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Development of cavefish eyes

Here's a story that Darwin got completely wrong. He had observed that certain species had profoundly reduced or rudimentary organs, and he explained them not as a consequence of natural selection, but as evidence of the inheritance of acquired characters.

But we learn from the study of our domestic productions that the disuse of parts leads to their reduced size; and that the result is inherited. It appears probable that disuse has been the main agent in rendering organs rudimentary. It would at first lead by slow steps to the more and more complete reduction of a part, until at last it became rudimentary, as in the case of the eyes of animals inhabiting dark caverns, and of the wings of birds inhabiting oceanic islands, which have seldom been forced by beasts of prey to take flight, and have ultimately lost the power of flying.

It's easy to feel mildly embarrassed for Darwin on reading this now; it was an honest error, though, and since he had no good model for inheritance, he fell back on an old idea, that the use or disuse of an organ in the parent would have an effect on its progeny. Blind fish lost their eyes because Mom and Dad fish lived in the dark and never used their eyes, so Junior inherited weaker eyes.

As it is difficult to imagine that eyes, though useless, could be in any way injurious to animals living in darkness, their loss may be attributed to disuse.

Well, actually, Charles…it's not difficult to imagine at all. Eyes are fragile, pulpy things that represent a significant investment in energy. I could imagine that there would be a slight selective advantage to jettisoning something an animal isn't using, that costs it effort to develop or is a weak or sensitive point of attack. Since we've long discarded the hypothesis of the inheritance of acquired characters, that's one of the primary explanations for the loss of eyes in cave animals—they're absence was an advantage.
Another explanation is that eyes are effectively a neutral character in dark environments, and that there is therefore no selective advantage in maintaining them. Cave organisms acquired mutations that knocked out the eyes, and in the absence of selection to maintain sight, these mutations accumulated until the entire population was lacking eyes.

There is a third possibility, now supported by observations in blind cave fish of the genus *Astyanax*. Despite being wrong on the mechanisms of inheritance, Darwin was no dummy, and he almost figured this one out. If he'd had just a little more intuition about development, he might have suggested this idea. Here's the tantalizingly close passage:

By the time that an animal had reached, after numberless generations, the deepest recesses, disuse will on this view have more or less perfectly obliterated its eyes, and natural selection will often have effected other changes, such as an increase in the length of the antennae or palpi, as a compensation for blindness.

The third possibility requires that one recognize that development is not infinitely plastic, that characters are linked in development, and that maybe the only way to develop these compensatory structures is at the expense of the eyes—that is, that there is a selective advantage to developing long antennae or palpi or other organs, but that the simplest developmental process to do so involves cannibalizing eye tissue. This explanation is an example of the way knowledge of developmental biology can inform our understanding of evolutionary biology.

Here, for example, are two species of a Mexican fish, *Astyanax*. The one on the left is found in surface streams, and the one on the right is found in caves, and has lost most of its pigment as well as its eyes. These two are sufficiently closely related that they can be interbred, and are thought to have diverged within the last ten thousand years. One has to wonder what is the cause of the differences between them. One answer is found in their development.
Here's how those two look as embryos; the surface fish is again on the left and the cave fish is on the right. The cave fish *starts* to form an optic cup (oc), but it never develops as far, and actually begins to regress, starting at the ventral edge, which is where the optic stalk is located (the optic stalk is the tissue connecting the embryonic eye to the brain.)

Looking earlier, when the optic cup has not yet formed the the primordium of the eye is called the optic vesicle (ov), we can see an obvious difference: the optic vesicle of the cave fish is much smaller than that of the surface fish. In addition, we can stain for various molecules present at this time, in particular *pax2*, which is expressed only in the optic stalk, and *pax6* found in the optic vesicle itself. Below, the fish have been stained for *pax2*, and the cave fish is expressing it much more strongly.
Another molecular player here is *hedgehog*, which is expressed in the midline. The authors have stained embryos for *hedgehog* and for other molecules downstream of it, looking for differences. Below are embryos stained for *ptc2*, a *hedgehog* receptor, and *nkx2.1a*, a transcription factor that is regulated by *hedgehog*. What we see in the cavefish on the right is an expansion of *hedgehog* expression in the midline, and an expansion of the regions of expression of genes regulated by *hedgehog*.

