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REVIEW

Utilizing the blind cavefish *Astyanax mexicanus* to understand the genetic basis of behavioral evolution

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ABSTRACT

Colonization of novel habitats often results in the evolution of diverse behaviors. Comparisons between individuals from closely related populations that have evolved divergent behaviors in different environments can be used to investigate behavioral evolution. However, until recently, functionally connecting genotypes to behavioral phenotypes in these evolutionarily relevant organisms has been difficult. The development of gene editing tools will facilitate functional genetic analysis of genotype–phenotype connections in virtually any organism, and has the potential to significantly transform the field of behavioral genetics when applied to ecologically and evolutionarily relevant organisms. The blind cavefish *Astyanax mexicanus* provides a remarkable example of evolution associated with colonization of a novel habitat. These fish consist of a single species that includes sighted surface fish that inhabit the rivers of Mexico and southern Texas and at least 29 populations of blind cavefish from the Sierra Del Abra and Sierra de Guatemala regions of Northeast Mexico. Although eye loss and albinism have been studied extensively in *A. mexicanus*, derived behavioral traits including sleep loss, alterations in foraging and reduction in social behaviors are now also being investigated in this species to understand the genetic and neural basis of behavioral evolution. *Astyanax mexicanus* has emerged as a powerful model system for genotype–phenotype mapping because surface and cavefish are interfertile. Further, the molecular basis of repeated trait evolution can be examined in this species, as multiple cave populations have independently evolved the same traits. A sequenced genome and the implementation of gene editing in *A. mexicanus* provides a platform for gene discovery and identification of the contributions of naturally occurring variation to behaviors. This review describes the current knowledge of behavioral evolution in *A. mexicanus* with an emphasis on the molecular and genetic underpinnings of evolved behaviors. Multiple avenues of new research that can be pursued using gene editing tools are identified, and how these will enhance our understanding of behavioral evolution is discussed.

KEY WORDS: Behavior, Cavefish, CRISPR, Behavioral genetics, Neurogenetics

Introduction

A central challenge in behavioral neuroscience is understanding the molecular and genetic mechanisms underlying behavioral evolution. Functional studies, possible because of the availability of genetic tools in classic model organisms such as flies, worms, mice and zebrafish, have provided fundamental insights into the genes, neurons and neural circuits that underlie behaviors. However, these classic model organisms are not well suited for evolutionary studies, or to understand the molecular basis of the vast amount of behavioral diversity that exists in nature. Studying populations of animals in which variation in behaviors can be linked to the ecology and evolutionary history of these organisms is key to understanding how behaviors evolve. Although decades of research have documented behavioral variation in natural populations, the molecular mechanisms and genetic changes that underlie this behavioral variation are still poorly understood. This is due in large part to an inability to genetically manipulate these ecologically and evolutionarily relevant organisms.

The recent advent of gene editing technologies such as transcription activator-like effector nucleases (TALENs) and clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 has provided unprecedented genetic access to non-traditional model organisms (Gilles and Averof, 2014; Kratochwil and Meyer, 2015; Chen et al., 2014; Bono et al., 2015). Indeed, these technologies have been applied to many organisms used to study behavior in which genetic manipulation was previously limited (e.g. Li et al., 2014; Erickson et al., 2016; Friedman et al., 2017; Kohno et al., 2016). Gene editing approaches have now been used to examine the contributions of genes and neuronal populations to complex behaviors in an array of organisms, including mating behaviors in cichlids and flies, social and repetitive behaviors in prairie voles, circadian rhythms in monarch butterflies and olfactory-mediated social behaviors in ants (Yan et al., 2017; Trible et al., 2017; Horie et al., 2019; Juntti et al., 2016; Tanaka et al., 2017; Zhang et al., 2017). Thus, widespread application of tools for genetic manipulation to non-traditional model organisms has the potential to provide fundamental insights into the genes and neural circuits that underlie behaviors, as well as the evolutionary mechanisms that lead to behavioral diversity.

Crucial to elucidating the genetic basis of behavioral diversity is identifying species conducive to genetic studies. Ideally, species used to study behavioral evolution would possess quantifiable behaviors, have populations with different behavioral phenotypes that are interfertile to allow for identification of putative loci that underlie these behavioral differences, and would survive and thrive in a laboratory setting. The blind cavefish, *Astyanax mexicanus* (De Filippi 1853) syn. *Astyanax fasciatus*, is an emerging model organism for studying the genetic and molecular underpinnings of behavioral evolution that possesses all of these characteristics. Here, I review the advantages of using *A. mexicanus* to examine behavioral evolution and highlight areas of cavefish research that have contributed to the understanding of how and why behaviors evolve. In doing so, I will demonstrate how this emerging model system is primed for gene editing studies to further the field of behavioral neuroscience by elucidating the molecular and genetic basis of behavioral evolution.

Astyanax mexicanus is an emerging model system to investigate behavioral evolution

Astyanax mexicanus is a single species of fish consisting of two morphs: an ancestral-like, eyed surface morph that lives in rivers and

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streams, and a derived, blind cave morph, which inhabits at least 29 caves in northeastern Mexico (Mitchell et al., 1977) (Fig. 1). Cavefish, populations of which derive their names from their caves of origin (Fig. 1), have evolved numerous morphological and physiological traits, including regression of eyes and reduction or loss of pigmentation, alterations to metabolism, and enhanced lateral line and olfaction (Wilkens, 1988; Rasquin, 1946; Moran et al., 2014; Teyke, 1990; Bibliowicz et al., 2013; Protas et al., 2008). Cavefish have also evolved a number of behavioral changes, including vibration attraction behavior (VAB) (Yoshizawa et al., 2010), loss of schooling and shoaling (Kowalko et al., 2013b; Parzefall and Fricke, 1991), disrupted circadian rhythms (Beale et al., 2013), reduced sleep (Duboué et al., 2011; Yoshizawa et al., 2015), altered feeding posture (Schemmel, 1980), changes in larval prey capture (Lloyd et al., 2018), reduced stress behavior (Chin et al., 2018), reductions in aggressive behavior (Burchards et al., 1985; Elipot et al., 2013), changes in temperature preference (Tabin et al., 2018), and enhanced food finding ability in the dark (Hüppop, 1987). Further, some populations of cavefish with similar behaviors derive from independent colonization events (Gross, 2012; Herman et al., 2018), allowing for examination of repeated evolution of behaviors. Thus, cavefish are an excellent system for studying how and why behaviors evolve.

