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Adaptive Evolution of Eye Degeneration in the Mexican Blind Cavefish

W. R. JEFFERY

Department of Biology, University of Maryland, College Park, MD 20742

Address correspondence to William R. Jeffery at the address above, or e-mail: jeffery@umd.edu

Abstract

The evolutionary mechanisms responsible for eye degeneration in cave-adapted animals have not been resolved. Opposing hypotheses invoking neural mutation or natural selection, each with certain genetic and developmental expectations, have been advanced to explain eye regression, although little or no experimental evidence has been presented to support or reject either theory. Here we review recent developmental and molecular studies in the teleost *Astyanax mexicanus*, a single species consisting of a sighted surface-dwelling form (surface fish) and many blind cave-dwelling forms (cavefish), which shed new light on this problem. The manner of eye development and degeneration, the ability to experimentally restore eyes, gene expression patterns, and comparisons between different cavefish populations all provide important clues for understanding the evolutionary forces responsible for eye degeneration. A key discovery is that Hedgehog midline signaling is expanded and inhibits eye formation by inducing lens apoptosis in cavefish embryos. Accordingly, eyes could have been lost by default as a consequence of natural selection for constructive traits, such as feeding structures, which are positively regulated by Hh signaling. We conclude from these studies that eye degeneration in cavefish may be caused by adaptive evolution and pleiotropy.

The unusual phenotypes of cave animals, including the absence of eyes and pigmentation, have been a source of amazement since the Middle Ages. For example, in Slovenian folklore the blind and ghostly pale cave salamander *Proteus anguinus* is thought to be the larval stage of a dragon (Habic 1993). Likewise, the evolutionary processes that gave rise to these phenotypes have been the subjects of considerable uncertainty and debate. In the *Origin of Species*, Darwin found no reason to invoke natural selection to explain the loss eyes in cave animals, remarking, "As it is difficult to imagine that eyes, though useless, could in any way be injurious to animals living in darkness, I attribute their loss solely to disuse" (Darwin 1859). Since then many different theories have been advanced to account for eye loss in cave animals, but little or no experimental evidence has been obtained to support or reject any of them. Today, the mystery still persists, although the field of possibilities has been narrowed to two opposing hypotheses.

The neutral mutation hypothesis (Kimura and Ohta 1971) suggests that eye regression is caused by random mutations in eye-forming genes, which accumulate in cave animals under relaxed selective pressure (Culver 1982; Wilkens 1988). Stated another way, this idea says that given enough time and a sufficiently high mutation rate, the eye is doomed to eventually disappear because it is not necessary for survival in the dark cave environment. In contrast, the adaptation hypothesis suggests that loss of eyes is adaptive and has a selective advantage in the cave environment (Culver 1982; Poulson 1963; Poulson and White 1969). As implied in the Darwin quote, however, the actual benefits of blindness have been difficult to understand. Accordingly, different versions of the adaptation hypothesis attribute eye regression to energy economy, citing the high cost of making an eye (Culver 1982), to pleiotropic effects (Wright 1964), in which sensory organs beneficial to survival in the cave environment are enhanced at the expense of eyes (Barr 1968), or even to active destruction of eyes because of their potential liability in caves. However, little or no experimental evidence has been obtained in support of any version of either the neutral mutation or adaptation hypotheses.

Here I review recent experimental studies of eye degeneration in the Mexican cavefish *Astyanax mexicanus* that shed new light on the mechanisms responsible for the evolution of regressive and constructive features in cave animals.

The *Astyanax* System

The teleost *A. mexicanus* is an excellent model for experimental studies on the evolution of eye degeneration (Jeffery 2001). It is a single species consisting of two morphological types: an eyed and pigmented surface-dwelling form (surface

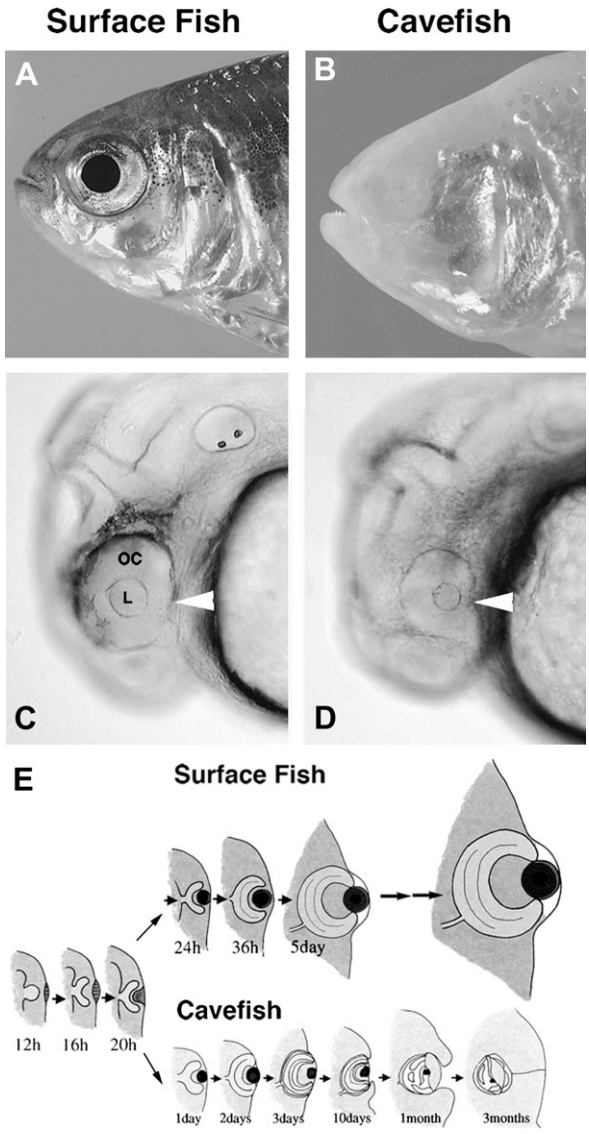


Figure 1. Eye development and degeneration in *Astyanax mexicanus*. Surface fish **(A)** and cavefish **(B)** adults. Surface fish **(C)** and cavefish **(D)** embryos at the 24 h stage showing eye primordia with lens (L) and optic cup (OC). Arrowheads indicate the ventral sector of the OC. **(E)** Diagram showing the timing of eye growth and development in surface fish (top) and eye degeneration in cavefish (bottom). **A–D** from Yamamoto et al. (2004).

fish) (Figure 1A) and at least 30 different eyeless and depigmented cave-dwelling (cavefish) forms (Figure 1B) (Avise and Selander 1972; Espinasa et al. 2001; Mitchell et al. 1977; Wilkens and Burns 1972). The cavefish populations originated at different times, and some may have evolved eye regression independently (Dowling et al. 2002; Strecker et al. 2003, 2004; Wilkins and Strecker 2003). Thus, *Astyanax* cavefish populations are an attractive resource to investigate the evolutionary forces driving eye degeneration in subterranean animals (Jeffery et al. 2003).

