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Evolution and development in cave animals: from fish to crustaceans

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Abstract

Cave animals are excellent models to study the general principles of evolution as well as the mechanisms of adaptation to a novel environment: the perpetual darkness of caves. In this article, two of the major model systems used to study the evolution and development (evo–devo) of cave animals are described: the teleost fish *Astyanax mexicanus* and the isopod crustacean *Asellus aquaticus*. The ways in which these animals match the major attributes expected of an evo–devo cave animal model system are described. For both species, we enumerate the regressive and constructive troglomorphic traits that have evolved during their adaptation to cave life, the developmental and genetic basis of these traits, the possible evolutionary forces responsible for them, and potential new areas in which these model systems could be used for further exploration of the evolution of cave animals. Furthermore, we compare the two model cave animals to investigate the mechanisms of troglomorphic evolution. Finally, we propose a few other cave animal systems that would be suitable for development as additional models to obtain a more comprehensive understanding of the developmental and genetic mechanisms involved in troglomorphic evolution.

INTRODUCTION

Cave animals are attractive models for evo–devo research because they have evolved an unusual suite of troglomorphic (cave related) traits, including the reduction or loss of eyes and pigmentation, the lengthening of appendages, and the elaboration of nonvisual sensory systems.¹ Troglomorphic traits have evolved by convergence in a diverse array of cave-adapted invertebrates and vertebrates. Cave adapted species exhibit several additional attributes making them exceptional models in evo–devo. First, cave animals were originally derived from surface dwelling ancestors, and therefore the direction of troglomorphic changes can be understood. Second, the cue for troglomorphic evolution is the absence of light and its consequences on primary productivity, allowing trait changes to be attributed to a specific environmental factor. Third, animals resembling the surface ancestors of cave animals are sometimes extant, providing a dual system of surface and cave forms in which the starting and ending points of evolution can be compared. In this article, we review two cave species that have been most successfully developed as model systems to understand troglomorphic evolution: the teleost fish *Astyanax mexicanus* and the isopod crustacean *Asellus aquaticus*.

REQUIREMENTS OF AN EVO–DEVO CAVE SYSTEM

Certain characteristics can be regarded as integral components for investigating the genetic basis of evolution and development in a cave model system.^{2,3} Both *A. mexicanus* and *A. aquaticus* fulfill the characteristics necessary to study both microevolutionary genetics and comparative developmental biology (see Figure 1). However, many other cave species that do not fulfill all of these characteristics have already provided interesting insights about the evolution and development of cave animals. Still other systems have the potential to fulfill all of these characteristics and some of these will be described as up-and-coming systems in the final section of this review.

Species with Cave and Surface Morphotypes

To address both genetic and developmental questions, a cave model must harbor both cave and surface forms with different phenotypes that can interbreed to produce fertile offspring. Therefore, all cave animals where the surface counterpart is extinct or unknown must be excluded. In addition to *A. mexicanus* and *A. aquaticus*, species with surface and cave forms capable of interbreeding include the amphipod *Gammarus minus*, the fishes *Poecilia mexicana* and *Garra barreimiae*, leeches of the genus *Dina*, the freshwater limpet *Ancylus fluviatilis*, the amphipod *Synurella ambulans*, and planthoppers of the genus *Oliarus*.^{5–12}

Collecting Animals

Some caves in which *A. mexicanus* can be found are relatively easy to approach, but others require a rope descent.¹³ Furthermore, some of these caves have large amounts of bat guano, raising the risk of spore inhalation of the disease-causing fungus, *Histoplasma capsulatum*. *A. aquaticus* can be found in the Planina cave, in Slovenia, which has a very easy collecting environment, and in the Movile cave in Romania, which is much less hospitable with high concentrations of hydrogen sulfide and ammonia.¹⁴

Another challenge to studying certain species is that unfortunately many cave animals are endangered. Factors contributing to the risk of extinction of cave animals include high endemism of some cave species; human interference with the physical habitat; and water quantity and quality issues resulting from mining, urbanization, deforestation, flooding, storm water drainage, erosion, and dumping.^{1,15,16} Fortunately, both *A. mexicanus* and *A. aquaticus* are found over an extensive geographic range and are relatively abundant within the caves where they are found.^{13,17} In addition, both *A. mexicanus* and *A. aquaticus* are easy to collect; *A. mexicanus* swims to the surface of disturbed water and *A. aquaticus* can be collected with a dip net in shallow water.

Practical Considerations: Rearing Animals in the Laboratory, Generation Time, and Number of Offspring

The next challenge is to breed and rear the animals in the laboratory. It is necessary to know what to feed the animals, cues to breeding, and necessary physical characteristics of the environment. Some systems might be too impractical to pursue further. Researchers began breeding *A. mexicanus* in the laboratory in the 1940s¹⁸ and since then, several groups have devoted significant time and effort to raise individuals from different populations in the laboratory environment. *A. aquaticus* had a promising start as a laboratory organism from some crosses done in the 1940s.^{5,6} However until recently, raising both cave and surface populations of *A. aquaticus* (and crosses between them) had not been revisited.⁴

Cave animals are known to have extremely long generation times. For example, the average lifespan of the cave salamander, *Proteus anguinus*, is predicted to be 68.5 years.¹⁹ Fortunately, the time to sexual maturity of *A. mexicanus* is relatively short, around 4–6

months,²⁰ and in *A. aquaticus* is population dependent but can be as fast as 2 months in one particular Italian population.²¹

In addition, both *A. aquaticus* and *A. mexicanus* produce large numbers of easily accessible embryos. *A. mexicanus* has external fertilization and the embryos can be cultured in a dish until they are free-swimming.²² *A. aquaticus* females deposit embryos into an external brood pouch and it is possible to remove the embryos without harming the female and culture them *in vitro*.²³

Convergent and Parallel Evolution in Multiple Cave Populations

Multiple cave populations allow for the study of convergent and parallel evolution. At least 29 cave populations of *A. mexicanus* have been discovered.¹³ Likewise, there are many cave populations of *A. aquaticus*.^{17,24–26}

Genomic Information

The genome has not yet been sequenced for either *A. aquaticus* or *A. mexicanus*, but many other genomic tools have been generated. For both species, there are linkage maps and hundreds of genetic markers.^{4,25,27–31} In addition, a BAC library is available for *A. mexicanus* and there is evidence of synteny with several other fish genomes.^{32,33} Transcriptome sequencing has been generated for both systems (Protas, Speiser, Patel and Oakley, unpublished; Furterer and Gross, unpublished). All of these molecular tools have aided and will aid in the identification of pathways, genes, and mutations responsible for different phenotypes.

Functional Assays

The final requirement for a cave animal evo–devo system is the ability to determine the relationship of genes to phenotypes using functional assays. Functional assays are well-developed in *A. mexicanus* and involve gene ‘knock up’ via introduction of synthetic mRNAs or conditionally expressed DNA expression constructs and gene ‘knock down’ using anti-sense oligonucleotides.³⁴ In these functional assays, molecules are injected into the egg or early cleaving embryo and effects can be seen as long as 5–6 days post-fertilization. In some cases, functional analysis of *A. mexicanus* genes can also be carried out in zebrafish (*Danio rerio*) or in cultured mouse cell lines. Functional analyses have not yet been attempted in *A. aquaticus*, although they have been generated for another pericardian crustacean, *Parhyale hawaiiensis*.^{35,36}

ASTYANAX MEXICANUS

The teleost *A. mexicanus* consists of a surface dwelling form (surface fish) and 29 known cave (cavefish) dwelling populations¹³ (see Figure 1(a)). The cavefish are native to limestone caves in the Sierra de El Abra region of northeastern Mexico and named according to their caves of origin: for example, Pachón cavefish are from La Cueva de El Pachón, Molino cavefish from El Sotano del Molino, and so forth. The cave-fish populations have arisen independently at least three times when ancestral surface fish entered limestone cave systems and became isolated from their above ground counterparts.^{20,34,37–42} Estimates for the length of time since the initial cavefish and surface fish divergence vary from about 100,000 to several million years.^{13,43} After their separation from surface fish, the cave adapted fish dispersed through underground channels and subsequently became isolated in different caves. The repertoire of troglomorphic features that evolved during adaptation to the dark cave environment includes both constructive and regressive traits.^{44,45} Regressive traits were present in the surface ancestors of cavefish but subsequently were reduced or lost

during adaptation to subterranean life. Constructive changes can entail either the enhancement of pre-existing traits or the evolution of novel traits.

Regressive Traits

The most prominent regressive traits in cavefish are the loss of eyes and pigmentation. During the last decade, significant progress has been made in understanding the developmental and genetic basis of eye and pigment regression in *A. mexicanus*.

Developmental Basis of Eye Regression—Normal development of the lens, retina, photoreceptors, neural connections, retinal pigment epithelium (RPE), and optic processing areas in the brain are required to form a correct visual image. The loss of vision in cavefish is due to developmental defects in these components. Eyes initially develop in the cavefish embryo,⁴⁶ but optic defects appear later during larval development and the eye subsequently degenerates^{47–49} (see Figure 2(k)). Although appearing eyeless, adult cavefish actually have small degenerated eye rudiments buried within their orbits. Different cavefish populations show various degrees of eye reduction, and variations in the size of vestigial eyes can also occur within a single cavefish population.⁴⁴ The developmental and genetic mechanisms of eye degeneration have been most extensively studied in Pachón cavefish; these experiments are described below.

When the eye primordium first appears in cavefish, the lens and optic cup are smaller than those of their surface fish counterparts⁴⁶ (see Figure 2(a), (b), (i), and (j)). As development proceeds, the lens fails to differentiate, gradually decreases in size, and in some cases may eventually disappear.^{48,49,53} In contrast, the embryonic retina initiates growth, neuronal, glial and photoreceptor cell layers are formed, and retinal ganglion cells project neural connections to the optic tectum via the optic stalk.^{46–49,53,54} The photoreceptor cells are transient and disappear later in development.⁵⁵ Eventually, however, the retinal layers become disorganized and further growth ceases. The cavefish RPE is poorly differentiated and usually lacks black pigment^{44,48} (see Figure 2(c) and (d)). The cornea, iris, and ciliary body also fail to differentiate, and the sclera, the partially ossified tissue surrounding the eyeball, remains cartilaginous.^{55,56} During the larval period, the degenerating eye sinks into the orbit and is covered by epidermis and connective tissue (see Figure 2(k)).

