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SYMPOSIUM

Trait Loss in Evolution: What Cavefish Have Taught Us about Mechanisms Underlying Eye Regression

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Synopsis Reduction or complete loss of traits is a common occurrence throughout evolutionary history. In spite of this, numerous questions remain about why and how trait loss has occurred. Cave animals are an excellent system in which these questions can be answered, as multiple traits, including eyes and pigmentation, have been repeatedly reduced or lost across populations of cave species. This review focuses on how the blind Mexican cavefish, *Astyanax mexicanus*, has been used as a model system for examining the developmental, genetic, and evolutionary mechanisms that underlie eye regression in cave animals. We focus on multiple aspects of how eye regression evolved in *A. mexicanus*, including the developmental and genetic pathways that contribute to eye regression, the effects of the evolution of eye regression on other traits that have also evolved in *A. mexicanus*, and the evolutionary forces contributing to eye regression. We also discuss what is known about the repeated evolution of eye regression, both across populations of *A. mexicanus* cavefish and across cave animals more generally. Finally, we offer perspectives on how cavefish can be used in the future to further elucidate mechanisms underlying trait loss using tools and resources that have recently become available.

Introduction

Evolution of traits often occurs as a response to an environmental change, such as the colonization of a novel environment. While some of these evolved traits are enhanced or novel, others are reduced or lost. Trait loss can occur to varying degrees (Sadier et al. 2022): from a complete loss of a structure, for example, the loss of teeth in birds (reviewed by Louchart and Viriot 2011), to structures that are present at some stage during development, like the lost digits in jerboa and some ungulate limbs (Cooper et al. 2014), to structures that are present as vestigial structures that persist throughout adulthood, like the hindlimb vestiges retained in some snake species (Leal and Cohn 2018). While evolutionary losses or reductions of traits are common (Ellers et al. 2012), many questions remain about how and why traits are lost.

Cave environments are defined by lack of light, environmental stability, and, in some cases, a reduction in food availability (Culver and Pipan 2019). Under these

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environmental conditions, many cave taxa have independently evolved suites of traits, some of which are enhanced, such as non-visual sensory systems, and other traits that are reduced or lost, such as eyes and pigmentation (Culver and Pipan 2019). One species containing cave populations is *Astyanax mexicanus*, a single species of freshwater fish that has a surface-dwelling form that lives in Southern Texas and Mexico and over 30 populations of a blind cave-dwelling form that live in caves located in Mexico (Fig. 1) (Mitchell et al. 1977; Espinasa et al. 2020). *Astyanax mexicanus* cavefish have evolved a multitude of traits, including regression of eyes, reduction of melanin pigmentation, decreases in sleep, increases in the lateral line, changes in foraging behavior, and decreases in aggression (Şadoğlu 1957; Şadoğlu and McKee 1969; Teyke 1990; Jeffery et al. 2003; Yoshizawa et al. 2010; Duboué et al. 2011; Elipot et al. 2013; Yoshizawa et al. 2014; Lloyd et al. 2018). Additionally, some populations of cavefish have evolved independently over the past ∼200,000 years (Ornelas-García et al. 2008; Bradic et al. 2012; Herman et al. 2018), providing an opportunity to examine the repeated evolution of cave traits in this species. Further, *A. mexicanus* offers a number of advantages as a model system, including that they can be bred and raised in the laboratory and that the two morphotypes are interfertile, allowing for the study of the genetic basis of traits through genetic mapping (reviewed in Jeffery 2020). Additionally, a set of genetic tools that include morpholinos, Tol2 transgenesis, CRISPR-Cas9 and TALENs for genetic manipulation are now available in this species (Yamamoto et al. 2009; Ma et al. 2015; Klaassen et al. 2018; Stahl et al. 2019; Ma et al. 2020). These tools, combined with the recently sequenced genomes of both surface fish and cavefish from the Pachón cave (McGaugh et al. 2014; Warren et al. 2021), provide researchers with resources for gene discovery and ways to identify and functionally assess the contributions of naturally occurring variation to particular traits.

The evolution of eye regression in *A. mexicanus* cavefish populations makes this an ideal system for examining how and why trait loss evolves. Surface fish have large, well-functioning eyes. In contrast, while cavefish initially develop eyes, their eyes begin to degenerate at early stages of development, resulting in adult fish with no external eye structures (Fig. 2). This striking phenotypic difference, which has evolved repeatedly in multiple *A. mexicanus* cavefish populations (Wilkens and Strecker 2003; Borowsky 2008; Sifuentes-Romero et al. 2020), has led to many studies being conducted to investigate the mechanisms contributing to eye regression in cavefish. Here, we will review what is known about the developmental, genetic, and evolutionary mechanisms that lead to eye regression in cavefish. Further, we will expand this discussion to review what is known about how the evolution of the eyes impacts other traits in this species and about the repeated evolution of eye loss across cave animals. Finally, we will comment on future research directions this field could take through employing new tools and resources now available in this species.

Developmental pathways contributing to the evolution eye regression

The developmental pathways that contribute to eye degeneration are one of the most well-studied aspects of *A. mexicanus*, and many key alterations in development in cavefish relative to surface fish have been identified (Figs. 2 and 3). The majority of this research has been conducted using the Pachón population of cavefish, and this section refers to this group unless otherwise specified. Eye development in surface fish is comparable with other vertebrate species, giving rise to a complete and functional eye. However, even though many adult cavefish do not have external eyes, all the cavefish populations studied to date form an eye primordium during early stages of development, which differs from surface fish in the size and proportions of its parts (Figs. 2 and 3) (Jeffery and Martasian 1998; Wilkens and Strecker 2003). In both morphotypes, the optic vesicles form as evaginations from the developing brain. While similar in form, optic vesicles are smaller in cavefish compared to surface fish (Fig. 2) (Cahn 1958; Strickler et al. 2001; Yamamoto et al. 2004). The cells in the optic vesicles undergo a complex set of movements to form the optic cup, composed of the neural retina and the retinal pigmented epithelium, and the optic stalk, which connects the optic cup to the forebrain. In both forms, the lens placode is formed in the overlying ectoderm and detaches and moves into the optic cup to form the lens vesicle. In surface fish, fiber cells in the lens vesicle then differentiate to form the crystallin lens (Jeffery and Martasian 1998).

