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

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Abstract

Recent colonization events to extreme environments provide unique opportunities to study the early steps of adaptation and the potential for rapid convergent evolution. However, phenotypic shifts in 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 of paired surface, subterranean, and facultatively subterranean Mexican Tetra, *Astyanax mexicanus* from recent introductions in two separate watersheds outside of their native range. We find a variety of phenotypic and behavioral shifts between subterranean and surface populations that mimic more established subterranean populations in Mexico. Despite this rapid morphological divergence, we find that most of these traits are due to plasticity in response to rearing environments, as common-garden, lab-raised fish do not maintain the phenotypes from the parental populations, and lab-born fish resemble each other for most traits more than any wild population. Interestingly and similar to wild-caught fish, subterranean-derived, lab-born subterranean fish exhibit more wall-following behavior than their lab-born surface counterparts, suggesting that this trait is genetically determined and rapidly diverging between subterranean and surface populations. Thus, our study sheds light on the early steps to subterranean evolution, is indicative of potential rapid behavioral evolution of navigational tactics and suggests that plasticity in traits involving exploratory behavior may facilitate or be in response to subterranean invasions.

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Introduction

Rapid phenotypic response to environmental shifts may be the result of phenotypic plasticity or adaptation. Instances of such rapid shifts in phenotype have been documented in phenology of feral rye (Burger, Holt, and Ellstrand 2007), cold tolerance of anole lizards (Campbell-Staton et al. 2017), and behavioral differences in urbanized animals (Miranda 2017). But whether or not these are the result of genetic adaptation, or phenotypic plasticity has been the subject of some debate. Evolution and adaptation are traditionally thought of as a long processes of genetic trial and error acted upon by selection. But given the right conditions, genetic adaptation can occur at a rapid pace (Qu et al. 2019; Campbell-Staton, et al. 2017). In contrast, rapid phenotypic change may be the result of phenotypic plasticity due to rearing environment. Plasticity can provide an important source of trait variability, promoting range expansion and allowing populations to persist in the face of extreme conditions (Moriuchi and Winn 2005; Pettit, Greenlees, and Shine 2016). Similar plasticity in response to environmental cues has been observed in response to predator presence in cichlids (Meuthen et al. 2018) and larval anurans (Relyea 2001), nutrient availability in Eurasian perch (Olsson, Svanbäck, and Eklöv 2007), and the presence of potential mates in Australian black field crickets (Kasumovic, Chen, and Wilkins 2016). The important distinction is that adaptation is the result of genetic change and is therefore passed down through generations, while plastic responses are not the result of genetic change and are thus entirely dependent on the environment. With this critical difference in mind, we can determine the relative roles of plasticity and genetic adaptation through phenotypic responses to rearing environments.

Here, we test whether rapid phenotypic changes observed in recent subterranean colonizations are the result of evolution or phenotypic plasticity. The Mexican Tetra, *Astyanax mexicanus*, is a well-studied evolutionary model species with several populations that have colonized subterranean environments throughout eastern and central Mexico hundreds of thousands of generations ago (Herman et al. 2018). *Astyanax* cavefish in Mexico exhibit striking traits associated with the subterranean environment including albinism (O'Gorman et al. 2021), reduction or complete loss of eye development (Krishnan and Rohner 2017; Rétaux and Casane 2013; McGaugh et al.

2014), and increases in superficial neuromasts (Protas et al. 2008), all which breed true in a laboratory environment. The physiology of Mexican cavefish has also shifted dramatically, exemplified by muscle cell reprogramming (Olsen et al. 2022) and differences in immune investment (Peuß et al. 2020). Genetically based cave-derived traits also tend to include a suite of behavioral shifts including reductions in aggression (Burchards, Dölle, and Parzefall 1985; Elipot et al. 2013; Elipot et al. 2014; Hinaux, Rétaux, and Elipot 2015), stress (Chin et al. 2018), total sleep (Duboué, Keene, and Borowsky 2011; Jaggard et al. 2017; Jaggard et al. 2018), and schooling behavior (Kowalko et al. 2013), as well as increased wall following (Sharma et al. 2009), and variations in levels of food consumption (Aspiras et al. 2015).

Interestingly, *A. mexicanus* were introduced from their native range in far south Texas to several streams and isolated pools in central Texas during the past century for use in the baitfish industry. Since their introduction, these fish have colonized multiple rivers, springs, and caves throughout the area (Brown 1953; McGaugh et al. 2020). In our previous work, we documented rapid phenotypic and behavioral shifts between wild caught fish from a pair of recently established populations of *A. mexicanus*: one subterranean (Honey Creek Cave) and the other epigean (Honey Creek) (McGaugh, et al. 2020). In this work, we explore a separate drainage with two additional populations of fish that occasionally or primarily occupy subterranean habitats. These two spring sites, Blue Hole (aka San Antonio Springs) and San Pedro Springs, and an appropriate surface comparison (the San Antonio Zoo) in San Antonio, Bexar County, Texas are considered subterranean aquatic environments with intermittent connectivity to surface habitats and the *A. mexicanus* populations are facultative stygobionts (i.e., ground-water dwelling). Blue Hole, San Pedro Springs, and San Antonio Zoo (a branch of the headwaters is located on zoo grounds) are part of the San Antonio River system, which extends 364 km before its confluence with the Guadalupe River, thus, these sites are thought to be the result of independent introductions from the Honey Creek populations.

Here we test wild-caught fish from this independent Texas introduction of *A. mexicanus* to discern if there are similar shifts in behavioral and morphological traits between subterranean fish and surface fish, as we observed in our previous work for the

Honey Creek populations (McGaugh, et al. 2020). Next, we test whether genetic changes or phenotypic plasticity led to the shifts in behavioral and morphological traits, by conducting the same assays on lab-born fish. We find that most traits are governed by plastic responses to the developmental environment, suggesting that phenotypes observed in recent subterranean invasions rely predominantly on developmental plasticity. This work has important implications for our understanding of subterranean evolution, as some enhanced sensory capabilities seem to be under genetic control, suggesting that one of the first phenotypes to evolve in dark environments may be sensory compensation.

Materials and Methods:

Populations

The Honey Creek and San Antonio River populations are the result of independent introductions of surface fish from Rio Grande River populations. These two clusters of sites are located in separate river basins and *Astyanax mexicanus* was first documented in these river systems in 1952 and 1908, respectively.

Honey Creek is part of the Guadalupe River basin, where *A. mexicanus* was first documented in 1953. Nearby Honey Creek Cave was likely colonized by surface fish from this creek sometime between their introduction and when they were first observed in the cave in the early 1980s.Honey Creek Cave is generally separated from Honey Creek, with little more than a trickle of water entering the creek from one of the two entrances within the Honey Creek Natural Area. While this separation traps fish in the cave, surface fish likely continue to be washed into the cave during infrequent extreme flood events (Brown 1953). In the cave, most individuals are encountered in the twilight zone of the cave and proximate dark zone although fish have been encountered over 100 m upstream from the entrance area (McGaugh, et al. 2020).

Blue Hole (otherwise known as San Antonio Springs) and San Pedro Springs are paired with the surface population from the San Antonio Zoo in San Antonio, Bexar County, Texas. All three populations are part of the San Antonio River system, and the total length of the river's path connecting all three sites is approximately 4.5 km. Both spring sites have resident populations of *A. mexicanus,* likely descendants of individuals

that were introduced at San Pedro Springs in 1908 (Brown 1953). The Blue Hole and San Pedro Springs populations have experienced long periods of drought or intermittent flow in past decades and fish at these sites occupy both surface and subsurface habitats. When water levels are high, small pools form at these spring sites which then flow into the San Antonio River but, for much of the year the pools are completely dry, requiring fish to retreat to groundwater refugia at the springs. 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 that there is a high degree of connectivity between those sites under normal flow conditions.

