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A Natural Animal Model System of Craniofacial Anomalies: The Blind Mexican Cavefish

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Abstract

Natural model systems evolving under extreme environmental pressures provide the opportunity to advance our knowledge of how the craniofacial complex evolves in nature. Unlike traditional models, natural systems are less inbred, and, therefore, better model the complex variation of the human population. Owing to the nature of certain craniofacial aberrations in blind Mexican cavefish, we suggest that this organism can provide new insights to a variety of craniofacial changes. Diverse cranial features have evolved in natural cave-dwelling *Astyanax* fish, which have thrived in the unforgiving darkness and nutrient-poor environment of the cave for countless generations. While the genetic and environmental underpinnings of various cranial anomalies have been investigated for decades, a comprehensive characterization of their molecular and developmental origins remains incomplete. Cavefish provide numerous advantages given the availability of genomic resources, developmental and molecular tools, and the presence of a normative surface-dwelling “ancestral” surrogate for comparative studies. By leveraging the frequency of abnormal and asymmetric cranial features in cavefish, we anticipate advances in our knowledge of the etiologies of irregular cranial features. Extreme adaptations in cavefish are expected to offer new insights into the complex and multifactorial nature of craniofacial disorders and facial asymmetry.

Keywords

adaptation; evolutionary; *Astyanax mexicanus*; intramembranous bone; asymmetry

In recent years, natural “evolutionary model systems” have played an important role in connecting genetic changes to phenotypic evolution. Examples of such model systems include the icefish, which models human conditions such as osteopenia and anemia (Albertson et al., 2009); and reptiles which model heterotopic ossification— a condition wherein normally soft tissues become mineralized (Dubansky and Dubansky, 2018). Additionally, studies in other natural model systems, such as the rock pocket mouse (*Chaetodipus intermedius*), have informed the genetic basis of pigmentation changes associated with living on lighter- versus darker-colored substrates (Nachman et al., 2003). Genomic studies in the rock pigeon (*Columba livia*) have shed light on the geographic

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origins of numerous breeds, as well as identified the genetic regulators of the “head crest” phenotype (Shapiro et al., 2013). Darwin’s finches helped identify *calmodulin* as a novel regulator of the development and morphology of the craniofacial complex (Abzhanov et al., 2006); and, genetic studies in sticklebacks enabled discovery of the role for *pitx1* and *ectodysplasin* in pelvic fin development and armor plate development, respectively (Shapiro et al., 2004; Colosimo et al., 2005).

Another emerging model system that will continue to yield new discoveries in genomic research is the blind Mexican cavefish, *Astyanax mexicanus* (Pennisi, 2016) (Fig. 1A,B). The blind Mexican cavefish has already provided key insights to a variety of cranial traits, alongside other natural systems. For example, studies in cichlid fish have led to gene discoveries associated with adaptive variation of the lower jaw (Powder et al., 2014), and candidate genomic regions associated with cranial asymmetry in scale-eating forms (Raffini et al., 2017).

This organism was discovered in the mid-1930s (Hubbs and Innes, 1936) and, owing to the persistence of extant surface-dwelling forms (Fig. 1D,E), continues to provide novel insights into a variety of biological problems. This unique system has enhanced our understanding of the nature of sleep (Duboué et al., 2011), feeding behaviors (Kowalko et al., 2013), and attraction to mechanical vibrations (Yoshizawa et al., 2014). Genetic studies have informed the molecular underpinnings of albinism (Protas et al., 2006), retinal degeneration (O’Quin et al., 2013), and starvation resistance (Aspiras et al., 2015).