In cavefish, the eyes are smaller throughout development, with a noticeable reduction of the ventral part of the optic cup, as well as an enlargement of the optic stalks (Figs. 2 and 3) (Jeffery et al. 2003; Yamamoto et al. 2004; Pottin et al. 2011). The neural retina is able to differentiate; however, retinas in cavefish show a reduction in lamination, appear disorganized, and the outer segments of the photoreceptors do not form (Jeffery and Martasian 1998; Alunni et al. 2007; Emam et al. 2020). The cells in the retinal pigmented epithelium have an abnormal morphology and reduced or no pigment (Jeffery and Martasian 1998; Ma et al. 2020). The size of the lens is significantly reduced in cavefish compared

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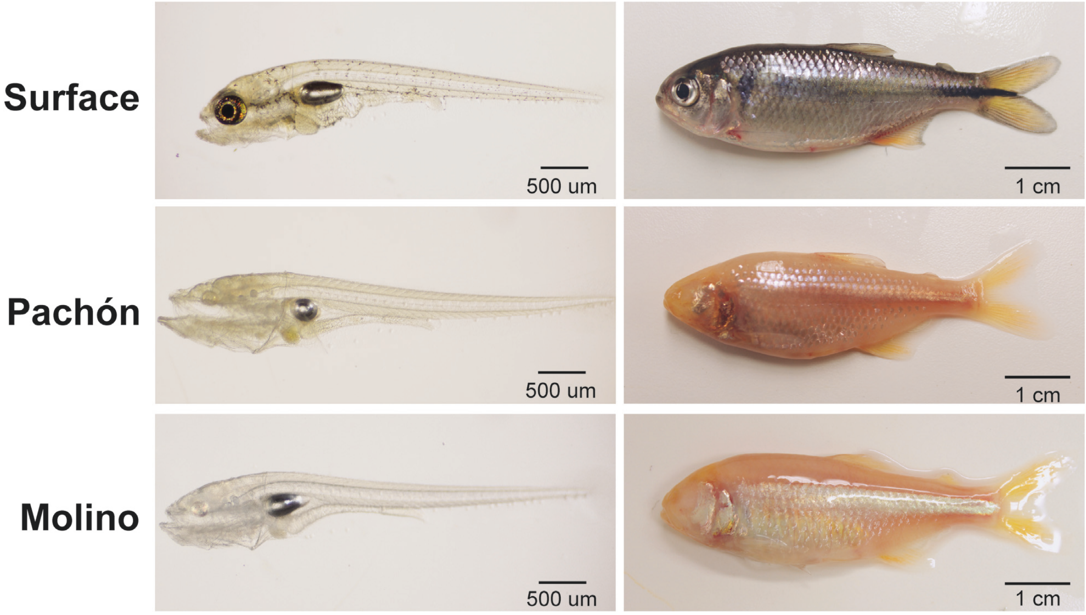

Fig. 1 *Astyanax mexicanus* surface and cave fish. Surface fish and fish from Pachón and Molino caves were imaged at 12 days post fertilization (left) and adult stages (right). Photographs by Charlotte Mulliniks, Aubrey Beck, and Briley Mullin.

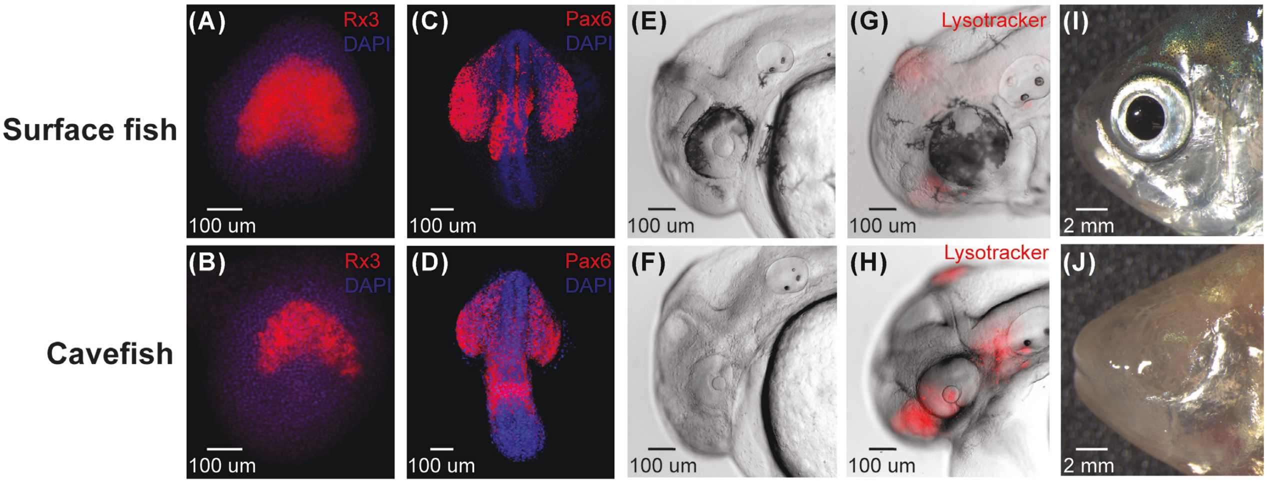

Fig. 2 Eye development in surface fish and Pachón cavefish. (A&B) The expression domain of *rx3* is reduced in cavefish compared to surface fish in the anterior neural plate during specification of the eye field (11 hpf). (C&D) The optic vesicles form in cavefish, but are smaller compared to surface fish (17 hpf). (E&F) The optic cup and the lens are both reduced in size in cavefish relative to surface fish, and the ventral portion of the optic cup is reduced in cavefish (36 hpf). (G&H) Apoptosis, visualized with lysotracker red, occurs in the lens in cavefish, but not in surface fish (60 hpf). (I&J) By adult stages, the eye has regressed and is no longer visible externally in cavefish compared to surface fish. Gene expression visualization for *rx3* and *pax6* where performed by fluorescent *in situ* hybridization using previously published probes (Sifuentes-Romero et al. 2020).

