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Developmental environment contributes to rapid trait shifts among newly colonized subterranean habitats

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ABSTRACT

Recent colonization of extreme environments provides unique opportunities to study the early steps of adaptation and the potential for rapid convergent evolution. However, phenotypic shifts during recent colonization may also be due to plasticity in response to changes in the rearing environment. Here, we analyzed a suite of morphological and behavioral traits in paired surface, subterranean, and facultatively subterranean Mexican tetras (*Astyanax mexicanus*) from recent introductions in two separate watersheds outside of their native range. We found a variety of phenotypic and behavioral shifts between subterranean and surface populations that are similar to those observed in relatively ancient populations in Mexico. Despite this rapid morphological divergence, we found that most of these trait differences were due to plasticity in response to rearing environments. While most trait assays in common-garden, lab-raised fish indicated that phenotypic shifts in wild fish were the result of plasticity, we also found evidence of genetic control in several traits present in subterranean populations. Interestingly, wall-following behavior, an important subterranean foraging behavior, was greater in lab-born subterranean fish than in lab-born surface fish, suggesting rapid divergence of this trait between subterranean and surface populations. Thus, this study sheds light on the early steps of subterranean evolution, identifies potential rapid behavioral evolution, and suggests that plasticity in traits involving exploratory behavior may facilitate subterranean colonization.

Keywords: Plasticity; Adaptation; Subterranean; Cavefish; Evolution; Common garden

INTRODUCTION

Rapid phenotypic responses to environmental shifts have been documented in various species, such as invasive *Betta splendens* (Brand et al., 2021), mangrove killifish (Edenbrow & Croft, 2013), and *Astyanax mexicanus* from Texas (McGaugh et al., 2020). However, whether these phenotypic shifts are the result of adaptative evolution or phenotypic plasticity remains unclear. Although evolution is traditionally thought of as a long process of genetic trial and error acted upon by selection (Barghi et al., 2020; Bomblies & Peichel, 2022), recent studies suggest that adaptation can occur rapidly under sudden environmental shifts (Campbell-Staton et al., 2017; Qu et al., 2020). However, rapid phenotypic change may also result from phenotypic plasticity, a crucial source of trait variability that enables range expansion and population persistence under extreme conditions (Behera & Nanjundiah, 2004; Fox et al., 2019; Lande, 2009; Moriuchi & Winn, 2005; Pettit et al., 2016). Trait variability resulting from phenotypic plasticity preserves existing genetic diversity, providing more material for selection to act upon over time, thereby facilitating adaptive evolution (Lande, 2009; Pfennig et al., 2010).

The Mexican tetra (*A. mexicanus*) is a well-studied evolutionary model organism, which has, over hundreds of thousands of generations, colonized and established more than 30 known subterranean populations throughout eastern and central Mexico (Herman et al., 2018). *Astyanax* cavefish in Mexico exhibit striking traits associated with their subterranean environment, including albinism (O'Gorman et al., 2021), reduction or loss of eye development (Krishnan & Rohner, 2017; McGaugh et al., 2014; Rétaux & Casane, 2013), and increased superficial neuromasts (Protas et al., 2008), which are heritable under laboratory conditions. These genetically based cave-derived traits are often accompanied by a suite of behavioral shifts, such as reduced aggression (Burchards et al., 1985; Elipot et al., 2013, 2014; Hinaux et al., 2016), stress (Chin et al., 2018), total sleep (Duboué et al., 2011; Jaggard et al., 2017, 2018), and schooling behavior (Kowalko et al., 2013), as well as increased wall following (Sharma et al., 2009) and variations in food consumption levels (Aspiras et al., 2015).

Despite the well-documented, genetically driven shifts in

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phenotype between cave and surface populations, various studies have suggested that plasticity plays an important role in influencing cave-related traits in *Astyanax* (Bilandžija et al., 2020; Reyes, 2015; Rohner et al., 2013). For example, when rearing surface fish in a dark environment, a range of plastic responses can be observed, including increased fat stores, starvation resistance, and cortisol levels, along with decreased serotonin and metabolic rate and changes in hormone expression (Bilandžija et al., 2020). Additionally, variation in eye morphology in surface *A. mexicanus* may be masked by a chaperon protein, HSP90, enabling plastic environmental responses when HSP90 is inhibited (Rohner et al., 2013). Skeletal morphology can also be affected by differences in current (Reyes, 2015), and neuromast density exhibits plasticity following disruption in facial bone development (Fernandes et al., 2018). These studies provide evidence that surface populations may display sufficient plasticity in subterranean-related traits to survive initial invasion, offering clues to the mechanistic origin of cave-related traits.

Interestingly, *A. mexicanus* was introduced from its native range in South Texas to spring-fed rivers in Central Texas during the past century for use as fish bait. Since its introduction, this species has colonized multiple rivers, springs, and caves throughout the region (Brown, 1953; McGaugh et al., 2020). We previously documented rapid phenotypic and behavioral shifts between wild-caught fish from a pair of recently established *A. mexicanus* populations, one subterranean (Honey Creek Cave) and one epigean (Honey Creek) (McGaugh et al., 2020). However, whether these changes represent rapid evolutionary change or phenotypic plasticity is uncertain.

Here, we studied two additional populations of fish that occasionally or primarily occupy subterranean habitats in the Upper San Antonio River Watershed in the San Antonio River Basin (San Antonio, Bexar County, Texas), including the Blue Hole (aka San Antonio Springs) and San Pedro Springs (Figure 1). These groundwater springs are connected to the San Antonio River only when aquifer levels are high enough for the springs to flow but provide no surface aquatic habitat during low aquifer conditions. Thus, we classified these sites as subterranean aquatic environments with intermittent connectivity to surface habitats, and the associated *A. mexicanus* populations as facultative stygobionts (i.e., groundwater dwelling). During periods of low aquifer levels (spanning months or years), fish at these sites likely experience similar conditions (total darkness, stable water temperature and chemistry, and low food availability) as many Mexican cavefish populations. We also included a surface population comparison from the San Antonio River on the grounds of San Antonio Zoo, which experiences perpetual flow from a groundwater well. These sites are part of the San Antonio River system, which extends 364 km before its confluence with the Guadalupe River. The first documented occurrences of this species in these adjacent watersheds are separated by nearly 50 years, with each attributed to different individuals (Brown, 1953). Therefore, we consider the San Antonio River populations to be the result of introductions independent from the Guadalupe River (Honey Creek) populations.

In this study, we examined wild *A. mexicanus* fish from the independent Texas introduction, which were captured and maintained in our lab (hereafter referred to as wild). We explored whether there are comparable shifts in behavioral and morphological traits between subterranean and surface fish as observed in our previous work with Honey Creek populations (McGaugh et al., 2020). We also determined whether genetic changes or phenotypic plasticity contributed to shifts in behavioral and morphological traits, achieved by conducting the same assays on lab-born fish (derived from wild fish) and comparing their results with those of the wild fish. By comparing behavioral and morphological traits in populations with subterranean and surface genetic backgrounds, reared in a controlled common-garden environment, we elucidated the relative roles of plasticity and genetic change in the trait shifts observed in wild-sourced populations. Our findings indicated that most traits are governed by plastic responses to the developmental environment, suggesting that the phenotypic shifts observed in recent subterranean invasions are predominantly caused by developmental plasticity. These results have important implications for our understanding of subterranean evolution. Notably, some enhanced sensory capabilities appear to be under genetic control, suggesting that phenotypes related to sensory compensation may initiate evolution in subterranean environments.

MATERIALS AND METHODS

Populations

The Honey Creek and San Antonio River populations are the result of independent introductions of surface fish into Central Texas. These two clusters of sites are located in separate river basins (Figure 1), with the first documentation of *A. mexicanus* in their parent river systems occurring in 1952 and 1908, respectively.

We initially focused on populations of fish known to exhibit phenotypic differences. Honey Creek, located within the Guadalupe River basin, was first documented as an *A. mexicanus* habitat in 1953. Neighboring Honey Creek Cave, the longest surveyed cave in Texas, spans over 32 km in length and consists of stream passages that provide extensive aquatic habitat. Honey Creek Cave was likely colonized by surface fish from the creek after an extreme flooding event. Honey Creek Cave is generally separated from Honey Creek, with groundwater flowing out from the lower of two cave entrances and cascading through a steep spring run to the headwater pool of the creek below. Within the cave, most individuals are found in the twilight zone and proximate dark zone, although fish have been encountered more than 100 m upstream from the entrance area (McGaugh et al., 2020). We collected surface fish approximately 1 600 m downstream of the cave.