The blind cavefish provides a number of experimental advantages. For example, cavefish are robust experimental animals that can be raised and bred in captivity (Hinaux et al., 2011). Many of the same molecular approaches used in zebrafish can also be applied to cavefish, including the development of transgenic lines (Elipot et al., 2014; Ma et al., 2015) and the use of morpholino knock-downs (Bilandžija et al., 2013). In 2014, the first draft of the cavefish genome was published (McGaugh et al., 2014), ushering in a new era of genomic biology for this organism. Furthermore, since several isolated cave populations have evolved over recent geologic time, these “natural experiments” have enabled a deeper understanding of convergence and parallelism for derived phenotypes (Gross, 2016). Perhaps one of the strongest attributes of this model system is the fact that surface-dwelling forms still exist in nature, permitting hybridization experiments (Wilkens and Strecker, 2003), QTL studies (Protas et al., 2008), and analyses of gene sequence and function in a “surrogate” ancestral form (Hinaux et al., 2013). Here, we provide a short review of aberrant cranial traits that have so far been discovered in blind Mexican cavefish, and review analogous traits in other vertebrate taxa that may benefit from further studies in the blind Mexican cavefish. The availability of a sequenced genome, combined with the frequency with which these extreme traits are observed in *Astyanax* cavefish, will enable new insights to the development, genetic, and morphological bases of these incompletely understood cranial phenotypes.

Cavefish Manifest Numerous Cranial Aberrations as a Consequence of Life in an Extreme Environment

One of the earliest phenotypes formally described in the blind Mexican cave tetra were craniofacial aberrations (Hubbs and Innes, 1936). In their description of the original holotype specimen (from the Chica cave locality), the authors described the circumorbital bone series as small, “irregular and asymmetrical,” with different numbers of bones on the left versus the right side of the face (p. 6; Hubbs and Innes, 1936). The most unusual cranial abnormalities affect the circumorbital bones, a series of intramembranous bones that encircle the eye orbit (Yamamoto et al., 2003). In surface fish, these dermal bones are stereotypical and symmetric across the left–right axis (Gross and Powers, 2015) (Fig. 2B). However, in cavefish, these bones are oftentimes fused, or “fragmented” (into numerous pieces; Figs. 1C 2B), and highly asymmetric (Gross et al., 2016a). Based on the original holotype specimen, Hubbs and Innes (1936) reasoned that the peculiar “fragmented” facial bones of the cranium potentially arose as a consequence of injury, either in life or during preservation. Years later, however, Alvarez (1946, 1947) discovered the same phenotypes were present in two other geographically distinct cave populations (Pachón and Sabinos) located several kilometers north in the Antiguo Morelos valley of Northeastern Mexico. This discovery led to the belief that suborbital bony fusion and fragmentation (Fig. 2B) were not the consequence of injury, but rather “normal” phenotypes in the skulls of blind cave-dwelling *Astyanax* fish. At the time, each cave population was regarded as an entirely different genus and species, compared to the surface-dwelling form (*Astyanax mexicanus*). Based on subtle differences, Alvarez (1946, 1947) employed patterns of fragmentation and fusion as a means of designating each cave population as a distinct species (reviewed in Mitchell et al., 1977). At present, most researchers regard the cave and surface populations as members of a single species supporting different morphotypes (i.e., a surface-dwelling form with multiple cave-dwelling forms).

Little additional scientific research was carried out on the skulls of Mexican cave tetra until a landscape survey of cave populations was performed from the late 1960s until the late 1970s. Mitchell et al. (1977) discovered and characterized 29 populations of *Astyanax* cavefish still known today. In this seminal publication, the authors also performed the first quantitative analysis of suborbital bone anomalies (Fig. 2B)—counting the number of fragmentation and fusion events impacting individuals from eight distinct cave localities. Using a posterior probability classification approach, the authors found significant enough differences between cave populations to correctly classify individuals based on a variety of morphologies. Among these morphologies included anatomical features of the skull: maxillary length, number of suborbital bones, number of fragments of the first suborbital bone (SO1), head width, and number of fragments of the third suborbital bone (SO3) on the right side of the head (Mitchell et al., 1977) (Fig. 2B). The widespread presence of bone fragmentation has since provided the rich opportunity to explore the molecular, developmental, and genetic bases of cranial bone abnormalities.