Behavioral evolution in cavefish

Foraging behaviors

Loss of light and reduced food availability are characteristic of many cave environments, and have resulted in robust changes in the foraging behavior of cave-adapted animals. *Astyanax mexicanus* cavefish have evolved multiple changes in foraging behaviors, including methods of capturing prey and amount of food consumption. Adaptation to an environment that lacks light has resulted in improved food finding ability in the dark in cavefish relative to surface fish (Hüppop, 1987). Although enhanced non-visual sensory systems undoubtedly contribute to this advantage under cave-like conditions, behavioral changes in foraging are also likely to benefit cavefish in the dark. For example, cavefish have evolved changes in what they approach in the dark. VAB is an attraction to movements in the water in the dark (Yoshizawa et al., 2010, 2015). Cavefish from multiple populations exhibit this behavior, whereas few surface fish display VAB (Yoshizawa et al., 2010). Small invertebrates, which can serve as prey for these fish within their natural habitats (Espinasa et al., 2017), elicit vibrations of a frequency that elicit this behavior (Montgomery and Macdonald, 1987; Yoshizawa et al. 2010), suggesting that VAB evolved to help cavefish find food in the dark. Indeed, this behavior is advantageous in cave-like conditions; fish with VAB are more successful at prey capture in the dark compared with fish without VAB (Yoshizawa et al., 2010).

Cavefish have also evolved differences in how they forage. Larval surface fish strike prey head on, whereas cavefish strike prey from a wider angle, and begin striking from a longer distance away from prey. This change in behavior is due to a shift away from visually mediated prey capture (Lloyd et al., 2018). Adult cavefish have also evolved differences in foraging. In the dark, cavefish have an altered posture while foraging relative to surface fish, feeding at a lower angle relative to the ground (Schemmel, 1980). This behavioral change has evolved repeatedly (Schemmel, 1980; Kowalko et al., 2013a), consistent with altered feeding posture being advantageous in the cave environment. However, whether or how this change in foraging benefits cavefish is still unknown.

In addition to darkness, food availability may be a driving force in the evolution of cave populations. Because caves lack light, photosynthesis cannot occur within caves, and obligate cave-dwellers are often dependent on food brought in from outside of the cave (Culver and Pipan, 2009). This can result in intermittent and/or reduced food availability in caves. *Astyanax mexicanus* cavefish have evolved multiple traits that could be advantageous in an environment with low nutrient availability and/or fluctuations in food availability, including loss of circadian rhythm in metabolism (Moran et al., 2014), reduced weight loss when food deprived

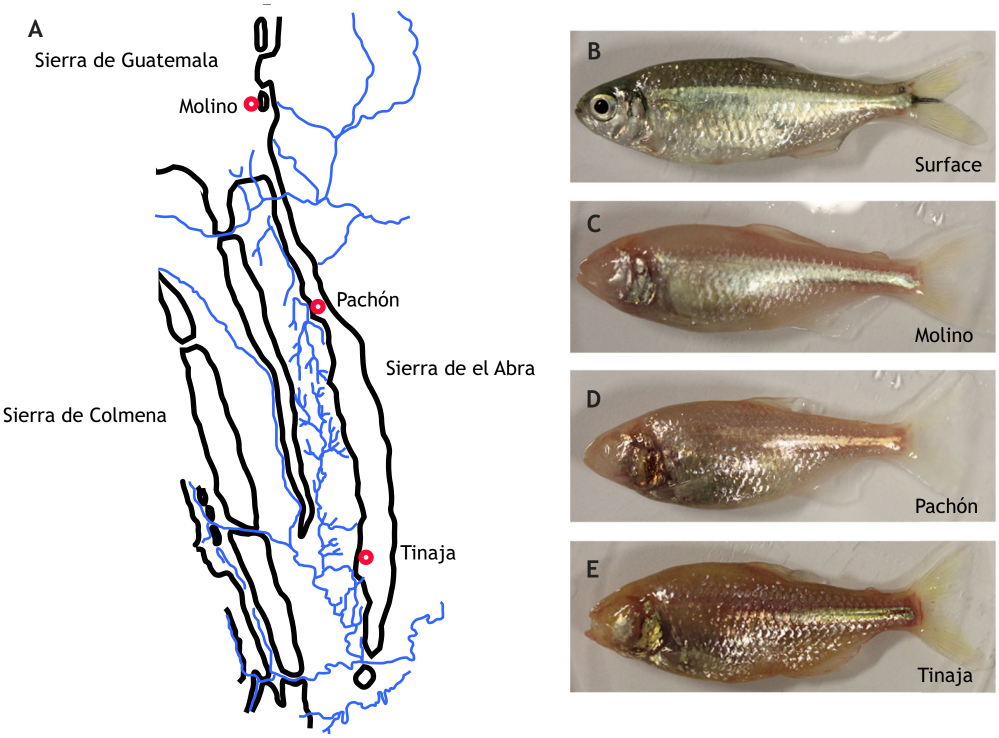

Fig. 1. *Astyanax mexicanus* is an emerging model system for understanding behavioral evolution. (A) Map of the region in Mexico containing cavefish caves. Cavefish are named after the cave from which they originate. The three cavefish populations discussed in this Review are Pachón, Tinaja and Molino. Images of adult (B) surface fish and (C–E) cavefish from different caves discussed in this Review.

(Aspiras et al., 2015), early development of fat stores, increased fat stores and increased body weight (Aspiras et al., 2015; Riddle et al., 2018; Xiong et al., 2018), and in some cave populations, differences in appetite regulation (Aspiras et al., 2015). Laboratory-raised cavefish from two independently evolved cave populations, Molino and Tinaja, are hyperphagic, consuming more than surface fish under fed conditions (Aspiras et al., 2015). Thus, cavefish may forage more when food is available as an adaptation to an environment characterized by long periods of low nutrient availability.