We also examined previously uninvestigated populations from a separate river drainage, including subterranean sites (Blue Hole and San Pedro Springs) and a surface population from San Antonio Zoo. These formerly large-volume, perennial springs are part of the San Antonio River system, with a total river length of approximately 4.5 km connecting all three sites. Both spring sites contain resident populations of *A. mexicanus,* likely descendants of individuals introduced at San Pedro Springs in 1908 (Brown, 1953). The populations at Blue Hole and San Pedro Springs have experienced long periods (often several years at a time) of drought or intermittent flow over the past several decades (data accessed 11/6/2022) (Eaa, 2020) and fish at these sites occupy both surface and subsurface habitats. When water levels are high (>204 m

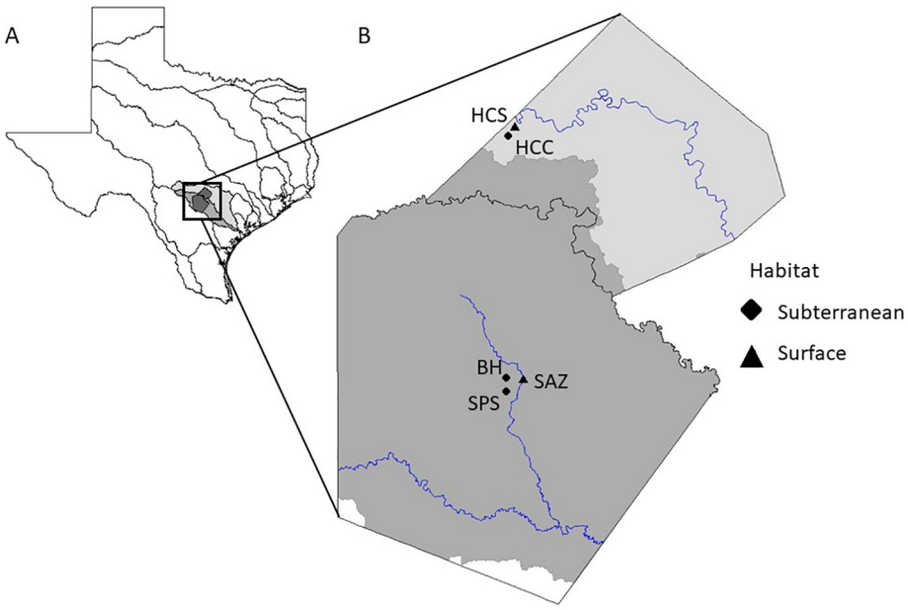

Figure 1 Map of field collection sites

A: State of Texas (USA), with river basin boundaries depicted in black. Guadalupe River basin is shaded in light gray, San Antonio River basin is shaded in medium gray, and Comal and Bexar counties where the study sites are located are shaded in dark gray. B: Close-up of Comal (top) and Bexar (bottom) counties in Texas. County boundaries are depicted in black. Guadalupe River (lighter) and San Antonio River (darker) basins are shaded in gray. Rivers are depicted as blue lines. Study site coordinates for Honey Creek surface (HCS), Honey Creek Cave (HCC), Blue Hole (BH), San Antonio Zoo (SAZ), and San Pedro Springs (SPS) are marked and labeled with subterranean populations marked with a diamond and surface sites marked with a triangle.

above mean sea level (AMSL)), spring pools appear at these sites, which then flow into the San Antonio River. When the aquifer level is low (<204 m AMSL), fish retreat to groundwater refugia. Interestingly, this species was first documented in the San Antonio River (on the grounds of San Antonio Zoo) the same year it was introduced to San Pedro Springs (Brown, 1953), suggesting a high degree of surface connectivity between those sites under normal flow conditions. San Antonio River flow is maintained by a large groundwater well on the zoo grounds.

Sampling locations and methods

Sampling occurred in February 2020 at the Blue Hole, San Pedro Springs, and San Antonio Zoo. Fish were trapped using either collapsible "umbrella traps" baited with sardines or a monofilament cast net. Fish were transported to San Antonio Zoo, kept in temporary tanks, and then shipped via Delta Cargo on a direct flight to Minneapolis St. Paul, where they were placed in tanks at the University of Minnesota.

Fish housing and husbandry

Aquariums were maintained at 21–23 °C, similar to the temperature range of their natural habitat, in rooms under a 1410h light:dark cycle (lights on 0800h CST, lights off 1800h CST). Fish were housed at a density of one fish per 5.3–7.6 L. A 20% water change was performed weekly for all tanks, and filter media were changed approximately once a month. All fish were fed frozen bloodworms, brine shrimp, or Tetra® Cichlid flakes 1–2 times a day *ad libitum*. All procedures and housing conditions were approved by University of Minnesota Institutional Animal Care and Use Committee (UMN IACUC), protocol 2002-37827A.

To compare the phenotypes of wild and lab-raised individuals, the Blue Hole, San Antonio Zoo, Honey Creek Cave, and Honey Creek surface fish were bred in the lab. Fish from San Pedro Springs were not bred in the lab due to limited space and because this population likely spends less time in subterranean environments than either the Blue Hole or Honey Creek Cave fish. Fish breeding was accomplished following the protocols of Borowsky et al. (2008). For the first 30 days post fertilization, fish were fed Hikari First Bites Fish Food. At approximately 30 days post fertilization, the juvenile fish were transferred to heated (26 °C) fully filtered tanks at a density of one fish per 8–18 L and switched to a diet of crushed flake, frozen brine shrimp, and frozen bloodworms. Heat was removed six months post fertilization, and the lab-born fish were treated as adults. All animal care and housing practices were carried out in accordance with the IACUC guidelines (UMN IACUC protocol 2002-37827A).

Experimental overview

Behavioral trials were conducted in the same order and not randomized. First, we examined behavior upon introduction to a new environment, as a proxy for stress (Chin et al., 2018). Second, we assayed aggression in response to a mirror. These first two trials were conducted on the same day in 19 L tanks separated by opaque dividers to prevent fish from seeing conspecifics during experiments. All trials were recorded continuously using one Wyze Cam v2 for each tank.

Next, the fish were acclimated to circular arenas for 70 h to test for wall-following behavior over a 1 h window. The fish were then returned to their 19 L experimental tanks, starved for three days, and tested for food consumption. After all behavioral measurements, fish were weighed, and photographs were taken to measure neuromast number, melanophore number, standard length, head depth, and eye diameter. All trials were conducted with tanks at room temperature (20–22 °C).

All video recordings of the trials were analyzed using EthoVision XT 15 (Noldus) behavioral tracking software to

track subjects and calculate variables of interest. To ensure accurate tracking, each recording was reviewed manually, with erroneous data removed and subsequently interpolated using EthoVision XT 15 (Noldus). Fish videos with greater than 20% missing data were removed from their respective datasets prior to statistical analysis.

Stress assay

The fish were removed from their home tanks and placed into small porous plastic containers filled with home-tank water, following previous research (McGaugh et al., 2020). In a dark room, one container was floated in each 19 L trial aquarium for 5 min to allow water temperature equilibration and exchange. After 5 min of acclimation in the plastic containers, fish were released from the containers into the tanks. After 12 min in the dark, the lights were turned on (without a researcher entering the room), and fish were recorded for an additional 12 min. The first 10 min of videos from both the light and dark trials were analyzed, with time spent immobile, distance traveled, average velocity, and time spent in either the top or bottom half of the tank measured. The final sample sizes included recordings of 224 individuals (wild Blue Hole, *n*=46; wild San Pedro Springs, *n*=55; wild San Antonio Zoo, *n*=40; wild Honey Creek Cave, *n*=16; wild Honey Creek surface, *n*=32; lab-born Blue Hole, *n*=13; lab-born San Antonio Zoo, *n*=9; lab-born Honey Creek Cave, *n*=6; lab-born Honey Creek surface, *n*=7).