The next major analysis into cranial biology in cavefish was carried out using a lentectomy approach. To determine how eye loss impacts the cranial architecture in cavefish, Yamamoto

et al. (2003) used an experimental embryological approach of transplanting the developing lens from a cavefish donor into a surface fish host, and vice versa. Strikingly, the authors discovered that a surface fish lens promoted the development of a structurally normal eye in cavefish hosts. However, a cavefish lens did not promote the development of a normal eye—surface fish hosts underwent visual system degradation and did not harbor structural eyes as adults (Yamamoto et al., 2003).

Interestingly, the authors concluded that certain cranial changes are attributable to the presence of the eye; however, others were not dependent on an eye. Among those features dependent on an eye included the size of the olfactory pits (as defined by the positions of the nasal and antorbital bones), and the size and positioning of the circumorbital bones. However, other craniofacial features including maxillary tooth number, SO3 bone fragment number, and the opercular bone shape did not change based on the presence of a structural eye (Yamamoto et al., 2003). The authors concluded that cavefish craniofacial complex is controlled by diverse developmental events, and some features are a consequence of eye degeneration, but other are unrelated to eye loss (Yamamoto et al., 2003).

Until this point, little was known of the possible genetic contributions to derived cavefish cranial morphologies. Using an experimental F₂ pedigree derived from Pachon cavefish and surface fish, Gross et al. (2014) scored variation in 33 of these eye-independent craniofacial phenotypes. This led to the discovery of several traits that harbor a genetic basis, many of which are related to the suborbital bone series. These include the bony area of the SO2, SO3, SO4, and SO5 bones. In addition, a detectable genetic architecture was observed for fusion of SO1 and SO2, and fusion of SO4 and SO5 (Gross et al., 2014). Additionally, SO3 bone fragmentation revealed two significant QTL peaks. Interestingly, however, these genetic signals were identified based on phenotypic analyses of the right SO3 bone, but not the left SO3 bone (Gross et al., 2014). One of these markers, 206A, maps to the same genomic scaffold as the genes *tgfb3* and *bmp4*, two genes with well characterized roles in craniofacial biology (Gross et al., 2016b).

Cavefish cranial abnormalities are not limited to abnormal bony fusions and fragmentations. For instance, as cavefish advance through their life history, they demonstrate a cranial “bend”—most often to the left (Powers et al., 2017) (Fig. 2B). In rare instances when this bend is observed in surface fish, it is very subtle (Fig. 2A). Interestingly, when a morphometric analysis was carried out across earlier developmental stages, two different cavefish populations (Pachon and Tinaja) were not found to be different from surface dwelling fish. Moreover, the lateral asymmetry present in osteocranial shape was not apparent at juvenile stages of development, specifically in the chondrocranium (Powers et al., 2017). The high frequency with which these anomalies occur across the lateral axis in cavefish provides the opportunity to evaluate how the left and right sides of the cranium become “decoupled” across ontogeny.

An important difference for the role of the cavefish system in craniofacial biology is that many phenotypes in this organism have evolved in the absence of an *obvious* adaptive function. For instance, the orbit of the skull in several cavefish populations is virtually collapsed, likely as an indirect consequence of lacking a structural eye (Mitchell et al.,

1977). As our understanding of the genetic and developmental underpinnings of craniofacial abnormalities in cavefish improves in the coming years, so too will our ability to determine if they arose under neutral pressures (e.g., genetic drift), or for an adaptive (but currently unknown) function.

Natural Cavefish May Provide Insight to Craniofacial Aberrations Occurring in Other Vertebrate Taxa

Facial asymmetry.

Facial symmetry has long been appreciated for its important role in the perception of beauty, irrespective of geography or culture. Early studies suggested potential environmental factors that can impact facial and dental symmetry, such as exposure to bacterial pathogens (Yates, 1928). More recent studies have argued that prenatal exposure to varying stress hormones also impacts facial symmetry (Fink et al., 2004). The complex participation of genetic and environmental factors in the regulation of human facial symmetry imposes a challenge to understanding how symmetry is maintained (and lost) in individuals. The precise genetic, molecular, and environmental changes that contribute to loss of symmetry remain incompletely understood. Natural model systems, such as cavefish, provide an opportunity for insight to this complicated biological problem. For example, in order to characterize a phenomenon, a suitable model system must *frequently* express the trait of interest (Trainor and Richtsmeier, 2015). In the case of facial asymmetry, blind cavefish demonstrate cranial asymmetry in virtually every specimen (Fig. 2B) (Powers et al., 2017).