Most of the lens and parts of the retina and RPE undergo apoptotic cell death during cavefish eye development^{48,54,55,57} (see Figure 2(c) and (d)). Cell death begins in the eye just after larval hatching and continues for at least 3 months.⁴⁸ Apoptosis is detectable in the lens at least a day prior to its initiation in other eye tissues, suggesting that lens death could trigger optic degeneration. Lens transplantation was developed to test this possibility⁵⁵: a surface fish lens was transplanted into a cavefish optic cup, and a cavefish lens was transplanted into a surface fish optic cup (see Figure 3(a)). The transplanted surface fish lens continued to grow in the cavefish host and mediated the restoration of a large eye (see Figure 3(b) and (c)) with a growing retina, permanent photoreceptors, neural connections to the optic tectum, and an ossified sclera. In contrast, the cavefish lens underwent apoptosis on schedule, and growth and development of the surface fish eye was decreased (see Figure 3(d) and (e)). Sophisticated experiments have not been performed to determine whether the rescued cavefish eye or the retarded surface fish eye are functional and can form visual images. However, the results given above indicate that the lens has an important role in cavefish eye degeneration.

How does lens apoptosis affect overall eye development in cavefish? The absence of the cornea, iris, and ciliary body is simple to explain because a functional lens induces these tissues during normal eye development. The role of the lens in controlling retinal growth is more complex. The teleost retina grows continuously throughout life by adding new cells

produced in two stem cell niches: the inner nuclear layer, which replenishes the recycling photoreceptor cells, and the ciliary marginal zone, which contributes to the growing retinal layers and RPE.^{58,59} Thus, the defective lens could possibly curtail retinal differentiation and growth by affecting stem cell proliferation. However, this is not the case: cell proliferation continues for at least 3 months in both stem cell niches after retinal growth is arrested^{48,51,54} (see Figure 2(e) and (f)). Instead, retinal growth stops because the cells produced by mitosis die before they can differentiate and contribute to the retina.⁴⁸ Thus, cavefish undergo repeated cycles of retinal cell division followed by death rather than differentiation, a process occurring throughout the period of larval development and into the adult. Transplantation of a surface fish lens into the cavefish optic cup protects the retinal stem cells from apoptosis, allowing retinal differentiation and growth to continue.⁴⁸

Lens dysfunction and its secondary effects on the retina are not the only defects in the cavefish eye, as revealed by two further observations. First, as mentioned above, the cavefish optic vesicle (the precursor of the optic cup) is smaller than in surface fish, and later in development the ventral sector of the optic cup is reduced (see Figure 2(a) and (b)). It has been demonstrated that premature (heterochronic) *fgf8* expression is involved in decreasing the ventral sector of the optic cup.⁶⁰ Accordingly, treatment of cavefish embryos with a fibroblast growth factor (FGF) inhibitor can restore the optic cup to normal size.⁶⁰ Second, when a surface fish lens is deleted during embryogenesis, there is no induction of retinal apoptosis, as would be expected if the lens were the sole protector of the retina.⁴⁸ This result suggests that a part of the eye besides the lens is capable of protecting the retina from apoptosis and this eye part is also defective in cave-fish. A good candidate for this second protector is the RPE, which is known to influence retinal growth and differentiation during vertebrate eye development. The protective factor(s) secreted by the lens and possibly the RPE remains to be identified.

Genetic Basis of Eye Regression—How many genes are involved in cavefish eye regression, what is their identity, and how do they function? Crosses between cavefish and surface fish and their F1 progeny have addressed the question of gene number. The eyes of the F1 progeny are fairly uniform in size, although smaller than normal, but the F2 generation shows a wide range of eye sizes, indicating that eye reduction is a multigenic trait.^{8,44,61} Genetic linkage mapping has identified 13 quantitative trait loci (QTL) responsible for eye formation: seven affecting eye size and six affecting the lens²⁸ (see Figure 4). In addition, some lens and eye size QTL overlap, suggesting the possibility of single genes with effects on both the lens and retina. Interestingly, some of the genes responsible for eye reduction are unique to different cavefish populations, as demonstrated by genetic complementation experiments:^{34,61–63} when cavefish from two different populations are interbred, eye size can be significantly larger in the F1 hybrids than their parents, and in some cases vision is restored.⁶³ The loss or alteration of three or four genes in each cavefish population is thought to be enough to abolish vision.⁶³ The genetic complementation results imply that eye regression can be accomplished differently, at least partially, in independently evolved cavefish lineages.

The identity of genes involved in eye regression has been explored by candidate gene analysis and QTL characterization. In some cases, these studies have also provided clues about gene function. Since many genes are known with roles in vertebrate eye development, candidate gene analysis focused on previously identified eye genes whose expression is modified in cavefish relative to surface fish. Most of these genes showed no changes in expression, but a few were substantially decreased or increased.⁵² For example, the expression of *α A-crystallin*, which encodes an anti-apoptotic factor, is greatly reduced in the cavefish lens.^{65,66} In contrast, the expression of *hsp90a*, which encodes a pro-apoptotic factor, is activated in the cavefish lens just prior to apoptosis.⁶⁷ Therefore, it is possible that

lens apoptosis is controlled by an antagonistic interaction between *αA -crystallin* and *hsp90a*.

Other changes in gene expression are subtler and observed by *in situ* hybridization as an expansion or contraction of expression domains. For example, the expression domains of *pax6* are diminished, whereas those of *pax2a* and *vax1* are expanded in the cave-fish optic vesicle and cup^{50,68} (see Figure 2(f) and (g)). During vertebrate eye development, the optic vesicle is subdivided into preoptic cup and preoptic stalk domains via antagonistic interactions between the Pax6, Pax2, and Vax1 transcription factors: Pax6 directs optic vesicle development and suppresses optic stalk development, whereas Pax2 and Vax1 promote optic stalk and suppress optic vesicle development.⁶⁹ Accordingly, expansion and contraction of the three transcription factor domains can account for a smaller optic cup in cavefish.

The anterior embryonic midline is also a location of expanded gene expression in cavefish.^{50,60,68,70} The midline expression domains of the two teleost *sonic hedgehog* (*shh*) genes, *shhA* (see Figure 3(h)–(k)) and *shhB* (once called *twiggy winkle hedgehog* or *twhh*) are expanded in cavefish relative to surface fish.⁵⁰ During vertebrate development, Sonic Hedgehog (Shh) signals emanating from this region inhibit *pax6* expression, producing an expression gap of the latter gene in the overlying neuroectoderm.^{71,72} As a result of Shh expansion, the gap in *pax6* expression is larger in cavefish relative to surface fish (see Figure 3(f) and (g)). The role of enhanced Shh signaling in cavefish eye development was further investigated by increasing *shh* expression in surface fish embryos.⁵⁰ Overexpression of *shhA* induced lens apoptosis and caused larvae to develop lacking external eyes (see Figure 3(l)). Thus, a blind cavefish phenocopy was produced in surface fish (see Figure 3(m)), demonstrating a key role for increased Shh signaling in eye degeneration.

No known eye QTL are located near the *pax6*, *shhA*, or *shhB* genes on the genetic map,²⁸ suggesting that they are not mutated and may be an indirect rather than a direct cause of eye degeneration. In contrast to candidate analysis, QTL characterization has the potential to identify those genes and their mutations that directly cause eye degeneration. In the current absence of a sequenced *A. mexicanus* genome (see Figure 1), however, this identification is extremely difficult. Nevertheless, progress has been made by anchoring the existing genetic map to syntenic regions in the sequenced zebrafish genome.³³ This approach revealed four promising genes located near eye QTL: *αA -crystallin* and *hsp90a*, which were also predicted to be involved in eye degeneration by candidate gene analysis,^{65–67} *shroom2*, which has a role in the synthesis and localization of melanin granules in the RPE,⁷³ and *rom1*, which encodes a structural protein required for differentiation of photoreceptor cells.⁷⁴ Further work will be necessary to investigate whether mutations in these genes actually cause eye degeneration. In addition, future candidate genes to be considered include genes that upregulate the Shh midline signaling system.

Developmental Basis of Pigmentation Regression—Pigmentation is one of the most commonly studied morphological traits in evo–devo studies because it is easily observed and much is known about the genetic pathways responsible for pigmentation in vertebrate and invertebrate model systems.^{75–77} Pigmentation in fish involves multiple types of pigment cells;⁷⁸ most of the focus on pigmentation in *A. mexicanus* involves melanophores, which are the pigment cells that produce black melanin.

The developmental basis of albinism was investigated in Pachón cavefish. To address whether melanophores were absent in albino animals or whether they were present but did not produce melanin, the experimenters added L-DOPA, a substrate of tyrosinase in the melanin pathway, which resulted in melanin production in melanophores⁷⁹ (see Figure 5(a)).

However, addition of a substrate that is active immediately upstream in the same biochemical pathway, L-tyrosine, did not result in melanin production. Therefore, the investigators identified the location of the defect in the melanin pathway that causes the albino phenotype in Pachón cavefish.

Genetic Basis of Pigment Regression—Early experiments documented two recessive monogenic traits, albinism and the ‘brown’ mutation, which generated brown instead of black pigment.^{81,82} A genetic approach was used to map the location of the monogenic trait responsible for albinism.³⁰ An albinism candidate gene, *ocular and cutaneous albinism 2* (*oca2*), was mapped to the albino locus in crosses from two different cavefish, Pachón and Molino. Two deletions were observed in the coding region of *oca2*, one in Molino cavefish and the other in Pachón cavefish (see Figure 5(f)). A mouse melanocyte cell line deficient in *oca2* was used to demonstrate that these deletions resulted in nonfunctional OCA2 proteins. A third albino cavefish, Japonés, which did not contain either deletion, did not complement when crossed to either Pachón or Molino cavefish. Therefore, albinism has evolved independently in three different cavefish through the same gene, *oca2*, via two different coding mutations and possibly a regulatory mutation.