Sleep

Nearly all animals sleep, and obtaining an appropriate amount of sleep is crucial; sleep deprivation has negative consequences on multiple aspects of health (reviewed in Keene and Duboue, 2018). However, examination of species-specific differences in sleep reveal that sleep duration is highly variable (Keene and Duboue, 2018; Siegel, 2005; Capellini et al., 2008a). Some species sleep for the majority of the day, and others sleep only a few hours (Siegel, 2005; Capellini et al., 2008a). Although little is known about the ecological factors and evolutionary forces driving these differences, foraging strategy and food availability may influence inter-specific sleep differences (Capellini et al., 2008b; Capellini, 2010). For example, large herbivores sleep less than small herbivores and carnivores, presumably because large herbivores require greater foraging time to meet their energy needs (Siegel, 2005; Capellini et al., 2008a; Capellini, 2010). Additionally, sleep and feeding may be functionally linked. Sleep deprivation is associated with hyperphagia, and increased sleep is a proposed mechanism of energy conservation (Berger and Phillips, 1995; Siegel, 2009; Knutson and Van Cauter, 2008; Masek et al., 2014). Cavefish, which have evolved a number of traits associated with living in an environment with low and/or irregular food availability, sleep for dramatically less time than surface fish (Duboué et al., 2011; Yoshizawa et al., 2015). Further, reduced sleep in cave populations persists from early stages of larval development through adulthood and occurs independently from light–dark cues present during testing (Duboué et al., 2011; Yoshizawa et al., 2015). Thus, cavefish may have evolved reduced sleep duration owing to the need for increased foraging time in a low-nutrient environment.

Social behaviors – aggression and schooling

Social behaviors, such as mating behaviors, aggression and collective motion, are critical for survival and reproduction. Although the neural and molecular underpinnings of these behaviors have been subject to intense study, much less is known about how these behaviors evolve. Aggressive behaviors are nearly ubiquitous across the animal kingdom and play roles in competition for resources and mates, and defense. Surface *A. mexicanus* are highly aggressive, while cavefish from multiple populations have evolved reduced aggression (Wilkens, 1988; Burchards et al., 1985; Breder, 1943). Why loss of aggression has evolved in cavefish is currently unknown. However, it has been proposed that cavefish have undergone a shift from aggressive behaviors to foraging (Elipot et al., 2013).

Fish form social groups called shoals and schools. Shoaling and schooling play a role in predator avoidance and food finding (Landeau and Terborgh, 1986; Partridge, 1982). However, when food is scarce, fish from a number of species reduce shoaling, demonstrating that this behavior can be regulated by food availability (Landeau and Terborgh, 1986; Partridge, 1982; Magurran et al., 1985; Baird et al., 1991). Surface fish form schools and shoals in the laboratory and in the field (Parzefall and Fricke, 1991; Parzefall, 1983). In contrast, cavefish from multiple cave populations exhibit reduced schooling and shoaling behaviors (Kowalko et al., 2013b; Parzefall and Fricke, 1991; Parzefall, 1983, 1985; Gregson and Burt De Perera, 2007). Reduced collective behaviors could have evolved as a result of selection against shoaling in a nutrient-poor environment. Alternatively, the lack of macroscopic predators in the cave environment may have removed the selective pressures to maintain shoaling once ancestral fish entered caves (Kowalko et al., 2013b).

Lessons about behavioral evolution derived from cavefish studies

Genetic underpinnings of behavioral evolution

Identifying the genetic architecture and specific genetic changes that underlie behavioral evolution is critical to understanding how behaviors evolve. Studying behavioral evolution in species with distinct ecomorphs that are interfertile, yet differ in behaviors, has led to insights into the genetic underpinnings of behavioral evolution. For example, crosses of individuals from closely related populations can be used to approximate the number of genes and type of inheritance underlying evolution of behaviors (e.g. Schemmel, 1980; Dawson et al., 1988). Further, crosses can be used to identify regions of the genome associated with evolved behaviors, allowing for identification of candidate genes that may underlie behavioral evolution. For example, quantitative trait loci (QTL) analysis has led to the identification of regions of the genome associated with reduction of schooling behavior in sticklebacks and evolution of parental care and burrowing behavior in *Peromyscus* (Greenwood et al., 2013; Bendesky et al., 2017; Weber et al., 2013). Additionally, crosses can be used to determine whether different behavioral components have evolved due to a shared genetic basis (e.g. Greenwood et al., 2013; Bendesky et al., 2017; Weber et al., 2013; Metz et al., 2017).

Research in *A. mexicanus* has leveraged crosses to determine heritability of behaviors and to estimate modes of inheritance (Schemmel, 1980; Parzefall, 1985). More recently, mapping studies have identified QTL for a number of behavioral changes in cavefish, including schooling (Kowalko et al., 2013b), feeding posture (Kowalko et al., 2013a), VAB (Yoshizawa et al., 2015, 2012b) and locomotor activity (Yoshizawa et al., 2015; Carlson et al 2018). These studies have demonstrated that behavioral evolution in cavefish often occurs through multiple genetic changes, each with small effect sizes rather than single genes of large effect (Kowalko et al., 2013a,b; Yoshizawa et al., 2015; Yoshizawa and Jeffery, 2011). Additional studies will be needed to reveal whether evolving through multiple, small-effect genetic changes is a general principle underlying cavefish behavioral evolution.

QTL mapping can be also used to identify candidate genes that may contribute to the evolution of traits. In cavefish, QTL analysis has been used to identify the genes responsible for morphological evolution, such as loss of pigmentation (Gross et al., 2009; Protas et al., 2006). These methods can also be applied to identify candidate genes associated with behavioral evolution. For example, QTL mapping of parental behaviors in two species of *Peromyscus* identified the gene encoding arginine vasopressin within a QTL for nest building, and pharmacological and genetic experiments confirmed that arginine vasopressin can modulate nest building behaviors (Bendesky et al., 2017). Candidate genes underlying behavioral QTL in *A. mexicanus* have not yet been identified and examined. However, the cavefish genome has been sequenced (McGaugh et al., 2014), and can be used in future studies to identify candidate genes underlying behavioral QTL that can be examined using functional analyses.

In addition to mapping studies, other methods, including transcriptomics and population genetics, have been used to identify candidate genes associated with differences in behavior in *A. mexicanus*. For example, Yoshizawa et al. (2018) reported that genes associated with autism spectrum disorder (ASD) in humans are enriched for differential expression between cavefish and surface fish. Further, evaluation of molecular evolution of these genes by whole genome sequencing of multiple individuals from surface and multiple cave populations demonstrated that this ASD-associated gene set in *A. mexicanus* is enriched for genes under positive selection (Yoshizawa et al., 2018).