What this is saying is that at the molecular and developmental level, eyelessness in the cave fish may not be a loss at all. Midline genes like *hedgehog* are in a balancing act with eye genes like *pax6*, and the eyelessness may be a side effect of tipping the balance towards wider expression of *hedgehog*, which secondarily represses eye formation.

This hypothesis can be tested by taking a surface fish embryo and artificially increasing the level of expression of *hedgehog* by injecting it with *hedgehog* RNA. The top two diagrams below are examples of surface fish embryos that were injected with *hedgehog* RNA on just the left side, and then stained with *pax6*. The eye on the injected side is visibly smaller.
The lower two images are older surface fish that had received the same kind of *hedgehog* RNA injection—the one on the left is reduced, while the one on the right has completely lost its eye, and is an excellent phenocopy of the cavefish.

What all this is telling us is that the failure of the eye to form in the blind cavefish isn't the result of a passive loss of eye genes, but the expansion of expression of genes that actively oppose eye formation. Other work from the Jeffery lab suggests that the expanding genes are responsible for an increase in jaw size and the number of gustatory receptors. The enlargement of sensory and manipulatory structures isn't to compensate for the loss of eyes, as Darwin suggested, but may actually be the developmental *cause* of the organism's blindness.

To See or Not to See: Evolution of Eye Degeneration in Mexican Blind Cavefish

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INTRODUCTION

In 1872 Charles Darwin wrote, "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." This statement launched more than a hundred years of speculation and debate on the evolutionary mechanisms responsible for the loss of eyes in cave animals (Culver, 1982). Today this problem is still unresolved, but prevailing opinions usually support one of two hypotheses.

The neutral mutation hypothesis suggests that eye degeneration is caused by random mutations in eye forming genes, which gradually accumulate in the absence of selective pressure. In contrast, the adaptation hypothesis suggests that natural selection causes the loss of eyes due to advantages in losing eyesight. As exclaimed in Darwin's famous quotation, the actual benefits of blindness are uncertain. Thus, different versions of the adaptation hypothesis have attributed the loss of eyesight to energy conservation, citing the high cost of making an eye, or to enhancement of other sensory organs that are highly beneficial to survival in the cave environment. Through the years, however, little or no experimental verification has been leveled in support of any version of either hypothesis. To understand the evolution of eye degeneration, it is necessary to determine the molecular and cellular mechanisms of the degenerative process, and whether the same or different genes and mechanisms are involved in loss of vision.
We study the mechanisms of visual degeneration in the Mexican Tetra, Astyanax mexicanus, a single species consisting of a surface-dwelling form (surface fish) (Fig. IA) and many cave dwelling (cavefish) forms inhabiting different caves (Fig. IB-E) (Jeffery, 2001). The Mexican tetra is easy to raise in the laboratory and exhibits many of the attributes that have made zebrafish a popular model system in developmental biology. These features include external fertilization, frequent and abundant spawning, transparent embryos, a 4-6 month generation time, and the opportunity for molecular, developmental, and genetic analysis. The surface and cave forms of A. mexicanus are interfertile, and successful mating is also possible between different cavefish populations (Sadoglu, 1957; Wilkens, 1971). Because of these attributes Astyanax cavefish represent one of the few cave animals in which laboratory experiments can be conducted on the mechanisms of eye degeneration and these mechanisms can be compared in the same species from different caves. Here we review current progress on the evolution and development of Astyanax cavefish and discuss how these studies have contributed to understanding the evolutionary basis of eye degeneration.

CAVEFISH EVOLUTIONARY HISTORY

To evaluate differences or similarities in the mechanisms of eye degeneration, it is first necessary to understand the evolutionary history of cavefish populations. Did all cavefish populations originate from a common ancestor and lose their eyes only once or did they evolve many times and lose their eyes independently? Different approaches have been used to determine the evolutionary relationships of cavefish, including allozyme analysis, biogeography, and phylogenetic reconstruction using molecular sequences. We will briefly consider the results obtained from the first two approaches and then describe the phylogenetic studies in more detail.
Figure 2 shows a map of the Sierra de El Abra region in northeastern Mexico illustrating the locations of known caves harboring Astyanax cavefish populations. The major cavefish region consists of the Sierra de El Abra, the Sierra de Guatemala, the Micos region (Fig. 2), and the valleys lying between these limestone ridges in the states of Tamaulipas and San Luis Potosi, Mexico (Wilkens and Burns, 1972; Mitchell et al., 1977). An outlying cavefish population has also been discovered in the state of Guererro in south central Mexico (Espinasa et al., 2001).