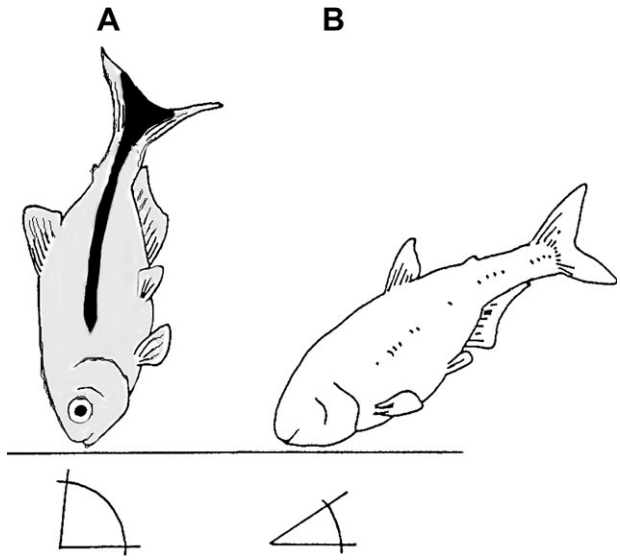


Figure 2. Bottom feeding behavior and orientation angles in surface fish **(A)** and cavefish **(B)**. After Schemmel (1980).

As expected of the same species, *Astyanax* surface fish and cavefish, as well as the different cavefish populations, are completely interfertile (Sadoglu 1957; Wilkens 1971, 1988), although usually isolated from each other in nature. However, conspecific surface fish and cavefish populations exhibit dramatic morphological and behavioral differences (Wilkens 1988, Jeffery 2001). The polarity of these evolutionary differences is known with certainty because cavefish were originally derived from surface fish ancestors, probably about 10,000 years ago (Mitchell et al. 1977). In addition to regressive changes, such as the loss of eyes and pigment, cavefish also show less conspicuous constructive changes. I will briefly describe some of the major constructive and regressive changes in cavefish, other than loss of vision and pigment, because they will be important in our considerations of eye degeneration.

Cavefish have reduced the size of their optic tecta (Soares et al. 2004; Voneida and Fish 1984; Voneida and Sligar 1976), the region of the brain responsible for interpreting visual information. Likewise, the strong schooling behavior characteristic of surface fish has disappeared. These changes are likely to be secondary consequences of eye degeneration.

A significant change in feeding behavior could be a key evolutionary alteration in cavefish (Schemmel 1980). Surface fish usually feed in the water column using visual cues. When feeding on the bottom in complete darkness, however, they orient their anteroposterior body axis perpendicular to the bottom and pivot to scan a limited area of the substrate (Figure 2A). In contrast, cavefish, which seldom feed in the water column, orient with their anteroposterior body axis at about a 45° angle to the substrate when bottom feeding (Figure 2B). This orientation, along with changes in head and oral structure (Jeffery 2001), allows them to scan a larger substrate area than surface fish without pivoting, which is probably a more effective strategy for finding potential food

items within detritus on the floor of cave pools. Likely in relation to feeding preferences, cavefish have also increased the size of the their jaws, the number of oral and pharyngeal tastebuds, and the number of maxillary teeth (Schemmel 1967, 1974; Jeffery et al. 2000; Jeffery 2001; Yamamoto et al. 2003). Accordingly, laboratory experiments have shown that cavefish are very efficient in feeding and will outcompete surface fish for a limited amount of food when they share a darkened aquarium (Hüppop 1987).

Other morphological changes, some related to behavioral differences, have evolved in cavefish. The size and number of cranial neuromasts has increased (Jeffery et al. 2000; Teyke 1990), possibly to more effectively avoid obstacles while swimming in darkness. There are also several differences in skeletal organization that do not have any obvious adaptation to the cave habitat. The size, shape, and organization of craniofacial bones, particularly those surrounding the eye socket, are altered in cavefish (Alvarez 1947; Yamamoto et al. 2003). Cavefish show one or two fewer rib-bearing vertebrae and a more compressed body shape than surface fish (Dowling et al. 2002). Finally, cavefish body scales are also compressed relative to surface fish scales (Wilkens 1988), perhaps to conform to their compact body. In summary, many regressive changes in cavefish seem to be related to loss of sight, whereas most constructive changes seem to be related to feeding and/or swimming behavior, and there are a few presumably neutral changes that presently defy explanation.

Astyanax is an excellent laboratory animal. It exhibits many of the favorable attributes that have made the zebrafish a model system in genetics and developmental biology, including external fertilization, frequent and abundant spawning, large transparent embryos, and the opportunity to combine molecular, developmental, and genetic analysis (Jeffery 2001). Therefore, the *Astyanax* system is extremely well suited to experimental studies of the evolutionary mechanisms responsible for eye degeneration.

What Does Development Tell Us?

Evolutionary changes proceed through alterations in development (Gould 2002). Thus, developmental studies might provide important clues about the evolutionary mechanisms underlying eye degeneration.

Cahn (1958) carried out the first detailed comparative analysis of eye development in *Astyanax* surface fish and cavefish. In her histological studies of Chica and Los Sabinos cavefish, she found that embryos of both populations actually develop eye primordia, which gradually disappear during the larval stage. Her work has been confirmed many times and in additional *Astyanax* cavefish populations (Langecker et al. 1993; Jeffery et al. 2003). The recent studies on eye development described herein have been carried out with the Pachón cavefish population.

Similar to other vertebrate embryos, the first sign of eye development in both forms of *Astyanax* is the appearance of optic vesicles, lateral bulges extending from the left and right margins of the developing brain (Figure 1E, also see Figures 5G, 5H, and 6G later). As the optic vesicles expand and extend toward the surface ectoderm, they constrict proximally to form the optic stalks and invaginate at their distal margins to form the optic cups (Figure 1E). Meanwhile, the surface ectoderm lying immediately distal to the optic vesicles thickens to form the lens placode, which subsequently pinches off to form a lens vesicle, which protrudes into the optic cup (Figure 1E). The eye primordia consist of the lens vesicle, an optic cup with an inner retinal layer and the outer retinal pigment epithelium (RPE) layer, and the optic stalk, which later channels the retinal axons toward visual centers in the brain (Figure 1E).