Aggression assay

Upon completion of the stress assay recordings, mirrors were positioned along the entire width of the shorter side of each individual's trial tank, with a 5 cm gap between the top of the mirror and the water surface. The placement of the mirror on the tank side was randomized for each fish. The aggression assays were conducted for 1 h, after which the mirrors were removed. Time spent within 6.77 cm of the mirror (i.e., closest 1/6 section of the tank) was measured for each fish. The final sample sizes included 186 individuals (wild Blue Hole, *n*=33; wild San Pedro Springs, *n*=37; wild San Antonio Zoo, *n*=35; wild Honey Creek Cave, *n*=15; wild Honey Creek surface, *n*=29; lab-born Blue Hole, *n*=13; lab-born San Antonio Zoo, *n*=10; lab-born Honey Creek Cave, *n*=6; lab-born Honey Creek surface, *n*=8).

Wall-following assay

To assess wall-following behavior, 1 h of data (starting at 1200h CST) was analyzed for each fish. Individual fish were acclimated to 20 L buckets with a water depth of 12.7 cm for at least 70 h. EthoVision arenas were established to cover the entire area of the water in the bucket within a perfect circle. While the bottom diameter of the bucket was 26 cm, the diameter of the water surface appeared slightly larger due to the proximity of the camera. Therefore, the arena diameter for each fish was 30.5±0.5 cm. Two concentric zones were then created: one with the same diameter as the arena and one with half the diameter of the bucket bottom (13 cm). Based on these arenas, EthoVision was used to assess time spent by individuals in the center zone of the tank, time spent outside the center zone of the tank (i.e., near the walls), frequency of visits to the center, and frequency and duration of mobility states (i.e., immobile, mobile, and highly mobile). The final sample sizes included 221 individuals (wild Blue Hole, *n*=46; wild San Pedro Springs, *n*=46; wild San Antonio Zoo, *n*=37; wild Honey Creek Cave, *n*=16; wild Honey Creek surface, *n*=34; lab-born Blue Hole, *n*=18; lab-born San Antonio Zoo, *n*=10; lab-born Honey Creek Cave, *n*=6; lab-born Honey Creek surface, *n*=8).

Feeding assay

After their sleep trials (see Supplementary Materials), individuals were returned to the 19 L trial tanks. During transfer, no leftover food from the circular arenas was transferred to the fasting tanks. Fish were fasted for 72 h in preparation for the feeding trials. As per previous research (McGaugh et al., 2020), after fasting, the fish were given 50 pre-counted bloodworms and allowed to eat undisturbed for 10 min. The fish were then removed from the trial tanks, and the remaining bloodworms were counted to determine the number eaten by each individual fish. The fish were weighed, so that the number of bloodworms eaten could be corrected for fish size. In total, food consumption was analyzed in 244 individuals (wild Blue Hole, *n*=41; wild San Pedro Springs, *n*=62; wild San Antonio Zoo, *n*=48; wild Honey Creek Cave *n*=17; wild Honey Creek surface *n*=34; lab-born Blue Hole, *n*=18; lab-born San Antonio Zoo, *n*=10; lab-born Honey Creek Cave, *n*=6; lab-born Honey Creek surface, *n*=8).

Neuromast assay

Fish were placed in conditioned water with a 0.025 g/L solution of DASPEI fluorescent stain (2-4-dimethylamino-N-ethylpyridinium iodide; Sigma Aldrich, USA) (Yoshizawa et al., 2010). After at least 1 h of stain absorption, the fish were anesthetized in an ice bath and sex was determined based on anal fin shape and presence/absence of anal fin denticles. The fish were then photographed in full light (Figure 2) alongside a size standard to ensure standard length could be measured and analyzed, as described in McGaugh et al. (2020). Fluorescent images of the head and whole body were taken for each individual using a Nikon TE2000 inverted fluorescence microscope with 1.0× magnification and a green fluorescent protein filter (Figure 2) (McGaugh et al., 2020). Using a custom macro in FIJI (Schneider et al., 2012), the number and average size of neuromasts on the surface of the third suborbital (SO-3) bone on the right side of the skull were analyzed for each fish, as described in McGaugh et al. (2020). The counts produced by the macro were hand-corrected to minimize the number of off-target counts and missed neuromasts. In total, neuromasts were analyzed in 203 individuals (wild Blue Hole, *n*=35; wild San Pedro Springs, *n*=45; wild San Antonio Zoo, *n*=57; wild Honey Creek Cave, *n*=13; wild Honey Creek surface, *n*=12; lab-born San Antonio Zoo, *n*=10; lab-born Blue Hole, *n*=18; Honey Creek Cave, *n*=6; lab-born Honey Creek surface, *n*=7). Following imaging, fish were returned to their home tanks.

Melanophore assay

Images were taken of the right side of each fish to capture the narial region, fourth suborbital (SO-4) bone, anterior insertion point of the anal fin, and dorsal insertion point of the caudal fin (Supplementary Figure S1) using a Nikon SMZ Zoom Stereoscope.

The number and size of melanophores within the designated collection area were ascertained using two modified versions of the same macro used in the neuromast assay. These modifications included an additional step to convert color images to 8-bit and adjust the thresholding, size, and circularity parameters. One macro was calibrated to measure large, widely spaced melanophores, while the other

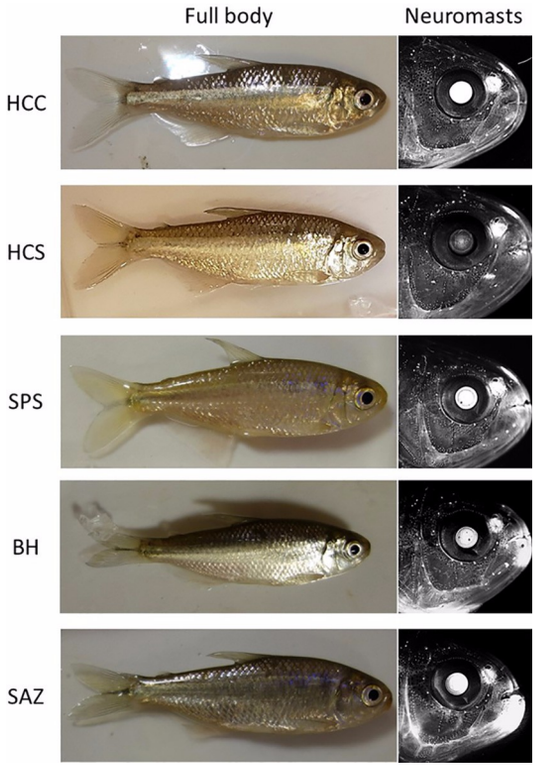

Figure 2 Reference images of wild *A. mexicanus* populations
Each row contains two images of a single randomly selected individual from each population, denoted by row names. Images in the left column are full body images used for general body measurements, such as eye diameter, standard length, and head depth. Images in the right column are fluorescence microscopy images used for neuromast quantification. Original fluorescence images were switched to grayscale and adjusted for brightness and contrast. For abbreviations see Figure 1 and text. Photos by N. E. Swanson.

was calibrated to measure small, tightly clustered melanophores. The areas for melanophore counts were specified as a ~1 mm^2 polygon within the designated sample location. Overcounts or missed melanophores were manually corrected. Total melanophore area within each collection area was divided by the area of the polygon to determine the percent coverage of the area. In total, melanophores were analyzed in 183 individuals (wild Blue Hole, *n*=25; wild San Pedro Springs *n*=49; wild San Antonio Zoo, *n*=31; wild Honey Creek Cave *n*=2; wild Honey Creek surface *n*=38; lab-born San Antonio Zoo, *n*=10; lab-born Blue Hole, *n*=16; lab-born Honey Creek Cave, *n*=6; lab-born Honey Creek surface, *n*=6), although data may be missing for some individuals due to poor image quality.

The identity of each fish was known for all behavioral trials through neuromast imaging. To minimize fish stress, however, photos for melanophore counts were conducted several weeks after the original trials. Thus, melanophore counts cannot be linked to individual behavioral trials or neuromast counts.