Moreover, cranial asymmetry in cavefish is influenced by both genetic (Gross et al., 2014) and environmental (Yamamoto et al., 2003) factors, and, therefore, provides a model for understanding the complex, multifactorial nature of this trait. For instance, the use of a lentectomy technique, which was carried out early in development, demonstrated that environmental factors intersect with abnormal facial bone development in cavefish. Specifically, by removing the lens from developing *Astyanax* fish, Yamamoto et al. (2003) demonstrated that primary disruption of the structure of the eye across ontogeny has dramatic effects on the resultant cranial skeleton. A less studied component of the approach used by Yamamoto et al. (2003) is the craniofacial impact that eye degeneration had on surface-dwelling hosts. This experimental approach may enable careful analysis of the interdependence of the bony orbit and the eye. This approach could provide an experimental test of the “functional matrix hypothesis,” proposed by Moss (1961), which would predict that the structural eye acts as a growth center influencing surrounding bones of the skull as it grows (Moss, 1961). By utilizing surface dwelling forms, subjected to lens transplantations, this study could provide new insights to the nature of craniofacial abnormalities in cavefish that arise through the absence of a structural eye, rather than genetic changes.

Blind Mexican cavefish may also provide deeper insight to certain human clinical disorders. Among these is the condition of craniosynostosis, a serious clinical condition that occurs when developing intramembranous skull bones fuse prematurely (De Pollack et al., 1996). During development, premature fusion occurs at the suture—an anatomically simple structure residing at the interface of two intramembranous bones of the cranial vault. The

suture normally contains rapidly dividing osteogenic stem cells, which are recruited to become mature bone (Opperman, 2000). Blind cavefish similarly demonstrate frequent premature fusions of intramembranous bones in the circumorbital bone series (Fig. 2B). Presumably these fusions are caused by changes in the spatial relationships of orbital bones during osteogenesis. For instance, bony fusion may result from two ossification “fronts” that are set too close to one another, perhaps as an indirect consequence of other shape changes associated orbital collapse in cavefish. At present, it is unclear if fusion of facial bones in cavefish recapitulates the aberrant tissue and molecular underpinnings of craniosynostosis in humans. As these important details become clear, it will also become clear if cave tetra may serve as a suitable model for understanding the basis of, and interventions for, premature cranial bone fusion in humans.

Cavefish enable craniofacial disorders to be evaluated in the context of other diverse phenotypes.

Cavefish demonstrate a number of features beyond modifications to the cranial complex that have evolved as a consequence of life in complete darkness. Among the traits that vary between cave and surface dwelling forms, and harbor a genetic basis, include: melanophore numbers, rate of weight loss in response to fasting, rib numbers, eye size, and threshold sensitivity to dissolved amino acids (Protas et al., 2008). By evaluating craniofacial phenotypes in light of these diverse morphologies, one may discover associations between two traits that were not previously appreciated.

One potential example of this type of interaction may be between cranial neuromasts and cranial bones of cavefish. Neuromasts are mechanosensory structures, comprising the lateral line system that enables certain aquatic species to sense movement in the water. Cavefish harbor many more neuromasts than surface-dwelling forms, and the anatomical position wherein these sensory structures are most densely populated is on the SO3 bone. To determine if an interaction may exist, Gross et al. (2016a) quantified the positions of surface neuromasts overlying the SO3 bones and compared their positions on the left and right sides of the face. This study revealed that cave populations, which harbor asymmetric facial bones, also harbor asymmetric patterns of cranial neuromasts. Although, this study did not reveal whether bone shape impacts neuromasts, or vice versa, it does reveal a potential interaction between the expansion of the lateral line system and alterations to the cranial complex. Future studies that aim to understand craniofacial features in cavefish in light of the diverse morphologies co-evolving in these animals, may shed new light on the origin and evolution of extreme cranial features.