Candidate gene approaches, facilitated by the existence of the cavefish genome (McGaugh et al., 2014), have been used to identify cave and surface fish coding variants in candidate genes for behavioral evolution. Hyperphagia in cavefish is associated with coding mutations in the *melanocortin 4 receptor* (*mc4r*) gene. MC4R plays a role in energy homeostasis, and mutations in the *mc4r* gene are associated with obesity in humans (Tao, 2010). The cavefish-associated allele of *mc4r* decreases the activity of the MC4R protein in cell culture. To examine the role of this variant *in vivo*, Aspiras et al. (2015) studied the effects of the cave-associated variant in fish from the Pachón cavefish population, which harbors both ancestral and derived alleles of *mc4r*. Pachón cavefish homozygous for the cavefish-associated allele exhibit hyperphagia and increased starvation resistance compared with cavefish heterozygous for the allele, supporting a role for *mc4r* in the evolution of hyperphagia (Aspiras et al., 2015). This cavefish-associated *mc4r* allele is also present at low levels in surface populations. Thus, some cave behaviors may evolve from selection on standing genetic variation present in the ancestral population (Aspiras et al., 2015).

Another coding variant identified by comparing candidate genes between cave and surface fish was found in the *monoamine oxidase* (*mao*) gene, which encodes the enzyme that catalyzes the oxidation of monoamines. This variant is hypothesized to contribute to the evolution of serotonin-regulated behaviors such as reduced aggression in cavefish. Consistent with this hypothesis, MAO enzymatic activity is lower in Pachón cavefish brains relative to surface fish brains, and inhibition of MAO in fish in both populations results in increased brain serotonin levels. Further, mammalian MAO mutated to produce the Pachón cavefish-associated substitution reduces MAO activity relative to the wild-type sequence in cell culture (Elipot et al., 2014a). Cave-associated mutations in the *mao* gene may also affect schooling, as pharmacological inhibition of MAO reduces schooling and shoaling in surface fish (Kowalko et al., 2013b). Thus, some behaviors in cavefish may have evolved as a result of a shared genetic basis.

Role of sensory systems in cave-evolved behaviors

Animals execute behaviors based on both internal and external cues. As external cues are often mediated through one or more sensory systems, understanding the role different sensory inputs play in modulating behaviors is crucial. In addition to evolved behaviors, *A. mexicanus* cavefish have evolved changes in sensory systems, including an enhanced lateral line, loss of vision, increases in the number and distribution of taste buds, and enhanced olfaction (Wilkens, 1988; Teyke, 1990; Bibliowicz et al., 2013; Protas et al., 2008; Schemmel, 1980; Blin et al., 2018; Jeffery, 2001). Thus, how behaviors evolve in the context of evolving sensory systems can be examined in this species.

Eye degeneration evolved in cavefish following multiple generations spent in the lightless cave environment. Thus, any behaviors in surface fish requiring visual cues either evolved to utilize other sensory cues in cavefish, or can no longer occur in the blind cavefish. There is evidence that different behaviors evolved in both of these ways in cavefish. For example, whereas surface fish use vision to hunt, cavefish must capture prey using non-visual cues. This has been demonstrated in larval fish; differences in strike angle and strike distance during prey capture between cavefish and surface fish are likely due to an evolutionary shift from vision-dependent prey capture in surface fish to lateral-line-dependent prey capture in cavefish (Lloyd et al., 2018).

Loss of vision played a major role in evolution of reduced schooling behavior in cavefish. Vision is required for schooling in this species. Surface fish cannot school without visual cues, and there is a strong correlation between two proxies of vision loss, eye size and dark preference, and schooling in surface–cave hybrid fish. Together, these data suggest that cavefish did not evolve to utilize non-visual cues to school (Kowalko et al., 2013b; Gregson and Burt De Perera, 2007). Genetic mapping studies further support an association between loss of schooling and vision. QTL for dark preference and schooling overlap. However, some hybrid fish with large eyes and a strong preference for the dark still do not school, and mapping studies identified additional, visual-system independent QTL for schooling behavior, suggesting that other, sensory system-independent genetic changes may also contribute to loss of schooling in cavefish (Kowalko et al., 2013b).

Non-visual sensory systems such as the olfactory system, the lateral line and taste are enhanced in cavefish relative to surface fish (Teyke, 1990; Protas et al., 2008; Schemmel, 1974). Thus, any behaviors mediated by these sensory systems could be affected by their enhancement in cavefish. The lateral line has been implicated in schooling in other fish species (Partridge, 1982; Partridge and Pitcher, 1980), and ablation of the lateral line in saithe causes fish to swim closer to their neighbors, suggesting that the lateral line could provide a repulsive force in schooling behavior (Partridge and Pitcher, 1980). Thus, an enhanced lateral line could play a role in loss of schooling in cavefish. Indeed, a minor, but statistically significant, negative correlation between number of superficial neuromasts, sensory organs of the lateral line, and tendency to school was found in cave–surface hybrid fish. However, ablation of the lateral line is not sufficient to alter schooling or shoaling behaviors in surface or cavefish, suggesting that an enhanced lateral line does not play a major role in the evolutionary loss of schooling in cavefish (Kowalko et al., 2013b).

Lateral line enhancement plays a more significant role in the evolution of other cave behaviors, including sleep and VAB. Ablation of the lateral line in Pachon cavefish, but not surface fish, increases sleep duration, suggesting that enhanced sensitivity to movements in the water plays a role in evolution of sleep loss in this cavefish population (Jaggard et al., 2017). Additionally, the superficial neuromasts of the lateral line are required for VAB (Yoshizawa et al., 2010, 2012b). Further, eye size, number of neuromasts in the orbital region and VAB are correlated in cave–surface hybrids, and QTL for these traits overlap. Thus, selection for enhancement of VAB and an enhanced lateral line may have contributed to the evolution of reduction in eyes in cavefish through a shared genetic basis (Yoshizawa et al., 2012b). In contrast, sleep duration and VAB are not correlated in hybrid fish, suggesting that these behaviors do not share a genetic basis in spite of their reliance on the same sensory system (Yoshizawa et al., 2015).

Connecting brain evolution to behavioral evolution

One challenge in behavioral neuroscience is identifying the changes in neural anatomy that underlie behavioral variation. In *A. mexicanus*, brain anatomy of closely related individuals that differ in behavior can be compared to determine what evolved brain differences underlie behavioral evolution. Comparative approaches have revealed a number of evolved differences in brain anatomy in cavefish. These include a reduction in the optic tectum, which is involved in visual processing, and an expansion of the hypothalamus (Soares et al., 2004; Menuet et al., 2007; Loomis et al., 2019 preprint), a region of the brain involved in regulating a number of cave-evolved behaviors, including sleep, feeding, stress and social behaviors (Kowalko et al., 2013b; Duboué et al., 2011; Chin et al., 2018; Elipot et al., 2013; Aspiras et al., 2015; Yoshizawa et al., 2015). Notably, although the cavefish hypothalamus is expanded in size, not all regions of the hypothalamus are larger relative to surface fish. Detailed measurements of hypothalamic nuclei in an adult *A. mexicanus* brain atlas show that some hypothalamic subnuclei are larger in cavefish relative to surface fish, while others are similar in size between morphs (Loomis et al., 2019 preprint). Quantification of numbers of neuropeptide-expressing neurons in the hypothalamus and preoptic regions of cavefish and surface fish during development reveals a similar pattern. Although some neuropeptinergic cell types are greater in number in cavefish, others are the same and still others are reduced (Alié et al., 2018). Thus, the hypothalamus is not simply expanded uniformly in cavefish, but instead, particular subnuclei and cell types have evolved changes.