Eye size

ImageJ was used to measure the eye diameter of each individual, using a size reference from photographs (Figure 2) taken during the neuromast and melanophore assays (Schneider et al., 2012). In total, eye size was analyzed in 228 individuals (wild Blue Hole, *n*=35; wild San Pedro Springs *n*=45; wild San Antonio Zoo, *n*=63; wild Honey Creek Cave *n*=13; wild Honey Creek surface *n*=32; lab-born San Antonio Zoo, *n*=9; lab-born Blue Hole, *n*=18; lab-born Honey Creek Cave, *n*=6; lab-born Honey Creek surface, *n*=7).

Statistical analysis

All video data with greater than 20% erroneous tracking were excluded from further analysis. The Shapiro-Wilk normality test confirmed that the data were non-normally distributed. Thus, we employed non-parametric Wilcoxon tests to compare traits across specific populations using R statistical software (R Core Team, 2020).

The populations of wild surface fish were significantly longer than the paired subterranean populations (HCC vs. HCS: W=83, *P*<0.005; SPS vs. SAZ: W=2 380.5, *P*<0.001; BH vs. SAZ: W=445, *P*<0.001), but head depth was virtually identical among all wild populations (HCC vs. HCS: W=214.5, *P*=0.88; SPS vs. SAZ: W=1 632.5, *P*=0.181; BH vs. SAZ: W=1 059.5, *P*=0.753). Therefore, head depth was used for standardization where applicable. Where not applicable, analysis of variance (ANOVA) was used to explore the effects of sex or fish length on dependent variables, and their interaction with population identity.

RESULTS

We compared phenotypes and behaviors of wild fish from surface populations in Texas with those from recently colonized subterranean sites. Similar to previous research (McGaugh et al., 2020), we found evidence for rapid phenotypic and behavioral changes between fish inhabiting these distinct environments. In contrast, and as expected, phenotypes among different populations of wild surface fish exhibited less divergence than that observed between wild surface fish and their respective paired subterranean and facultatively subterranean populations. We did not observe significant differences between males and females (unless stated otherwise). To determine whether the rapid shifts observed between subterranean and surface populations were due to phenotypic plasticity or genetic change, we tested the suite of traits in 11–13-month-old lab-raised individuals bred from the stock of wild adults (Figure 3). Based on these results, rearing environment appears to play an important role in the development of behaviors and morphological phenotypes.

Stress assay

Behavior upon introduction to a new environment is a reliable proxy for stress (Chin et al., 2018). Previous work reported that behavioral shifts between dark and light environments are different between Honey Creek Cave and Honey Creek surface fish (McGaugh et al., 2020), suggesting that the two populations exhibit light-dependent differences in stress response.

In accordance with our previous study (McGaugh et al., 2020), wild Honey Creek Cave fish displayed several behaviors in the stress trials under light, suggesting they experience less stress in novel environments. Honey Creek Cave fish traveled significantly further (Supplementary Figure S2; Table 1), spent more time at the top of the tank (Supplementary Figure S2; Table 1), and less time in an immobile state (Supplementary Figure S2; Table 1) than Honey Creek surface fish in light stress trials (see Supplementary Table S1 for statistical output). In contrast to previous work, however, we did not observe differences in

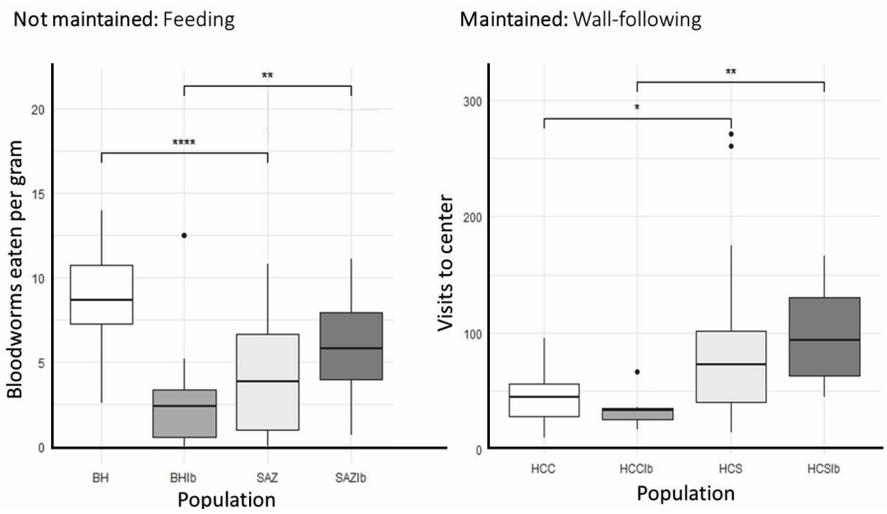

Figure 3 Excerpts of feeding (from Supplementary Figure S6) and wall-following assays (from Supplementary Figure S5), providing graphical representation of the trends indicative of plasticity ("Not Maintained") and putative genetic control ("Maintained")

Plasticity is reflected in the trait shift observed in wild populations (BH and SAZ) that is either absent or reversed in lab-born fish from the same populations (BHIb and SAZIb). Putative genetic control is reflected by the observation of the same or a more exaggerated trend in both wild (HCC and HCS) and lab-born fish from the same population (HCCIb and HCSIb). See Supplementary Materials for fully detailed figures depicting the observations for each trait assay. *: P<0.05; **: P<0.01; ****: P<0.0001. For abbreviations see Figure 1 and text.

Table 1 Summary of observed relationships and significance between paired subterranean and surface populations of wild fish

Assay	Variable	HCC vs. HCS	BH vs. SAZ	SPS vs. SAZ
Stress: Dark	Percent time in top half of tank	Cave>Surface	Cave>Surface	Cave>Surface*
	Percent time immobile	Cave<Surface	Cave<Surface	Cave>Surface
	Distance traveled	Cave>Surface	Cave<Surface	Cave<Surface
	Average velocity	Cave>Surface	Cave<Surface	Cave<Surface
Stress: Light	Percent time in top half of tank	Cave>Surface*	Cave<Surface	Cave>Surface
	Percent time immobile	Cave<Surface*	Cave>Surface	Cave>Surface*
	Distance traveled	Cave>Surface*	Cave>Surface	Cave<Surface
	Average velocity	Cave>Surface*	Cave>Surface	Cave<Surface
Aggression	Percent time near mirror: all	Cave>Surface	Cave>Surface	Cave>Surface***
	Percent time near mirror: females	Cave>Surface*	Cave>Surface	Cave>Surface*
	Percent time near mirror: males	Cave<Surface	Cave<Surface	Cave>Surface
Wall-following	Percent time in center zone	Cave<Surface	Cave>Surface*	Cave<Surface**
	Visits to center zone	Cave<Surface*	Cave>Surface	Cave<Surface***
Feeding	Consumption per cm fish length	Cave>Surface	Cave>Surface***	Cave>Surface
	Consumption per g fish weight	Cave>Surface	Cave>Surface***	Cave>Surface*
Eye size	Eye diameter per cm fish length	Cave>Surface	Cave>Surface**	Cave>Surface***
	Eye diameter per cm head depth	Cave<Surface***	Cave<Surface*	Cave<Surface*
Neuromasts	Density on SO-3 bone	Cave>Surface	Cave>Surface***	Cave>Surface***
Melanophores	Density dorsal to nare	Cave<Surface	Cave>Surface**	Cave>Surface***
	Density near SO-4 bone	Cave<Surface	Cave>Surface***	Cave>Surface***
	Density at anterior insertion of anal fin	Cave<Surface	Cave<Surface	Cave>Surface***
	Density dorsal insertion of caudal fin	Cave<Surface	Cave>Surface***	Cave>Surface***

Phenotypic shifts in subterranean populations paralleling those in Mexican cavefish are in light gray, those that do not are in dark gray. Level of significance is indicated with asterisks in each cell (*: P<0.05; **: P<0.01; ***: P<0.001). For full statistical output see Supplementary Tables S1 and S2 for Honey Creek and San Antonio River drainages, respectively. For abbreviations see Figure 1 and text.

how Honey Creek Cave and Honey Creek surface fish shifted their behavior between light and dark trials, with both being more active in the light trials than in the dark trials (Paired Wilcoxon tests: HCC: V=10, P=0.0013; HCS: V=161, P=0.055).