Sampling locations and methods

Sampling took place during February 2020 at Blue Hole, San Pedro Springs, and San Antonio Zoo. We followed similar procedures as performed in (McGaugh, et al. 2020), except all fish were trapped using collapsible "umbrella traps" baited with sardines 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

Animal care and housing practices were carried out in accordance with all IACUC guidelines (UMN IACUC protocol 2002-37827A). Aquariums were kept between 21°C and 23°C, which is similar to the temperature of the natural habitat from which the fish were captured. All rooms were kept on a 14:10 light cycle, with lights on at 8:00 CST and off at 18:00 CST. Fish were housed at a density of between one fish per 5.3L - 7.6L. A 20% water change was performed weekly on all tanks, and filter media was changed as needed, approximately once a month. All fish were fed frozen bloodworms, brine shrimp, or Tetramin flakes 1-2 times a day, *ad libitum*. Individuals were monitored for health issues and were quarantined and treated with commercially available treatments (e.g., API Fin and Body Cure) when needed.

Blue Hole, San Antonio Zoo, Honey Creek Cave, and Honey Creek surface fish

were bred in the lab. Breeding of fish was accomplished following protocol from Borowski et al. (2008), with minor adaptations (Borowsky 2008). At ~30 days post fertilization, juvenile fish were transferred to heated (26°C) fully filtered tanks at a density of one fish per 9-18L and switched to a diet of crushed flake, frozen brine shrimp, and frozen bloodworms. Fish care and tank maintenance was carried out in accordance with all IACUC guidelines as above. Heat was removed 6 months post fertilization, and lab-born fish were treated as adults.

Experimental overview

Behavioral trials were conducted in the same order and not randomized. First, we examined behavior upon introduction to a new environment, which can be 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. Trials were conducted in 19L tanks that were separated by opaque dividers to prevent fish from seeing conspecifics during behavioral experiments. All trials were recorded continuously using one Wyze Cam v2 for each tank. Cameras were loaded with empty MicroSD HC Ultra Uhs-1 Class 10 memory cards, turned on, placed in front of their respective tanks, and checked for functionality. Cameras were turned on before fish were placed in tanks and were shut down after the completion of the final trial of the day.

Next, fish were acclimated to circular arenas for 48 hrs. to test for wall-following behavior over a one-hour window and sleep duration and number of bouts over a 24hr period (see supplemental material). Finally, fish were placed back into their 19-L experimental tanks, starved for three days, and then were tested for food consumption. After the conclusion of 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 trial videos were analyzed using Ethovision XT 15 (Noldus) behavioral tracking software to track subjects and calculate variables of interest. To assure individuals were tracked accurately, each recording was reviewed manually, and

erroneous data was removed. After manual review all missing data was interpolated, and trial statistics were exported. Fish with greater than 20% missing data were removed from their respective data sets prior to statistical analysis.

Stress assay

Following (McGaugh, et al. 2020), fish were removed from their home tanks and placed into small porous plastic containers filled with water from their home tank. In a dark room, one container was floated in each 19L trial aquarium for five minutes to allow water temperatures to equilibrate and some water exchange. After the five-minute acclimation in plastic containers, fish were released from the containers into their respective 19L trial tanks. After 12 minutes in the dark, the lights were turned on (without a researcher entering the room), and fish were recorded for an additional 12 minutes. The first 10 minutes of videos from both the light and dark trials were analyzed using Ethovision XT 15 (Noldus). For the light and dark trials separately, we measured time spent immobile, distance traveled, average velocity, and time spent in either the top or bottom half of the tank. After removing data for individuals which exceeded a 20% threshold of missing tracks from Ethovision processing, our final sample sizes included recordings for 224 individuals (Blue Hole, N = 46; San Pedro Springs, N = 55; San Antonio Zoo, N = 40; Honey Creek Cave, N = 16; 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 recordings for the stress assays, mirrors were placed on a short side of each individual's trial tank. The entire width of the short side of the aquarium was covered by the mirror, leaving about a 5 cm gap between the top of the mirror and the surface of the water. The side of the tank on which the mirror was placed was randomized for each fish. Aggression assays lasted for one hour, after which mirrors were removed.

Time spent within 6.77cm of the mirror was measured for each fish using

Ethovision XT 15. After removing individuals which exceeded the > 20% threshold of missing data from Ethovision processing, our final sample sizes included 186 individuals (Blue Hole, N = 33; San Pedro Springs, N = 37; San Antonio Zoo, N = 35; Honey Creek Cave, N = 15; and Honey Creek Surface, N = 29; lab-born Blue Hole, N = 13; lab-born San Antonio Zoo, N = 10; Honey Creek Cave, N = 6; lab-born Honey Creek Surface, N = 8).

Wall following assay

In order to assess wall following behavior, a one-hour excerpt was taken from the sleep trial at 12:00pm CST for each fish. Since the assay was taken from the sleep trials, individual fish were already acclimated to their 20L buckets for at least 70 hrs., with a water depth of 12.7cm. The Ethovision arenas were set up such that they encompassed the entire surface area of the water in the bucket in a perfect circle. The diameter of the bottom of the bucket was 26cm, but due to the proximity of the camera to the water's surface, the diameter of the water surface was slightly larger. Therefore, the arena diameter for each fish was 30.5 ± 0.5cm. Two concentric zones were then placed, one zone was an exact copy of the arena, and one that was exactly half the diameter of the bottom of the bucket (13cm). Using these arenas Ethovision was used to assess the amount of time individuals spent in the center zone of the tank, amount of time not in the center zone of the tank (i.e., near the walls), the frequency with which they visited the center, and frequency and duration of mobility states (immobile, mobile, and highly mobile). After removing individuals which exceeded the > 20% threshold of missing data from Ethovision processing, our final sample sizes included 221 individuals (Blue Hole, N = 46; San Pedro Springs, N = 46; San Antonio Zoo, N = 37; Honey Creek Cave, N = 16; and Honey Creek Surface, N = 34; lab-born Blue Hole, N = 18; lab-born San Antonio Zoo, N = 10; Honey Creek Cave, N = 6; lab-born Honey Creek Surface, N = 8).

Feeding assay

After their sleep trials, individuals were returned to the 19L trial tanks. During transfer, no leftover food from sleep arenas was transferred to fasting tanks. Fish were

fasted for 72 hours in preparation for feeding trials. Similar to (McGaugh, et al. 2020), after the 72 hour fast, fish were given 50 pre-counted bloodworms and allowed to eat undisturbed for 10 minutes. After the 10-minute period, fish were removed from their trial tanks, and the remaining bloodworms were counted to determine the number of blood worms eaten by each individual. Fish were weighed, so that the number of bloodworms eaten could be corrected for the size of the fish, and fish were also photographed so that standard length could be included in analyses. In total, we analyzed feeding from 244 individuals (Blue Hole, N = 41; San Pedro Springs, N = 62; San Antonio Zoo, N = 48; Honey Creek Cave N = 17; and Honey Creek Surface N = 34; lab-born Blue Hole, N = 18; lab-born San Antonio Zoo, N = 10; Honey Creek Cave, N = 6; lab-born Honey Creek Surface, N = 8).

