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TESTING THE CAVEFISH MODEL: AN ORGANISM-FOCUSED THEORY OF BIOLOGICAL DESIGN

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ABSTRACT

The Institute for Creation Research (ICR) is performing controlled experiments to test the response of an organism to different environmental conditions. The animal model is *Astyanax mexicanus* (Mexican tetra), a freshwater fish with two well-differentiated, interfertile morphotypes: eyed surface-dwelling fish (surface fish) with a distinct pigmentation pattern, and eyeless cave-dwelling fish (cavefish) with minimal pigmentation. For this research, we have established and equipped a new biology laboratory to investigate the mechanisms and process of adaptation in this model. Preliminary results from experiments with mature adult *A. mexicanus* include the following: (1) Cavefish increase pigmentation across their body when exposed to high-intensity light; (2) Cavefish exhibit behavioral and physiological acclimation to high CO_2 (low pH) water; (3) Surface fish decrease pigmentation across their body and labor during respiration in high CO_2 (low pH) water; (4) Adult cavefish and surface fish respond to experimental treatments within weeks of treatment; and (5) Responses to treatments by both morphotypes are not limited to multigenerational genetic inheritance. The first result implies that UV light may stimulate melanosome production in adult cavefish through biochemical induction of a latent melanin synthesis pathway. Second, pre-acclimation by cavefish to acidic water chemistry likely reflects conditions within their native cave environments. Third, the comparative loss of pigmentation and associated respiratory challenges in adult surface fish exposed to darkness and high CO_2 (low pH) suggest they actively self-adjust. And in contrast to cavefish, non-acclimated surface fish indicate they are outside of their native environment. The significance of this research is multifaceted. At the 8th ICC, Guliuzza and Gaskill (2018) introduced a novel paradigm: Continuous Environmental Tracking (CET). This model infers that organisms actively and continuously track conditions within specific environments to self-adjust through internal mechanisms that integrate molecular, biochemical, cellular, physiological and behavioral functionality of the whole organism. These mechanisms are predicted to operate by the same integrative principles that govern human-engineered control systems, suggesting that fish and other animals make highly-regulated responses in order to compensate for changes in external conditions that may exceed their routine efforts to maintain homeostasis. Moreover, the model also predicts that organisms can modify the course of their development; that adaptive larval and adult traits are sometimes reversible; that epigenetic modifications are heritable across multiple generations; and that common phenotypic traits will be observed among a diversity of organisms living in similar environments. Our predictions are testable.

KEYWORDS

Astyanax, cavefish, CET, pigmentation, adaptation, self-adjustment, environment, neo-Darwinian theory

I. INTRODUCTION

A primary purpose of scientific experimentation is to provide data that helps researchers develop new models, and confirm, refine or reject existing theories. Theory is paramount in science since it sets research agendas, becomes the guiding interpretative framework of observations, and forms the basis for explanatory models. Our intent is to use experimental results to test whether several widespread theoretical assumptions are valid. Here, we apply new data from pigmentation experiments on multiple mating pairs of *Astyanax mexicanus* (Mexican tetra) to principally test theoretical assumptions of three models explaining the extreme morphological differences observed in fish living in profoundly different environments. Model one is the standard Neo-Darwinian Theory (NDT). Evolutionists prefer studying cavefish as a vertebrate model of adaptation in caves because surface-dwelling (ancestor) and cave-dwelling (descendant) forms of the same species (Fig. 1) are available for comparative research (Jeffery 2019). Model two is the historical non-evolutionary interpretation for the origin of blind cavefish. Model three is Continuous Environmental Tracking (CET), a new organism-focused, engineering-based model of adaptation (Guliuzza and Gaskill 2018).

A. Conventional neo-Darwinian explanation for the origin of blind cavefish

Blind Mexican cavefish (*Astyanax mexicanus*), "Darwin's finches," peppered moths, and "Lucy" are icons of Darwinian evolution. Because the loss of eyes and pigmentation following cave migration are dramatic transformations of anatomy, blind cavefish have become a notable example of Darwinian selectionism. Icons are important because they are seen as accurate illustrations of the core ideas (or basic

Figure 1. Comparative morphology of Mexican tetra (*A. mexicanus*) surface fish and cavefish. **A.** Surface fish in left-lateral view, showing a common pattern of melanin pigmentation along dorsal sides of head, back and posterior regions (white arrows), posterior to the gill chamber (yellow arrow), and along the lateral line, with high concentrations toward the posterior end at the junction of the lateral line and tail (dashed white arrow). Xanthophores produce bands of yellowish pigment extending along the lateral line and into rays of the caudal fin. The eye is functional, with a pigmented iris surrounding the lens. **B.** Cavefish in left-lateral view with much lower numbers and spatial expression of melanophores in all regions where melanin is produced in the surface fish. The eye is absent due to apoptotic degeneration and removal of the primary components of eye anatomy during larval development and juvenile growth stages. Macrophotographic images by Scott Arledge and Michael J. Boyle.

assumptions) of a hypothesis about how some natural phenomenon operates. Thus, they are held as strong affirmative evidence supporting a scientific theory. For instance, advocates of Intelligent Design (ID) theory made icons of the bacterial flagellum and a mousetrap by using them as definitive examples of the key concept of irreducible complexity (Behe 1996). Critics of intelligent design, therefore, strove to invalidate central tenets of design theory by discrediting such models as valid evidence for ID (Miller 2004; Clements 2009).

NDT explains the origin of traits specific to the blind cavefish as a series of gradually accumulating adaptations derived from features of their ancestral surface fish relatives. Those adaptations are postulated as the products of random unguided genetic mutations. Purposeless adaptive variability was incorporated as a foundational assumption of neo-Darwinian theory, which is also known as the Modern Synthesis (MS). The NDT, with its subsequent interpretations of observable changes in blind cavefish, is heavily dependent upon several bedrock assumptions that are best articulated by evolutionary researchers themselves.

"The random occurrence of mutations with respect to their consequences is an axiom upon which much of biology and evolutionary theory rests [Futuyma 1986]. This simple proposition has had profound effects on models of evolution developed since the modern synthesis, shaping how biologists have thought about and studied genetic diversity over the past century" (Monroe 2022). "The core tenet of the MS is that adaptive evolution is due to natural selection acting on heritable variability that originates through accidental changes in genetic material. Such mutations are random in the sense that they arise without reference to their advantages or disadvantages…" (Charlesworth 2017). "A classical or Darwinian evolutionary system embodies a basic principle: purposeless genetic variation of reproductive individuals, united by common descent, coupled with…natural selection of those rare individuals that fortuitously express the traits that complement or thwart the contemporary selective pressures…it's a process replete with chance" (Greaves and Maley 2012).

Stephen J. Gould (2002) summarizes three criteria for genetic variability within neo-Darwinian theory by stating, "Variation, in short, must be copious, small in extent, and undirected. A full taxonomy of non-Darwinian evolutionary theories may be elaborated by their denial of one or more of these central assumptions." Gould adds that the most important criterion is undirected variation. He emphasized that wholly unbiased variation is *fundamental* to evolutionary theory, going on to say "in a sense, the specter of directed variability threatens Darwinism even more seriously than any putative failure of the other two postulates [copious, small in extent]" and he clarifies the meaning of directed variation as "…adaptive pressures [that] automatically trigger heritable variation in favored directions…" Automatic triggers of specific responses would have much in common with the components and outcomes corresponding to human-engineered systems, and thus, "Darwin clearly understood the threat of directed variability to his cardinal postulate of creativity for natural selection" (Gould 2002).

Precisely because cavefish are believed to validate the vital interpretive assumptions of Darwin's selectionist theory of evolutionary adaptation (Table 1), and specifically because these core assumptions should be reexamined, but are rarely questioned, we took on the study of cavefish to separate fact from myth for this notably persuasive icon.

B. Historical non-evolutionary interpretation for the origin of blind cavefish

Interpretive assumptions are also integral to historical non-evolutionary interpretations for the origin of blind cavefish. Accordingly, we recognize that it is time to reassess these assumptions in light of our findings, and over two decades of molecular and genetic data compiled by a growing cavefish research community. A comparison of our findings with historical non-evolutionary and evolutionary assumptions is presented in Table 1. Because widely-popular explanations are an important focus of our research, we located an accurate representative example that clearly delineates interpretive assumptions of the historical approach. A prime illustration of a historical non-evolutionary response to Darwinian evolution is currently displayed in The Creation and Earth History Museum in Santee, CA., the original site of ICR's Creation Museum. Their interpretation for the origin of blind cavefish is as follows:

> As genetic information is copied and passed on generation after generation, occasionally there are copying 'mistakes' known as mutations. Mutations have been observed to destroy, damage, or corrupt genetic information or to be neutral, but have never been observed to add new information. This is true even of so-called 'beneficial' mutations that may be advantageous to the surviving organism in some

Table 1. Condensed outline summarizing the main elements of Neo-Darwinian Theory (NDT). (**a**) Darwin's revolutionary shift from preceding theories of evolution was the initiation of an externalistic interpretive framework[1]. Externalism is the belief that dynamic environmental changes set the course of adaptive change in the organism-environment relationship. Environments are viewed as active agents and organisms are seen as passive modeling clay[2]. Externalism is the framework for the assumptions and interpretations within NDT. (**b**, green) NDT projects onto the environment a pseudo-agency as a causal explanation of adaptation in evolutionary scientific literature[3]. Nature is conferred an ability to govern verbs as a causal agent[4] in a blind, unconscious manner. (**c**, gray) NDT has three core assumptions of what genetic and phenotypic change necessarily will be during adaptation. (**d**, white) The core assumptions of NDT dictate how a genetic or phenotypic change must be interpreted and characterized in evolutionary literature. (**e**, magenta) Inferences about how evolution and increases in biological complexity and diversification happen. 1. Gould, S.J. 2002. *The Structure of Evolutionary Theory*. Cambridge, Massachusetts: Harvard University Press, p. 141-145. 2. Kirschner, M. and J. Gerhart. 2005. *The Plausibility of Life*. Yale University Press; New Haven and London. 3. Lewontin, R.C. 1983. *Gene, Organism, and Environment*. From *Evolution from Molecules to Man*, D.S. Bendall, ed. Cambridge: Cambridge University Press. 4. Hodge, M.J.S. 1992. Natural selection: Current usages. In *Keywords in evolutionary biology*, eds. E.F. Keller and E.A. Lloyd. Cambridge, Massachusetts: Harvard University Press.

(**a**) Externalistic framework of Neo-Darwinian Theory: nature drives the organism-environment relationship.					
(**b**) NDT empowers the Environment to:	1. Act. Nature can act like a human breeder.				
	2. Select. Environmental conditions are "selective pressures" that drive relatively passive creatures through lethal filters.				
	3. Cobble. Phenotypic outcomes over time should bear characteristic signs of a "cobbling" or "tinkering' evolutionary process.				
	4. Direct. Nature's selective actions are the overriding directional factor in evolution.				
(**c**) NDT assumptions:	(**d**) Resulting interpretations of any type of changes are viewed as:				(**e**) Inferences:
Undirected Genetic Change: No automated activation of genetic variation. No preset production of useful genetic outcomes.	Mistakes: Genetic changes are mutational errors that offer principal sources of genetic variety for adaptive changes.	Random: Changes are blind, unpredictable and aimless to the future needs of organisms.	Losses: Genetic change most often produces a loss of function. Broken products will be the dominant outcome.	Non-Lamarckian: No transfer of information from somatic cells to germ cells across generations. The "Weisman Barrier" is impenetrable.	Genetic inheritance is the principle means for transmitting selectable variation across generations. Random genetic recombination and drift are secondary mechanisms to produce genetic variation and sort through a diversity of traits.
Gradual Changes to Traits: No large-scale arrival of new traits. Phenotypic changes are imperceptibly small.	Slow: Each variant with slight phenotypic effects over extended timeframes.	Linear: Consistent rate of change over time—not long periods of stasis punctuated by episodes of conspicuous change.	Continuous: Any trait to represent descent with modification from a universal common ancestor.		Incremental accumulations of variation explain phenotypic differentiations between higher taxa. New species arise by prevention of gene flow between separate populations that evolve differently.
Copious Offspring: Genetic variation is assumed to be plentiful within a population.	Trial and error: Natural selection works on a large pool of random mutations in an unpredictable and haphazard manner with hit and miss outcomes.				Evolution requires study both within and across populations. Most animal species are now extinct. Populations evolve by changes in gene frequency brought about by natural selection, gene flow and drift.

circumstances…When a mutation occurs in a light environment that causes animal's offspring not to have eyes, it is an enormous disadvantage…so natural selection eliminates this flaw…When the eyeless defect occurs here [in a cave], it does not give any disadvantage so it is not eliminated. In fact, it gives advantages. Those with eyes can crash into things, injuring the eyes, and can get diseases of the eyes, possibly leading to death…Eventually, selective pressures ensure that all are eyeless…These ghostly fish and amphibians swim blindly as prime examples of how mutation and natural selection lead to a reduction of functioning systems as complex genetic information has been corrupted or lost, not gained…These adaptations are no evidence at all for the belief that complexity has arisen by such processes – they only show how information can be lost in a fallen world.

In sum, the historical non-evolutionary explanation of how surface-dwelling ancestors gradually transitioned into populations of cave-dwelling descendants consists of a process whereby: random genetic mutations result in a loss of information, which, in turn, produces such traits as depigmentation and blindness that are subsequently fractioned out and reproduced through natural selection. Over time, selection pressures ensure that all cavefish are eyeless.

It's enlightening to note that both non-evolutionary and evolutionary explanations are identical and use the same evolutionary assumptions to interpret the same biological phenomena (Table 1). Thus, resolving complex details of *A. mexicanus* pigmentation mechanisms is essential to evaluate whether historical non-evolutionary explanations are scientifically accurate and effective in altering our current, and future, perceptions of Darwinian selectionism.

C. Continuous Environmental Tracking (CET) interpretation for the origin of blind cavefish

Since cavefish can be directly compared to surface fish (mimicking the way that mutants are compared to wild-type phenotypes in other model organisms,) *A. mexicanus* is also an appropriate model organism to evaluate the interpretive assumptions and predictions of the Continuous Environmental Tracking (CET) model of adaptation. CET is a recent organism-focused, engineering-based model of adaptation (Guliuzza and Gaskill 2018) that was presented at the 8th ICC. The significance of this research is multifaceted. This model of adaptation infers that organisms actively and continuously track conditions within their specific environments to self-adjust through

internal mechanisms that integrate molecular, biochemical, physiological and behavioral functionality of the whole organism. These mechanisms are predicted to operate by the same integrative principles that govern human-engineered tracking systems, suggesting that fish and other animals make highly-regulated responses in order to compensate for changes in external conditions that may exceed their routine efforts to maintain homeostasis. Moreover, the theory also predicts that organisms can modify the course of their development; that adaptive larval and adult traits are sometimes reversible; that many epigenetic modifications are heritable across multiple generations; and that phenotypic traits will trend toward convergence among a diversity of organisms living within similar environments.

Collectively, there are multiple major points of departure in assumptions and predictions between the CET model of adaptation and the (essentially synonymous) conventional evolutionary and non-evolutionary interpretations for the origin of blind cavefish. First, in terms of how adaptation is characterized (descriptors of how the mechanism of adaptation operates) CET expects adaptive outcomes to be tightly regulated, rapid, repeatable, sometimes reversible, and highly targeted – even predictable – responses. Second, in terms of the extent of resulting adaptations, CET expects that "adaptation" should be viewed as a temporal continuum where an organism's adaptations can range from very rapid physiological self-adjustments, to intra-lifetime, to multi-generational. Third, the environment is viewed as a range of conditions to which organisms are variably exposed, and to which organisms themselves control their variable responses. It is the traits of organisms that specify which environmental conditions are "stimuli" and the extent to which each individual organism can relate to its environment and solve environmental challenges. Environments are not personified with intelligent agent-like powers to "select," "favor," or "act on" creatures. Fourth, in terms of the organisms themselves, CET would view organisms as active, problem-solving entities that successfully navigate environmental challenges and fill new niches. Thus, creatures are not viewed as passive modeling clay that is constantly being shaped by their environment; they are actively in control of adapting to their environment.