The events occurring up to this point are known as the proliferative (or embryonic) phase of eye development because all cells in the lens and optic cup are still undergoing cell division (Hu and Easter 1999). Cell differentiation occurs in this phase of eye development as well, particularly in the retina, which begins to show subdivision into layers. Initially, cell differentiation appears to be normal in the cavefish eye, as indicted by the formation of precursors of the first fiber cells (primary lens fiber cells) in the lens and the inner nuclear, outer nuclear, and photoreceptor layers of the retina. Except for size and the proportion of parts (see later discussion), there are no remarkable differences in the surface and cavefish eye primordia during the embryonic phase. The cavefish eye primordia, including the optic vesicles, optic cups, and lens vesicles are smaller than their surface fish counterparts (Figure 1C, D; Strickler et al. 2001; Yamamoto et al. 2004). The smaller size of the optic cup is due to reduction of its ventral sector (Figure 1D; Jeffery et al. 2003, Yamamoto et al. 2004), a significant alteration that I will return to later in this review.

The second phase of eye development is known as the growth phase because the eye gets larger as new cell division occurs in stem cells located in specific regions of each tissue. The stem cells divide asymmetrically to form another stem cell and a cell that will ultimately cease dividing and differentiate. The growth phase of eye development continues throughout life in teleosts as the eye increases in size in proportion to the body (Figure 1E; Harris and Perron 1998; Johns and Easter 1977). In the lens, cell division occurs continuously in its marginal epithelial zone to produce secondary fiber cells, which join the primary fiber cells as concentric layers within the central core of the growing lens. Cell division also occurs at the distal margin of the optic cup (retina and RPE), a region known as the ciliary marginal zone (CMZ). As new cells are formed at the CMZ, they are gradually displaced to a more central location in the retina and RPE, and they subsequently differentiate into specific cell types.

New cells are also added to the developing eye from the cranial neural crest, which migrates into the developing optic region and contributes mainly to tissues outside the lens and optic cup. For example, the iris and ciliary body, which form at the margins of the optic cup, are partial neural crest derivatives, and the bilayered cornea develops from a combination of surface ectoderm and migratory neural crest cells infiltrating the region between the surface ectoderm and lens.

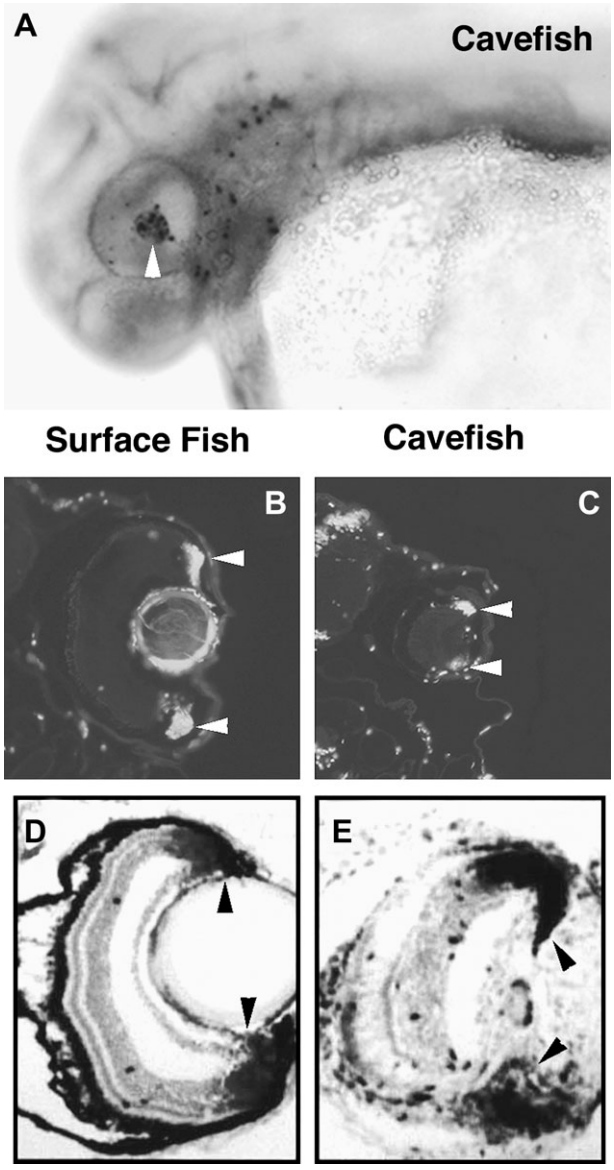


Figure 3. Cell death and proliferation in the degenerating cavefish eye. **(A)** A cavefish embryo at the 36 h stage showing TUNEL detected lens apoptosis (arrowhead); from Jeffery and Martasian (1998). Sections of surface fish **(B**, **D)** and cavefish **(C**, **E)** eyes showing cell division detected by BrdU labeling **(B**, **C)** or PCNA antibody staining **(D**, **E)** in the CMZ (arrowheads). **B–E** from Strickler et al. (2002).

Finally, the outer layer of connective cells surrounding the interior portions of the eye, a thin cartilaginous or bony tissue known as the sclera, is formed entirely from migratory neural crest cells.

The cavefish eye begins to diverge from its surface fish counterpart during the growth phase. First, the primary and secondary lens fiber cells do not complete differentiation, and the central core of crystallin containing fibers does not form. Second, although retinal ganglion and glial cells are present, there is an obvious deficiency in rhodopsin-expressing rod photoreceptor cells, and pigment cells do not differentiate in the RPE (Jeffery et al. 2000; Langecker et al. 1993; Yamamoto and Jeffery 2000). Finally, the cornea, iris, and ciliary body do not develop, although neural crest cells migrate into the eye region, and the sclera, although present, remains cartilaginous, rather than partially ossified, as it is in surface fish (Yamamoto et al. 2003). The lack of overall eye growth and rapid increase in body size during this period lead to "swamping" of the cavefish eye by the growing body, which is manifested by disappearance of the eye into the orbit and sealing by a flap of skin.

The critical information that can be obtained by comparing eye development in surface fish and cavefish is that the cavefish eye develops normally for a considerable length of time before finally disappearing due to failure to keep up with overall body growth. Therefore, the developmental evidence does not support an evolutionary model that proposes loss of function of the genes involved in early eye development and/or eradication of the embryonic eye to conserve energy. This conclusion is substantiated and further extended by experimental results described in the following sections.

What Does the Process of Eye Degeneration Tell Us?

Clues about the evolutionary processes involved in eye loss can also be obtained from studying the manner of degeneration. Although earlier studies described degeneration of various eye parts in cavefish based on histological or ultrastructural analysis (Cahn 1958; Langecker et al. 1993), they did not report the timing or spatial parameters of this process. More recent studies using TUNEL analysis to detect apoptotic cell death revealed that eye degeneration occurs in several steps (Jeffery and Martasian 1998; Strickler et al. 2002; Strickler and Jeffery unpublished data). The first step is apoptosis of the embryonic lens (Figure 3A). Lens cell death is first detected early in cavefish development, about a half-day after the lens vesicle has pinched off from the surface ectoderm, and prior to the first differentiation of fiber cells in the surface fish lens. Apparently, lens cells are programmed to die rather than differentiate. No other eye tissues consistently show apoptosis at this early stage in eye development. Next, the retina begins to show cell death, although primarily in a restricted zone between the CMZ and the differentiating cell layers produced during the embryonic phase of eye development. Once the cell death process begins, however, it is detected continuously in both the lens and retina throughout subsequent development (Soares et al. 2004; Strickler and Jeffery unpublished data).