Neuromast assay

Fish were placed in conditioned water with a 0.025g/L solution of DASPEI (2-4-dimethylamino-N-ethylpyridinium iodide; Sigma Aldrich) fluorescent stain(Yoshizawa et al. 2010). After at least an hour of stain absorption, fish were anesthetized in an ice bath, sex was determined based on anal fin shape and presence/absence of anal fin denticles. Fish were then photographed in full light alongside a size standard. Fluorescent images of the head and the whole body were taken for each individual using a Nikon TE2000 inverted fluorescence microscope, using 1.0x magnification and a green fluorescent protein filter, as described in (McGaugh, et al. 2020). Using a custom macro in FIJI (Schneider, Rasband, and Eliceiri 2012), fish were analyzed for the number and average size of neuromasts on the surface of the third suborbital (SO-3) bone on the right side of the skull. The counts produced by the macro were hand-corrected to minimize the number of off-target counts and missed neuromasts. In total, we analyzed neuromasts from 203 individuals (Blue Hole, N = 35; San Pedro Springs, N = 45; San Antonio Zoo, N = 57; Honey Creek Cave, N = 13; and 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 nares, fourth suborbital (SO-4) bone, anterior insertion point of the anal fin, and dorsal insertion point of the caudal fin (see supplemental figure X), using a Nikon SMZ Zoom Stereoscope.

The number and size of melanophores within the collection area were determined using two modified versions of the same macro used in the neuromast assay. These included an added step that converted the color images to 8-bit, and adjusted thresholding, size, and circularity parameters. One macro was adjusted to measure large, widely spaced melanophores, while the other was adjusted to measure small, tightly clustered melanophores. The collection areas for melanophore counts were specified as a ~1 mm^2 polygon within the designated sample location. Overcounts or missed melanophores were corrected by hand. The summed area of the melanophores in each collection area was divided by the area of the polygon outlining the collection area to determine the percent coverage of the area.

We analyzed melanophores from 183 individuals (Blue Hole, N = 25; San Pedro Springs N = 49, San Antonio Zoo, N = 31, Honey Creek Cave N = 2, and Honey Creek Surface N = 38; lab-born San Antonio Zoo, N = 10; lab-born Blue Hole, N = 16; Honey Creek Cave, N = 6; lab-born Honey Creek Surface, N = 6), though some individuals may be missing data for some locations due to poor image quality.

We used the photographs taken during the neuromast and melanophore assays to compare eye size among fish. In total, we analyzed eye size from 202 individuals (Blue Hole, N = 35; San Pedro Springs N = 45, San Antonio Zoo, N = 57, Honey Creek Cave N = 13, and Honey Creek Surface N = 12; lab-born San Antonio Zoo, N = 9; lab-born Blue Hole, N = 18; Honey Creek Cave, N = 6; lab-born Honey Creek Surface, N = 7). Identity of the fish was known for all behavioral trials through neuromast imaging. To minimize fish stress, however, photos for melanophore counts were conducted several weeks after these original trials. Thus, melanophore counts cannot be linked to individual behavioral trials or neuromast counts.

Statistical analysis

After data collection, trial statistics were extracted from Ethovision, and raw tracking data was used to calculate the percentage of missing data. Individuals with greater than 20% missing raw data were excluded from statistical analyses. Data were non-normally distributed due to low sample size, confirmed by Shapiro-Wilk's normality test. We therefore used non-parametric Wilcoxon tests to compare traits across specific populations as implemented in the R statistical package (Team 2015). ANOVAs were used to explore the effects of sex or fish length on dependent variables, and their interaction with population identity.

Results:

Throughout this work, we compared phenotypes and behaviors for wild-caught fish from surface populations in Texas with those from recently colonized subterranean locations. Similar to (McGaugh, et al. 2020), we find evidence for rapid phenotypic and behavioral change between fish inhabiting these distinct environments. In contrast and as expected, phenotypes among the two locations of wild-caught surface fish exhibit less divergence than phenotypic differences between wild-caught surface fish and their respective paired subterranean and facultatively subterranean populations. Additionally, we analyzed potential differences between sexes within each population. Unless stated otherwise, sex we did not observe significant behavioral differences between males and females. To determine if the rapid shifts we observe between subterranean and surface populations are due to phenotypic plasticity or genetic change, we tested the suite of traits in 11-13 month-old, lab-raised individuals bred from our stock of wild-caught adults. Based on these results, it appears that the rearing environment plays an important role in the development of the behaviors and morphological phenotypes.

Stress assay

Behavior upon introduction into a new environment is a reliable proxy for stress (Chin, et al. 2018). Previous work indicates 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 differ in the stress responses

in a light-dependent manner.

In accord with our previous study, wild-caught Honey Creek Cave fish displayed several behaviors in lighted stress trials that suggest they experience less stress in a novel environment. Honey Creek Cave fish traveled significantly further, spent more time at the top of the tank, and less time in an immobile state than Honey Creek Surface fish in lighted stress trials (see Table S1). In contrast to previous work, here we did not observe differences in how Honey Creek Cave and Honey Creek surface fish shifted their behavior between light and dark trials, both were more active in the lighted 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. For all of the newly tested populations (i.e. San Antonio Zoo surface, Blue Hole, San Pedro Springs), fish spent more time at the bottom of the tank and spent more time immobile in lighted 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 lights were on than off. Wild-caught Blue Hole fish, however, increased their distance traveled and velocity once lights were turned on (Distance: $V = 187$, $p = 0.002$; Velocity: $V = 188$, $p = 0.002$), suggesting a reduction in stress and complicating this trend. San Pedro Springs fish spent more time immobile than San Antonio Zoo fish in the lighted trials, and spent more time at the top of the tank than San Antonio Zoo in dark trials (see Table S2). This indicates that San Pedro Springs are more stressed in the light, but less stressed in the dark in comparison to their surface counterparts. Surface fish from the two drainages were nearly identical except in lighted conditions where San Antonio Zoo fish spent nearly 3x more time immobile than Honey Creek Surface fish ($W = 413$, $p = 0.010$).

While trends in stress related behaviors in the dark were inconclusive, we did find a clear trend of reduced stress in Honey Creek Cave fish relative to Honey Creek Surface. The observed effect is in concordance with previous observations of reduced stress in subterranean *A. mexicanus* from Mexico (Chin, et al. 2018). Additionally, increases in stress under lighted conditions for the newly tested populations is in accord with

scotophilic behaviors (preference for shade) of *A. mexicanus* observed in the wild.

The observed differences in stress-associated behaviors between wild-caught 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 dark trials (see Table S1), suggesting these fish potentially experienced less stress than lab-born Honey Creek Surface fish. Lab-born fish from Blue Hole and San Antonio Zoo (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 traveled significantly further than lab-born Blue Hole (see Table S2), suggesting reduced stress in San Antonio Zoo fish which contrasts with wild caught fish. Paired testing of individuals across light and dark trials revealed no significant differences in stress-associated behaviors between light and dark trials for any lab-born population. The distributions of results for the majority of these traits between lab-born subterranean and lab-born surface fish were almost entirely overlapping, suggesting that this is likely not an artifact of small sample sizes.

The lack of differentiation in captive-bred fish is likely due to a significant elevation in stress for all fish born in the lab in comparison to their paired wild populations. In both light and dark trial conditions, lab-born fish from Honey Creek Cave and surface exhibited significantly elevated measures of stress associated behaviors than both of their paired wild populations (see Table S1). Lab-raised fish from both San Antonio Zoo and Blue Hole also showed elevated stress levels compared to their wild counterparts during light and dark trials for most measures (see Table S2). One exception was that lab-born Blue Hole fish spent significantly less time immobile than wild-caught Blue Hole and San Antonio Zoo fish in the dark (see Table S2).

In sum, we found evidence that wild-caught Honey Creek Cave and Blue Hole fish exhibit less stress than their paired surface populations (consistent with (McGaugh, et al. 2020)), while wild-caught San Pedro Springs exhibited more stress than wild-caught San Antonio Zoo in the light, but less in the dark. In contrast, lab-born subterranean and

surface populations from each drainage showed few differences in stress-associated behaviors in comparison to one another. We found instead that all captive-bred populations demonstrated elevated markers of stress when compared to their paired wild populations, suggesting potent responses to developmental environments.