Overall, for the newly tested populations, trends were less clear for stress-related behaviors and habitat of origin. Individuals from the San Antonio River drainage (i.e., San Antonio Zoo, Blue Hole, and San Pedro Springs wild fish) spent more time at the bottom of the tank and more time immobile under light conditions than in the dark (SAZ: V=1161, P<0.001; BH: V=701, P=0.015; SPS:V=608, P=0.007), suggesting they experienced more stress when the lights were on than off. However, increased distance traveled and velocity in wild Blue Hole fish once lights were turned on (Distance: V=187, P=0.002; Velocity: V=188, P=0.002) suggests a reduction in stress under light, complicating this trend. San Pedro Springs fish spent more time immobile than

San Antonio Zoo fish in the light trials (Supplementary Figure S2; Table 1) and spent more time at the top of the tank than San Antonio Zoo in the dark trials (Supplementary Figure S1; Table 1; see Supplementary Table S2 for statistical output). This suggests that San Pedro Springs fish are more stressed under light conditions and less stressed under dark conditions compared to their surface counterparts. Surface fish from the two drainages showed nearly identical responses, except under light conditions, where San Antonio Zoo fish spent nearly three times more time immobile than Honey Creek surface fish (W=413, P=0.010).

While trends in stress-related behaviors for the San Antonio populations were inconclusive, we found a clear trend of reduced stress in the Honey Creek Cave fish relative to Honey Creek surface fish. The observed effect is congruent with previous observations of reduced stress in subterranean *A. mexicanus* from Mexico (Chin et al., 2018) and previous work in our lab with these populations (McGaugh et al., 2020). Additionally, increases in stress under light conditions for the San Antonio populations are consistent with the scotophilic behaviors (preference for shade) observed in *A. mexicanus* in the wild.

The observed differences in stress-associated behaviors between wild subterranean and surface populations were not maintained in their lab-born progeny. Stress responses in lab-born Honey Creek Cave and surface fish (HCClb and HCSlb, respectively) were not significantly different from one another regardless of lighting conditions, except that Honey Creek Cave lab-born fish spent more time in the top half of the tank during the dark trials (Supplementary Figure S3; Table 2; see Supplementary Table S1 for statistical output). These findings suggest that lab-born Honey Creek Cave fish potentially experienced less stress than lab-born Honey Creek surface fish. Blue Hole and San Antonio Zoo lab-born fish (BHlb and SAZlb, respectively) exhibited a similar trend. Lab-born fish did not differ in measures of stress-associated behaviors in either light or dark trials, except that San Antonio Zoo lab-born fish spent significantly more time immobile than lab-born Blue Hole fish in the dark (Supplementary Figure S3; Table 2; see Supplementary Table S2 for statistical output). These findings suggest reduced stress in Blue Hole lab-born fish, similar to the results of wild fish from the same population.

In summary, we found evidence that wild Honey Creek Cave and Blue Hole fish exhibit less stress than their paired surface populations (consistent with McGaugh et al. (2020)), while wild San Pedro Springs fish exhibited more stress than wild San Antonio Zoo fish under light conditions, but less under dark conditions. In contrast, lab-born subterranean and surface populations from each drainage showed few differences in stress-associated behaviors. Notably, all captive-bred populations demonstrated elevated stress behaviors compared to their paired wild populations, suggesting potent responses to developmental environment (see Supplementary Table S2 for statistical output).

Aggression assay

In the long-established Mexican populations, surface fish are more aggressive than cavefish (Elipot et al., 2013). The use of mirrors in an experimental tank simulates the arrival of a size-matched conspecific, which can potentially elicit aggressive responses (Elipot et al., 2013, 2014; Espinasa et al., 2005; Hinaux et al., 2016; Way et al., 2015). We previously found that Honey Creek Cave fish spent 1.3 times more time in the 1/6th portion of the tank closest to the mirror, serving as a proxy for aggression, compared to surface fish (McGaugh et al., 2020).

Table 2 Summary of observed relationships and significance between paired subterranean and surface populations of lab-born fish

Assay	Variable	HCClb vs. HCSlb	BHlb vs. SAZlb
Stress: Dark	Percent time in top half of tank	Cave>Surface*	Cave>Surface
	Percent time immobile	Cave<Surface	Cave<Surface*
	Distance traveled	Cave<Surface	Cave>Surface
	Average velocity	Cave<Surface	Cave>Surface
Stress: Light	Percent time in top half of tank	Cave<Surface	Cave<Surface
	Percent time immobile	Cave>Surface	Cave>Surface
	Distance traveled	Cave<Surface	Cave>Surface
	Average velocity	Cave<Surface	Cave>Surface
Aggression	Percent time near mirror: all	Cave>Surface	Cave>Surface*
	Percent time near mirror: females	Cave>Surface* (HCSlb n=1)	No SAZlb Females
	Percent time near mirror: males	Cave>Surface	Cave>Surface*
Wall-following	Percent time in center zone	Cave<Surface*	Cave>Surface
	Visits to the center zone	Cave<Surface**	Cave>Surface
Feeding	Consumption per cm fish length	Cave>Surface*	Cave<Surface**
	Consumption per g fish weight	Cave>Surface	Cave<Surface**
Eye size	Eye diameter per cm fish length	Cave>Surface	Cave>Surface***
	Eye diameter per cm head depth	Cave<Surface	Cave>Surface**
Neuromasts	Density on SO-3 bone	Cave<Surface*	Cave>Surface**
Melanophores	Density dorsal to nare	Cave>Surface	Cave<Surface
	Density near SO-4 bone	Cave>Surface	Cave<Surface
	Density at anterior insertion of anal fin	Cave<Surface	Cave>Surface
	Density dorsal insertion of caudal fin	Cave>Surface*	Cave>Surface

Phenotypic shifts in subterranean populations paralleling those in their wild counterparts are in light gray, those that do not are in dark gray. Level of significance is indicated with asterisks in each cell (*: P<0.05; **: P<0.01; ***: P<0.001). For full statistical output see Supplementary Tables S1 and S2 for Honey Creek and San Antonio River drainages, respectively. For abbreviations see Figure 1 and text.

In our current study, exploratory ANOVA revealed a significant interaction between fish length, sex, and population in relation to the amount of time spent in proximity to the mirror for wild fish (F=7.163, df=1, 34, *P*=0.011). In wild Honey Creek Cave fish, larger males were less aggressive than smaller males (Spearman rank correlation: S=154, *P*=0.015), but this relationship was not significant for females (S=42, *P*=0.714). In contrast, in the Honey Creek surface fish, male size was not associated with aggressiveness (S=432, *P*=0.541), while larger females were less aggressive than smaller females (S=843.3, *P*=0.054). Honey Creek Cave females spent more time (1.3 times) near the mirror than Honey Creek surface females (Supplementary Figure S4; Table 1), while males showed no significant differences between populations (Supplementary Figure S4; Table 1; see Supplementary Table S1 for statistical output). Notably, sex was not included as a factor in our previously published work.

In contrast, for the newly evaluated wild populations, no interactions were observed among sex, fish length, and population of origin for the Blue Hole or San Pedro Springs fish compared to San Antonio Zoo fish. Wild San Pedro Springs fish spent more time (1.3 times) near the mirror than San Antonio Zoo fish (Supplementary Figure S4; Table 1; see Supplementary Table S2 for statistical output). To explore whether the observed trend is an artifact of the San Antonio Zoo fish being larger than the San Pedro Springs fish, we truncated the dataset to include only those San Antonio Zoo fish that were smaller than the largest San Pedro Springs fish (SAZ=16 fish, SPS=36 fish), with results remaining consistent (SPS-SAZ truncated: W=172, *P*=0.021). Comparisons between wild Blue Hole and San Antonio Zoo fish showed no significant differences (see Supplementary Table S1 for statistical output), although wild Blue Hole fish did spend slightly more time (1.1 times) near the mirror than wild fish from San Antonio Zoo (Supplementary Figure S4; Table 1). The two wild surface populations did not differ significantly from one another (HCS-SAZ W=581, *P*=0.327).

