybridized to surface fish cDNA probe. Filter B was hybridized to cavefish cDNA probe. Filter C was hybridized to surface fish driver/cavefish tester probe. Finally, filter D was hybridized to cavefish driver/surface fish tester probe. Circled spots represent clones chosen from filters probed with non-selected probe (A and B, numbers 1-9). Asterisks represent clones chosen from filters probed with selected probe (C and D, numbers 10-12).



Figure 3-3



**Figure 3-3. Filter hybridizations for the original cavefish tester and surface fish driver experiment.** Each filter represents a replica spotting of the cavefish tester differentially subtracted library. Filter A was hybridized to surface fish cDNA probe. Filter B was hybridized to cavefish cDNA probe. Filter C was hybridized to surface fish driver/cavefish tester probe. Finally, filter D was hybridized to cavefish driver/surface fish tester probe. Circled spots represent clones chosen from filters probed with non-selected probe (A and B, numbers 1-5). Asterisks represent clones chosen from filters probed with selected probe (C and D, numbers 6 and 7).

Table 3-1

## Tester/Driver Experiment – Up Regulated Surface Fish (Fig. 2)

- 
1. Unknown
  2. Unknown (Loc 402883 protein [*Danio rerio*])
  3. Ribosomal Protein L27a
  4. Unknown
  5. Gamma M Crystallin
  6. Goosecoid
  7. Unknown
  8. Unknown
  9. Unknown
  10. Unknown (Unnamed Protein Product)
  11. 40s Ribosomal Protein S20
  12. Unknown

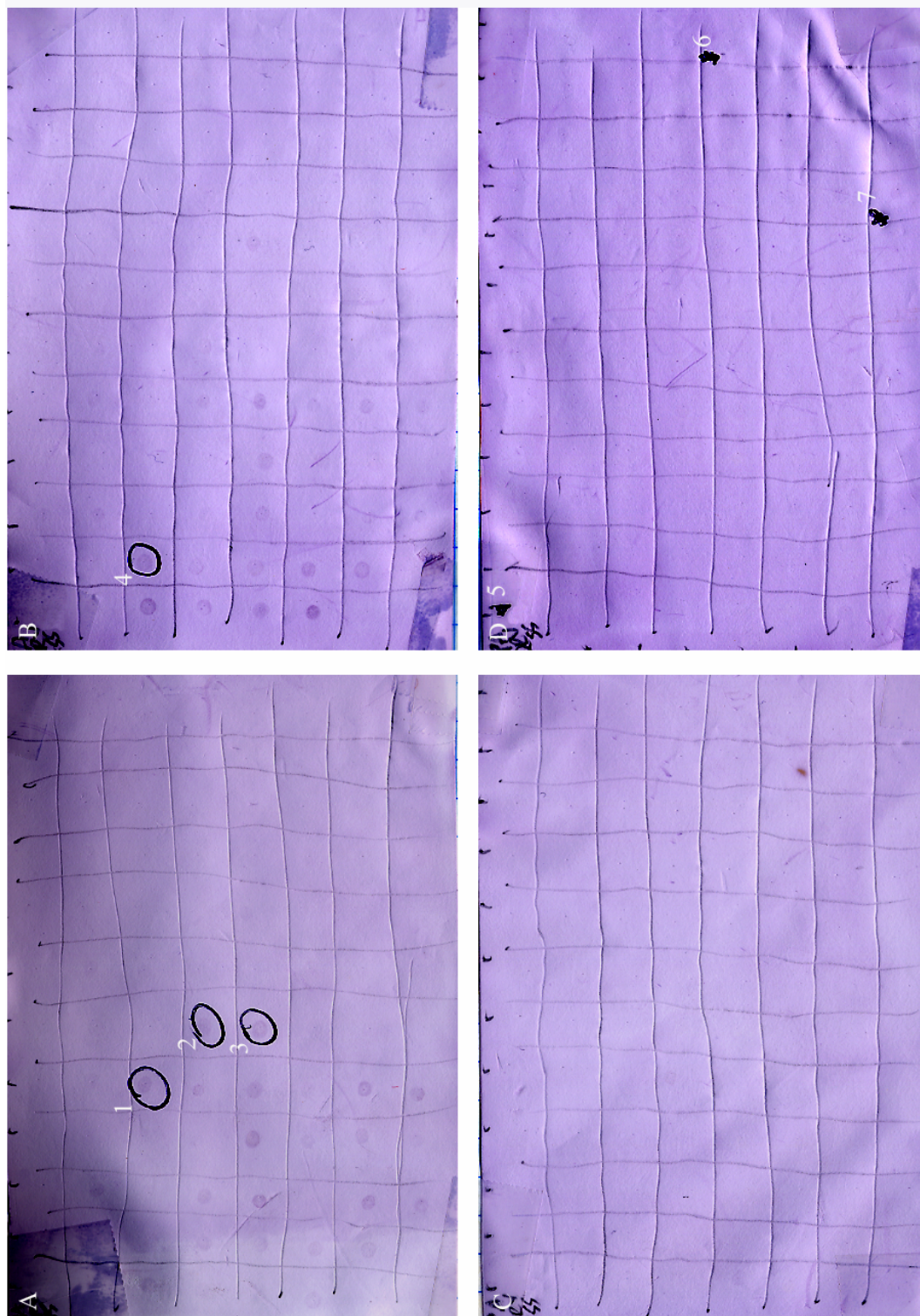
## Tester/Driver Experiment – Up Regulated Cavefish (Fig. 3)

- 
1. Unknown (Possible similarity to Alcohol Dehydrogenase)
  2. Unknown (Possible similarity to Gag-Pol Polyprotein Precursor)
  3. Unknown (Possible similarity to Formate Dehydrogenase)
  4. Unknown
  5. Unknown
  6. Eukaryotic Translation Elongation Factor 2
  7. Goosecoid

**Table 3-1. Individual sequenced clones from the original tester/driver experiment.** The top list of genes correspond to the numbered spotted clones in Figure 3-2 (surface fish tester library). The bottom list corresponds to the numbered spotted clones in Figure 3-3 (cavefish tester library).



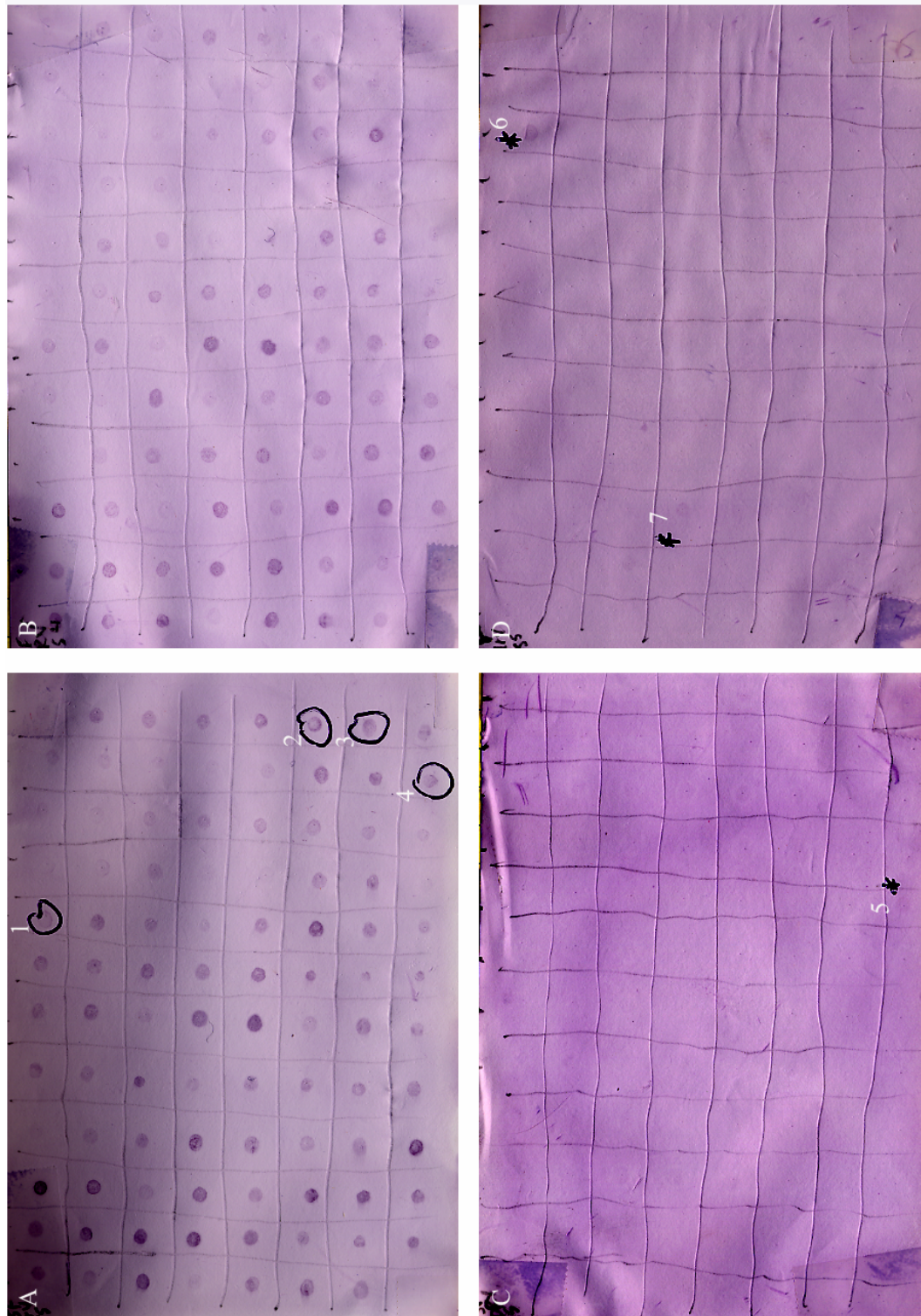
Figure 3-4



**Figure 3-4. Filter hybridizations for the gel extracted/size selected surface fish tester and cavefish driver experiment.** Each filter represents a replica spotting of the surface fish gel extracted/size selected tester differentially subtracted library. Filter A was hybridized to surface fish cDNA probe. Filter B was hybridized to cavefish cDNA probe. Filter C was hybridized to surface fish driver/cavefish tester probe. Finally, filter D was hybridized to cavefish driver/surface fish tester probe. Circled spots represent clones chosen from filters probed with non-selected probe (A and B, numbers 1-4). Asterisks represent clones chosen from filters probed with selected probe (C and D, numbers 5-7).



Figure 3-5



**Figure 3-5. Filter hybridizations for the gel extracted/size selected cavefish tester and surface driver experiment.** Each filter represents a replica spotting of the cavefish gel extracted/size selected tester differentially subtracted library. Filter A was hybridized to surface fish cDNA probe. Filter B was hybridized to cavefish cDNA probe. Filter C was hybridized to surface fish driver/cavefish tester probe. Finally, filter D was hybridized to cavefish driver/surface fish tester probe. Circled spots represent clones chosen from filters probed with non-selected probe (A and B, numbers 1-4). Asterisks represent clones chosen from filters probed with selected probe (C and D, numbers 5-7).



Table 3-2

Tester/Driver Size Selected Experiment – Up Regulated Surface Fish (Fig. 4)

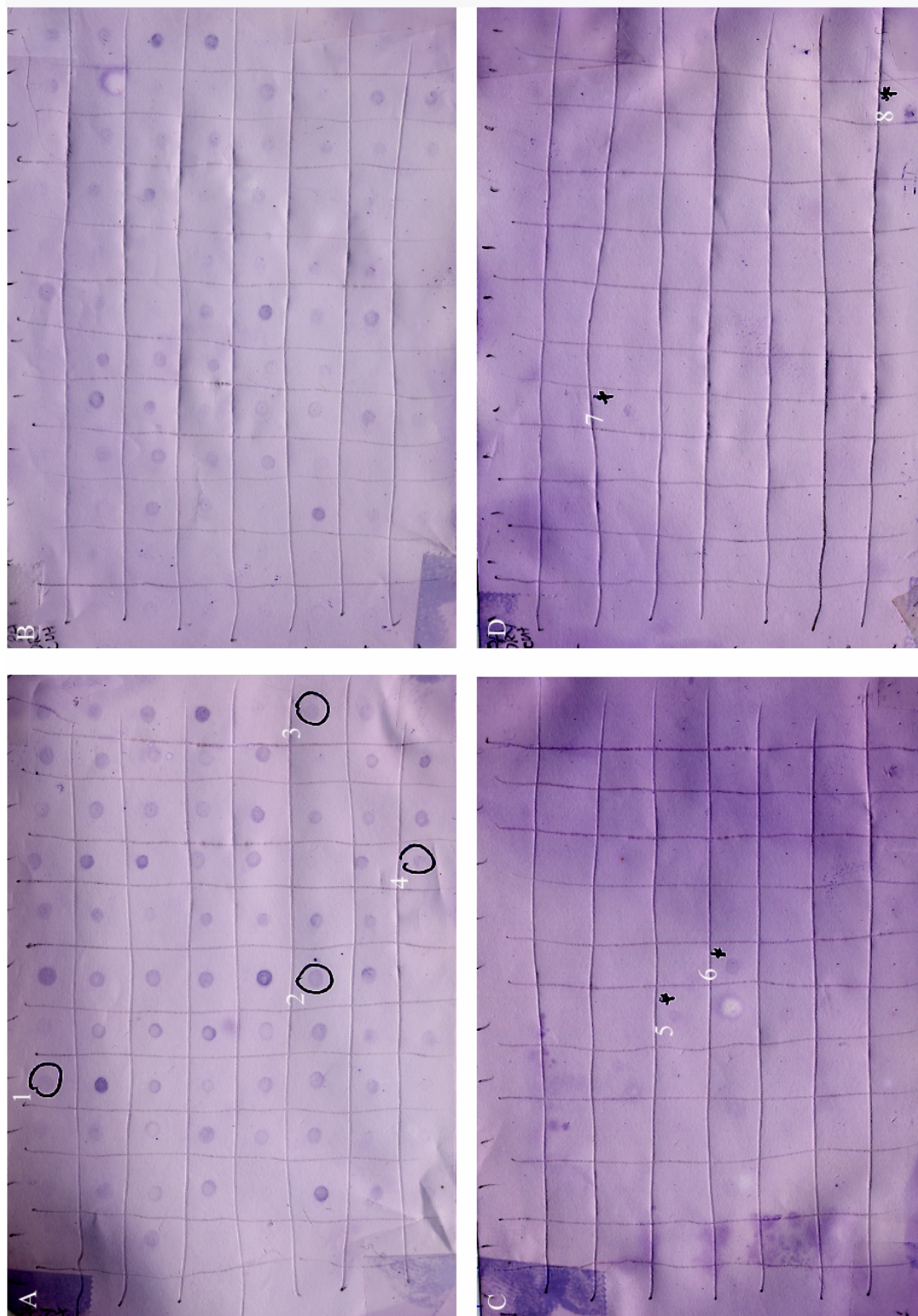
- 
1. Unknown (Unnamed Protein)
  2. Fast Muscle Troponin T Isoform TnnT3b
  3. Gamma M Crystallin
  4. Apolipoprotein A
  5. Unknown
  6. Unknown
  7. Goosecoid

Tester/Driver Size Selected Experiment – Up Regulated Cavefish (Fig. 5)

- 
1. Mannosyl (Alpha-1,6-)-Glycoprotein Beta-1,2-N-Acetylglucosaminyl Transferase
  2. Goosecoid
  3. Goosecoid
  4. Similar to Alcohol Dehydrogenase
  5. Goosecoid
  6. Cytochrome B
  7. Sox 3

**Table 3-2. Individual sequenced clones from the gel extracted/size selected experiment.** The top list of genes correspond to the numbered spotted clones in Figure 3-4 (surface fish gel extracted/size selected tester library). The bottom list corresponds to the numbered spotted clones in Figure 3-5 (cavefish gel extracted/size selected tester library).

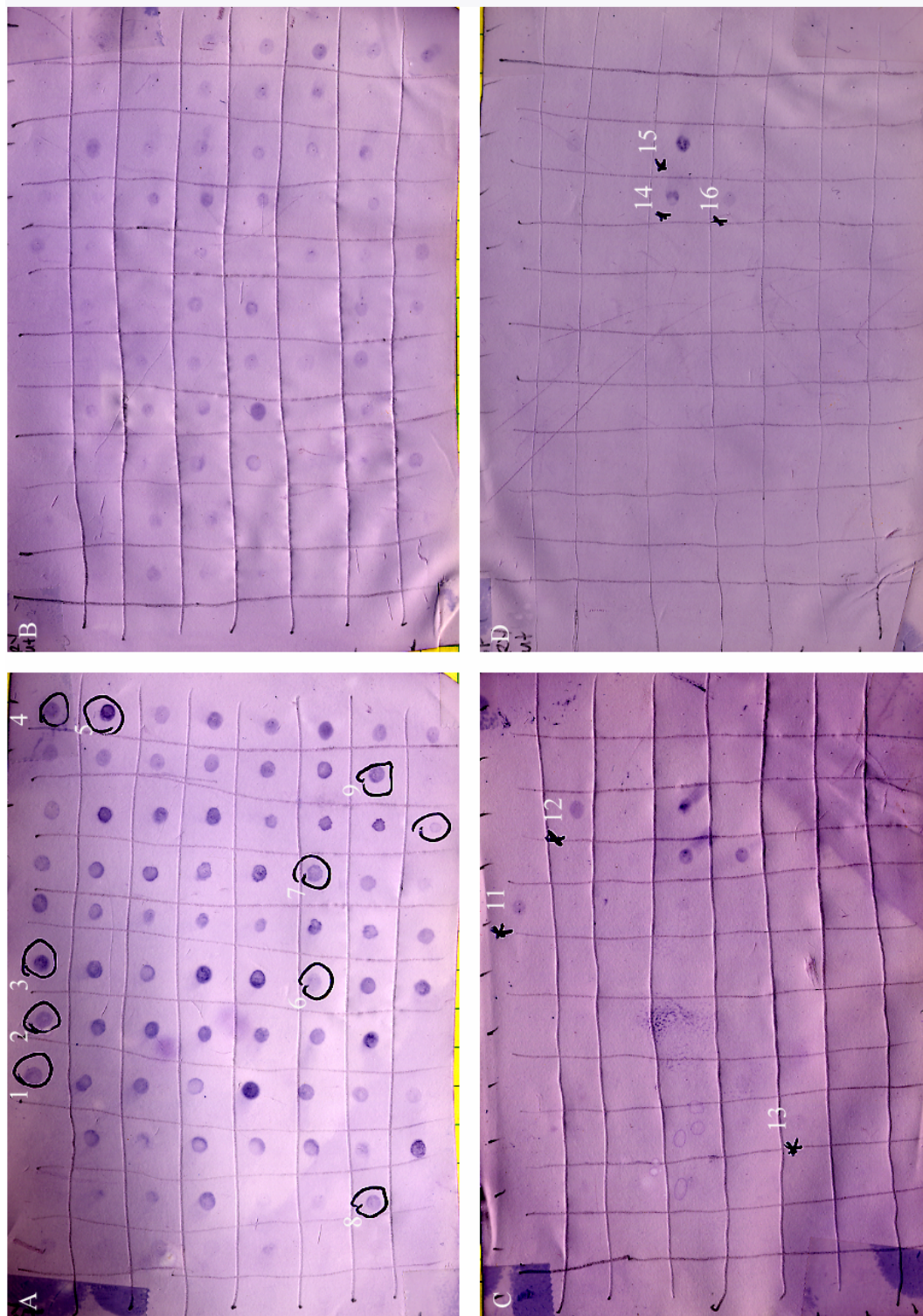
Figure 3-6



**Figure 3-6. Filter hybridizations for the restriction digest selected surface fish tester and cavefish driver experiment.** Each filter represents a replica spotting of the surface fish restriction digest selected tester differentially subtracted library. Filter A was hybridized to surface fish cDNA probe. Filter B was hybridized to cavefish cDNA probe. Filter C was hybridized to surface fish driver/cavefish tester probe. Finally, filter D was hybridized to cavefish driver/surface fish tester probe. Circled spots represent clones chosen from filters probed with non-selected probe (A and B, numbers 1-4). Asterisks represent clones chosen from filters probed with selected probe (C and D, numbers 5-8).



Figure 3-7



**Figure 3-7. Filter hybridizations for the restriction digest selected cavefish tester and surface fish driver experiment.** Each filter represents a replica spotting of the cavefish restriction digest selected tester differentially subtracted library. Filter A was hybridized to surface fish cDNA probe. Filter B was hybridized to cavefish cDNA probe. Filter C was hybridized to surface fish driver/cavefish tester probe. Finally, filter D was hybridized to cavefish driver/surface fish tester probe. Circled spots represent clones chosen from filters probed with non-selected probe (A and B, numbers 1-10). Asterisks represent clones chosen from filters probed with selected probe (C and D, numbers 11-16).

Table 3-3

Tester/Driver Restriction Digest Experiment – Up Regulated Surface Fish  
(Fig. 6)

- 
1. Unknown
  2. Similar to NADH Dehydrogenase
  3. Gamma M Crystallin
  4. 40s Ribosomal Protein S2
  5. Unknown
  6. Unknown
  7. Eukaryotic Translation Initiation Factor 4A, Isoform 2
  8. 40s Ribosomal Protein S26-1

Tester/Driver Restriction Digest Experiment – Up Regulated Cavefish  
(Fig. 7)

- 
1. Ribosomal Protein L17
  2. Beta Globin Type-3
  3. Probable Transmembrane Protein
  4. Ribosomal Protein S12
  5. Similar to Translation Elongation Factor
  6. Unknown
  7. Basic Transcription Factor 3
  8. Unknown
  9. Unknown
  10. Ribosomal Protein L-37
  11. Ribosomal Protein S-23
  12. Manganese-Containing Superoxide Dismutase Precursor
  13. Goosecoid
  14. Unknown
  15. Unknown
  16. Unknown

**Table 3-3. Individual sequenced clones from the restriction digest selected experiment.** The top list of genes correspond to the numbered spotted clones in Figure 3-6 (surface fish restriction digest selected tester library). The bottom list corresponds to the numbered spotted clones in Figure 3-7 (cavefish restriction digest selected tester library).



Probe sets similar or homologous to known genes based on the Affymetrix batch query are outlined in Table 3-5, Table 3-6, and Table 3-7. The identification numbers of these probe sets are provided for cross referencing purposes. Of the 67 putative significant probe sets, 31 had known similarities or homologies according to the Affymetrix database. All 31 of these probe sets were upregulated in surface fish. Known gene ontology descriptions are listed with their corresponding ID numbers and gene names in Tables 3-5, 3-6, and 3-7.

Finally, probe sets from the original analysis with no known similarities based on the Affymetrix database were subjected to a second round of sequence analysis using their probe set sequence details. These probe set sequences were subjected to both BLAST and web search analysis. A list of these probe sets is given in Table 3-8 with corresponding identification numbers and any additional information if it was encountered in the further BLAST and web analyses. After the initial Affymetrix expression batch query, which identified 31 probe sets mentioned previously, 36 unidentified probe sets remained consisting of 30 upregulated in surface fish probe sets, and the original six (ID's 62-67 of table 3-4) cavefish upregulated probe sets. I found further information for 16 of the 30 surface fish probe sets and three of the six cavefish probe sets.

Thus, Table 3-4 represents all probe sets revealed by my analysis (IDs 1-61 were upregulated in surface fish and IDs 62-67 were upregulated in cavefish). Of these original probe sets, the Affymetrix database could identify gene homology for 31

of the 61 surface fish upregulated probe sets and none of the six upregulated cavefish probe sets. These 31 putative upregulated surface fish probe sets are shown in Tables 3-5, 3-6 and 3-7. Information concerning the biological process, molecular function and cellular component of identified genes is provided when applicable based on information provided by the Gene Ontogeny (GO) database. However, the Affymetrix database positively identified several probe sets with genes for which there was no GO database information, or only partial information. For example, ID number 47 was identified as the *fbxl3a* gene by the Affymetrix database, but the GO database could not provide additional information on this gene (Table 3-7). The original probe sets that could not be identified as homologous to known genes are listed in Table 3-8. Some sequence information from the probe sets provided gene homology information not included in the Affymetrix database, and is indicated where appropriate.