to surface fish (Cahn 1958; Jeffery et al. 2003; Yamamoto et al. 2004; Ma et al. 2014), and lens fibers are not produced (Langecker et al. 2009).

After this, the eye in surface fish undergoes a growth and differentiation process culminating in the formation of a complete and functional eye (Jeffery and Martasian 1998). However, in cavefish, the eyes degenerate through an apoptotic process that starts in the lenses and later spreads to the retina (Figs. 2 and 3) (Jeffery and Martasian 1998; Yamamoto and Jeffery 2000; Alunni et al. 2007; Strickler et al. 2007). Retina cells are produced during early development and cell proliferation is proportional in cavefish relative to surface fish retinas, suggesting apoptosis, rather than defects in proliferation, plays a major role in the reduced growth of the eye (Strickler et al. 2002; Alunni et al. 2007; Strickler et al. 2007). Eye degradation continues into larval stages, and results in

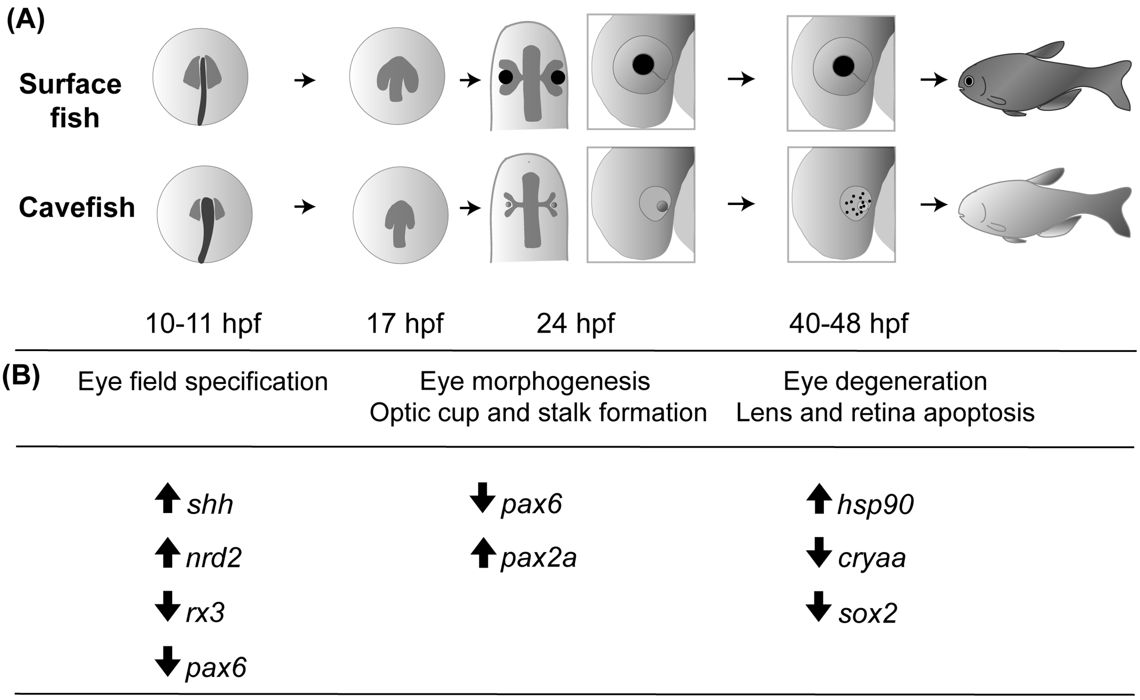

Fig. 3 Eye development comparison between surface fish and cavefish. (A) Schematic representation of the main developmental mechanisms from eye field specification to eye degeneration in surface fish (top) and cavefish (bottom). Compared to surface fish, cavefish have an expansion of *shh* expression at the midline and a smaller eye field, as well as an anterior expansion of the prechordal plate (10–11 hpf), smaller optic vesicles (17 hpf), and subsequently, smaller optic cups and smaller lenses, and an expanded optic stalk (24 hpf dorsal view, left) and a reduction of the ventral portion of the optic cup (24 hpf lateral view, right). Finally, apoptosis begins in the lenses and is later extended to the retina (40–48 hpf). Note that the developmental differences outlined here extend across time points in development. (B) A number of gene expression differences in cavefish relative to surface fish have been identified at different stages of eye development. A subset of gene expression differences in cavefish relative to surface fish are indicated. Arrows represent either up-regulated/increased expression domain size (↑) genes or down-regulated/reduced expression domain size (↓) genes in cavefish relative to surface fish. See the text for areas of the developing fish where expression differences are found.

a highly degraded eye primordia which sinks into the orbit (Jeffery and Martasian 1998; Jeffery et al. 2003).