Some of these evolved differences in the hypothalamus have been directly linked to the evolution of behavior. Enhancement of Hypocretin (HCRT) signaling, a critical regulator of arousal (reviewed in Tyree et al., 2018), has been implicated in the evolution of sleep loss in cavefish. Pachón cavefish have more HCRT-expressing neurons and increased levels of HCRT mRNA and protein compared with surface fish. Further, pharmacological treatments that inhibit HCRT signaling, genetic silencing of HCRT-expressing neurons and knockdown of *hcrt* all promote sleep in cavefish, strongly suggesting that evolution of enhanced HCRT signaling contributes to sleep loss in cavefish (Jaggard et al., 2018). Manipulations that alter sleep in Pachón cavefish such as neuromast ablation or starvation (Jaggard et al., 2017) also affect HCRT levels, suggesting that observed changes to sleep following these perturbations are integrated by HCRT-expressing neurons (Jaggard et al., 2018). The developmental basis for evolution of increased numbers of HCRT cells in cavefish has also been investigated. Cavefish have evolved changes in expression of the genes encoding the signaling molecules Fibroblast Growth Factor 8 (FGF8) and Sonic Hedgehog (SHH), and these gene expression changes have been linked to forebrain evolution in cavefish (Menuet et al., 2007; Pottin et al., 2011). Inhibition of either FGF8 or SHH signaling during development reduces the number of *hcrt*-positive cells in cavefish, consistent with alterations in early gene expression of *fgf8* and *shh* contributing to evolution of increased HCRT signaling in cavefish (Alié et al., 2018).

Evolution of monoamines system, catecholamines and serotonin, has also been associated with evolution of cavefish behavior. Pachón and Tinaja cavefish have evolved increased levels of the catecholamines noradrenaline and dopamine (Elipot et al., 2014a; Bilandžija et al., 2013, 2018). Treating larval Pachón cavefish with propranolol, a β-adrenergic receptor antagonist, increases sleep, while surface fish sleep is unaffected by this drug (Duboué et al., 2012). Thus, enhanced catecholamine levels could contribute to the evolution of sleep loss in cavefish. Additionally, aggression in *A. mexicanus* has been linked to serotonin, and the serotonergic system has evolved in cavefish, both at the neuroanatomical level and at the level of total serotonin levels (Elipot et al., 2013, 2014a; Bilandžija et al., 2019 preprint). Further, inhibition of SHH signaling both reduces the size of two serotonin-positive nuclei and increases the number of aggressive attacks by cavefish (Elipot et al., 2013), suggesting that evolved differences in SHH signaling in early development could affect the evolution of aggressive behavior in cavefish through altering the number of serotonergic neurons in the brain. As enhanced SHH signaling in cavefish has also been linked to increased numbers of HCRT-positive neurons (Alié et al., 2018), it is possible that evolution of sleep loss and reduced aggression share a genetic basis. However, the relationship between sleep and aggression has not been examined in cavefish. Finally, Yoshizawa et al. (2012b) overexpressed *shh* in surface fish to determine whether eye loss owing to enhanced SHH signaling was sufficient to enhance VAB. Although *shh* overexpression reduced eye size, VAB and neuromast number were unaffected in these fish, suggesting that the relationship between eye size, VAB and neuromast number is independent of enhanced *shh* expression (Yoshizawa et al., 2012b). These studies demonstrate that multiple cell types and regions of the *A. mexicanus* cavefish brain have evolved, and they suggest that evolution of different behaviors has occurred through evolution of at least some different regions of the brain.

Repeated evolution of behaviors

Adaptation to similar environmental conditions can lead to convergence on the same evolved traits in independent lineages. Whether the same or different mechanisms underlie the repeated evolution of behavioral traits is largely unknown. However, studies in cavefish have begun to address this question. Fish from independently evolved cave populations exhibit many of the same behaviors, including sleep loss (Duboué et al., 2011; Yoshizawa et al., 2015), loss of schooling (Kowalko et al., 2013b), altered feeding posture (Schemmel, 1980; Kowalko et al., 2013a) and reduced stress behaviors (Chin et al., 2018), allowing for examination of repeated evolution of these behavioral traits. Some behaviors in *A. mexicanus* cavefish appear to have evolved through different genetic changes in different cave populations. For example, quantification of feeding posture in Tinaja–Pachón hybrids and QTL mapping both suggest that feeding posture in these two cave populations has evolved through at least some different genetic changes (Kowalko et al., 2013a). Additionally, while multiple *A. mexicanus* cavefish populations have evolved enhanced lateral lines (Teyke, 1990; Kowalko et al., 2013b; Yoshizawa et al., 2012a, 2013a; J.E.K., unpublished), lateral line ablation increases sleep only in Pachón cavefish, indicating that different mechanisms underlie the evolution of sleep loss in different cavefish populations (Jaggard et al., 2017).

Other repeatedly evolved behavioral traits may share at least some underlying genetic changes. The presence of the cave-associated alleles of two candidate behavioral genes, *mao* and *mc4r*, were examined in natural populations of cavefish. Cave-associated alleles of both of these genes were present in multiple cave populations, suggesting that the derived behaviors associated with these genes may have evolved through at least some of the same genetic changes in independently evolved cave populations (Aspiras et al., 2015, Elipot et al., 2014). These studies highlight the power of cavefish to identify different molecular pathways underlying repeated evolution.