The genetic basis of the ‘brown’ mutation⁸² was investigated using similar genetic methods.⁶⁴ The *melanocortin receptor 1* (*mc1r*) gene mapped to the brown locus. Two coding mutations were found.⁶⁴ One was a 2 base pair (bp) deletion resulting in a frameshift, found in Pachón cavefish and at a low frequency in the connected Japonés and Yerbaniz Caves. The other was a point mutation leading to an amino acid change, also found in humans with red hair and pale skin, present only in Japonés and Yerbaniz cavefish.⁶⁴ When an F2 cross of fish originating from Pachón cavefish was observed, those animals with two copies of the *mc1r* surface allele had larger and darker melanophores, those fish with one copy of the Pachón *mc1r* allele had fewer and lighter melanophores, and those fish with two copies of the Pachón *mc1r* allele had still fewer and lighter melanophores (see Figure 5(b)–(d)). Individuals who had two copies of the Pachón *oca2* allele were albino regardless of the genotype at *mc1r*⁶⁴ (see Figure 5(e)). Functional analysis in *Danio rerio* showed that both the amino acid change and the 2bp deletion altered the function of *mc1r*.⁶⁴ In addition, other cavefish expressed the ‘brown’ phenotype based on complementation tests,⁸⁰ but coding changes were not observed in the *mc1r* open reading frame in representatives from these caves. This may indicate that *cis*-regulatory changes in cavefish from these populations lead to reduced expression of the MC1R receptor (see Figure 5(f)).

Several cave populations only express the brown mutation, while Molino cavefish harbor albinism in the absence of brown (see Figure 5(f)). Interestingly, Pachón, Yerbaniz, and Japonés cavefish all express both brown and albinism. Furthermore, many other QTL were mapped for the trait of melanophore number²⁸ showing that multiple genes are responsible for pigmentation differences in cave versus surface *A. mexicanus*.

Other Regressive Traits—Other regressive traits in cavefish are listed in Table 1. Some of these may be a direct consequence of the absence of functional eyes and vision. For example, reduction of the optic tectum is probably due to decreased neuronal targeting from the retina.⁵³ The loss of aggressive and schooling behaviors has also been attributed to the absence of eyes,⁴⁴ although there is no compelling evidence that this is the case. In fact, Molino cavefish have lost eyesight but continue to show aggressive behavior.⁸³ Other behaviors that are modified or missing in cavefish are the dorsal light reaction,⁸⁴ which orients the dorsal side of a fish at a constant 90° angle to a light source, and the alarm or fear behavior,⁸⁵ which is mediated by a substance released from the skin in the presence of a predator or a sustained physical disturbance. The alarm substance triggers rapid swimming, taking cover, or a sudden freeze in motion, presumably to avoid or confuse predators.

Interestingly, despite their inability to respond to the alarm substance, cavefish appear to have conserved the ability to produce it.⁸⁵ Genetic analysis shows that a minimum of 3–6 genes are responsible for changing the dorsal light reaction,⁸⁴ and two dominant genes may cause loss of the alarm response.⁸⁵

Sleep is another behavioral trait that has regressed in cavefish.⁸⁶ Cavefish show dramatic reductions in the duration of sleep, as measured by daily activity patterns. Sleep regression has converged in three independently evolved cavefish populations and is controlled by a small number of genes with dominant effects.⁸⁶

Some cavefish populations show a reduction in anal fin rays,²⁹ smaller body scales,⁴⁴ and have one or two fewer rib-bearing thoracic vertebrae than surface fish.³⁸ The loss of rib-bearing vertebrae reduces body length in Pachón cavefish (see Figure 1(a)) and is a polygenic trait.²⁹ QTL analysis (see Figure 4) and anchoring to the zebrafish genome show that one of the rib QTL is tightly linked to the *hoxc8a* gene,³³ which patterns rib numbers in other vertebrates.⁸⁷

Constructive Traits

Most constructive traits fall into one of two categories: those associated with (1) food detection, feeding, or energy storage and (2) changes in brain organization (Table 1). The developmental and genetic basis of constructive changes is just beginning to be studied, and consequently much less is currently known about them compared to regressive traits. Constructive traits are extremely important, however, because in contrast to regressive traits they were probably directly involved in the adaptation of *A. mexicanus* to life in caves.

Food Detection, Feeding Activities, and Energy Storage—Compensation for the loss of visual feeding is a major challenge for survival in a dark cave. One of the ways in which ancestral surface fish may have adapted to caves is by the evolution of vibration attraction behavior (VAB), which is defined as the movement of cavefish toward oscillating objects in cave pools.⁸⁸ VAB is advantageous for detecting moving prey in darkness, heritable, and dependent on an enhanced neuromast system. *A. mexicanus* contains two types of lateral line sensory organs: canal neuromasts located in the lateral line proper, and superficial neuromasts (SN) distributed outside the lateral line. The size of both types of neuromasts is increased in cavefish, but SN specifically show a several fold increase in number compared to surface fish, which is particularly evident in the cranial region^{88,89} (see Figure 6(a) and (b)). When cranial or trunk SN are ablated, VAB also disappears indicating that SN are the VAB receptors.⁸⁸ While most surface fish lack VAB, a small proportion of laboratory raised animals exhibit low levels of VAB,⁸⁸ suggesting that there may be standing genetic variation for this trait in surface fish populations. VAB is not expected to be adaptive in lighted environments because of the risk of predation. In caves, however, where there is a much lower risk of predation, the benefits of selection for increased food detection via VAB may be quite strong. Therefore, continuous selection for SN-mediated VAB enhancement in cavefish may be a major adaptation to life in darkness.

A change in feeding posture has evolved in cavefish to facilitate foraging on the substratum of cave pools⁹² (see Figure 6(c)). Surface fish usually feed in the water column under lighted conditions. When forced to feed from the substratum in darkness, however, they position themselves at a 90° angle with respect to the bottom. Then, by swiveling their body, they pick up pieces of food by a rather awkward and inefficient process. In contrast, under the same conditions, cavefish position their bodies at a 45° angle with respect to the substratum and move steadily forward to more efficiently collect food particles. The feeding posture of cavefish is likely to be adaptive for feeding in the cave environment. When surface fish and cavefish are placed in the same lighted tank and required to compete for

limited food, surface fish obtain most of it and thrive, but in darkness cavefish have better food finding ability and flourish.⁹³ Genetic analysis suggests that the regulation of feeding posture behavior is controlled by only a few genes.⁹⁵

Possibly to accompany their novel feeding posture, cavefish have evolved wide shovel-like jaws with several additional maxillary teeth, more taste buds, and higher sensitivity to dissolved amino acids compared to their surface fish counterparts^{29,56,91} (see Figure 6(h), (i), (j) and (k)). In surface fish, taste buds are located along the lips and in the pharynx, whereas in cavefish they are increased in these locations but also found in large numbers in the ventral skin of the head.^{91,94,95} Taste bud formation begins relatively early in *A. mexicanus* embryos, just after the opening of the larval mouth, and their number increases continuously during subsequent development.^{91,94} In addition to its role in eye degeneration, the Shh signaling system mediates enhancement of mouth/jaw size and taste bud number.⁹¹ The midline expansion of *shhA* expression continues during later development, encompassing the developing mouth and pharynx (see Figure 6(d) and (e)), and is eventually attenuated to the taste buds (see Figure 6(f) and (g)). The expression of *shhA* has been shown to be necessary and sufficient (see Figure 6(l) and (m)) for increasing mouth/jaw size and taste bud number.⁹¹ Although 11–12 genes were originally predicted to be involved in determining taste bud numbers,⁴⁴ this has been a difficult trait to quantify and consequently only three taste bud QTL have been detected so far²⁸ (see Figure 4). Similar to eye QTL, however, none of the taste bud QTL are closely linked to the *shh* genes.²⁸ Perhaps, upstream regulation of Shh signaling by modulating genes could also be important in oral and taste bud enhancement.

In addition to changes in the abundance and types of food, the availability of food also fluctuates in caves with major inputs probably corresponding to sporadic flooding. Thus, cavefish are faced with the challenge of survival during periods of low food input. To accomplish this feat, they have evolved capacities for increased lipid metabolism and fat deposition.⁹⁶ The developmental and genetic basis of increased fat formation is unknown.

Brain Organization—The cavefish brain is organized much differently from that of surface fish.⁹⁷ In addition to a reduced optic tectum,⁵³ it has larger olfactory lobes, telencephalon, and hypothalamus than the surface fish brain.^{70,97,98} These constructive changes may be in part caused by amplifications in the senses of taste (gustation) and olfaction. The gustatory relay centers, which are located in the three enlarged areas of the cavefish brain, may have increased to accommodate excess inputs from more taste buds. Although the possibility of changes in the olfactory epithelium has not been investigated in cavefish, their nasal volume (nares) is much larger than surface fish,⁵⁶ suggesting more internal space to accommodate receptors. Moreover, a specific population of GABAergic interneurons that are part of the olfactory bulb neural circuitry is enhanced in cavefish.⁷⁰ Thus, although further investigation is necessary, it seems likely that the olfactory epithelium has been enhanced in cavefish.