Table 3-4

	ID No.	Probe Set	GenBank Accession No.	Lower Confidence Bound	Upper Confidence Bound	Fold Change
SF	1.	Dr.25729.1.S1_at	BM573934	43.89	99.73	61.21
	2.	Dr.19583.1.S1_at	AL915133	11.12	1E+08	33.48
	3.	Dr.9899.1.S1_at	NM_131868.1	15.64	134.78	29.53
	4.	Dr.16934.1.A1_at	BI325578	7.55	1E+08	25.9
	5.	Dr.354.1.S1_at	NM_131084.1	13.68	55.35	22.97
	6.	Dr.9881.1.S1_at	NM_131869.1	9.55	56.28	17.24
	7.	Dr.11355.1.A1_at	BG308730	6.39	1E+08	15.54
	8.	Dr.16690.1.A1_at	BI980401	3.56	1E+08	13.57
	9.	Dr.19653.1.S1_at	AL919725	7.4	28.81	12.26
	10.	Dr.3789.1.A1_at	BI890279	3.44	1E+08	9.75
	11.	Dr.26486.1.S1_at	BC053123.1	3.18	1E+08	9.1
	12.	Dr.10440.1.A1_at	BE605864	2.22	1E+08	7.79
	13.	Dr.16053.1.S1_at	BM531631	2.54	1E+08	7.6
	14.	Dr.7467.1.S1_at	BC044159.1	3.85	19.03	7.48
	15.	Dr.2401.1.A1_at	CD604147	3.01	153.57	7.01
	16.	Dr.22157.1.A1_at	AW019059	3.03	1E+08	6.98
	17.	Dr.9911.1.A1_at	AW826769	2.74	1E+08	6.69
	18.	Dr.9457.1.A1_at	BI671269	2.06	1E+08	6.27
	19.	Dr.25949.1.A1_at	CD605589	2.14	56.77	6.18
	20.	Dr.20815.2.S1_at	BQ783659	3.53	12.6	6.17
	21.	Dr.21025.1.A1_at	AF043903.1	3.26	20.11	6.13
	22.	Dr.12482.1.S1_x_at	BM036408	2.23	1E+08	6.03
	23.	Dr.11534.2.A1_at	AL729194	2.29	1E+08	5.96
	24.	Dr.11388.1.S1_at	BI889248	2.45	20.86	5.93
	25.	Dr.16478.1.A1_at	BI705297	2.13	92.88	5.93
	26.	Dr.7543.1.A1_at	AW019494	2.69	35.56	5.9
	27.	Dr.10103.1.A1_at	CB890995	2.66	46.89	5.85
	28.	Dr.11073.1.A1_at	AI588427	2.43	1E+08	5.83
	29.	Dr.26329.1.A1_at	BG728815	2.15	1E+08	5.82
	30.	Dr.16610.1.S1_at	BI983598	2.6	34.27	5.48
	31.	Dr.14072.1.S1_at	BC045370.1	2.36	24.34	5.4
	32.	Dr.12806.1.A1_at	BI879720	2.36	1E+08	5.26
	33.	Dr.5408.1.S1_at	BC046050.1	2.7	22.75	5.1
	34.	Dr.2197.1.A1_at	AI721729	2.48	19.13	4.9
	35.	Dr.15478.1.A1_at	BM316135	2.26	40.82	4.82
	36.	Dr.19902.2.A1_x_at	AW116196	2.33	24.1	4.75
	37.	Dr.22153.1.A1_at	AW019053	2.13	1E+08	4.72
	38.	Dr.4773.1.A1_at	AL715316	2.67	10.71	4.65
	39.	Dr.14662.1.S2_at	NM_131880.1	2.38	17.45	4.55
	40.	Dr.19425.1.S1_at	AB064939.1	2.23	14.93	4.46
	41.	Dr.9914.1.S1_at	BM181827	2.5	10.63	4.32
	42.	Dr.10326.1.S1_at	BC053234.1	2.05	1E+08	4.3
	43.	Dr.1002.1.S1_at	BM858769	2.24	11.02	4.21
	44.	Dr.14191.1.A1_at	BQ109774	2	19.97	4.19
	45.	Dr.22021.1.A1_at	AI957759	2.37	11.89	4.08
	46.	Dr.25151.1.S1_at	BC052115.1	2.33	8.94	3.97
	47.	Dr.16658.1.S1_at	BF937803	2.43	8.98	3.95
	48.	Dr.12300.1.A1_at	BG728602	2.18	8.32	3.85
	49.	Dr.25536.1.A1_at	BG306502	2.13	14.94	3.82
	50.	Dr.9662.1.S1_at	BQ261450	2.12	13.1	3.81
	51.	Dr.14840.1.A1_at	BM071859	2.12	9.74	3.79
	52.	Dr.18540.3.A1_at	BI883285	2.22	8.65	3.76
	53.	Dr.3338.1.S1_at	BC053268.1	2.01	6.47	3.63
	54.	Dr.26420.1.A1_at	CD606196	2.01	7.64	3.47
	55.	Dr.10904.1.S1_a_at	BC050486.1	2.2	6.32	3.4
	56.	Dr.16362.1.A1_at	BI430090	2.21	5.7	3.36
	57.	Dr.3523.1.A1_at	AI964241	2.01	7.23	3.32
	58.	Dr.9411.1.A1_at	AL909228	2	6.4	3.25
	59.	Dr.25595.1.A1_at	BM958030	2.15	4.92	3.22
	60.	Dr.16127.1.S1_at	BI887495	2.05	5	3.22
	61.	Dr.25707.1.A1_at	CD605002	2.01	5.76	3.13
CF	62.	Dr.16871.3.A1_at	AI544824	-2.01	-4.05	-2.82
	63.	Dr.18477.1.A1_at	BM154222	-2.03	-16.02	-4
	64.	Dr.18973.1.A1_at	BI709496	-2.56	-1E+08	-8.46
	65.	Dr.7488.1.S1_at	BC044544.1	-2.55	-1E+08	-10.78
	66.	Dr.20823.1.A1_at	BI326634	-4.02	-1E+08	-42
	67.	Dr.14618.1.A1_at	BM185013	-13.29	-1E+08	-1474.26

**Table 3-4. List of putative genes by probe set obtained from the surface fish versus cavefish microarray experiment.** The top list comprising ID numbers 1-61 represents probe sets that were upregulated in surface fish (SF). ID numbers 62-67 represent probe sets that were upregulated in cavefish (CF). The lower 90% and upper 90% confidence bound are given for each fold change. Entries are listed in descending order by absolute value fold changes with ID number 1 representing the putative surface fish gene with the highest fold expression increase over cavefish and ID number 67 representing the putative cavefish gene with the highest fold expression increase over surface fish.



Table 3-5

ID No.	Gene Title	Gene/Locus Symbol	GO Biological Process Description	GO Molecular Function Description	GO Cellular Component Description
1.	similar to Gamma crystallin B (Gamma crystallin 1-2)	zgc:86750 zgc:92724 zgc:112327 LOC558501 LOC561929 LOC566558 LOC569000	homophilic cell adhesion blood coagulation signal transduction G-protein coupled receptor protein signaling pathway	transmembrane receptor activity calcium ion binding signal transducer activity GTP binding guanyl nucleotide binding	membrane
2.	crystallin, gamma M4	crygm4			
3.	guanine nucleotide binding protein (G protein), alpha transducing activity polypeptide 1	gnat1			
4.	similar to BarH	zgc:113948 BarH	regulation of transcription, DNA-dependent regulation of transcription signal transduction G-protein coupled receptor protein signaling pathway sensory perception visual perception phototransduction rhodopsin mediated phototransduction protein-chromophore linkage	DNA binding transcription factor activity	nucleus
5.	rhodopsin	rho		rhodopsin-like receptor activity signal transducer activity receptor activity G-protein coupled receptor activity photoreceptor activity retinal binding	integral to membrane
6.	guanine nucleotide binding protein (G protein), alpha transducing activity polypeptide 2	gnat2	signal transduction G-protein coupled receptor protein signaling pathway detection of light stimulus during visual perception regulation of transcription, DNA-dependent development regulation of transcription actin cytoskeleton organization and biogenesis	signal transducer activity GTP binding guanyl nucleotide binding	
11.	similar to Six3/Six6/Optx2	zgc:63871		DNA binding transcription factor activity	nucleus
12.	similar to F-actin capping protein alpha-1 subunit (CapZ alpha-1)	zgc:101755 LOC555818 LOC556325		actin binding	F-actin capping protein complex

**Table 3-5. Genes from the *Astyanax* microarray analysis with homology to the Gene Ontogeny database (all surface fish upregulated).** Probe sets with known gene homology from Table 3-4 are represented by their corresponding ID number. In addition to the gene title and catalogued gene or locus symbol, any known gene ontology (GO) data based on biological process, molecular function, and cellular component is included. Blank spaces indicates no information in the GO database.

Table 3-6

ID No.	Gene Title	Gene/Locus Symbol	GO Biological Process Description	GO Molecular Function Description	GO Cellular Component Description
13.	Transcribed locus, weakly similar to heparin-binding EGF-like growth factor [Gallus gallus]				
15.	upstream transcription factor 2, c-fos interacting	usf2	regulation of transcription	transcription regulator activity	nucleus
18.	egl nine homolog 3 (C. elegans)	egl n3	protein metabolism	oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen, 2-oxoglutarate as one donor, and incorporation of one atom each of oxygen into both donors	
20.	crystallin, gamma M2c	crygm2c		calcium ion binding	membrane
21.	protocadherin a	pcdha	homophilic cell adhesion	catalytic activity	membrane integral to membrane
24.	ATPase type 13A	atp13a	cation transport metabolism	ATPase activity, coupled to transmembrane movement of ions, phosphorylative mechanism hydrolase activity, acting on acid anhydrides, catalyzing transmembrane movement of substances ATPase activity nucleotide binding magnesium ion binding hydrolase activity metal ion binding	
29.	clathrin, heavy polypeptide (Hc)	cltc		binding	clathrin coat of coated pit
31.	ATPase, H+ transporting, V1 subunit D	atp6v1d	ATP synthesis coupled proton transport	hydrogen-transporting ATP synthase activity, rotational mechanism	proton-transporting two-sector ATPase complex

**Table 3-6. Continuation of the table of genes from the *Astyanax* microarray analysis with homology to the Gene Ontogeny database (all surface fish upregulated).** Probe sets with known gene homology from Table 3-4 are represented by their corresponding ID number. In addition to the gene title and catalogued gene or locus symbol, any known gene ontology (GO) data based on biological process, molecular function, and cellular component is included. Blank spaces indicates no information in the GO database.



Table 3-7

ID No.	Gene Title	Gene/Locus Symbol	GO Biological Process Description	GO Molecular Function Description	GO Cellular Component Description
33.	RAD54-like ( <i>S. cerevisiae</i> )	rad54l	DNA repair response to DNA damage stimulus	nucleic acid binding DNA binding DNA binding helicase activity ATP binding DNA-dependent ATPase activity hydrolase activity nucleotide binding	nucleus
35.	similar to RAD21 homolog	LOC571082			
39.	transcription factor 2, hepatic	tcf2	regulation of transcription, DNA-dependent proteolysis anterior/posterior pattern formation positive regulation of transcription	DNA binding transcription factor activity subtilase activity transcriptional activator activity protein dimerization activity zinc ion binding	nucleus
40.	polyhomeotic Ph1 homolog	ph1			nucleus
42.	jun B proto-oncogene	junb	regulation of transcription, DNA-dependent isoprenoid biosynthesis	DNA binding transcription factor activity	nucleus
46.	geranylgeranyl diphosphate synthase 1	ggps1			
47.	F-box and leucine-rich repeat protein 3A	fbx3a			
49.	similar to Heat shock protein HSP 90-alpha (HSP 86)	LOC565155			
53.	hydroxymethylbilane synthase	hmbs	porphyrin biosynthesis	hydroxymethylbilane synthase activity	
55.	adducin 3 (gamma)	add3			
56.	similar to SRR1-like protein	LOC562374			
57.	similar to Acyl-Coenzyme A dehydrogenase, very long chain	LOC573723			
58.	similar to protein tyrosine phosphatase CRYP-2	LOC572179			
59.	synaptophysin-like protein	sypl	transport	transporter activity	synaptic vesicle membrane
60.	similar to phosphodiesterase 4B	LOC570353			

**Table 3-7. Continuation of the table of genes from the *Astyanax* microarray analysis with homology to the Gene Ontogeny database (all surface fish upregulated).** Probe sets with known gene homology from Table 3-4 are represented by their corresponding ID number. In addition to the gene title and catalogued gene or locus symbol, any known gene ontology (GO) data based on biological process, molecular function, and cellular component is included. Blank spaces indicates no information in the GO database.

Table 3-8

	ID No.	Probe Set	Similarity, Homology, or Other Notes
SF	7.	Dr.11355.1.A1_at	Unknown - Obtained from adult zebrafish retina cDNA library
	8.	Dr.16690.1.A1_at	WRNIP1 - Werner helicase interacting protein 1
	9.	Dr.19653.1.S1_at	NF-M - Middle-molecular weight neurofilament gene
	10.	Dr..3789.1.A1_at	Unknown
	14.	Dr.7467.1.S1_at	TPO1 - Topoisomerase 1
	16.	Dr.22157.1.A1_at	BBX - Bobby sox homolog
	17.	Dr.9911.1.A1_at	Cytokine inducible SH2-containing protein
	19.	Dr.25949.1.A1_at	Unknown
	22.	Dr.12482.1.S1_x_at	Unknown
	23.	Dr.11534.2.A1_at	BRD8 - Bromodomain-containing protein 8
	25.	Dr.16478.1.A1_at	Unknown
	26.	Dr.7543.1.A1_at	Similar to F-box leucine rich repeat protein 3A
	27.	Dr.10103.1.A1_at	Stromal cell derived factor receptor 1 isoform a isoform 3
	28.	Dr.11073.1.A1_at	Unknown
	30.	Dr.16610.1.S1_at	Unknown
	32.	Dr.12806.1.A1_at	Unknown - Obtained from adult zebrafish retina cDNA library
	34.	Dr.2197.1.A1_at	Unknown
	36.	Dr.19902.2.A1_x_at	Putative protein family member (XC177)
	37.	Dr.22153.1.A1_at	ATP-binding cassette transporter sub-family G member 2a
	38.	Dr.4773.1.A1_at	cGMP-gated cation channel, rod photoreceptor
	41.	Dr.9914.1.S1_at	Srprb - Signal recognition particle receptor, B subunit
	43.	Dr.1002.1.S1_at	Unknown
	44.	Dr.14191.1.A1_at	Unknown
	45.	Dr.22021.1.A1_at	Unknown
	48.	Dr.12300.1.A1_at	Unknown
	50.	Dr.9662.1.S1_at	MHC class II receptor activity
	51.	Dr.14840.1.A1_at	Unknown
	52.	Dr.18540.3.A1_at	Unknown
	54.	Dr.26420.1.A1_at	Unknown
	61.	Dr.25707.1.A1_at	Zinc finger protein subfamily 1A 5
CF	62.	Dr.16871.3.A1_at	Unknown
	63.	Dr.18477.1.A1_at	Unknown
	64.	Dr.18973.1.A1_at	Neurologin
	65.	Dr.7488.1.S1_at	TMEM33 - Transmembrane protein 33
	66.	Dr.20823.1.A1_at	Unknown
	67.	Dr.14618.1.A1_at	Ubiquitin specific protease 53

**Table 3-8. List of probe sets without confirmed gene homology.** Remaining probe sets from Table 3-4 not represented in Table 3-5, 3-6, and 3-7 are listed according to their ID number. The probe set sequence data was analyzed further, and resulting data is displayed in the right column. The top portion of the table represents probe sets up regulated in surface fish (SF), and the bottom portion (62-67) represents probe sets up regulated in cavefish (CF).

### **Rhodopsin , $\gamma$ -Crystallins, and Genes Involved in Programmed Cell Death**

I obtained several genes in my analyses that are associated with PCD. I performed PCD assays on developing surface and cavefish previously (see I and J of Figure 2-13 of Chapter 2). My differential analyses also indicated that  $\gamma$ -crystallin may be upregulated in surface fish. As with PCD, I examined the expression of  $\gamma M$ -crystallin in previous studies (see I and J of Figure 2-8 of Chapter 2). Based on my *in situ* experiments, the spatial distribution and expression level of  $\gamma M$ -crystallin transcripts are reduced in the cavefish lens compared to surface fish at the 72 hpf stage, supporting the differential hybridization and microarray results.

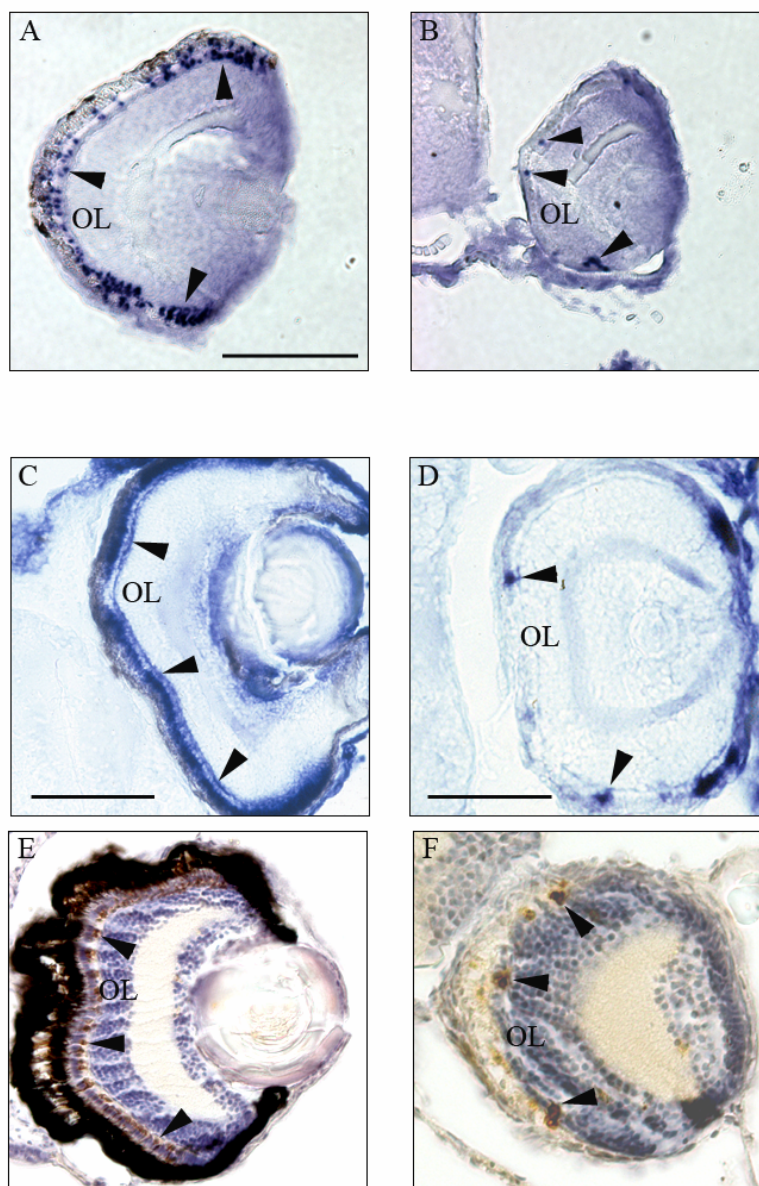
In addition to  $\gamma M$ -crystallin *in situ* hybridization experiments, I also checked the expression of *rhodopsin* in surface fish and cavefish. The results are shown in Figure 3-8. At the 72 hpf stage, abundant *rhodopsin* transcripts are found in the developing outer layer of the surface fish retina (arrowheads in Fig. 3-8, A). In contrast, the spatial distribution of *rhodopsin* in the developing outer layer of the cavefish retina is severely reduced (arrowheads in Fig. 3-8, B).

I also checked rhodopsin mRNA and protein expression at the 10 dpf stage to confirm that the trend seen at 72 hpf continues throughout development. In the 10 dpf surface fish retina, a solid line of expression can be seen just inside the retinal pigmented epithelium (arrowheads in Fig. 3-8, C). Only small areas of expression

domain could be found in the cavefish retina at this stage, confirming the microarray analysis results (arrowheads in Fig. 3-8, D). To confirm that rhodopsin mRNA is being transcribed into protein, I subjected 10 dpf sections of *Astyanax* to immunohistochemistry using an antibody to rhodopsin. I found that the distribution of rhodopsin protein is similar to areas of mRNA expression in the outer layer of both the surface fish retina (arrowheads in Fig. 3-8, E) and cavefish retina (arrowheads in Fig. 3-8, F).



Figure 3-8



**Figure3-8. Rhodopsin mRNA and protein expression in surface fish and cavefish.** A, C, E, Surface fish. B, D, F, Cavefish. Larva were subjected to whole mount *in situ* hybridization and subsequently embedded and cross sectioned at the 72 hpf (A,B) and 10 dpf (C,D) stages. Larva were also subjected to rhodopsin immunohistochemistry staining , embedded, and cross sectioned at the 10 dpf stage (E,F). Arrowheads indicate areas of staining. Abbreviation: ONL, outer nuclear layer. Scale bar in A, 100um; magnification is the same in A,B. Scale bar in C, 200um; magnification is the same in C,E. Scale bar in D, 100um; maginification is the same in D,F.



## **Discussion**

### **Differential Hybridization and Microarray Analysis**

I used differential hybridization techniques to reveal genes overexpressed in surface fish compared to cavefish, and vice versa. The studies were done on the 72 hpf larval stage. I chose this stage for several reasons. At this time, there is a significant difference in eye size between the two forms. Furthermore, melanin pigmentation is visible in the surface fish, but not the cavefish (see Figure 3-1). I was also aware of elevated amounts of PCD in the cavefish eye at the 72 hpf stage (unpublished data). An initial differential hybridization experiment yielded a library which was enriched with surface fish up regulated  $\gamma$ -M-crystallin sequences. Despite the relevance of this gene to changes in lens development, I hoped to find other significant genes as well. Thus, in addition to producing surface fish and cavefish differential libraries by repeating the original experimental procedures, I performed two additional experimental manipulations to produce two other differential libraries. The latter treatments were performed in an attempt to enrich the applicable library with other gene fragments. Although the differential libraries of the additional experimental treatments still yielded  $\gamma$ -crystallin clones, I also obtained other sequences of interest.

The three pairs of differential library screening experiments yielded a total of 57 putative significant clones, consisting of 27 putative upregulated surface fish clones, and 30 putative upregulated cavefish clones. Of the putative upregulated

clones, 14 surface fish and 11 cave fish sequences could not be identified by homology or similarity to other known sequences.

In addition to producing and screening differential libraries, I also performed a microarray analysis on 72 hpf surface fish and cavefish RNA. *Astyanax mexicanus* microarray chips do not exist. However, based on previous experiments showing that zebrafish sequences can hybridize to *Astyanax* sequences, I chose to use Affymetrix Zebrafish Genome chips to perform the experiments (Meijer et al., 2005). It appears this specific type of microarray cross hybridization experiment is viable based on my results and the work of others (Kayo et al., 2001). As mentioned previously, *Astyanax* and zebrafish are fairly closely related and zebrafish sequences have been used previously in *Astyanax* studies (Jeffery and Martasian, 1998).