Aggression assay

In the long-established Mexican populations, surface fish are more aggressive than cavefish (Elipot, et al. 2013). Introduction of mirrors into the tank simulates the arrival of a size-matched conspecific, potentially eliciting aggressive responses (Way et al. 2015; Elipot, et al. 2013; Elipot, et al. 2014; Hinaux, Rétaux, and Elipot 2015; Espinasa, Yamamoto, and Jeffery 2005). In our past work, we found that Honey Creek Cave fish spent 1.3x more of their time in the 6.77cm zone closest to the mirror (a proxy for aggression), compared to surface fish (McGaugh, et al. 2020).

For wild-caught fish in our current work, an exploratory ANOVA indicated a significant fish length by sex by population interaction term on the amount of time spent near the mirror ($F = 7.163$, $df = 1, 34$, $p = 0.011$). In wild-caught Honey Creek Cave fish, larger males were less aggressive than smaller males (Spearman rank correlation: $S = 154$, $p = 0.015$), and 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 in surface fish ($S = 432$, $p = 0.541$), but larger females were less aggressive than smaller females ($S = 843.3$, $p = 0.054$). Females from Honey Creek Cave spent 1.3x more time adhered to the mirror than females from Honey Creek Surface, while males were not significantly different between populations (see Table S1). Notably, analyzing the impact of sex was not included in our previously published work.

In contrast, for the newly evaluated wild-caught populations, there was no interaction between sex, fish length, and population of origin for Blue Hole or for San Pedro Springs fish compared to San Antonio Zoo fish. Wild-caught San Pedro Springs fish spent more time near the mirror than San Antonio Zoo fish (SPS - SAZ: $W = 363.5$, $p = 0.001$). To confirm this trend was not an artifact of San Antonio Zoo fish being larger than San Pedro Springs fish, we truncated the dataset to only those San Antonio Zoo fish

which were smaller than the largest San Pedro Springs fish (SAZ = 16 fish, SPS = 36 fish), and results were consistent (SPS - SAZ truncated: W = 172, p = 0.021). Comparisons between wild-caught Blue Hole and San Antonio Zoo fish showed no significant differences (see Table S1), though wild-caught Blue Hole fish did spend slightly (~1.1x) more time near the mirror than wild-caught fish from San Antonio Zoo. The two wild-caught surface populations were not significantly different from one another (HCS-SAZ W = 581, p = 0.327).

Though contrary to trends of reduced aggression in subterranean fish from Mexico (Elipot, et al. 2013), the observed increases in aggression in wild-caught subterranean fish from Texas matches the results from our previous work in this system. By including sex as a factor, we were also able to determine the relative influence of sex in each of these populations and found that wild-caught Honey Creek Cave females spent more time near the mirror than those from the Honey Creek Surface population.

Similar to wild-caught populations, lab-born fish from Honey Creek Cave tended to spend more time (~1.2x) in the mirror zone than lab-born Honey Creek Surface, though the trends were not significant, likely due to sample size (see Table S1). These trends were consistent for both males and females in Honey Creek lab-born populations. Lab-born Blue Hole fish also spent significantly more time near the mirror (~1.3x more) than lab-born San Antonio Zoo, regardless of sex (see Table S2).

To summarize, we found evidence that wild-caught Honey Creek Cave (consistent with (McGaugh, et al. 2020)) and wild-caught San Pedro Springs populations exhibit more aggressiveness than their paired surface wild-caught populations. Though not significant, lab-born Honey Creek Cave fish were also slightly more aggressive than lab-born Honey Creek Surface. Additionally, we observed that lab-born Blue Hole fish were significantly more aggressive than the San Antonio Zoo lab-born population, as well as wild-caught Blue Hole and San Antonio Zoo.

Wall-following behavior

In previous work wall-following behavior was documented to be more prevalent in subterranean than surface populations (McGaugh, et al. 2020). Here, we also found

wild-caught Honey Creek Cave fish visited the center of the arena significantly fewer times, and spent slightly more of the trial time (97% versus 94%) in the outer zone of the arena than Honey Creek Surface fish (see Table S1), suggesting that subterranean fish preferred the outer edge of the arena. Likewise, wild-caught San Pedro Springs fish visited the center of the arena significantly fewer times, and spent significantly more of the trial time in the outer zone of the arena than wild-caught San Antonio Zoo surface fish (see Table S2).

While there was no impact of sex on most populations, Blue Hole wild-caught fish, were an exception. Blue Hole female fish spent significantly less of the 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). On the whole, Blue Hole fish spent significantly less time in the outer zone, and visited the center of the arena slightly more times than wild-caught San Antonio Zoo fish (see Table S2). This is opposite of what was observed in other wild-caught subterranean-surface comparisons and suggesting that wild-caught Blue Hole fish do not exhibit exceptional wall-following behaviors.

Increased wall following behavior in all wild-caught subterranean populations except Blue Hole are consistent with previous observations from subterranean fish in Mexico (Patton, Windsor, and Coombs 2010; Sharma, et al. 2009). The two wild-caught surface populations differed in that San Antonio Zoo fish visited the center of the arena fewer times than Honey Creek Surface fish (W = 833, p = 0.01), but did not differ in percent of the trial spent in the outer zone (W = 546, p = 0.172).

The differences observed in wall following between lab-born populations mirror those found in wild-caught individuals. Neither lab-born population from Honey Creek drainage differed from their paired wild-caught fish (see Table S1), and phenotypic relationships between surface and subterranean fish were maintained in lab-bred populations. Honey Creek Cave lab-born fish spent significantly less time in, and made significantly fewer visits to, the center zone of the arena over the course of the one hour trial than Honey Creek Surface lab-born fish (see Table S1).

The relationships between lab-born and wild-caught fish from the San Antonio River drainage were less clear. Lab-born fish from Blue Hole and San Antonio Zoo were

not significantly different from one another, though lab-born Blue Hole spent about 1.5x more time in the center zone (6.2% vs 4.2%) than lab-born San Antonio Zoo fish (see Table S2), which is consistent with observations of wild populations.

Overall, we found that wild-caught fish from Honey Creek Cave and San Pedro Springs appeared to stay towards the outer zone of the trial arena, relative to their paired wild-caught surface populations. This is consistent with wall following behavior documented in previous work on Texas cavefish (McGaugh, et al. 2020), as well as in Mexican cavefish (Sharma, et al. 2009). Furthermore, we observed that wall following behavior of lab-born fish was similar to that of their respective wild-caught counterparts, suggesting that this behavior, or morphological correlates of the behavior, may be genetically determined.

Feeding assay

Mexican cavefish from Pachón cave exhibit reduced appetites compared to surface fish after a three week fast (Aspiras, et al. 2015). In our previous work, we documented Honey Creek Cave fish ate less than Honey Creek surface fish after a five-day fast (McGaugh, et al. 2020). Here we assayed whether the fish from Blue Hole and San Pedro Springs also exhibited lower consumption post-fast than San Antonio Zoo fish.

In contrast to our previous work which found Honey Creek Surface fish consumed significantly more than the Honey Creek Cave population (McGaugh, et al. 2020), our results here show that wild-caught Honey Creek Cave fish consumed 1.5x more bloodworms per unit fish weight than wild-caught Honey Creek Surface populations though this test was not significant (see Table S1), likely due to lower sample size.

