

# Dark world rises: The emergence of cavefish as a model for the study of evolution, development, behavior, and disease

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## Abstract

A central question in biology is how naturally occurring genetic variation accounts for morphological and behavioral diversity within a species. The Mexican tetra, *Astyanax mexicanus*, has been studied for nearly a century as a model for investigating trait evolution. In March of 2019, researchers representing laboratories from around the world met at the Sixth *Astyanax* International Meeting in Santiago de Querétaro, Mexico. The meeting highlighted the expanding applications of cavefish to investigations of diverse aspects of basic biology, including development, evolution, and disease-based applications. A broad range of integrative approaches are being applied in this system, including the application of state-of-the-art functional genetic assays, brain imaging, and genome sequencing. These advances position cavefish as a model organism for addressing fundamental questions about the genetics and evolution underlying the impressive trait diversity among individual populations within this species.

## KEY WORDS

6th *Astyanax* international meeting, 2019, *Astyanax mexicanus*, cavefish, meeting report

## 1 | INTRODUCTION

Since the publication of Darwin's *Origin of Species*, cave animals have been subjects of conjecture about the forces driving evolution (Cartwright, Schwartz, Merry, & Howell, 2017; Darwin, 1859). Among over 200 cavefish species identified to date, the Mexican tetra, *Astyanax mexicanus*, harbors a number of advantages that have positioned it as a leading model system (Borowsky, 2018). First, *A. mexicanus* consists of an extant eyed surface species and at least 30 cave populations of the same species that are mostly eyeless with reduced body pigmentation (Gross, 2012; Jeffery, 2009; Keene, Yoshizawa, & McGaugh, 2015). Multiple waves of cave colonization have resulted in convergence on cave-derived morphological and

behavioral traits across these cave populations (Bradie, Beerli, Garcia-de Leon, Esquivel-Bobadilla, & Borowsky, 2012; Coghill, Darrin Hulsey, Chaves-Campos, Garcia de Leon, & Johnson, 2014; Herman et al., 2018). The cave and surface populations of *A. mexicanus* are interfertile in a laboratory setting, allowing examination of the genetic basis of changes in morphology and behavior over the course of cavefish evolution (Kowalko, Rohner, Linden, et al., 2013; Protas et al., 2006, 2008). Perhaps most importantly, this species can be readily bred in the laboratory (Borowsky, 2008). These factors enable the cavefish community to take advantage of advances in genomics and genetic technology.

The Sixth *Astyanax* International Meeting, which took place March 17–20, 2019 in Santiago de Querétaro, Mexico, was attended

by 73 researchers representing 10 countries (Figure 1). The meeting began with a Keynote talk by Horst Wilkens, Curator Emeritus at the University of Hamburg, in which he described the rich history of *A. mexicanus* investigating the genetic underpinnings of trait evolution and provided a conceptual bridge between some of the earliest genetic studies carried out in this system and the genome-level analyses currently being conducted. Wilkens is a pioneer in *Astyanax* research and has published on a range of topics, from olfaction and spawning (Wilkens, 1988) to the genetic basis for albinism (Protas et al., 2006). In addition, he discovered the Micos cave locality that has been critical for understanding the evolution of *A. mexicanus* (Wilkens & Burns, 1972). Blind Mexican cavefish were first described in 1936 by researchers from the University of Michigan. They originally identified these cavefish as a new genus, *Anoplichthys* ("bony fish with no eyes"), based on individuals collected from the type locality at the Chica Cave (which contains surface and cavefish hybrid fish; Hubbs & Innes, 1936). This was followed by pioneering work by Charles Breder, an ichthyologist at The American Museum of Natural History during the Second World War, who provided a detailed description of cavefish phenotypes and their ecology (Breder, 1942). Wilkens was a contemporary of Charles Breder, Curt Kosswig, and Perihan Sadoglu, who created the first genetic pedigrees of cave × surface crosses in Breder's lab at the New York Aquarium (Sadoglu, 1957; Sadoglu, 1957). Sadoglu built on these initial discoveries to perform classical genetic experiments that defined the monogenic basis for albinism versus brown pigmentation in this species (Sadoglu & McKee, 1969; Sadoglu, 1957). The ability of these crosses to generate fertile offspring was quite surprising, given that the early pioneers in our field assumed that cavefish were members of an entirely different genus. In the 1970s, this line of investigation was further advanced by Horst Wilkens.