The microarray analysis resulted in 67 putative significant probe sets, with each probe set corresponding to a specific zebrafish gene. Of these, I obtained 61 putative upregulated surface fish probe sets, and 6 putative upregulated cave fish probe sets. Of the surface fish up regulated probe sets, the highest mean fold change samples included  $\gamma$ -crystallin B at 61.21 fold change over cavefish,  $\gamma$ -crystallin M4 at 33.48, guanine nucleotide binding protein alpha transducing activity polypeptide 1 (Gnat1) at 29.53, a BarH-like sequence at 25.9, and rhodopsin at 22.97. The highest fold changes for probe sets upregulated in cavefish consisted of a set similar to ubiquitin specific protease 53 at a 1474.26

change, an unidentified probe set at 42, and probe set similar to transmembrane protein 33 (TMEM33) at 10.78.

By using lower 90% confidence of fold changes in addition to two fold change comparison in mean probe intensity values I feel I have achieved a sufficient amount of stringency in significant probe set identification. During the comparison analysis, probe set error analysis is performed resulting in confidence bound values defined as the 90<sup>th</sup> percentile value above or below the mean fold change. Thus for a given probe set comparison, the lower confidence bound value is the lower 90% cutoff range of fold change based on average intensities of probe pairs within a probe set. This means that for different probe sets with similar absolute fold changes, the probe set with the smaller total confidence interval is more reliable. This type of analysis accounts for probe intensity irregularities that may result from inherent hybridization dynamics or experimental anomalies. For example, ID numbers 19 and 20 have fold changes of 6.18 and 6.17, respectively. However, 19 has a confidence interval of 54.63 (upper confidence bound – lower confidence bound = confidence interval), while 20 has a confidence interval of 9.07. Based on this analysis, ID number 20 is a more reliable probe set specific to my experiment. Thus, when interpreting possible importance of probe sets, one must consider both the absolute mean fold change and its upper and lower bound confidence interval.

During the comparison analysis, I also performed random permutation comparisons to determine false discovery rates. Each permutation comparison consisted of 50 random permutations of chip data. This was repeated 20 times to determine a mean false discovery rate of 13.4%. With 67 total probe sets, this translates into nine of the probe sets being false discoveries. There are no criteria to further determine which probe sets may be false discoveries other than performing experiments such as *in situ* hybridizations or real time PCR. However, it is possible to use this data, along with fold change and confidence bounds, to determine which probe sets may be more likely to reveal genes having higher significance relating to *Astyanax* development and evolution.

It is important to remember that upregulation of a gene in one form of *Astyanax* is a relative term. This designation is analogous to the downregulation of that same gene in the other form. For example, when we speak of a gene that is upregulated in surface fish, the possibility exists for the gene to not truly be upregulated in surface fish, but to instead be downregulated in the cavefish. Regardless of how it is stated, the end meaning is that there are more copies of mRNA for that gene in the 'upregulated' form and fewer copies in the form where it is 'downregulated'.

### Specific Genes Identified by Differential Hybridization

The analyses revealed several genes with possible roles in surface fish and cavefish development. The tester/driver differential hybridization experiment indicated  $\gamma$ -M-crystallin as a gene upregulated in surface fish compared to cavefish. The lens is primarily made up of crystallins, of which  $\gamma$ -crystallins are the most abundant in teleosts (Pan et al., 1995). It is not surprising that levels of  $\gamma$ -M-crystallin RNA could be lower in the cavefish lens when compared to the surface fish. Despite the fact that I performed specific experimental procedures to exclude  $\gamma$ -M-crystallin from the size selected and restriction digest differential libraries, it still appeared in these libraries as an upregulated gene in surface fish.

Goosecoid is another possible interesting gene uncovered in the differential hybridization experiments. During embryonic stages, goosecoid is expressed in the organizer (Eivers et al., 2004). In the post hatching stage larva, goosecoid is expressed in the ventral head region which eventually forms the cartilage of the developing lower jaw (Miyoshi et al., 2006). The lower jaw is larger in cavefish, and there are differences in craniofacial structures in surface fish versus cavefish (Jeffery, 2001). Goosecoid has been isolated from *Astyanax* by another investigator, and has been shown to be upregulated along the cavefish anterior midline during embryogenesis (Yamamoto, unpublished data). Embryogenesis occurs until approximately 24 hpf, but my analysis involved 72 hpf larva. However, I found the goosecoid gene in several of the cavefish differential

libraries. Surprisingly, I found a goosecoid clone in the upregulated surface fish tester/driver library. Based on this information, it is possible that goosecoid is a false positive. Other than performing further experiments such as *in situ* hybridization, there is no way to determine if any gene obtained from the differential hybridization analysis is truly upregulated in surface fish or cavefish. Despite these inconclusive results, it may be interesting to study goosecoid gene expression and functionality in *Astyanax* development at the 72 hpf stage.

In the upregulated surface fish size selected library, I found a gene fragment representing Troponin T isoform 3b (*tnnt3b*). This isoform is expressed in fast muscle, but not slow muscle (Hsiao et al., 2003). There is evidence that *sonic hedgehog* (*shh*) is involved in slow muscle development (Barresi et al., 2000; Blagden et al., 1997). Furthermore, anterior midline expression of *shh* during early development is increased in cavefish compared to surface fish (Yamamoto et al., 2004). This increase in *shh* expression has been implicated as a pleiotropic mechanism in *Astyanax* development (Jeffery, 2005). Based on the information that *tnnt3b* may be upregulated in surfacefish and that *shh* mediates slow muscle development in teleosts, it is possible that cavefish have more slow muscle than surface fish. This would not be surprising based on observed lifestyles of the two forms. Cavefish are found in stagnant pools of water, while surface fish are found in fast moving waters of surface creeks and streams. It is possible that the development of slow muscle may be influenced by differences in *shh* expression in cavefish.

In this same surface fish upregulated library, I found apolipoprotein A. During larval zebrafish stages, apolipoproteins are expressed in the brain and eyes (Babin et al., 1997). Like other genes expressed in the eye, this has potential to be significant in relation to surface fish versus cavefish development.

Apolipoprotein may reveal important correlative trends. This gene is involved in lipid metabolism, which has been shown to be a phenotypic difference in cavefish versus surface fish (Jeffery, 2001). It is also expressed in the retina of zebrafish during a comparable developmental stage to the 72 hpf stage of *Astyanax*.

Additionally, there is evidence that a high lipid diet linked with a certain allele of apolipoprotein is sufficient to cause retina degeneration (Malek et al., 2005).

Thus a connection between visual systems and lipid metabolism exists.

Furthermore, there are studies in mouse suggesting a link among lipid metabolism, nitric oxide synthase, and apolipoprotein (Miyoshi et al., 2006).

Nitroxidative stress caused by nitric oxide is a hallmark of a series of cellular events involving Rad54, an apoptosis related gene discussed under the microarray analysis results. It will be interesting to determine if there are links among retina development, programmed cell death, and lipid metabolism in *Astyanax*.

In the upregulated cavefish size selected library I discovered Sox3. Sox3 is important for neural development and is specifically involved in forebrain development (Ryan et al., 1998; Wood and Episkopou, 1999). It has been determined that the telencephalon (forebrain) of the cavefish has undergone constructive evolution (Peters et al., 1993). Sox3 may play a developmental role

in this constructive change. Although not previously studied, it is possible that the olfactory system and corresponding portions of the telencephalon are enhanced in cavefish.

Another gene from the upregulated cavefish size selected library was alcohol dehydrogenase (adh). This gene is involved in numerous physiological processes including nitric oxide homeostasis as well as cellular nitrosative and oxidative stress responses (Canestro et al., 2003). Furthermore, nitric oxide and oxidative stress have been implicated in apoptotic processes (Li and Wogan, 2005; Naoi et al., 2005). Therefore, it is feasible that adh may be upregulated in cavefish as a response to programmed cell death occurring in the eye at the 72 hpf stage. In relation to oxidative stress responses, I obtained a gene fragment representing manganese-containing superoxide dismutase precursor from the upregulated cavefish restriction digest library. Superoxide dismutase is an indicator of oxidative stress and serves a role in ameliorating tissue damage due to such stress (Munoz-Casares et al., 2006; Valko et al., 2006). It is possible that this gene is upregulated in 72 hpf cavefish larva in response to the increased levels of programmed cell death in the eye.



## Specific Genes Identified by Microarray Analysis

### *Genes Expressed in the Eye*

To extend my studies on genes differentially expressed between surface fish and cavefish at the 72 hpf stage, I used microarray analysis. Despite the fact that there are no *Asytanax* chips available due to a lack of a large number of known cDNA sequences, I performed the analysis using zebrafish genome chips. Using stringent comparison criteria, I discovered several putative genes that are likely related to differences in phenotype between surface fish and cavefish.

Two genes with high fold increases in surface fish expression were *gnat1* and *gnat2*. Gnat1 and gnat2 are guanine nucleotide binding proteins that make up the transducin  $\alpha$ -subunit of the G-proteins of rods and cones respectively (Ray et al., 1997; Shen and Raymond, 2004). Another probe set similar to a rod receptor cGMP gated cation channel was also indicated as being upregulated in surface fish. Protocadherin was another putative upregulated surface fish gene.

Protocadherin has been shown to be necessary for organization of the intercalation of photoreceptor cells with the retinal pigment epithelium (Seiler et al., 2005). I have shown at 72 hpf stage, there are fewer rhodopsin expressing photoreceptor cells in the cavefish. It is not surprising that there may be fewer cone opsin expressing photoreceptor cells in cavefish as well. It follows that there

may be less mRNA of corresponding proteins involved in phototransduction mechanisms in the cavefish.

I also discovered BarH as a putative upregulated surface fish gene. In the rat, the BarH gene has been implicated in nervous system development. Specifically, BarH is expressed in a subset of retinal cells including photoreceptors (Saito et al., 1998). A probe set showing similarity to the Six3/Six6/Otx2 homeobox genes was also indicated as significantly upregulated in surface fish. The Six gene family is divided into three subfamilies (Ghanbari et al., 2001). Six3, Six 6, and Otx2, all members of the Six3 subfamily, have been implicated in either lens development, retina development, or both (Lopez-Rios et al., 1999). It is possible that a Six3 family gene may be a cause or consequence of the smaller eye size of cavefish.

Two other surface fish upregulated probe sets of interest were protein tyrosine phosphatase (CRYP-2), and middle molecular weight neurofilament gene (NF-M). These genes are interesting because they may have some function in axon guidance. It has been shown that there are significantly fewer axon bundles in the optic nerve of the cavefish (Soares et al., 2004). CRYP-2 is involved in axon outgrowth and guidance (Stepanek et al., 2001). NF-M expression has been correlated with projection length and neural arborization (Zopf et al., 1990). Thus, these genes may be downregulated in cavefish as a cause of or a response to the existence of fewer retinal-tectal projections.

*Genes Involved in Programmed Cell Death*

I showed previously that programmed cell death (apoptosis) is a significant cellular process involved in cavefish versus surface fish eye development. At the 72 hpf stage, there is a large amount of programmed cell death occurring in the cavefish eye, while the surface fish eye experiences no programmed cell death. In accordance with this, several putative genes from my analysis have been implicated in programmed cell death processes. Of the probe sets similar to genes involved in programmed cell death, five are upregulated in surface fish, and one (Ubiquitin specific protease 53) is upregulated in cavefish. The upregulation of genes involved in programmed cell death in surface fish is interesting. From an initial standpoint, it seems that programmed cell death genes should be upregulated in cavefish rather than surface fish. However, it is important to remember the varied roles of genes involved in the proliferation, differentiation, and death of cells. Genes have been discovered that promote programmed cell death as well as inhibit cell death (St Clair et al., 1997). It is possible that there are groups of apoptotic inhibitory genes that are downregulated in cavefish (eg, upregulated in surface fish). As a result, specific tissues or even all cells of cavefish may have a natural propensity for undergoing programmed cell death, perhaps due to stresses encountered in the cave environment.

A probe set similar to RAD21 was the highest fold upregulated gene in surface fish that was linked with programmed cell death. RAD21 is involved in the cell

division cycle, which is intimately linked with programmed cell death. The precise role this gene may have in the control of cell division and how this may tie in with programmed cell death is unclear, but it appears to be involved in DNA stabilization and DNA strand break repair, which are both involved in normal cell maintenance, but are also involved in programmed cell death (Pati et al., 2002).

Phosphodiesterase 4B was another probe set obtained from the analysis.

Suppression of this gene has been linked to growth inhibition and apoptosis (Ogawa et al., 2002). It follows that cells with more phosphodiesterase may be more resistant to programmed cell death, explaining a possible upregulation this mRNA in surface fish.

A probe set with similarity to heparin-binding EGF-like growth factor (HB-EGF) showed upregulation in surface fish. This gene has been shown to prevent apoptosis (Zhang et al., 2004). Oxidative stress is one mechanism that may indicate programmed cell death. HB-EGF can be activated by reactive oxygen species as a result of oxidative stress (Kim et al., 2005). Furthermore, HB-EGF is important in nervous system development; including cell migration, survival, and differentiation (Xian and Zhou, 1999). Presumptive neural cells of the cavefish retina die by programmed cell death around the 72 hpf stage. It is possible that downregulation of HB-EGF could be important in this process. SM-20 is a vertebrate homolog of Egl-9, to which an upregulated surface fish probe set showed similarity. This gene, like HB-EGF, has been shown implicated in nerve growth factor dependent survival (Taylor, 2001). Again, a downregulation of this

gene in cavefish may be indicative of elevated levels of programmed cell death in the eye.

Rad54, a gene having similarity to an upregulated surface fish probe set, is involved in DNA stabilization (Wesoly et al., 2006). More specifically, it is involved in homologous recombination, an important mechanism responsible for DNA repair after damage. DNA damage and fragmentation can be a direct cause, as well as a result, of apoptosis (Belyaev, 2005; Janssens et al., 2005). This is exemplified by nitroxidative stress, which has been linked to both DNA damage and apoptosis (Rachek et al., 2006). Nitric oxide damage to mitochondrial DNA may lead to faulty electron transport chain proteins. This in turn can cause reactive oxygen species to accumulate which can initiate apoptosis. It is possible that a gene that helps prevent this DNA damage may be upregulated in surface fish.

The probe set with the highest fold upregulation in cavefish was similar to ubiquitin specific protease 53. This probe set had the highest mean fold change of any probe sets in this study. Ubiquitin acts as a tag marking proteins for degradation (Li et al., 2002). Protein degradation by ubiquitin-mediated proteasome is the major form of eukaryotic proteolysis along with lysosomal mediated degradation (Roos-Mattjus and Sistonen, 2004). One of the functions of the ubiquitin proteasome system is the break down of proteins involved in apoptosis along with substrates that have already been cleaved by caspases

(Melino, 2005). By nature of the function of this protein, one might expect its mRNA to be upregulated in a system undergoing a significant amount of programmed cell death, such as the cavefish eye. For example, proteins in cells of the cavefish eye undergoing PCD are tagged with ubiquitin, and there is an abundance of ubiquitin specific protease 53 to cleave these tagged proteins.

### *Other Genes*

In addition to genes involved with eye development and programmed cell death, I found probe sets with similarities to neurological genes, genes involved with metabolism, genes involved with blood development, and several other miscellaneous genes. I found surface fish upregulated probe sets with similarity to Topoisomerase I (TPO1), and synaptophysin which are involved in myelination and synaptic vesicle formation, respectively (Fukazawa et al., 2006; Johnson et al., 1996). In addition to upregulated surface fish genes, I found an upregulated cavefish probe set that was similar to neuroligin, which is responsible for synapse recognition and formation (Song et al., 1999).

I found several significant probe sets with similarities to genes involved in metabolism and fat processing, including Acyl-CoA dehydrogenase, clathrin, ATPase V1 subunit D, hepatic transcription factor 2 (tcf2), and geranylgeranyl diphosphate synthase 1 (ggps1). Acyl-CoA dehydrogenase is involved

specifically in the  $\beta$ -oxidation of fatty acids (Seol et al., 2005). Geranylgeranyl diphosphate synthase 1 is another gene that may provide hints about underlying mechanisms of cavefish development and evolution. This gene encodes for an enzyme that is near the beginning of a biochemical cascade that results in retinol (vitamin A) and sterol metabolism. Retinol is important for rhodopsin mediated visual transduction (Bridges et al., 1987). Thus, a possible link between lipid metabolism and vision exists. Vitamin A also has important roles in development. Zebrafish injected with morpholinos to a gene encoding an enzyme that forms vitamin A developed malformed eyes and craniofacial skeletons (Lampert et al., 2003). These phenotypes are reminiscent of developmental processes occurring in the cavefish (Jeffery, 2001).

Clathrin is a component of lipid transporting vesicles (He et al., 2002). V-type ATPases have been shown to be involved in receptor mediated endocytosis, lysosomal hydrolase activity, and hormone processing (Crider and Xie, 2003). Tcf2 is involved in the transcriptional regulation of several genes that control metabolism of glucose, lipids, steroids, and amino acids (Gong et al., 2004). Finally, ggps1 is involved in isoprenoid biosynthesis which leads to carotenoid synthesis, retinal synthesis, and the production of Ras, RhoB, and Rac. The isoprenoid enzymatic pathway is also linked to sterol metabolism (Santos and Lehmann, 2004).

These probe sets were all upregulated in surface fish. It has been shown that cavefish are evolutionarily regressive in metabolism and constructive for fat content relative to surface fish (Huppopp, 1986; Rose and Mitchell, 1982). These genes may be downregulated in cavefish in relation to their specific ecological niche. Surface fish, which live in streams with flowing water, are more active and are exposed to a more continuous food supply, while cavefish lead a more sedentary lifestyle in stagnant cave pools without a continuous supply of food. Therefore, a slower metabolism with more fat stores is advantageous to their survival. It follows that genes promoting more metabolism and fewer fat stores, like the aforementioned genes, may be downregulated in cavefish. There may also be genes upregulated in cavefish which promote fat storage and slow metabolism. However, I did not find genes fitting this description in the present study.

I found two probe sets representing genes involved in blood development. Both genes, adducin and hydromethylbilane synthase (HMBS), are specifically involved in erythropoiesis (Gregor et al., 2002; Yenerel et al., 2005). It is unclear why genes in a blood cell differentiation pathway may be upregulated in surface fish. One possibility for upregulation of genes involved in erythrocyte development in surface fish (or downregulation of genes in cavefish) may involve aforementioned metabolism rates. Certain cavefish populations are reported to have a lower oxygen consumption rate than surface fish (Hueppopp, 1986). This difference may be reflected in erythrocyte differentiation between the two forms.



Another possibility is that components of blood cell development share common mechanisms with programmed cell death. It has been shown that mutations in the zebrafish transcriptional intermediary factor 1 $\gamma$  gene causes differentiating blood cells to express lower levels of hematopoietic transcription factors, eventually undergoing programmed cell death (Ransom et al., 2004). Thus there may be a link with blood cell development, lens and retina development, and apoptosis in *Astyanax*. Changes in a gene common to blood cell development and lens and/or retina development may cause lowered downstream gene expression levels in differentiating cavefish blood cells, but not enough to cause abnormal development. Alternatively, there may be a slower net production of red blood cells in cavefish, which correlates with the lowered need for oxygen. This same gene may go below a certain threshold, or cause other cellular components to fall below a certain threshold in lens or retina cells, causing programmed cell death.

Finally, I found probe sets related to various miscellaneous genes such as polycomb homolog 1 (ph1), heat shock protein 90 $\alpha$  (hsp90 $\alpha$ ), F-box leucine rich repeat protein 3A (fbxl3A), and the immediate early genes Jun B and c-Fos. Ph1 is involved in anterior-posterior axis formation (Isono et al., 2005). Hsp90 $\alpha$  is important in muscle development in *Astyanax* at the 72 hpf stage, but also plays a role in cavefish lens degeneration at earlier stages of development (Hooven et al., 2004). Hsp90 $\alpha$  has been shown to protect ribosomal proteins from ubiquitination and subsequent degradation and may play a similar role in protecting other

proteins from ubiquitination and degradation (Kim et al., 2006). Fbxl3A plays a role in cell cycling, while the immediate early genes Jun B and c-Fos are involved in numerous gene activating mechanisms (Bonifas et al., 2001; Chiaur et al., 2000; Ohta et al., 2005).

### **Programmed Cell Death, *$\gamma$ M-Crystallin* expression, and Rhodopsin Expression**

To support the significance of several genes from my analysis, I provided cellular and expression data of programmed cell death and  *$\gamma$ M-crystallin*. In addition, I studied the expression of Rhodopsin at the 72 hpf and 10 dpf stages.

Several differential hybridization gene sequences and significant probe sets from the microarrays provided clues about actual cell morphological differences or gene expression patterns that may be upregulated in the 72 hpf surface. ID number 67, which was the highest upregulated probe set in cavefish, showed sequence similarity to ubiquitin specific protease 53 (see Table 3-8). Ubiquitin specific proteases are involved in protein turnover and degradation. They have also been implicated in nuclear factor- $\kappa$ B activation, which has a role in promoting apoptosis (Radhakrishnan and Kamalakaran, 2006; Tzimas et al., 2006). Thus, there is a possible link between this gene and programmed cell death occurring in the cavefish eye primordia at this stage. I've previously shown

the existence of extensive cavefish lens and retina programmed cell death at the 72 hpf stage.

Both the differential hybridization and microarray experiments revealed  $\gamma M$ -*crystallin* as a gene that is upregulated in surface fish. Based on my previous studies, I confirmed that there are fewer  $\gamma M$ -*crystallin* transcripts in the smaller lens of the cavefish at 72 hpf. The microarray results indicated that rhodopsin is another gene having a significant mean fold increase in expression in surface fish. A probe set corresponding to rhodopsin represented a putative upregulated gene in surface fish (ID number 5). This prompted me to clone the *Astyanax* rhodopsin sequence and examine its expression pattern in surface fish versus cavefish. I performed *in situ* at the 72 hpf stage. I found fewer cells expressing rhodopsin in the cavefish retina. I also studied rhodopsin expression at the 10 dpf and determined that downregulation of rhodopsin expression in cavefish persists at this stage. However by using immunohistochemical techniques, I determined that the few cells producing rhodopsin transcripts are also capable of producing rhodopsin protein at the 10 dpf stage.

While there is more programmed cell death in the cavefish eye at the stage I studied, I am not sure if the genes obtained from the differential analyses are directly involved in this programmed cell death. Furthermore, while I confirmed that  $\gamma M$ -*crystallin* and Rhodopsin expression are reduced in the cavefish at the 72 hpf stage, it is unclear if these genes are directly responsible for cavefish eye

degeneration or a consequence of some other developmental mechanism. For example, rhodopsin expression in the cavefish retina may be a result of the existence of fewer photoreceptor cells and all cells that are present and would be expressing rhodopsin do so. However, it may also be a result of faulty transcription regulation where there are cells of the cavefish retina that would normally possess rhodopsin transcripts but do not. It will take further studies to determine the exact roles these genes play in *Astyanax* development.

I originally expected to find genes involved in development of pigment, but did not find any such genes in either differential analysis. There may be several reasons for this. It is possible that a change in a gene seemingly unrelated to pigment formation has an effect on the downstream cellular physiology of pigment formation. Furthermore, despite the fact that there are obvious melanin pigmentation differences at 72 hpf, the major changes associated with pigment development may occur at a different time during development. Also, differences in genes affecting the melanin pathway may be small enough that they were not detected by either of the analysis techniques. Lastly, in the case of the microarray analysis, there is the possibility that there were problems with the cross-hybridization of *Astyanax* sequences to zebrafish sequences. We must remember this fact when considering the microarray data in general.