D. Biological observations are inconsistent with the assumptions of neo-Darwinian theory

Before the Linnean Society on July 1st 1858, Sir Charles Lyell and J. D. Hooker read papers by Charles Darwin and Alfred Wallace highlighting their respective deductions on the "Perpetuation of Varieties and Species by Natural Means of Selection". Their philosophical 'convergence' on similar proposals reflected an emphasis on nature as the ultimate creative agency behind the diversity of all life forms on earth. This perspective has changed little over the ensuing years, and remains as the standard academic view today. However, evolutionists are struggling to reconcile a surging number of observable mechanisms for adaptive change that are not compatible with NDT, which has led to heated discussions over the future of evolutionary theory (Lewontin 1983; Gould 2002; Koonin 2009; Laland et al. 2014; Laland et al. 2015; Bateson et al. 2017; Muller 2017; West-Eberhard 2019; Jablonka and Lamb 2020; Sultan 2021). And on top of such discussion comes a remarkable new emphasis, from a review of research on the extended evolutionary synthesis (EES), which proposes, "more sources of biological innovation and adaptations" in order to update the "structure of evolutionary theory" (Chiu 2022). With the EES, there is an apparent interest to move past a "gene-centric" view, toward recognizing "more agency for organisms to affect their own evolution", or in other words, a growing interest toward an "organism-centered perspective" (Chiu 2022). Yet, both NDT and EES perspectives are distinctly evolutionary syntheses. Importantly, we observe the same body of evidence and it prompts us to question whether interpretive assumptions derived from the NDT, or the EES, are valid for anything more than trivial cases.

For instance, which tests in the scientific literature enable us to confidently identify the sources of genetic or epigenetic changes underlying adaptive phenotypes that originate from copying mistakes? Additionally, what studies have demonstrated that genetic changes are random or undirected, or that altered genetic sequences should be classified as "broken" or as "loss-of-function" rather than being precisely modified to produce purposeful changes in function? Is it observational data or NDT that constrains researchers to expect that phenotypic changes will be very slight in extent, and sorted out through "hit and miss" or "trial and error" processes that only advance through very gradual rates of change? Is the Weisman Barrier physically identifiable or is it a necessary interpretive assumption of NDT? The lack of details in the literature to support these assumptions makes them more conspicuous as dogmatic declarations rather than experimental demonstrations.

E. The cavefish *Astyanax mexicanus* is a suitable research model to test theoretical assumptions

Organisms that live in caves for their entire life are known as troglobites. Cave animals share an astonishingly consistent set of sensory, morphological, physiological and behavioral traits. Though not expressed identically in all organisms, or even between cavefish, shared troglomorphic traits are highly similar across insects, crustaceans, centipedes, millipedes, spiders and salamanders, all animals that are permanent cave inhabitants (Borowski 2018). Borowski adds, "in fact, as far as we know, whenever a surface species comes to live in a cave, given enough time, it changes in the same way. Thus, cave animals are a natural model for the study of convergent and adaptive evolution…" Importantly, even different types of cavefish with shared traits are known to be unrelated and geographically widespread.

Cavefish are not uncommon and their range is remarkably diverse. Over a nine-year period from 2011 to 2020 an average of eight new species per year were documented (Maldonado 2020). The number of fish species characterized as full-time cave dwellers likely exceeds 230, and worldwide they have been found on all continents except Antarctica (Borowski 2018). Borowski suggests that, "all of them have evolved independently from surface ancestors." Independent lines are important for studying differences in genetic expression, physiologic pathways or mechanisms that produce very similar phenotypic outcomes. The Mexican cavefish *Astyanax mexicanus* is the most studied vertebrate model for troglomorphic traits. Other genera of cavefish that significantly add to the body of knowledge are the Chinese cavefish *Sinocyclocheilus* (Yang 2016), the Somali cavefish *Phreatichthys andruzzii* (Cavallari 2011) and the Northern cavefish *Amblyopsis spelaea* (Hart 2020). In Southeast Asia and southern China, even though there are many cavefish, most are in the loach and cyprinid (carp) families, yet in South America, most cavefish are in the catfish family.

Our model, *A. mexicanus* (Characid Mexican tetra), is abundant, robust and easily maintained in the laboratory. Mexican tetras are freshwater fish with well-differentiated, interfertile morphotypes: eyed surface-dwelling fish (surface fish) with a distinct pigmentation pattern, and eyeless cave-dwelling fish (cavefish) with minimal pigmentation (Fig. 1). Surface fish and cavefish reach sexual maturity in only 4-6 months and produce hundreds of relatively large translucent

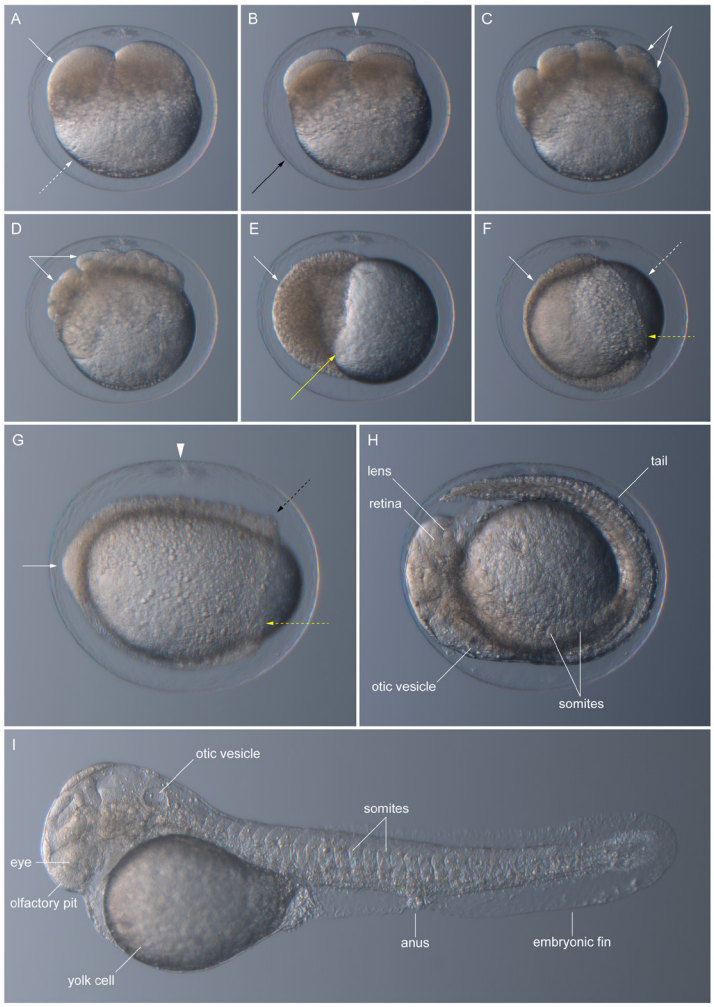

Figure 2. Selected stages of embryonic and larval development in an *A. mexicanus* surface fish. **A.** ~1.0 hpf (hours post fertilization); 1st cleavage stage embryo with two blastomeres (white arrow) extending from the nutritive yolk cell (dashed white arrow). Animal pole is to the top, vegetal pole is to the bottom. **B.** 1.25 hpf; 2nd cleavage stage with four blastomeres. A spherical, semi-transparent chorion (black arrow) surrounds the embryo. The original site of sperm penetration (micropyle) is visible on the animal pole (white arrowhead). **C.** 1.5 hpf; 3rd cleavage stage with eight blastomeres (white arrows). The embryo has begun to rotate toward the left side (relative to the micropyle). **D.** 2.0 hpf; embryo with more than ~ 30 undifferentiated blastomeres. **E.** 4.0 hpf; sphere stage undergoing epiboly, where the blastoderm (white arrow) is an enveloping layer (EVL) of motile cells that spread over the yolk syncytial layer (YSL, yellow arrow). Embryonic rotation is almost 90˚. **F.** 7.0 hpf; ~60% epiboly where the blastoderm is advancing (dashed yellow arrow) over the yolk cell (dashed white arrow) toward the vegetal pole. A bulge of blastoderm cells under the micropyle now provides a dorsal-ventral axis, with dorsal toward the outer edge (white arrow). **G.** 10 hpf; ~80% epiboly with notable elongation of the blastoderm and yolk cell. The future head end (white arrow) and tail end (dashed black arrow) are now defined. The micropyle (white arrowhead) has remained in the same original orientation during all stages of development thus far. **H.** 23 hpf; late pre-hatch larval stage. Relative to the image in 'G', this larva has rotated horizontally 180˚, with the anterior-posterior axis extending from the head in a counterclockwise orientation with the tail pointed back toward the head at the top left corner of the panel. Almost all larval organ systems are fully developed. **I.** 28 hpf; the swimming, non-feeding larva remains dependent on nutrition from the yolk cell. The developmental staging and process identification follow Hinaux et al. 2011. Stereomicrographs were extracted as individual files from a timelapse video series of images produced by Scott Arledge and Michael J. Boyle.

embryos and larvae in a single spawning event. Therefore, access to early stages of development (Fig. 2) are readily available for experimental applications on comparative developmental morphology, genetic and transcriptomic expression patterns, environmental treatment conditions, and a diversity of micrographic imaging techniques – all of which facilitate in-depth research on the biochemistry and physiology underlying the process of adaptation.

The cavefish forms of *A. mexicanus* are found within 29 known caves across the Sierra del Abra of Northeastern Mexico (Krishnan and Rohner 2017; Jeffery 2020). Often, cave-dwelling populations live in close proximity to conspecific surface-dwelling morphotypes. Genetic studies suggest there have been at least five cave invasions by surface-dwelling fish along with persistent gene-flow among cave-dwelling populations (Maldonado 2020; McGaugh 2020). Both surface fish and cavefish genomes have been sequenced and assembled. Although each morphotype exhibits a unique set of anatomical, physiological and behavioral traits, there is a limited amount of overall genetic variability between the surface and cave variants, and their genomes are nearly identical. As Jeffery (2019) states, "based on the molecular studies and the ability to produce fertile offspring, all cavefish populations and nearby surface fish are usually considered to be a single genetic species: *A. mexicanus*".

Tomkins discussed a widely-divergent range of evolutionary time intervals published by NDT advocates for the development of dark-adaptive traits in *Astyanax* (Tomkins 2022). NDT frames the adaptation of those traits as a gradual, haphazard or "hit and miss" process. Early estimates suggested that blind *Astyanax* morphotypes diverged from sighted surface populations between 3.1 – 8.1 million years ago (Strecker 2004; Ornelas-Garcia 2008). A decade later, Jeffery reported a much younger age, stating that "surface fish and cavefish split from a common ancestor very recently, within the past million years or less" (Jeffery 2019). Several additional studies suggested more recent deep-time estimates. And there is increased recognition for relatively fast rates of morphological change in which "recent studies have shown that the cave-dwelling form evolved rapidly within the last 200,000 years from an ancestor that lived at the surface" (Bilandzija 2020). Yet another contemporaneous study arrived at a divergence time between 115,000 – 190,000 years ago (Herman 2018). Although such estimates may reflect differences in methodology, they clearly indicate a decreasing trend in the time of production for cavefish adaptations.

However, a new analytical approach considering the geographic distribution of mitochondrial and nuclear DNA polymorphisms are raising questions about all of the age estimates for development of troglomorphic traits, as well as the current assignment of cavefish into the so-called "old" and "new" lineages, and whether several caves have been independently populated at different times (Fumey 2018). Fumey "found that microsatellite polymorphism strongly supports a very recent origin of cave populations (<20,000 years)" adding that "the only safe conclusion is that these cave populations are not millions of years old. The large uncertainty associated with these [prior] estimations is probably the reason why they are rarely cited by investigators working on these cavefish." (Fumey 2018).

In this manuscript we present a series of preliminary (single test) and controlled (treated and untreated specimens) experiments designed to test for phenotypic responses in *A. mexicanus* cavefish and surface fish morphotypes. Experimental treatment conditions consisted of exposure to high intensity light, elevated CO_2 (low pH) and low dissolved O_2 levels. In three different cavefish populations (strains), light exposure stimulated a noticeable increase in chromatophore

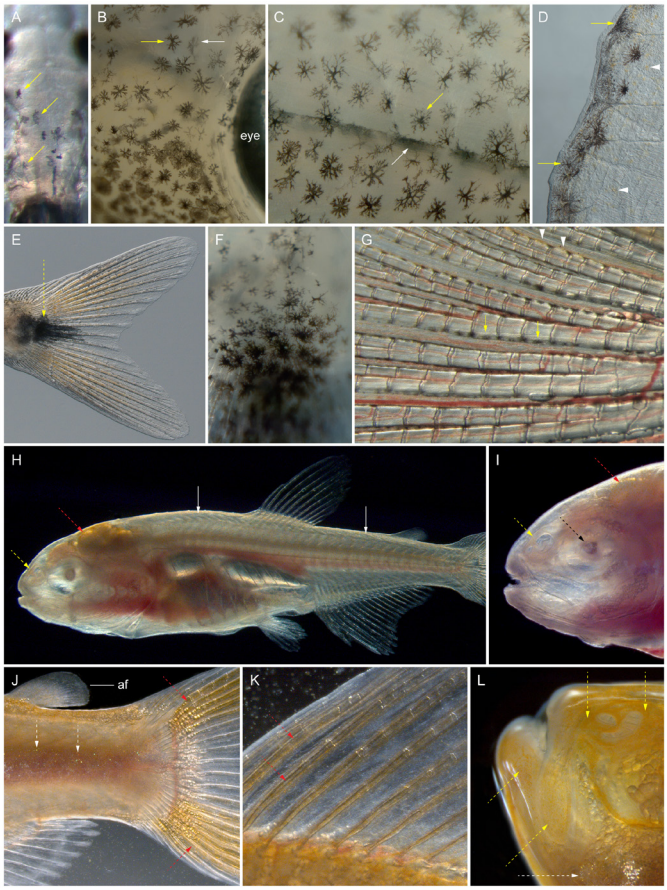

Figure 3. Localized patterns and morphology of pigmentation in Mexican tetras (*A. mexicanus*). **A.** Surface fish larva after two days of development showing melanic chromatophores (yellow arrows); dorsal view with anterior to the top. **B.** Dendritic melanin granules along the circular margin of a juvenile surface fish eye, and within a field of melanophores anterior to the eye in both surface (yellow arrow) and subsurface cells (white arrow). **C.** Melanophores along the lateral line of a juvenile surface fish showing typical dendritic morphologies within (yellow arrow) and below (white arrow) the epidermis. **D.** Terminal edge along the posterior field of an adult surface fish scale, pigmented with melanophores (yellow arrows) and xanthophores (white arrowheads). **E.** Pigmentation in the caudal fin of a juvenile surface fish showing a highly concentrated area of melanophores extending from the lateral line into the tail (dashed yellow arrow), and linear patterns melanophores and xanthophores aligned along the branched fin rays. **F.** Magnified view of dendritic melanophores in the concentrated area shown in '**E**'. **G.** Magnified view of the branched caudal fin rays of an adult cavefish. Blood vessels are visible (red), and both melanophores (yellow arrows) and xanthophores (white arrowheads) are detectable along lateral margins of the fin rays. **H.** Molino cavefish juvenile in left-lateral view. Black melanophores are not visible in any regions of the body. Xanthophores are discernable as yellow-orange spots on margins of the olfactory pit (dashed yellow arrow), on the optic tectum (dashed red arrow) and along dorsal sides (white arrows) and flanks of the body. **I.** Magnified, lateral view of the head of a Molino cavefish juvenile. A remnant of the embryonic eye is centered within a transparent eye orbit (dashed black arrow), and xanthophore pigmentation is visible within tissues of the olfactory pit (dashed yellow arrow) and optic tectum (dashed red arrow). **J–L.** Pigmentation in different body regions of a Molino cavefish juvenile exposed to daily treatments of high-intensity light for 15 days. Overall body coloration is noticeably orange due to increased xanthophore pigment along body and fin rays (**J**,**K**, dashed red arrows) and regions of the head and mouth (**L**, dashed yellow arrows). There is also an increase in the presence of iridescent cells (iridophores) along the lateral stripe and sides of the head (**J**,**L**, dashed white arrows) and other areas (not shown). af, adipose fin. Stereomicrographs by Michael J. Boyle.