The lens plays an important role in eye degeneration (Yamamoto and Jeffery 2000; Strickler et al. 2007). Transplanting the lens from a surface fish into a cavefish optic cup early in development results in the loss of retinal apoptosis in cavefish. Further, these transplants result in an eye with normal external morphology, although smaller than a surface fish eye (Yamamoto and Jeffery 2000; Strickler et al. 2007). Surface-to-cave lens transplants also increase the size of the optic nerve projections into the optic tectum, although not to the extent that is seen in surface fish (Soares et al. 2004), and it is thought that the eye is not functional, since cavefish with transplanted lenses do not respond to light in behavioral assays measuring scototaxis (Romero et al. 2003). Conversely, transplantation of a cavefish lens into a surface optic cup results in the degeneration of the eye in surface fish (Yamamoto and Jeffery 2000). Together, these results suggest that while apoptosis of the lens plays a critical role in eye degeneration in cavefish, lens apoptosis does not explain the full cavefish eye phenotype. Indeed, other factors, such as defects in the retinal pigmented epithelium (Ma et al. 2020), contribute to eye regression in cavefish.

Beyond the lens, other developmental changes have been identified that contribute to eye regression in cavefish. For example, transplantation of neural crest cells from surface fish into cavefish results in increases in eye size but not in lens size, suggesting a role for the neural crest in the evolution of the eyes in this species (Yoshizawa et al. 2018). Furthermore, differences in early developmental events contribute to differences in eye development between cavefish and surface fish. For example, heterochronies during gastrulation have been linked with evolved differences in eye size in cavefish, and these differences during gastrulation have a strong maternal effect, which likely plays a role in differences in eye development between morphs (Torres-Paz et al. 2019). Consistent with this, there is a maternal effect on eye size, lens size, and the reduction of the ventral side of the retina in surface × cave reciprocal hybrids from

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multiple populations (Ma et al. 2018; Torres-Paz et al. 2019; Sifuentes-Romero et al. 2020). In addition, recent work suggests a role for downregulation of gene expression in the eye through DNA methylation in the evolution of eyes in cavefish (Gore et al. 2018).

Genetic underpinnings of evolution of the eyes in cavefish

Understanding the genetic underpinnings of trait loss is essential for understanding how and why traits are lost. The genetic basis of eye regression in *A. mexicanus* has been an active area of research (Fig. 3). Similar to other vertebrates, the specification of the eye field, from which the retina, retinal pigmented epithelium, and optic stalk originate, in *A. mexicanus* occurs during the regionalization of the anterior neural plate and involves the expression of multiple transcription factors. Multiple genes expressed in the eye field show smaller areas of expression in cavefish relative to surface fish, including *pax6* and *rx3*, suggesting that the specification of a smaller eye field plays a role in eye evolution in cavefish (Strickler et al. 2001; Sifuentes-Romero et al. 2020; Agnès et al. 2022). In addition to being reduced in expression in cavefish, the *rx3* gene is located under a quantitative trait locus, or QTL, for eye size identified in genetic mapping studies, and the generation of mutations in *rx3* in surface fish through the CRISPR-Cas9 gene editing results in reduced or absent eyes in injected fish, demonstrating the conserved requirement for *rx3* in eye development and suggesting the possibility that cis-regulatory mutations in *rx3* contribute to the evolution of eyes in cavefish (McGaugh et al. 2014; Sifuentes-Romero et al. 2020; Warren et al. 2021).

Changes in multiple molecules secreted from the prechordal plate (positioned beneath the neural plate) during eye field specification have been implicated in the evolution of eye regression in cavefish. *Sonic hedgehog* (*shh*) is a morphogen that is expressed at and diffuses from the midline and influences the cell fate within the eye primordia (Ekker et al. 1995). In cavefish, the *shh* expression domain at the midline is expanded relative to surface fish, and this expansion of *shh* has been associated with a number of evolved eye changes in cavefish (Yamamoto et al. 2004). Overexpression of *shh* in surface fish results in a downregulation of *pax6,* smaller optic vesicles, the elongation of the optic stalk, a reduction of the ventral part of the retina, apoptosis in lenses, and arrested eye growth. Conversely, chemical inhibition of Shh signaling in cavefish results in a partial rescue of the eye (Yamamoto et al. 2004; Yamamoto et al. 2009). Ren et al. (2018) found that the prechordal plate is expanded anteriorly in cavefish, and the area of expression of the *nodal-related 2 (ndr2)* gene in the prechordal plate is expanded in cavefish relative to surface fish (Ren et al. 2018). Nodal is a known regulator of *shh* expression (Rohr et al. 2001), and modulation of nodal signaling in *A. mexicanus* affects how anteriorly expanded the prechordal plate is, levels of *pax6* expression in the anterior neural plate, and the size of the optic cup and lenses, suggesting a role for nodal signaling in the evolution of the eyes in cavefish (Ren et al. 2018). The expansion of *shh* has been linked to an earlier expression of the *fgf8* in cavefish relative to surface fish. This heterochrony in *fgf8* expression may affect eye development in cavefish, as inhibition of Fgf receptor signaling in cavefish restores the ventral retina (Pottin et al. 2011). Thus, modulation of early signaling pathways impacts eye development and likely contributes to the evolution of the cavefish eye.

As discussed above, the lens plays a critical role in cavefish eye degeneration. The *alpha-A-crystallin* (*cryaa*) gene encodes an antiapoptotic heat-shock chaperone protein that is expressed in the lens (Aoyama et al. 1993; Mehlen et al. 1996). Expression levels of *cryaa* are downregulated in cavefish, suggesting a role for this gene in lens apoptosis in cavefish (Behrens et al. 1998; Strickler et al. 2007). Indeed, apoptosis is significantly reduced in lens cells expressing a *cryaa* transgene, suggesting that CRYAA acts in a cell-autonomous manner to protect the lens cells from apoptosis (Hinaux et al. 2015). While knockdown of *cryaa* induces lens apoptosis in surface fish, genetic analysis suggests that cis-regulatory mutations do not drive the differences in *cryaa* expression observed between cavefish and surface fish (Ma et al. 2014). However, the SRY-box transcription factor 2 (*sox2*) gene is downregulated in lenses in cavefish relative to surface fish, and knockdown of *sox2* in surface fish reduces *cryaa* expression and induces lens apoptosis, suggesting that *cryaa*-dependent apoptosis is downstream of *sox2* (Ma et al. 2014).