In an electrophoretic study showing minimal divergence in 17 allozyme loci, Avise and Selander (1972) concluded that the Sierra de El Abra cavefish had a common origin. However, a limited number of cavefish populations (Pachon, Los Sabinos, and Chica; Fig. 2) were sampled in this study. In contrast, Mitchell et al. (1977), who surveyed 29 different cavefish populations in the Sierra de El Abra, Sierra de Guatemala, and Micos region, proposed several different origins of Astyanax cavefish. Mitchell et al. (1977) also estimated the divergence between surface fish and cavefish to have occurred about 10,000 to 100,000 years ago in the Sierra de El Abra region. The possibility of multiple cavefish origins is strongly supported by the recently discovered Guerrero cavefish from a cave located several hundred miles southwest of the main cavefish region (Espinasa et al., 2001).

The first phylogenetic studies of cavefish populations were done using DNA polymorphisms amplified by arbitrary primers (RAPDs) (Espinasa and Borowsky, 2001). This analysis supported a single origin of Sierra de El Abra cavefish and an independent origin of Subterraneo cavefish in the Micos region (Fig. 2). The limited number of RAPD markers scored in this study, however, left some uncertainty about the true relationships among the Sierra de El Abra cavefish. Thus far, it has proved difficult to obtain sufficiently variable sequence information from nuclear genes to
construct robust phylogenetic trees, presumably due to the recent divergence of surface fish and cavefish. Thus, Dowling et al. (2002) were prompted to use NAD1 dehydrogenase-2 (ND-2), a rapidly evolving mitochondrial gene, to infer cavefish relationships (Fig. 3).

Before discussing the resulting ND-2 mitochondrial DNA (mtDNA) phylogeny, it is necessary to comment on the currently unresolved taxonomy of A. mexicanus and related forms. Some taxonomists recognize two separate Astyanax species in Mexico: A. mexicanus in northern Mexico and Astyanax aeneus in southern Mexico (Obregon-Barbosa et al., 1994). Others believe that all Mexican and Central American Astyanax are a single species, Astyanax fasciatus (see Wilkens, 1988). Here, we defer to the first classification, designating the northern Mexican form as A. mexicanus, the southern Mexican form as A. aeneus, and the Central American form as A. fasciatus. Our justification is that these taxa are strongly supported by the mtDNA phylogeny (Fig. 3).

The mtDNA phylogeny infers at least two separate origins of cavefish, one before the divergence of the present day A. mexicanus and A. aeneus, and the other after the bifurcation of these taxa (Fig. 3). Accordingly, two distinct mtDNA lineages are recognized: the A lineage, including A. mexicanus and A. aeneus surface fish and Pachon and Subterraneo cavefish, and the B lineage, including Tinaja, Los Sabinos, and Curva cavefish (Dowling et al., 2002). The A lineage exhibits one of more than 20 different Type A ND-2 haplotypes, which vary from each other in only a few nucleotide positions and are mostly represented in surface fish. The B lineage exhibits one or two of only a few Type B ND-2 haplotypes, which differ in 7 or more nucleotide sites from the Type A haplotypes and are present in cavefish but not in any nearby surface fish populations. Sampling from Texas to Costa Rica failed to find any surface fish populations with Type B haplotypes.
(Dowling et al., 2002), suggesting that the surface fish stock that established the B lineage cavefish may be extinct.

Although the mtDNA tree has strong bootstrap support, our interpretation of these data must be treated with caution. First, the tree is based on only a single gene. However, a recent phylogenetic analysis has confirmed the topology of this tree using a different mitochondrial gene, cytochrome b (Strecker et al, 2003). Second, mtDNA trees could be influenced by hybridization, which is known to have occurred between some of the cavefish populations and nearby surface fish (Mitchell et al, 1977; Romero, 1983; Langecker et al., 1991). Third, a recent phylogeny using microsatellite loci is more consistent with a common origin of the Sierra de El Abra cavefish (Strecker et al., 2003), suggesting replacement of mitochondrial DNA may have occurred by hybridization in Pachon cavefish. It is clear from the mtDNA data, however, that A and B lineage cavefish are genetically isolated populations.