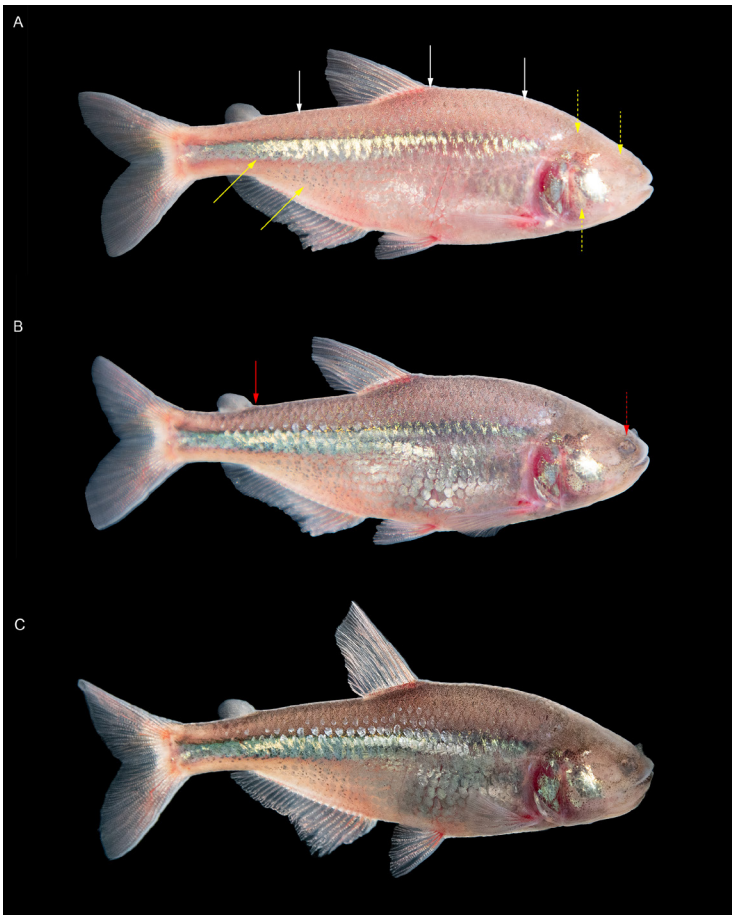

Figure 4. Comparative pigmentation of an *A. mexicanus* cavefish after exposure to high light. **A.** Adult male cavefish in right-lateral view. This fish was maintained in an aquarium under ambient light for several months. Regions of pigmentation along the dorsal side of the body (white arrows), within surface tissues of the head (dashed yellow arrows), and posterior flank (yellow arrows) point to areas for direct comparison during and after experimental treatments. **B.** The same fish as shown in 'A' after 45 days of daily exposure to high light treatments. All areas pointed to in '**A**' have increased in the level and distribution of melanin production. Other areas of melanin increase include the pigmented band dorsal to the lateral line, cells along the base of the adipose fin (red arrow), and around the mouth, and olfactory pits (dashed red arrow). **C.** The same fish as shown in 'A' (and 'B') after 72 days of daily exposure to high light treatments. Macrophotographic images by Scott Arledge and Michael J. Boyle.

pigmentation (melanophores, xanthophores, iridophores). Cavefish were not adversely affected by low pH or lowered O_2 levels; surface fish showed signs of stress under those treatments. The primary interpretation of our test results indicate that *Astyanax* cavefish exhibit the capacity to adapt rapidly to significant abiotic changes in aquatic environments. As they show relatively rapid responses to light exposure, they may have shown similar response times when adapting to cave environments in the past.

II. MATERIALS and METHODS

A. Animal model and husbandry

The research model tested for all experiments is the freshwater teleost fish, *Astyanax mexicanus* (Mexican tetra). This species consists of one surface-dwelling (SF, surface fish) morphotype, and multiple cave-dwelling (CF, cavefish) morphotypes across stream systems. All SF stocks were collected from the Guadalupe River, Texas.

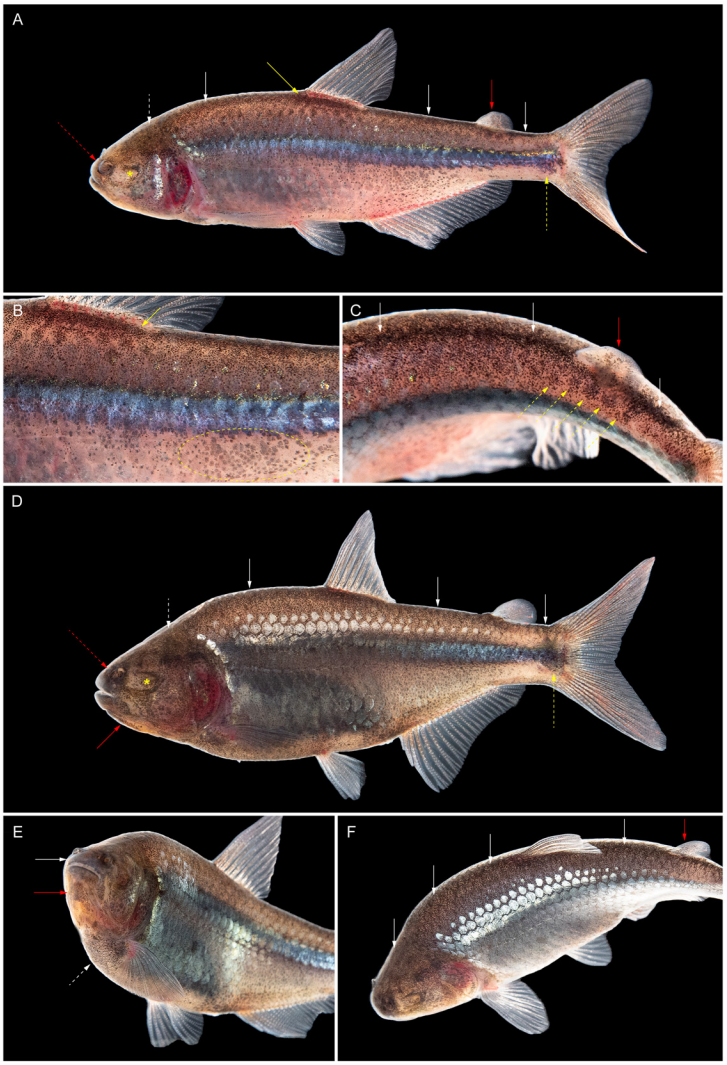

Figure 5. Increase in melanic pigmentation of *A. mexicanus* cavefish from sustained exposure to treatments of high-intensity light. **A–C.** Adult male cavefish in left-lateral views. This fish was exposed to daily cycles of high intensity, full-spectrum light for 5 months, followed by daily cycles of LED lighting for 4 months. Pronounced increases in melanin pigment production (relative to adult cavefish in Fig. 1B) are detectable along the dorsal sides and dorsal midline (**A**, **C**, white arrows), the base of the dorsal fin (**A**, **B**, yellow arrows), dorsal flanks of head (**A**, dashed white arrow), within the adipose fin (**A**, **C**, red arrows), within and around olfactory pits (dashed red arrow), posterior end of the lateral line (**A**, dashed yellow arrow), in clusters of pigment along the dorsal body (**C**, dashed yellow arrows), and in fields along posterior body flanks (**B**, dashed yellow ellipse). Numerous iridescent cells also reveal linear accumulations of green and gold iridophores along the upper edge of the lateral line (**A**, **B**) and in clusters above the lateral line. **D–F.** First generation (F1) young adult offspring of the fish in '**A**' in left-lateral views. This fish developed under exposure to high intensity, full-spectrum light for ~40 days, followed by daily cycles of LED lighting for 4 months. The F1 fish is approximately 20–30% smaller in overall size than the adult fish in '**A**'. There are general similarities in the amount and coverage of melanin pigment production between parent and offspring. However, In the F1, there is a more uniform distribution of pigment on the dorsal side and dorsal midline (**D**,**F**, white arrows), on the sides of the head (**D,** dashed white arrow), around the mouth (**E**, white arrow), chin (**D**, **E**, red arrows), underside (**D**, and **E**, white dashed arrow), olfactory pits (**D**, dashed red arrow; **E**, **F**), and posterior body flanks (**D**), and at the posterior end of the lateral line (**D**, dashed yellow arrow). The adipose fin also contains melanophores (**D**, **F**). In both fish (**A**, **D**) there is a common semi-circular pattern of melanophores following the contour of the apoptotic site of eye degeneration (yellow asterisks). Macrophotographic images by Scott Arledge and Michael J. Boyle.

The primary CF stocks were purchased commercially from Florida. A secondary stock of Molino CF was generously provided by Dr. William R. Jeffery, University of Maryland, College Park. A third stock of CF was purchased commercially from Arizona. Original cave populations for the commercial CF stocks will be identified by PCR (DNA barcoding). SF and CF are maintained and cultured within individual aquarium tanks or a recirculating aquaculture system (RAS) of multiple aquaria. Prior to entering any tanks, source water is pumped through a multi-stage, 1.0 µm reverse osmosis and deionization filtration system (Bulk Reef Supply, RO Plus 200 GPD; LiquaGen 150 GPD) into a pre-RAS sump reservoir. Chemical treatments are added to pre-RAS water, which is then pumped into the RAS system following removal of equal amounts of system water (water changes). RAS water is further treated through continuous inline mechanical (200 µm ring-filter socks) and biological (AQUAMAXX) filtration. All water (individual tanks and RAS) is UV sterilized (Aqua Ultraviolet, Bulk Reef Supply). Conductivity, temperature and pH is monitored (bluelab® guardian) continuously. Water temperature is maintained at ~75 ˚F by automated regulation of temperatures measured below (EHEIM Thermocontrol^{e}150) and above (ARCTICA Titanium Chiller) the desired set point. Water chemistry (e.g. ammonia, nitrites, nitrates) is tested weekly and adjusted as required. Breeding and pre-experimental SF and CF stocks are maintained within individual 340L/90gal aquaria with dedicated water recirculation (FLUVAL 407), and temperature regulation systems. Individual tanks also receive periodic chemically-treated water exchanges as required.

Surface fish and cavefish are fed daily with food pellets for adults (Xtreme NICE™ Aquatic Foods®) early juveniles and fry (Xtreme Nano™ Aquatic Foods®) or larvae (First Bites™ KYORIN Co., LTD). Fish are supplemented with brine shrimp cultured in the laboratory (www.brineshrimpdirect.com). Selected male and female SF and CF are maintained in pairs or small groups during, or outside of, experiments to monitor them for spawning events. When spawning occurs, embryos are collected from tank bottoms with serological pipettes and maintained in smaller tanks with regular water changes. Larval fish provide material for studies of development (Fig. 1) and for tracking morphological and genetic changes that may have been transferred to them through experimental treatments performed on their parental stages (Fig. 5). In vitro fertilization experiments are in progress to obtain reliable sources of lineage-specific materials for morphological, molecular, and epigenetic experiments during early development. All animals are treated humanely, as per the IACUC Handbook.

B. Preliminary experiments

Three preliminary experiments were performed to test for morphological responses of adult CF and SF to contrasting environmental conditions.

First experiment: a pair of adult CF (male and female) were maintained in a 76L/20gal tank under continuous daily exposure to combined illumination from wide-spectrum LED (Hydra 32 HD Reef light, Bulk Reef Supply) and full-spectrum, high-intensity (VIVOSUN 10,000K metal halide bulb, Grower's Choice) light sources. The pair of adult CF were exposed to daily cycles of high intensity,

Figure 6. Comparative pigmentation of *A. mexicanus* surface fish under different environmental conditions. **A.** Adult surface fish in left-lateral view (see Fig. 1) maintained under ambient light. Notable areas of melanic pigmentation include the dorsal side (white arrows), posterior end of lateral line (dashed yellow arrow), posterior flank of body (yellow arrow), pigmented iris (red arrow) and a prominent arrow-shaped patch posterior to the gill chamber (dashed white arrow). **B.** Adult surface fish maintained under minimal light, lower than normal oxygen (0.9–4.0 mg/L) and moderately high CO_2 (pH 5.8–6.0) for ~ 3 months. This 'treated' fish shows a visibly lower overall amount of melanic pigmentation (i.e. reduction of melanin) when directly compared with all body regions noted in '**A**'. Xanthophore pigments (yellow) are noticeably lower along the lateral line and tail in '**B**'. Macrophotographic images by Scott Arledge and Michael J. Boyle.

full-spectrum light for 5 months, followed by daily cycles of LED lighting for 4 months. A group of their progeny (F1) were exposure to the same high intensity, full-spectrum light for ~40 days, followed by daily cycles of LED lighting for 4 months. The fish used for this experiment were from the original commercial (Florida) stock of cavefish. Morphological results of the first experiment are presented in Figure 5.

Second experiment: five SF adults were maintained in a 340L/90gal tank with recirculating water and temperature regulation. Experimental conditions included minimal light, lower than normal levels of dissolved O_2 (0.9–4.0 mg/L) and moderately high levels of CO_2 (pH 5.8–6.0). Treatment was sustained for approximately 3 months. Morphological results of the second experiment are presented in Figure 6.

Third experiment: four CF (Florida) were maintained in a 76L/20gal tank under ambient light, normal levels of dissolved O_2 (6.5–8.0 mg/L) and high levels of CO_2 (pH 5.3–5.5) for 6 weeks. See Results section for descriptive observations (not shown).

CF and SF in all three preliminary experiments received a similar diet (see section on husbandry), regular water changes, and daily observations to assess health and changes in morphology.

C. Controlled experiments

Two controlled experiments were performed, each with a specific set(s) of untreated controls for direct visual and morphological comparison with the experimental groups.

First experiment: a series of eight 76L/20gal tanks each contained one pair of adult CF (Florida) or SF, consisting of a male and female for each *A. mexicanus* morphotype. Four tanks were maintained under 8-hour daily treatments of high light conditions with 2500 cm^2 LED grow lamps (Mars Hydro TS 3000) suspended ~15 cm above the water surface of the tanks (experimental group); four tanks were maintained under 8-hour daily exposure to ambient light conditions (control group). Light intensities at the water surface measured 696–702 lux at the four treatment tanks, and 36–40 lux at the four ambient tanks. All tanks were connected to the RAS water (see above). Within each set of four tanks, pairs of CF and SF alternated, providing two tanks of each morphotype under experimental and control conditions. All fish were imaged live at the start of the experiment. The experiment was run for 72 days. All fish were imaged live at the end of the experiment. One additional 76L/20gal tank containing a group of 3 adult CF (Florida) was maintained under each of the two lighting conditions to provide biological materials for genetic and molecular data (see next section). Two 38L/10gal tanks were also maintained as additional no-light (dark) controls, with one tank containing a pair of adult CF (Florida), and a second tank containing a pair of adult SF.