Cell death is often triggered when cells are prevented from undergoing normal divisions. Therefore, it was expected that the lens and retina are stimulated to undergo programmed cell death because of arrested stem cell division. However, this is not the case. Analysis of cell proliferation in the cavefish lens and retina using the thymidine analog BrdU (Figure 3B, C), the DNA polymerase cofactor PCNA (Figure 3D, E), and the *Rx* and *Vsx* homeobox genes, which are expressed by recently

divided CMZ cells, show that both tissues continuously produce new cells in the correct spatial location (Strickler et al. 2002). Because the cavefish lens and retina do not increase appreciably in mass, the newly divided cells must be targets of apoptosis. Accordingly, growth is not retarded because cell proliferation is inhibited. Instead, rounds of cell proliferation continue in the lens and retina, but the newly born cells do not contribute to the lens and retinal mass because they die prior to differentiation. Eventually, apoptosis outperforms cell division, at least in the lens, which eventually disappears in most adult cavefish (Soares et al. 2004). However, a small retina remains with little or no net contribution by new cell division during the growth phase.

Recycling of lens and retinal cells during cavefish development provides another important clue to understanding the evolutionary forces responsible for eye loss. Once again, because the cavefish eye continues to experience the high cost of growth and development, these forces are not likely to be related to energy conservation.

What Do Lens Transplantation Experiments Tell Us?

The early cell death of the lens suggests that this tissue may have a central role in regulating cavefish eye degeneration. We developed an embryonic lens transplantation assay to test this possibility (Yamamoto and Jeffery 2000, 2002). The lens vesicle was removed from a surface fish embryo shortly after its formation and transplanted into the optic cup of a cavefish embryo at the same stage of development after the host lens had been removed. We also carried out the reciprocal experiment, transplantation of a cavefish lens vesicle into a surface fish optic cup. These transplantations were carried out unilaterally so that one eye served to assay the eye-forming ability of the donor lens, whereas the unoperated eye of the host embryo served as a control.

We initially asked whether the donor lens lived or died in the host embryo (Yamamoto and Jeffery 2000). By TUNEL assay it was demonstrated that the transplanted lens developed as it would have in the donor embryo: the cavefish lens vesicle died on schedule in the surface fish host, and the surface fish lens vesicle underwent normal differentiation and further growth in the cavefish host. The results show that the cavefish lens is already programmed for apoptosis at the time of transplantation, a day before death is detectable by TUNEL. Furthermore, they indicate that lens cell death is autonomous, at least at the time of the transplantation, and not induced by the optic cup, which is known to signal the lens during eye development (Saha et al. 1992).

Because lens development is autonomous, we were able to determine its effect on overall eye formation in the host embryo (Yamamoto and Jeffery 2000). The eye of cavefish embryos containing a transplanted surface fish lens did not degenerate (Figure 4A, B). Instead it continued to grow; retinal photoreceptor cells differentiated; the cornea, ciliary body, and iris appeared; and the sclera became ossified (Figure 4D). All of these features are hallmarks of the surface fish but not the cavefish eye. Reciprocally, the eye of surface

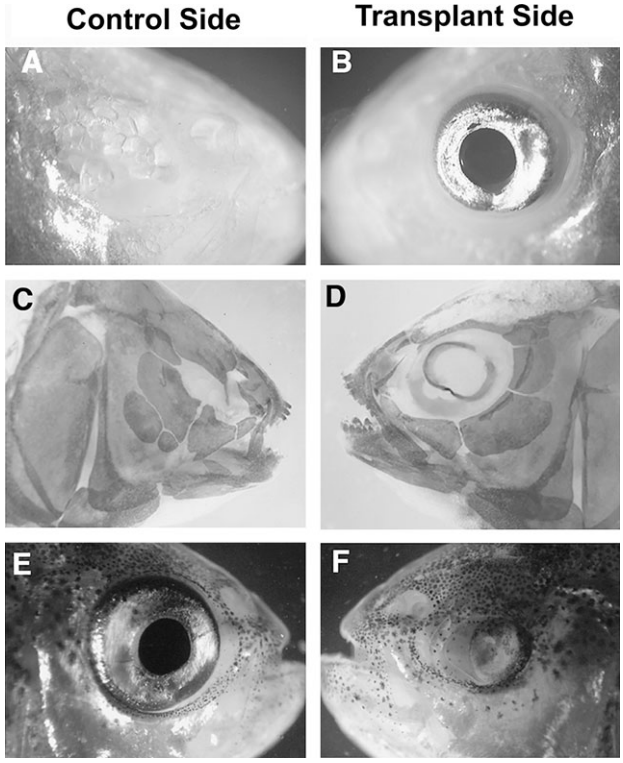
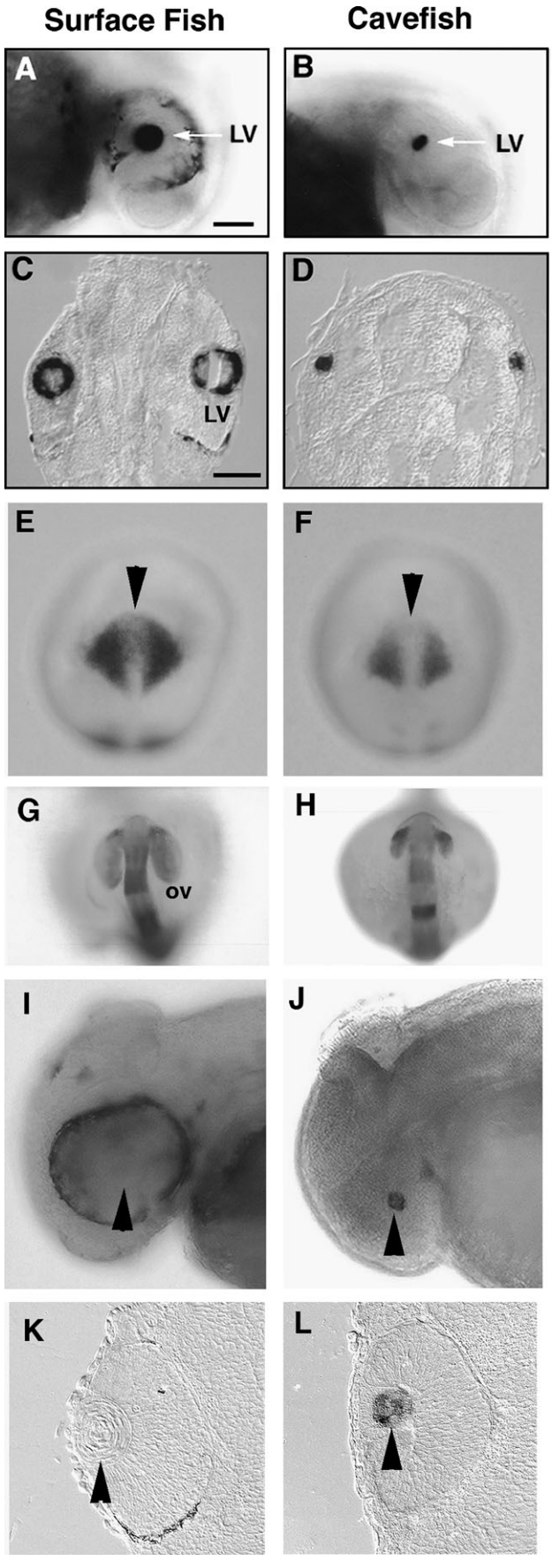