The new wild-caught, subterranean populations consumed more blood worms per unit fish weight than the surface populations, after a 3-day fasting period. More specifically, Blue Hole fish consumed 2x more bloodworms than San Antonio Zoo fish per unit fish weight, and San Pedro Springs consumed 1.5x more bloodworms than San Antonio Zoo fish per unit fish weight (see Table S2).

Though inconsistent with our previous work on fish from the Honey Creek

drainage, increased food consumption is consistent with previous observations in fish from Pachon cave in Mexico (Aspiras, et al. 2015). There are, however, varying trends in food consumption between Mexican caves with some subterranean fish consuming more and some less than their surface counterparts.

Fish length influenced blood worm consumption, even after standardization for weight of the fish. Smaller, wild-caught fish from subterranean populations ate more bloodworms per unit fish mass than smaller fish from surface populations and this interaction term was significant for San Antonio Zoo-Blue Hole and San Antonio Zoo-San Pedro Springs ANOVAs (SAZ -BH : $F = 5.17$, $df = 1, 82$, $p = 0.026$; SAZ -SPS : $F = 5.53$, $df = 1, 96$, $p = 0.021$; HCC -HCS : $F = 1.716$, $df = 1, 43$, $p = 0.197$). Many fish from surface populations were much larger than those of subterranean populations, thus, we truncated the dataset to include only fish 7.55cm or smaller to understand if the fish at the upper end of the distribution were driving this interaction, and 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 72-hour fast also differed significantly between paired populations of lab-born fish. Similar to wild populations, lab-born fish from Honey Creek Cave ate significantly more bloodworms per unit fish mass than lab-born Honey Creek Surface fish (see Table S1). In contrast, Blue Hole lab-born fish ate ~3x fewer bloodworms per unit fish mass than lab-born San Antonio Zoo when corrected for weight (see Table S2). This is a reversal of the trends seen in their wild counterparts, suggesting some impact of developmental environment on this trait.

Nearly all lab-born fish ate fewer bloodworms than their wild-caught counterparts from the same populations. Honey Creek Cave lab-born fish ate ~2x fewer bloodworms than their wild-caught relatives, while Honey Creek Surface lab-born fish ate ~3x fewer bloodworms per unit fish weight than wild-caught Honey Creek Surface (see Table S1). Similarly, Blue Hole lab-born fish ate significantly fewer bloodworms per unit fish weight than all of their paired wild-caught populations, most notably consuming ~4x fewer bloodworms than their parental lineage (see Table S2). In contrast, lab-born San Antonio Zoo fish ate more bloodworms than wild-caught San Antonio Zoo fish (see

Table S2).

Overall, we found evidence that wild-caught Honey Creek Cave (inconsistent with (McGaugh, et al. 2020)), San Pedro Springs, and Blue Hole fish consumed more bloodworms per unit fish mass than their paired, wild-caught surface fish. In San Pedro Springs and Blue Hole fish, smaller fish ate significantly more bloodworms per unit fish mass than larger fish. We also observed differences in food consumption in lab-born Honey Creek Cave and Surface fish mirroring those found in wild-caught fish, though they ate significantly less than wild-caught fish. In contrast, we saw a reversal in trends in lab-born San Antonio Zoo and Blue Hole fish, with lab-born surface fish eating significantly more bloodworms per unit fish weight. In all, our results suggest that feeding is highly plastic based on the developmental environment.

Morphology: Eye size

Standardized eye size of Mexican cavefish is often much smaller than surface fish or the eye is completely absent. In our previous work, we documented that eyes of Honey Creek Cave fish were significantly larger than Honey Creek surface fish (McGaugh, et al. 2020). Here, we found no significant difference in fish length standardized eye size between wild-caught Honey Creek surface fish and wild-caught Honey Creek Cave fish (see Table S1).

In the newly-evaluated, subterranean, wild-caught populations, we consistently found that eye size standardized by fish length was larger than in surface population (see Table S2), and the two wild-caught surface populations did not differ in eye size (HCS vs SAZ: $W = 1202.5$, $p = 0.127$). We found no impact of sex on standardized eye size in Blue Hole, Honey Creek Cave, or Honey Creek Surface fish. San Antonio Zoo and San Pedro Springs males exhibited larger standardized eye size than females (SAZ : $W = 99$, $p < 0.001$; SPS : $W = 95$, $p < 0.005$).

When standardized by head depth, we found all subterranean populations exhibited smaller eyes than surface populations (see Tables S1 and S2) and Honey Creek Surface exhibited significantly larger eyes than San Antonio Zoo fish ($W = 1851$, $p < 0.001$). When standardized by head depth, male eye size was only significantly larger

than females in San Antonio Zoo fish ($W = 135.5$, $p < 0.001$). Therefore, the quantification of eye size is highly dependent on the standardization method for size of the fish, making drawing firm conclusions difficult.

A reduction in head-depth standardized eye size is consistent with reduced investment in eye development. While the subterranean fish from Texas are certainly not eyeless like their Mexican counterparts, reductions in eye size parallel such adaptations to subterranean environments.

In contrast to wild populations, lab-born fish from Honey Creek Cave and Honey Creek Surface did not differ in eye size after either correction method. Interestingly, lab-born San Antonio Zoo fish had significantly smaller eyes than lab-born Blue Hole fish, using both head-depth (~1.3x smaller) and fish length (~1.2x smaller) standardization methods (see Table S1), and we suspect this is due to a significant increase in relative eye size in lab-born Blue Hole and San Antonio Zoo fish compared to paired wild-caught populations.

In sum, we found that wild-caught fish from subterranean habitats exhibit reduced head-depth standardized eye diameter in comparison to wild-caught surface fish. Interestingly, the direction of this relationship was reversed when standardized by fish length, with subterranean populations having increased eye diameter per unit fish length. Trends remained consistent in lab-born fish from the Honey Creek drainage, though they were not statistically significant. Lab-born fish from Blue Hole exhibited the largest eyes of any of their paired populations regardless of standardization method, a reversal of the trend in wild-caught populations from the same drainage. Thus, as documented previously, eye size appears to be highly plastic (Rohner et al. 2013).

Morphology: Neuromast number

Using fluorescent microscopy, we counted the superficial neuromasts on the third suborbital bone (SO-3) on the right side of the skull. It has been documented that Mexican cavefish exhibit increased size and density of superficial neuromasts near suborbital bone 3 (SO-3)(Yoshizawa, et al. 2010). These particular neuromasts are directly associated with vibration attraction behavior and food finding capabilities

(Yoshizawa, et al. 2010). Likewise, Honey Creek Cave fish were shown to have an increased number of superficial neuromasts at the SO-3 bone compared to surface fish (McGaugh, et al. 2020). In our analysis here, Honey Creek Cave wild-caught fish exhibited 1.2x more neuromasts than Honey Creek Surface fish after correcting for area of the SO-3 bone, though the effect was marginally non-significant (see Table S1). We did not correct for fish length, since SO-3 bone area and fish length are tightly correlated and the size distributions of Honey Creek Cave fish and Honey Creek Surface fish are similar.

Both Blue Hole and San Pedro Springs wild-caught fish exhibit higher neuromast density than San Antonio Zoo wild-caught fish (see Table S2). After correcting for the area of the SO-3 bone, Blue Hole fish exhibit 1.2x higher neuromast density and San Pedro Springs fish exhibit 1.4x higher neuromast density than San Antonio Zoo fish.

This trend is complicated by the size of the fish. We found a significant interaction term between population and fish length, such that both Blue Hole and San Pedro Springs wild-caught fish exhibit a steeper negative slope for the fish length - neuromast density relationship than San Antonio Zoo (BH v SAZ: df = 1, 92, F = 4.4782, P = 0.0374; SPS v SAZ: df = 1, 92, F = 6.8693 P = 0.0103). In other words, smaller sized Blue Hole and San Pedro Springs wild-caught fish exhibit higher neuromast density than San Antonio Zoo surface wild-caught fish, and this density drops more quickly for the subterranean populations than the surface fish.