Second experiment: Three experimental groups of CF (Molino, Arizona, Florida) were each maintained separately in three 76L/20gal tanks, where they were exposed to daily cycles of combined illumination from wide-spectrum LED and full-spectrum, high-intensity halide light sources (see B; previous section). The experimental tanks were housed together in a separate room. Molino CF are juveniles that do not express black melanic pigmentation (eumelanin). Arizona CF are young adults that do express black melanin. Florida CF are adults from the first controlled experiment (72-day light treatments) that received additional exposure to combined illumination treatments. All three CF sources express other pigments (e.g. xanthophores, iridophores; see Figure 3). Additionally, the Molino CF group was exposed to three, 15-minute treatments per week under a 4-bulb tanning array (Sperti, FIJI SUN, KBD, Inc.) to stimulate pigment production. Control stocks for each CF group under combined illumination treatments were regularly maintained in ambient light for direct comparison with their respective treatment groups.

D. Molecular biology

Tissue and organ samples were collected on the 1st and 72nd day of the first controlled experiment (see previous section). On day 1, a dorsal subsection of the caudal fin was clipped from each CF and SF in the experimental groups, and from each CF and SF in the 38L/10gal dark tanks. Caudal fin samples were preserved in 95% EtOH at -20 ˚C. These samples will be processed for CF population identification through PCR and sequencing of barcoding genes (e.g. *MT-CO1*, *MT-CYB*, 16S rDNA). On the 1st and 72nd day of the same experiment, one adult fish was sacrificed from each of the two groups of three CF (Florida) under each condition. The dorsum at the dorsal fin, caudal peduncle, caudal fin, gill and brain were removed by dissection and preserved in RNA*later*™ (Invitrogen, Thermo Fisher Scientific) at -20 ˚C. These dissections provide molecular resources for the purification of DNA, purification of total RNA to synthesize cDNA templates for gene expression experiments, and as tissue samples for analyses with mass spectrometry. On the 1st day of the second controlled experiment with CF undergoing combined illumination treatments, dorsal subsections of the caudal fin were clipped and preserved from each of six CF (Arizona) for population identification; CF (Florida) were clipped previously. One adult CF (Arizona) was sacrificed at the end of illumination treatments to perform tissue and

organ dissections (as above), and for comparison with corresponding samples from one adult CF from a stock tank; One month after arrival and acclimation of the Molino cavefish to the ICR, one juvenile fish was preserved in 95% EtOH at -20 ˚C, and one juvenile fish was preserved in RNA*later*™ at -20 ˚C. These samples will be processed for gene-specific identification analyses, cDNA template synthesis, and mass spectrometry to assess presence or absence of chromatophores and proteins directly integral to the melanin synthesis pathway.

E. Image acquisition and processing

Macrophotographic images were captured with a Panasonic Lumix GH5 camera body, through a Cannon EF 24-70mm f/2.8L lens (camera settings are available upon request). Photographic lighting included two amaran P60c RGBWW LED Panels and a LS C300d II (Aputure Imaging Industries Co., Ltd.). Live SF and CF were individually placed within a 1.0 liter glass aquarium to record pre- and post-experimental morphology. The position of each fish within the aquarium was restricted toward one side, enabling photography of lateral, full-body profiles (Figs. 1, 4-6). Micrographs were captured with a Jenoptik Gryphax PROKYON digital microscope-dedicated camera, through a Zeiss SteREO Dicovery.V20 stereomicroscope under multiple objectives; these images were obtained as single captures, or as single image files extracted from a video series (see figure captions). Image editing and figure layouts were performed with Adobe Photoshop CC; all figures were formatted with Adobe Illustrator CC; video files were edited and extracted with Adobe Premiere Pro CC (Adobe.com; San Jose, CA).

III. RESULTS

A. General observations

Spawning events were detectable in both stock and laboratory aquariums. Prior to spawning, male and female cavefish (CF) and surface fish (SF) begin to follow each other in close proximity, and at times in parallel profiles. Their coordinated movements indicate they are a mated pair. At the peak of their 'dance-like' behavior they simultaneously release a burst of gametes (sperm and eggs) and then either move off in different directions, or repeat the event before doing so. The egg cells are semi-transparent and tan (CF) or whitish-grey (SF) in color. Embryos have an approximate diameter of 960 µm, and reside within a larger egg envelope (chorion) with a diameter of ~1100–1200 µm (Fig. 2). Time of development from fertilization to the hatching of larval fish is ~25–28 hours (hrs) at 23 ˚C (see Materials and Methods for husbandry of larval and juvenile fish). Our commercial stock of *Astyanax mexicanus* CF from Florida come from a cave-dwelling population that exhibits melanic (black) pigmentation in early and late larval stages. The commercial CF from Arizona also produce black melanin, and observations of their larvae following spawning events confirm melanic pigmentation during early development.

CF and SF under single or combined illumination treatments have not exhibited signs of stress, or either visible or behavioral symptoms of disease, during experimental periods. Similar observations have been made for both morphotypes when maintained in ambient light or in the dark. The multi-tank recirculating aquaculture system (RAS) is modifiable and accommodates a series of contrasting illumination treatments under shared, identical water conditions (chemistry, conductivity, temperature, pH). Regulation of narrow target ranges of CO_2 (pH) and O_2 are performed within individual, free-standing aquariums. We observed no obvious behavioral or physiological differences of CF or SF between these systems, or when moving fish from one system to another.

B. Pigmentation

Three chromatophores are visually identifiable on and within tissues of experimental surface fish (Guadalupe River, Texas) and cavefish (Molino, Arizona, Florida) models in this study. All surface fish specimens exhibit typical patterns of melanic pigmentation in *A. mexicanus* (see Fig. 1A). Under moderate stereoscopic magnification, patterns of dendritic melanocytes in SF (Fig. 3A–F) are observed within the head (encircling the eye, on upper and lower mouth parts, gill opercula), along the midbody (dorsum, behind gill chamber, along lateral stripe, scale margins) and within fins (e.g. adipose fin, caudal fin). The melanocytes are visible at both surface and subsurface positions along the body (Fig. 3B, C, F) and also deeper within regions of the brain cavity and heart (not shown). Xanthophores are most noticeable along the lateral stripe, in the adipose fin, and rays of the caudal fin (Fig. 1A; Fig. 3E). Iridescent iridophore pigments are primarily visible along the lateral stipe and peduncle (Fig. 1A, dashed white arrow).

Along and within body regions of the cavefish models, black melanocytes are present in almost all body domains as observed in SF, with the exception of Molino CF. However, respective levels of expression in the commercial CF (Arizona, Florida) are visibly lower (Fig. 1B; Fig. 3G). In the Molino CF, regions of yellow-orange pigmentation are spatially similar to black melanic pigmentation patterns observed in commercial CF, and SF (Fig. 1A, B; Fig. 3H–L; Fig. 4B–C; Fig. 5; see Discussion). After high-light treatments, the expression and distribution of yellow-orange pigments in Molino CF are pronounced in head, body and fin cells (Fig. 3J–L). In the Molino CF, yellow orange pigments on the body are dendritic (not shown), and these same pigments are in dorsal cells of the optic tectum, and brain (Fig. 3H, I). As shown in SF, xanthophores are also expressed along the body in all three commercial CF, and in similar locations (Fig. 1B; Fig. 3G, H, J; Fig. 4A, B; Molino not shown), as are iridophores (Fig. 1B; Fig. 3J; Fig. 4A–C; Fig. 5A–C). Additionally, all three chromatophores are observable during early development of the Florida CF model (eye cup/pre-retinal tissues, head, olfactory pit, optic tectum, dorsum, viscera) in larval and pre-juvenile stages from 5–26 days (Fig. 7F–M) and beyond. We have not yet reared Arizona CF to juvenile stages, and have not induced or observed spawning in Molino CF.

C. Preliminary experiments

Surface fish (SF) from the Guadalupe River exhibit the common suite of external morphology, coloration and pigmentation patterns (Fig. 1A; Fig. 3) observed on SF from populations found in Mexico. These include dark melanic pigmentation along the dorsum, extending from their head to the tail. They express melanophores in a band between the dorsum and the lateral line that extends from their 'shoulder' to end of the peduncle (attachment site of caudal fin) at the tail. Melanophores are also expressed within the caudal fin, extending along central fin rays at the junction of dorsal and ventral sections of the forked tail, and along upper and lower fin rays at dorsal and ventral margins. Numerous melanophores are observed across posterior flanks, along the mid-body region, on the opercula (gill covers), in a dense vertical streak posterior to each operculum, on both upper and lower mouth sections, and encircling the iris (Fig.1A). All surface fish have a pair of functional eyes.

In contrast, although melanophores are expressed to some extent within almost all analogous regions of the commercial cavefish (CF) model from Florida (Fig. 1B), the extent, density and overall pattern of melanic pigmentation is noticeably less. Their relatively size-con-

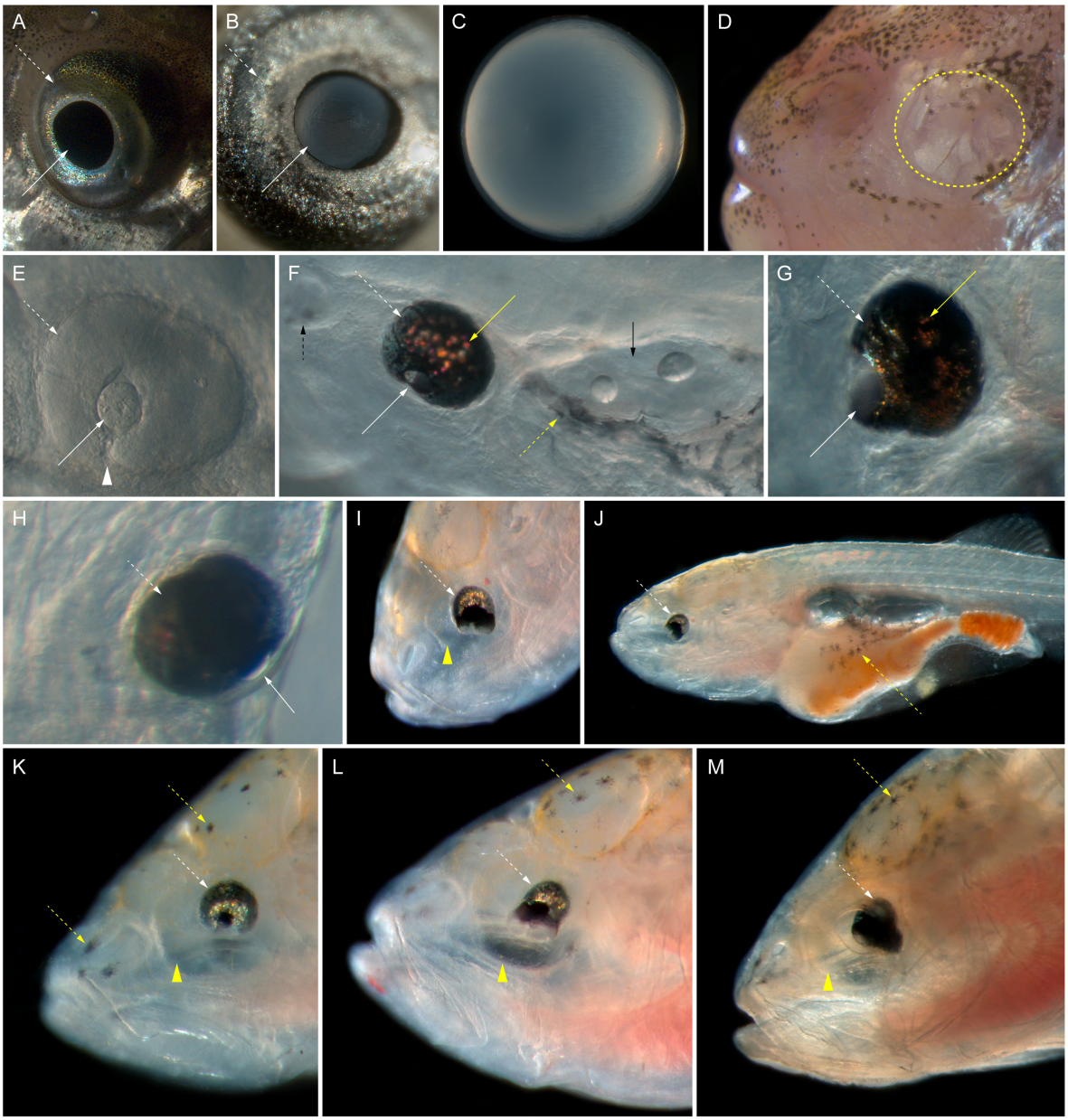

Figure 7. Morphological development and degeneration of the eye in *A. mexicanus* cavefish. **A.** The eye of a live juvenile surface fish showing a pigmented iris (dashed white arrow) encircling a dark central region containing a cornea and lens (white arrow). Dorsal to the top. **B.** Fixed eye showing a blueish lens (white arrow) within retracted iris tissue (dashed white arrow). The iris is pigmented with melanophores (black) and iridophores (iridescent dots). **C.** Isolated lens extracted from the fixed eye in '**B**'. **D.** Anterior left-lateral view of an adult cavefish showing the region of complete eye loss (dashed yellow ellipse). melanophores form a semicircle of pigmentation along the posterior edge of the eye field, which is completely closed by a cartilaginous sclera. **E.** Optic cup (dashed white arrow) and lens (white arrow) in a cavefish after 1d (day) of development. The ventral sector (white arrowhead) of the optic cup is morphologically reduced during eye formation in cavefish. **F.** left-lateral views of a larval cavefish eye undergoing apoptotic degeneration at 5d. The lens (white arrow) extends from an optic cup (dashed white arrow) pigmented with melanophores and iridophores (yellow arrow). Melanic pigment is visible in the olfactory pit (dashed black arrow) and margins (dashed yellow arrow) of the otic vesicle (black arrow). **G.** The lens has dropped into the ventral sector of degenerating optic cup (dashed white arrow) at 5d. Iridophores (yellow arrow) cover the optic cup. **H.** Lens (white arrow) and cornea attached to the optic cup (dashed white arrow) of a larval cavefish at 7d. **I.** Left-lateral view of larval cavefish head of at 15d with a remnant of the pigmented optic cup (dashed white arrow) nested in the center of the orbit (yellow arrowhead). **J.** Left-lateral view of the larval fish in '**I**' at 15d, showing the fixed angle of the degenerate optic cup (dashed white arrow) and melanin pigmentation (dashed yellow arrow) on the larval body covering the viscera. **K.** Left-lateral view of larval cavefish head at 19d. Melanin is visible around the olfactory pit anterior to the eye, and within the left lobe of the optic tectum (midbrain) that is dorsal to the eye (dashed yellow arrows, respectively). The optic cup (dashed white arrow) is split open on its ventral side and fixed within the orbit (yellow arrowhead). **L.** Larval cavefish at 19d, with similar orientation and patterns of pigmentation and degeneration as shown in '**K**'. Note the ventral extrusion of pigmented and non-pigmented matter from the optic cup (dashed white arrow). **M.** Left-lateral view of a larval cavefish head at 26d of development. The optic cup and its contents (dashed white arrowhead) have been displaced into the orbit (yellow arrowhead). Melanic pigmentation on the optic tectum has increased in the number and spatial coverage of dendritic melanophores (dashed yellow arrow). Overall, the degenerative remnants of cavefish eyes remain visible during larval development through late stages of juvenile growth (not shown). Stereomicrographs in A–D by Michael J. Boyle; Stereomicrographs in E–M were prepared by M. J. Boyle, and obtained from video frames produced by Scott Arledge.

stricted melanophores are spread widely across the body presenting the overall appearance of a semi-transparent, orange-colored cavefish morphotype, distinct from the SF. Both morphotypes are genetically interfertile. This CF, as in all other known *A. mexicanus* CF, does not have a functional eye, or any of the major components of eye anatomy observed in the SF (Fig. 1B). Differences between functional anatomy and appearance of SF and CF morphotypes provide a foundation for the design and implementation of preliminary and experimental treatments, and for the direct comparison of results in this study.