In summary, separate origins with accompanying episodes of eye degeneration may have occurred in the Guerrero, Sierra de Guatemala (Molino), Micos (Subterraneo), and Lineage A and B Sierra de El Abra cavefish populations. Below we will compare the developmental mechanisms of eye degeneration in some of these cavefish.

**THE LENS AS AN ORGANIZER OF EYE DEVELOPMENT**

To determine the mechanisms of eye regression, we focused on the nature and timing of degenerative processes in the embryonic eye primordia. In every cavefish population we have studied, the eye primordium appears to be smaller than its surface fish counterpart. However, the cavefish eye seems to develop normally up to about the hatching stage, forming a lens and optic cup. Subsequently, development gradually arrests, the retina becomes disordered, and the
degenerating eye disappears into the orbit (Cahn, 1958; Langecker et al., 1993). The cavefish lens does not differentiate arrays of aligned crystallin fibers and the retina, although at first layered normally, eventually shows disorganization and complete or partial loss of photoreceptor cells. In many developing systems, an alternative to cell differentiation is apoptosis: programmed cell death (White, 1996). Therefore, we first investigated whether apoptosis occurred during cavefish eye development.

If cell death is restricted to a single eye tissue, or begins in one tissue and later spreads to others, then the tissue that dies first is a strong candidate to initiate the degeneration process. Apoptosis was compared in surface fish and in Pachon cavefish embryos using the TUNEL assay (Jeffery and Martasian, 1998), which detects DNA fragmentation. Surface fish embryos showed little or no programmed cell death in the developing eye (Fig. 4A), except in the isthmus that temporarily forms between the budding lens and the surface ectoderm, as has been previously described in the mammalian eye (Silver and Hughes, 1968). Cavefish showed the same apoptotic event in a small number of isthmus cells as the lens vesicle pinched off from the surface ectoderm. About a day after the cavefish lens vesicle was formed, however, an additional and more extensive episode of apoptosis was detected in its central core (Fig. 4B), the region where lens fiber cells would normally differentiate from lens epithelial cells. No apoptosis was detected at this time in the surface fish lens (Fig. 4A), and no other cavefish eye tissue died at this stage of development. A few days later, the retina began to undergo apoptosis. Retinal cell death is restricted to the outer nuclear layer and the region adjacent to the ciliary marginal zone (CMZ) (A.G.S., unpublished), where most new retinal cells are produced in the teleost retina (Johns and Easter, 1977; Harris and Perron, 1998). Thus, the lens is the first tissue to undergo cell death during eye degeneration in Pachon cavefish.
Does the embryonic lens also die in other cavefish populations? Using the TUNEL assay, we showed that the Los Sabinos cavefish lens also dies before any other tissue in the degenerating eye (Fig. 4C). The results suggest that lens apoptosis may be responsible for triggering eye degeneration in both A and B lineage cavefish.

The cessation of retinal growth in cavefish could be caused by the failure of the dying lens to produce a growth-promoting factor or it could be due to an independent event in the retina. A reasonable candidate for an independent retinal event would be interference with cell proliferation. Surface fish have an active CMZ. Proliferating cells can be detected by incorporation of labeled nucleotides into DNA, the presence of the DNA polymerase cofactor PCNA, and the expression the homeobox genes RxI and Vsx2 (Fig. 4D, G), throughout the period of eye growth (Strickler et al., 2002; A.O.S., unpublished results). all of these cell proliferation markers were expressed in the Pachon cavefish CMZ (Fig. 4E, H), although the retina does not markedly increase in size during this period (Strickler et al., 2002). Presumably, new cells are removed from the retina soon after they are formed by the apoptotic events that begin a few days after the initiation of lens cell death.

We next asked whether the surprisingly wasteful process in which retinal cells appear to cycle quickly between birth and death also occurs in other cavefish populations? As shown in Figure F, I, RxI and Vsx2 are also expressed in the CMZ of Los Sabinos cavefish, despite a comparable lack of net growth. Thus, we conclude that arrest of cell proliferation is not the major cause of eye degeneration in A and B lineage cavefish populations.