Although it is clear that multiple troglomorphic traits arose repeatedly in different cavefish populations, the fundamental mechanisms underlying the evolution of these traits remain to be uncovered. Wilkens' overview of these classic studies sparked a colorful debate on the roles of pleiotropy, selective pressure, and neutral mutations in the evolution of cavefish traits. Richard Borowsky (New York University) outlined our growing appreciation of gene flow between the surface and cave populations (Badic et al., 2012; Herman et al., 2018), requiring revision of old notions that cave and surface morphs represent discrete morphotypes. Despite these questions about evolutionary mechanism, there is no doubt that early hybridization experiments laid the groundwork for much of the gene mapping and functional analyses of today.

For many years, studies on cavefish have focused on reductions in eye size and body pigmentation; more recently, however, the set of identified trait differences between surface and cave populations has expanded dramatically to include differences in the development and regenerative abilities of various tissues, neural circuitry, and a vast array of behavioral differences. Comparative studies of *A. mexicanus* populations have the potential to elucidate the biology of these traits, their underlying development, and their evolutionary basis. Emerging areas of study in cavefish include social behavior, acoustic communication, epigenetics, metabolic regulation, modulation of wake-activity cycles, gut morphologies and physiology, craniofacial bone structure and tooth patterning (Carlson & Gross, 2018; Gore et al., 2018; Jaggard et al., 2017; Powers, Kaplan, Boggs, & Gross, 2018; Riddle, Boesmans, Caballero, Kazwiny, & Tabin, 2018), and the role of plasticity and maternal effects in shaping phenotypes (Bilandžija et al., 2019). Notably in this regard, Misty Riddle (Harvard University) presented compelling data comparing gut morphologies across caves in response to different diets, and Li Ma (University of



**FIGURE 1** A growing community. Since the inaugural meeting in 2009 which was held in Ciudad Valles, subsequent meetings have seen an increasing number of participants, culminating in the attendance of 73 researchers at the Sixth Astyanax International Meeting, which took place March 17–20, 2019 in Santiago de Querétaro (bottom right). Credits: Bill Jeffery, Josh Gross, Patricia Ornelas García, Nicolas Rohner, Ernesto Maldonado

Maryland) explored the potential phenotypic impacts of differences in maternally provisioned transcripts between cave and surface fish. Many of these evolved differences arose in multiple independently evolved cavefish populations, suggesting that the repeated evolution of anatomical traits extends to physiology and brain function. Further, there is growing evidence that some morphological traits pleiotropically impact behavioral and physiological traits. Albinism, for example, has evolved in cave populations due to deletions in the pigmentation gene *oca2* (Klaassen, Wang, Adamski, Rohner, & Kowalko, 2018; Protas et al., 2006). Knockdown of *oca2* causes elevated catecholamine levels, raising the possibility that loss of pigmentation underlies catecholamine-dependent changes in sleep regulation (Bilandžija, Abraham, Ma, Renner & Jeffery, 2018; Bilandžija, Ma, Parkhurst, & Jeffery, 2013). Therefore, a growing understanding of the genetic basis of the evolution of apparently simple traits may provide insight into the broader “troglomorphic syndrome.”