Differences in cavefish brain organization first appear during early development and are controlled by the Shh and FGF pathways.^{60,70,98} In addition to its expansion along the anterior embryonic mid-line, Shh is also increased in the floor plate of the developing anterior neural tube.⁷⁰ There are two lines of evidence suggesting that Shh signals have important consequences on cavefish brain organization.⁷⁰ First, the large hypothalamus is probably due to Shh-induced increase in cell proliferation. Second, Shh is responsible for an increase in the development of GABAergic olfactory interneurons via the downstream activation of NKX and LHX transcription factors. These GABAergic interneurons are first produced in the ventral telencephalon and later migrate into the olfactory bulbs

Pleiotropic Tradeoffs between Constructive and Regressive Traits

On the basis of developmental studies, it was predicted that tradeoffs based on pleiotropy could occur between constructive and regressive changes in cavefish.⁹⁹ Consistent with this idea, genetic linkage analysis has revealed numerous overlaps both among different regressive trait QTL and between constructive and regressive trait QTL that are hard to reconcile simply by chance.²⁹ For example, some eye QTL are linked to taste bud QTL (see Figure 4). The possibility of an eye/oral/taste bud antagonism was substantiated by developmental methods, and once again expansion of the Shh signaling system was found to be a key factor. Evidence for coupling between oral size/taste bud enhancement and eye degeneration via Shh signaling was obtained by experiments in which *shhA* was conditionally expressed at specific times during surface fish development.⁹¹ Overexpression at early developmental stages resulted in subsequent eye degeneration and mouth and taste bud enhancement in the same surface fish larvae, but had no effects when carried out later in development (see Figure 6(n) and (o)). It was concluded that the critical developmental period for eye degeneration and oral enlargement/taste bud expansion is the same and likely to be controlled by an antagonistic tradeoff based on pleiotropic Shh signaling.

Convergent/Parallel Evolution and Evolutionary Forces

Depending on the identity of the founder surface fish, troglomorphic traits could have evolved by convergent or parallel evolution. Whichever the case, an understanding of the evolutionary forces responsible for troglomorphy is a major goal in the *A. mexicanus* model system.

The forces responsible for the evolution of constructive traits seem obvious: natural selection is acting on standing genetic variation to produce adaptive phenotypes with the most fitness for cave life. A good example is the evolution of VAB.⁸⁸ The evolutionary forces responsible for regressive traits are less clear and have been the subject of considerable debate.^{1,100} Two major hypotheses have been proposed: (1) the accumulation of neutral (loss of function) mutations and their fixation via genetic drift and (2) natural selection acting either directly based on the benefits of energy conservation or indirectly via antagonistic pleiotropy. In the case of eye regression, some of the available evidence supports the first hypothesis, whereas other evidence seems to favor the second hypothesis. Thus, it has been proposed that both neutral mutation and indirect selection may be acting in concert.⁹⁰ Evidence for the neutral hypothesis is primarily based on the observation that vestigial eye sizes vary within cavefish populations, which seems hard to account for by selection.⁶² Evidence for selection comes from QTL studies in which the direction of eye size changes is always negative,²⁸ whereas random genetic drift would predict both positive and negative QTL. Other information also supports selection over neutral mutation.¹⁰¹ First, most of the genes involved in eye development do not appear to have mutated to a degree in which they have lost function. Second, the restoration of eyes by lens transplantation suggests that many of the genes acting downstream of lens function are present and potentially active in cavefish. Third, most genes with modified expression patterns, such as those in the Shh signaling pathway and *hsp90a*, increase rather than decrease activity in cavefish. If natural selection is actually involved, what are the benefits of losing eyes? Direct evidence for a role of energy conservation is lacking, and perhaps this explanation is questionable considering the costly cycles of cell division and death that occur in the cavefish retina.⁴⁸ Another explanation may be pleiotropy involving the expanded Shh midline signaling system.^{90,91}

In contrast to eye regression, individual QTL governing the number of pigment cells can either increase or decrease,²⁸ supporting the role of neutral mutation and genetic drift in this regressive trait. In contrast, convergent or parallel evolutionary changes in the *oca2* gene

suggest the possibility that the block in melanin synthesis (albinism) could be adaptive, although its beneficial aspects remain to be discovered. Further progress in understanding the evolutionary forces involved in regressive evolution awaits the identification of the specific genes and mutations involved in this process.

ASELLUS AQUATICUS

A. mexicanus is an excellent species for the study of evolutionary and developmental biology of troglomorphic evolution. However, to understand if there are common themes in the evolution of cave related characteristics, it is critical that multiple species be explored. Ideally one would investigate several cave species, both similar (perhaps other fish) and dissimilar (invertebrates). Because of the significant time and resources needed for genetic and developmental studies, one invertebrate system was first chosen.

Arthropods are very common cave dwellers (see Figure 7) and, in particular, crustaceans have been successful in adapting to the cave environment with greater than 2900 known obligate species.¹⁰² The isopod crustacean, *A. aquaticus*, has recently been developed as an emerging invertebrate cave model.

A. aquaticus is commonly found throughout much of Europe, in freshwater habitats including ponds, rivers, lakes, caves, and wells. This species is a good model for studying evolutionary and developmental biology because cave and surface populations are inter-fertile,^{4,5} the surface form is easy to breed in the laboratory and is widely used for ecotoxicological studies, the life cycle is relatively short, and there are many morphological differences between cave and surface populations.^{103,104} Furthermore, the phylogenetic history of *A. aquaticus* has been well studied, despite its remarkable complexity, describing multiple different invasions of the cave environment both within a relatively small geographic location in Slovenia as well as in Italy and Romania^{17,24–26} (Figure 8(a)).

Regressive Traits

Eye Regression—The surface form of *A. aquaticus* has four ommatidia, each having between 1 and 4 crystalline cone cells.¹⁰⁶ Different cave populations of *A. aquaticus* indicate a high diversity of phenotypes within caves, between caves, and even within individuals^{6,107} (see Figure 8(b)). Some animals have fragmented ommatidia with disassociated crystalline cone cells of different sizes, shapes, and different numbers of fragments. Other individuals have no visible ommatidial remnants.

Genetic Basis of Eye Regression—Genetic mapping analyses were performed in this species to identify regions of the genome responsible for the morphological differences. Genetic markers were isolated, including anonymous genomic markers and those associated with candidate genes in eye, pigment, and appendage development.⁴ Backcrosses were generated using individuals from the Planina cave and individuals from the surrounding surface waters. Based on a molecular clock approach, these populations are thought to have been separated for less than 100,000 years.¹⁷ A linkage map was generated with eight linkage groups, which is the karyotypic number in *A. aquaticus*.¹⁰⁸

The Planina cave population has great variability in the eye phenotype (see Figure 8(b)). Eye size was measured as a quantitative trait and a QTL was observed accounting for 30% of the variance (see Figure 9(k)). As the analysis was fairly small, it is expected that with a larger cross size, more regions of the genome would be discovered accounting for the rest of the variation. A qualitative trait was also measured, presence of ommatidia or ommatidial remnants versus absence of ommatidial remnants. A single region of the genome, potentially even a single gene, is responsible for the observed variation (see Figure 9(k)). Therefore, as

was predicted from earlier genetic crosses,⁶ multiple genes are responsible for the variation in eye phenotypes in the Planina cave population.

Pigment Regression—Researchers first described the body pigment in *A. aquaticus* as a melanin⁵ but more recent evidence indicates that the pigments could be ommochromes (one of the pigments present in *Drosophila melanogaster* eyes).¹⁰⁹

Early work on pigmentation variation in surface and cave populations of *A. aquaticus* described six levels of body pigmentation where the faintest levels were only present in cave populations.⁶ Furthermore, in certain cave populations, different colors of eyes were observed including unpigmented, red, light purple, purple, and black. Genetic crosses were performed with individuals from caves that had great variation in pigmentation phenotypes and the experimenters concluded that multiple genes were responsible for the variation. However, not all cave populations demonstrated variability in pigmentation; the Planina cave was documented to contain only unpigmented individuals.¹⁰⁵

Genetic Basis of Pigment Regression—The same methods and crosses used to analyze the eye phenotype were also utilized for pigmentation.⁴ Backcross offspring fell into five different color categories: no pigment, light red eye pigment and no head pigment, red eye and head pigment, orange eye and head pigment, and brown eye, head and body pigment (see Figure 9(c)–(f)). The genetic bases of several qualitative traits were examined: presence versus absence of pigment, light red and red versus orange and brown pigment, light red and orange versus red and brown pigment, and stellate versus diffuse pigment. For each of these qualitative traits, a different single region of the genome was discovered to be responsible for the majority of the variation (see Figure 9(k)). Further work investigating genotypes of these different regions of the genome has generated a model where it appears that there are two ways to generate the unpigmented phenotype—either by a single region of the genome or the combined effect of two other regions of the genome. Although, the Planina population is described as having a uniform loss of pigment, it is likely that multiple genes are involved in pigmentation. The fact that there are multiple ways to achieve albinism in the same population might support the neutral mutation theory. However, it is also possible that pleiotropy is responsible for some or all of the pigmentation mutations. Further experiments will examine additional constructive traits and whether the genetic basis of these traits is linked to the pigmentation loci.

Constructive Traits: Appendage and Sensory Phenotypes

Other traits compared between surface and cave individuals of different populations include the common constructive traits of appendage length and enhancement of sensory systems.^{24,103,104} In these studies, multiple cave populations from Slovenia were examined in addition to the Romanian cave population (see Figure 8(a)). Comparisons between the Planina cave population and the neighboring surface population showed that the relative length of antennae II, the relative length of the seventh thoracic leg, and the number of aesthetascs (thought to be chemosensory cells) were increased in the cave population¹⁰⁴ (see Figure 8(c)–(f)).

Future Directions

Genetic Analyses—It will be very interesting to investigate if QTL for the constructive traits are located near QTL for the eye and pigmentation traits. By investigating these additional traits it will be possible to address the question of pleiotropy in the evolution of cave characteristics in *A. aquaticus*.

Furthermore, many behavioral traits have been pursued in *A. mexicanus* and likewise it would be fascinating to expand the analysis to the genetic basis of behavioral evolution in *A. aquaticus*. For example, photobic/photophilic behavior has already been examined in differentially pigmented individuals of *A. aquaticus*.¹¹⁰

Developmental Analyses—The embryonic development of *A. aquaticus* has also been investigated¹¹¹ (see Figure 10). The method of *in situ* hybridization has been performed in this species^{111,112} and, therefore, it should be possible to compare expression patterns of promising candidate genes between cave and surface embryos.

A. aquaticus demonstrates many of the same advantages present in *A. mexicanus*. Through continued development of *A. aquaticus* as a system for evolutionary and developmental biology research, more discoveries will be enabled, allowing a much better understanding of the evolution of cave characteristics in these isopods. Furthermore, comparing *A. mexicanus* and *A. aquaticus* will allow for a broader view of how and why cave-associated traits evolve.