## Summary

I used differential gene analyses to determine specific genes that may be responsible for changes in the phenotypic development of surface fish versus cavefish. While there are several genes that are interesting, it is also important to remember the trends that these genes reveal. For instance, when considering the evolutionary history of *Astyanax*, it is attractive to consider genes or mechanisms affecting more than one developmental process. The pleiotropic effect of *shh* is an example of this phenomenon. It has been proposed that *shh* overexpressed along the anterior midline of the cavefish promotes the development of a larger jaw, more teeth, and a larger forebrain at the cost of smaller eyes (Jeffery, 2005). It is attractive to postulate that other mechanisms involved in cavefish development affect more than one characteristic in a similar fashion. Some of the genes obtained from the present study allude to such trends. The abundance of genes involved in apoptosis and ubiquitination-dependent proteolysis are examples. I found two genes involved in erythrocyte development. As mentioned previously, there may be a link between erythrocyte development and programmed cell death. Moreover, lens cells undergo programmed cell death in the cavefish at the 72 hpf stage. It was shown that bovine lens cells undergo ubiquitin-dependent proteolysis in a normal ‘attenuated’ form of programmed cell death (Huang et al., 1993).

There may be significant associations among genes obtained from my analyses. For example genes involved in ubiquitin-dependent proteolysis may be linked with other programmed cell death genes and erythrocyte differentiation genes. A difference in a gene common to these mechanisms may allow retention of normal development of certain cells and tissues, such as the cavefish lens and retina, and faulty development of others. For example, instead of cells of the cavefish lens undergoing typical lens cell attenuated programmed cell death, an alteration may occur causing them to undergo complete programmed cell death. An alteration in the balance or threshold of certain programmed cell death associated mRNA or proteins may be responsible. As mentioned previously, there may be a set of anti-apoptotic genes that are downregulated in the cavefish, resulting in a natural propensity to undergo programmed cell death, while certain genes responsible for the downstream execution of programmed cell death, like ubiquitin-specific proteases, may be upregulated.

It will be interesting to see exactly how genes revealed by the differential analyses are involved in *Astyanax* development. Several genes may play very important roles in affecting a phenotypic change in surface fish versus cavefish. Furthermore, like *shh*, certain genes may be affecting more than one cavefish phenotypic change. This is an interesting way to view evolution of development since it designates multiple 'phenotypic modules', sometimes seemingly unrelated, as the objects of evolutionary change (Franz-Odegaard and Hall, 2006). The elegance of this model becomes apparent when we can connect each module

of a single pleiotropic network with a possible causatory developmental and/or evolutionary force. We hope to uncover these phenotypic modules and their underlying evolutionary and developmental mechanisms by integrating knowledge obtained by differential gene expression experiments with other work, such as molecular biology, genomics, and research with other model organisms.

## Chapter 4: The Role of the Lens in Retina Development in the Blind Cavefish *Astyanax mexicanus*.

### Abstract

I am interested in developmental and evolutionary mechanisms involved in *Astyanax mexicanus* eye development. This species consists of a surface stream dwelling form (surface fish) and a cave dwelling form (cavefish). The two forms possess strikingly different adult phenotypes, making them excellent subjects for comparative evo-devo studies. Cavefish form an embryonic lens and retina, but these structures arrest in development and eventually degenerate. I found that the cavefish retina retains its ability to proliferate, but undergoes elevated levels of programmed cell death (PCD), resulting in little or no net growth. BrdU pulse/chase incubation experiments indicated that cells of the cavefish retina proliferate normally, but are later removed by this PCD as they move back into the retina. To test the role of the lens in *Astyanax* retina PCD, I performed surface fish lens deletions and surface fish to cavefish lens transplants. I subsequently studied the effects these manipulations had on cell proliferation and PCD in the retina. I found that replacing a faulty cavefish lens with a surface fish lens rescues the cavefish retina from PCD. However, removing the surface fish lens did not cause PCD in the surface fish retina. Based on these results, I conclude that the lens has a permissive role in *Astyanax* retina development, but that there is another component of the eye, most likely the retinal pigment epithelium (RPE), that may also be responsible for retina development.



### **Introduction**

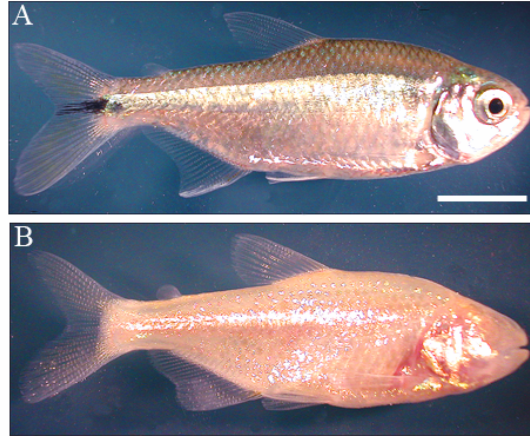
The field of evolution of development (evo-devo) has contributed much to our understanding of how certain phenotypes have arisen. Frequently, the goal of evo-devo research is to dissect apart genetic pathways and understand how these pathways affect development, while considering the evolutionary ramifications connected to the development of the resulting phenotype(s) (Cracraft, 2005). I am specifically interested in eye development and how changes in development have been affected by evolution.

I use a comparative microevolutionary approach to understand how evolution has affected phenotypic changes through alterations in genetics and development by using the Mexican tetra, *Astyanax mexicanus* as a model system in evo-devo.

*Astyanax mexicanus* consists of two forms: a surface stream dwelling form (surface fish; see Figure 4-1, A) and a cave dwelling form (cavefish; see Figure 4-1, B). Surface fish possess functional eyes as adults. In contrast, cavefish lack functional eyes.

The teleost retina is an excellent subject for growth and differentiation studies since the eyes of fish continue to grow throughout life, by addition of new cells and by stretching of existing tissue (Julian et al., 1998; Perron and Harris, 2000). There are two areas of proliferation in the teleost retina. The first area, comprised of cells where the retina meets the iris, is known as the ciliary marginal zone (CMZ). Cells proliferate in this area and migrate back into the retina as they

Figure 4-1



**Figure 4-1. Adult forms of *Astyanax mexicanus*.** A, Surface fish. B, Cavefish. Adult surface fish have normal body melanin pigmentation and normal eyes. Cavefish lack body melanin pigmentation, and only small vestigial eyes remain under layers of fat and epidermis. Scale bar in A, 1cm; magnification is the same in A,B.

differentiate into their prospective cell types. Cells from this region contribute both to the neuroretina and the retinal pigment epithelium (RPE). The second region of proliferation consists of rod progenitor cells in the outer nuclear layer of the retina (Kwan et al., 1996). Studies involving the incorporation of cell birth markers such as tritiated thymidine and BrdU showed that the descendants of cells of the CMZ eventually migrate away from the margin of the retina (Harris and Perron, 1998).

It is known that certain pathways involving secreted proteins and growth factors, such as IGF-I, EGF, and the Wnt genes, are necessary for stimulation of cell proliferation in the CMZ (Fischer and Reh, 2000; Kubo et al., 2003). However, it is still unclear how different parts of the eye interact with one another to direct proper development. For example, there is limited knowledge of the role the lens plays in eye development. Zebrafish transgenic for a diphtheria toxinA fragment driven by an  $\alpha$ A-crystallin promoter developed malformed lenses. As a result, the retina did not develop correctly and exhibited improper lamination and either abnormal expression or total lack of expression of molecular markers of the inner and outer plexiform layers (Kurita et al., 2003). A similar experiment in mouse resulted in abnormal growth and development of the neuroretina, sclera, cornea, ciliary body, vitreous body, and iris (Harrington et al., 1991).

The importance of the lens in directing eye development was shown in previous experiments in *Astyanax* (Yamamoto and Jeffery, 2000). When a normal surface fish lens was transplanted into the optic cup of the cavefish during early larval stages, the eye of the cavefish recovered ocular structures that normally would not develop. Furthermore, the normal lens promoted a significant improvement in patterning, growth, and differentiation of the retina. I am interested in investigating this further in order to help obtain clues about the evolution of mechanisms of eye degeneration in *Astyanax* cavefish. Specifically, I wanted to determine the processes that may be involved in the recovery of the cavefish retina after lens transplantation and the surface fish retina in the absence of a lens.

To accomplish these tasks, I performed initial assessments of proliferation and programmed cell death (PCD) on the eyes of *Astyanax*. I followed this with experiments involving surface fish lens deletions, and transplants of surface fish lenses into cavefish, repeating the proliferation and PCD studies on these deletion and transplant samples.

I show that the rate of cell proliferation in the cavefish is similar to surface fish, despite its reduction in size. I then show that the cavefish retina undergoes elevated levels of PCD. Through subsequent BrdU studies I provide evidence that the eradication of cells in the retina is responsible, at least in part, for the smaller size of the cavefish retina. Finally, I show that introduction of a normal lens into the cavefish retina confers protection against retinal PCD, contributing to a

recovery of the adult cavefish eye approaching that of the normal surface fish phenotype. However, I also demonstrate the removing the lens from surface fish eyes does not promote retinal apoptosis, indicating that the cavefish eye phenotype may depend on factors in addition to the lens. These results provide further understanding of the role of the lens in directing development and growth of the retina, as well as giving us clues about how evolution has affected development of the cavefish eye.

## **Materials and Methods**

### **Biological Materials**

Animal collection and care are as stated in part A of Materials and Methods in Chapter 1.

### **PNCA Immunohistochemistry**

PCNA expression in the eye was detected according to the procedures outlined in section D of Materials and Methods in Chapter 2.

### **PCNA and Retina Area Measurements**

After PNCA immunohistochemistry was performed on 3 month old cross-sections of surface fish and cavefish, both the area of PCNA staining and total retina area were measured. Measurements were made using a Zeiss Axiocam and the AxioVision Software, version 4.5.0.0 (Carl Zeiss MicroImaging, Inc., Thornwood, NY). The measurement outline feature was used to measure, in square micrometers, the PCNA labeling in the two ciliary marginal zones of the retina cross-section, in addition to the total retinal area. The two ciliary marginal zone staining values were added to give total PCNA staining area. The total

PCNA staining area and the total retinal area values of surface fish versus cavefish were subjected to statistical analysis using Student's unpaired t-test.

### **Detection of PCD by TUNEL**

PCD was detected in 72 hpf larva based on the whole mount TUNEL procedure outlined in section E of Materials and Methods in Chapter 2.

### **Lens Transplantation and Deletion**

Lens transplantations and deletions were performed on 30 to 36 hpf *Astyanax* larvae according to the methods outlined by Yamamoto and Jeffery (2002). Briefly, for lens deletion experiments, surface fish larvae were embedded in 1.2% agar, and the lens from one side of the individual was removed using a sharpened tungsten needle. For transplantation experiments, surface fish and cavefish were embedded in 1.2% agar. The lens was removed from both surface fish and cavefish optic cups on one side of the individual. The surface fish lens was then placed into the cavefish optic cup. Lens deleted surface fish and lens transplanted cavefish were subsequently allowed to recover and were removed from the agar. A schematic of the lens deletion and transplantation procedures is shown in Figure 4-6.

## **BrdU Incubation Experiments**

Non-surgically manipulated surface fish and cavefish, lens deleted surface fish, and lens transplanted cavefish were raised to three months post fertilization (mpf) before I began BrdU incubation experiments. At this time, I initiated one of three types of incubation protocols lasting 50 days. Incubations were performed in either fresh water or a solution of 1g/liter BrdU. A general representation of the three treatment protocols is shown in Figure 4-4.

The first protocol (treatment 1) involved an initial 40 day fresh water incubation initiated at the three mpf stage. This was followed by a 10 day incubation in BrdU, immediately followed by fixation. The second protocol (treatment 2) involved 20 day fresh water incubation. This was followed by a 10 day BrdU incubation, and finally another 20 day fresh water incubation before fixation. The third protocol (treatment 3) consisted of an initial 10 day BrdU incubation, followed by a 40 day fresh water incubation and subsequent fixation.



**Detection of BrdU incorporation**

BrdU incorporation was detected using a fluorescein linked antibody to BrdU (Roche Applied Science, Indianapolis, IN, USA). After BrdU incubation experiments and fixation, samples were embedded in paraffin and cross sectioned at 10um. These sections were dewaxed, rehydrated, and subjected to the BrdU detection procedure.

Slide glasses with sections were incubated twice for five minutes in PBSAT (PBS with 0.5% BSA and 0.1% Tween20). They were then treated with Trypsin solution (0.05% trypsin and 0.05% Calcium Chloride in PBS) for three minutes at room temperature. This was followed by a 15 minute incubation in trypsin inhibitor (Roche Applied Science, Indianapolis, IN, USA). Samples were washed twice for five minutes in PBSAT followed by a 15 treatment in 4M HCl at room temperature. This was followed by three washes in PBSAT, assuring that the pH of the final wash solution was 6.5 or higher after incubation. The sections were then covered with BrdU antibody solution (diluted to 50ug/ml in PBSAT) for one hour at 37°C in a humid chamber. After BrdU antibody incubation, sections were washed three times for five minutes in PBSAT and cover-slipped. Finally, samples were viewed and photographed using fluorescence microscopy.

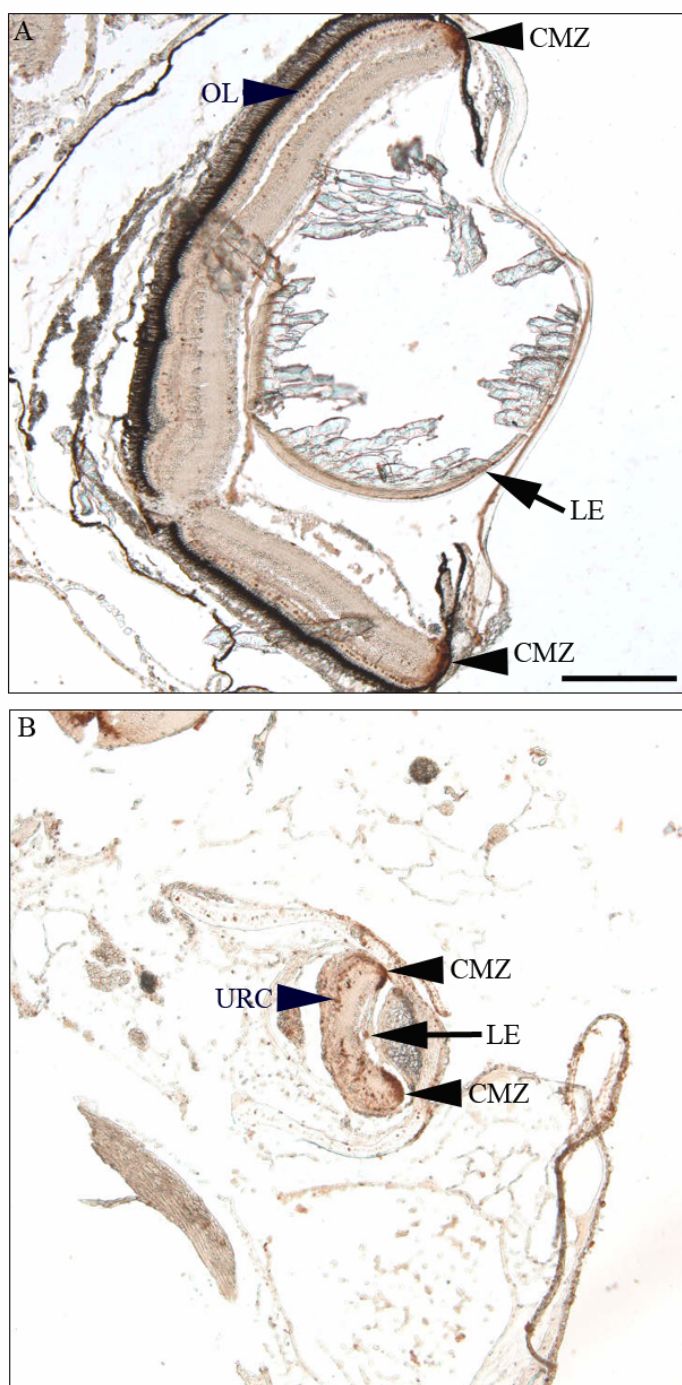
## **Results**

### **Cell Proliferation in Retinal Development**

To determine the role of cell proliferation in retinal growth and development, I used PCNA to label the CMZ. At the three mpf stage, PCNA labeling is seen in the CMZ and the lens epithelium in surface fish and cavefish (Figure 4-2). I also see labeled cells in the outer layer of the surface fish retina, which are most likely photoreceptor progenitor cells. In addition, there are labeled cells outside the CMZ of the cavefish retina, but it is difficult to determine which cell type these may be as a result of the disorganized structure of the cavefish retina.

I was interested in determining the likelihood that cell proliferation has a role in retinal degeneration of the cavefish. To accomplish this, I made measurements of cross sections of the surface fish and cavefish retina. One random cross-section of each individual was chosen to measure the area of PCNA staining of the two CMZ areas of the section (see second and third column of Table 4-1). The cross-section was chosen as close as possible to what was determined the central sectioned plane of the eye to avoid differences in size near the eye periphery. I recorded both the area of PNCA staining and total area of the retina. These values are given in Table 4-1. The first column of the table designates each of the 10 individuals (five surface fish and five cavefish) for which data was recorded.

Figure 4-2



**Figure 4-2. PCNA expression in the eye of *Astyanax mexicanus*.** A, Surface fish. B, Cavefish. Three month old surface fish and cavefish were fixed, embedded, and cross sectioned. These samples were subjected to immunohistochemistry using an antibody to PCNA. Abbreviations: CMZ, ciliary marginal zone; LE, lens; OL, outer layer; URC, unidentified retinal cells. Scale bar in A, 200um; magnification is the same in A,B.

These values were added together for each sample to give a total PCNA staining area shown in the fourth column. Finally, the last column of the table indicates values for the total area of the retina.

The mean for each type of measurement for surface fish and cavefish is given below each data group. I found that the mean total PCNA staining area was similar in surface fish and cavefish ( $1357.39\mu\text{m}^2$  and  $1512.61\mu\text{m}^2$ , respectively). In contrast, I found that the mean cross sectioned area of the surface fish retina was approximately five times larger than the mean cross sectioned area of the cavefish retina ( $95474.61\mu\text{m}^2$  and  $18596.77\mu\text{m}^2$ , respectively).

To determine if recorded areas between surface fish and cavefish were significantly different, I subjected the total PCNA and total retina measurements to a statistical test. I obtained P values of 0.532 and 0.001 for the PCNA and total retinal measurements respectively. This indicates that the area of total PCNA staining between surface fish and cavefish is not significantly different, while the total retina size is significantly different.

Table 4-1				
Retina PCNA Measurement Data				
Sample	PNCA half 1	PCNA half 2	total PCNA	total retina
SF1	659.17 $\mu\text{m}^2$	686.87	1346.04	89784.65
SF2	884.83	383.07	1267.90	71123.55
SF3	563.24	396.58	959.82	79592.80
SF4	658.72	519.77	1178.49	81447.59
SF5	875.37	1159.35	2034.72	155424.47
	mean = 728.27	629.13	1357.39	95474.61
CF1	520.22	625.62	1145.84	18158.84
CF2	890.01	677.86	1567.87	14528.38
CF3	908.02	1056.21	1964.23	20137.31
CF4	890.23	801.28	1691.51	21139.92
CF5	666.15	527.43	1193.58	19019.40
	mean = 774.93	737.68	1512.61	18596.77
P value =			0.532	0.001

**Table 4-1. Area measurements of retina and retinal PCNA staining in *Astyanax mexicanus*.** Sections of three month old surface fish and cavefish were subjected to immunohistochemistry using an antibody to PCNA. Measurements of area in square micrometers were taken for PCNA staining in each half of the retina. These were added together for each retina to give a total PCNA value for each sample. A measurement was also taken for the total area of the retina for each corresponding individual. A t-test was performed on the total PCNA staining area and retina size for each individual. All measurement values are given in square micrometers. Abbreviations: SF, surface fish; CF, cavefish.

## Programmed Cell Death in Retinal Development

In addition to cell proliferation, I was also interested in studying programmed cell death in the *Astyanax* retina using the TUNEL assay. At one mpf, I did not find cells undergoing programmed cell death in the surface fish retina (Figure 4-3, A). A portion of the surface fish retina is shown, but no TUNEL labeled cells were seen in any portion of the retina. At this time point, I found cells undergoing PCD in the cavefish retina (arrows in Figure 4-3, B).

I also tested for PCD at the three mfp stage and obtained similar results. No cells undergoing PCD were seen in the surface fish retina (Figure 4-3, B), but I detected numerous TUNEL labeled cells in the cavefish retina (arrows in Figure 4-3, D). Areas of PCD in the cavefish retina were sometimes restricted to a single cell, at least in section. They may also involve several adjacent cells as exemplified by the middle arrow in C of Figure 4-3 and the top arrow in D of Figure 4-3. The dying cells did not appear to be restricted to a certain region or layer of the cavefish retina.

Figure 4-3



**Figure 4-3. Programmed cell death in the retina of *Astyanax mexicanus*.** A, B, Surface fish. C, D, Cavefish. Sections of surface fish and cavefish were subjected to the TUNEL assay to detect programmed cell death at the one month stage (A,C) and the three month stage (B,D). Arrows indicate cells or areas of cells undergoing programmed cell death. Abbreviation: CMZ, ciliary marginal zone. Scale bar in A, 100um; magnification is the same in A,C. Scale bar in B, 200um; magnification is the same in B,D.



### **Persistence of Newly Born Cells in the Surface Fish and Cavefish Retina**

I developed three BrdU labeling protocols to test for the persistence of newly born cells in the surface fish and cavefish retina (see Materials and Methods, section F and Figure 4-4). One surface fish and one cavefish were subjected to each of the three treatments. The results are shown in Figure 4-5. For treatment 1 (Figure 4-4, A), individuals were fixed immediately after BrdU incubation. Cells labeled for BrdU are seen in the CMZ and the outer layer of the retina in both surface fish and cavefish for this treatment (arrows in Figure 4-5, A and B, respectively).

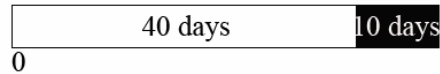
Treatment 2 consisted of incubations in water, then a pulse of BrdU, which was followed by a chase in water (Figure 4-4, B). The eyes of surface fish subjected to treatment 2 possessed a well defined group of labeled cells that had moved away from the CMZ.

I also found labeled cells in the outer layer of the retina (arrows in Figure 4-5, C). In the cavefish, only a few labeled cells that have moved away from the CMZ remain in the retina. Furthermore, only a few cells can be seen in the outer layer of the retina (arrows in Figure 4-5, D). Treatment 3 consisted of an initial pulse of BrdU labeling followed by a long water chase (Figure 4-4, C). This treatment resulted in an area of cells that moved even further away from the CMZ in surface fish than the group of cells for treatment 2. Labeled cells persist in the outer layer of the retina (arrows in Figure 4-5, E). No labeled cells were found in the cavefish retina for treatment 3.