Figure 4. Control of eye formation and craniofacial development by embryonic lens transplantation. **(A, B)** Restoration of the eye in a cavefish containing a transplanted surface fish lens **(B)**. **(C, D)** Phenocopy of surface fish craniofacial morphology in a cavefish containing a transplanted surface fish lens **(D)**. **(E, F)** Degeneration of the eye in a surface fish containing a transplanted cavefish lens **(F)**. **(B, D, F)** Transplantation sides. **(A, C, E)** Control unoperated sides in the same specimens as **B, D, F**. **A, B, E, F**, from Yamamoto and Jeffery (2000); **C, D** from Yamamoto et al. (2003).

fish embryos containing a transplanted cavefish lens was retarded in growth and eventually disappeared into the orbit, thus mimicking cavefish (Figure 4E, F). These results show that the lens is a central regulator of eye growth and differentiation and that lens apoptosis controls eye degeneration in cavefish. During surface fish development, the lens signals other eye parts to define the pace of normal growth and differentiation. In cavefish, however, this signal(s) is probably absent due to lens cell death.

As a result of lens transplantation, adult cavefish were obtained with a completely differentiated eye on one side of their head (Jeffery et al. 2003; Yamamoto and Jeffery 2000). Remarkably, orbital bone structure resembling the surface fish phenotype is also induced on the side of the head containing the restored eye, whereas the other side of the head has the craniofacial structure typical of cavefish (Figure 4C, D; Yamamoto et al. 2003). These results suggest that the lens dictates craniofacial morphology by controlling eye growth. Recent studies have shown that more retinal nerve fibers project from the restored eye to the contralateral optic



tectum, which is also increased in size, suggesting that cavefish with a transplanted eye may be capable of vision (Soares et al. 2004).

In summary, lens transplantation indicates that cavefish have the capacity of form a complete eye and that they possess and are capable of using all the genetic factors necessary for later eye development.

What Do Gene Expression Patterns Tell Us?

Gene expression has been compared between cavefish and surface fish in several different ways. First, expressed DNA sequences have been compared by differential display analysis (Heyser and Jeffery unpublished data). Second, large numbers of transcripts have been directly compared between surface fish and cavefish embryos by microarray analysis (Strickler and Jeffery unpublished data). Unexpectedly, both approaches suggest that many genes are up-regulated in cavefish relative to surface fish, rather than vice versa. Of course, many of these genes are probably not expressed in the eye. Third, the prior identification of many different eye development genes in vertebrates permits a candidate gene approach to be used to identify changes in eye gene expression. Using this approach, we have assayed the expression of genes encoding transcription factors that function upstream in eye gene hierarchies, such as *pax6*, as well as structural genes encoding proteins that function at the bottom of gene cascades, such as various lens crystallin genes. Thus far, we have surveyed about 150 individual genes using this approach (Strickler et al. unpublished data). The expression patterns of some of these genes are discussed next.

Most of the candidate genes did not show any detectable changes in expression patterns between surface fish and cavefish embryos when assayed by *in situ* hybridization. For example, upstream in the eye gene cascade, the Prox1 transcription factor is expressed normally in the developing cavefish lens and retina (Jeffery et al. 2000), and downstream in the cascade, the gammaM crystallin gene (Figure 5A–D; Jeffery et al. 2000) and protein (Strickler and Jeffery unpublished data) are expressed in the cavefish lens. Moreover, Langecker et al. (1993) showed that the opsin gene is expressed in the outer nuclear layer of the cavefish retina.

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Figure 5. Gene expression determined by *in situ* hybridization in surface fish **(A**, **C**, **E**, **G**, **I**, **K)** and cavefish **(B**, **D**, **F**, **H**, **J**, **L)** embryos. **(A–D)** GammaM crystallin expression in whole mounts **(A**, **B)** and sections **(C**, **D)** at the 36 h stage; from Jeffery et al. (2000). LV: lens vesicle. Scale bar in **A** is 10 μm and **B** is 20 μm. **(E**, **F)** *Pax6* expression in bilateral optic vesicle primordia at the neural plate stage; from Strickler et al. (2001). Arrowheads indicate the embryonic midline. **(G**, **H)** *Pax2a* expression at the base of the optic vesicles (ov) at the 5-somite stage; from Yamamoto et al. (2004). **(I–L)** *Hsp90α* expression in whole mounts **(I**, **J)** and sections **(K**, **L)** of the cavefish lens at the 36 h stage; from Hooven et al. (2004).

Thus, gene expression data suggest that loss of function mutations have not occurred in cavefish eye genes, including those structural genes that function at the bottom of regulatory cascades.

In contrast to loss of gene function, recent studies have identified a distinct gain of function in one of the genes expressed in the cavefish lens. The gene encoding the molecular chaperone *hsp90α* is expressed in both surface fish and cavefish embryos under nonstress conditions (Hooven et al. 2004) as part of the normal program of muscle development (Sass et al. 1996). However, *hsp90α* expression is activated specifically in the cavefish lens beginning just prior to apoptosis (Figure 5I–L; Hooven et al. 2004). *Hsp90α* expression was not detected in the surface fish lens or in any other tissue of the surface fish or cavefish eye during embryonic development. Hooven et al. (2004) showed that when Hsp90α function was specifically inhibited, lens apoptosis was suppressed and normal lens differentiation was restored to a certain extent. These results suggest that activation of the *hsp90α* gene may be required for lens apoptosis and eye degeneration in cavefish. The role of Hsp90α in cavefish eye degeneration is currently unknown. However, it has been postulated that Hsp90α chaperones and thereby activates cell death factors in the lens (Hooven et al. 2004). Owing to the recent studies showing that Hsp90α can be secreted outside cells (Eustace et al. 2004), it is also possible that this molecular chaperone may have effects throughout the deteriorating cavefish eye.