Similarities and Differences between *Astyanax* and *Asellus*

Cave animals represent a unique system in which it is possible to investigate the evolution of the same characteristics in very different species from vertebrates to invertebrates. We will compare eye and pigmentation in *A. mexicanus* and *A. aquaticus*.

Eye and Pigment Reduction—Monogenic or Polygenic?—Eye reduction appears to be a polygenic trait in both *A. aquaticus* and *A. mexicanus*.^{4,27,44} On the other hand, the phenotype of ommatidial absence in *A. aquaticus* appears to be encoded by a single locus of large effect.

Pigmentation seems to involve multiple monogenic traits in both *A. mexicanus* and *A. aquaticus*. In *A. mexicanus*, there appears to be three monogenic recessive traits involved in pigmentation phenotypes: the yellow, albino, and brown phenotypes.^{81,82,100} Similarly, in *A. aquaticus*, four qualitative pigmentation traits are each determined primarily by a single region of the genome. However, melanophore number is a polygenic trait in *A. mexicanus* and has not yet been described for *A. aquaticus*. Therefore, it seems that both pigmentation and eye reduction can be either monogenic or polygenic traits.

Eye Reduction and Pigment Reduction—Multiple Mechanisms or One Favored Method?—Within the single *A. aquaticus* population examined, there appears to be multiple genetic modes of eye reduction—a potentially monogenic mode of ommatidial absence and a polygenic mode of fragmented ommatidia.⁴ Multiple methods also are apparent in *A. mexicanus* where complementation studies between different populations result in individuals with eyes more developed than either parent.^{34,62,63,80} This might indicate that there are multiple possible ways to cause eye reduction and not a single favored genetic path in both species.

If one looks at pigmentation as a whole, it seems as though there are multiple methods in both systems to degenerate the amount and types of pigments. For example, in *A. mexicanus*, there are the brown, albino, yellow, and iridophore mutations^{81,82,100} and in *A. aquaticus* there are mutations that cause albino, orange, red, or stellate chromatophores.^{4,6}

However, in *A. mexicanus*, the most extreme phenotype in both species, albinism, the same gene, *oca2*, appears to be responsible in three different cave populations with two discovered coding mutations and potentially an unknown regulatory mutation³⁰ (see Figure 11(a)). Contrastingly, in *A. aquaticus*, within a single cave population there appears to be two

different ways to achieve albinism⁴ (see Figure 11(b)). Within the *A. mexicanus* populations examined, there seems to be a bias in the method of evolving albinism but it does not appear that there is such a bias in *A. aquaticus*. Perhaps, loss of *oca2* could confer some kind of advantageous pleiotropic effect in the fish populations, or other genes in the pigmentation pathway when mutated have adverse effects. Investigation of additional populations and species will be needed to further conclude whether there are more constraints in complete pigment loss in vertebrates versus invertebrates.

Similar Degree of Reduction?—The degree of reduction in pigment seems similar in *A. mexicanus* and *A. aquaticus*; in both there are either populations or individuals that exhibit complete loss of melanin (*A. mexicanus*) or melanin/ommochrome (*A. aquaticus*).^{6,81} However, the degree of eye reduction can be different in *A. aquaticus* and *A. mexicanus*. In all of the populations of *A. mexicanus* investigated, a rudimentary eye is present. Although some vertebrate cave animals have no exterior evidence of an eye, when the face is dissected or sectioned, there is evidence of a rudimentary eye. It has been suggested that complete eye loss in vertebrates is not possible due to developmental constraints.⁸⁰ In *A. aquaticus* some individuals are missing all ommatidia. These animals were described as blind with undifferentiated photoreceptors and an optic nerve.⁶ However, other cave arthropod species have been described as eyeless (reviewed in¹¹³). Therefore, the same developmental constraints present in vertebrates may not be present in arthropods. Another method of addressing the degree of reduction in cave animals is to sequence the transcriptome of putatively eyeless animals and ask whether any transcripts involved in phototransduction are present. Recently such an analysis found many transcripts involved in phototransduction in the cave beetle *Ptomaphagus hirtus*, which had been previously described as blind.¹¹⁴ In terms of structures, it seems that invertebrates can have a greater degree of eye reduction than vertebrates. However, whether this is a functional reduction (as evidenced by transcriptome sequencing), as well as a structural reduction, remains to be seen.

Unresolved Questions and Future Comparisons—Many questions and comparisons remain to be addressed either because of the complexity of the question or the recent development of *A. aquaticus* as a model for evolutionary and developmental biology.

First, are the same genes affected in both *A. mexicanus* and *A. aquaticus*? For pigmentation it is unlikely because many genes in the melanin pathway are not conserved between vertebrates and arthropods and the type of pigment present in the isopods could be ommochromes instead of melanin.¹⁰⁵ However, there are many conserved genes in eye development in both vertebrates and invertebrates. Therefore, it could be possible to see the same genes affected in eye reduction in *A. mexicanus* and *A. aquaticus*.

Second, what are the evolutionary mechanisms leading to eye and pigment loss in both systems? In *A. aquaticus*, additional phenotypic traits must be investigated to determine whether phenotypes such as antennal length and sensory cell elongation are genetically or developmentally linked to the eye and pigment loss characteristics. Furthermore, once the actual mutations responsible for eye and pigment loss are found in both systems, researchers can investigate whether or not there are signatures of selection around causal mutations. With this information, it should be possible to understand if there is a ‘universal answer’ in cave evolution of pigment and eye loss, whether a single loss phenotype within a population can be governed by multiple varied mechanisms, or whether the answer lies between these two extremes.

Overall Comparisons between *A. mexicanus* and *A. aquaticus*—There are many similarities and differences in the evolution of cave-associated characteristics of *A. mexicanus* and *A. aquaticus*. To truly understand the evolution of cave-related

characteristics in cave animals it will be necessary to examine many other species. Only through examination of additional systems will it be possible to identify trends unique to certain types of cave animals or to a particular trait.

UP AND COMING SYSTEMS: OTHER CAVE ANIMALS AS EVO-DEVO MODELS

The following are just a few examples of cave animals that might be appropriate as additional model systems. Different cave adapted species have different advantages. For example, the cave beetle *P. hirtus* profits by its phylogenetic relationship to the genetic model organism *Tribolium castaneum*.¹¹⁴ The primary consideration in selecting the animals described below, however, is the existence of closely related surface and cave-dwelling forms.

Planarians have surface dwelling and cave-adapted representatives, the latter lacking eyes and pigmentation.¹¹⁵ Although constructive traits are not obvious, cave-adapted planaria are an attractive system because of their basal position in metazoan phylogeny and their intense current use as a developmental model. The Hawaiian planthopper genus *Oliarus* has closely related surface and numerous cave dwelling forms, and the latter have lost eyes and pigmentation, reduced their wings, and increased the size of their antennae.¹² Albino cave planthoppers, like *Astyanax* cavefish, have interrupted melanin synthesis at its first step (the conversion of L-tyrosine to L-DOPA), suggesting convergence in the evolution of albinism between a cave adapted insect and a vertebrate.¹¹⁶ The planthopper system is also attractive because of its ecology. Hawaiian cave-adapted planthoppers inhabit lava caves, a different type of habitat than the limestone caves typical of most other troglomorphic cave animals. In addition, they feed on the roots of surface foliage that extend into the relatively shallow lava caves and thus may not be food limited, making it possible to address the role of energy conservation in troglomorphic evolution.

Two other systems would be attractive for further development because of their close phylogenetic affinities to *A. aquaticus* and *A. mexicanus*. The amphipod *Gammarus minus* has already provided insights as a model system in understanding cave animal evolution.^{3,7} Side by side comparisons of *A. aquaticus* and *G. minus* would give useful insights into the convergent evolution of troglomorphic features in cave crustaceans. Likewise, the teleost *Poecilia mexicanus* is being developed to examine the evolution of cave adapted traits, particularly in the extreme environment of a sulfurous cave system.¹¹⁷ *P. mexicanus* has small eyes and reduced pigmentation and is interesting to compare with *A. mexicanus* due to its less pronounced troglomorphic characters. Further progress in developing these and other systems could significantly increase our overall understanding of the comparative evolutionary biology of cave animals and take maximum advantage of the cave environment as a true evolutionary laboratory.

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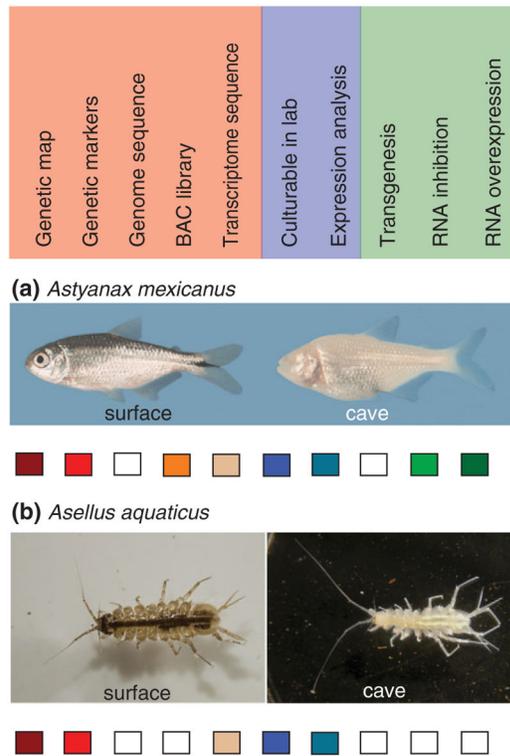


FIGURE 1. Requirements of an evo–devo cave system. In red are genetic requirements, in blue developmental requirements, and in green requirements for functional analysis. (a) The cavefish *Astyanax mexicanus*. (b) The isopod crustacean *Asellus aquaticus*. Boxes filled with the appropriate color indicate that the above tool is present for the particular species. An empty box indicates that the tool has not yet been generated for the species. (Reprinted with permission from Ref 4. Copyright 2011 National Academy of Sciences)