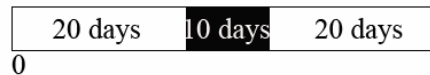
Figure 4-4

## BrdU Incubation Experiments

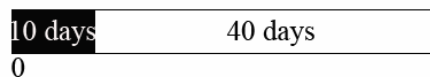
### A. Treatment 1 (T1)



### B. Treatment 2 (T2)



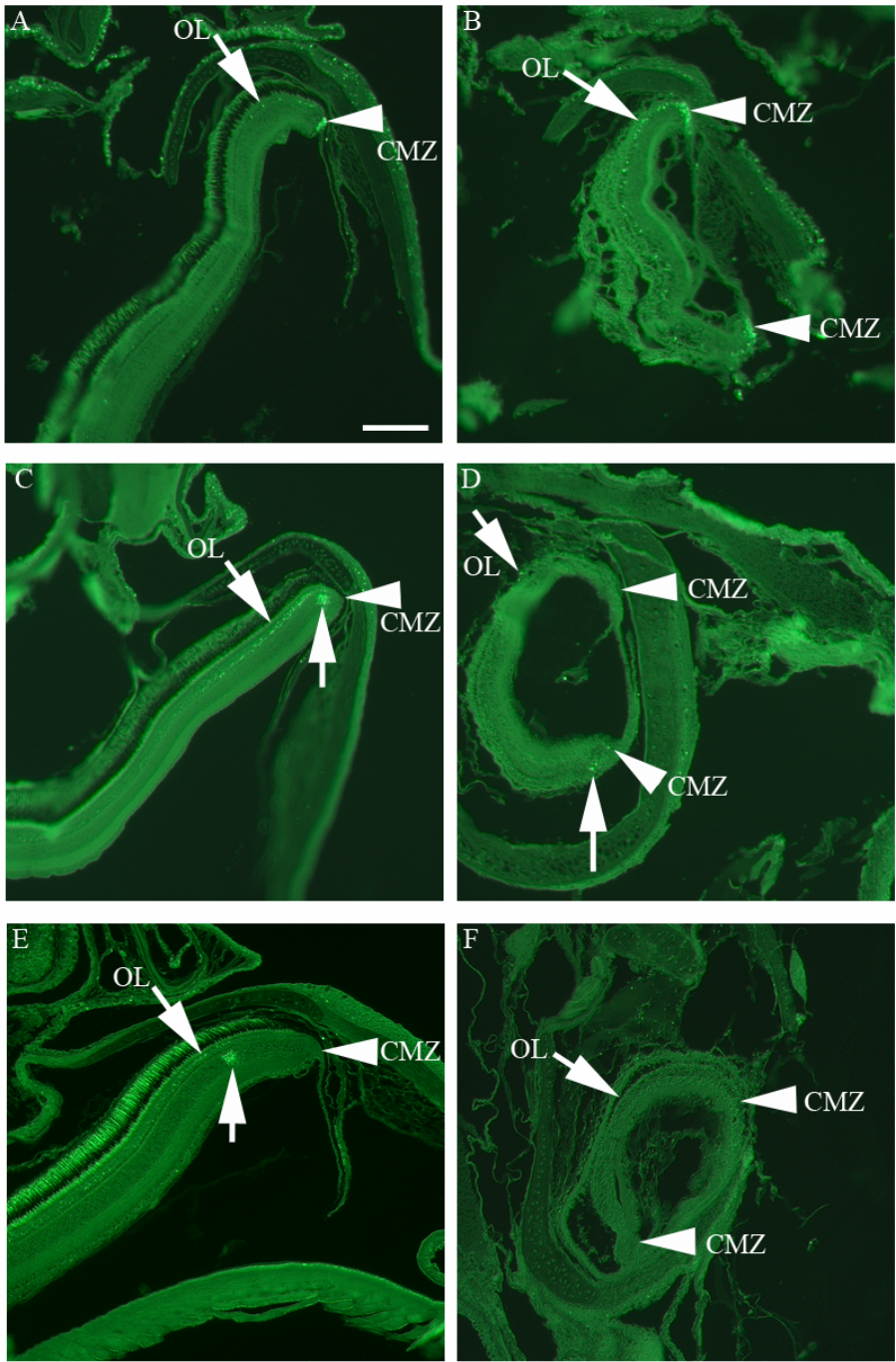
### C. Treatment 3 (T3)



**Figure 4-4. Schematic representation of BrdU incubation experiments.**

Surface fish and cavefish were raised until three months of age. At this time, incubations were initiated in different BrdU and fresh water treatments over 50 day periods, ending with fixation and analyzation. In the timeline bars, white areas consist of fresh water incubations and black area indicate BrdU incubations. The initiation of the treatments at the three month post fertilization stage is indicated by '0'. Treatment 1 (A) consisted of the first 40 days in fresh water followed with a 10 day BrdU pulse. Treatment 2 (B) consisted of 20 days in fresh water, followed by a 10 day BrdU pulse, followed by a 20 day chase in fresh water. Treatment 3 (C) consisted of a 10 day BrdU pulse followed by a 40 day chase in fresh water.

Figure 4-5



**Figure 4-5. BrdU labeling in the eye of *Astyanax mexicanus*.** A, C, E, Surface fish. B, D, F, Cavefish. Surface fish and cavefish were subjected to incubations in BrdU and fresh water. After fixation, samples were embedded, cross sectioned, and subjected to immunohistochemistry with a fluorescein linked antibody to BrdU. Experimental incubations were performed based on the treatments in Figure 4: A and B were subjected to incubation treatment 1. C and D were subjected to treatment 2, and E and F were subjected to treatment 3. Abbreviations: CMZ, ciliary marginal zone; OL, outer layer. Scale bar in A, 200um; magnification is the same in A-F.

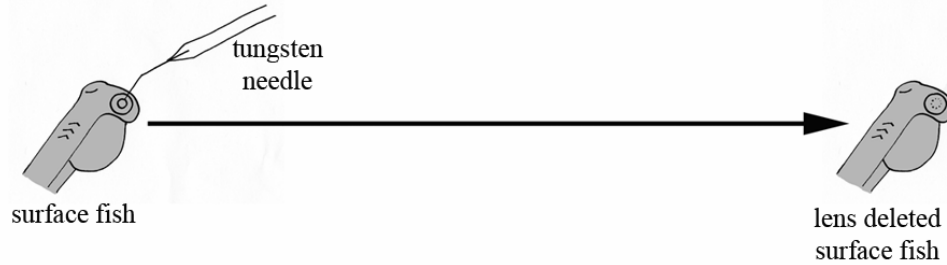
## Lens Deletion and Transplantation

In order to study the effects of the lens on retinal proliferation and PCD in *Astyanax*, surface fish lens deletions were performed, in addition to surface fish to cavefish lens transplant experiments. These experiments were done at the 36 hpf stage. Individuals were subsequently raised to larval stages to assess the effects of embryological lens manipulations on long term retina growth.

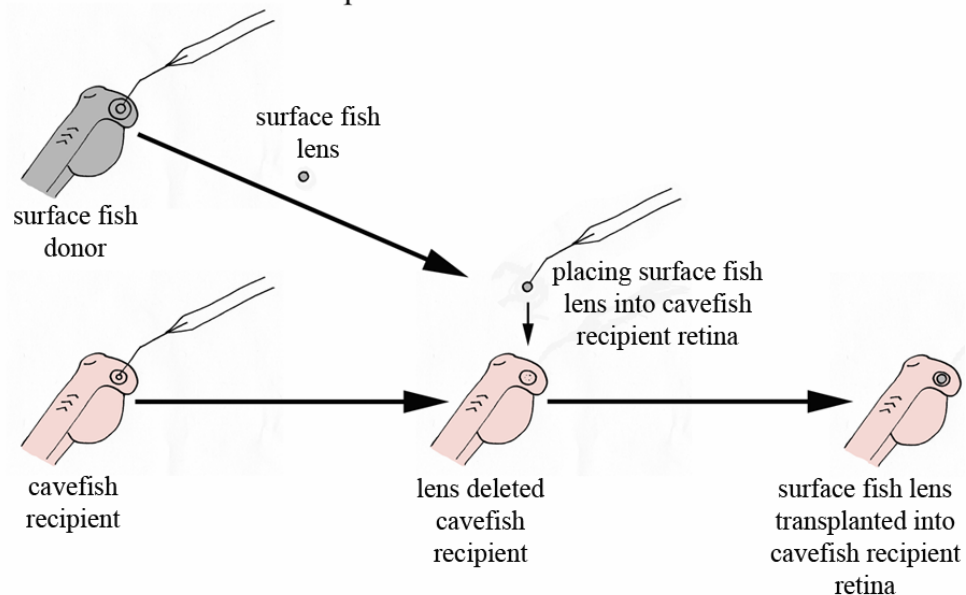
A surface fish subjected to lens deletion is shown in Figure 4-7, A and B (control side, A; deletion side, B). A cavefish subjected to lens transplantation is shown in Figure 4-7, C and D (control side, C; transplantation side, D). Not all cavefish receiving surface fish lens transplants result in a well formed eye. Half of the surgically manipulated larvae survive the procedure. Of these, two of five show some type of gross improvements to eye morphology, with approximately one in five showing a significant improvement to eye size and morphology. As demonstrated previously, structures that are absent from the control side cavefish eye, such as the pupil, anterior chamber, cornea, and iris, are present in the restored cavefish eye (Yamamoto and Jeffery, 2000).

Figure 4-6

## A. Surface fish lens deletion

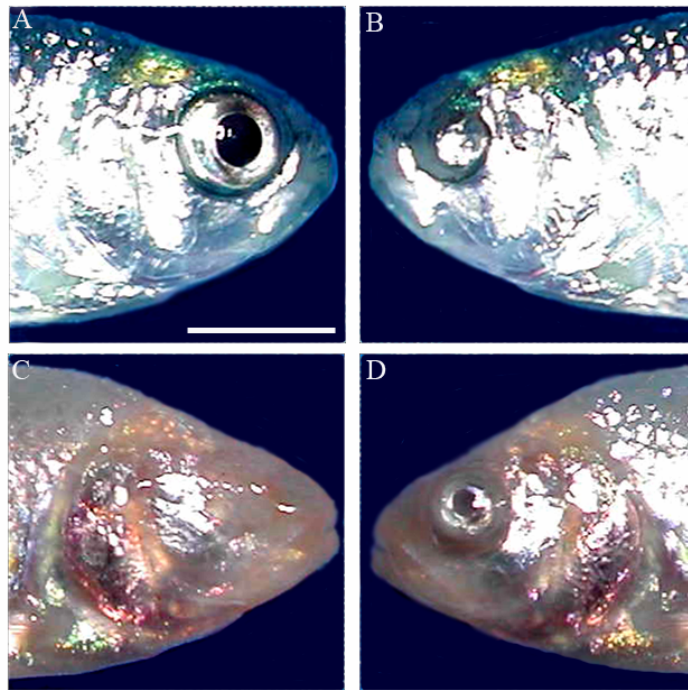


## B. Surface fish lens transplant to cavefish retina



**Figure 4-6. Schematic of the experimental procedures used to delete and transplant lenses.** The surface fish lens deletion experiment is diagrammed in A. A tungsten needle was used to remove the lens from 30-36 hpf stage surface fish larvae. Cavefish lens transplants are outlined in B. A tungsten needle was used to remove the lens from 30-36 hpf surface fish and cavefish. The surface fish lens was subsequently placed into the cavefish retina.

Figure 4-7



**Figure 4-7. Three month old *Astyanax mexicanus* juveniles previously subjected to lens deletion or tranplantaion experiments.** A, B, Surface fish. C, D, Cavefish. Lens deletions and transplants were performed on 36 hpf surface fish and cavefish respectively. These were raised to the 3 mpf stage. A and B represent the same individual with no experimental procedures performed on the right side of the individual (A), and a lens deletion performed on the left side (B). C and D represent the same individual with no experimental procedures performed on the right side (C), and a lens transplant performed on the left side (D). Scale bar in A, 500mm; magnification is the same in A-D.

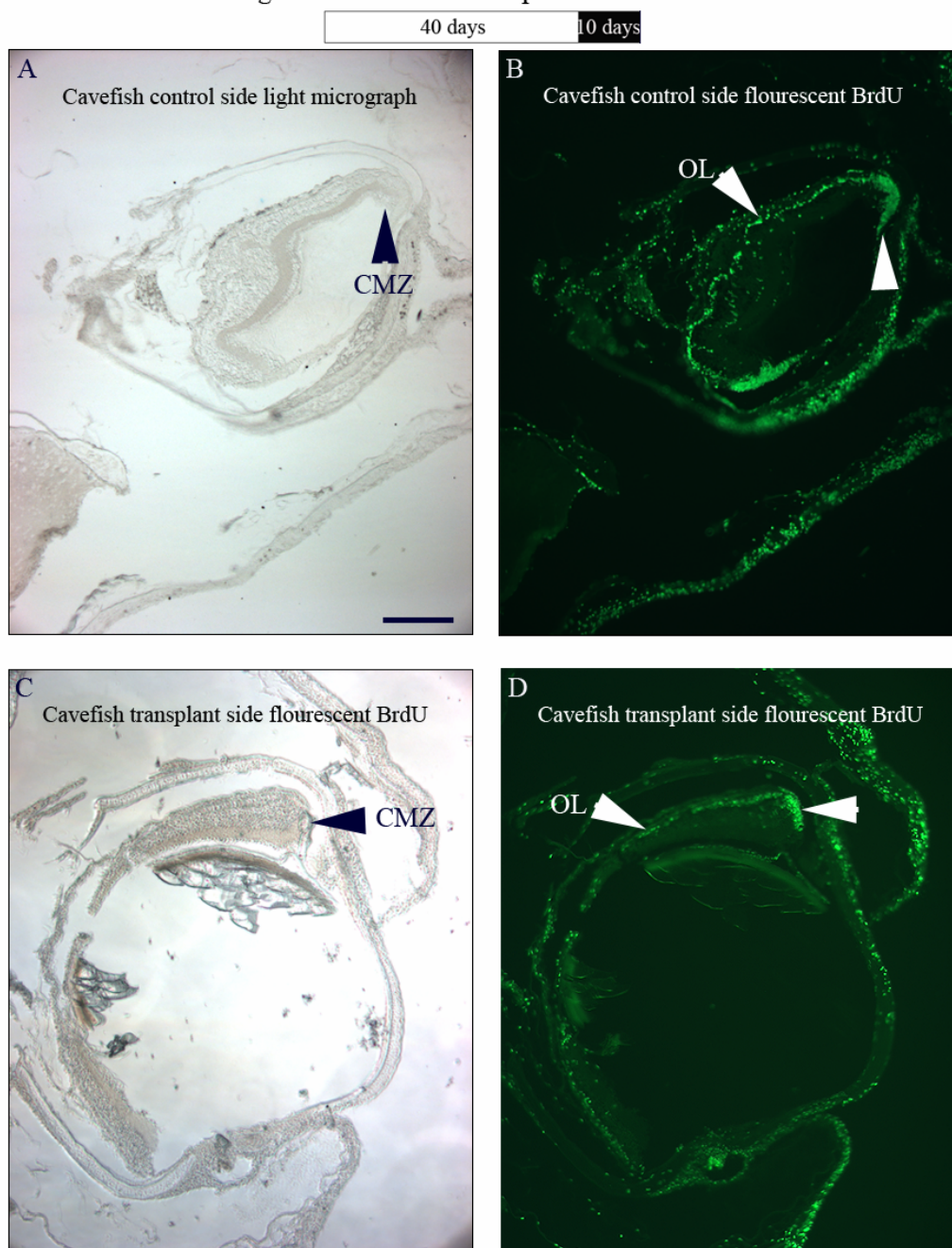
### **Role of the Lens in Cavefish Retinal Growth**

To test the effects of the lens on the developing retina, I performed the three BrdU labeling protocols on cavefish with a transplanted lens. For BrdU treatment, the same individual was used as both an experimental treatment and control. Thus, one side of the individual served as the non-transplanted control, while the other side served as the experimental transplanted treatment. I raised one lens transplanted individual for each of the three BrdU treatments.

Light and fluorescence micrographs of the treatment 1 lens transplanted cavefish are seen in Figure 4-8. BrdU labeling occurs in the CMZ of both the control and transplant side cavefish (arrowhead in Figure 4-8, B and D respectively). I also saw labeling in the outer layer of the retina in the control and transplant side (arrowheads labeled OL in Figure 4-8, B and D). I saw a very small area of possible BrdU labeled cells in the control side eye for the cavefish subjected to treatment 2 (arrowhead in Figure 4-9, B; corresponding light micrograph shown in Figure 4-9, A). However, I feel that this is background fluorescence since it is difficult to discern individual cells in the area of staining. In the cavefish eye with a transplanted lens (treatment 2), an area of BrdU labeled cells persists and has moved away from the CMZ. (arrowheads in Figure 4-9, D; corresponding light micrograph is shown in Figure 4-9, C). I detected some BrdU labeled cells in the outer layer of the retina in the transplant side (arrowhead labeled OL in Figure 4-9, D). There are no labeled cells in the cavefish treatment 3 control



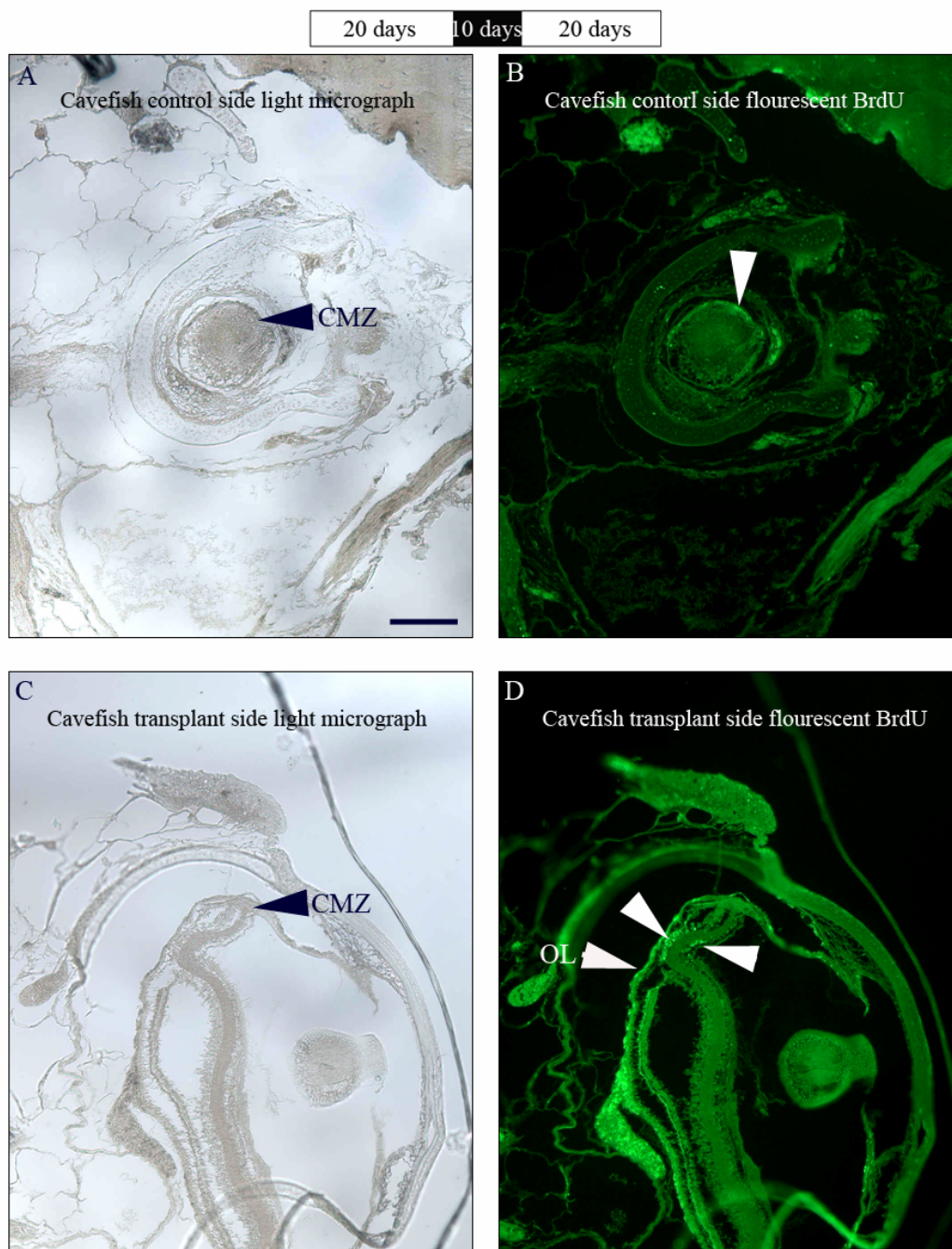
Figure 4-8 Cave fish transplant treatment 1



**Figure 4-8. Transplant cavefish subjected to BrdU incubation treatment 1.**

A, B, control side eye. C, D, lens transplantation side of the same individual. A surface fish lens was transplanted to one side of a cavefish at 36 hfp. This individual was raised to 3 mpf, then subjected to a treatment of 40 days in fresh water, followed by a 10 day BrdU pulse. The sample was fixed, embedded, cross sectioned, and immunohistochemistry was performed using a flourescein linked antibody to BrdU. Light micrographs are shown in A and C. Flourescent BrdU detection micrographs are shown in B and D. Arrowheads in B and D indicate areas of BrdU labeling. Abbreviations: CMZ, ciliary marginal zone; OL, outer layer. Scale bar in A, 200um; magnification is the same in A-D.

Figure 4-9 Cavefish lens transplant treatment 2

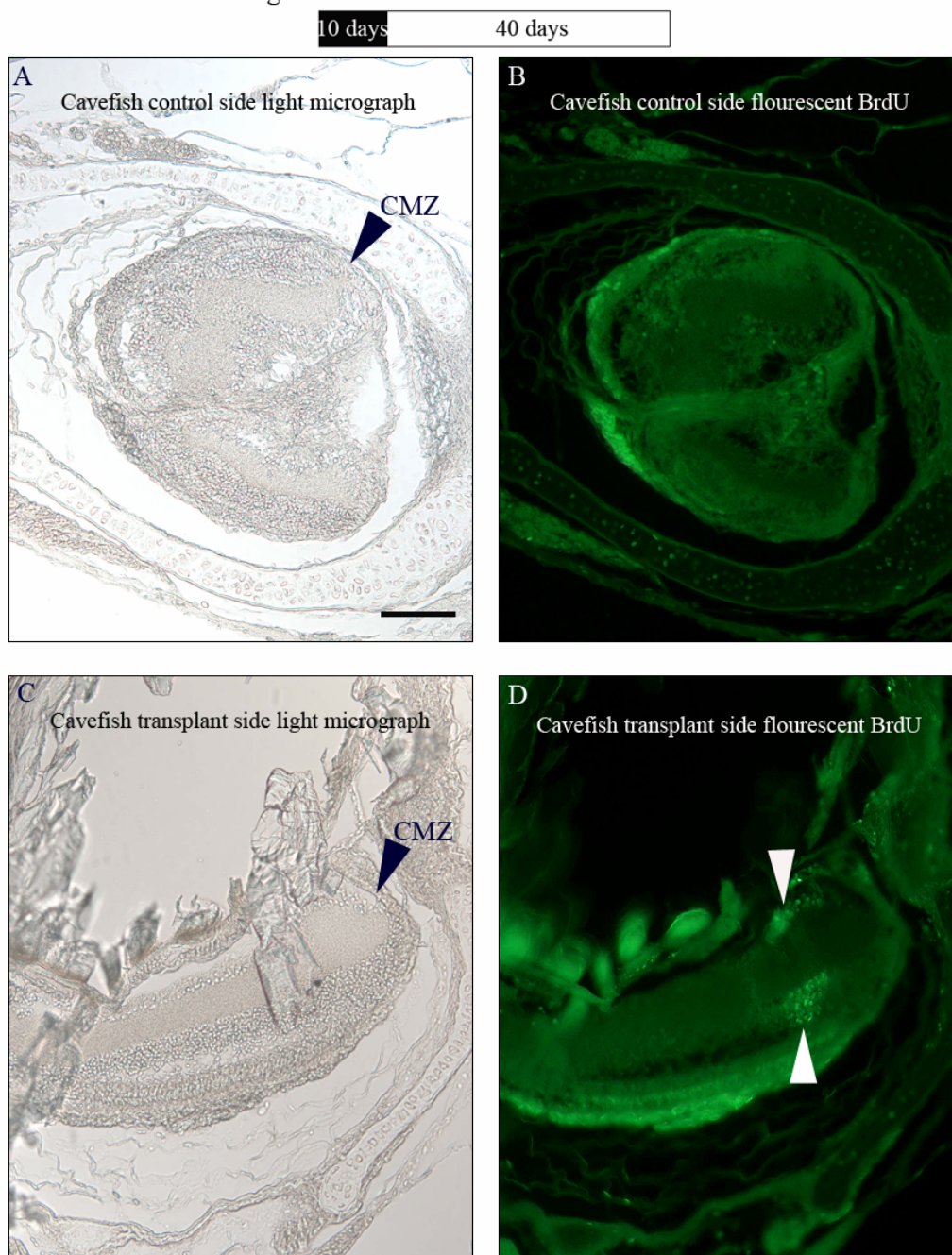


**Figure 4-9. Transplant cavefish subjected to BrdU incubation treatment 2.**

A, B, control side eye. C, D, lens transplantation side of the same individual. A surface fish lens was transplanted to one side of a cavefish at 36 hfp. This individual was raised to 3 mpf, then subjected to a treatment of 20 days in fresh water, followed by a 10 day BrdU pulse and a subsequent 20 day fresh water incubation. The sample was fixed, embedded, cross sectioned, and immunohistochemistry was performed using a fluorescein linked antibody to BrdU. Light micrographs are shown in A and C. Fluorescent BrdU detection micrographs are shown in B and D. Arrowhead in B and D indicate area of BrdU labeling. Abbreviations: CMZ, ciliary marginal zone; OL, outer layer. Scale bar in A, 200um; magnification is the same in A-D.