More subtle changes in other gene expression patterns have also been detected in cavefish embryos. The *pax6* gene encodes a transcription factor with a major role in regulating eye formation (Gehring and Ikeo 1999). In vertebrates, including zebrafish (Krauss et al. 1991; Püschel et al. 1998), *pax6* is expressed in lens placodes and optic vesicle primordia at the neural plate stage. Yamamoto et al. (2001) showed that *pax6* expression is similar in *Astyanax* surface fish and zebrafish embryos (Figure 5E). However, they found subtle differences in *pax6* expression in cavefish (Figure 5F). First, *pax6* expression was reduced in cavefish lens placodes. Second, the size of the bilateral *pax6*-expressing domains was diminished in cavefish, correlating with the smaller size of the optic vesicles (Figure 5E, F). Third, the left and right *pax6*-expressing domains corresponding to the future optic vesicles lacked a distinct connecting zone spanning the embryonic midline in cavefish, as they do in surface fish, thus producing a midline gap between the cavefish optic vesicle fields (Figure 5E, F). These results show that *pax6* expression patterns are modified in cavefish.

The patterning of optic vesicles into two parts, the optic cups and optic stalks, is controlled by reciprocal repression between the Pax6 and Pax2 transcription factors (Schwarz et al. 2000). Pax6 directs optic cup development, whereas Pax2 controls optic stalk development. Thus, if the changes in *pax6* expression domains observed by Yamamoto et al. (2001) are important in cavefish eye development, then reciprocal changes would be expected in *pax2* expression. Accordingly, Yamamoto et al. (2004) have recently shown that the *pax2a* expression domain at the base of the optic vesicles is increased in size in cavefish embryos (Figure 5G, H). Because of mutual antagonism with *pax6, pax2a* enhancement is also predicted to increase the size of the optic stalk at the expense of the ventral optic cup. This is the phenotype seen in cavefish embryos, which show optic cups missing their ventral sectors and more extensive optic stalks (Figure 1D; Jeffery et al. 2003; Yamamoto and Jeffery unpublished data). Therefore, part of the mechanism of cavefish eye reduction involves a reciprocal change in *pax6* and *pax2* expression, resulting in modified optic vesicle patterning.

The wide gap between *pax6* expressing domains in the cavefish neural plate provides a further clue about how eye development is controlled in cavefish. During vertebrate development, the presumptive optic cup is initially determined as a single medial domain, which is subsequently split into two bilateral eye domains by Hedgehog (Hh) signals emanating from the underlying embryonic midline (Ekker et al. 1995; Macdonald et al. 1995). Thus, during normal eye development, Hh signaling inhibits *pax6* expression in the midline to create two lateral eyes. In fact, mutants in *sonic hedgehog*, the single Hh midline signaling gene in humans (Belloni et al. 1996) and mice (Chiang et al. 1996), result in cyclopia, the development of a single medial eye. Teleost genomes have two *hh* midline signaling genes, *shh* and *tiggy winkle hedgehog* (*twhh*), with overlapping expression patterns (Ekker et al. 1995). Thus, the activity of both *shh* and *twhh* may be required to completely separate the eyes during teleost development (Chow and Lang 2001).

Shh and *twhh* expression patterns were compared during surface fish and cavefish development (Yamamoto et al. 2004). The results showed that midline expression domains of both genes were expanded in cavefish relative to surface fish, as determined by comparing expression of the *hh* genes to that of *pax2a* and *dlx3b*, marker genes that define the boundaries of the anterior neural plate. At the neural plate stage, *shh* and *twhh* expression was expanded laterally along the anterior midline of cavefish embryos (see Figure 6A–D for *shh*). The *shh* expression domain is about 10 cells wide in cavefish at its greatest width, whereas it is only about 6 cells wide in surface fish (Yamamoto et al. 2004). Later in cavefish development, *hh* expression was expanded anteriorly, curling around the rostrum in the presumptive oral area. Yamamoto et al. (2004) also showed that expression patterns of genes acting downstream of *shh* and *twhh* in the Hh midline signaling pathway, such as *patched*, encoding a Shh receptor, and *nkx2.1*, encoding an Shh-dependent transcription factor, are expanded, suggesting that a general increase in midline signaling has evolved in cavefish. These results also imply that suppression of *pax6* and eye development in cavefish is controlled by Hh midline signaling genes that function outside the developing eye.

In summary, analysis of gene expression patterns has not identified nonfunctional genes during early cavefish eye development, as might be predicted by the neutral mutation hypothesis. Of course, those genes expressed in eye tissues that completely degenerate, such as the lens, are gradually down-regulated after the onset of apoptosis, but only after