In all wild-caught populations except Honey Creek Surface fish, males exhibit ~1.3x greater neuromast density after correcting for SO-3 bone area (Wilcoxon test between males and females per population: BH: W = 189, p = 0.001; SAZ: W = 189, p = 0.001; SPS: W = 94, p = 0.004; HCC: W = 8, p = 0.073). Males tend to be smaller than females, and smaller fish exhibit higher neuromast density than larger fish. We ran an ANOVA for each population separately, with neuromast density at the SO-3 bone as the dependent variable and length of fish, sex, and the interaction between the two as the independent variables. After accounting for length of fish in the model, sex was significant in the ANOVA only for the Blue Hole wild-caught population (df = 1, 31, F = 6.25, p = 0.018). Thus, this trend largely reflects the size differences of the sexes.

Increased superficial neuromast density in wild-caught subterranean populations from Texas relative to their surface pairings mirrors previous observations in subterranean Mexican fish (Yoshizawa, et al. 2010). The two wild-caught surface populations did not differ in their neuromast number corrected for SO-3 area (HCS v SAZ: $W = 327$, $p = 0.818$); we found no significant effect of sex or population, on neuromast density and no significant length by sex by population interaction terms. We did, however, find that larger fish exhibited 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-caught populations from Honey Creek Cave and Surface, lab-born fish from Honey Creek Surface exhibited ~1.5x higher neuromast density than lab-born Honey Creek Cave fish (see Table S1). The relationships between subterranean and surface populations from the San Antonio River drainage remained consistent in lab-born populations. Blue Hole lab-born fish had ~1.4x greater neuromast density on the SO-3 bone than San Antonio Zoo lab-born fish (see Table S2).

Despite some shifting in relationships between lab-born populations in comparison to those seen in their wild-caught counterparts, all lab-born populations exhibited increased neuromast density relative to their paired wild-caught populations. Both lab-born Honey Creek Surface and Honey Creek Cave had significantly greater superficial neuromast density than their paired wild-caught populations (see Table S1). Blue Hole lab-born fish had significantly higher neuromast density than all paired wild-caught populations (see Table S2). San Antonio Zoo lab-born fish had significantly greater neuromast density than their paired wild-caught fish (see Table S2).

Overall, it appears that wild-caught subterranean and facultatively subterranean populations exhibit 1.2-1.4x higher density of neuromasts in the SO-3 area than their respective wild-caught surface populations. Though, we approach these results with some caution since larger fish appear to have lower neuromast density. Observations of neuromast density in lab-born populations revealed conflicting relationships. Lab-born fish from Honey Creek Cave had lower neuromast density than lab-born Honey Creek Surface fish (a reversal of what is observed in the wild-caught fish from the same population), while Blue Hole lab-born fish had significantly higher neuromast density

than lab-born San Antonio Zoo (mirroring relationships in paired wild populations). Lab-born fish also tended to have significantly increased neuromast density than their paired wild-caught populations. In sum, it appears that our data support some degree of plasticity in neuromast density, but interpretations are complicated by the impact that size of fish has on density of neuromasts.

Morphology: Melanophore density

We tested whether cavefish and facultatively subterranean populations exhibited reduced pigmentation relative to surface fish, as is similar to the Mexican subterranean populations of *Astyanax*. In our previous work, we found that Honey Creek Cave fish were lighter than surface fish, though we did not quantify melanophores (McGaugh, et al. 2020). Here, we analyzed the number of melanophores divided by the size of the sample polygon to obtain a measure of melanophore density in four different locations on the fish body (Figure S1). Due to concern for fish health, we did not have images suitable for melanophore measurements of wild-caught Honey Creek Cave fish.

Blue Hole fish exhibited a higher melanophore density than San Antonio Zoo fish for most locations on the body (see Table S2). Likewise, San Pedro Springs fish exhibited a higher melanophore density than San Antonio Zoo fish for all body locations (see Table S2). While additional factors influenced melanophore density, the overall conclusion is that, contrary to expectations, San Antonio Zoo fish exhibited lower melanophore density than either subterranean population from the same drainage.

The observed increase in melanophore density in wild caught subterranean populations from the San Antonio River drainage runs counter to expectations. While Mexican *A. mexicanus* cavefish are known to be albino (O'Gorman, et al. 2021), evidence of increased melanophore density as a plastic response in surface fish raised in a dark environment has been found (Bilandžija et al. 2020).

When comparing the two surface populations, San Antonio Zoo and Honey Creek Surface, there were no significant factors in the models for sampling locations near the SO-4, eye, or anal fin melanophore counts. However, at the base of the caudal fin, Honey Creek Surface exhibited significantly lower melanophore density than San Antonio Zoo

(Wilcoxon rank sum: W = 248, p = 0.002, Supplementary Material). In sum, the two surface populations were overall more similar than San Antonio Zoo fish were to either subterranean population from the same drainage.

Lab-born fish from the Honey Creek Cave or Honey Creek Surface 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 (see Table S1). The change in this relationship is likely due to significantly lower melanophore density in lab-born Honey Creek Surface fish compared to wild-caught Honey Creek Surface fish (see Table S1). Lab-born Blue Hole showed slightly reduced melanophore density compared to lab-born San Antonio Zoo, but no relationships were significant (see Table S2). The change in this relationship is likely due to significantly lower melanophore density in lab-born Blue Hole fish compared to wild-caught Blue Hole fish (see Table S2).

In summary, we did not find support for previous work that suggested that wild-caught Honey Creek Cave fish were lighter than Honey Creek Surface fish (McGaugh, et al. 2020) and fish from wild subterranean populations in the San Antonio River basin had significantly increased melanophore density in the body locations analyzed relative to the San Antonio Zoo surface fish. Lab-born Honey Creek Cave fish had significantly more melanophores in the caudal region than lab-born Honey Creek Surface fish, but otherwise no relationships between paired lab-born populations were significant. Thus, we failed to replicate previous work showing that cave fish were lighter, and we found, similar to other studies, that the rearing environment plays an important role in melanophore density (Bilandžija, et al. 2020).

Discussion

The populations analyzed in this paper present a unique opportunity to study the phenotypic shifts that may occur early in subterranean invasions. Our previous work documented trait shifts among wild populations that had been established within a few generations (McGaugh, et al. 2020). Building on this work, we re-examined paired subterranean and surface populations from Honey Creek, as well as two other recent subterranean colonizations in a separate drainage. *Astyanax mexicanus* were introduced

into a small branch of the San Antonio River system at San Pedro springs in 1908 and spread rapidly, appearing on surveys of the main river channel and the Blue Hole spring site in the same year (Brown 1953). To determine whether phenotypic shifts observed in wild-caught individuals are maintained in a laboratory setting, we bred and raised fish from paired subterranean and surface populations in the laboratory. While maintenance of phenotypic differences between subterranean and surface populations would suggest these trait shifts are encoded by genetic differences between the subterranean and surface populations, our data suggest that phenotypic plasticity has played a large role in the trait shifts we observed.