Similar to wild populations, lab-born fish from Honey Creek Cave tended to spend more time (1.2 times) in the mirror zone than lab-born Honey Creek surface (Supplementary Figure S4; Table 2), although the trends were not significant, which may be due to the low sample size (see Supplementary Table S1 for statistical output). These trends were consistent for both males and females in the Honey Creek lab-born population (Supplementary Figure S4; Table 2). Lab-born Blue Hole fish spent significantly more time (1.3 times) near the mirror than lab-born San Antonio Zoo fish (Supplementary Figure S4; Table 2; see Supplementary Table S2 for statistical output).

Consistent with our earlier study (McGaugh, et al., 2020), we found evidence that the wild Honey Creek Cave and wild San Pedro Springs populations exhibited more aggressiveness than their paired wild surface populations. While not significant, lab-born fish maintained the trend observed in wild fish, whereby Honey Creek Cave fish were also slightly more aggressive than lab-born Honey Creek surface fish. Additionally, lab-born Blue Hole fish were significantly more aggressive than lab-born San Antonio Zoo fish, mirroring the qualitatively more aggressive wild Blue Hole fish relative to wild San Antonio Zoo fish.

Wall-following behavior

We previously reported that wall-following behavior is more prevalent in subterranean than surface populations (McGaugh et al., 2020), as also observed in subterranean fish in Mexico (Patton et al., 2010; Sharma et al., 2009). Here, we found wild Honey Creek Cave fish visited the center of the arena significantly fewer times (Supplementary Figure S5; Table 1) and spent slightly more trial time (97% vs. 94%) in the outer zone of the arena (Supplementary Figure S5 ; Table 1) than Honey Creek surface fish (see Supplementary Table S1 for statistical output), suggesting that subterranean fish prefer the outer edge of the arena. Similarly, wild San Antonio Zoo fish executed approximately twice as many visits to the center of the arena (Supplementary Figure S5; Table 1) and spent 1.6 times more trial time in the center zone of the arena compared to wild San Pedro Springs fish (Supplementary Figure S5; Table 1; see Supplementary Table S2 for statistical output).

Sex had no impact in the fish populations, except for the wild Blue Hole fish. Blue Hole female fish spent significantly less trial time in the outer zone of the arena than Blue Hole male fish (F=90.7%, M=92.7%; W=87, *P*=0.04). Overall, Blue Hole fish spent significantly less time in the outer zone (Supplementary Figure S5; Table 1) and visited the arena center slightly more often (Supplementary Figure S5; Table 1) than wild San Antonio Zoo fish (see Supplementary Table S2 for statistical output). This pattern is opposite of that observed in other wild subterranean-surface comparisons, suggesting that wild Blue Hole fish do not exhibit preferential wall-following behavior.

The two wild surface populations exhibited a difference in their visitation to the center of the arena, with San Antonio Zoo fish visiting the center fewer times compared to Honey Creek surface fish (W=833, *P*=0.01). However, there was no significant difference between the two populations in terms of the percentage of time spent in the outer zone (W=546, *P*=0.172).

The differences observed in wall-following behavior between lab-born populations mirrored those found in wild individuals. Lab-born Honey Creek Cave fish spent significantly less time in (Supplementary Figure S5; Table 2) and made significantly fewer visits to (Supplementary Figure S5; Table 2) the center zone of the arena over the 1 h trial period than lab-born Honey Creek surface fish (see Supplementary Table S1 for statistical output). Lab-born Blue Hole and San Antonio Zoo fish did not differ significantly, although lab-born Blue Hole fish spent more time (1.5 times) in the center zone (Supplementary Figure S5; Table 2) than lab-born San Antonio Zoo fish (6.2% vs. 4.2%) (see Supplementary Table S2 for statistical output), consistent with the observations of wild populations.

Feeding assay

Mexican cavefish from Cueva Pachón exhibit reduced appetites compared to surface fish after fasting for two months (Aspiras et al., 2015). In contrast to our previous work showing that Honey Creek surface fish consume significantly more than Honey Creek Cave fish (McGaugh et al., 2020), our current results indicated that wild Honey Creek Cave fish consumed about 1.5 times more bloodworms per unit fish weight than the wild Honey Creek surface populations (Supplementary Figure S6; Table 1), although this result was not significant (see Supplementary Table S1 for statistical output).

Likewise, in wild fish from the San Antonio River drainage, facultatively subterranean populations consumed significantly

more bloodworms per unit fish weight (Supplementary Figure S6; Table 1) than the surface populations after a 3 day fasting period. Specifically, Blue Hole fish consumed two times more bloodworms than San Antonio Zoo fish per unit fish weight, while San Pedro Springs fish consumed 1.5 times more bloodworms than San Antonio Zoo fish per unit fish weight (see Supplementary Table S2 for statistical output).

Fish length had a significant effect on bloodworm consumption, even after standardization of fish weight. Smaller, wild fish from subterranean populations exhibited higher bloodworm consumption per unit fish mass than smaller fish from surface populations, with significant ANOVA results for San Antonio Zoo-Blue Hole and San Antonio Zoo-San Pedro Springs (SAZ-BH F=5.17, df=1, 82, *P*=0.026; SAZ-SPS: F=5.53, df=1, 96, *P*=0.021; HCC-HCS: F=1.716, df=1, 43, *P*=0.197). To determine whether larger fish from the surface populations were driving the observed interaction, we truncated the dataset to include only fish shorter than the longest subterranean fish (<7.55 cm). However, our results remained consistent (SAZ-BH: F=5.85, df=1,61, *P*=0.019; SAZ-SPS: F=5.14, df=1, 75, *P*=0.026; HCC-HCS: F=3.44, df=1, 32, *P*=0.073).

Bloodworm consumption after a 3 day fast also showed significant differences between paired populations of lab-born fish. Similar to wild populations, the lab-born Honey Creek Cave fish ate significantly more bloodworms per unit fish mass (Supplementary Figure S6; Table 2) than the lab-born Honey Creek surface fish (see Supplementary Table S1 for statistical output). In contrast, lab-born Blue Hole fish consumed three times fewer bloodworms per unit fish mass than lab-born San Antonio Zoo when corrected for weight (Supplementary Figure S6; Table 2; see Supplementary Table S2 for statistical output). This is a reversal of the trends seen in their wild counterparts, suggesting some environmental influence on this trait.

Nearly all lab-born fish ate fewer bloodworms than their wild counterparts from the same populations. Lab-born Honey Creek Cave fish consumed two times fewer bloodworms than their wild relatives, while lab-born Honey Creek surface fish ate approximately three times fewer bloodworms per unit fish weight than the wild Honey Creek surface fish (see Supplementary Table S1 for statistical output). Similarly, lab-born Blue Hole fish ate significantly fewer (about four times) bloodworms per unit fish weight than their wild counterparts (see Supplementary Table S2 for statistical output). In contrast, lab-born San Antonio Zoo fish ate more bloodworms than wild San Antonio Zoo fish (see Supplementary Table S2 for statistical output).

Overall, our results suggested that wild subterranean populations consumed more bloodworms per unit fish mass than the paired, wild surface fish populations. Lab-born Honey Creek Cave and surface fish feeding mirrored that of wild fish, although each population ate significantly less than the wild fish populations.

Morphology: Eye size

In our previous work, we documented that the eyes of Honey Creek Cave fish were significantly larger than that of Honey Creek surface fish when standardized by fish length (McGaugh et al., 2020). Here, when standardized by head depth, all wild subterranean populations exhibited smaller eyes than wild surface populations (Supplementary Figure S7; Table 1; see Supplementary Tables S1 and S2 for statistical output). Wild Honey Creek surface fish possessed significantly larger eyes than wild San Antonio Zoo fish (W=1 851, *P*<0.001). Male head depth-standardized eye size was only significantly larger than females in San Antonio Zoo fish (W=135.5, *P*<0.001).

As the lab-born fish were better size-matched, we considered both head depth and length standardizations. When corrected for head depth, lab-born Honey Creek Cave fish had smaller eyes (1.06 times) compared to lab-born Honey Creek surface fish (Supplementary Figure S7; Table 2), although this effect was not statistically signfcaint (see Supplementary Table S1 for statistical output). Interestingly, lab-born San Antonio Zoo fish had significantly smaller eyes than lab-born Blue Hole fish based on both head depth (1.3 times smaller; Supplementary Figure S7; Table 2) and fish length (1.2 times smaller; Supplementary Figure S7; Table 2) standardization (see Supplementary Table S2 for statistical output).