## 2 | ECOLOGY AND VARIATION WITHIN THE CAVE ENVIRONMENT

A central strength of the cavefish system is the ability to associate trait evolution with well-defined ecological changes in local environment. That said, surprisingly little is known about the ecological differences among caves that underlie trait evolution, or whether the cave-derived traits are truly adaptive in the cave environment. The ecologies of many of the caves in which *A. mexicanus* resides were documented by a series of expeditions led by Robert Mitchell, William Russell, and Bill Elliot in the 1970s (Elliot, 2018; Mitchell, Russell, & Elliott, 1977). The meeting attendees reached a consensus regarding the need to revisit this classic work and examine cavefish in their natural setting. Multiple studies have examined behaviors within the cave environment, including olfaction and feeding, and others have investigated microbiome differences in wild-caught animals (Bibliowicz et al., 2013; Espinasa et al., 2017; Ornelas-García, Pajares, Sosa-Jiménez, Rétaux, & Miranda-Gamboa, 2018). In particular, work from Sylvie Rétaux's laboratory (Paris-Saclay Institute of Neuroscience), which combined lab and field approaches, has revealed dramatic differences in olfactory threshold among natural populations (Bibliowicz et al., 2013; Blin et al., 2018). However, major questions about the caves' ecology and its impact on trait evolution remain unanswered. For example, there is no consensus on whether some caves are nutrient-poor (and if so, which ones), the degree of seasonal variation, and the differences in abiotic and biotic factors across caves (Culver & Pipan, 2009; Keene et al., 2015). Several significant impediments to research on these issues were discussed, including geopolitical barriers to accessing the caves and the need to adapt behavioral assays commonly used in laboratories to the natural environment. Such obstacles in the cave environment could be overcome by technological innovations, including the application of long-term tracking (Fortune et al., 2019), the use of water monitors to track seasonal changes in conductivity, pH, and temperature

(Tabin et al., 2018), and the application of environmental DNA sequencing to identify differences in biodiversity among different cave habitats (Stat et al., 2017). Helena Bilandžija (Ruđer Bošković Institute, Zagreb) presented a laboratory study showing that troglomorphic phenotypes are strongly impacted by developmental environment (Bilandžija et al., 2019), emphasizing the importance of characterizing differences between cave environments (Bilandžija et al., 2019). In addition, Andrew Gluesenkamp (San Antonio Zoo) reported on invasive populations of *A. mexicanus* surface fish that have recently colonized caves in Central Texas, providing the opportunity to examine the steps of early cave colonization in a geographically accessible karst system (McGaugh et al., 2019). The adoption of novel geographic locations and field ecology experiments has the potential to provide unprecedented insight into the relationship between environment and evolution of traits and genomes.

## 3 | INCREASED SOPHISTICATION OF BEHAVIORAL APPROACHES

Cavefish are emerging as a system for investigating how neural circuits and behavior change over evolutionary time. A wide range of behaviors differs between *A. mexicanus* surface and cavefish forms, including sleep and circadian rhythms (Beale et al., 2013; Duboué, Keene, & Borowsky, 2011; Moran, Softley, & Warrant, 2014; Yoshizawa et al., 2015), schooling (Kowalko, Rohner, Rompani, et al., 2013), aggression (Elipot, Hinaux, Callebert, & Rétaux, 2013), feeding (Aspiras, Rohner, Marineau, Borowsky, & Tabin, 2015; Kowalko, Rohner, Linden, et al., 2013; Yoshizawa, Gorički, Soares, & Jeffery, 2010), and stress (Chin et al., 2018). Several presentations highlighted powerful approaches for examining behavioral evolution in cavefish. Johanna Kowalko (Florida Atlantic University) discussed her lab's work examining the role of the *oculocutaneous albinism type 2* (*oca2*) gene, previously shown to underlie albinism in multiple cavefish populations (Klaassen et al., 2018; Ma, Jeffery, Essner, & Kowalko, 2015), in modulating catecholamine levels and behavior. Her lab is pioneering methods for implementing CRISPR/Cas9 in *A. mexicanus*, and has generated surface fish with mutations in the *oca2* gene (Klaassen et al., 2018; Stahl, Jaggard, et al., 2019). Multiple labs discussed their efforts to develop a brain atlas for larval and adult *A. mexicanus* and described how these could be applied to map neuronal regions implicated in sleep and feeding (Gallman, Rivera, & Soares, 2019; Loomis et al., 2019). This study, which expands on several publications from these groups and others (Alié et al., 2018; Jaggard et al., 2017, 2018), demonstrates robust differences in sleep between cave and surface fish. Carole Hyacinthe (Harvard Medical School) presented innovative work conducted in the Rétaux lab demonstrating acoustic communication differences between cave and surface fish (Hyacinthe, Attia, & Rétaux, 2019). This portion of the meeting raised several central questions, including whether behavioral differences are present throughout development, and the relationship between behavioral evolution and the environmental differences within each cave.