The results described above focus our attention back to the lens. Does the lens organize the whole eye and could its removal by apoptosis result in the arrest of eye formation? The central role of the lens in eye
formation has recently been appreciated (Beebe and Coats, 2000; Thut et al., 2001), due largely to developmental studies with cavefish (Yamamoto and Jeffery, 2000). We developed a lens transplantation assay to determine the role of the lens in surface fish eye development and in cavefish eye degeneration (Yamamoto and Jeffery, 2000, 2002).

The embryonic lens was removed from a donor embryo shortly after it pinched off from the surface ectoderm, about a day before the first detection of largescale apoptosis in the cavefish lens, and it was transplanted into the optic cup of a host embryo. Lens transplantation was done unilaterally, with the unoperated eye of the host serving as a control. The first transplantation experiments were carried out reciprocally between surface fish and Pachon cavefish: a surface fish lens was transplanted into a cavefish optic cup and vice versa (Yamamoto and Jeffery, 2000). These experiments also addressed the autonomy of programmed cell death in the cavefish lens: is cell death determined by the lens itself or is it induced by another tissue, for instance the retina? When a cavefish lens was transplanted into a surface fish optic cup it died on schedule, just as if it had not been removed from the donor embryo. Likewise, when a surface fish lens was transplanted into a cavefish optic cup it continued to grow and differentiated as it would have in the surface fish host. Together, these results indicate that the Pachon cavefish lens is autonomously fated for apoptosis, at least by the time of the transplantation (Yamamoto and Jeffery, 2000).

The autonomy of surface fish lens development in the cavefish host is the key part of the transplantation experiment. After obtaining a surface fish lens, the Pachon cavefish eye reversed its fate and began to grow and develop (Yamamoto and Jeffery, 2000). Eventually, the cornea and iris appeared, which are normally missing in cavefish, and the retina enlarged and became more organized. Further growth resulted in the presence of a highly developed eye containing all of the expected eye tissues, including the cornea, iris, and photoreceptor cells, in the adult
Pachon cavefish host (Fig. 5B). When the donor lens was labeled with GFP no labeled cells appeared in the restored tissues of the host (Yamamoto and Jeffery, 2000). Thus, the rescued eye tissues arise from the host and not the donor. The cornea and iris are derived in part from optic neural crest cells, indicating that cavefish neural crest cells are present and located in the proper positions to be induced by the lens. In contrast to the eye with a transplanted lens, the unoperated eye of the cavefish host degenerated and disappeared into the orbit according to its usual schedule (Fig. 5A). Likewise, after obtaining a cavefish lens, development of the surface fish eye was retarded, the cornea and iris did not differentiate, and the size and organization of the retina was reduced. The degenerate surface fish eye eventually disappeared into the orbit (Fig. 5D), mimicking the cavefish eye. In contrast, the unoperated eye developed normally (Fig. 5C), resulting in a one-eyed surface fish.

Several conclusions can be made from the lens transplantation experiments. First, the lens is an organizer of optic development, mediating differentiation of the cornea and iris and survival and growth of the retina and cornea. Whether the lens sends an instructive or a permissive signal to these tissues is currently under investigation. Second, the cavefish lens has lost the ability to organize the eye, presumably as a result of apoptosis. Third, despite the loss of its own lens, the cavefish eye and accessory tissues have retained the ability to respond to signals generated by a normal surface fish lens. Thus, the lens plays a key role in eye degeneration in Pachon cavefish.

We next asked whether the lens is central to eye degeneration in other cavefish populations. The lens transplantation experiments were repeated in Los Sabinos cavefish (Fig. 5E-H). The results were the same: a surface fish lens was able to restore eye formation in a Los Sabinos cavefish host, and the lens from a Los Sabinos cavefish transplanted into a surface fish optic cup was unable to mediate eye development in the
surface fish host. Thus, the eye degeneration process appears to be very similar, if not identical, in Los Sabinos and Pachon cavefish. In both cases, evolutionary changes have targeted the lens.