Multiple quantitative trait locus (QTL) studies for eye-related traits have been published and include QTL for eye size, lens size, and size of different regions of the retina (Protas et al. 2007; Protas et al. 2008; Yoshizawa et al. 2012; Kowalko et al. 2013; Kowalko et al. 2013; O'Quin et al. 2013; Borowsky 2016). These studies demonstrate that multiple genes were involved in eye evolution in cavefish. Sequencing of the cavefish and surface fish genomes has led to the identification of a set of candidate genes that could contain causative variants that contribute to eye degeneration in cavefish (McGaugh et al. 2014; O'Quin and McGaugh 2016; Warren et al. 2021). In spite of this, few studies have identified causative genes and genetic variants for eye degeneration in *A. mexicanus.* A notable exception is the identification of Cystathionine β-synthase a *(cbsa)* (Ma et al. 2020). The *cbsa* gene, which encodes

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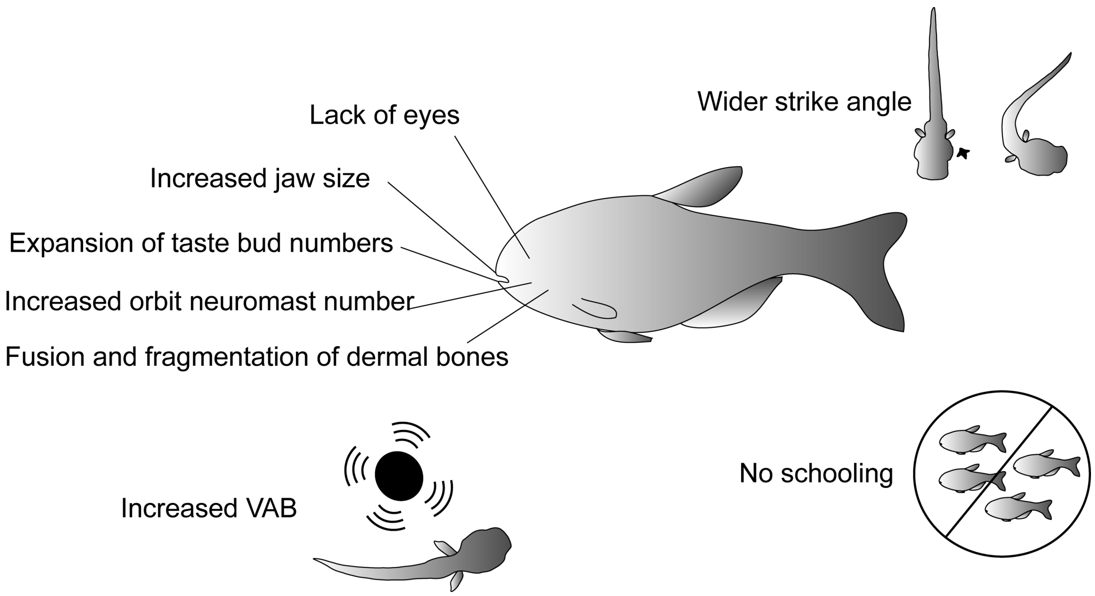

Fig. 4 Morphological and behavioral changes that have been linked to eye regression in cavefish. Morphological traits associated with the loss of eyes in cavefish include craniofacial changes and sensory organ modifications. Several behavioral traits have been associated with the loss of eyes. These include vibration attraction behavior (VAB), the tendency to swim towards a vibrating object, which represents an advantage for cavefish when hunting in the dark and has been genetically linked to the loss of eyes, but cannot be induced by placing surface fish in the dark. Other behavioral changes include that cavefish capture prey using a wider strike angle than surface fish and tend to not forms shoals or schools. These behaviors are vision-dependent in surface fish and are also altered when surface fish are placed in the dark.

an enzyme involved in the transsulfuration pathway, is located near a QTL for eye size, and *cbsa* expression is downregulated in several cavefish populations (Ma et al. 2020). Furthermore, a cis-regulatory variant has been identified in an enhancer of this gene in cavefish, and knockdown of *cbsa* leads to a reduction in eye size and defects in optic vasculature, which are also found in cavefish, demonstrating that mutations in this gene contribute to eye degeneration in cavefish (Ma et al. 2020).

Functional and genetic relationships between eye regression and morphological traits

Loss of eyes in cavefish has been linked to a number of cave-evolved traits (Fig. 4), providing an opportunity to study the mechanisms underlying the evolution of suites of cave-evolved traits. Cavefish have evolved changes to a number of craniofacial structures relative to surface fish, including reduced eye orbit size, fusions and fragmentations of dermal bones, and expansion of the number and distribution of tastebuds (Schemmel 1980; Jeffery et al. 2000; Varatharasan et al. 2009; Gross and Powers 2020). Removal of lenses in surface fish produces a number of cave-like changes to the craniofacial bones, including a reduction in the size of the eye orbit, changes to the shapes of orbital dermal bones, and increases in the size of the olfactory pits (Yamamoto et al. 2003; Dufton et al. 2012). In addition, the relationship between eyes and craniofacial traits has been examined by manipulating early signaling. Manipulation of Shh signaling affects eye size, as well as both taste bud number and jaw size in surface fish (Yamamoto et al. 2004; Yamamoto et al. 2009). Further, manipulation of Shh signaling alters regions of the brain that differ in size and number of types of neurons between cavefish and surface fish, suggesting that the endogenous differences in *shh* expression between cavefish and surface fish contribute to the evolution of the brain (Menuet et al. 2007; Alié et al. 2018). Together, these data suggest that some of the same genes may contribute to the evolution of eyes and other cave-evolved traits. Indeed, genetic studies have revealed correlations between eye size and a variety of other traits in cave-surface hybrids, and genetic mapping studies have shown that QTL for eye and lens size overlap QTL for other traits (Protas et al. 2008; Yamamoto et al. 2009; Yoshizawa et al. 2012; McGaugh et al. 2014; Gross et al. 2016; O'Quin and McGaugh 2016).