The participants also enthusiastically discussed how best to compare behavioral differences observed between the laboratory and the wild and concluded that a particularly powerful approach is to discover behavioral differences in the lab, and then to confirm these differences in the wild. For example, Masato Yoshizawa (University of Hawai'i) and William Jeffery (University of Maryland) found that several populations of laboratory-bred cavefish are attracted to vibrations at 35 Hz (Yoshizawa et al., 2010); they discussed findings demonstrating that this may reflect vibrations generated by food. Luis Espinasa (Marist College) presented compelling comparisons of intra-cave variation in vibration attraction behavior and related these phenotypic differences to the abiotic and biotic variables. Similar approaches may be useful for defining how other behaviors that are commonly studied in the lab, such as sleep, feeding, and aggression, relate to differences in the wild. In addition, there was broad agreement for the need to standardize protocols and fish stocks used for behavioral analysis, and to report the stocks that are used in each study. This is an exciting time for the *Astyanax* system, especially for groups using it to examine how behaviors evolve. Collectively, these talks not only galvanized the community, but also led to important discussions aimed at improving an already stimulating field.

#### 4 | SEQUENCING PROVIDES NEW OPPORTUNITIES FOR GENE DISCOVERY

Whole-genome sequencing of cavefish populations allows predictions of evolutionary history, selection, and association of traits with loci in the genome. The cavefish community currently has access to a Pachón female reference genome assembled from Illumina short-reads from libraries with inserts of different sizes (McGaugh et al., 2014), and Wes Warren (University of Missouri) presented information about a newly created reference genome for a surface fish. Both the cavefish (referred to as v1.02) and surface fish reference genomes (referred to as v2) are available on NCBI and Ensembl (as of v98 of the Ensembl browser). The surface fish reference was created using long-read technology and optical mapping, and consequently contains fewer gaps than the cavefish reference genome. The surface fish genome was constructed from a female that is 25% Río Sabinas and 75% Río Valles, generated by backcrossing a female hybrid between a Río Sabinas female and a Río Valles male with the original Río Valles male. These two populations are geographically disparate surface populations from regions near the El Abra cave populations. An upcoming surface genome paper will include comparisons between each reference, and users of the surface fish genome should be aware that elevated heterozygosity may result in an expanded genome assembly. The cave and surface genome references provide the basis for identifying evolved differences in genes that are causally related to developmental or behavioral phenotypes that can be validated with a second recently developed technology, gene editing.

The community agreed that going forward, data should be shared on the short-read archive and that the code for genomic processing should be published with manuscripts. In addition, metadata

regarding population or stock of origin, extraction protocol, as well as sequencing library preparation, sequencing lane, sex, age, preservation methods, sequencing technology and library preparation method, and barcodes should be provided in the data repository. To emphasize this point, Suzanne McGaugh (University of Minnesota) presented data showing that the method of tissue preservation had an effect on RNA-seq data that dwarfed any biological signal (Passow et al., 2019).

Another major impediment to cavefish research is lack of sex-specific markers. Such markers would allow the sex of larval fish to be incorporated into analyses, thereby facilitating the detection of sex-specific phenotypes. Boudjema Imarazene from the Rétaux lab described advances in understanding the sex determination systems of cave and surface fish. Further, Alejandro Gil Galvez (University of Seville) presented the first application of Assay for Transposase-Accessible Chromatin with high-throughput sequencing (ATAC-seq) in *Astyanax*, providing proof of concept that chromatin accessibility sites can be assayed genome-wide in this species. In the same vein, the community expressed a strong motivation to create a database of cavefish stocks across laboratories, so that genetic backgrounds can be kept consistent for the application of transgenic technologies.