**GENES INVOLVED IN EYE DEGENERATION**

Many different eye development genes have been identified in vertebrates. This resource prompted us to take a candidate gene approach to characterize the genes involved in cavefish eye degeneration. The approach involves obtaining the sequences of known eye genes by Reverse Transcription PCR with degenerate primers and comparing their expression patterns in surface fish and cavefish embryos by in situ hybridization.

Our candidate gene survey includes genes encoding transcription factors that function near the top of eye gene hierarchies, as well as structural genes encoding proteins that function at the bottom of the gene cascades. Most of the surveyed genes did not show any changes in expression in surface fish and cavefish embryos. For example, the transcription factor Proxl is expressed normally in the developing lens and retina of Pachon and Los Sabinos cavefish until after the eye begins to degenerate (Jeffery et al, 2000), indicating that it could not have a causal role in regression. Likewise, prior to lens degeneration, the [beta] and gammaM crystallin genes are expressed in the cavefish lens (Jeffery et al, 2000), despite lack of lens fiber cell differentiation and diversion into a cell death pathway. The gamma crystallin protein is also synthesized in the cavefish lens (A.G.S., unpublished). Langecker et al. (1993) noted a similar pattern of opsin gene expression in the outer nuclear layer of the cavefish retina. The changes that did occur in cavefish gene expression in our survey were subtle and appeared very early in eye development (A.G.S. and Y.Y., unpublished). Below we describe the early changes in Paxo gene expression and their implications for the regulation of cavefish eye development.
Paxo encodes a transcription factor (Gehring and Ikeo, 1999) that is expressed in the lens placodes and optic primordia (presumptive retina and retinal pigment epithelium) early in teleost eye development (Krauss et al., 1991; Puschel et al., 1998). This well studied gene is known to play a fundamental role in many aspects of eye development in both invertebrates and vertebrates (Halder et al., 1995; Ashley-Padan et al., 2000). Interestingly, the morphology of the small eye primordium in cavefish embryos resembles the Small eye phenotype in mouse, which is caused by a mutation in the Paxo gene (Hill et al., 1991). Our PCR analysis and library screen detected a single Paxo gene in Astyanax (Strickler et al., 2001), although two genes have been described in zebrafish (Nornes et al., 1998). Astyanax Paxo is expressed in the lens placode, presumptive retina, and in parts of the central nervous system during early development. Later, Paxo expression becomes restricted to the lens epithelial cells, the ganglion and amacrine cells of the retina, and the corneal epithelium. Below we consider Paxo expression patterns in the neural plate of cavefish embryos.

The teleost eye arises from two regions at the neurula stage: the bilaterally symmetric optic fields, which are located in the anterior neural plate, and the lens placodes, which are located in the surface ectoderm just outside the anterior margin of the neural plate. After the neural tube appears, each optic field forms an optic vesicle. The optic vesicle then rotates through an angle of about 90° to form a lateral optic cup and a medial optic stalk (see Fig. 7A-B). Next, the lens placode buds into the space within the optic cup to form the embryonic lens. Finally, the retina and retinal pigment epithelium differentiate from the optic cup, and the optic nerve develops from the optic stalk.

In surface fish embryos, bilateral Paxo expression domains, which coincide with the optic fields in the anterior neural plate, connect across
the midline at their anterior margins (Fig. 6A, B). In Pachon cavefish embryos, however, the Paxo domains are slightly diminished in size and show a gap across the midline (Fig. 6C, D). Paxo expression in the lens placodes was also diminished in Pachon cavefish embryos (Fig. 6C, D). Controls showing that Dlx-3 and Pax2 expression were unchanged at the same developmental stage (Strickler et al, 2001, Y.Y., unpublished) indicated that Pax6 expression is downregulated in cavefish. The decrease in Pax6 expression may explain the decreased size of the cavefish lens and optic cup.

We next examined Pax6 expression in Los Sabinos and Curva cavefish embryos (Fig. 6E-H). Similar results were obtained: Pax6 expression was reduced in the optic fields, a midline gap was present between the bilateral expression domains, and expression was reduced or absent in the lens placodes. The results indicate that similar changes in Pax6 expression occur in the eye primordia of A and B lineage cavefish embryos.