Overall, when standardized by head depth, we found that wild fish from subterranean habitats exhibited reduced eye diameter in comparison to wild surface fish. Trends remained consistent, albeit non-significant, in lab-born Honey Creek drainage fish. Lab-born Blue Hole fish exhibited the largest eyes of any of their paired populations regardless of the standardization method, a reversal of the trend in wild populations from the same drainage. Thus, as documented previously, eye size and development are highly plastic (Bilandžija et al., 2020; Rohner et al., 2013).

Morphology: Neuromast density

Mexican cavefish exhibit increased size and density of superficial neuromasts near the SO-3 bone, which are associated with vibration attraction behavior and food finding capabilities (Yoshizawa et al., 2010). Likewise, we previously showed that Honey Creek Cave fish have an increased number of superficial neuromasts at the SO-3 bone compared to surface fish (McGaugh et al., 2020). Here, wild Honey Creek Cave fish contained 1.2 times more neuromasts than Honey Creek surface fish after correcting for SO-3 bone area (Supplementary Figure S8; Table 1), though the effect was marginally non-significant (*P*=0.087; see Supplementary Table S1 for statistical output). We did not correct for fish length, as SO-3 bone area and fish length were tightly correlated and the size distributions of the Honey Creek Cave and surface fish were similar.

Both wild Blue Hole and San Pedro Springs fish exhibited higher neuromast density than wild San Antonio Zoo fish (see Supplementary Table S2 for statistical output). After correcting for SO-3 bone area, the Blue Hole and San Pedro Springs fish still exhibited higher neuromast density (1.2 and 1.4 times, respectively) in comparison to the San Antonio Zoo fish (Supplementary Figure S8; Table 1).

This trend was complicated by fish size. A significant interaction was observed between population and fish length, with wild Blue Hole and San Pedro Springs fish displaying a more pronounced negative correlation between fish length and neuromast density compared to San Antonio Zoo fish (Supplementary Figure S9) (BH vs. SAZ: df=1, 92, F=4.4782, *P*=0.0374; SPS vs. SAZ: df=1, 92, F=6.8693 *P*=0.0103). In other words, smaller wild Blue Hole and San Pedro Springs fish exhibited higher neuromast density than wild San Antonio Zoo surface fish, and this density declined more quickly for the subterranean populations than the surface populations.

To confirm differences in neuromast density between populations, we truncated the dataset to include only those wild San Antonio Zoo fish that were smaller than the largest wild Blue Hole or San Pedro Springs fish (depending on the population being compared). Based on this truncated dataset, the wild San Pedro Springs fish showed higher neuromast density compared to the wild San Antonio Zoo fish (see Supplementary Table S2 for statistical output), but no significant differences in neuromast density were observed between the wild Blue Hole and San Antonio Zoo fish (see Supplementary Table S2 for statistical output), although a very slight increase in mean neuromast density was observed in wild Blue Hole fish compared to San Antonio Zoo fish. We did not analyze the truncated dataset for the Honey Creek drainage because size distributions between the Honey Creek Cave and surface fish were similar.

No significant difference in neuromast number (when corrected for the SO-3 area) was observed between the two wild surface populations (HCS vs. SAZ: W=327, P=0.818); neuromast density was not significantly affected by sex or population, nor were there significant interaction terms between length, sex, and population. However, larger fish did exhibit a significantly lower density of neuromasts within the SO-3 bone area (df=1,63; F=89.1280, P<0.0001).

In contrast to wild Honey Creek Cave and surface populations, lab-born Honey Creek surface fish exhibited significantly (1.5 times) higher neuromast density than lab-born Honey Creek Cave fish (Supplementary Figure S8; Table 2; see Supplementary Table S1 for statistical output). The relationships between subterranean and surface populations from the San Antonio River drainage remained consistent in the lab-born populations. Lab-born Blue Hole fish showed significantly greater (1.4 times) neuromast density on the SO-3 bone than lab-born San Antonio Zoo fish (Supplementary Figure S8; Table 2; see Supplementary Table S2 for statistical output). While truncated analysis of wild fish yielded a non-significant result, the existence of this trait shift in better size-matched, lab-born individuals suggests a level of genetic control.

Morphology: Melanophore density

We investigated whether cavefish and facultatively subterranean populations exhibited reduced pigmentation compared to surface fish, as observed in Mexican subterranean populations of *Astyanax* (Gross et al., 2009). Our previous study indicated that Honey Creek Cave fish are paler than surface fish, although melanophores were not quantified (McGaugh et al., 2020). Here, we assessed melanophore density by analyzing the number of melanophores divided by the size of the sample polygon in four different locations on the fish body (Supplementary Figure S1). Due to concern for fish health, we lacked suitable images for melanophore measurements of wild Honey Creek Cave fish.

Blue Hole fish exhibited significantly higher melanophore density than San Antonio Zoo fish for all body locations except one (Supplementary Figure S10; Table 1; see Supplementary Table S2 for statistical output). Likewise, San Pedro Springs fish showed significantly higher melanophore density than San Antonio Zoo fish for all body locations (Supplementary Figure S10; Table 1; see Supplementary Table S2 for statistical output). Although additional factors influenced melanophore density, our overall findings contradict expectations, as San Antonio Zoo fish exhibited lower melanophore density than either subterranean population from the same drainage.

When comparing the San Antonio Zoo and Honey Creek surface populations, no significant factors were found in the models for melanophore counts in areas near the SO-4, eye, or anal fin. However, at the base of the caudal fin, Honey Creek surface fish exhibited significantly lower melanophore density than San Antonio Zoo fish (Wilcoxon rank sum: W=248, P=0.002, Supplementary Material). Overall, the two surface populations were more similar than San Antonio Zoo fish were to the facultatively subterranean populations from the same drainage.

Lab-born Honey Creek Cave and Honey Creek Surface fish did not differ from one another in melanophore density, except in the region near the caudal fin, where lab-born Honey Creek Cave fish showed increased neuromast density (Supplementary Figure S10; Table 2; see Supplementary Table S1 for statistical output). Lab-born Blue Hole fish showed slightly reduced melanophore density compared to lab-born San Antonio Zoo fish, but the relationships were not significant (see Supplementary Table S2 for statistical output).

Hence, our results did not corroborate previous research showing that cavefish possess lighter coloration. Consistent with other studies, we found that rearing environment plays an important role in melanophore density (Bilandžija et al., 2020).

DISCUSSION

Plastic responses to environmental cues have been documented in a variety of organisms (Meuthen et al., 2018; Moriuchi & Winn, 2005; Olsson et al., 2007; Pettit et al., 2016), as has rapid evolution (Campbell-Staton et al., 2017; Qu et al., 2020). Several studies have investigated the plasticity of traits associated with cave adaptation in *A. mexicanus* (Bilandžija et al., 2020; Fernandes et al., 2018). Notably, *A. mexicanus* surface fish from Mexico raised in complete darkness have been shown to develop cave-related traits in a single generation through phenotypic plasticity (Bilandžija et al., 2020). However, certain plastic responses observed in dark-reared surface fish are incongruent with canonical cave-associated traits, highlighting that plasticity can be beneficial but also highly variable (Bilandžija et al., 2020). As expected for such a recent invasion, our results showed that many traits were predominantly governed by developmental plasticity (e.g., Figure 3), which may facilitate colonization of novel environments and fixation of adaptive traits (Behera & Nanjundiah, 2004; Lande, 2009; Pfennig et al., 2010). In addition to the predominant role of plasticity in this system, we found several phenotypic shifts that bred true in the lab (e.g., Figure 3), providing compelling evidence for rapid evolution in key cave-derived traits.

In our previous work, we documented trait shifts among wild populations established within a few generations (McGaugh et al., 2020). Building upon this work, we re-examined paired subterranean and surface populations from Honey Creek, as well as two other recently colonized subterranean populations from a separate drainage. To determine whether phenotypic shifts observed in wild individuals represented potential evolutionary shifts, we bred and raised fish from paired subterranean and surface populations in the laboratory. While our data suggested that phenotypic plasticity accounted for many of the observed trait shifts, the persistence of phenotypic differences between subterranean and surface populations suggests that certain trait shifts are representative

of rapid evolutionary change (Figure 4), in this case, occurring within just 70–115 years.