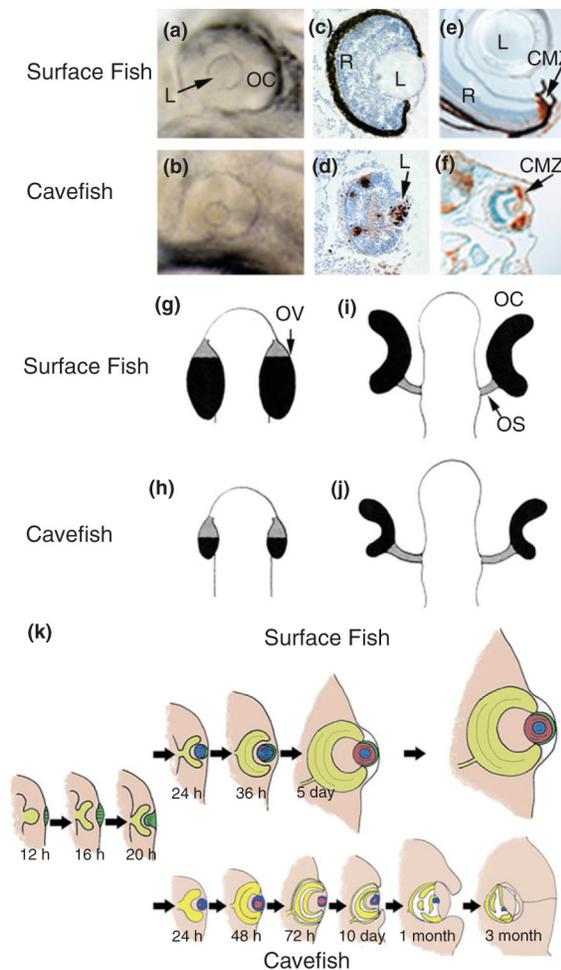


FIGURE 2.

Eye development in *Astyanax mexicanus* surface fish and cavefish. (a and b) Surface fish (a) and cavefish (b) eye primordia viewed laterally at 1 day post-fertilization (dpf) showing the small lens (L) and reduced ventral optic cup (OC) in the latter. (Reprinted with permission from Ref 50. Copyright 2004 Nature Publishing Group). (c and d) Section through surface fish (c) and cavefish (d) eye primordia at 1.5 dpf showing apoptotic cells (brown color) detected by the terminal deoxynucleotidyl transferase (TUNEL) assay in the lens and retina in cavefish but not surface fish. (Reprinted with permission from Ref 48. Copyright 2007 Elsevier Limited). (e and f) Sections through surface fish (e) and cavefish (f) eyes at 10 dpf showing dividing cells in the ciliary marginal zone (CMZ) stained with anti-proliferating cell nuclear antigen (PCNA) (brown color). (Reprinted with permission from Ref 51. Copyright 2002 University of the Basque Country Press). (g–j) Diagram showing OC morphogenesis in surface fish (g and i) and cavefish (h and j). OV, optic vesicle; OS, optic stalk. Black and gray areas represent OC and OS domains, respectively. (Reprinted with permission from Ref 52. Copyright 2003 Oxford University Press). (k) Diagram comparing eye development and growth in surface fish (top) with eye degeneration in cavefish (bottom). (Reprinted with permission from Ref 34. Copyright 2009 Annual Reviews)

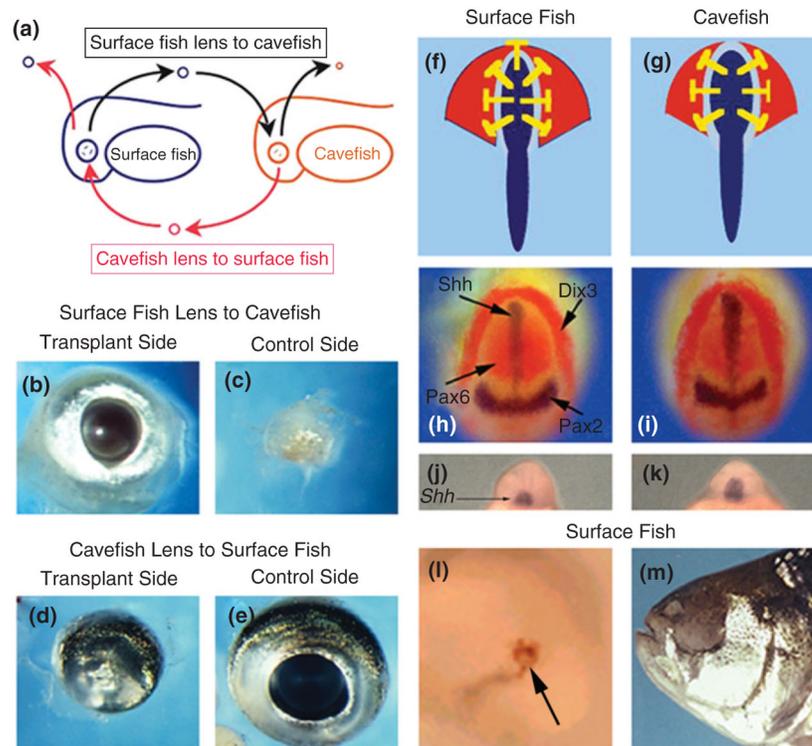


FIGURE 3. Role of the lens and *shh* expression in *Astyanax mexicanus* eye degeneration. Left columns. Rescue of the cavefish eye by lens transplantation. (a) Diagram showing transplantation of a surface fish embryonic lens to a cavefish embryo (top) and a cavefish embryonic lens to a surface fish embryo (bottom). The host lenses were removed prior to transplantation. (b and c) Dissected eyes from an adult cavefish that received a surface fish lens transplant on one side as an embryo. (b) Transplant side. (c) Control side. (d and e) Dissected eyes from an adult surface fish that received a cavefish lens on one side as an embryo. (d) Transplant side. (e) Control side. Photos (b)–(e) courtesy of Yoshiyuki Yamamoto. Right columns. Role of *Shh* in cavefish eye degeneration. (f and g). Diagram showing expanded *shhA* gene expression along the embryonic midline (blue) and inhibitory effects (yellow blocked lines) on *pax6* expression (red) in the optic domain of the neural plate in surface fish. (h and i) Four-gene *in situ* hybridization showing expanded *shhA* expression at the cavefish (h) midline (blue) and corresponding reduction of *pax6* expression relative to surface fish (i) at the neural plate stage. No changes were seen in *pax2a* (blue) or *dlx3* (red) gene expression. (j and k) *In situ* hybridization showing expanded *shhA* expression in the cavefish (j) compared to surface fish (k) rostrum. (l and m) Overexpression of *shhA* causes lens apoptosis (l, arrow) and eye degeneration (m) in surface fish. (Reprinted with permission from Ref 50. Copyright 2004 Nature Publishing Group)

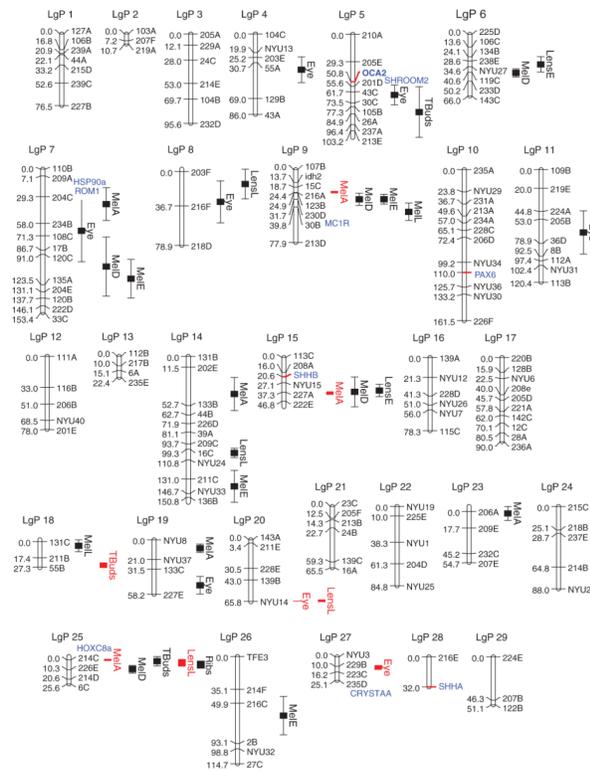
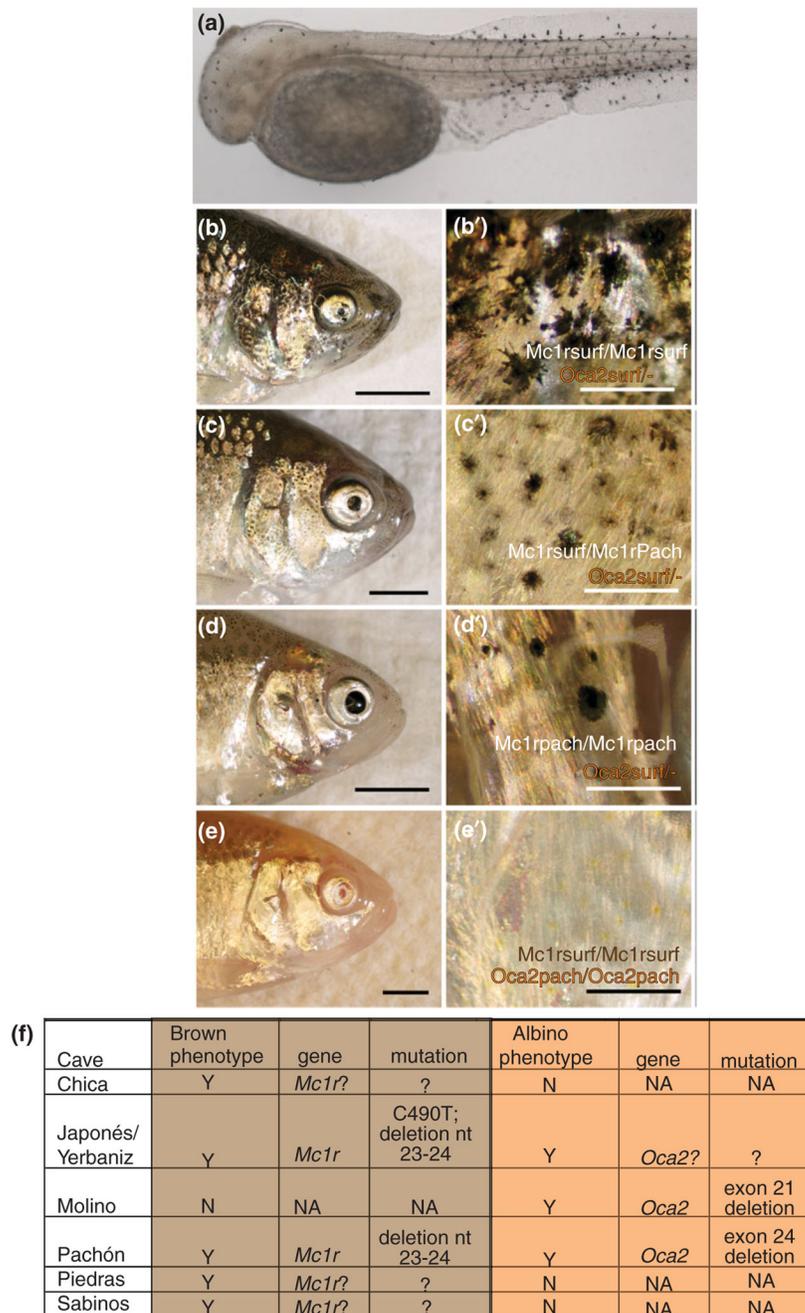