The division of the optic vesicle into the optic cup and stalk 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. Accordingly, a reduction of Pax6 levels (or an increase in Pax2 levels) is expected to increase the optic stalk at the expense of the optic cup, leading to reduction in the size of the ventral optic cup. The antagonism between Pax6 and Pax2 function during optic primordium development is illustrated in Figure 7A, B. Consistent with a diminished Pax6 expression domain, the optic cup is ventrally reduced and its ventral sector is replaced by optic stalk in Pachon, Tinaja, and Curva cavefish embryos (Fig. 7C-F). Thus, reduction of the Pax6 expression domains has the same phenotypic effects on eye formation in A and B lineage cavefish.
DISCUSSION

We compared eye degeneration in A lineage Pachon cavefish and several B lineage cavefish populations. Based on the mtDNA tree, A and B lineage cavefish were inferred to evolve at different times from distinct surface fish ancestors, implying that they lost their eyesight independently. Multiple origins of blind cavefish in the genus Astyanax would be consistent with convergent reduction and loss of eyes that has been described in many different species of cave adapted fishes (Romero and Paulson, 2001). Although there is still uncertainty about the reliability of mtDNA for inferring the phylogenetic relationships between closely related taxa (Shaw, 2002), the mtDNA phylogeny is supported by genetic complementation in the progeny of a cross between Pachon and Los Sabinos cavefish (Wilkens, 1971). Therefore, at least some of the genes responsible for eye degeneration must be different in A and B lineage cavefish populations. Current studies are consistent with at least four (Guerrero, Sierra de Guatemala, Subterraneo, Sierra de El Abra cavefish populations) and possibly five (A and B lineage Sierra de El Abra cavefish populations) showing independent origins and visual degeneration episodes in Astyanax cavefish.

Eye development pathways are modified in the same or very similar ways in A and B lineage cavefish. Embryos of both types of cavefish initially form optic primordia consisting of a small lens and a ventrally reduced optic cup. Retinal cell differentiation begins on schedule but eye growth and development are gradually arrested, and the degenerating eye sinks into the orbit. Surprisingly, cessation of cell proliferation is not the primary cause of arrested retinal development in Faction or Los Sabinos cavefish (Strickler et al., 2002). The Vsx2 and Rx1 genes, positive indicators of retinal cell division, are expressed strongly in the CMZ, implying that degenerative events may cancel the addition of new cells.
In A and B lineage cavefish, lens cell death is a prelude to general optic arrest and degeneration, suggesting that the lens plays a central role in the loss of vision. Indeed, a surface fish embryonic lens can rescue eye development, including the induction of the cornea and iris and the restoration of retinal growth, after transplantation into the optic cup of a Pachon or a Los Sabinos cavefish embryo. Thus, lens apoptosis mediates eye degeneration in A and B lineage cavefish populations.

The following scenario is proposed for loss of vision in cavefish. The developing lens normally produces a factor(s) that is responsible for inducing differentiation of the anterior eye segment (e.g., cornea and iris) and sustaining retinal growth by suppression of apoptosis. The signal(s) is either greatly reduced or absent in the cavefish lens after it switches to an apoptotic pathway. Although generation of new cells in the retina (and other eye parts?) is not prevented, cell death triggered by the absence of the lens signal prohibits net growth, degeneration begins, and the cavefish eye is overwhelmed by rapid growth of the body. Therefore, cavefish eye degeneration does not appear to be an economic process: considerable metabolic energy must be expended by the continuous generation of new retinal cells that are eventually bound to die. These results appear to be inconsistent with any theory of cavefish eye regression that assigns a positive selective value to energy conservation.

Because apoptosis can result from changes in different genes, it was important to compare the expression patterns of many genes in different cavefish populations. Genetic crosses show that 3 to 6 genes are responsible for eye regression in Pachon cavefish (Wilkens, 1988). Although still in its early stages, our eye candidate gene survey has not revealed any genes with complete loss of function or gross changes in expression patterns in cavefish embryos (Jeffery et al., 2000; Strickler et al, 2001, 2002; A.G.S. and Y.Y., unpublished results). Instead, the changes we have seen are modest, spatial rather than temporal, and
occur early in development, allowing them to be magnified into larger changes as development continues.