#### 5 | DEVELOPMENT OF GENETIC TOOLKITS FOR FUNCTIONAL INTERROGATION OF TRAIT EVOLUTION

The molecular mechanisms contributing to the evolution of cave traits can be identified through comparative and genetic mapping approaches. However, a central impediment to validating genomic findings has been the lack of tools for functional assessment of identified differences between cavefish and surface fish. Several presenters described recent applications of genetic engineering technologies to *A. mexicanus*, which will empower functional studies in this species. Multiple laboratories discussed the successful application of CRISPR-Cas9 to produce surface fish harboring mutations for candidate genes hypothesized to underlie the evolution of cave traits. This method can be used to definitively demonstrate the causal roles of candidate genes in cave-evolved traits (Klaassen et al., 2018), and is currently being applied to understand the role of specific genes in behavioral and morphological evolution. Furthermore, development of these methods in *A. mexicanus* will open avenues of investigation beyond production of loss-of-function alleles including knock-in of transgenes for imaging of specific cell types and allele-swapping to interrogate the role of specific genetic changes in the evolution of cave traits.

In addition to gene editing, the attendees discussed advances in applications of transposase-mediated transgenesis, building on initial work from Rétaux lab (Elipot, Legendre, Père, Sohm, & Rétaux, 2014). Collaborative efforts between multiple cavefish laboratories have yielded standardized procedures for *Tol2* transgenesis, leading to the ability to produce stable transgenic lines that mark specific cell types (Stahl, Jaggard, et al., 2019; Stahl, Peuß, et al., 2019). In addition to

visualizing specific cell types, these methods can be extended into multiple areas of functional analysis, including performing comparative fate mapping, comparing brain activity to determine how sensory inputs affect brain activity in cave and surface fish, and activating or silencing different neuronal subtypes to dissect the neural circuits underlying behavioral evolution. Future use of these and other transgenic lines will facilitate multiple lines of investigation into the molecular mechanisms underlying cave trait evolution.

## 6 | IMPROVEMENTS IN HUSBANDRY ARE CRITICAL FOR EXPANDED USE

The increased use of *A. mexicanus* brings the need for improved breeding efficiency and the standardization of husbandry practices. Currently, each laboratory has its own methods of rearing fish, feeding regimens, and breeding protocols. This situation could lead to differences in maturation rates, which are often a bottleneck for research. Participants emphasized the importance of inter-lab communication to optimize husbandry practices. The majority of laboratories have adopted many of the systems and rearing techniques used in zebrafish (Lawrence, 2007, 2011), including the use of RO purification systems, dosing with artificial sea salt and/or sodium bicarbonate, and feeding fish high-quality commercial aquaculture diets. However, protocols for optimal rearing pH, salinity, and temperature still differ significantly between laboratories. As we continue to develop new rearing strategies, it is important that each laboratory is not unnecessarily repeating the same rearing experiments. In addition, it was noted that the widespread application of transgenic approaches requires efficient breeding, which in turn allows for reliably timed-matings to facilitate injections at the single-cell stage.

Beyond expressing their consensus view in favor of developing standardized protocols, the participants also noted that the unique evolutionary history of each population may result in differences in responsiveness to different breeding protocols (e.g., different conductivity ranges, sex ratios, etc.). In many cases, difficulties with breeding are associated with specific individual populations of cavefish. This may relate to differences in water chemistry between wild and laboratory conditions; a clearer understanding of these issues may allow us to maintain fish under conditions that most closely resemble their natural environments. Ultimately, improved husbandry protocols will be essential to facilitate expanded use of this system.