In our assays of wild-caught individuals we found several phenotypic shifts in subterranean populations relative to paired surface populations that were consistent across drainages. Consistent shifts in isolated populations provide insights into the first changes in phenotypes that may arise in the transition to subterranean life. Among these shifts in subterranean populations were increases in superficial neuromast density, decreased eye-size relative to head depth, increased wall following behavior (decreased in Blue Hole). Wall following behavior is mediated by superficial neuromasts (Yoshizawa, et al. 2010). Thus consistency in these observed phenotypic differences indicate a reliance on auxiliary sensory systems for primitive behavioral navigation strategies, paralleling established changes in Mexican cavefish (Fernandes et al. 2018; Teyke 1990; Yoshizawa, et al. 2010). Furthermore, significantly increased post-fast food consumption, coupled with slight increases in aggression suggest increased boldness facilitating foraging and prey location. Propensity to binge eat when food is available in subterranean populations of fish is likely necessary in their nutrient poor environment, and has been observed in subterranean populations of *A. mexicanus* from their native range (Aspiras, et al. 2015). Observed consistency in phenotypic shifts that mirror those in established subterranean populations is an encouraging sign that these populations are adjusting to their (relatively) new environment.

In contrast, several other shifts in traits associated with subterranean populations did not remain consistent between drainages. Established subterranean populations of *A. mexicanus* in Mexico exhibit reduced stress relative to surface fish (Chin, et al. 2018),

which was mirrored in our results from lighted stress trials in wild-caught fish from the Honey Creek drainage. Inconsistency in these trends across drainages, however, may be indicative of differing environmental pressures between localities. Additionally, increased pigmentation (melanophore density) in subterranean populations from the San Antonio River drainage is a striking departure from the albinism that is characteristic of established Mexican cavefish (O'Gorman, et al. 2021). Though concerns for fish health prevented melanophore quantification in wild-caught Honey Creek Cave fish, our previous work suggested reduced pigmentation in this population relative to wild-caught Honey Creek Surface (McGaugh, et al. 2020). Such inconsistency further reinforces evidence of differences in environmental pressures across drainages.

Retesting lab-born fish from paired subterranean and surface populations in a common garden experiment allows the potential for inferences of phenotypic plasticity or genetic coding of phenotypic shifts. Phenotypic plasticity allows populations to rapidly (in as little as one generation) adjust to changes in environmental condition, facilitating range expansion and colonization of novel environments (Pamela J. Yeh and Trevor D. Price 2004; Pettit, Greenlees, and Shine 2016). Behavioral and morphological assays revealed that several observed phenotypic differences between wild-caught subterranean and surface fish were not maintained when fish from the same populations were raised in the lab, suggesting environmental influence. Lab-born subterranean and surface pairings did not show differences in stress associated behaviors, head-depth standardized eye diameter, or melanophore density indicating that they are the result of plastic responses. Interestingly, the majority of the traits exhibiting evidence of plasticity are morphological rather than behavioral. This suggests that behavioral shifts may be genetically coded, while morphological changes are the result of developmental plasticity.

In contrast, several of the phenotypic shifts observed in wild-caught subterranean individuals were maintained in lab-born individuals of the same populations. Most consistently, shifts in wall-following and aggression (though aggression response differences were not significant in wild-caught fish) remained consistent or became more pronounced in lab-born individuals, though some sex specific differences were lost.

Maintenance of phenotypic shifts in a new generation raised in a common-garden provides evidence of a level of genetic control for these traits. Both of these genetically coded traits are behavioral, further reinforcing evidence that behavioral trait shifts are more well established in subterranean populations than morphological ones.

While the above observations in lab-born fish were consistent across drainages, some phenotypic differences from wild-caught pairings were maintained in lab-born fish from one drainage, but not the other. Differences in plasticity vs genetic control across drainage may indicate a difference in the sequence of phenotypic shifts, as genetic responses take longer to arise than plastic ones. In particular, the observed differences superficial neuromast density appears to be under genetic control in the San Antonio River drainage but is the result of plasticity in the Honey Creek drainage. Increased investment in non-visual senses may have happened sooner in subterranean populations from the San Antonio River drainage than it did in Honey Creek Cave. Contrastingly, increased post-fast food consumption appears to be under plastic control in subterranean populations from the San Antonio River drainage, but is not as dependent on environmental cues in fish from Honey Creek Cave. Lab-born subterranean populations maintaining the phenotypic shifts observed in wild-caught fish from the same population in only one of two drainages suggests differential levels of genetic control and plasticity between colonization events. These differences in response mechanism may also indicate a difference in the environmental pressures experienced by wild populations.

Plastic responses to environmental cues have been found in a variety of organisms (Moriuchi and Winn 2005; Pettit, Greenlees, and Shine 2016; Meuthen, et al. 2018; Olsson, Svanbäck, and Eklöv 2007), but so too has evidence of rapid evolution (Qu, et al. 2019; Campbell-Staton, et al. 2017). In *A. mexicanus*, there have been several studies investigating the relative genetic control of traits involved in cave adaptation (Bilandžija, et al. 2020; Fernandes, et al. 2018). Some such studies have shown that evolution in Mexican cavefish is the result of standing genetic variation (e.g., Rohner et al., 2013). Yet others have suggested that phenotypic plasticity plays a pivotal role (Bilandžija, et al. 2020). For example, superficial neuromasts have been shown to be developmentally plastic in Mexican *A. mexicanus* (Fernandes, et al. 2018). Most strikingly, *A. mexicanus*

surface fish from Mexico have been shown to develop cave-related traits in a single generation as a result of phenotypic plasticity (Bilandžija, et al. 2020). The evidence presented here indicates that phenotypic plasticity has caused similar phenotypic shifts in wild populations of these fish living in subterranean environments. But we have also found evidence that several of these traits are under genetic control, even after such a short period of time.

Future studies are needed to further examine the relative role of plasticity and genetic differentiation in the phenotypic shifts found in populations of recent invaders. As behavioral phenotypes are often highly variable between individuals, a large sample is needed in order to detect differences, especially in closely related populations. Though over 260 fish were assayed, the present study suffered from a lack of samples for lab-born individuals, reducing our power to detect differences in behavior and morphology. This was mainly due to difficulty in initial implementation of breeding protocols. Furthermore, we found that several traits assayed here were influenced by length. Size matching of wild-caught individuals was difficult as populations in subterranean habitats are small and their size seems highly restricted and generally smaller than surface fish. Future studies will use size-matched groups to eliminate confounding length by population interactions. Additionally, strategies to quantify melanophores need to be refined. We noted that to the naked eye, wild-caught Blue Hole and San Pedro Springs fish appear much lighter in color than wild-caught San Antonio Zoo. Melanophore quantification methods presented here are effective, but location selection and improvement of automatic counting may reveal different results. Finally, similar to McGaugh and colleagues (2019), we observed a greatly reduced cold tolerance in both wild-caught and lab-born fish from Honey Creek Cave(McGaugh, et al. 2020). Though anecdotal, this is the second time such evidence has been presented on this specific phenomenon, warranting further study.

In conclusion, we found several behavioral and morphological shifts in wild-caught fish from subterranean and facultatively subterranean populations relative to fish from paired surface populations. Increases in superficial neuromasts, wall following behavior (except in BH), aggression, and post-fast food consumption, along with a

decrease in head-depth standardized eye size were consistent between both drainages. Reductions in behavioral indicators of stress and increases in pigmentation were present in subterranean fish from one drainage but not the other, suggesting differential influence by developmental environment. Some of these shifts, including increased wall following, and aggression remained consistent in lab-born fish from the same populations, indicating that they may be genetically controlled. But shifts in stress, relative eye size, and melanophore density observed in wild-caught populations were absent or reversed in lab-born fish of the same populations. We found conflicting evidence of environmental influence in post-fast food consumption, and neuromast density, where relationships between lab-born fish from one drainage mirror that of their parents (HCClb>HCSlb in feeding; BHlb>SAZlb in neuromasts), while the other reverses that trend (HCClb<HCSlb in neuromasts; BHlb<SAZlb in feeding). The evidence presented here leads to the conclusion that plasticity plays an important role in the phenotypic variation between subterranean and surface populations in certain traits. We do, however, provide evidence of apparent genetically encoded differences resulting from recent colonization events.