The subtle modifications in Pax6 expression suggest a role in generating the cavefish eyeless phenotype. The important differences in early cavefish embryos are reduced Pax6 expression in the bilateral optic fields and a wider gap between the expression domains at the anterior midline. The prechordal mesoderm lies immediately beneath the anterior neural plate during optic field determination. Hedgehog (Hh) proteins diffusing from the prechordal mesoderm regulate the size of the optic primordia by suppressing Pax6 expression in the overlying neural plate (Ekker et al., 1995; Macdonald et al., 1995; Li et al., 1997). Likewise, reduced Pax6 expression in the optic fields is expected to change the fate of the ventral optic cup via relaxing the normal transcriptional repression of Pax2, which directs optic stalk development (Schwarz et al., 2000). A ventrally reduced optic cup was observed in A and B lineage cavefish embryos.

Diminutive Pax6 domains and optic cups without ventral sectors in cavefish embryos may be a consequence of hyperactive Hh midline signaling. Experiments in progress (Y.Y., unpublished results) show that expression of the midline signaling gene Sonic hedgehog (Shh) is increased and expanded in the prechordal mesoderm of both Pachon and Los Sabinos cavefish embryos. Furthermore, Pax2 expression is also expanded in cavefish optic vesicles and upregulation of midline signaling by injecting Shh mRNA into surface fish embryos results in a phenocopy of the cavefish eye, including a change of fate in the optic cup, lens apoptosis, arrest of eye development, and loss of vision.

The developmental and gene expression studies have revealed a negative relationship between midline signaling and eye formation, which could have major implications for cavefish eye regression. Thus,
eyes could be lost as a secondary consequence of expanded midline signaling, which could promote enhancement of other sensory organs that may be advantageous to the survival of blind cavefish. We have recently shown that Shh is both sufficient and necessary for the differentiation of taste bud primordia (Y. Y., unpublished), which could be one of the affected sensory organs.

We opened this paper with Darwin's quote discounting a role of natural selection in blinding cave animals. The difficulty in explaining eye regression by natural selection resulted in the popularity of the neutral mutation hypothesis. The results of the developmental studies described here are largely inconsistent with neutral mutation as the only force responsible for eye degeneration. First, loss of the eye by the same mechanisms in different cavefish populations would not be expected according to the neutral mutation hypothesis. Second, continued expression of genes with functions restricted to the eye, such as the [beta] and gamma crystallins (Jeffery et al., 2000) and other genes encoding lens proteins (A.G.S., unpublished), would not be predicted. According to the neutral mutation hypothesis, loss of function mutations would be expected to accumulate in these genes over time. Behrens et al. (1998) also concluded that the cavefish alphaA crystallin gene is structurally intact, although they were unable to detect transcripts in the lens. Third, parallel changes in gene expression in different cavefish populations would not be expected according to the neutral mutation hypothesis. The developmental results are more adequately explained by natural selection acting through pleiotropic genes that simultaneously promote some of the constructive features and suppress some of the regressive features of the cavefish phenotype. Recent QTL analysis has also suggested a possible role for pleiotropy in the co-evolution of constructive and regressive traits in Astyanax cavefish (Borowsky and Wilkens, 2002). QTL analysis may eventually lead to the discovery of more candidates for genes controlling eye degeneration in cavefish.
Previous attempts to explain cavefish eye degeneration invoke either the neutral mutation or adaptation hypotheses, which are not mutually exclusive. In addition to the developmental studies reviewed here, any attempt to understand the evolution of eye degeneration also must take into account the results of genetic crosses showing that some of the genes involved in eye degeneration are different in A and B lineage cavefish (Wilkens, 1971). Some of the progeny of Pachon X Los Sabinos cavefish crosses were found to have less degenerate eyes than either parent, suggesting different eye genes have been modified in these cavefish populations. Similar results have been obtained by crossing Pachon and Subterraneo cavefish (Y.Y., unpublished). Accordingly, we propose that both natural selection and neutral mutation may have contributed to the loss of eyes in Astyanax cavefish.

Eye degeneration could have occurred in two steps, the first mediated by natural selection and the second by neutral mutation. Natural selection could have initiated the eye degeneration as a tradeoff between forming complete eyes and enhancing taste buds and other cranial sensory organs. The tradeoff may be controlled by Shh and other pleiotropic genes, whose midline signaling domains are expanded in cavefish embryos. Subsequently, neutral mutations may have accumulated in different eye genes as eye regression continued under relaxed selection in the cave environment.

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