## 7 | MODELING DISEASE AND RESILIENCE IN CAVEFISH

Interest has been growing in the use of cavefish as models for human disease. Previous genetic and developmental studies have shown that retinal phenotypes during cavefish eye degeneration mimic phenotypes observed in retinitis pigmentosa (O'Quin, Yoshizawa, Doshi, & Jeffery, 2013), and that one of the genes that causes albinism in humans is responsible for pigmentation loss in cavefish (Protas

et al., 2006). Recent work in cavefish has considerably expanded the repertoire of cave traits that could be used to study human disease. However, while cavefish display phenotypes that resemble certain pathological states including insomnia, diabetes, autism, and obesity, it is also possible that cavefish may have evolved mechanisms for resilience against these states (Aspiras et al., 2015; Riddle, Aspiras, et al., 2018; Yoshizawa et al., 2018). A subset of presentations at the meeting dealt with this emerging topic in the field. These disease models have been extended to morphological development through studies demonstrating interactions between sensory neuromasts and facial bones, indicating that developmental patterning can have far-reaching impacts on cranial asymmetry—a common dysmorphic feature in humans (Gross, Gangidine, & Powers, 2016; Gross, Krutzler, & Carlson, 2014; Powers, Boggs, & Gross, 2018). Work by Tamara Franz-Odendaal's research group (Mount Saint Vincent University) has shown that tooth and jaw development differ between cave and surface fish (Atukorala & Franz-Odendaal, 2015). Moreover, Devi Atukorala (University of Manitoba) demonstrated that the expression pattern of the gene underlying tooth and eye anomalies in humans with Axenfeld-Rieger's syndrome is altered in cavefish relative to surface fish, providing a further demonstration of the usefulness of the cavefish model in understanding disease (Atukorallaya & Franz-Odendaal, 2018).

Comparative approaches are rapidly being developed to establish *A. mexicanus* as a model for studying heart regeneration and development. Mathilda Mommersteeg's research group (University of Oxford) has reported that while the hearts of surface fish regenerate similarly to those in other fish models, cavefish hearts scar after amputation or injury (Stockdale et al., 2018). This is very similar to the scarring seen in human hearts after a heart attack. Understanding the differences in regenerative capacity between cave and surface fish could provide important insights into the treatment of cardiovascular disease. Bill Jeffery presented work on asymmetric heart looping, a phenotype that occurs in cavefish but not in surface fish, illustrating the potential of this system as a model for the development of normal and abnormal (heterotaxic) organ arrangement in humans. Together, these findings demonstrate that cavefish provides an accessible system for studying developmental and functional processes related to heart disease.

The modeling of disease states extended to how cavefish respond to dietary and environmental stressors. Masato Yoshizawa presented work showing how a ketogenic diet affects some of the social phenotypes of cavefish and argued that studying the social behaviors of cavefish could provide unique insights into autism (Yoshizawa et al., 2018). Jacqueline Chin from the Duboué lab (Florida Atlantic University) explored how early-life stress impacts later-life anxiety (Chin et al., 2018). Shaolei Xiong from the Rohner lab (Stowers Institute) presented evidence that cavefish store more visceral fat than surface fish after consuming the same amount of food, and that Pachón cavefish have faster onset of adipocyte development and larger adipocytes than surface fish (Xiong, Krishnan, Peuß, & Rohner, 2018). Robert Peuß, also from the Rohner lab, reported that cavefish have evolved resilience against certain autoimmune traits (Peuß et al., 2019;

Xiong et al., 2018). Taken together, their work highlights the usefulness of evolutionary model systems for studying processes that distinguish between health and disease, and may yield new insights into human pathologies. The identification of genetic variants that allow cavefish to remain seemingly healthy despite possessing traits typically associated with human pathology could open new avenues to the study of disease resilience.

## 8 | APPLICATION OF APPROACHES DEVELOPED IN CAVEFISH TO OTHER EMERGENT MODELS

Cavefish represent one of many aquatic species in which trait evolution can be studied. The African turquoise killifish has emerged as a model for the study of aging (Valenzano et al., 2015), and African cichlids and the three-spine stickleback have long been leading models for investigating trait evolution and speciation (Peichel & Marques, 2017; Seehausen, 2006). Like cavefish, researchers using these models have recently implemented transgenic and gene-editing approaches to their investigations of trait function (Erickson, Ellis, & Miller, 2016; Harel, Valenzano, & Brunet, 2016; Juntti, Hu, & Fernald, 2013). The ability to laterally transfer genetic and behavioral approaches between these fish species, combined with the unique advantages of each model, provides a platform for studying genetic variation in humans and guiding efforts to personalize medicine.

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### CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

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