Observations from the first preliminary experiment reveal an increase in spatial coverage of melanic pigmentation across all body regions of CF (Florida) where melanin is expressed in SF (Fig. 5A–C). These areas include sides and center line of the dorsum, at the bases of dorsal, adipose and caudal fins, on posterior flanks of the body, on the head, olfactory pits and gill opercula. Distinct patches of melanin pigment align with the positions of scales along both sides of the dorsum in the adult CF, and also in their F1 progeny, although less distinctly (Fig. 5D–F). The distribution of melanic pigmentation in F1 CF (Florida) is increased, with a comparatively broader spatial pattern of melanophores than in parent fish. Additionally, melanophores are concentrated along ventral undersides of the F1 (Fig. 5D, E), but do not show similar expression in the analogous ventral regions of their adult form. In both the adult and F1, melanophores are expressed in a semi-circular pattern around the areas where functional eyes are located in SF (Fig.5A, D). Another observable chromatophore pigment includes iridophores that are expressed along the dorsal side of the pigmented lateral stripe and along sides of the dorsum in the adult (Fig. 5A, B). Iridophores are noticeable where the lateral stripe meets the caudal fin, and on lateral sides of the head and opercula in F1 cavefish. Xanthophores and iridophores are both observed along fin rays of the caudal fin in the adult and F1. Among the F1 progeny, there is a noticeable range in the amount, distribution and overall expression level of melanophores, as also observed with the xanthophores and iridophores.

Observations from the second preliminary experiment show that when surface fish (SF) are exposed to conditions of lower oxygen and pH levels, their behavior and morphology are altered. Behaviorally, these experimental SF exhibit comparatively rapid movements of mouth and gill opercula, they appear disoriented, and at times collide with walls of aquarium (see Discussion, Pleiotropy and genetic integration).

Morphologically, there is noticeable reduction of melanic pigmentation along the dorsum, lateral stripe, head, and posterior flanks of the body (Fig. 6A, B). Xanthophoric pigments are visibly less pronounced compared to levels observed in non-treated SF (Fig. 6A). Pigmentation also appears uniform and considerably lighter around the surface of the iris, which surrounds the lens region (Fig. 6B).

Results of the third preliminary experiment were not photographed. After 6 weeks of treatment under high levels of dissolved CO_2 (pH 5.3–5.5), melanic pigment in these CF (Florida) was clearly reduced from non-treated CF stocks. The expression of melanin in all regions of the head, along the dorsum, posterior flanks of the body, and within rays of the caudal fin, was undetectable without a microscope. Additionally, there were no observed indications of stress, accelerated respiratory activity, agitated swimming or complications with behavioral navigation within the aquarium (see Discussion, Pleiotropy and genetic integration).

D. Controlled experiments

Results of the first controlled experiment show an increase in the expression of melanic pigmentation. After 45 days of light treatments, the distribution, densities and expression levels of melanic chromatophores is higher in all regions where melanin was observed in the same CF at the start of the treatments (Fig. 4A, B). Notable areas of melanin concentration include the head (olfactory pits, gill opercula), dorsum (bases of dorsal and adipose fins), and posterior dorsal midlines and flanks. The same contrasting pattern of pigmentation is observed when comparing the treated fish directly with untreated CF (Florida) in our stock tanks. After 72 days, the pattern of melanin expression is similar but more intense in the same adult cavefish as observed after 45 days of light treatment (Fig. 4B–C). In general, the underlying orange coloration of CF was diminished over the duration of treatment. All other adult CF (Florida) in this experiment showed a similar increase in chromatophore expression. The general positions of iridophores along the body line are similar after 45 days, and after 72 days, although the amounts or levels of these iridescent chromatophores appear to have increased in those positions. Melanophore and xanthophore distributions along rays of the caudal fin are visually similar from day 1 to day 72. Quantitative differences in pigment expression have not been measured.

Results of the second controlled experiments show that pigment levels and distributions have increased in all three CF groups (Molino, Arizona, Florida). In particular, iridescent chromatophores (iridophores) show an increase in level of expression and spatial distribution across the bodies of Molino cavefish, relative to their untreated stocks. And, xanthophore numbers and distributions in the head, body and fins of Molino CF exhibit increases (Fig. 3H–L; see discussion). Melanic pigmentation on and within tissues of the other cavefish (Florida, Arizona) models also increased. When compared to the overall coverage prior to the combined illumination treatments, and specifically in comparison with previous 72d light treatments, there was high contrast in pigment expression across the body and head. The level and pattern of increased melanic pigmentation in treated fish was substantially different from their respective stocks.

IV. DISCUSSION

How creatures adapt and diversify through time are central questions about the origins of animals on earth. As with Darwin's celebrated theory, *On the Origin of Species by Means of Natural Selection* (1859), Neo-Darwinian Theory (NDT, 1895) and the Modern Synthesis (MS, 1942) share a common perspective: adaptation can be, and is, caused by natural selection. All of the iterations of Darwinism promote environmental (*natural*) selection of mutation-derived trait differences as the primary driver of adaptation in all animals. Because of this, externalism has become the fundamental interpretive framework for all mainstream biology (Lewontin 1983; Gould 2002). And although the Extended Evolutionary Synthesis (EES, 2010) amends externalism with newer "organism-centered" predictions that include 'epigenetic inheritance', 'ecological inheritance' and 'non-Mendelian inheritance', natural environments are ultimately given agency as the inducers of adaptation, speciation and diversification. In direct contrast to conferring transformative agency upon natural, environmental or external resources, we continue to advocate for recognition of the only known creative power – Jesus! As introduced above, ICR's model of continuous environmental tracking (CET) infers that all organisms were created with purpose and intention, and are therefore divinely 'engineered' to adapt rapidly and appropriately to every environmental condition they encounter. If and when those conditions change, they are prepared to respond. Here, we discuss preliminary results of testing the cavefish model of adaptation, consider

molecular, genetic and physiological mechanisms that may account for those results, in cavefish, and other organisms, and present both ongoing experiments and future directions for research with the *A. mexicanus* model organism.

A. Reproduction and development provide experimental access to early mechanisms of adaptation

Development from fertilization through embryogenesis to the formation of larvae is considered one of the most critical periods in the life of fishes, as also observed in most major lineages across the Metazoa. During this period, numerous genomic elements are expressed, and distinct types of cells, tissues and organ-systems of the body plan become specified, differentiated and functional. It is also an influential interval of time when genetic and epi-genetic architectures are inherited, and/or initiated in a new generation. Thus, molecular and cellular signatures of adaption(s) would likely be detectable during development. Furthermore, the physical scale of cell types (neuron, blood), tissues (epidermis, muscle) and organs (brain, eye) are optimal for experimental manipulation and micrographic evaluation and imaging during early stages of development (Fig. 2).

We have begun to take advantage of spawning events from both surface fish and cavefish morphotypes; however, such events have been unpredictable. Therefore progress is underway to establish breeding tanks and associated infrastructure for collecting, rearing and handling embryos and larvae based upon published protocols (Riddle et al. 2018; Baumann and Ingalls 2022). As mentioned above, the primary targets of experimental research include changes in pigmentation (next section) and restoration of sight, both of which are predicted by CET as repeatable and reversable adaptations. Through applied techniques in molecular biology, riboprobe synthesis, and both whole mount in situ hybridization (WMISH) and immunohistochemistry (IHC), we will initiate experiments to characterize gene expression during embryonic development of the visual system. This approach includes a microscopic comparison of eye development and eye degeneration in surface fish and cavefish, respectively. Primary molecular targets include genes that are shown to regulate optic cup and stalk development (*pax6*, *shh*, *pax2*), genes effecting loss and rescue of retinal tissue (*Fgf8*, *Lhx2, rx3*), and genes that are essential during lens formation (*sox2*, *cryaa*, *crybb1c*, *cryba1l*) that inhibit the apoptotic process following initial eye development in late embryonic and early larval stages (Yamamoto et al. 2004; Pottin et al. 2011; Krishnan and Rohner 2017; Sifuentes-Romero et al. 2020; Warren et al. 2021).

There is a four-fold purpose in pursuing these and other molecular experiments. First, we intend to repeat multiple experiments that were published from the conventional perspective, and thus motivated by evolutionary thinking. This is not a redundant exercise, for we approach our questions from a completely different worldview with an original model that is formulated to test adaptations as pre-engineered systems in accord with that worldview. Second, the CET model proposes that adaptative mechanisms integrate multiple functions across different scales (molecular, cellular, physiological). For example, the loss of eyes in cavefish is not organ-specific, but involves the dual effect of *shh* expression on eye loss with a concomitant role in the enhancement of feeding anatomy (Yamamoto et al. 2009). Third, eye loss or eye restoration should be investigated to assess comparative gene expression patterns between generations. This will require WMISH of 'eye genes' within early developmental stages obtained from parental cavefish reared under high light treatments, the progeny of their subsequent F1 generation, the progeny of untreated cavefish from the same strain, and similar stages of development from different strains (e.g. commercial, Molino). And fourth, because conventional research with the *Astyanax* model is focused on "the gain and loss of traits" in cavefish (Jeffery 2001, 2020), random genetic mistakes are commonly invoked to explain those changes, whether they are regressive or constructive. Although, we also find conventional explanations that are illuminating: "To our knowledge, there is no case of a viable vertebrate embryo that would never develop eyes." (Pottin et al. 2011). This is partly acknowledged in the fact that, "tight temporal regulation of signaling systems during early embryogenesis has a crucial impact on the size and shape of a structure . . . the neural plate-derived component of the CF eye defect." (Pottin et al. 2011). Is there another explanation for eye loss, and the "gain and loss" of other traits in cavefish? Through molecular developmental experiments we aim to assess the conventional narrative that loss-of-function mutations (e.g. indels, frameshift, transposition) and natural selection have produced the natural cave mutants of *Astyanax mexicanus*. We think that CET would account for a series of highly-integrated molecular and developmental mechanisms regulating adaptive eye loss and restoration.

B. Patterns of pigmentation demonstrate rapid and distinct responses to environmental change

Experimental targets of chromatophore expression include light-induced changes in the amount, density and pattern distribution of melanophores – the melanin-producing chromatophores in fish. The size and shape of melanophores is known to be correlated with the extent of aggregation or dispersal of their melanosomes, which are the pigment-containing organelles within melanophores. In surface fish, melanosomes are abundant and widely dispersed within melanophores. In all cavefish that have been thoroughly examined, melanophores are detectable, even when they do not convey an obvious pigmentation pattern, or even lack melanin (see below). This leads many investigators to describe cavefish as unpigmented, having reduced pigmentation, or exhibiting a loss of melanin pigmentation (Klaassen et al. 2018; Jeffery 2020). The term 'albinism' is also utilized to indicate the absence of melanin or low production of melanin. But it does not imply the absence of pigmentation produced by other chromatophores. Before we initiated any experimental treatments, we observed a detectable distribution of black pigment in the form of small dendritic (stellate or star-shaped) melanophores in our commercial cavefish stocks (Fig 1, Fig. 3A–G, Fig. 4A, Fig. 5). The expression of melanin is notably low in these cavefish, and geographical locations for the cave systems of our commercial cavefish – purchased through suppliers in Florida and Arizona – have not yet been identified. However, they do possess both epidermal and subdermal melanocytes across their bodies that produce melanin pigment, as well as distinct expression patterns of pigments from other chromatophores (xanthophores, iridophores). It is possible that sources of ambient and/or incandescent room lighting on proprietor stocks, and on our own stock tanks after purchase, may have induced some pigmentation prior to experimentation.

When we treat these cavefish with daily cycles of combined high intensity, full-spectrum and LED light sources, there is a pronounced increase in melanin pigment production. The pattern does vary among cavefish under treatment, which may indicate that source fish stocks are a genetic mosaic of cavefish populations, and/or there are undisclosed or untraceable patterns of hybridization events across surface fish and cavefish leading to the stocks we possess (Fumey et al. 2018; Jeffery 2020; Moran et al. 2022). Yet the response patterns are unmistakable. Those patterns include increased melanic pigmentation in the adult fish over short periods of weeks (Fig.

4), and continual increase in the sizes and distribution patterns of melanophores with continued treatment for several months (Fig. 5). If melanic pigmentation is an adaptive trait, then our observations support the possibility that it is not only a rapid response to an environmental stimulus, but also that pigmentation is a reversable and potentially repeatable trait in cavefish (Fig. 6). Note, we observed melanic pigmentation increases along the dorsum, at the bases of fins, around the mouth, olfactory pits and gill opercula. And there is a consistent increase in subdermal pigmentation surrounding the brain – multiple cell layers below epidermal cells – in both cavefish stocks. This deeper pattern suggests a requirement for protection of the optic tectum and its primary cavity, and that the other patterns likely provide a similar protective function. Furthermore, when we allowed the F1 generation to develop under the same light treatment, they produced a more pronounced pattern of increased pigmentation in the same areas as their parent cavefish within less than half the time (Fig. 5). These F1 fish also expressed melanic pigmentation in new areas, including undersides of the head and belly. Their visible increase in the density of melanophores may be due in part to their concentrations within and upon a smaller body, although specific areas of comparative increase suggest otherwise. Collectively, the F1 cavefish progeny exhibit a putative phenotypic transition between their original untreated commercial cavefish stock, and the *A. mexicanus* surface fish morphotype (Fig.1, Fig. 5).

We also maintain a stock of Molino cavefish, and have reared them from post-larval stages to early juveniles. These fish were obtained from cultures research stocks (see Materials and Methods), and do not exhibit any evidence of melanic pigmentation (Fig. 3H–L). They were bred from one of only two original known cavefish populations that exhibit melanic albinism – a complete absence of melanin (Protas et al. 2006; Klaassen et al. 2018). Both Pachón and Molino cavefish are missing part or all, respectively, of an exon in the *oculocutaneous albinism type 2* (*oca2*) gene and the inferred 'deletions' are not from the same exon (see below). Importantly, these cavefish do have melanophores (melanoblasts); however those cells are not producing melanin pigment (Klaassen et al. 2018). As stated above, our Molino cavefish juveniles do not produce black melanin, but they do produce both yellow-orange and iridescent pigments on and within their body (Fig. 3H–L). We intend to purify and analyze *oca2* allele sequences from multiple specimens within our Molino cavefish stocks, and all other stocks (SF and CF), and also submit samples for mass spectrometry to identify the yellow-orange pigments and any other pigments they produce. Under a high-magnification stereomicroscope, the yellow-orange pigments appear to be small dendritic xanthophores; the identity of iridophores is not in question. We have also treated our Molino cavefish juveniles with high intensity, full-spectrum and LED light sources, along with bi-weekly pulses of 15-minute tanning treatments. This combination of light treatments has notably increased the amount and distribution of yellow-orange pigmentation in multiple regions, including oral, olfactory, brain-cavity, fin and in multiple epidermal cells and tissues (Fig. 3J–L). There has been no evidence yet of melanin production, although prominent yellow pigmentation patterns increased in similar locations where melanin increased in commercial cavefish under the same treatment protocols. Are they performing similar or related functions in cavefish as melanin? Does pheomelanin or another pigment (e.g. xanthophores) provide an alternative response to downregulation of black or brown melanic pigmentation? Thus far, pheomelanin has not been definitively confirmed in fish (Adachi et al. 2005; Kottler et al. 2015; Cal et al. 2017; Stocker et al. 2020). Comparative histology, mass spectrometry and developmental transcriptomics are appropriate next steps in order to confirm the presence or absence of eumelanin and other pigments responding to treatments in cavefish.