Examining behavioral evolution through application of gene editing

Gene editing in *A. mexicanus*: a case study for examining the role of the *oculocutaneous albinism 2* gene in the evolution of albinism

Functional manipulations are key to identifying the genes and neurons underlying behavioral evolution. Until recently, the genetic

Fig. 2. Gene editing demonstrates that mutations in *oca2* cause albinism in *A. mexicanus* cavefish. Surface fish were engineered to contain *oca2*-mutant alleles harboring a 2 bp deletion ($oca2^{2bpdel}$). Incrosses of $oca2^{2bpdel/+}$ surface fish produce (A) $oca2^{+/+}$, pigmented surface fish, and (B) $oca2^{2bpdel/2bpdel}$, albino surface fish.

tools available for making functional connections between genotypes and behavioral phenotypes in *A. mexicanus* were approaches such as morpholinos, transient overexpression, pharmacology or transgenics (e.g. Kowalko et al., 2013b; Elipot et al., 2013, 2014b; Alié et al., 2018; Jaggard et al., 2018; Bilandžija et al., 2018; Duboué et al., 2012; Stahl et al., 2019b preprint). Gene editing complements and, in some cases, improves upon these other approaches by providing the opportunity to make targeted knockouts and knock-ins. Knocking out genes is more specific than pharmacology, providing the opportunity to associate loss of a specific gene product with a behavior. Morpholinos can target specific genes, but are transient, and cannot be used to study adult phenotypes. Further, morpholino-induced phenotypes can be different than knockout phenotypes targeting the same gene, likely owing to compensation (Rossi et al., 2015; Kok et al., 2015). In contrast, gene knockouts, such as those generated using CRISPR/Cas9, can be used to study the phenotypic effects of loss-of-function alleles, and the contribution of specific genes to behavioral traits at later stages in development.

Gene editing techniques have recently been utilized in *A. mexicanus* to functionally assess the role of candidate genes in the evolution of cave traits (Stahl et al., 2019a; Kowalko et al., 2016; Ma et al., 2015; Klaassen et al., 2018). This research has focused on investigating the genetic basis of albinism, i.e. the complete loss of melanin pigmentation, in cavefish. Both genetic crosses and QTL mapping are consistent with a single locus being responsible for albinism in cavefish (Protas et al., 2006; Şadoglu, 1957). Further, complementation studies suggest that the same gene underlies albinism in at least three cavefish populations (Protas et al., 2006). The *oculocutaneous albinism 2* (*oca2*) gene lies within the QTL for albinism, and different deletions in coding regions of this gene are found in Pachón and Molino cavefish, suggesting convergence on albinism through mutations in *oca2* in cavefish (Protas et al., 2006). Mutations in the *oca2* gene are responsible for loss or reduction of pigmentation in multiple species, including humans, mice, zebrafish and medaka (Grønskov et al., 2007; Beirl et al., 2014; Brilliant et al., 1994; Fukamachi et al., 2004). Mutating *oca2* in surface fish using both TALENs and CRISPR/Cas9 demonstrates that mutations in this gene cause albinism in this species (Kowalko et al., 2016; Klaassen et al., 2018) (Fig. 2). One advantage of the *A. mexicanus* system is that surface fish and cavefish hybridize. Capitalizing on this, a complementation test was performed to definitively demonstrate that cavefish *oca2* alleles are responsible for albinism in cavefish. Surface fish harboring CRISPR/Cas9 engineered mutant *oca2* alleles were crossed to fish from two albino cave populations, Pachón and Molino. Clutches from both of these crosses contained albino offspring, demonstrating that mutations in *oca2* are solely responsible for albinism in these cave populations (Klaassen et al., 2018). This example demonstrates the power of gene editing for examining the genetic basis of trait evolution, and future applications of similar methods can be used to investigate a number of important questions about how behaviors evolve (Fig. 3).

Establishing genotype-to-phenotype connections

Gene editing technologies can be used to make functional connections between genotypes and behavioral phenotypes. For example, the analysis of CRISPR/Cas9 generated knockouts of the receptor for prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), a hormone involved in spawning behavior in fish (e.g. Villars et al., 1985, Stacey and Peter, 1979), demonstrated a role for $PGF_{2\alpha}$ signaling in the initiation of mating behavior in the cichlid species *Astatotilapia burtoni*, revealing a critical mechanism by which complex social behaviors are regulated in this species (Juntti et al., 2016). Using similar methods, researchers studying other non-traditional model organisms can investigate the functional role of specific genes in behavioral evolution.

One application of these techniques is to test whether candidate genes harboring mutations hypothesized to cause behavioral variation

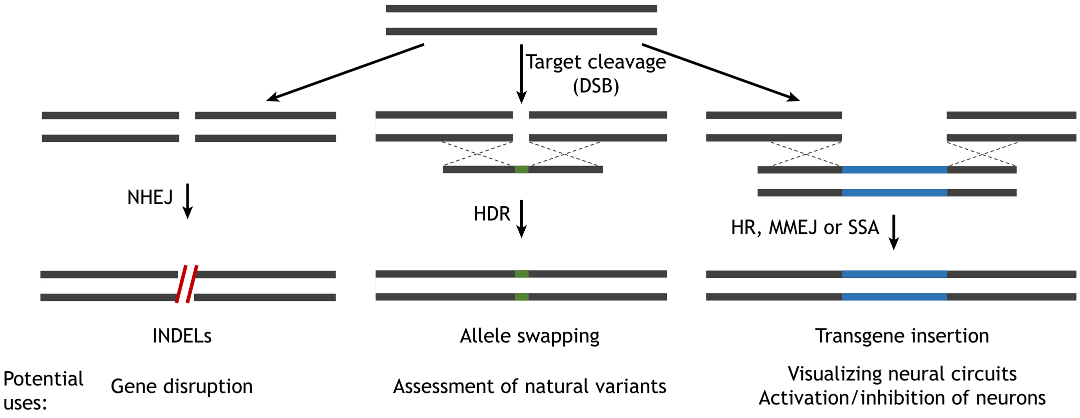

Fig. 3. Schematic of the types of genome engineering that can be performed using CRISPR/Cas9 or TALENs and the applications to understanding behavioral diversity. DSB, double-strand break; NHEJ, non-homologous end joining; INDELs, insertion or deletions; HDR, homology-directed repair; HR, homologous recombination; MMEJ, microhomology-mediated end joining; SSA, single strand annealing.

do indeed alter behaviors when functionally perturbed. *Astyanax mexicanus* is ideal for these types of studies, as a number of candidate genes with mutations hypothesized to be responsible for evolution of cavefish behaviors have been identified in this species. Potential targets include previously identified candidate genes for the evolution of cavefish behaviors, such as *mc4r* and *mao* (Aspiras et al., 2015; Elipot et al., 2014a), and additional candidate genes that could be identified by examining the genes underlying QTL identified by previous mapping studies.