CONCLUSIONS

In this article, we review the current knowledge of craniofacial anomalies present in the blind Mexican cavefish, an organism that lives amidst extreme environmental subterranean conditions. We believe that *Astyanax* cavefish can serve as a useful model for understanding of the genetic, developmental, or tissue-level bases for a variety of cranial anomalies. Cavefish demonstrate a spectrum of cranial features, ranging in severity and penetrance. Natural genetic variation, overlaid with influences from an extreme environment, provides

the opportunity to understand the complex multifactorial interactions that govern cranial abnormalities.

Using animals to model human craniofacial disorders poses challenges as no animal system, even phylogenetically close mammalian models, perfectly recapitulates human development. For example, while Old world monkeys exhibit similar facial structures to humans, their dentition with respect to canine tooth development is more similar to dogs (Siegel and Mooney, 1990). Additionally, non-human primates pose an issue of tractability in terms of generation time and number of offspring. Rodents provide an abundance of offspring, can be genetically manipulated and exhibit similar cranial shape to other primate models such as macaques (Hallgrímsson et al., 2004). However, rodent models offer poor resolution in terms of natural genetic variation as they are purposely inbred to reduce genetic variability. Nontraditional animal models allow for comparisons across evolutionary lineages as well as understanding mechanisms of cranial diversification (Hanken, 1993). For example, cichlid fishes have evolved extensive modifications to the cranium across closely related species and thus comparisons across morphs provides the opportunity to understand gene-by-environment interactions involved in craniofacial patterning (Albertson et al., 2009).

Fish model systems also enable us to characterize phenotypes across life history, as they are tractable developmental models for *in vivo* imaging and analysis. This allows researchers the ability to manipulate genes and tissues at precise spatial and temporal intervals to better understand the intersections of developmental, cellular, and genetic mechanisms underlying facial patterning. Taken together, cavefish provide the opportunity to understand cranial anomalies by capturing natural phenotypic and genetic variation. Functional testing of candidate genes identified in natural animal models, as well as characterizing the precise cellular and tissue level interactions, will lay the foundation for improved understanding of cranial variation in nature.

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Fig. 1.

Morphological differences between cave- and surface-dwelling forms of *Astyanax mexicanus*. The Mexican tetra includes two distinct morphotypes: an eyeless, albino cavefish (**A** and **B**) and an eyed, pigmented surface-dwelling fish (**D** and **E**). Cavefish harbor craniofacial aberrations affecting the shape, positioning and development of dermal bones, particularly bones surrounding the collapsed eye orbit (**C**). Surface fish (**F**), however, rarely exhibit alterations to the cranium, making this system a tractable, comparative model for developmental, and genetic mechanisms underlying craniofacial formation. Photo credits (**A** and **D**) to Daniel J. Berning. Light images (**B** and **E**) were taken at 9.5× magnification with a stereomicroscope (Leica; Wetzlar, Germany) and the scale bar (white) represents 1 mm. Cavefish (**C**) and surface fish (**F**) skulls were rendered as images from x-ray micro-computed tomography (micro-CT) scans.

Fig. 2.

Cavefish demonstrate bilateral cranial asymmetry compared to symmetric surface-dwelling fish. Surface fish (**A**) illustrate bilateral symmetry, as well as global symmetry as demonstrated by the frontal and dorsal views of the skull. In contrast, cavefish (**B**) exhibit lateral asymmetries associated with suborbital (SO) bones encircling the eye orbit. The largest of the suborbital series, the suborbital #3 (SO3) bone (highlighted in green), spontaneously subfragments into multiple discrete bony elements (3 elements on the left side of the face and 4 on the right). The SO4 (blue) and SO5 (red) bones in cavefish can fuse (purple) as shown on the right side of the representative cavefish individual. Lastly, the smallest and terminal SO bone, the SO6 (orange), is frequently absent as shown on the right side of the representative cavefish. In addition to bilateral asymmetries at the level of individual bony traits, cavefish also demonstrate global and directional asymmetry, which can be visualized in the frontal and dorsal views as a cranial bend biased toward the left.