FIGURE 4. *Astyanax mexicanus* genetic map showing locations of quantitative trait loci (QTL) and candidate genes. Shown is a linkage map generated from a Pachón F2 cross.²⁸ QTL are shown for eye size, lens size (Lens E, L), melanophore number (Mel A, D, E, L) in four different places on the body, and number of tastebuds (Tbuds). QTL with more precise locations are shown in red. The location of a rib QTL from Ref 28 is also shown. Candidate genes are shown in blue. Candidate genes with red tick marks were mapped in Ref 28. Candidate genes without red tick marks were mapped in a different analysis⁶⁴ and are shown next to the most closely linked marker in common between the two analyses. (Reprinted with permission from Ref 28. Copyright 2007 Elsevier Limited)

**FIGURE 5.**

Pigmentation in *Astyanax mexicanus*. (a) Tyrosinase positive cells in a Pachón cavefish embryo at 72 hpf. (b–e′) F2 animals from a cross between a surface and a Pachón cave individual. (b–e) Side views of each fish head. (b′–e′) Higher magnification of (b–e). Genotypes of each individual are written in (b′–e′) for both *mc1r* and *oca2*. (f) Table describing certain cave populations of *A. mexicanus*: whether the albino and/or brown phenotypes are present, what genes are responsible, and what mutations are responsible. nt, nucleotide. ‘*Mc1r?*’ or ‘*Oca2?*’ shows that complementation tests indicate that the particular gene is involved but no coding mutation has been observed. (b–e′) from Gross et al.⁶⁴ (f) Summarizing results in Gross et al.⁶⁴ Protas et al.³⁰ and Wilkens and Strecker.⁸⁰

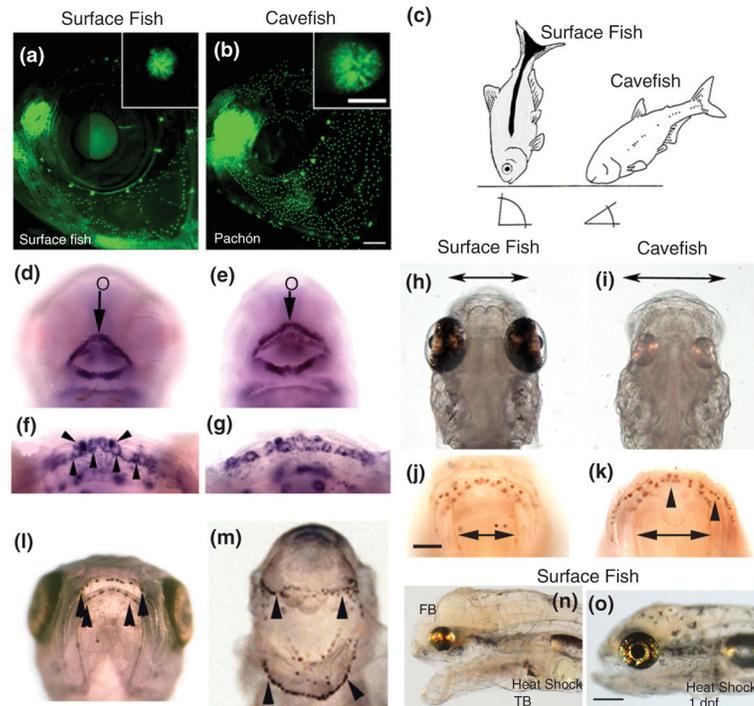


FIGURE 6.

Constructive traits in *Astyanax mexicanus* cavefish. (a and b) Superficial neuromast number and diameter (insets) is increased in cavefish relative to surface fish (a). (Reprinted with permission from Ref 88. Copyright 2010 Elsevier Limited). (c) Differences in feeding posture behavior in surface fish and cavefish. (Reprinted with permission from Ref 90. Copyright 2005 Oxford University Press). (d–g) Cavefish embryos (e) have a larger mouth (o) than surface fish (d). The mouth is encircled by *shhA* expression (d and e). Later in development (f and g), *shhA* expression is attenuated to tooth germs (downward arrowheads) and developing taste buds (upward arrowheads). (h–k) Cavefish larvae (i) show increased jaw span (double headed arrows) and anti-calretinin-stained taste buds (arrowheads) on their lips (k) relative to surface fish (h and j). (l and m) Overexpression of *shhA* by mRNA injection into cavefish embryos causes the formation of a large gaping mouth with excessive taste bud development. (l) Surface fish control and (m) *shhA*-injected cavefish. (n and o) Conditional heat shock *shhA* overexpression at the tailbud stage (n) but not at 1 dpf (o) induces eye degeneration, forebrain enlargement, and a larger mouth with more taste buds (not shown) in surface fish larvae. (Reprinted with permission from Ref 91. Copyright 2009 Elsevier Limited)

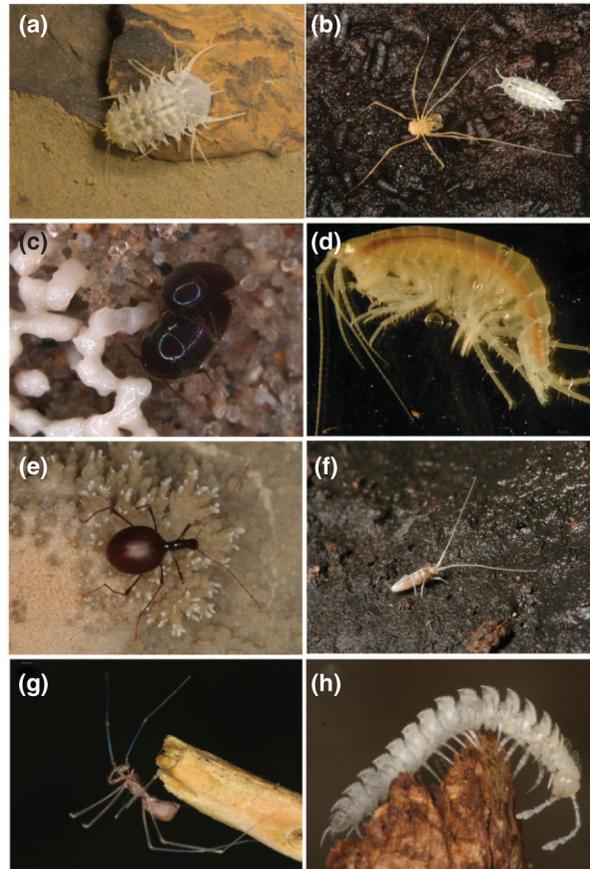


FIGURE 7.

Cave arthropods. (a) The crustacean, *Monolitra monstruosa*, photo courtesy of Helena Bilandžija. (b) The spider, *Travunia jandai* and the isopod crustacean, *Aegonethes cervinus*, photo courtesy of Helena Bilandžija. (c) The beetle, *Ptomaphagus hirtus*, photo courtesy of Markus Friedrich. (d) The crustacean, *Gammarus minus*, photo courtesy of Dan Fong. (e) The beetle, *Leptodirus hohenwartii*, photo courtesy of Helena Bilandžija. (f) The collembolan, *Verhoeffiella longicornis*, photo courtesy of Marko Luki. (g) The spider, *Sulcia nocturna*, photo courtesy of Martina Pavlek. (h) The millipede, *Brachidesmus sp.*, photo courtesy of Helena Bilandžija.