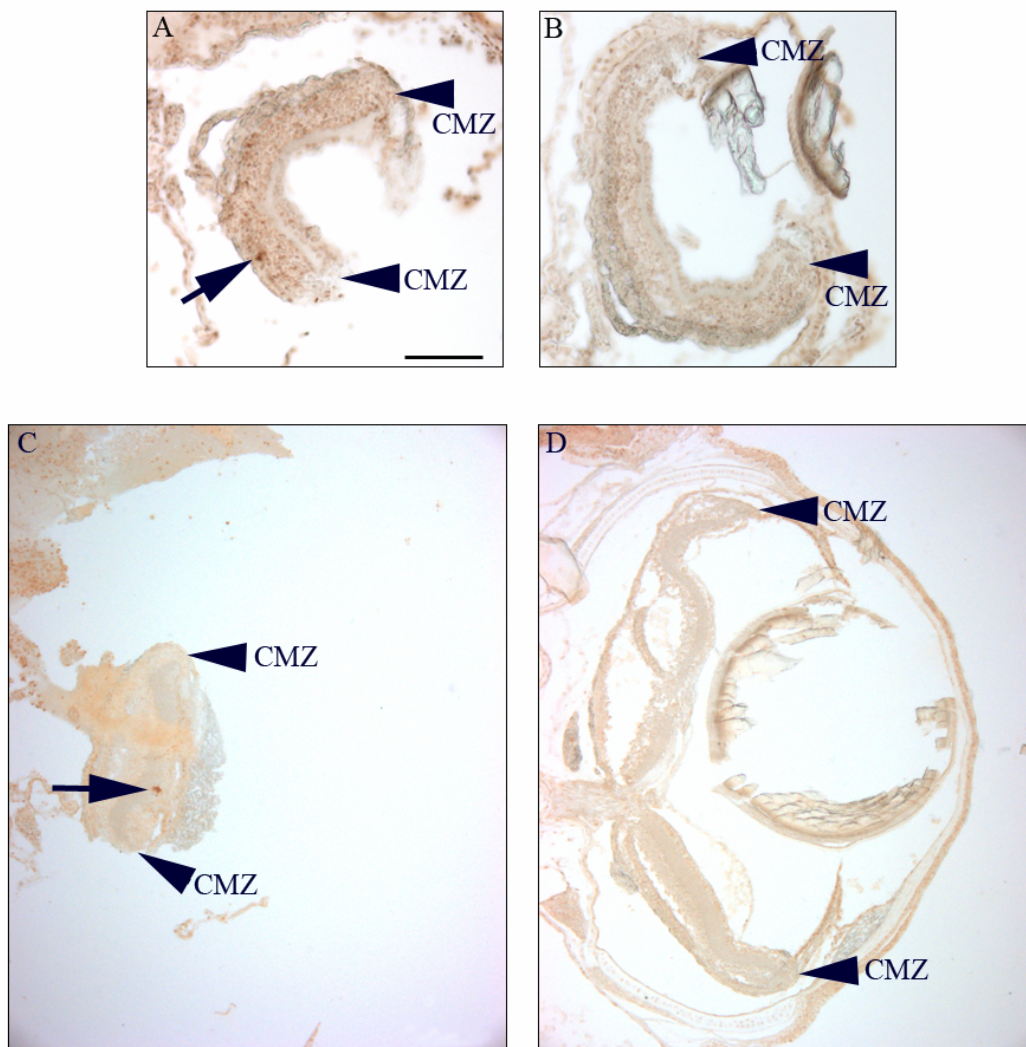
Figure 4-10 Cavefish lens deletion treatment 3



**Figure 4-10. Transplant cavefish subjected to BrdU incubation treatment 3.**

A, B, control side eye. C, D, lens transplantation side of the same individual. A surface fish lens was transplanted to one side of a cavefish at 36 hpf. This individual was raised to 3 mpf, then subjected to a treatment of 10 days in BrdU followed by a 40 day fresh water incubation. The sample was fixed, embedded, cross sectioned, and immunohistochemistry was performed using a fluorescein linked antibody to BrdU. Light micrographs are shown in A and C. Fluorescent BrdU detection micrographs are shown in B and D. Arrowhead in D indicates area of BrdU labeling. Abbreviations: CMZ, ciliary marginal zone; OL, outer layer. Scale bar in A, 200um; magnification is the same in A-D.

Figure 4-11



**Figure 4-11. Programmed cell death in cavefish subjected to lens transplant.**

A, B, One month post fertilization. C, D, Three months post fertilization. Cavefish were subjected to lens transplantations at the 30 to 36 hpf stage. These individuals were raised to 1 mpf or 3 mpf and subsequently fixed, embedded, sectioned, and subjected to programmed cell death detection by the TUNEL method. The 1 mpf control cavefish side is shown in A. The lens transplant side of the same individual is shown in B. The 3 mpf control cavefish side is shown in C. The lens transplant side of the same individual is shown in D. The arrows in A and C point to cells undergoing programmed cell death. Abbreviation: CMZ, ciliary marginal zone. Scale bar in A, 200um; magnification is the same in A-D.



side eye (light and fluorescence micrographs seen in Figure 4-10, A and B).

However, a group of BrdU labeled cells remains in the retina of the cavefish eye with a transplanted lens for this treatment (arrowhead in Figure 4-10, D; corresponding light micrograph shown in Figure 4-10, C). Although faint fluorescence around the periphery of the retina is seen in these samples, I believe this is background fluorescence since discrete cell labeling can not be detected in these areas.

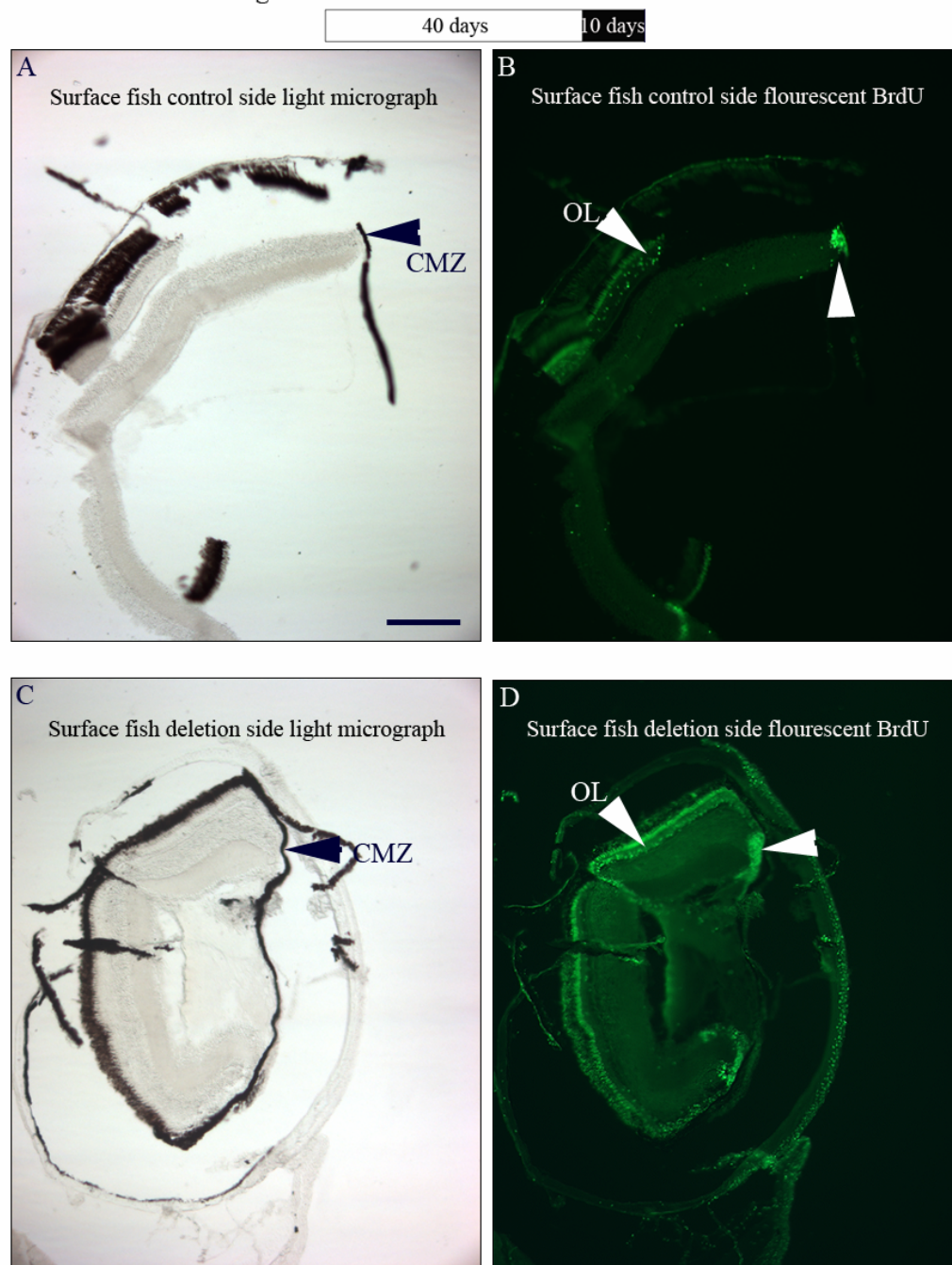
I tested for the presence of PCD at the one mpf and three mpf stages after lens transplantation. I found TUNEL labeled cells in the eye on the cavefish control side (arrowheads in Figure 4-11, A and B). However, there were no cells undergoing PCD in the lens transplanted side eye of the cavefish at either stage (Figure 4-11, B and D). It should be noted that at the one and three mpf larval stages, PCD does not occur in the normal cavefish retina to the extent it does earlier in development (see Figures 15 and 16 of Chapter 2). Typically, two to five cells are seen undergoing PCD in the whole cavefish retina at later stages. Since I only examined one sample for PCD at each of the one and three mpf time points, it is possible that PCD still occurs in the cavefish lens transplanted retina. However, if PCD does exist in the lens transplanted retina, I believe it is occurring at a reduced rate compared to the control cavefish retina.

### **Role of the lens in Surface Fish Retinal Growth**

To investigate the effects of the absence of the lens on normal eye development, lenses were removed from surface fish. These surface fish were allowed to develop to three mpf and subjected to one of the three BrdU labeling protocols. Again, one side of a surface fish individual served as the experimental deletion side, and the other, unoperated side served as the control. One surface fish individual was used for each of the three labeling treatment protocols.

The results of surface fish treatment 1 are shown in Figure 4-12. The light and fluorescence micrographs for the control side retina are shown in A and B. I saw a group of BrdU labeled cells at the CMZ in this treatment in surface fish (arrowhead in Figure 4-12, B). The lens deleted side light and fluorescent micrographs are shown in Figure 4-12, C and D respectively. Like the control side, I see a group of BrdU labeled cells localized to the CMZ (arrowhead in Figure 4-12, D). I also saw labeling in the outer layer of the retina (arrowheads labeled OL in Figure 4-12, B and D). The surface fish subjected to treatment 2 is shown in Figure 4-13. A group of BrdU labeled cells can be seen in both the control and lens deleted sides (arrowheads in Figure 4-13, B and D respectively; corresponding light micrographs shown in Figure 4-13, A and C respectively). Like treatment 1, I saw labeling in the outer layer of these samples (arrowheads labeled OL in Figure 2-13, B and D).

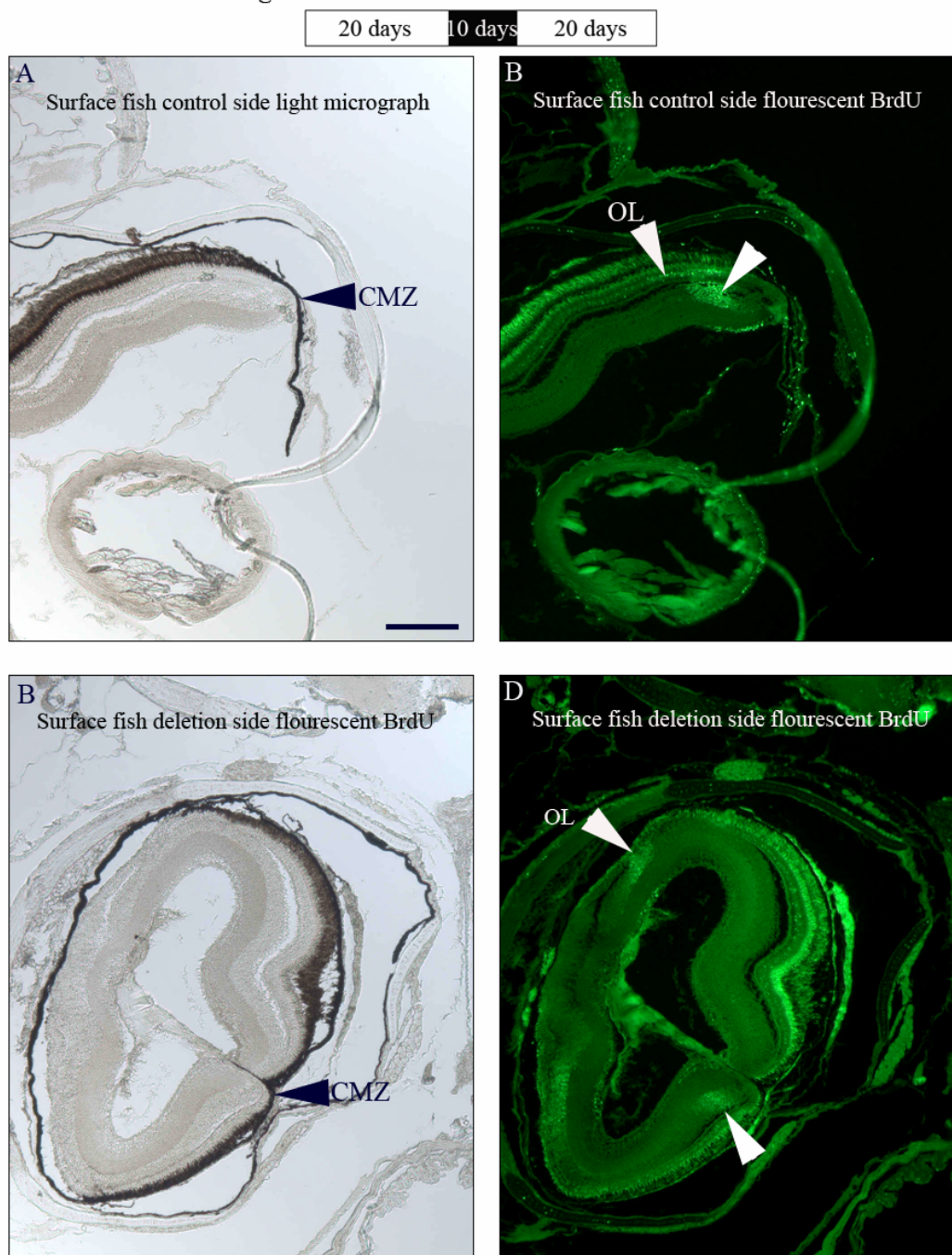
Figure 4-12 Surface fish deletion treatment 1



**Figure 4-12. Deletion surface fish subjected to BrdU incubation treatment 1.**

A, B, control side eye. C, D, lens deletion side of the same individual. The lens was deleted from one side of a surface fish at 36 hfp. This individual was raised to 3 mpf, then subjected to a treatment of 40 days in fresh water, followed by a 10 day BrdU pulse. The sample was fixed, embedded, cross sectioned, and immunohistochemistry was performed using a flourescein linked antibody to BrdU. Light micrographs are shown in A and C. Flourescent BrdU detection micrographs are shown in B and D. Arrowheads in B and D indicate areas of BrdU labeling. Abbreviations: CMZ, ciliary marginal zone; OL, outer layer. Scale bar in A, 200um; magnification is the same in A-D.

Figure 4-13 Surface fish deletion treatment 2

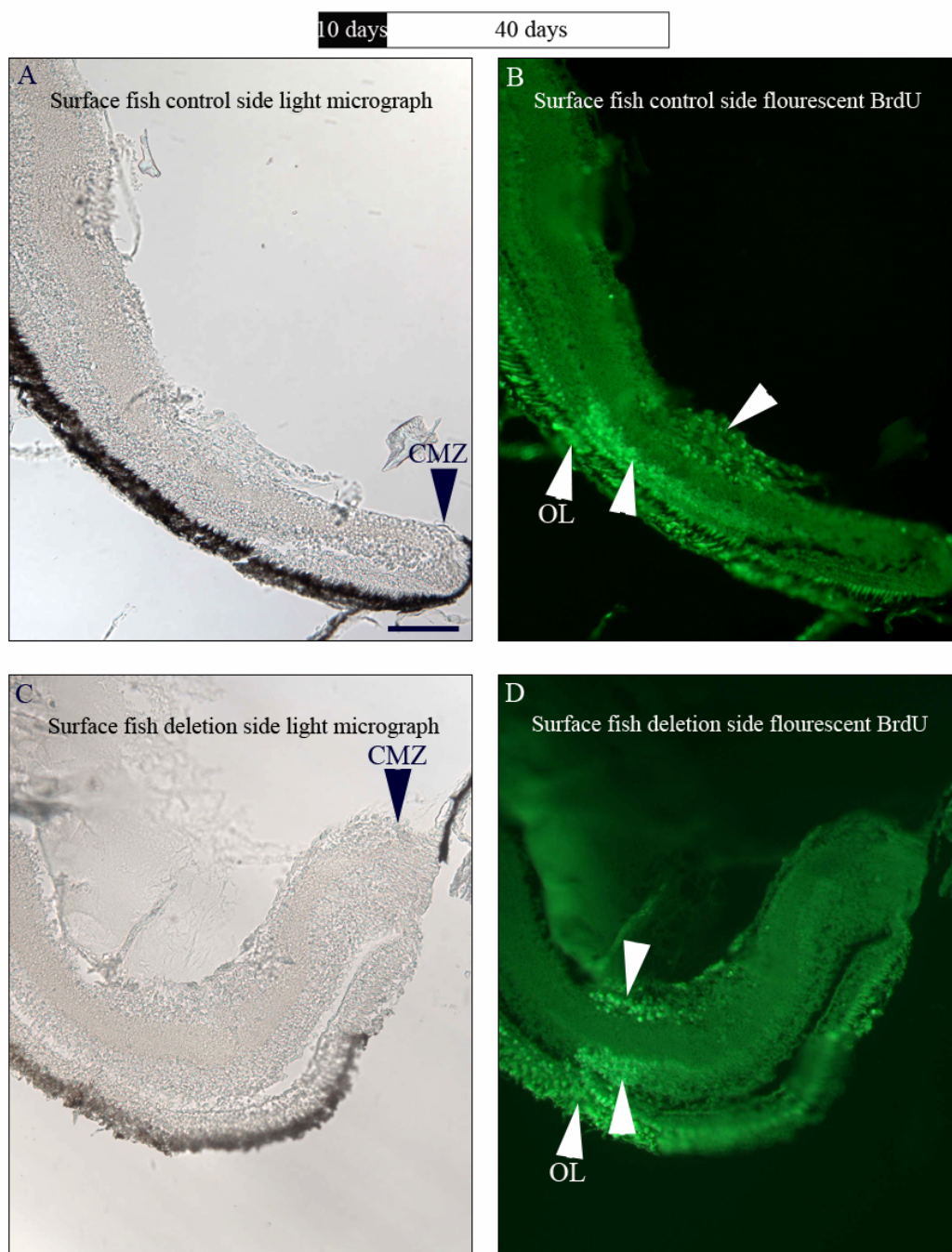


**Figure 4-13. Deletion surface fish subjected to BrdU incubation treatment 2.**

A, B, control side eye. C, D, lens deletion side of the same individual. The lens was deleted from one side of a surface fish at 36 hfp. This individual was raised to 3 mpf, then subjected to a treatment of 20 days in fresh water, followed by a 10 day BrdU pulse and a subsequent 20 day fresh water incubation. The sample was fixed, embedded, cross sectioned, and immunohistochemistry was performed using a flourescein linked antibody to BrdU. Light micrographs are shown in A and C. Flourescent BrdU detection micrographs are shown in B and D. Arrowheads in B and D indicate areas of BrdU labeling. Abbreviations: CMZ, ciliary marginal zone; OL, outer layer. Scale bar in A, 200um; magnification is the same in A-D.



Figure 4-14 Surface fish lens deletion treatment 3

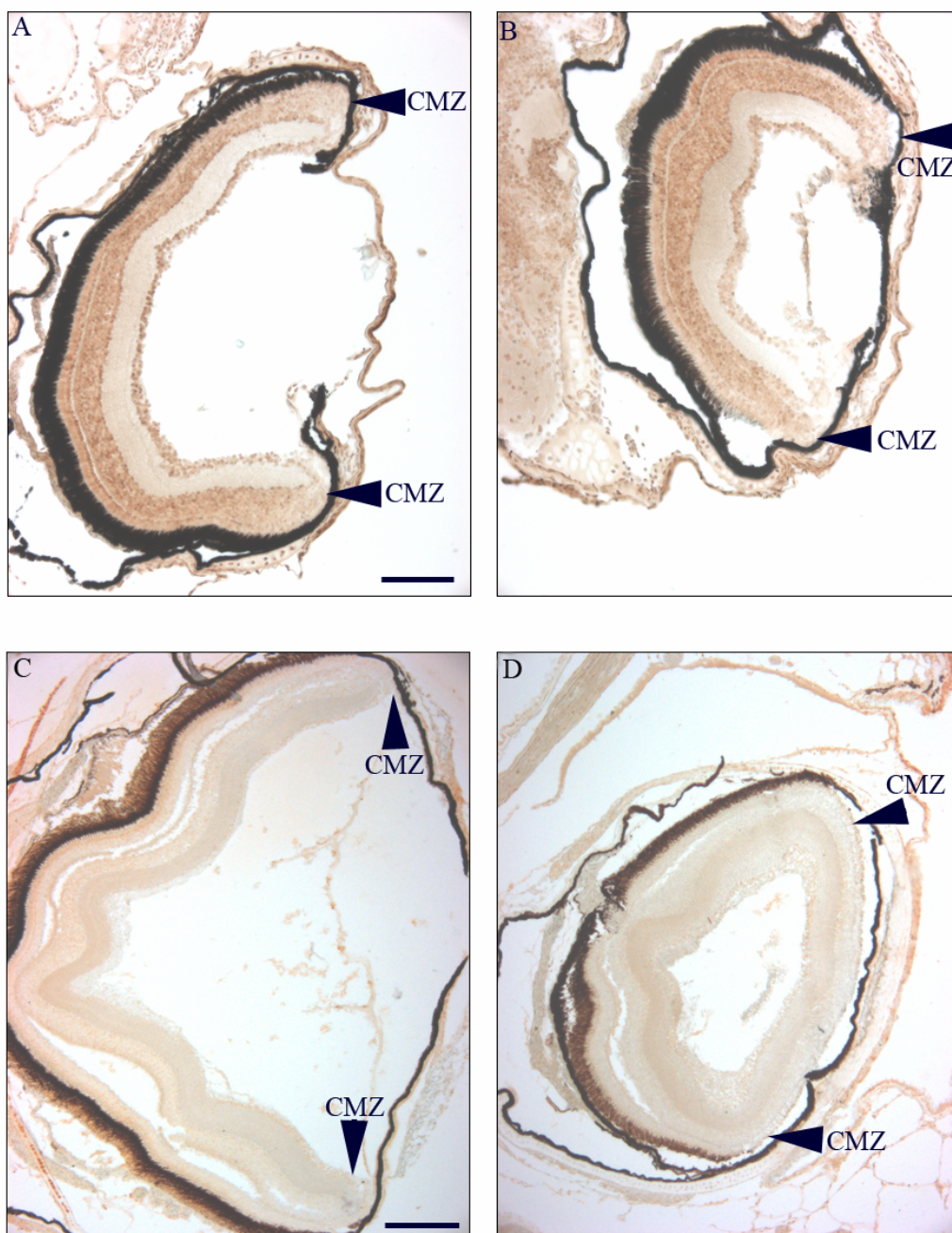


**Figure 4-14. Deletion surface fish subjected to BrdU incubation treatment 3.**

A, B, control side eye. C, D, lens deletion side of the same individual. The lens was deleted from one side of a surface fish at 36 hfp. This individual was raised to 3 mpf, then subjected to a treatment of 10 days in BrdU followed by a 40 day fresh water incubation. The sample was fixed, embedded, cross sectioned, and immunohistochemistry was performed using a flourescein linked antibody to BrdU. Light micrographs are shown in A and C. Flourescent BrdU detection micrographs are shown in B and D. Arrowheads in B and D indicate areas of BrdU labeling. Abbreviations: CMZ, ciliary marginal zone; OL, outer layer. Scale bar in A, 200um; magnification is the same in A-D.