Alleles of genes associated with evolution of traits are not always coding mutations predicted to be loss-of-function (e.g. Aspiras et al., 2015; Riddle et al., 2018; Elipot et al., 2014a; Manceau et al., 2010). Gene editing has been used successfully in other species to generate precise knock-ins utilizing a repair template including an exogenous DNA sequence or a single nucleotide polymorphism, which can be integrated into the genome through homology-directed repair (reviewed in Chen et al., 2014; Doudna and Charpentier, 2014) (Fig. 3). Application of these approaches in non-traditional model organisms will allow for examination of the functional consequences of the precise genetic changes identified in these natural populations. For example, these methods can be used in *A. mexicanus* to perform allele swapping to generate cavefish with a surface allele or surface fish with a cavefish allele in genes such as *mao*, which harbor derived variants predicted to reduce, but not ablate, protein function. Further, some causative mutations underlying the evolution of traits are regulatory changes, not coding changes (e.g. Young, 2003; Steiner et al., 2007; O'Brown et al., 2015; Sucena et al., 2003; Shapiro et al., 2004; Wittkopp et al., 2002). Establishment of technologies for allele swapping in non-model organisms will be critical to evaluating the contribution of these regulatory changes to behavioral evolution, as specific changes to regulatory regions can be performed.

Examining the contributions of sensory systems to behaviors

Critical to examining behavioral evolution is understanding the sensory inputs that affect behaviors. Gene editing can be used to manipulate genes known to be required for sensory system function to examine the role sensory systems play in modulating behaviors. For example, in ants, the role of the olfactory system in social behaviors was demonstrated through generation of olfactory deficiencies by CRISPR/Cas9 knockout of *orco*, a gene encoding an odorant receptor co-receptor that is required for function of all odorant receptors in ants (Yan et al., 2017; Trible et al., 2017). The *orco* mutant ants of two species, *Ooceraea biroi* and *Harpegnathos saltator*, have deficiencies in olfaction (Yan et al., 2017; Trible et al., 2017). Additionally, *orco* mutants from both of these species have defects in social behaviors. *Ooceraea biroi* individuals mutant for *orco* are deficient in trail-following and nesting behavior (Trible et al., 2017). In *H. saltator* ants, young individuals mutant for *orco* spend longer outside of their nests, *orco*-mutant workers display reduced social behavior necessary for increasing reproductive status to become gamergates in the absence of the queen, and *orco*-mutant females do not perform mating behaviors (Yan et al., 2017).

Application of gene editing could also contribute to a better understanding of the role sensory systems play in behavioral evolution in *A. mexicanus*. For example, QTL analysis has been performed for eye size, vision, enhanced sensitivity to amino acids, and number of superficial neuromasts in the lateral line (Protas et al., 2008, 2007; Kowalko et al., 2013b; Yoshizawa et al., 2012b). Thus, candidate genes for these traits identified through mapping studies could be genetically manipulated to examine their effect on both sensory systems and behaviors.

Pleiotropy and behavioral evolution

A central question in evolutionary genetics is the role pleiotropy plays in the evolution of traits. Pleiotropy could have multiple effects on evolution depending on the fitness consequences on the different traits affected. Pleiotropy could impose restrictions on a gene's use in evolution if a mutation within that gene has a negative impact on fitness owing to one or more of the traits it affects (Fisher, 1930). Alternatively, pleiotropy could result in the evolution of a trait as a secondary effect of positive selection for another, unrelated trait affected by the same genetic variant. Finally, pleiotropy could enhance adaptation through positive fitness impacts of multiple traits resulting from the same mutation (reviewed in Dittmar et al., 2016). Given the complex effects of pleiotropy on evolution, it is critical to understand the role specific alleles play in all of the phenotypes they affect.

Pleiotropy has been hypothesized to play a role in multiple aspects of *A. mexicanus* cavefish evolution (Protas et al., 2008; Yamamoto et al., 2009). One of these is loss of pigmentation, a hallmark characteristic of cave organisms. Classically, it was hypothesized that pigmentation loss in cave animals was due to genetic drift (reviewed in Culver and Pipan, 2009). In surface habitats, pigmentation has multiple functions, including camouflage, visual cues for conspecifics and UV protection (reviewed in Protas and Patel, 2008). In cave habitats, where light is absent, both UV protection and visual cues become irrelevant. Under these ecological conditions, loss of pigmentation could evolve following the loss of purifying selection to maintain functional copies of the genes underlying pigmentation traits and accumulation of neutral mutations over time. However, recent studies in *A. mexicanus* suggest that mutations in the pigmentation gene *oca2* may contribute to other cave traits, raising the intriguing possibility that loss of pigmentation could be adaptive (Bilandžija et al., 2013, 2018). Albino Pachón cavefish have evolved elevated levels of the catecholamines dopamine and noradrenaline, and knockdown of *oca2* in surface fish using morpholinos results in increased total dopamine in larval fish (Bilandžija et al., 2013). A number of behaviors associated with enhanced catecholamine levels have evolved in cavefish populations, including sleep and shoaling (Kowalko et al., 2013b; Duboué et al., 2011, 2012; Ouyang et al., 2004; Scerbina et al., 2012). Thus, these results provide a potential link between evolution of albinism and evolution of cave-associated behaviors. Indeed, one behavior, anesthesia resistance, which is enhanced in cavefish, is also enhanced in *oca2*-morphant larval surface fish, suggesting that *oca2* plays a role in the evolution of at least one catecholamine-regulated behavior (Bilandžija et al., 2018). Although morpholinos have provided intriguing insights into how *oca2* affects catecholamines and behavior (Bilandžija et al., 2013, 2018), these types of functional studies are limited to investigations of early developmental stages and cannot be used to examine the relationship between *oca2* and traits that manifest later in development, such as adult brain catecholamine levels or catecholamine-regulated behaviors such as sleep and schooling. Examination of behaviors and catecholamines in recently published *oca2* mutant surface fish (Klaassen et al., 2018) should resolve the role *oca2* plays in the evolution of these traits.

QTL analysis has also identified loci that may contain genes that play a pleiotropic role in cavefish evolution. QTL for multiple cavefish traits overlap, consistent with either pleiotropy of the genetic changes responsible for these traits, or close physical linkage between different genetic changes each responsible for a different trait (Protas et al., 2008; Yoshizawa et al., 2012b, 2013b). For example, QTL for eye size, number of the intraorbital superficial neuromasts of the lateral line, and VAB overlap in two locations in the *A. mexicanus* genome (Yoshizawa et al., 2012b). Identification of the genes and genetic changes within these QTL, and functionally manipulating them through methods such as gene editing, can resolve whether these overlapping QTL are due to pleiotropy or linkage of multiple causative alleles. Thus, gene editing has the potential to answer long-standing questions about the role of pleiotropy in evolution.