Melanin synthesis and correlated biosynthetic pathways are fundamental and extensive across animals. Functionally, pigmentation is clearly required in all vertebrate classes inhabiting sunlit environments, and is apparently either nonessential or necessarily downregulated in cave habitats, as observed among global cavefish varieties and a broad diversity of terrestrial representatives found within troglobitic communities (White et al. 2019). In the context of pigment-specific cave adaptations the most pertinent questions include (1) whether melanin synthesis is truly 'lost' and irreversible in cavefish, or (2) is the downregulation of melanin production one of several reversible traits within a system of adaptive responses by cavefish to changing environments? Molino and Pachón are the only two *A. mexicanus* populations with experimentally characterized "loss-function-mutations" that inactivate *oca2* genes in cavefish (Protas et al. 2006). The proposed mutations have been traced to separate deletions of exons 21 and 24 in Molino and Pachón cavefish, respectively. In both cases, these 'mutations' cause albinism (no eumelanin production), with no evidence of genetic complementation through reproduction by fish that possess them. And although both of these cavefish populations have melanophores with functionally intact melanosomes, the first substrate in the melanin synthesis pathway – conversion of L-Tyrosine to L-DOPA – is blocked (Bilandžija et al. 2013). Melanophores can be induced to produce melanin in these cavefish with the addition of exogenous L-DOPA (Klaassen et al. 2018). The product of the *oca2* gene "encodes a putative 12-pass membrane protein" (Bilandžija et al. 2013) that is considered "solely responsible for the evolution of albinism in multiple cavefish populations" (Klaassen et al. 2018). Of special interest, an *oca2* deletion in exon 24 through the 3′ UTR (untranslated region) was also found in captive-bred Micos cavefish where it causes albinism (Gross and Wilkens 2013). It is the same deletion as found in albino Pachón cavefish. However, this particular loss-of-function *oca2* allele does not cause albinism in the wild population of Micos cavefish (Gross and Wilkins 2013). Their explanation? "Perhaps a loss-of-function *oca2* allele harbors a 'cryptic' selective value for cave-dwelling fish". The same authors also imply that, "albinism can arise remarkably quickly in captive-bred fish drawn from cave populations that do not express albinism in nature." (Gross and Wilkins 2013). And it has been suggested that many cave-related traits can appear within a single generation by phenotypic plasticity (Bilandžija et al. 2020). These interpretations suggests that *oca2* may not act alone, but within larger networks of rapid and distinct responses to environmental change. Accordingly, although the "loss or modification of melanin" may indicate the action of single genes, reduction in the overall numbers of melanophores appears to require the action of "ten or more genes in each population" (Borowsky 2018). Thus, there is much more to the story on how this and other genes influence a diversity of traits in cavefish.

C. Genomic and genetic regulatory architecture indicate multi-level controls underlying CET

1) Epigenetic mechanisms and transposable elements

In a recent groundbreaking study on epigenetic mechanisms of eye loss in *Astyanax* cavefish (CF), the authors state that, "recent sequencing of the Pachón cavefish genome and other studies revealed no inactivating null mutations in essential eye development genes" (Gore et al. 2018). Null 'mutations' are defined as changes that interrupt gene transcription (nonsense or frameshift) or lead to the absence of gene products**.** Without such 'mutations', epigenetic

regulation and modification of the genome are most likely to be involved in cavefish eye degeneration. Thus, mechanisms for shutting down eye development in cavefish as an adaptive response to cave conditions must be due to built-in systems that *regulate* traits at the level of the genome. One of the more easily determined epigenetic modifications involves cytosine methylation where specified regulatory sections of the genome surrounding genes will have methyl groups attached to cytosine nucleotides along the genetic code. This type of site-directed methylation effectively downregulates or silences certain types of gene activity. Gore et al. (2018), determined that methylation-based epigenetic silencing was an adaptive mechanism for eye degeneration in Pachón cavefish. By performing parallel analyses in both blind cavefish and surface fish, and using zebrafish (*Danio rerio*) as a comparative sighted fish model, they discovered that DNA methylation of specific genomic sites confers eye-specific gene repression, and also regulates early eye development. Also, multiple cavefish genes with "promoter hypermethylation" were reported to be associated with eye disorders in humans and mice (Gore et al. 2018). These epigenetic data suggest that blindness in cavefish is inherent, pre-programmed and adaptive. Therefore, 'mutations' (random genetic errors) should be ruled out as having any selective value as credible explanations for eye loss in *Astyanax* cavefish. And in relation to our observations of rapid melanic pigmentation in commercial cavefish when exposed to light, it is probable that removal of methylation from euchromatin surrounding regulators of melanin production (e.g. *oca2*) may serve to upregulate the melanin synthesis pathway. It does not imply that the same epigenetic mechanism is active in cavefish where *oca2* exon deletions have been confirmed (Klaassen et al. 2018). However it does mean that epigenetic methylation or acetylation patterns can inactivate or activate, respectively, specific gene loci (epialleles) or multiple phenotypic traits that are deployed in different environments (Cubas et al. 1999; Bertozzi and Ferguson-Smith 2020). In known cases of epigenetic inheritance, distinct regions of hypermethylated chromatin can be transferred between generations by epialleles. To assess generational transfer of genomic signatures of eye development and pigment regulation, we will need to characterize pan-epigenetic and specific epiallelic states in adult and larval cavefish, and in our Molino cavefish where melanic albinism is observed.

Genetic deletions and insertions by transposable elements may indicate intentional regulatory events. With their known ability to rewire regulatory circuits (Feschotte, 2008), transposons could initiate or deactivate particular traits in cavefish during development. Transposable elements (e.g. *Alu* SINES), contain many binding sites for transcription factors that allow them to regulate developmental processes (Polak, 2006; Lynch, 2011). Indeed, research has shown that a very high proportion of cis-regulatory changes associated with development and adaptation are connected with transposition events (Chenais, 2012). The Zebrafish model genome has provided a wealth of information for not only animal genetics in general, but also *Astyanax* research as well (Chang, 2022). Recent research on the spatial and temporal expression of transposable elements during Zebrafish development provides a valuable reference resource for interpreting genomic regulation in cavefish. As developmental programs of zebrafish and *Astyanax* are very similar, we anticipate exploiting that resource for interpreting cavefish variation in developmental and adaptive morphotypes, especially as they relate to transposon-mediated genomic regulation. A number of specified changes to the genome may be occurring during the development of *Astyanax* cavefish. In the literature, we found one study that performed genome sequencing of a variety of different *Astyanax* cave morphotypes with up to nine different individuals sequenced per morphotype (Warren, 2021). In the supplemental data, alignments of *oca2* loci showed three specific deletion events (see section B) thought to be associated with loss of melanin pigmentation. Two of the smaller deletions in the Tinaja and Pachón morphotypes had specific deletion signatures showing distinct five and three prime (5´and 3´) boundaries. Another morphotype (Molino) had a larger *oca2* deletion for which the specific boundaries have not yet been defined. These signatures may be the result of targeted transposon-mediated deletion activity associated with cave adaptations. Specific genome modifications that are regulated by transposons, including deletion events, are a well-documented phenomenon associated with a variety of developmental processes in animals (Bourque, 2018). At the ICR, genetic research on molecular mechanisms that may regulate adaptive traits in cavefish will include transposon activity.

2) Pleiotropy and genetic integration

Another important component of trait development in cavefish comes from observations that multiple traits are influenced by the expression of single genes. The term 'pleiotropy' refers to the production of two or more unrelated effects produced by one gene. Our model of continuous environmental tracking (CET) would lead us (1) to question whether the effects are truly unrelated, and (2) to interpret the connectivity of multiple trait-specific effects as being coordinated, pre-programmed responses to changing environmental conditions. A prime example of inferred pleiotropy in cavefish describes the coupling of eye degeneration with enhancement of feeding anatomy (Yamamoto et al. 2004, 2009). Underlying the connectivity of these traits is a network of gene expression patterns. Upregulation or "hyperactivity" of the sonic hedgehog (*shh*) gene in the embryonic neural plate and dorsal anterior midline of cavefish meditates the expression levels of other genes. Specifically, increased levels of *shh* downregulates (represses) expression of *pax6* and upregulates (induces) the expression of *pax2* and *vax1* (Yamamoto et al. 2009; Krishnan and Rohner, 2017). The relative expression of the *pax6*, *pax2* and *vax1* genes under the influence of hyperactive *shh* initiates the process of apoptosis (cell death) in the lens and retina, which causes degeneration and loss of eyes (Fig. 7). Importantly, correlated increase of *shh* expression in oral ectoderm and pharyngeal endoderm, also during embryonic cavefish development, induces the enhanced development of tastebuds and jaws (Yamamoto et al. 2009; Jeffery 2020). Following the onset eye degeneration, these cavefish embryos produce more tastebuds, at a faster rate (Varatharasan et al. 2009), and larger jaws than surface fish, which have functional eyes. And when *shh* is experimentally overexpressed in surface fish, there is a similar coupling of eye degeneration with enhancements of the gustatory system (taste buds and jaws) as observed in cavefish (Yamamoto et al. 2009). This complex pleiotropic effect is interpreted as a "developmental trade-off between these regressive and constructive traits" (Jeffery 2010). From our perspective of CET, coupled, coordinated, purposeful responses of vision and feeding within cave environments, both of which are clearly adaptive traits, would not imply some form of "trade-off" through an unguided natural mutation-selection process. Especially when such traits are also verified through reciprocal experiments with surface morphs of the same species. But there is more to pleiotropy and supposed 'trade-offs'. As mentioned above, reduction of melanophores in cavefish is controlled by *oca2* (Protas et al. 2006; Klaassen et al. 2018), along with the melanocortin-1 receptor gene (*mcr1*), and others (Gross et al. 2009). In *Astyanax* cavefish, the melanin and catecholamine synthesis pathways diverge after conversion of L-DOPA. With a

'loss-of-function' mutation of *oca2* in melanic albino cavefish (see Fig. 3H–L), the melanin pathway is interrupted prior to tyrosinase function, which prevents melanin synthesis (Bilandžija et al. 2013). This increases the availability of L-tyrosine, dopamine and norepinephrine in pre-feeding larval cavefish, which in turn increases the level of catecholamines (CAT) in the brain and kidneys, relative to surface fish (Bilandžija et al. 2013). As with the *shh* pathway, the CAT pathway could promote adaptive physiological and behavioral traits in cave environments, and therefore provides another inferred example of pleiotropy in *Astyanax*. Furthermore, *oca2* mutant surface fish present a pleiotropic function with duel effects on albinism and sleep loss (O'Gorman et al. 2021). This study implies that *oca2* has yet another role as a regulator of "adaptive evolution" in cavefish (O'Gorman et al. 2021). The trend of uncovering coupled adaptive processes in the cavefish model is growing. This will likely challenge conventional science to reconcile random, mutational, evolutionary trade-offs with inferences for purposeful, organism-centered deployment of complex adaptive traits, of which most, or all, will be confirmed to be experimentally reversible. Are pleiotropic events truly selective gain and loss modalities?

> Tradeoffs also link other trait gains and losses. The relationship between the olfactory and lens placodes is impacted by a tradeoff controlled by Shh, Fgf8, and BMP4 signaling, antagonism between eyes and number of teeth may be controlled by Fgf8, BMP4, and pitx2, the enlargement of the hypothalamus is mediated by re-deployment of cells from the ventral retina, and VAB, and increased cranial neuromast density may be facilitated by the extra space created by eye loss. The precise mechanisms responsible for sensory trait linkages are still poorly understood. (Jeffery 2020).

From an engineering perspective, "trait gains and losses", "tradeoffs", "antagonism", "re-deployments" and "sensory trait linkages" would actually point to highly-integrated, innate adjustments that are pre-programmed responses by organisms to changing environments. In other words, coupled pleiotropic effects reflect built-in adaptive mechanisms, not fortuitous unguided evolutionary by-products of natural selection. To use Dr. Jeffery's own words, "precise mechanisms" are certainly "responsible for sensory trait linkages" within cavefish and surface fish. However, they will *remain* "poorly understood" if their origins and functionality are pursued within the mutation-selection paradigm.

With regards to adaptation, interpretations from continuous environmental tracking (CET) necessarily include the premise that organisms become acclimated to their environments. When traits are coupled, or multiplexed as described above, then those trait adjustments regardless of their direction (e.g. reduced, latent, enhanced) contribute to the process of acclimation. Furthermore, we should expect to find evidence that trait adjustments are integrated across molecular, genetic, physiological and anatomical scales. This would also apply to a broad number of traits and their genetic regulatory mechanisms that have yet to be investigated. In our second preliminary experiment, surface fish (SF) were maintained under low light conditions at lower than normal levels of dissolved O_2 (0.9–4.0 mg/L) and moderately high levels of CO_2 (pH 5.8–6.0) for 3 months. These fish were agitated, bumped into aquarium walls on multiple occasions, and at the lowest O_2 levels they were 'gasping' for oxygen – gill opercula were flapping in coordination with rapid jaw movements. These fish also began to exhibit reductions in pigmentation around the iris, and along the dorsum and lateral flanks of the body (Fig. 6). In contrast, cavefish (CF) in our third preliminary experiment were maintained under ambient light, normal levels of dissolved O_2 (6.5–8.0 mg/L) and high levels of CO_2 (pH 5.3–5.5) for 6 weeks. These CF showed no outward signs of stress in feeding, breathing, swimming or navigation. Although O_2 levels were not intentionally low, pH levels were set low to emulate aquatic pH levels within natural cave systems. The pH setting was derived from expected limestone mineral contributions ($CaCO_3$) to the water, suggesting acidic levels in karst caves where CF are found. Accordingly, the CF in our experiment exhibited behavior indicative of acclimation to low pH, as predicted. However, the SF exhibited no evidence of acclimation to low oxygen or darkness. Several investigations provide fundamental examples of coupled trait adjustments that clearly point to the necessary process of acclimation in cave environments. In addition to the absence of light and limited food resources, karst caves commonly contain low oxygen environments – regions of hypoxia. Multiple studies report that *Astyanax* cavefish "likely consume less oxygen than surface fish" through "stable oxygen consumption" across a 24-hour day (Boggs and Gross 2021). Thus, cavefish are hypoxia-tolerant or hypoxia-acclimated. Compared with *Astyanax* surface fish, molecular and anatomical evidence supporting acclimation in karst caves include increased gill size for more efficient gas exchange, greater numbers of mature red blood cells (erythrocytes) during development, higher expression levels of *hemoglobin subunit adult alpha 1* (*hbaa1*), genes involved in "oxygen transport" and "oxygen binding", and resistance to hypoxia during "both development and adulthood" (Boggs and Gross 2021). We also learn from van der Weele and Jeffery (2022) that cavefish adjust to hypoxia with increased "erythrocyte development and constitutive overexpression" of multiple hypoxia-inducible factor one (hif1) gene subunits, and demonstrate the capacity to "carry and distribute essential oxygen to tissues and organs early in development". Furthermore, cave populations not only "harbor significantly higher blood hemoglobin concentrations" than surface fish, but also possess "significantly larger erythrocytes" than surface fish (Boggs et al. 2022). When compared with surface fish, these hemoglobin-rich erythrocytes bind, transport and deliver more oxygen per blood cell, thus optimizing respiration at genetic, molecular, cellular and physiological levels within hypoxic environments.