Figure 4-15



**Figure 4-15. Programmed cell death in surface fish subjected to lens deletion.** A, B, One month post fertilization. C, D, Three months post fertilization. Surface fish were subjected to lens deletions at the 30 to 36 hpf stage. These individuals were raised to 1 mpf or 3 mpf and subsequently fixed, embedded, sectioned, and subjected to programmed cell death detection by the TUNEL method. The 1 mpf control surface fish side is shown in A. The lens deletion side of the same individual is shown in B. The 3 mpf control surface fish side is shown in C. The lens deletion side of the same individual is shown in D. Abbreviation: CMZ, ciliary marginal zone. Scale bar in A, 200um; magnification is the same in A-D.

The results for the surface fish lens deletion subjected to treatment 3 are seen in Figure 4-14. There are areas of BrdU labeled cells that have moved away from the CMZ in both the control and lens deleted retinas (Figure 4-14, B and D respectively; corresponding light micrographs shown in Figure 4-14, A and C respectively). BrdU labeling persists in these samples in the outer layer (arrowheads labeled OL in Figure 4-14, B and D).

I also tested one and three mpf lens deleted surface fish for the presence of PCD in the retina. I did not find any cells undergoing PCD in the one mpf surface fish control or lens deletion eye (Figure 4-15, A and B respectively). I found PCD results at the three mpf stage to be similar to the one mpf stage, with no PCD occurring in the eye of the surface fish control or deletion side eye (Figure 4-15, C and D respectively). Only one sample for each time point was tested for PCD, so it is still possible that PCD occurs in the lens transplanted eye, but simply did not occur in the specific lens deletion samples in our studies.

## **Discussion**

### **Proliferation and Programmed Cell Death in the *Astyanax* Eye**

I began my study of *Astyanax* eye growth by checking proliferation and programmed cell death in the retina of surface fish and cavefish. By three mpf, individuals have progressed out of the larval stage to the young adult stage. At this time, the surface fish has a well developed eye. However, the cavefish eye has sunk into the orbit and is covered with layers of connective tissue and skin. There is variability in eye size of both surface fish and cavefish, but the actual morphology of the cavefish eye is also variable based on the fact that the eye does not achieve a significant level of organization. Some cavefish eye vestiges are degenerate to the point of not possessing identifiable lens tissue, and having only a small amount of retinal tissue proximal to a reduced sclera and choroid (Wilkens, 1971). While there is variability in eye size among surface fish as well as cavefish, there is a distinct difference in eye size between the two forms.

I initially checked PCNA to test the proliferative integrity of the CMZ, and found that expression remains in the cavefish retina at the three mpf stage. Thus it appeared that a lack of a proliferation zone was not a cause for the reduced size of the cavefish retina. In addition to staining in the CMZ, I saw staining in the outer layer of the surface fish retina. This staining is probably due to cells giving rise to new rod photoreceptors. I saw PCNA staining outside the CMZ in the cavefish retina as well. However, these cells are spread throughout the retina and due to

their disorganized nature, it is difficult to determine their precise identity. There is evidence that proliferating cells outside the CMZ arise in the inner nuclear layer and subsequently migrate to the outer nuclear layer, replenishing the population of rod cell precursors (Otterson et al., 2001; Raymond and Rivlin, 1987). This could be the case in the cavefish since the cells are spread throughout the retina. A more likely explanation is that these cells are analogous to the non-CMZ proliferating cells in the surface fish retina, but instead of remaining in the outer layer, they have intercalated into other retinal layers due to the general disorganization of the cavefish retina.

After I verified the presence of PCNA in the cavefish retina, the possibility remained that a smaller area of the cavefish CMZ retains the ability to proliferate. The presence of fewer proliferating stem cells could mean an overall slower rate of retinal cell proliferation, causing a reduction in size of the retina. For example, it was shown that mouse retinas lacking Notch1 are reduced in size due to a decrease in the number of proliferative cells (Jadhav et al., 2006). I performed PCNA immunohistochemistry to measure the area of PCNA staining in the CMZ in relation to the total area of the retina.

I then performed statistical t-tests on these data to determine significance. The t-test value for the total PCNA staining area was 0.532. This value indicates that I cannot safely reject the null hypothesis, which states that the difference in PCNA staining area between surface fish and cavefish is due to chance alone. I obtained

a p value of 0.001 for the total size of the retina meaning the null hypothesis can be rejected. Therefore there is a significant difference in the total cross sectional size of the retina between surface fish and cavefish, yet the area of PCNA staining in the CMZ is not significantly different. I conclude that an alteration in proliferative ability of the cavefish retina is not a likely cause of its size reduction

In response to PCNA proliferation data, I was interested in investigating PCD as a possible cause of the degenerate cavefish retina. PCD has been documented as a normal process involved in retinal development of fish, amphibians, birds, and mammals (Cole and Ross, 2001; Vecino et al., 2004). Despite this fact, I found no PCD labeled cells in the surface fish retina at the one or three month post fertilization stages. In contrast, I found several cells undergoing PCD in the cavefish retina at both stages.

There are several possibilities that may explain why PCD does not exist in the surface fish retina despite the fact that it is documented as a normal developmental process in other organisms. First, it was found previously that the appearance of apoptotic cells throughout development occurs in temporal waves (Biehlmaier et al., 2001; Candal et al., 2005). Thus, it is possible that I am not assaying at time points when PCD is occurring during normal development. Alternatively, PCD may not occur in the retina of *Astyanax* surface fish as a part of normal development as it does in other organisms. Finally, PCD may be happening within specific cells at a very high rate while any episode of cellular

PCD in the retina does not occur very often. Thus, I may not be able to detect PCD because it is temporally rare.

Although PCNA immunohistochemistry data suggested that the cavefish retina retains normal characteristics of proliferation, the possibility remained that cells of the CMZ were simply arrested in the cell cycle and not producing new retinal cells. Zebrafish mutants have already been characterized with CMZ progenitor cells arrested in the cell cycle (Wehman et al., 2005). Furthermore, proliferation being modulated by cell cycle length is supported by other work in zebrafish (Li et al., 2000). While PCNA can detect cells proliferating at a specific moment, BrdU can be used to track cells over time. I took advantage of this fact to investigate the fate of cells as they proliferate and migrate away from the CMZ in the *Astyanax* retina. Starting at the three mpf stage, I incubated surface fish and cavefish in BrdU according to one of three treatments.

I found areas of BrdU labeling in the CMZ of both the surface fish and cavefish in treatment one (40 days in water; 10 day BrdU pulse). The BrdU incubation pulse was short enough to only label cells in the area of the CMZ, and as expected, this data was very similar to what was seen in PCNA immunohistochemistry experiments. After being subjected to treatment two (20 days in water; 10 day BrdU pulse; 20 days in water), I found an intact group of BrdU labeled cells which migrated a short distance away from the CMZ. I did not see this in the cavefish. Instead, I found only a few remaining BrdU labeled cells. In treatment

three (10 days BrdU pulse, 40 days in water), I again found a group of cells proximal to one another which had migrated away from the CMZ. In accordance with a longer water incubation chase, this group of cells had migrated a greater distance away from the CMZ than the group in treatment two. In contrast, I found no labeled cells in the cavefish retina subjected to treatment three.

My BrdU experiment results suggest that cells of the cavefish retina proliferate normally, but must be cleared away by PCD as time progresses. The balance of cell proliferation and PCD in the retina has previously been explored in the zebrafish (Li et al., 2000). Furthermore, the possibility of an increase in PCD in the cavefish retina has been previously suggested (Wehman et al., 2005). At the three mpf stage, a 20 day migration time (treatment 2) was enough for most, but not all, of the cells to be wiped away by PCD. A 40 day migration (treatment 3) was a sufficient amount of time for all the cells originally labeled with BrdU to be eradicated from the cavefish retina. Most likely, the ratio of programmed cell death to proliferation is larger in the cavefish retina when compared to surface fish, resulting in an overall smaller retina in the cavefish. Since the rates of proliferation are similar between the two forms, the rate of PCD must be higher in cavefish, which is supported by my data.



## The Effect of the Lens on the Retina

Surface fish eye development progresses in a manner similar to the zebrafish eye. The lens does not undergo massive PCD in either teleost. However, the cavefish lens undergoes elevated levels of PCD throughout development. Furthermore, the cavefish lens never forms a substantial amount of differentiated lens fibers, and only a small lens vestige remains during larval stages (Soares, et al., 2004). The lens has been shown to have an important effect on retina development. The mutant lens of the *lens opaque (lop)* mutant in zebrafish, when transplanted to a normal retina, causes the wild type retina to take on characteristics of the typical *lop* mutant retina, including lack of a definite photoreceptor cell layer. However, a wild type lens could not rescue the *lop* mutant retina (Vihtelic et al., 2005). Studies in mouse have shown that the lens has a neurotrophic effect on the retina, allowing retinal ganglion cell outgrowth (Lorber et al., 2005). Finally, there is evidence that lens epithelium derived growth factor (LEDGF) has a protective effect on the cells of the retina (Inomata et al., 2003; Nakamura et al., 2000). LEDGF was originally isolated from a human lens epithelial cell line and was shown to support the survival of lens cells, keratinocytes, and fibroblasts (Singh et al., 2000).

I was interested in investigating the effect of the lens on cell proliferation and PCD in the *Astyanax* retina. To test the effect of a normal lens on cavefish retina development, lens transplants were performed by replacing the faulty cavefish

lens with a surface fish lens at the 36 hpf stage. To test the effect of the absence of the lens on retina development, lenses were removed from optic cups of surface fish at the same time. The side of the organism opposite the embryological manipulation served as a control. I raised lens transplanted cavefish and lens deleted surface fish to three mpf and subjected them to the three BrdU incubation treatments originally used on non-deletion/transplant *Astyanax*.

All eyes, including the control side eyes and the experimental side eyes, had areas of cells labeled with BrdU localized to the CMZ after being subjected to BrdU treatment 1. This is not surprising since the cavefish control eyes (possessing faulty cavefish lenses) retained this labeling in the original BrdU experiments. There was the possibility that the surface fish deletion eye had lost its ability to support BrdU labeling without a lens, but this was not likely to occur in BrdU treatment 1. If the lens contributes to the control of growth and differentiation of the retina, there are several possibilities as to how this is accomplished. The lens may be responsible for promoting the maintenance of proliferating cells in the CMZ. It may also be responsible for maintenance of cells after they have undergone proliferation and are differentiating into mature retinal cells. A lack of labeling in the CMZ of the treatment 1 surface fish deletion, would mean the lens is directly and entirely responsible for promoting the maintenance of the proliferating cells of the CMZ. Based on my previous data that the cavefish retina retains labeling in the CMZ despite the presence of a highly degenerate lens, it is

not surprising that the surface fish deletion CMZ retained BrdU labeling in this treatment.

Based on the original BrdU experiment, one would expect a normal retina subjected to BrdU treatment 2 to possess a group of cells that has migrated away from the CMZ. I saw this result in the surface fish control eye. A few labeled cells remained on one side of the cavefish control side retina. It is possible that this fluorescence is background and no BrdU labeling remains in the retina.

Although I saw a few labeled cells in the retina of the cavefish in the original BrdU treatment 2 experiment, it is possible that all the cells have already been wiped away by PCD in this control sample based on variance in retinal size, shape, and condition among individual cavefish. The cavefish transplanted side eye retained an area of BrdU labeling. This indicated that these cells did not undergo PCD. Like the surface fish control and lens transplanted cavefish, the surface fish lens deletion side eye retained a group of BrdU labeled cells.

Treatment 3 of the BrdU incubation experiments consisted of a BrdU pulse followed by a 40 day fresh water incubation chase. The surface fish control side eye retained an area of BrdU labeled cells, which agrees with the original BrdU experimental data. Similarly, no BrdU labeled cells could be found in the control side cavefish eye. Like treatment 2, the lens transplanted cavefish retina retained an area of BrdU labeled cells. Again, this suggested that these cells were

protected from PCD. Finally, the eye of the surface fish lens deletion also retained a group of BrdU labeled cells that had migrated away from the CMZ.

To verify the BrdU incubation treatment results, I tested deletion and transplant animals for PCD at the one and three month post fertilization stages. In both cases, I found no TUNEL labeled cells in the surface fish control side, the surface fish lens deleted side, or the cavefish lens transplant side eyes. However I still found cells labeled for PCD in the cavefish control side retina. Thus, the absence of a lens is not sufficient to cause PCD in the surface fish retina. However, when a cavefish retina receives a normal surface fish lens, cells of the retina are protected from PCD.

### **Eye Development and Differentiation**

It has been shown that both morphogenesis and lamination of the retina are affected by the RPE (Raymond and Jackson, 1995). Furthermore, the *mosaic eyes* gene has been shown to affect RPE and subsequent neural retina development (Jensen et al., 2001). My PCD data show that the outer layer of the retina experiences PCD during early larval stages (see Chapter 2, Figure 15, J, L, and N). Additionally, adult cavefish possess a small and rudimentary RPE (Jeffery, 2001). While the effect of the RPE on development of the cavefish retina has not been studied, it is possible that lack of a normal RPE may be partly responsible

for retina degeneration. In addition to the effect of the RPE on proper neural retina development, it has been shown that the lens is responsible for development of anterior eye structures, most likely through a signaling mechanism (Strickler et al., 2001; Thut et al., 2001; Yamamoto and Jeffery, 2000).

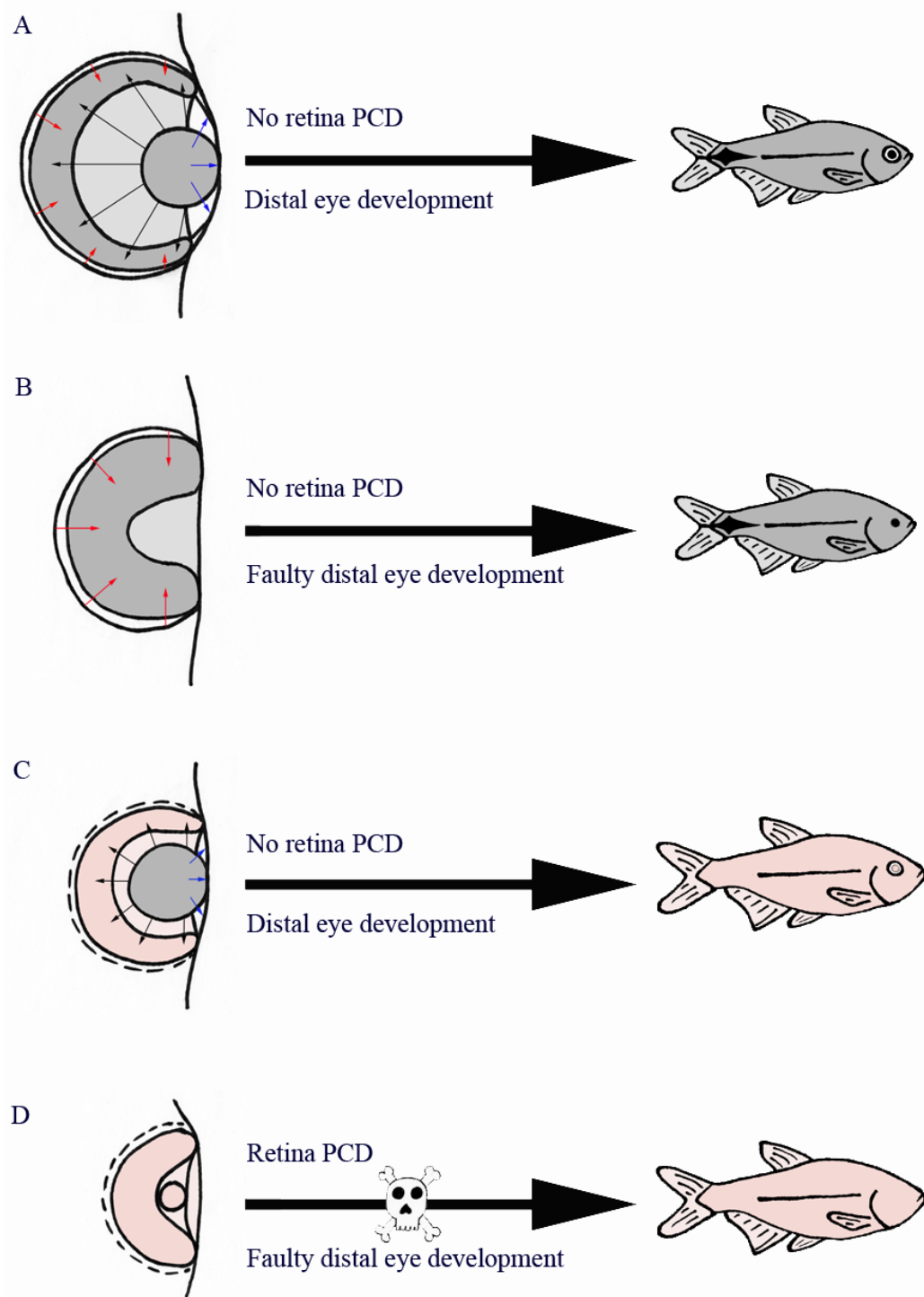
In the initial BrdU experiments in this study, the outer layer of the surface fish retina was labeled for BrdU incorporation for all three treatments. There were cells in the outer layer of the retina in treatment 1 cavefish. Only a few labeled cells were present in this region of the treatment 2 cavefish, and no labeled cells could be found in the retina outer layer of the treatment 3 cavefish. In the transplant and deletion experiments, BrdU labeled cells could be found in the outer layer of the retina of all surface fish specimens. BrdU labeled cells were found in both the control and transplant side of the treatment 1 cavefish. In treatment 2 cavefish, I found a few BrdU labeled cells in the transplant side retina, but none on the control side. I found no labeled cells in either side of the treatment 3 cavefish.

I theorize that to undergo proper development, the neural retina must receive signals from both the lens and the RPE, in an epistatic relationship. I term this the dual sub-module theory (DSM) of eye development. This theory treats the eye as a single module with the lens and RPE serving as the two prominent sub-modules within the eye which are responsible for normal development of other ocular structures.

The development of an ocular structure may be dependent on one or both of the sub-modules, such as the lens directing cornea development, or the lens and RPE both directing neural retina development. Furthermore, this theory suggests that PCD occurs in the developing retina when the RPE and lens sub-module are absent. If one of these sub-modules is present, cells of the retina are conferred protection against PCD. Based on my data, a surface fish eye normally consists of a functional lens and RPE (see Figure 4-16, A). In this case, both sub-modules develop normally and function to direct and support development of other eye structures, such as the neural retina. When the lens is deleted from the surface fish eye, the neural retina develops relatively well, but the eye lacks distal structures (see Figure 4-16, B). Additionally, PCD does not occur in the lens deleted surface fish retina.

Based on my theory, cells do not die in this case as a consequence of the support of the RPE. When a normal lens is transplanted into the cavefish retina, distal eye structures develop. The eye develops to a relatively normal state, but is not a direct phenocopy of the surface fish eye (see Figure 4-16, C). With a normal lens, cells of the neural retina do not undergo PCD. Based on the DSM theory, the eye is not fully restored because of the lack of a completely functional RPE. In normal cavefish eye development, it was shown that the lens is eradicated by PCD (Soares, et al., 2004). Furthermore, as previously mentioned, there is visual evidence that the RPE is rudimentary in the cavefish.

Figure 4-16



**Figure 4-16. A schematic of the dual sub-module theory of eye development in *Astyanax mexicanus*.** Normal surface fish eye development is represented in A. Lens deleted surface fish eyes are represented in B. Lens transplanted cavefish are shown in C. Normal cavefish eye development involving programmed cell death is shown in D. Surface fish components are designated by gray, and cavefish are designated by pink. The line surrounding the retina of the surface fish samples represents the RPE, while the broken line surrounding the outer portion of the retina of cavefish represent a proposed rudimentary RPE. Red arrows represent proposed signals from the RPE to the neural retina. Black arrows represent proposed signals from the lens to the neural retina. Blue arrows represent proposed signals from the lens to anterior structures of the eye. When lens and RPE signals are present (A and C), normal eye structures develop and retinal PCD does not occur. When one of these signals is present (B), the retina does not undergo PCD, but some morphological features of the eye are missing. When neither signal is present (D), the retina undergoes PCD and numerous eye features are absent.



Thus I predict neither sub-module is present in full capacity, resulting in faulty development of cavefish eye structures, and lack of retina PCD protection (see Figure 4-16, D).

I originally expected the surface fish retina to undergo PCD when the lens was removed. My proposed DSM theory may explain why this does not occur. The possibility remains that the faulty cavefish lens secretes a factor that causes PCD in the retina. This is doubtful, but future experiments may prove or disprove this. Furthermore, experiments showing the role of the RPE in the development of the retina of *Astyanax* should be performed. As stated, after lens transplantation, the eye of the cavefish recovers, but not fully. Obviously, the lens is not the sole cause of the cavefish program of eye development. As stated by the DSM theory, the RPE is a likely player in the process as well.

It is attractive to consider a gene that may affect cavefish lens and RPE development in a pleiotropic manner. This gene may affect the development of these ocular sub-modules, or it may be a gene that is responsible for a signal that is secreted by these two structures. Future experiments combining embryology, genetics, and molecular biology could address these issues. This information may help further explain how and why the cavefish has evolved to possess such a different mode of eye development in comparison to its surface fish counterpart.

## Chapter 5: Summary and Interpretation

My research goal is to understand how developmental and evolutionary forces influence phenotypic change. I accomplish this by studying the teleost fish *Astyanax mexicanus*. This species is ideal because both a surface dwelling form and cave dwelling form exist. Since both forms are extant and can be used for comparison studies, we are able to answer questions regarding phenotypic change on a microevolutionary scale.