Eye regression and behavioral traits

Cavefish are more successful than surface fish at finding food in the dark, and this advantage cannot be replicated by raising surface fish without visual cues (Hüppop 1987; Yoshizawa et al. 2010; Espinasa et al. 2014), suggesting other genetically encoded factors play a role in their success. Cavefish display vibration attrac-

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tion behavior (VAB), which is defined as the tendency to swim towards a source of vibration (Yoshizawa et al. 2010). VAB requires the superficial neuromasts of the lateral line and provides an advantage when hunting in the dark (Yoshizawa et al. 2010). Further, this trait has been linked to eye loss: QTL for VAB, number of superficial neuromasts in the orbit, and eye size overlap, suggesting these traits share genetic underpinnings (Yoshizawa et al. 2012). Larval prey capture is also different between cavefish and surface fish. Cavefish orient themselves beside live prey and strike at prey from wide angles, whereas surface fish in the light strike from head on. These differences in prey capture are due to a shift from vision-dependent prey capture in surface fish to lateral line-dependent prey capture in cave fish populations, and this cave-like method of capturing prey is utilized by surface fish hunting in the dark, demonstrating surface fish use visual cues to capture prey (Lloyd et al. 2018).

The surface form of *A. mexicanus* displays a number of social behaviors that are reduced in cavefish, including aggression and schooling (Gregson and Burt de Perera 2007; Elipot et al. 2013; Kowalko et al. 2013; Iwashita and Yoshizawa 2021; Patch et al. 2022). While aggressive behaviors can be induced in surface fish in the dark (Espinasa et al. 2005; Elipot et al. 2013; Rodriguez-Morales et al. 2022), a functional eye is required for schooling behavior (Wilkens 1988; Kowalko et al. 2013). However, while there is a strong correlation between both dark preference (scototaxis, used as a proxy for vision) and eye size and tendency to school in cave-surface hybrid fish, some non-schooling hybrid fish exhibit visual function, suggesting that while vision is required to engage in schooling behavior, loss of schooling in cavefish cannot be explained by loss of vision alone (Kowalko et al. 2013). This is supported by fine-scale analysis of social behavior in *A. mexicanus*, which suggests that while surface fish do not school in the dark, they do perform coordinated swimming in the dark when in close proximity to other fish, unlike cavefish from multiple populations (Iwashita and Yoshizawa 2021; Patch et al. 2022). Together, the evidence that loss of eyes correlates with or contributes to other cave-evolved traits suggests the possibility that pleiotropy may have played a role in the evolution of eye loss in this species.

Evolutionary forces contributing to eye degradation

Trait loss can occur when the source of selection that maintains a trait is removed or when loss of the trait becomes adaptive in a particular environment (Lahti et al. 2009; Ellers et al. 2012). Whether the regressive traits observed across cave animals have been lost through natural selection or the accumulation of neutral mutations following relaxation of selection to maintain these traits has been the subject of decades of debate (reviewed in Culver et al. 2023). The repeated evolution of eye regression in *A. mexicanus* has been a major focus for researchers interested in understanding the evolutionary mechanisms underlying trait loss in cave animals, and the evidence for different evolutionary mechanisms driving the evolution of eye loss has been the subject of extensive discussion (for example, see Jeffery 2005; Rétaux and Casane 2013; Borowsky 2016; Culver et al. 2023).

Proponents of the neutral evolution hypothesis for eye loss argue that once within the caves, selection that maintains a functional eye is relaxed, allowing for neutral, destructive mutations to accumulate in genes required for proper eye development and function (Wilkens 1988). The variability in eye degeneration across populations of cavefish and between population crosses is the primary data referred to in support of this hypothesis, with the idea that natural selection would reduce variability in eye size (Wilkens 2010), but this hypothesis has not been tested directly (see Culver et al. 2023).

Supporters of the hypothesis that natural selection has played a role in evolution of eye degeneration have argued that direct or indirect selection could lead to evolution of eye regression (Jeffery 2005). Direct selection could occur if there was an adaptive value of eye loss within the cave. The main hypothesis for direct selection suggests that regression of eyes could be beneficial within the cave if loss of eyes results in energy savings (Jeffery 2005). Experimental evidence supports this hypothesis from the perspective of energy savings from not maintaining an eye: estimates of the energetic cost of vision in surface fish obtained through measuring the oxygen consumption rates of brain and eye tissues in cave and surface fish suggest that there is a substantial energetic cost of vision in surface fish (Moran et al. 2015).

Indirect selection, or selection for a trait because it is linked with another trait that provides an adaptive benefit in a particular environment, may also contribute to the evolution of eye regression (Jeffery 2005; Yoshizawa et al. 2012). Genetic studies suggest that there could be genes that contribute to the evolution of eye regression and other cave-evolved traits (see “Functional and genetic relationships between eye regression and morphological traits”). Further, at least one of these linked traits, VAB, has been shown to provide an advantage under cave-like conditions (Yoshizawa et al. 2010). However, as few causative genes and alleles for eye regression have been

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identified, and overlapping QTL and trait correlations observed from crosses could be explained by genetic linkage rather than pleiotropic genes, additional work needs to be done to understand these relationships.