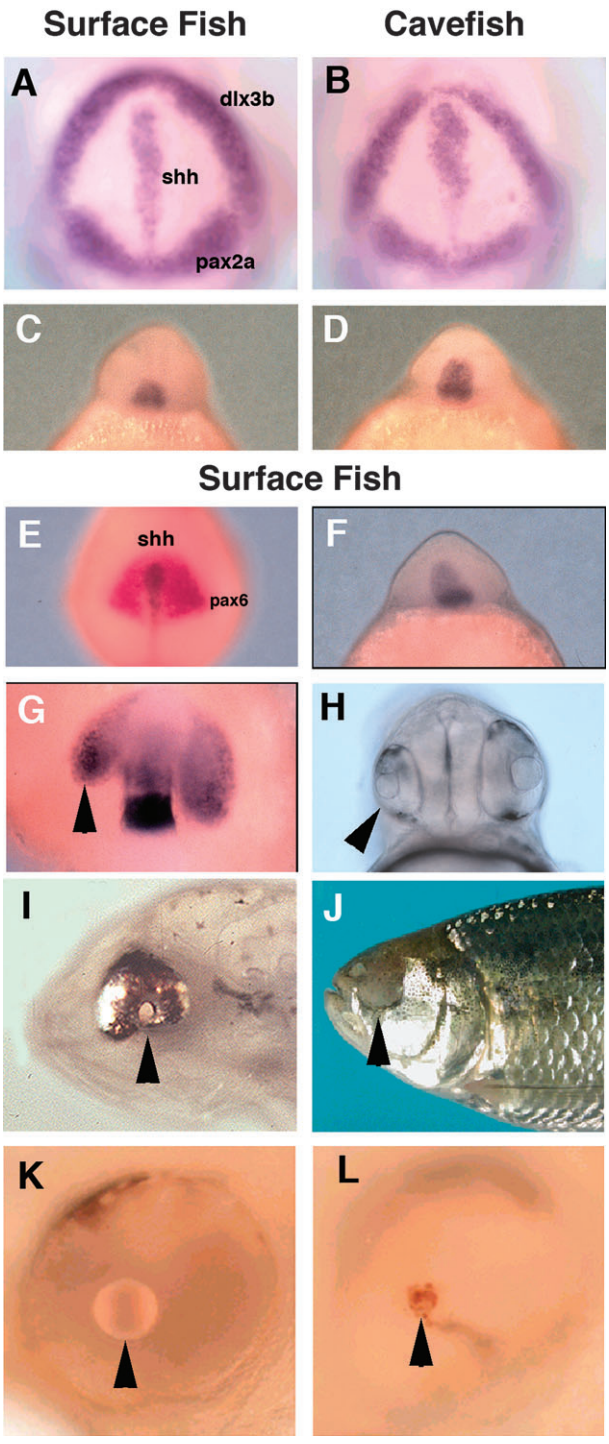

Figure 6. Role of Hh signaling in cavefish eye degeneration. **(A, C, E–L)** Surface fish. **(B, D)** Cavefish. **(A, B)** *In situ* hybridization showing expanded *shh* expression at the embryonic midline in neural plate stage cavefish embryos relative to *dlx3b* expression at the edge of the neural plate and *pax2a* expression at the future midbrain–hindbrain boundary. Dorsal views. **(C, D)** *In situ* hybridization showing expanded *shh* expression in the cavefish rostrum at the 10-somite stage. Rostral views. **(E–L)** Surface fish embryos developed from eggs injected with *shh/twhh* **(E–J, L)** or control GFP **(K)** mRNA. **(E)** *In situ* hybridization at the neurula stage showing *shh* expression (blue) skewed toward the left midline and corresponding reduction in *pax6* expression (red) in the left optic vesicle domain. **(F)** *In situ* hybridization showing skewed *shh* expression in the rostrum of a 10-somite embryo. **(G)** *In situ* hybridization with *pax6* showing a smaller optic vesicle (arrowhead) on the left side of a 10-somite embryo. **(H)** Rostral view of a 24 h stage embryo showing smaller eye primordium (arrowhead) on one side. **(I)** Lateral view of a 10-day larva showing eye primordium (arrowhead) lacking a ventral sector and no apparent lens. **(J)** Adult phenocopy of a blind cavefish showing degenerate eye (arrowhead). **(K, L)** TUNEL analysis of control mRNA- **(K)** and *shh* mRNA-injected embryos showing lens (arrowhead) apoptosis induced in the latter. From Yamamoto and Jeffery (2004).

being expressed earlier in development. Although the expression domains of some genes, such as *pax6*, may be slightly reduced during early eye development, others, such as *pax2a* and genes of the Hh midline-signaling pathway, are increased. Last, the *hsp90a* gene is specifically activated during cavefish eye development.

The Key Role of Hedgehog Signaling

Changes in gene expression patterns suggest that enhanced Hh signaling may have a key role in cavefish eye degeneration. To explore this possibility, Yamamoto et al. (2004) carried out functional experiments in which *twhh* and/or *shh* were overexpressed in surface fish embryos by injecting excess amounts of their mRNAs. If *hh* genes control eye degeneration, up-regulation of Hh signaling proteins in surface fish would be expected to mimic the cavefish phenotype.

The results confirmed this hypothesis (Figure 6E–L; Yamamoto et al. 2004). When *hh* mRNA was injected into one side of a surface fish egg or early cleaving embryo, *shh* expression was expanded along that side of the embryonic midline, and *pax6* expression was down-regulated unilaterally in the corresponding optic vesicle field (Figure 6E). Later in development, *shh* expression was also expanded asymmetrically in the rostrum of these surface fish embryos (Figure 6F). As a consequence of expanded *hh* expression, unilateral changes in eye development, a smaller optic vesicle (Figure 6G), a smaller eye (Figure 6H), and an optic cup (retina) lacking its ventral sector (Figure 6I), were induced. When these embryos developed into adults, the eye was missing on one side of the head, and there was no response to a light beam focused on the orbit with the missing eye (Figure 6J; Yamamoto et al. 2004). Thus, blind cavefish were phenocopied by increasing the levels of *hh* gene expression in surface fish, implying a key role for Hh midline signaling in eye degeneration.

Yamamoto et al. (2004) also used TUNEL to follow lens apoptosis after increasing *hh* expression in surface fish embryos. Lens apoptosis occurred on the side of the injected

Table 1. Events and processes associated with eye degeneration in different cavefish populations

Event or Process	Cavefish Population					Reference(s)
	Pachón	Los Sabinos	Tinaja	Curva	Chica	
Smaller eye primordium	+	+	+	+	+	Cahn (1958); Jeffery and Martasian (1998); Jeffery et al. (2003)
Loss of ventral optic cup	+	+	+	+	+	Jeffery et al. (2003); Yamamoto et al. (2004)
Lens apoptosis	+	+	+	?	+	Jeffery and Martasian (1998); Jeffery et al. (2003)
Continued cell division at CMZ	+	+	?	?	?	Strickler et al. (2002)
Eye restoration by lens transplantation	+	+	+	?	+	Yamamoto and Jeffery (2000); Jeffery et al. (2003); Yamamoto and Jeffery (unpublished data)
Hsp90α activation	+	?	?	+	+	Hooven et al. (2004)
Continued Prox1 expression	+					Jeffery et al. (2000)
Pax6 down-regulation in optic vesicle fields	+	+	?	+	?	Yamamoto et al. (2001)
Hh expansion at embryonic midline	+	+	?	+	+	Yamamoto et al. (2004); Yamamoto and Jeffery (unpublished data)

+ Event or process detected. ? Event or process has not been studied yet.

embryo with enhanced Hh signaling (Figure 6K, L), implying that *hh* genes control lens apoptosis and subsequent eye degeneration. These results are consistent with earlier studies in zebrafish embryos showing that enhancement of *shh* or *twhh* sometimes resulted in the complete absence of lenses (Ekker et al. 1995; Macdonald et al. 1995). Although exactly how *hh* genes expressed at the embryonic midline control lens cell death is currently unknown, it seems likely that activation of *hsp90a* may be an important downstream target of Hh signaling.

The involvement of midline signaling genes provides critical information regarding the evolutionary mechanisms of cavefish eye degeneration. First, it confirms the idea that gene activation rather than the loss of gene function controls eye degeneration. Second, and most important, it shows that eye degeneration is controlled by signals emanating from outside the eye itself. Thus, changes in midline signaling could link eye degeneration to constructive changes in the cavefish phenotype.

What Do Different Cavefish Populations Tell Us?