I began my work by performing comparative studies to gain insight into what developmental changes cause differences in adult surface fish and cavefish eye phenotypes. I initially compared the expression patterns of the homeobox genes *pax6*, *rx*, and *vsx*. I was especially interested in the CMZ of the retina, where all three genes are expressed. I did not find major changes in the expression patterns of these genes between surface fish and cavefish in the stages studied.

In addition to gene expression in the retina, I also compared the expression patterns of the lens genes *MIP*, *MP19*,  *$\alpha A$ -crystallin*,  *$\beta B$ -crystallin*, and  *$\gamma M$ -crystallin*. These genes form structural components of the lens. MIP and MP19 form intermembrane channels involved in water balance, and the crystallins provide the lens with a transparent, refractive environment. I found expression pattern differences between surface fish and cavefish in all lens genes studied. With the exception of  *$\alpha A$ -crystallin*, gene transcripts could be detected at the 24 hpf stage in surface fish and cavefish. The cavefish lens does not appear to

accumulate a crystalline structure similar to a typical lens. In agreement with this, transcripts of cavefish lens genes were found in a compact area within the lens, opposed to the ring-like expression pattern seen in surface fish. Presumably, the ring of gene expression occurs in the differentiating lens cells surrounding the differentiating lens fiber core. The onset of  *$\alpha A$ -crystallin* expression begins at approximately 36 hpf. The expression pattern takes on a typical ring shape in the surface fish lens. However, transcripts are not found in the cavefish lens, other than a few individuals where very low amounts of expression are seen.

After studying expression patterns of several retina and lens genes, I investigated cell proliferation and PCD in the eye of *Astyanax*. I found that even as late as three mpf, proliferative cells exist in both the lens and retina of the cavefish. Furthermore, I could not find PCD in the surface fish eye, other than at the 24 hpf stage, but PCD was present in the cavefish lens and retina at all stages studied up to three mpf. I performed an initial BrdU pulse/chase labeling experiment to track cell movements in the retina. In addition to labeling for BrdU incorporation, I also studied PCD in these samples. My results indicated that cells of the cavefish retina proliferate normally, but die as they move away from the CMZ.

After my initial comparative studies, I performed both differential hybridization and microarray analysis experiments on 72 hpf larvae. I obtained several significant genes from these experiments. Both analyses indicated that  *$\gamma M$ -crystallin* was upregulated in surface fish. This was verified by *in situ*

hybridization experiments on 72 hpf surface fish and cavefish. The microarray analysis also indicated that rhodopsin may have a higher level of expression in the surface fish. This was verified by *in situ* hybridization and immunohistochemistry experiments.

In addition to the genes I verified by expression experiments, I obtained several genes that may be relevant to differences between surface fish and cavefish. For instance, I found *gnat1* and *gnat2*, which are involved in photoreceptor signal transduction. I also obtained several genes involved with PCD, some of which were upregulated in surface fish (or downregulated in cavefish). I hypothesize a possible ubiquitous downregulation of PCD protection mechanisms in cavefish. For example, the analysis indicated that *RAD54*, which is involved in cellular protection against PCD, had higher expression levels in surface fish. Of genes with higher levels of expression in cavefish, ubiquitin specific protease 53 had the highest fold upregulation level. This protein functions by marking other proteins for degradation. It was not surprising to find a gene involved in the actual PCD degradation process to be upregulated in cavefish.

My initial comparative data indicated a faulty cavefish lens. This was reinforced by gene expression patterns, such as *γM-crystallin*, obtained by the differential analyses. While early proliferation appears normal in cavefish, gene expression and PCD analysis showed that shortly after formation, the cavefish lens degenerates and does not form the proper crystalline lens structure. Additionally,

my experiments indicated that the cavefish, but not the surface fish, retina undergoes PCD. I hypothesized that the lens may be necessary for retinal cell survival and were interested in determining the role of the cavefish lens in eye degeneration. To test this, I performed surface fish to cavefish lens transplants, and surface fish lens deletions.

To test for a link between lens and retina PCD, I performed lens transplants and deletions and subjected these samples to one of several BrdU labeling protocols. The results indicated that the cavefish lens is at least partially responsible for the degeneration of the retina by allowing PCD to occur. However, I cannot conclude that the lens is responsible for preventing PCD in the surface fish retina since the lens deleted surface fish retina did not undergo any changes in PCD when compared to the control surface fish retina.

My results indicate that there must be another aspect of the cavefish eye that contributes to eye degeneration. I believe this to be the RPE. There is evidence that the RPE supports the posterior portion of the retina (Rojas-Munoz et al., 2005; Jensen et al., 2001; Malicki et al., 1996). My initial comparative studies indicated that a large amount of PCD occurs in the presumptive RPE region of the developing cavefish retina. Furthermore, genes obtained from the differential analyses, such as rhodopsin, *gnat1*, and *gnat2*, suggested that the posterior portion of the retina may not develop correctly, supporting my hypothesis that the cavefish RPE may be faulty.

This suggests that to develop correctly the eye may require signals from both a distal module and a proximal module. This hypothesis led me to postulate a dual signaling module (DSM) theory of eye development. I believe the distal signaling center to be the lens and the proximal signaling center to be the RPE. Based on this theory, both modules must be present to promote proper eye development. If one module is present, cells of the retina do not undergo PCD and are allowed to develop in a relatively normal fashion, but the eye still fails to develop completely. If neither module is present, cells of the retina undergo PCD, causing the degenerate eye phenotype. Thus, surface fish possess both fully functional modules, promoting normal eye development. Although it has not been proven that the cavefish RPE is nonfunctional, I believe this is highly likely based on my data and the work of others (Wilkens, 1988). The cavefish may have neither functional module, resulting in retina PCD and eye degeneration. When a surface fish lens (functional distal module) is transplanted into the cavefish retina, PCD does not occur. This allows relatively normal eye formation. However, the eye is not fully restored. I hypothesize that this is a result of the absence of a functional proximal module. When the lens is extirpated from the surface fish retina, the eye does not form normally. However, the retina does not undergo PCD, presumably as a result of the presence of a functional proximal module (the RPE).

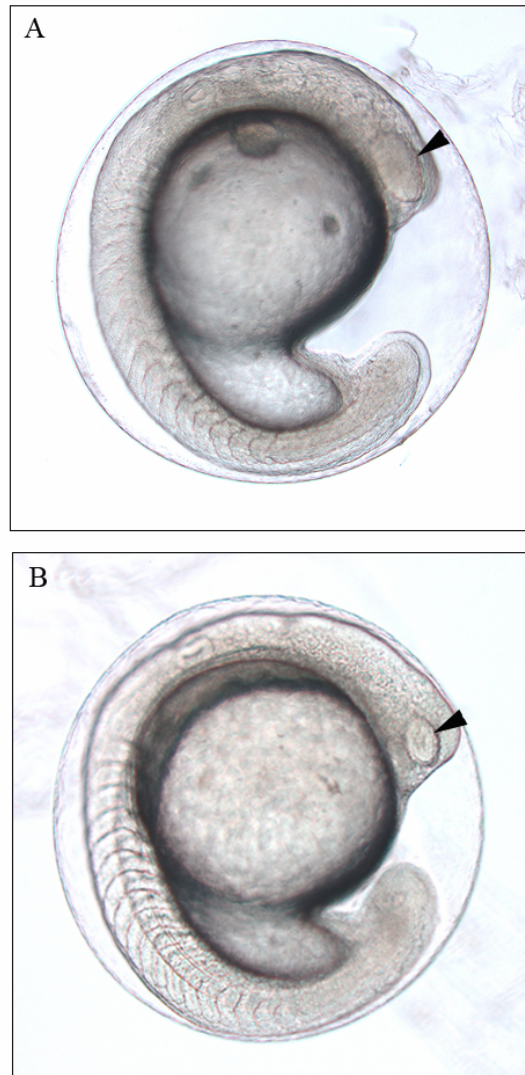
In addition to this spatial aspect of the degenerate eye phenotype of the cavefish, the temporal aspect of eye development must also be considered. There are

differences in eye size between cavefish and surface fish even before elevated levels of PCD occur in the cavefish. I initially detect PCD in cavefish and surface fish at 24 hpf, but this is a result of the lens pinching off from the surface ectoderm. This is a normal developmental process. At 36 hpf, PCD begins to occur in the cavefish eye but not the surface fish eye. However, differences in eye size exist between surface fish and cavefish from the onset of eye formation (the three to six somite stage or approximately 11 hpf). The difference in surface fish and cavefish optic bulb size at the 18 somite stage is shown in Figure 5-1. By the 24 hpf stage, the lens and retina primordia have formed. At this stage, both structures continue to be markedly smaller in the cavefish (Figure 5-2).

It is not entirely clear what causes this early difference in eye size. Moreover, early changes may influence differences that occur later in development.

Alternatively, they may be independent of one another. We may understand these phenomena more clearly if we consider pleiotropy as a causatory force. There may be a gene which, when its expression is altered, may affect more than one adult phenotype. This is in contrast to the hypotheses that cave animals lose eye structures based on genetic or developmental ‘economy’, or that relaxation of selective pressure

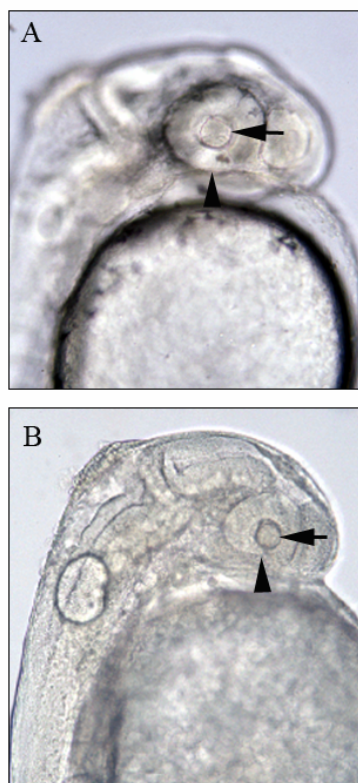
Figure 5-1



**Figure 5-1. Surface fish and cavefish embryos at the 18 somite stage. A.** Surface fish. **B.** Cavefish. Embryos are facing to the right with the head structures on the top portion of the yolk, and the tail at the bottom of the yolk. The arrowheads indicate the developing optic bulb.



Figure 5-2



**Figure 5-2. Surface fish and cavefish larvae at the 24 hpf stage.** A. Surface fish. B. Cavefish. The arrow points to the developing lens. The arrowhead points to the ventral portion of the developing retina.

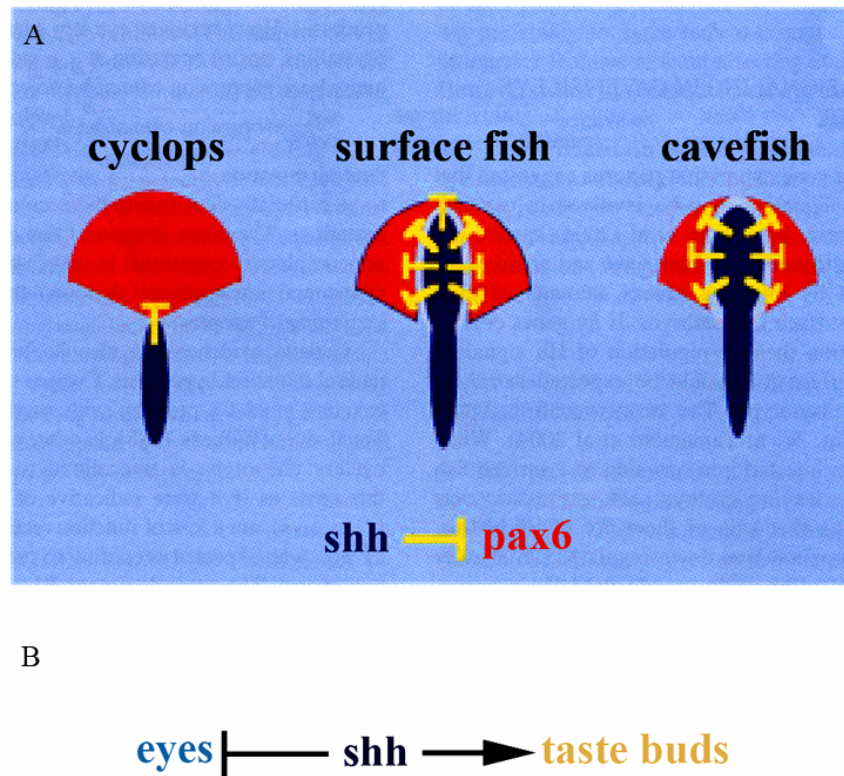
causes the accumulation of neutral mutations in eye development genes.

Specifically in *Astyanax*, the hedgehog (hh) genes may contribute to eye development in conjunction with affecting other phenotypes, such as taste bud development (Jeffery, 2005).

*Sonic hedgehog (shh)* and *tiggy winkle hedgehog (twhh)* genes function as midline signaling molecules in teleosts (Ekker, et. al., 1995). During the neural plate stage, *shh* and *twhh* expression domains are expanded at the anterior midline of the cavefish compared to the surface fish (Yamamoto, et. al., 2004). Additionally, expression domains of genes known to act downstream of hedgehog genes, such as *patched* and *nkx2.1a*, are expanded in the cavefish. It has been shown that increased anterior *shh* expression in the embryo suppresses anterior expression of *pax6*, resulting in a smaller anterior *pax6* expression domain (Ekker, et. al. 1995). At this stage, *pax6* is responsible for setting up the presumptive eye region. Therefore, a smaller *pax6* expression domain may result in smaller eyes in the cavefish. It is unclear what ultimately causes changes in hh gene expression patterns between surface fish and cavefish.

In addition to a role in early determination of the eye field, hh genes have been reported to be involved in taste bud development (Hall, et. al., 1999). There is still debate regarding what early factors promote taste bud development. The original model, known as the neural induction model, proposes that innervation must occur for taste buds to form (Barlow, 1999). However, more recent work in

Figure 5-3

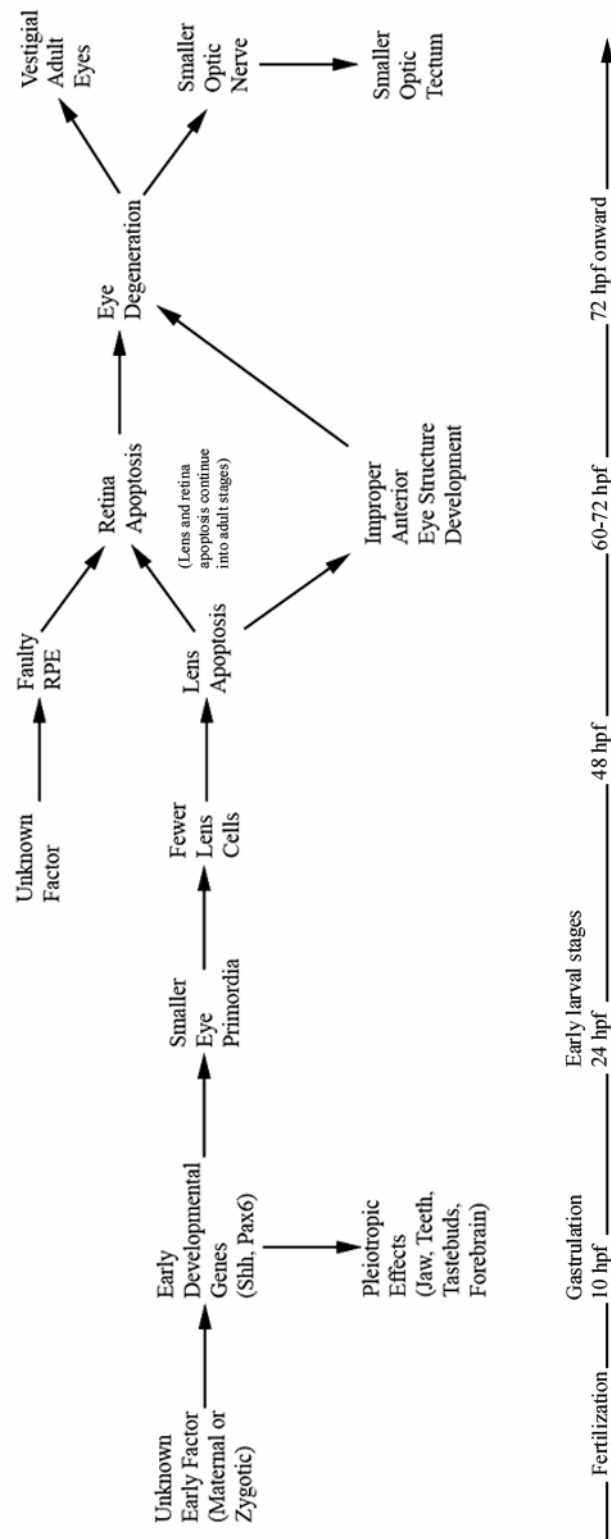


**Figure 5-3. Schematic overview of the pleiotropic action of shh in *Astyanax* development.** The interactions of shh and Pax6 are shown in A. In the case of cyclops, too little shh is present to split the anterior Pax6 field. In surface fish, a smaller anterior shh expression domain results in a larger Pax6 domain, promoting the development of larger eyes. The larger anterior domain of shh in cavefish promotes a smaller Pax6 domain and smaller eyes. A model of the pleiotropic actions of shh are shown in B. More shh represses eye development, while promoting the development of taste buds. (Adapted from Jeffery, 2005).

amphibians has shown that taste buds can develop independently of innervation. The oropharyngeal endoderm, which is specified by early signals from the axial mesoderm, is the origin of development (Barlow, 2003; Northcutt, 2004). Hedgehog genes may have a role in this early specification. When *shh* mRNA is injected into surface fish, a larger jaw and more taste buds form. Conversely, when *shh* morpholinos are injected into cavefish, a smaller jaw and fewer taste buds form (Yamamoto and Jeffery, unpublished data). Thus, it is possible that a larger anterior hedgehog expression domain promotes jaw and taste bud development at the expense of eye development through a pleiotropic mechanism (Jeffery, 2005b). A summary of this theory is presented in Figure 5-3.

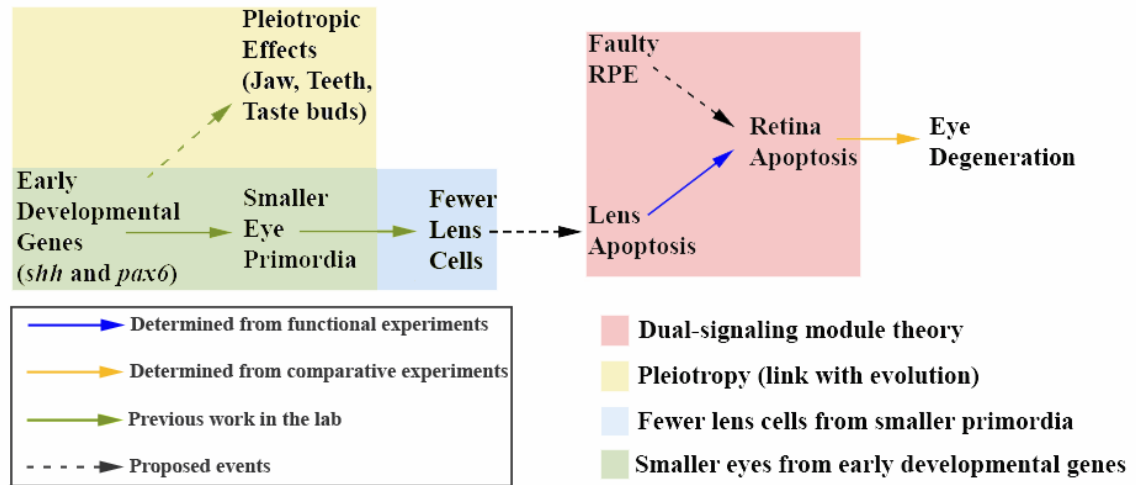
To place this in the context of evolution, we must consider the physical environments of surface fish and cavefish. Surface fish live in surface streams on natural light cycles, while cavefish live in complete darkness. Furthermore, surface streams experience a more constant food source in contrast to the cave, where food is often scarce. Thus, in an environment devoid of light, there is no need for the sense of sight, while more taste buds may better aid cavefish in food location. Therefore, changes in one or a few genes, such as hedgehog genes, may simultaneously alter the phenotype of the organism to fit its environment. In this case, increasing the anterior expression domain of *hh* genes during the neural plate stage may promote the development of an increased number of taste buds. Simultaneously, this may cause the development of a smaller eye, which is not structurally or functionally necessary in the cave environment.

Figure 5-4



**Figure 5-4. A model showing the course events that may lead to cavefish eye degeneration throughout development.** An unknown early factor may affect the expression of *shh* which in turn affects the expression of *Pax6*. Pleiotropic effects such as the development of an increased number of taste buds may result. This results in smaller cavefish eye primordia. Fewer lens cells result from this which may cause lens PCD. This in turn may cause development of anterior eye structures. In conjunction with a possible faulty posterior eye module (RPE), the faulty lens may also cause PCD in the retina along with improper retina development. This results in eye degeneration, a smaller optic nerve, and smaller optic tectum in cavefish. A basic timeline of *Astyanax* development is given at the bottom of the figure.

Figure 5-5



**Figure 5-5. A review of what we know concerning cavefish eye regressive evolution.** Early factors, most likely through a pleiotropic mechanism, cause the development of a smaller eye (and lens) in the cavefish. This may promote lens PCD which contributes to retina apoptosis and the eventual degeneration of the eye. with information obtained from other sources such as differential analyses, candidate gene studies, and genetics.

Based on functional experiments, an increase in *hh* gene expression in the surface fish can cause the development of a smaller lens that undergoes PCD (Yamamoto, 2004). It has been shown that lens cells in culture do not require other cell types for survival, but when the cell density is low, they will undergo PCD (Wride, 1996). Furthermore, low density cultures are rescued from PCD after the addition of medium from high density cultures. Thus, lens cell survival is dependent on cell density. It is possible that a larger *hh* expression domain in the anterior cavefish embryo may promote the development of smaller eye structures. The lens may be too small and possess too few cells to support survival, resulting in subsequent PCD. This lens degeneration, as shown by the lens deletion and transplantation experiments, can have significant effects on overall eye development. A schematic showing a possible scenario for cavefish eye degeneration is shown in Figure 5-4.

It is likely that a factor prior to the action of *hh* genes in the embryo is responsible for alterations in cavefish development. This factor may possibly be traced back to the maternal environment. It will be interesting to determine the identity of this factor if it exists. Furthermore, studies should be performed to determine the extent of RPE development in cavefish, followed by studies determining the role of the RPE in *Astyanax* eye development. Finally, studies should be performed to determine the specific cause of PCD in the cavefish lens. It will be important to integrate these data with other developmental studies in *Astyanax*.



In summary, previous work indicates that a pleiotropic mechanism may be responsible for the smaller eye of the cavefish, which may subsequently promote lens apoptosis. This study provides evidence that lens apoptosis is an indirect cause of retina apoptosis, and that retina apoptosis is a major contributor to eye degeneration in cavefish (See Figure 5-5). These results support the pleiotropic theory of natural selection as a cause of cavefish eye regressive evolution. It will be interesting to see if pleiotropic mechanisms are responsible for the evolution of other phenotypic characters in *Astyanax* and if these mechanisms are similar to those responsible for regressive evolution in other cave adapted animals. Ultimately, an understanding of regressive eye evolution may provide clues concerning eye evolution in general and how eye evolution may be linked to the evolution of other phenotypes.

We will eventually integrate *Astyanax* studies with the work of others and extrapolate the data to other systems. For example, when we understand the specific intraspecies developmental changes that have resulted in the evolution of cavefish from surface fish, we may start to answer questions about the forces which have caused interspecies phenotypic changes among closely related organisms. Finally, we can then understand how more diverse phenotypic forms have evolved by applying these concepts to developmental and evolutionary events on a larger scale.

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