The anatomy, morphology and function of their hearts are optimized as well. *Astyanax* cave-dwelling morphs exhibit a slower heart rate than river-dwelling morphs, with "shape and size differences of the heart" arising during early development, "suggesting that such traits are genetically determined" (Tang et al. 2018). Also during early development, there are noticeable differences between CF and SF in heart size, morphology, beating frequency, melanophores, and adipocyte cells, and they all "become increasingly apparent over life" (Tang et al. 2018). Further, cavefish exhibit spongier heart morphologies that correlate with rounder ventricles and lower wall-to-trabecula area ratios. According to Tang et al. (2018), "a heart with more trabeculae has a larger surface area exposed to the blood." And to emphasize the genetics behind many of the differences listed above, Tang et al. (2018) suggest there is an uncoupling of heart-related phenotypes "with atrial size and adipocyte number similar to surface fish, and ventricular size, shape and sponginess similar to Pachón [cavefish]". From all of the essential systems described above, we are told that *Astyanax* cavefish have evolved hypoxic adaptations *as a consequence* of life in a low-oxygen environment. Clearly, such a complex series of highly-specified adjustments must be pre-planned and integrated on multiple scales in order to facilitate the stringent mechanisms of gas exchange and circulation in hypoxic environments. *Astyanax* cavefish function efficiently with this circulatory

system, along with a profusion of other complex organ systems under extreme environmental conditions. It is incredulous to think that a random, unguided mutation-selection model of evolution could cobble together all functional components with such precision to establish this irreducible suite of adaptive traits, let alone create all of their precursor lineages, and more than ~30,000 teleost species found worldwide. What's more, almost all of the adaptations that enable these cavefish to thrive and reproduce within limestone caves are, and would have to be, promoted as evidence of convergent evolution.

3) Convergent evolution or engineered adaptability?

There is a growing trend in evolutionary biology to infer 'convergence' whenever similar character states (traits) arise *independently* between lineages and/or species. Such inferences most often derive from genetic, phylogenetic, geographic and biological analyses that incorporate estimates of time. Most often, phyogeographic or phylogenetic character mapping strategies are involved (Avise 2000). One of the world's leading college textbooks of biology defines convergent evolution as: "The evolution of similar features in independent evolutionary lineages"(Urry et al. 2020). Twenty-five years earlier, that textbook defined convergent evolution as: "The independent development of similarity between species as a result of their having similar ecological roles and selection pressures"(Campbell 1996). One scientific dictionary defines 'convergence' as: "The evolution of unrelated species occupying similar adaptive zones, resulting in structures bearing a superficial resemblance" (King and Stansfield 2002). A second scientific dictionary defined convergence as: "The independent evolution of structural or functional similarity in two or more unrelated or distantly related lineages or forms that is not based on genotypic similarity and common ancestry" (Lincoln et al. 1998). Only one of these definitions avoids the term 'evolution'; one avoids the term 'independent'; all of them include the terms 'similar' or 'similarity' and either 'species' or 'lineages'. Furthermore, we can safely imply that each definition is not limited in scope, but is applicable across all multicellular taxa (fungi, plants, animals). In the *Astyanax* cavefish model, rapid convergent evolution is the most prevalent explanatory hypothesis for their common array of traits (see previous discussion). However, they are presumed to have had a 'head start' as they could not depend upon slow and gradual production of numerous new mutations. Accumulation of standing genetic variation in ancestral surface fish populations is thought to explain not only their rapid response times, but also widespread convergence of the specialized adaptations they exhibit today.

As outlined in the Introduction, cave populations of blind *A. mexicanus* are estimated to have diverged from surface populations sometime between 8.1 million and ~20,000 years ago, with persuasive support for the lower reference date, and perhaps even younger (Fumey et al. 2018). At least five independent cave invasions (Gross 2012) have led to 29 distinct populations of cavefish in the El Abra region of Mexico (Fig. 8). At least ten or more of these populations are "significantly distant from one another" (Gross 2012). Thus, multiple cave colonization events over an evolutionarily short timeframe have resulted in "convergence on cave-derived morphological and behavioral traits" across multiple, geographically separated cavefish populations (Bradic et al. 2012; Coghill et al. 2014; Herman et al. 2018). And, Herman et al. (2018) have confirmed that cave populations are polyphyletic, and therefore derived "from more than one common evolutionary ancestor or ancestral group". This pattern of cave colonization and convergence on highly-similar integrated adaptations is not limited to Mexico.

At present, over 200 such cavefish species have been

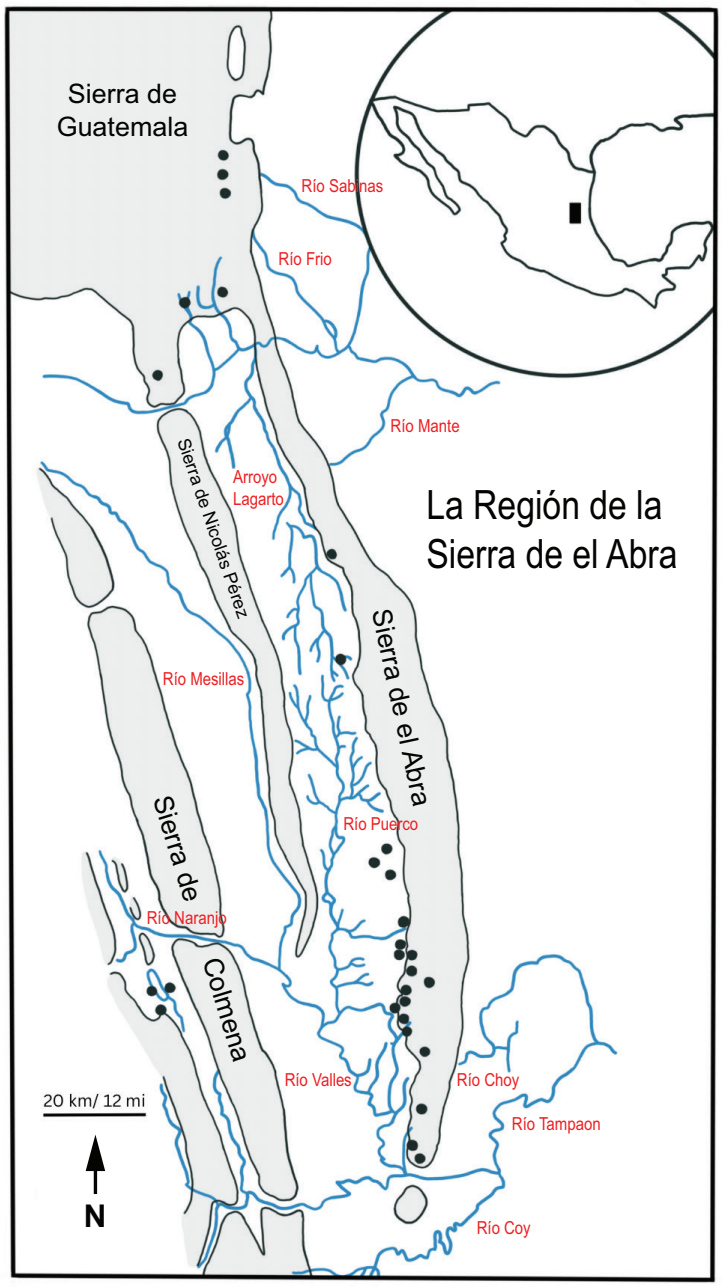

Figure 8. Location and distribution of known populations of *Astyanax mexicanus* in Mexico. There are 29 known populations of *A. mexicanus* cavefish within karst cave habitats across the Región de la Sierra de el Abra. Almost all of *Astyanax* surface fish and cavefish models utilized for research today were collected from the rivers and cave systems in this region. Solid black circles: cavefish populations. (Map and population distributions modified from Figure 2 in Gross, 2012; Figure 1 in Fumey et al. 2018).

described, and all of them have evolved independently from surface ancestors. Thus, each cavefish species is a replicate of the same natural experiment, testing the evolutionary response of a sighted surface fish to the absence of light and the limitations on food in a subterranean environment. The evolutionary *responses converge* on loss of eyes and pigmentation and the augmentation of other senses, such as taste, smell or mechanosensation, as well as a more efficient metabolism, changes in feeding behavior, altered activity

> levels, loss of circadian rhythmicity and increased wakefulness. (italics added; Borowski, 2018).

Furthermore, over the past ten years "about eight new species have been discovered per year", and there may by hundreds of undiscovered cavefish species (Borowski, 2018). Worldwide, the most recent estimates suggest there are almost 230 known species of cavefish (Maldonado et al. 2020). In Mexico's El Abra region, there is only one predominant *Astyanax* species. These fish have independently colonized multiple cave systems, leading to repeated phenotypic convergence of particular cavefish morphotypes. Interestingly, such pronounced convergences "have occurred in spite of gene flow from surface fish populations" (Bradic et al. 2012). These authors imply that "strong natural or sexual selection" for specific alleles are responsible for such convergences. Based upon analyses of 47 whole genomes, Herman et al. (2018) concluded that "troglomorphic traits are maintained despite gene flow with surface populations". They further conclude that "a key troglomorphic phenotype QTL" for cave phenotypes could be passed between caves by gene flow (Herman et al. 2018). Jeffery covers all possibilities with this statement: "the existence of cavefish populations evolving in parallel or by convergence from surface fish ancestors offers an excellent opportunity to study gene use during repeated evolution. (Jeffery 2020). And, although *Astyanax* populations are interfertile, Protos et al. (2006) combines definitions when stating that, "we have identified specific genetic lesions responsible for the parallel evolution of albinism in different cave populations of *Astyanax*, and found that they represent convergent genetic events in separate populations". From a synthesis of the conventional explanations above there is a discernable pattern of interpretation. Multiple independent cave colonization events by *Astyanax* surface fish have repeatedly led to the convergence of cave-adapted phenotypes under strong natural or sexual selection. Those adaptations are maintained despite gene flow from surface fish populations, and between cave populations.

However most, if not all, definitions of 'convergent evolution' require starting with unrelated or distantly related species and lineages. All *A. mexicanus* morphotypes are interfertile, and hybridize, because they are the *same species*. Cavefish researchers take advantage of such a highly reliable hybridization process. According to fundamental evolutionary theory, 'convergence' directly implies the absence of common ancestry. In this *Astyanax* system we find similar cave-adapted traits in one of two morphotypes of the same species; these traits are not the result of inheritance from two or more distant lineages sharing a common ancestor, which is defined as homology – the cornerstone of the entirety of evolutionary theory. Yet because a broad, common and necessary set of adaptive traits in multiple *Astyanax* cavefish populations is thought to be derived from an ancestral population of *Astyanax* surface fish (i.e. common ancestry), homology would be a more appropriate inference. The conventional research community cannot have it both ways. As similar troglomorphic trait adaptations are observed in animals as diverse as, for example, arachnids, myriapods, turbellarians, annelids, gastropods and teleosts, this pattern would indicate evolutionary convergence. So why does the *Astyanax* cavefish community uniformly infer trait convergence within a single species? And why do they infer two distinct evolutionary processes – parallelism and convergence – in the same species? Perhaps it is increasingly clear to them that as time marches on, a finite 'evolutionary language' (Gould 2002) is becoming fundamentally inadequate.

Collectively, we can see that evolutionary process conceptions are consistently invoked across a spectrum of cavefish studies. Interpretations for the production of *Astyanax* cavefish morphotypes include parallel, convergent and repeated evolution; gene flow or in spite of gene flow; natural selection and/or sexual selection; millions of years or less than twenty thousand years; multiple isolated cave invasions and/or introgression and transmission between populations; pleiotropy through hyperactive regulatory control and/or biosynthetic pathway cooption; constructive or regressive loss-of-function mutations through deletion and/or transposition; epigenetic activation and/or silencing, etc. Let us also not forget some of the evolutionary categorical conceptions of trait gains and losses, tradeoffs, antagonism, redeployments, and sensory trait linkages put forth as possible reasons for the development and integration of many 'exclusive' adaptations in *A. mexicanus*, and by extension, global cavefish populations. Again, when considering the broad list of cave-adapted traits observed within an impressive biodiversity of other troglomorphic animals across this planet, there is little doubt that – of all the conventional evolutionary concepts – convergent evolution is the most common explanatory mechanism applied to those traits.

Obviously, there are many outstanding questions: If there have been at least five independent colonization events into different cave populations over the past 1–2 million years, why do all cavefish arrive at nearly identical phenotypes? Why are the fish of different caves still completely interfertile with the ancestral surface form if they are interpreted as having been separated by convergent and/or divergent events? Can all tetra species become cave morphs when placed in cave environments? How do almost all other cave-adapted troglobites *converge* on similar adaptions when they possess such a disparate range of body plans? Why do all the cavefish in our experimental test groups respond in similar ways to treatment with high-intensity light, regardless of starting point or which pigmentation pathways are functional?

As previously noted, we hold a very different view on the origin, function and deployment of adaptations in the *Astyanax* cavefish model. Common adaptations are the result of common engineering. Within all *Astyanax* cavefish there is an internal system of preprogrammed adjustments that actively deploy in response to distinct sets of environmental stimuli. In essence, these fish continuously track a range of environmental parameters, assess those parameters on all levels (e.g. molecule, gene, cell, organ system, physiology, anatomy), and adjust rapidly, and appropriately. This process is similar to, but far more complex than human engineered systems that utilize a series of sensors, logic mechanisms, and responders. In all cases, the organism is the agent in control of each adaptive response. Each adaptation, correlated with all other adaptive responses, is repeatable and reversable. Thus, nature (the environment) has no agency, and therefore, there is no selective agent acting through random, mutational error-prone mechanisms over long durations of time. Hence, a proposed mutation-selection mechanism of regressive or constructive trade-offs in trait production, refinement and establishment is purely hypothetical. And as for advocating an evolutionary concept of *convergence* to explain similar adaptations across *Astyanax* cavefish populations – what appears to be convergent evolution, we model as similar biological systems of engineered solutions activated by organisms when confronted with similar environmental challenges. Our view presents an original organism-focused Theory of Biological Design (TOBD) that is predictive and testable. Respectively, we have outlined a condensed engineering-based framework of assumptions, tenets, expectations, interpretations, and major inferences for a biological research program that is guided by an organism-focused TOBD (Table 2).

4) Directed genetic change nullifies random mutation as the source for adaptation

Evolutionary biologist Jerry Coyne reiterates a central tenet in table 1, "true, the raw materials for evolution – the variations between individuals – are indeed produced by chance mutations. These mutations occur willy-nilly, regardless of whether they are good or bad for the individual" (Coyne 2009). The purpose of our experimental tests on cavefish is to assess if that assumption is based on real observations, or is Coyne reciting some form of a creed. A substantial body of literature identifies (i) an unexpectedly large number of ways to produce adaptive phenotypes that are decoupled from random mutation altogether, and (ii) highly-regulated genetic changes that appear to be directed toward specific adaptive outcomes. These findings are not consistent with current neo-Darwinian theory (NDT). The assumption that mutational processes were disconnected with changes in environmental conditions seemed to be demonstrated in 1943 by the widely-cited Luria–Delbruck experiment. Their experiments on bacteria infected by bacteriophages was taken as proof that mutations in bacteria conferring resistance to phages existed *before* phage exposure (Luria and Delbruck 1943). These results have, somehow, been continuously heralded as proof that mutations are random. Their results do indicate that genetic information for resistance to phages doesn't need to be associated with phage exposure (i.e. some bacterial populations also undergo genetic changes after exposure to phage (Foster 2004)). Yet, if a design-based explanation had been considered, then bacterial resistance prior to exposure to phages, antibiotics, or mechanisms that activate genetic change, could be interpreted to indicate that bacteria were engineered with potential solutions that existed prior to exposure to a variety of context-dependent challenges.