In our assessment of wild individuals, we identified several phenotypic shifts in subterranean populations compared to their paired surface populations, a pattern that was consistent across drainages. Consistent shifts in isolated populations provide insights into the initial phenotypic changes that may occur during the transition to subterranean life. These shifts in subterranean populations included increased superficial neuromast density, decreased eye-size relative to head depth, and increased wall-following behavior (although wall-following behavior was decreased in Blue Hole fish) (Figure 4). Wall-following behavior is mediated by superficial neuromasts (Yoshizawa et al., 2010) and is critical for navigating subterranean environments without the benefit of visual cues. The consistency in observed phenotypic differences across subterranean populations indicates shared environmental pressure promoting investment in auxiliary sensory systems for behavioral navigation strategies, similar to the patterns observed in Mexican cavefish (Fernandes et al., 2018; Teyke, 1990; Yoshizawa et al., 2010). Here, we found that wild Honey Creek Cave fish displayed a significant increase in post-starvation food consumption, coupled with a slight increase in aggression and decrease in stress, suggesting increased boldness that may facilitate foraging. Increased boldness and propensity to eat when food is available may aid survival in nutrient-poor environments, as observed in subterranean populations of *A. mexicanus* in their native range (Aspiras et al., 2015; Chin et al., 2018). While several traits differed between the new and well-established populations (e.g., stress assays, pigmentation), our observations indicated that the recently established subterranean populations exhibit consistent behavioral and morphological shifts toward the stereotypical Mexican cavefish phenotype.

Several phenotypic differences observed between wild subterranean and surface fish persisted when fish from these same populations were raised in the lab, suggesting a potential genetic basis for these traits. Cave-surface fish divergence in wall-following behavior, post-starvation feeding, and aggression (although not significant in wild fish) showed consistent or more pronounced divergence among lab-born individuals (Figure 4). Notably, the increase in neuromast density observed in wild Blue Hole fish relative to San Antonio Zoo fish became non-significant when the dataset was truncated to only include smaller fish, although lab-born Blue Hole fish exhibited a striking increase in neuromast density. The emergence of significant differences in lab-born individuals, where wild fish only showed qualitative differences, may be attributed to improved size matching, thus minimizing variation associated with differences in wild populations. Both wild and lab-born Honey Creek Cave fish consumed more bloodworms than their Honey Creek Surface counterparts, while the differences observed in wild fish from the San Antonio River drainage were not retained in the lab. Furthermore, all cave populations displayed higher aggression levels than surface populations, a trend that remained consistent in lab-born fish. Interestingly, similar to previous study (McGaugh et al., 2020), we observed a markedly reduced cold tolerance in both wild and lab-born Honey Creek Cave fish. Anecdotally, the wild and lab-raised fish exhibited extreme cold sensitivity when we used an ice water bath prior to photography, resulting in rapid death. This is notable given the differential responses of Mexican cavefish to other anesthetics (Bilandžija et al., 2020). The preservation of morphological and behavioral shifts in a common-garden setting strengthens the evidence for potential genetic control of these traits and points to rapid evolutionary changes (Figure 4).

In contrast, several observed phenotypic differences between wild subterranean and surface fish were not retained in lab-born fish from the same populations, suggesting a strong environmental influence on the development of those traits. Lab-born subterranean and surface pairings did not exhibit differences in stress-associated behaviors, head depth-

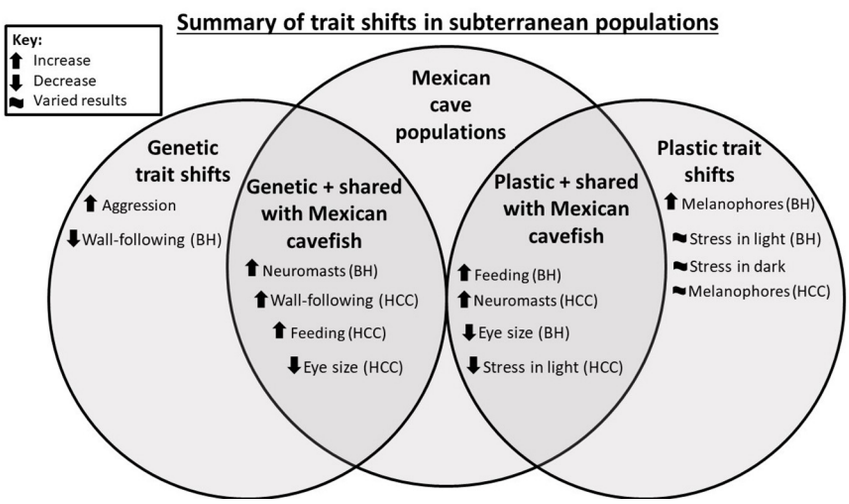

Figure 4 Venn diagram summarizing observed trait shifts in wild subterranean individuals and common-garden results of each trial

Trait shifts were identified as an increase, decrease, or varied result (see symbols in the "Key" box) based on cave populations relative to their respective paired surface population. Trait shifts in wild subterranean individuals that mirrored those seen in Mexican cavefish populations were placed within the center circle. Those that were maintained in lab-born populations were placed in the left (Genetic Trait Shifts) circle, while those lost in lab-born populations were placed in the right (Plastic Trait Shifts) circle. The overlapping area of the center, right, and left circles indicates trait shifts that are both shared with Mexican cavefish and are plastic or genetic (respectively). Trait shifts that differed across drainages are indicated in parentheses for each trait (HCC: Honey Creek Cave; BH: Blue Hole).

standardized eye diameter, or melanophore density, indicating that differences among wild populations were primarily the result of plastic responses (Figure 4). Overall, our findings provide evidence that certain morphological and behavioral traits are plastic in response to the developmental environment and suggest that plasticity may have played a crucial role in facilitating the persistence of facultative subterranean populations over long periods of drought.

Several limitations need to be considered when interpreting our results. Firstly, although we assayed over 260 fish, the sample size of the lab-born individuals was relatively small due to initial difficulties in implementing breeding protocols, which reduced our statistical power to detect significant differences in behavior and morphology. Secondly, we found that several traits were influenced by length, and size matching of wild individuals was difficult as populations in subterranean habitats were generally smaller than surface fish. To address this, we truncated the datasets to achieve similar length distributions between surface fish and cavefish, albeit at the cost of a reduced sample size. Finally, the methods used to quantify melanophores may require refinement. While the employed quantification methods were deemed effective, it is important to consider the potential influence of physiological color changes on the density counts of melanophores. Notably, wild Blue Hole and San Pedro Springs fish appeared visibly lighter in color compared to wild San Antonio Zoo fish when observed without magnification. Thus, it is possible that physiological color changes may have affected the accuracy of our melanophore density measurements (Fujii, 2000; Ligon & McCartney, 2016).

In conclusion, our study identified several behavioral and morphological shifts in wild fish from subterranean and facultatively subterranean populations compared to fish from paired surface populations. Some of these shifts, such as increased wall-following behavior, aggression, and post-fast food consumption, remained consistent in lab-born fish from the same populations, suggesting potential examples of rapid evolution in response to a new environment. However, subterranean-surface shifts in stress response, relative eye size, and melanophore density observed in wild populations were absent or reversed in lab-born fish of the same populations, indicating a high degree of plasticity for these traits. As expected for such a recent invasion, our findings suggest that many traits are predominantly governed by developmental plasticity, but we also found compelling evidence for potential rapid evolution in key cave-derived traits.

SUPPLEMENTARY DATA

Supplementary data to this article can be found online.

COMPETING INTERESTS

The authors declare that they have no competing interests.

AUTHORS' CONTRIBUTIONS

N.E.S., A.G.G., A.E.D., and S.E.M. designed and executed the experiments. N.E.S., A.G.G., and S.E.M. wrote and revised the manuscript. A.E.D. edited a draft of the manuscript. All authors read and approved the final version of the manuscript.

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