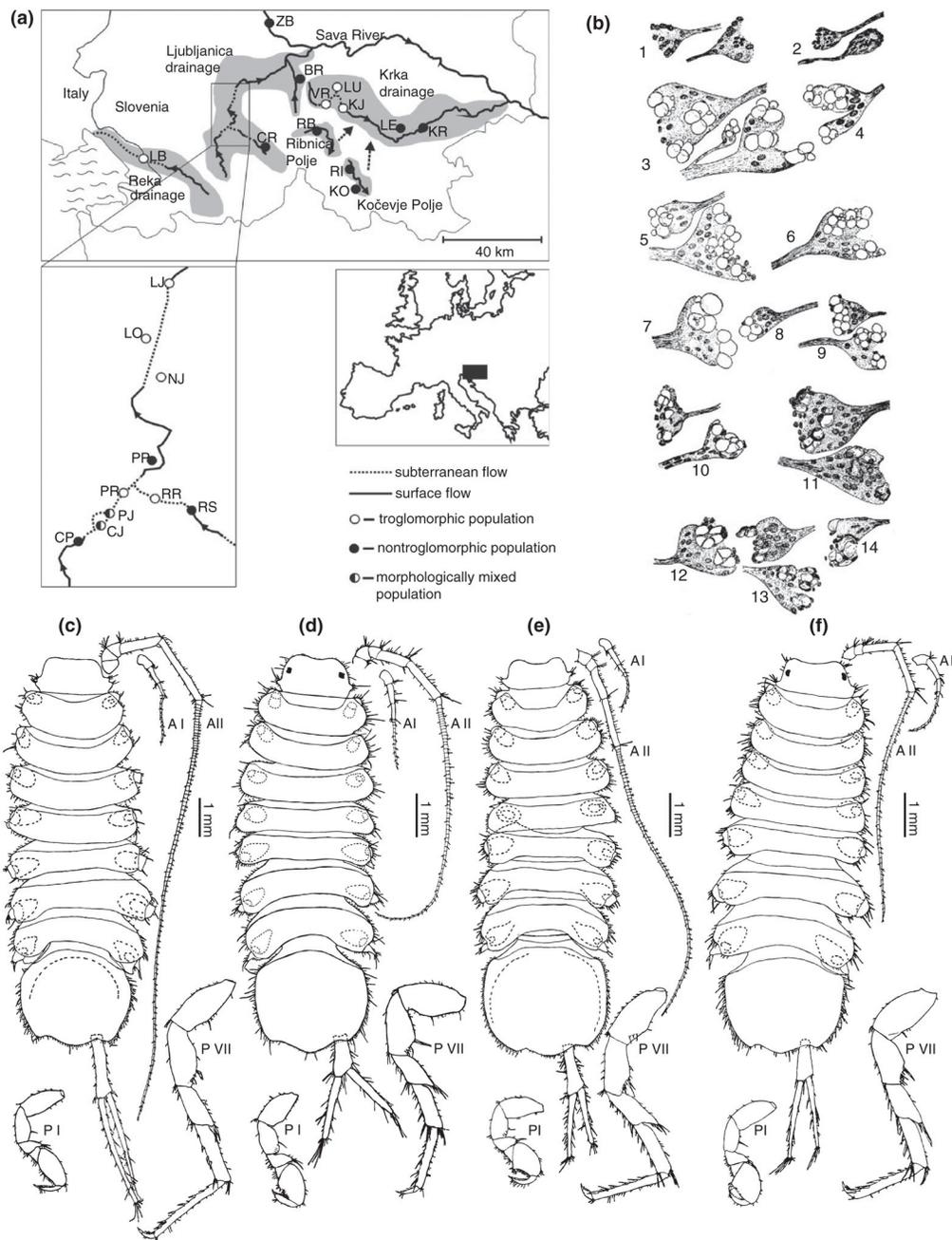


FIGURE 8.

Different cave and surface populations of *Asellus aquaticus*. (a) Map of cave and surface populations of *A. aquaticus*. (Reprinted with permission from Ref 17. Copyright 2010 John Wiley and Sons). (b) Drawings of eye rudiments from individuals from the Planina cave population. Pairs of eye rudiments depict right and left sides from the same individual. (Reprinted with permission from Ref 105. Copyright 1965 Guy Demortier). (c–f) Drawings of individuals from (c) Planina cave in Slovenia, (d) Planina polje (surface water) in Slovenia, (e) Mangalia well in Romania, and (f) Bucharest, Romania surface waters. Note differences in length of appendages and numbers of setae. P, pereopod (thoracic leg) and A, antennae. (Reprinted with permission from Ref 104. Copyright 1996 Springer)

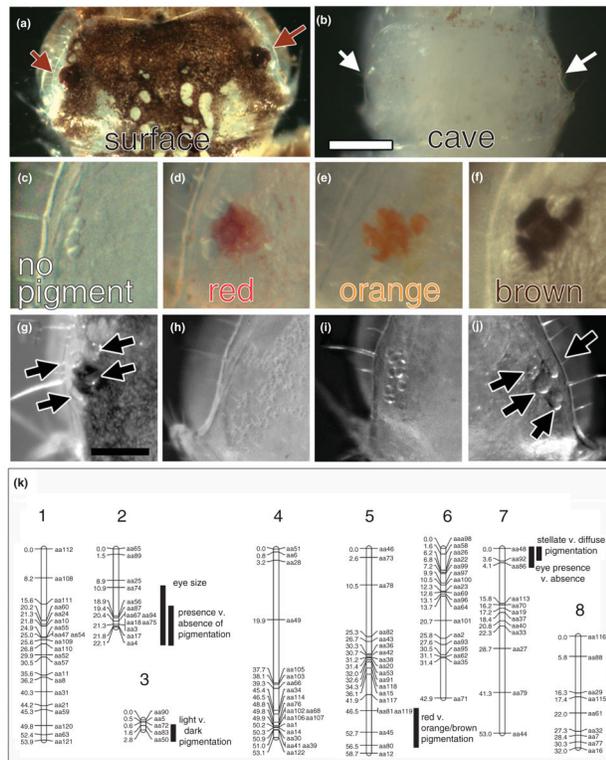
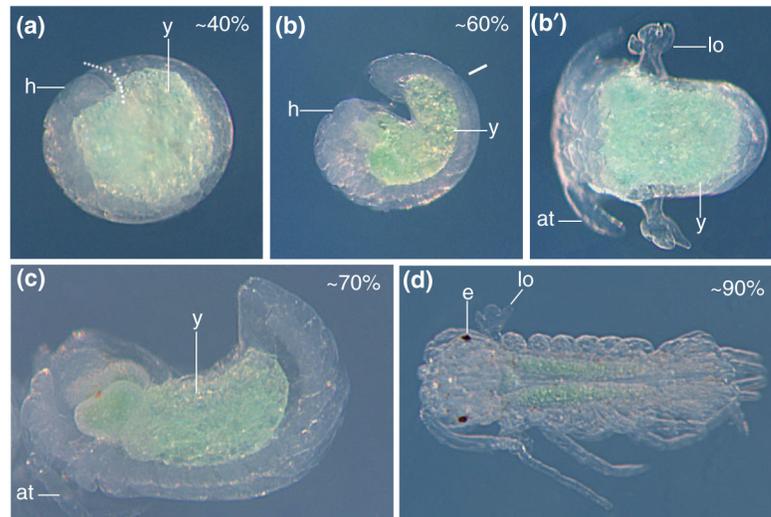
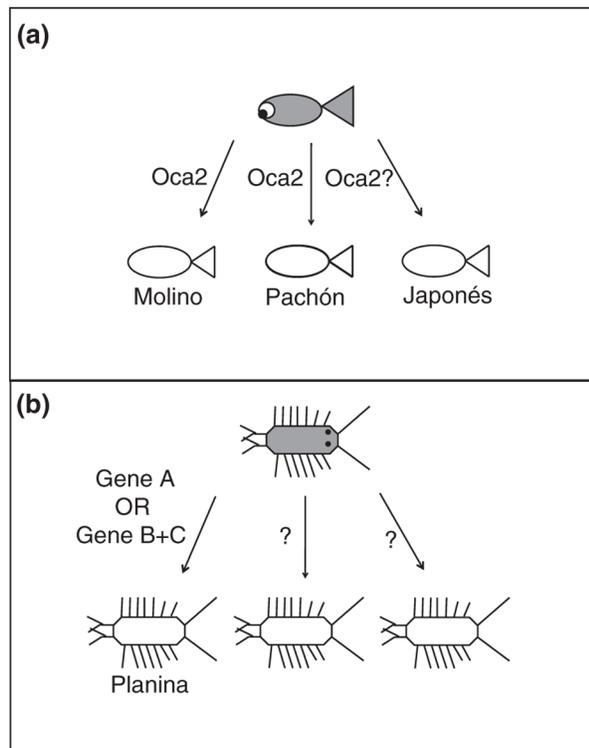


FIGURE 9. Quantitative trait loci (QTL) map, phenotypes, and mapped loci in a backcross of cave and surface *Asellus aquaticus*. (a) Surface head, arrows pointing to the eyes. Scale bar = 0.25 mm. (b) Cave head, arrows pointing to the eyes. (c–f) Four different eye colors present in the backcross offspring. (g) Surface eye. Scale bar = 0.125 mm. (h–j) Representative eye phenotypes in the backcross offspring. (k) Linkage map with eight linkage groups. Distance in centimorgans is on the left side of the linkage group and marker name is on the right side of the linkage group. Mapped locations of various QTL and loci for eye and pigment traits. The length of the black bar represents the 1.5 logarithm (base 10) of odds (LOD) interval of the QTL or locus. (Reprinted with permission from Ref 4. Copyright 2011 National Academy of Sciences)

**FIGURE 10.**

Embryonic development in the surface form of *Asellus aquaticus*. (a) Lateral view of an embryo at 40% of development. h, head; y, yolk. The dotted line shows the separation between the head and the posterior. (b) Lateral view of an embryo at 60% of development. (b'). Dorsal view of the same embryo. lo, lateral organs; at, antenna. (c) Lateral view of an embryo at 70% of development. (d) Dorsal view of an embryo at 90% of development, just before hatching. e, eye. (Reprinted with permission from Ref 111. Copyright 2010 Staatliches Museum für Naturkunde Stuttgart)

**FIGURE 11.**

Differences in albinism between *Astyanax mexicanus* and *Asellus aquaticus*. (a) Three different cavefish populations: Molino, Pachón, and Japonés have an albino phenotype and the causative gene appears to be *oca2*. (In Japonés, the actual mutation has not been mapped—the evidence for *oca2* as the responsible gene is by complementation test).³⁰ (b) In *A. aquaticus*, only one cave population has been examined but there are two different methods observed to cause albinism; either a single locus or mutations at two different loci.⁴

TABLE 1**Major Constructive and Regressive Troglomorphic Traits in *Astyanax mexicanus* Cavefish**

Constructive Traits	Regressive Traits
Jaws	Eyes (lens, cornea, iris, ciliary body, retinal photoreceptors, RPE, scleral bones)
Forebrain	Melanophores
Olfactory bulbs and interneurons	Melanin
Hypothalamus	Rib-bearing vertebrae
Feeding posture behavior	Sleep
Vibration attraction behavior	Aggressive behavior
Neuromasts	Dorsal light reaction
Fat deposition	Feeding angle behavior
Maxillary teeth	Scales
Nares	Fin rays
Orbit bones	—