Neural basis of natural variation in behaviors

Little is known about the neural basis of behavioral evolution. In model systems, genetic engineering has been crucial for understanding the neurons and neural circuits that underlie specific behaviors. Application of genetic engineering technologies in nontraditional model systems has the potential to elucidate the neural underpinnings of behavioral diversity. For example, gene editing has uncovered species-specific differences in the neural circuits underlying variations in mating behavior in different *Drosophila* species. The organization of the neural circuitry responsible for male courtship behavior in *Drosophila melanogaster* is regulated by the *fruitless* (*fru*) gene, and mating behavior in male *D. melanogaster* with null alleles of *fru* is reduced or absent (reviewed in Yamamoto et al., 2014). Males of another *Drosophila* species, *D. subobscura*, perform mating behaviors not observed in *D. melanogaster* (Immonen et al., 2009; Steele, 1986), providing the opportunity to investigate the role of *fru* in interspecific differences in mating behaviors. Mutations in *fru* in *D. subobscura* were generated using CRISPR/Cas9. *Drosophila subobsura* homozygous *fru*-mutant males had defects in courtship behavior, demonstrating conserved functions of this gene in courtship across *Drosophila* species (Tanaka et al., 2017). To determine whether *fru*-expressing neurons mediate *D. subobscura*-specific courtship behaviors, CRISPR/Cas9 was used to knock-in an optogenetic activator and a fluorescent reporter to the *fru* locus. Visualization of *fru*-labeled circuitry in *D. subobscura* revealed differences between *fru*-expressing neuronal circuitry in *D. subobscura* and *D. melanogaster* (Tanaka et al., 2017). Further, optogenetic activation of the *fru*-expressing neurons in *D. subobscura* males resulted in isolated males performing a subset of mating behaviors, including a *D. subobscura*-specific mating behavior, demonstrating that this species-specific mating behavior is mediated by *fru*-expressing neurons (Tanaka et al., 2017).

Genetic manipulations using gene editing methods such as those described above can be used to uncover the neural circuits that contribute to behavioral diversity in *A. mexicanus* through multiple methods. Functionally knocking out specific genes in the brain can determine whether these genes are required for an evolved behavior. Additionally, the ability to knock-in genes encoding fluorescent proteins to specific loci will allow for visualization of neural circuits and comparative anatomy. Finally, knock-ins provide a means for neuron or circuit-specific functional manipulation, such that behaviors can be examined following activation or silencing of specific neurons. For example, recent studies have identified evolved differences in the numbers of neurons in the hypothalamus expressing different neuropeptides between cavefish and surface fish (Alié et al., 2018; Jaggard et al., 2018). Tol2 transgenesis has been used successfully in *A. mexicanus* (Elipot et al., 2014b; Stahl et al., 2019a,b preprint), and transgenesis utilizing the Gal4/UAS system was used to silence HCRT-expressing neurons and establish a role for these neurons in sleep in cavefish (Jaggard et al., 2018). This work leveraged a previously identified promoter driving expression in *hcrt*-expressing neurons from another species. Using gene editing, the role of other neuropeptinergic cell types that vary in number between cavefish and surface fish can be investigated without requiring the use of transgenesis or the characterization of a promoter to drive cell-type-specific expression. For example, cavefish have more *neuropeptide Y* (*npy*)-expressing neurons in the hypothalamus compared with surface fish (Alié et al., 2018), and NPY has been shown to play a role in cavefish-evolved behaviors, such as sleep, in other species (e.g. Prober et al., 2006). To uncover the role of NPY and NPY-expressing neurons in the evolution of sleep loss in cavefish, the *npy* gene could be mutated using CRISPR/Cas9 mutagenesis, or genes encoding proteins to activate or silence neurons could be knocked-in to the *npy* locus to determine the role of these neurons in sleep in this species.

Establishing a role for evolved behavior in survival and fitness

Central to understanding why behaviors have evolved is establishing whether behaviors are adaptive in a specific environmental context. However, uncovering the fitness consequences of an evolved behavior is difficult, as it requires being able to disentangle the contribution of a particular behavior from the contributions of other traits present in an individual. For example, cavefish are better at finding food in the dark than surface fish (Yoshizawa et al., 2010; Hüppop, 1987). However, this could be due to one or multiple evolved traits, such as increased number of taste buds, enhanced olfaction, altered feeding posture, or VAB (Bibliowicz et al., 2013; Protas et al., 2008; Yoshizawa et al., 2010; Schemmel, 1980, 1974). The contributions of VAB specifically to food finding in *A. mexicanus* was investigated, leveraging the variability of this trait in both cave and surface fish in the laboratory. Thus, food competitions could be performed between fish of the same morph with and without VAB, and in this way an advantage of VAB in food-finding in the dark was established (Yoshizawa et al., 2010). For traits that do not vary within a population, being able to functionally alter behaviors can uncover their contributions to fitness. For example, CRISPR/Cas9-generated *orco* mutants in two ant species had reduced fecundity and offspring survival rates, suggesting that *orco* mutants have reduced fitness (Yan et al., 2017; Trible et al., 2017). Using similar methods, genetic manipulation of behaviors in *A. mexicanus* could provide insight into whether these behaviors are adaptive in cave-like conditions.

Evolutionary medicine

Animal models for human disease have been restricted to a few species. However, evolution has generated species that display a wide range of phenotypic diversity, the study of some of which may provide novel insight into the causes of human behaviors (Johnson and Young, 2018). *Astyanax mexicanus* has previously been proposed as a model for evolutionary medicine for understanding traits relevant to human health and disease, including retinal degeneration and insulin resistance (Albertson et al., 2009; Krishnan and Rohner, 2019). The ability to manipulate the genomes of these fish through transgenesis (Stahl et al., 2019b preprint) and gene editing (Kowalko et al., 2016; Klaassen et al., 2018) will further advance their use in understanding the genetic basis of medically relevant traits. Such techniques are already being used in other non-traditional model organisms to investigate the genetic basis of human behavioral variation. For example, CRISPR/Cas9 mutagenesis of *oxytocin receptor* in prairie voles revealed a role for this gene in behaviors potentially relevant to ASD, repetitive behaviors and social novelty (Horie et al., 2019). The generation of additional genetically altered nontraditional model organisms with diverse behaviors and the application of techniques such as CRISPR/Cas9-mediated knock-in within *A. mexicanus* has the potential for broad applications to human health and disease.

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