The studies described were carried out with the Pachón cavefish. As mentioned earlier, at least 30 different cavefish populations have been identified, providing an exceptional resource to study parallelism or convergence in eye regression. Based on geographical distribution and phylogenetic analysis, it is likely that eye loss has occurred independently in some of these cavefish populations (Espinasa et al. 2000; Dowling et al. 2002; Jeffery et al. 2003; Mitchell et al. 1977; Strecker et al. 2003, 2004). Therefore, to obtain further insights into the evolutionary forces involved in visual regression, I asked whether eye degeneration has occurred by the same or different processes in various cavefish populations. Currently, information exists for only the Pachón, Chica, Los Sabinos, Tinaja, and Curva cavefish populations. Because the available data for these cavefish populations has been reviewed recently (Jeffery et al. 2003) it is presented here as a summary (Table 1). The results suggest that eye degeneration is controlled by the same or similar mechanisms in different cavefish populations.

Conclusions

The Neutral Mutation Hypothesis

Experiments provide evidence against the neutral mutation hypothesis as an evolutionary mechanism for eye degeneration. According to this hypothesis, some of the genes involved in eye development, particularly those that act at the bottom of eye genetic cascades and are expressed at no other place in the embryo or adult, would become nonfunctional after a sufficient amount of time. However, all of the genes examined by candidate gene analysis appear to be expressed normally in cavefish, at least as mRNAs, and some as both mRNAs and proteins. Indeed, differential display and microarray approaches suggest that many genes are actually up-regulated in cavefish. Thus far, the changes in gene expression patterns detected are either activations (e.g., *hsp90α*) or subtle enhancements (e.g., *shh*) and reductions (e.g., *pax6*) in expression domains rather than loss of functions.

It could be argued that many eye genes are regulatory and pleiotropic, in the sense that they have multiple functions in development other than their role in forming eyes, and therefore would not be subject to neutral decay processes. However, even eye structural genes (e.g., lens crystallins, retinal opsin) that operate at the bottom of gene cascades and whose expression cannot be detected elsewhere in the embryo or adult are expressed during cavefish eye development (Jeffery et al. 2000; Yamamoto and Jeffery 2000). According to the neutral mutation hypothesis, these genes are expected to be especially sensitive to accumulation of neutral

mutations. Therefore, it appears that eye gene cascades are completely operational in cavefish embryos prior to the general transcriptional shutdown that occurs after the beginning of apoptosis.

Some observations and experiments on eye development are also inconsistent with the neutral mutation hypothesis. First, the eye does not completely disappear in cavefish, as would be expected over time if loss of function mutations accumulate in eye genes. Indeed, the eye is initially formed in a relatively complete state and advances to about the same developmental stage before degenerating in various cavefish populations (Jeffery et al. 2003). If eye genes were gradually being rendered nonfunctional and eye development were subject to drift, arrest at the same developmental stage would not have been expected in cavefish populations of different antiquities. Second, the ability of the eye to be restored by lens transplantation suggests that the genes necessary for later eye development are present and functional in cavefish. The ability of the cavefish eye to be restored (Jeffery et al., 2003; Soares et al. 2004; Yamamoto et al. 2000) is perhaps the strongest single piece of evidence against the neutral mutation hypothesis.

Adaptive Evolution Based on Energy Conservation

If neutral processes do not adequately explain the evolution of eye regression, then we must turn to explanations based on adaptive evolution and natural selection. A major impediment to the adaptive hypothesis is lack of evidence for a trait that could be subject to natural selection and lead to loss of eyes. Studies do not support the possibility that eyes are lost due to selection for energy economy, one of the most popular adaptive explanations for regressive evolution in cave animals.

Several lines of evidence argue against the possibility that cavefish eye development is blocked to conserve energy. First, cavefish males and females show the same degree of eye reduction, although the high cost of egg production might be expected to dictate a greater degree of eye reduction in females, as has been reported in cave-adapted beetles (Park 1951). Second, cavefish populations inhabiting pools under bat colonies do not appear to be food-limited, yet they show significant eye regression (Breder 1943, 1953). Third, the manner of eye degeneration in *Astyanax* cavefish does not appear to be economical. Instead of undergoing eye loss at a very early stage, the cavefish eye develops to a relatively mature stage prior to the beginning of degeneration, presumably at high energetic cost.

Alternatively, it could be argued that formation of a rudimentary eye is necessary, despite its energetic cost, because of a requirement for the normal development of other structures, such as the craniofacial skeleton (Yamamoto et al. 2003). Even after the embryonic stage, however, the cavefish eye does not stop developing. Both the lens and retina undergo dynamic cycles of cell division followed by cell death at least as long as the late larval or early adult stage. Clearly, these repetitive cycles of building and dismantling the eye are not energy efficient.

Adaptive Evolution Based on Pleiotropy

Pleiotropy (Wright 1964) is the other evolutionary force that has been proposed to drive adaptive evolution of eye loss in cave animals (Barr 1968). Pleiotropy is virtually a universal feature of many genes. Importantly, the form of pleiotropy meant here differs from that described earlier in this review in which the same gene positively regulates many different developmental events. The second form of pleiotropy is essentially a developmental trade-off, in which the same gene(s) regulates some phenomena positively and others negatively. Natural selection would act on the positive traits and therefore indirectly suppress the negative traits. There is a precedent for such developmental trade-offs, as exemplified by competition between horns and eyes in beetles (Nijhout and Emlen 1998).

I propose an adaptive hypothesis to explain cavefish eye regression based the pleiotropic activity of midline signaling genes. Gene expression data and functional studies involving the negative role of Hh midline signaling during cavefish eye development strongly support this hypothesis. Enhanced Hh midline signaling in cavefish has a negative effect on eye development based on its ability to change the patterns of *pax6* and *pax2* expression, reduce the size of the eye primordia, and promote lens apoptosis and arrest eye growth (Yamamoto et al. 2004). Conversely, Hh midline signaling is a positive regulator of many other developmental events based on its role as a morphogen (Ingham and McMahon 2001). Accordingly, I suggest that selection for a trait(s) that is positively controlled by the Hh midline signaling pathway results in eye regression by default because of constraints in the way in which bilateral eyes are formed in vertebrates.

The specific cavefish trait(s) that is positively regulated by Hh signaling and antagonistic to eye development is currently unknown. However, constructive features related to changes in cavefish feeding structures and behavior (Figure 2) are excellent candidates for such traits. In other vertebrates, *shh* is expressed in developing teeth (Gritli-Linde et al. 2002), in tastebuds (Hall et al. 1999, 2003), and in craniofacial structures (Helms et al. 1997), all of which are enhanced or modified in cavefish (Jeffery 2001). In preliminary studies, my colleagues and I have shown that *shh* expression is both sufficient and necessary for *Astyanax* tastebud development (Yamamoto and Jeffery unpublished data). The next challenge will be to identify the constructive features in cavefish that are subject to natural selection and regulated positively by the Hh signaling pathway.

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