That mutagens, copying errors, etc., are possible causes of true random mutations is not disputed by us. But, there has always been an absence of direct evidence that all mutations, especially genetic changes associated with suitable adaptations to environmental challenges, are fully random. Brundin (1986) noted this lack of evidence when stating, "The great primary problem is evidently set by the mutations. Are they random or nonrandom?" By the 1960's some evolutionists recognized the need for nonrandom genetic changes in order for the evolutionary process to plausibly explain all that it claimed. This was due to the objective inefficacy of the random mutation-selection mechanism as Barbara Wright identified, "The existence of such mechanisms [nonrandom genetic modification] has been pre-

Table 2. Condensed outline of a biological research program guided by a Theory of Biological Design (TOBD). (**a**) A TOBD functions as an interpretive framework of biological phenomena that is part of a paradigm which presumes that organisms look engineered because they are engineered. The organisms possess a unique quality called "life" that the environment as an aggregate does not possess. Two primary research implications are that organisms are viewed as active problem-solving agents, and their environment is an unconscious set of variable conditions to which the organisms are exposed. Thus, the environment is incapable of independently exercising agency. Internalism is a major element of the framework for assumptions and interpretations within a TOBD that stands in stark contrast to the externalism of NDT. (**b**, green) Four core assumptions within the rationale for initiating a biological research program that is consistent with an engineering project. Engineering-based biological research is limited to explaining biological functions and does not address consciousness. (**c**, gray) Three basic tenets of a TOBD are descriptive of engineering principles that are essential for pursuing a correct understanding of biological operation. (**d**, white) The core tenets of a TOBD that constrain how observations will be characterized, and what biological research should be focused on within an engineering-based framework. (**e**, magenta) Major inferences from explanations of engineered systems within a TOBD.

(**a**) Synopsis of a biological research program conducted within an engineering-based framework.					
(**b**) Research assumptions of a TOBD as applied to biology:	1. Integrated. Both basic research of biological functions and associated technical applications are within the domain of engineering practice.				
	2. Comprehensive. All biological systems related to development, metabolism, reproduction and adaptation can be reverse-engineered.				
	3. Instructive. Biological functions will only be accurately explained by models developed utilizing engineering principles.				
	4. Directive. Analyzing human engineering practices will inform predictions and point researchers to accurate characterizations of biological phenomena.				
(**c**) TOBD basic tenets:	(**d**) Resulting expectations of findings or interpretations of observations:				(**e**) Inferences:
Intentionalistic Purpose: Goal-directed activity on an organism-wide basis is expected at every research level.	Directed: Activity of systems is efficiently directed toward need-fulfilling ends. Embrace the reality of purpose.	Coherent: Coherence is pervasive. Explore for system elements functioning simultaneously as both ends and means.	Foresighted: Presume traits that function to attain goal-oriented states are indicators of experience-based planning.	Proactive: Expect anticipatory planning that integrates knowledge of present conditions, past experiences, and probability of future conditions, to produce models that step forward in time to direct up-front responses to handle them now.	Since systems are always engineered top-down, purposeful systems btwn all biological functions are expected. Deterministic outcomes are direct evidence of purpose, e.g., repeated, self-regulated attainment of a "final state" from a single cell *are* non-random scientific data.
Internalistic Operation: Identifiable control systems within an organism are the true cause for all operations including adaptations.	Unity: The organism is the directing program for all purposeful outcomes and cannot be reduced below the level of self, e.g. DNA and its machinery is a subsystem of the organism.	Precedence: An organism's traits determine all of its capabilities. The traits should be credited with success or failure—not external exposures.	Triggers: The actual triggers initiating all self-adjustments by organisms are integrated sensors that detect variable conditions.	Information: Organisms sense exposures and extract data. Environments can't send instructions.	Adaptation: Organisms optimize the suitability of their traits to the environment through an innate engineered control of the organism-environment relationship.	Corresponding elements are expected between human-designed contrivances and biological systems performing similar functions. Engineering causality eliminates non-observable external interventions. Both internal form and adaptability are governed by innate systems.
Individualistic Agency: Autonomous organisms are delineated by a definite "self" boundary and operationally are not to be conflated with "non-self."	Stimuli: Internal programming will specify any condition "to be" a stimulus. No condition in-and-of-itself is a "stimulus." Organisms have sensors tuned to each specified condition.	Interfaces: Individuals will relate to environments through identifiable interfaces. For two or more independent entities to work together they must be connected by an interface. Biological interfaces will have one to three subsystems: authentication; communication protocols; a common medium.		Organisms as Elements: Organisms themselves are discreet elements working together in broader systems. They are not absorbed into a collective. Individuality isn't abolished. "Seamless" operations always have identifiable seams.	Engineered controls will regulate organism-environment relationships. Entangling individual causal operation produces confused explanations. Information explaining ecosystems and interfaces is inferred as it is not found in information of the elements.

dicted by mathematicians – for example, Bernhard (1967) – who argued that, if every mutation were really random and had to be tested against the environment for selection or rejection, there would not have been enough time to evolve the extremely complex biochemical networks and regulatory mechanisms found in organisms today" (Wright 2000). In fact, the reassuring assumption that mutations occur at random has long been challenged by discoveries of molecular biology, which indicate that complex regulation of genetic change is at work. And because these findings are inconsistent with NDT, they are "ignored or side-lined", or have been outright marginalized (Shapiro and Noble 2021, p. 147). For instance, Barbara McClintock's discoveries in the 1940's of transposable "controlling elements" that could control gene expression and regulate adaptation were initially ostracized, and then disregarded (McClintock 1987). In the 1970's the SOS DNA-damage response and subsequent regulated, "inducible" genetic change mechanisms were clearly non-random processes (Witkin and George 1973; Radman 1975). Additionally, Barry Hall's early work indicated the prosses of regulated genetic change when he found rare, yet beneficial, mutations occurring sequentially within the same bacterium (Hall and Hartl 1974; Hall 2003). More evidence of regulated genetic change was identified following John Cairns' discovery that specific genetic changes appear in *Escherichia coli* only when needed (Cairns et al. 1988), and by his further proposition of "directed" or "adaptive" mutagenesis in starvation-stressed bacteria (Cairns and Foster 1991).

Detailing what we currently know about specific mechanisms that produce adaptive phenotypes would fill an enormous review article. James Shapiro has broadly characterized these particular mechanisms as "natural genetic engineering" and his books link to over a thousand references (Shapiro 2022). The 'big-picture' shows that there is a growing body of evidence that many mutations are not random in their formation (Hogeweg 2015). In fact, many genetic changes seem to be specifically programmed as targeted responses to specific external conditions. Adaptive responses in bacteria can result from the same independently occurring genetic change in different populations (Herron and Doebeli 2013). Short segments of DNA can be inverted to generate new patterns in human chromosomes (Löytynoja and Goldman 2017). When cells detect different environmental conditions, innate mechanisms that are not completely understood can change their chromosome state and alter DNA methylation patterns (Angers et al. 2010; Zhu et al. 2013). There is strong evidence that intracellular enzymes control the locations and events of genetic changes on chromosomes in humans. (Pinto et al. 2016). Significant work by Hull et al. (2017) indicates that yeast cells appear to direct greater variation to exact locations in their genome where it would protect them against a toxin, which therefore "provides cells with a remarkable and unexpected ability to alter their own genome in response to the environment". And, recent research on genetic changes in the plant *Arabidopsis thaliana* "found a lower mutation frequency inside gene bodies and certain essential genes, shattering the long-standing idea that mutations are entirely random across the genome" (Veitia 2022).

However, even with increasing evidence that targeted, nonrandom genetic mechanisms are involved in the production of adaptive traits, there is persistent opposition. Futuyma (2013) insists that "genetic variation arises by random mutation and recombination". Furthermore, he also holds to a fundamental, but now incorrect premise, that "environmental effects of an individual's phenotype do not alter the genes passed on to its offspring" (Futuyma 2013). Both statements adhere to the assumption that directed or purposeful genetic changes don't occur. Although the examples above (previous paragraph) do not indicate that 'random variation and recombination' are adequate mechanisms for generating the extensive, complex genetic variation observed in biological systems. And what about the transfer of environmentally induced genetic and regulatory effects? It is widely held that August Weismann's lecture in 1883 established the 'fact' of an impermeable barrier between "disposable" cells of the soma (body) and "immortal" cells of the germline (gametes) in animals (Weismann 1889). This 'Weismann barrier' is a longstanding central theme of the NDT. Futuyma tells us that "extensive subsequent research has provided no evidence that specific hereditary changes can be induced by environmental conditions under which they would be advantageous (Futuyma 2013, p. 10). Yet, assertions like Futuyma's are at least a decade out of date. We now know that epigenetic mechanisms can regulate and facilitate inheritance of the acquired characteristics of adaptive traits between parent and offspring (Jablonka 2017). Soma to germline informational transfers have been identified by several mechanisms (Sharma 2013), and chromosomes within mouse spermatozoa have been modified by interactions with non-germline DNA (Pittoggi et al. 2006). And several researchers have identified extracellular vesicles that can transfer genetic material from soma to germ cells (Eaton 2015). Furthermore, the germline descends from a somatic lineage in unicellular eukaryotes and in plants, and is designated from the soma in multicellular animals. Bacterial geneticist, James Shapiro (2022 p.16) sums up the hardened resistance of NDT theorists to incorporate this new path of research: "despite massive genomic evidence to the contrary, the philosophy of evolution by random processes – the neo-Darwinian Modern Synthesis – still reigns supreme in the public mind, in the classroom, and in the minds of many scientists and clinicians as well". Fitzgerald and Rosenberg (2019) summarize what they and other researchers have been discovering:

> …but this view [random mutation] is being revised by discoveries of molecular mechanisms…these mechanisms reveal a picture of highly regulated mutagenesis, up-regulated temporally by stress responses and activated when cells/organisms are maladapted to their environments – when stressed – potentially accelerating adaptation. Mutation is also nonrandom in genomic space, with multiple simultaneous mutations falling in local clusters, which may allow concerted evolution…assumptions about the constant, gradual, clock-like, and environmentally blind nature of mutation are ready for retirement.

Our results of rapid repigmentation in the cavefish model are an indicator that depigmentation in these fish is not the product of a 'broken' pigmentation pathway due to random mutations. The melanin synthesis pathway is clearly functional, responsive, reversible, and therefore adaptive. Based upon a large body of literature, and our experiments thus far, the current perception that random mutations provide the genetic variation required for adaptive change, is in error. CET is a predictive and testable substitute.

D) Future directions in cavefish research at the ICR

Within the span of one year, the Institute for Creation Research has established three new resources dedicated to experimental research. These resources include a (1) Biology Laboratory equipped with a recirculating aquaculture system of aquaria for maintenance and experimental treatment of *Astyanax mexicanus* cavefish and surface fish; a (2) Molecular Biology Laboratory equipped with technology for standard and advanced protocols in molecular research, including PCR, gene cloning, riboprobe synthesis and sample preparations

for external applications with transcriptomics, genomics, epigenetics and mass spectroscopy; and an (3) Imaging Center equipped with state-of-the-art stereo, compound and confocal microscopes, each with dedicated computer and digital camera systems. We will pursue multilevel research experiments with the *Astyanax* model to investigate genetic, cellular, organ-system and physiological evidence underlying organism-driven mechanisms of adaptation.

In addition to ongoing experiments on pigmentation, and the associated components of pigment synthesis pathways, we will focus on the developmental processes of eye degeneration (Fig. 7), and restoration, in different cavefish strains. To do this, we will move beyond testing commercial cavefish – currently used to explore and establish technical protocols – to culturing distinct lines of cavefish (e.g. Pachón, Molino, Tinaja) utilized by prominent academic laboratories (see: Riddle et al. 2018; Baumann and Ingalls, 2019). There is a considerable amount of literature available on the process of eye degeneration in cavefish, and eye development in *Astyanax mexicanus* and *Danio rerio* (Zebrafish). Although, very few, if any, of the treatments we are exploring have been pursued in these fish. Therefore, we will begin to repeat several of the conventional studies, which provide invaluable protocols and methods to guide our work. It is also our intention to investigate multiple organ systems that exhibit adaptive traits, including nervous, respiratory, circulatory, muscular, digestive, epidermal, and others, as discussed above (see: Jeffery 2020). Notably, we will characterize the expression of gene transcripts in embryonic and larval stages when all cell types, tissues and organs are moving from specification to differentiation and functionality. We will also begin to ramp up reproductive output in different cavefish lines, providing not only material for developmental protocols, but also to assess trans-generational (genetic, epigenetic, phenotypic) signatures of adaptation. All of the above will guide necessary adjustments to ongoing experiments and future directions with the cavefish model, and potential alternative models going forward (e.g. reptiles, birds, freshwater and marine invertebrates). Most importantly, we approach this research from the most essential perspective of all: The intimate scriptural and working knowledge of the Creator of life, and through careful application of an original model of engineered adaptability that serves to honor His creative power and wisdom.

V. CONCLUSIONS

Based upon qualitative investigations thus far, we are able to conclude the following. The *Astyanax* model is tolerant of broad variations in light intensity, pH, oxygen, physical handling and tissue sampling without obvious signs of stress in behavior or physiology. These observations are applicable when maintaining cavefish under high-intensity light, or maintaining surface fish in total darkness. Upon treatment with high-intensity light, three different strains of cavefish show an increase in the amount, distribution and expression level of pigment cells (chromatophores) across multiple body regions. Two of our commercial cavefish strains express melanin, in addition to xanthophores and iridophores. In those fish, all three chromatophores show increased expression and distribution within weeks to months after exposure to light. In one commercial strain, the F1 progeny of parents under light treatments, showed increased pigmentation in less time than their parents, when reared under the same treatment. Juvenile Molino cavefish do not express melanin under sustained high-intensity light treatments; however, they exhibit conspicuous increases in both xanthophore and iridophore pigmentation patterns. When cavefish are transitioned to lower pH (~5.3–5.5), there is a noticeable reduction in melanic pigmentation, but no adverse physiological reactions; these cavefish are acclimated to low pH environments. When surface fish transition into simulated cave environments with low pH and low O_2, they are stressed and do not show obvious signs of acclimation; they do exhibit decreases in melanic pigmentation. Commercial cavefish in controlled experiments under light reveal a range in the levels of increased melanin pigmentation across specimens, most likely due to genetic variation within unknown parent lineages from which they were reared. The above observations, and the conclusions drawn from them, indicate rapid responses to experimental treatments by *A. mexicanus* within short timeframes from days or weeks, to several months. These experiments imply that *A. mexicanus* may undergo relatively rapid transitions between surface and cave environments, suggesting the reversible character of adaptive traits in this model.

While we are still in the early days of cavefish research, it should not be surprising to find that animals are adaptive to specific conditions in contrasting environments. Moreover, that their responses are rapid, reversible and appropriate. Human engineered systems are prepared with forethought to sense and collect information, interpret that information, and respond in accordance with the intention and purpose for which they were constructed. How much more should we then expect divinely engineered creatures to be prepared with unapproachable precision to thrive in the environments for which they were created. In line with our model of Continuous Environmental Tracking (CET), we should expect that if and when surface fish migrated into caves, they would actively track conditions within such unique environments and self-adjust by reintegrating latent sources of biological functionality. We predict that because all animals are adaptive, they would all have this capacity. They would not adjust and adapt through a popularized evolutionary scheme promoting a random mutation-selection process under the trackless agency of nature. As noted by Shapiro (2022), "To give Natural Selection deterministic power over the evolutionary process, it was necessary to assume that genetic changes were random, of small phenotypic effect, and generated significant adaptive differences by accumulation over long periods of time due to selective advantages they conferred". The most notable outcome of our research thus far, is that adult cavefish and surface fish respond to experimental conditions within weeks of treatment, with responses not limited to multigenerational genetic inheritance. However, we envision that multigenerational *epigenetic* inheritance may confer certain capacities in subsequent generations that do not yet track with simple treatment conditions (i.e. light-induced restoration of eyes). As stated above, these are early days with the cavefish model, and we are already encouraged to present a new direction in experimental science for the ICR, and a vital new approach for Creation Science that envisions every organism as a divinely engineered creation with wondrous potential. Our research will confirm that life is thoughtfully and intentionally prepared by the infinite wisdom of our Creator, "in whose hand is the life of every living thing, and the breath of all mankind" (Job 12:10).

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