Together, this previous work suggests that both direct and indirect selection for eye reduction and degeneration play at least some role in the evolution of eyes in *A. mexicanus* cavefish. This is further supported by recent modeling work, which suggests that directional selection is likely required for the evolution of eye regression in cavefish (Cartwright et al. 2017; Herman et al. 2018). Further, population genetics analyses suggest that there is selection on a number of genes associated with eye development and function across cavefish populations (Moran et al. 2023), further supporting the idea that selection plays a role in the evolution of eye regression in this species.

Repeated evolution

Evolution in similar environments often leads to the repeated evolution of similar traits in different lineages. Examining whether traits evolve repeatedly through the same genetic and developmental mechanisms has the potential to reveal if there are developmental or genetic constraints that underlie the evolution of these traits, and may provide insight into the evolutionary pressures that contribute to trait evolution (Manceau et al. 2010). Many cave populations have independently evolved regression of their eyes (Culver and Pipan 2019), providing an opportunity to study repeated evolution in these organisms.

Repeated evolution: across populations of *A. mexicanus*

At least two separate colonizations of caves from surface populations have been identified, which have been described as old and new lineages, or lineages 2 and 1, respectively (Ornelas-García et al. 2008; Bradic et al. 2012; Gross 2012; Herman et al. 2018; Moran et al. 2023). The "old" lineage/lineage 2 includes fish originating from caves in the El Abra region, including Pachón, Tinaja, and Sabinos, while the "new" lineage/lineage 1 includes the Guatemala cave populations, including Molino (Gross 2012; Moran et al. 2023). While there is variation in eye regression between populations, adult cavefish from many populations have no external eyes (Fig. 1) (Wilkens 1988; Wilkens and Strecker 2003). Crosses between fish from different cavefish populations result in cave-cave hybrid offspring with eyes that are larger and more developed than those found in fish from either parental population, suggesting that at least some different genes and pathways underlie the evolution of eyes between these populations. However, cave-cave F1 hybrids do not have surface-like eyes, suggesting some shared and some distinct genetic changes (Wilkens and Strecker 2003; Borowsky 2008; Sifuentes-Romero et al. 2020). Genetic mapping studies support these conclusions: distinct and overlapping QTL for eye size were found when comparing surface-Pachón and surface-Tinaja crosses (Kowalko et al. 2013). Together, these results support the hypothesis that populations of *A. mexicanus* have evolved eye loss independently through some of the same and some different genetic factors.

Some similarities in the developmental processes underlying differences in eye development and degeneration are found across *A. mexicanus* cavefish populations. Reductions in the size of the eye field (assessed by *pax6* and *rx3* expression*)*, the optic vesicles and optic cups, and the partial loss of the ventral retina as well as apoptosis in the lenses are found during development in multiple cavefish populations (Cahn 1958; Jeffery and Martasian 1998; Strickler et al. 2001; Jeffery et al. 2003; Sifuentes-Romero et al. 2020). Further, many of the gene expression changes associated with these developmental differences are found across independently evolved populations, including expansion of *shh* at the midline and expression of *cryaa* in the lens (Yamamoto et al. 2004; Ma et al. 2014; Sifuentes-Romero et al. 2020). However, there are both similarities and differences in differential gene expression relative to surface fish for different cavefish populations at the level of the transcriptome during eye-relevant developmental stages (Stahl and Gross 2017) and differences between different cavefish populations in the degree to which they differ from surface fish during eye development (Sifuentes-Romero et al. 2020), further supporting that some genetic differences underlie similar developmental trajectories for eye loss across *A. mexicanus* cavefish populations.

Repeated evolution: across cave species

Like *A. mexicanus*, many other cave species have evolved eye regression (Culver and Pipan 2019). However, the extent to which eyes are reduced varies across populations. For example, eye size is variable in adult fish from *Poecilia mexicana* cavefish populations, and the eyes in these fish retain visual function (Parzefall 2001; Korner et al. 2006; Parzefall et al. 2007). Other cavefish, such as cave *Garra barreimiae*, have significant reductions in eyes, with some individuals lacking external eyes at adult stages, similar to *A. mexicanus* (Banister 1984). Reduced or absent eyes are also observed in cave-dwelling invertebrates. For example, cave populations of *Asellus aquaticus*, a freshwater crustacean, have individuals with both reductions in eyes

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and complete loss of external eyes, and developmental studies suggest there are individuals that never develop ommatidia (Protas et al. 2011; Mojaddidi et al. 2018).

While systematic analyses across many species have not been performed, existing research suggests that there may be some similarities and some differences in the developmental mechanisms underlying the evolution of eye regression across cave species. For example, in a Somalian cavefish, *Phreatichthys andruzzii*, an eye initially forms during development, regressing before the fish reach adult stages. However, how this eye develops and degenerates is dissimilar to what has been observed for *A. mexicanus* cavefish: the first signs of developmental defects occur after eye specification and optic cup morphogenesis, when differentiation in the retina is arrested during the development of early born retinal cell types. Further, apoptosis begins in the retina, rather than the lens (Stemmer et al. 2015). Similarly, eye transcriptomes and histology from closely related cave and surface species from the genus *Sinocyclocheilus* suggest that eye degeneration occurs in the absence of lens degeneration, unlike the process in multiple populations of *A. mexicanus* (Meng et al. 2013). However, there may be some shared genetic mechanisms underlying eye loss in at least some lineages of cave animals. For example, loss-of-function mutations in the *rhodopsin* gene, which encodes the opsin in rod photoreceptors, are found in multiple lineages of amblyopsid cavefish (Niemiller et al. 2013). Additionally, the same genomic regions are associated with eye loss in independently evolved cave populations of the isopod *A. aquaticus*, raising the possibility of the same genes underlying the evolution of eye loss in both of these populations (Re et al. 2018). More distantly related cave lineages may also share genetic mechanisms of eye degeneration. Analysis of gene expression differences in the cave molly *P. mexicana* found that many genes that were downregulated in cavefish relative to surface fish were also downregulated in other cave populations from different species, including *Sinocyclocheilus anophthalmus* and *A. mexicanus* (McGowan et al. 2019). Together, this suggests that there are multiple ways in which eye regression evolved in cave animals. However, the repeated use of genetic and developmental factors in at least some lineages may indicate that constraints or shared ecological pressures contribute to the evolutionary loss of eyes among these populations. Assessment of eye regression in additional cave lineages, and identification of the genetic changes underlying eye loss, along with their phenotypic consequences, will provide additional insight into how and why loss of eyes has repeatedly evolved.

Future directions

Studies in *A. mexicanus* have provided extraordinary insight into the developmental, genetic, and evolutionary mechanisms underlying the evolution of trait loss. These advances have occurred in large part because of tractability of the *A. mexicanus* as a model organism for laboratory studies. Recent advances in the tools and resources available for *A. mexicanus* research will now allow for future studies to further advance our understanding of trait evolution in cave species. For example, the cavefish genome was published in 2014, and a surface fish genome was published in 2021 (McGaugh et al. 2014; Warren et al. 2021). These resources have allowed for the identification of genes underlying QTL for different cave-evolved traits and are essential for rapid and easy identification of genes for genetic and developmental studies, advancing our understanding of genotype–phenotype relationships and evolutionary forces contributing to trait evolution. Furthermore, the population genetics work being done in this species has allowed for further resolution of the evolutionary histories of different cavefish populations, as well as the identification of genes under positive selection in different cave populations (Herman et al. 2018; Mack et al. 2021; Moran, Jaggard, et al. 2022; Moran et al. 2023). These studies have identified a number of candidate genes for the evolution of eyes and other traits that can be investigated through developmental and functional genetic studies in the future.

Recent applications of different functional genetic tools in *A. mexicanus* will now allow for functional interrogation of candidate genes identified from genomic studies like those mentioned above. CRISPR-Cas9 has now been used successfully in *A. mexicanus* to examine the role of candidate genes for the evolution of morphological and behavioral traits (Klaassen et al. 2018; Ma et al. 2020; Mack et al. 2021; O'Gorman et al. 2021; Warren et al. 2021). These phenotypic assessments have been performed in mutant lines (Klaassen et al. 2018; O'Gorman et al. 2021), as well as in individuals that have been injected with Cas9 and a gRNA targeting a gene of interest, or "crispant" individuals, which allows for rapid assessment of gene function (Ma et al. 2020; Mack et al. 2021; Warren et al. 2021). Further refinement of these methods in *A. mexicanus* could allow for rapid screening of candidate genes for cave-evolved traits, similar to studies done in other organisms, such as zebrafish (for example, Shah et al. 2015; Klatt Shaw and Mokalled 2021), to increase the speed at which candidate genes for cave-evolved traits can be identified. Further, CRISPR-Cas9 can be used to generate precise changes in genomic sequences (for example, Wu et al. 2013; Zhu et al. 2021). Application of this technology

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in *A. mexicanus* would allow for the assessment of specific genetic variants associated with the evolution of eye loss.

In addition to gene-editing tools, transgenic technologies have now been applied in *A. mexicanus* (Hinaux et al. 2015; Stahl et al. 2019). The ability to follow particular cell populations during development has provided unprecedented insight into development in other model organisms, and this type of analysis can be performed through tissue-specific expression of fluorescent proteins in *A. mexicanus*. As *A. mexicanus* embryos are clear, allowing for live imaging, the application of these technologies to examine the development and degeneration of the eye in *A. mexicanus* has the potential to reveal additional mechanisms underlying eye evolution in this species. Towards this end, recent work used CRISPR-Cas9 to establish lines to visualize eye cells during development, revealing defects in cell movements during early stages of optic cup morphogenesis in cavefish (Devos et al. 2021), demonstrating the power of such techniques to reveal mechanisms underlying trait evolution in this species.

Finally, tools to investigate the development and function of the brain have recently been established in *A. mexicanus*, which will allow for the expansion of investigation of loss of vision in cavefish to investigation of the visual processing centers of the brain. Brain atlases have recently been established for both adult and larval *A. mexicanus*, and have revealed differences in brain morphology between cave and surface fish, including alterations to the morphology and size of regions of the brain associated with vision (Loomis et al. 2019; Jaggard et al. 2020; Kozol et al. 2022). Additionally, transgenesis has now been used to generate lines of cave and surface fish expressing a Ca^{2+} indicator GCaMP6s panneuronally that allow for visualization of neural activity in the brain. Through examining neural activity in the optic tectum, it was established that blind cavefish still have light-invoked responses in the optic tectum (Lloyd et al. 2022). These tools can now be used to further understand visual function in surface fish and the impacts of eye loss in cavefish.

Conclusions

Cave animals have convergently evolved a number of traits, including reductions and losses of eyes and pigment, positioning them as powerful systems for understanding the mechanisms underlying trait loss. Indeed, the evolution of eye loss has been investigated in a number of cave-evolved organisms, and advances have been made in understanding how and why eye loss has evolved. Studies of eye loss in *A. mexicanus* cavefish have revealed genetic, developmental, and evolutionary factors that have contributed to eye loss in this species. Recent advances in genomic and functional genetic tools and resources provide promise for further understanding the processes that lead to the evolution of eye loss in *A. mexicanus* and beyond.

This review was written as a collaborative project by undergraduates in Honors Developmental Biology at Florida Atlantic University in Fall 2020. Students in the course worked collaboratively to decide the topic of the review, the direction the review would take, the review contents, and to write the initial drafts and revisions of the review. Following revisions and updates to include more recent work, student co-authors also edited the final manuscript. Thus, undergraduate co-authors were the driving force behind this review.

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Conflicts of interest

The authors declare no conflicts of interest.

Data availability

No new data was generated for this review.

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