Figures and Tables

Summary of Wild-caught Relationships

Table 1: Summary of observed relationships and significance between paired subterranean and surface populations of wild-caught fish. Phenotypic shifts in subterranean populations that parallel those in Mexican cavefish are colored in light blue, and those that do not are colored in dark orange. Level of significance is indicated with asterisks in each cell (*: p<0.05, **: p<0.01, ***: p<0.001). For full statistical output see Tables S1 and S2 for Honey Creek and San Antonio River drainages, respectively.

Assay	Variable	HCC vs HCS	BH vs SAZ	SPS vs SAZ
Stress: Dark	Proportion of Time Spent in Top Half of Tank	Cave > Surface	Cave > Surface	Cave > Surface*
	Proportion of Time Spent 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	Proportion of Time Spent in Top Half of Tank	Cave > Surface*	Cave < Surface	Cave > Surface
	Proportion of Time Spent Immobile	Cave < Surface*	Cave > Surface	Cave > Surface*
	Distance Traveled	Cave > Surface*	Cave > Surface	Cave < Surface
	Average Velocity	Cave > Surface*	Cave > Surface	Cave < Surface
Aggression	Proportion of Time Spent Near Mirror: All	Cave > Surface	Cave > Surface	Cave > Surface***
	Proportion of Time Spent Near Mirror: Females	Cave > Surface*	Cave > Surface	Cave > Surface*
	Proportion of Time Spent Near Mirror: Males	Cave < Surface	Cave < Surface	Cave > Surface
Wall Following	Proportion of Time Spent in the Center Zone	Cave < Surface	Cave > Surface*	Cave < Surface**
	Visits to the 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*

Table 1: Summary of observed relationships and significance between paired subterranean and surface populations of wild-caught fish. Phenotypic shifts in subterranean populations that parallel those in Mexican cavefish are colored in light blue, and those that do not are colored in dark orange. Level of significance is indicated with asterisks in each cell (*: $p<0.05$, **: $p<0.01$, ***: $p<0.001$). For full statistical output see Tables S1 and S2 for Honey Creek and San Antonio River drainages, respectively.

Assay	Variable	HCC vs HCS	BH vs SAZ	SPS vs SAZ
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***

Summary of Lab-born Relationships

Table 2: Summary of observed relationships and significance between paired subterranean and surface populations of lab-born fish. Phenotypic shifts in subterranean populations that parallel those in their wild-caught counterparts are colored in light blue, and those that do not are colored in dark orange. Level of significance is indicated with asterisks in each cell (*: p<0.05, **: p<0.01, ***: p<0.001). For full statistical output see Tables S1 and S2 for Honey Creek and San Antonio River drainages, respectively.

Assay	Variable	HCClb vs HCSlb	BHlb vs SAZlb
Stress: Dark	Proportion of Time Spent in Top Half of Tank	Cave > Surface*	Cave > Surface
	Proportion of Time Spent Immobile	Cave < Surface	Cave < Surface*
	Distance Traveled	Cave < Surface	Cave > Surface
	Average Velocity	Cave < Surface	Cave > Surface
Stress: Light	Proportion of Time Spent in Top Half of Tank	Cave < Surface	Cave < Surface
	Proportion of Time Spent Immobile	Cave > Surface	Cave > Surface
	Distance Traveled	Cave < Surface	Cave > Surface
	Average Velocity	Cave < Surface	Cave > Surface
Aggression	Proportion of Time Spent Near Mirror: All	Cave > Surface	Cave > Surface*
	Proportion of Time Spent Near Mirror: Females	Cave > Surface* (HCSlb N = 1)	No SAZlb Females
	Proportion of Time Spent Near Mirror: Males	Cave > Surface	Cave > Surface*
Wall Following	Proportion of Time Spent in the 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

Stress Dark Trial

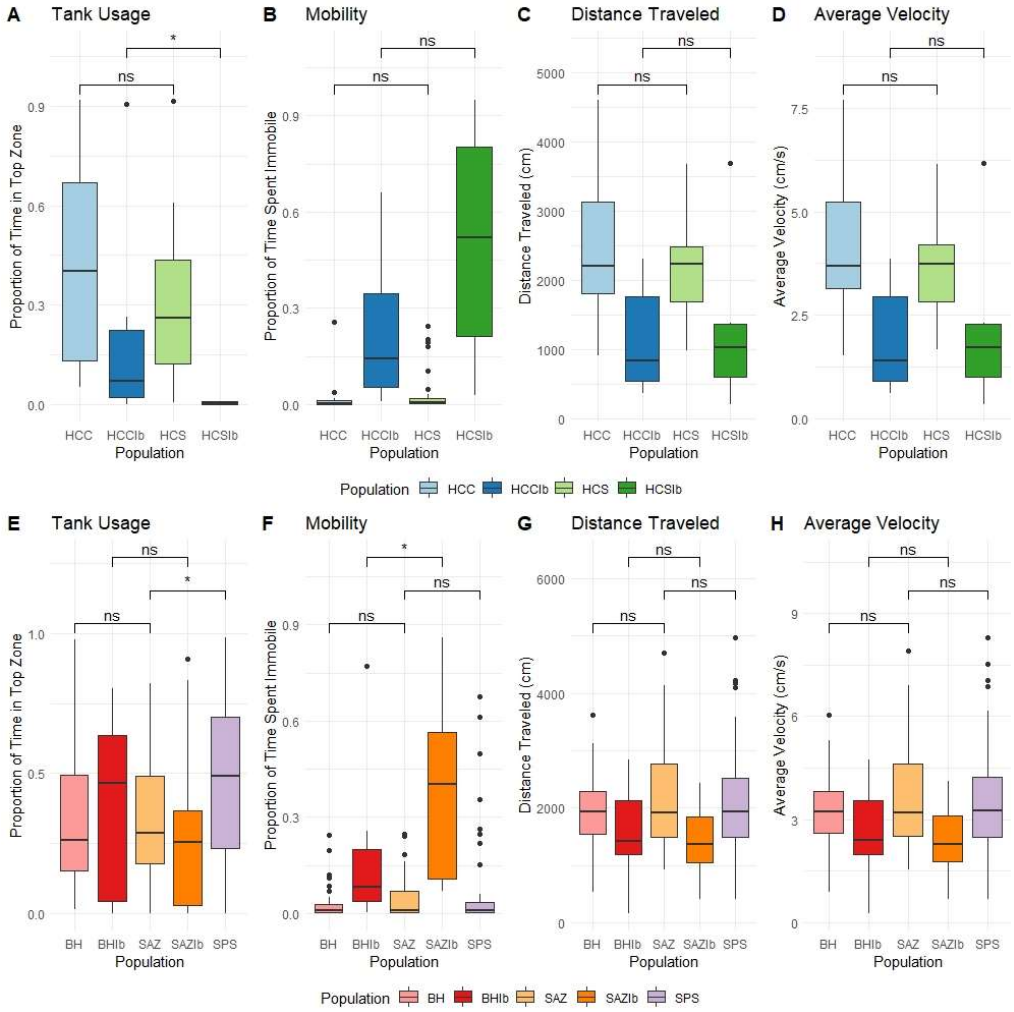

Figure 1: Box and whisker plot of stress assay, dark trial results for the Honey Creek (top row) and San Antonio River (bottom row) drainages. Each column of plots represents the results for a different behavioral indicator of stress. Elevated stress is indicated by a reduction in the proportion of time spent in the top half of the tank (plots A and E), increased proportion of time spent immobile (plots B and F), decreased distance traveled (plots C and G), and decreased average velocity (plots D and H). Significance codes for wild caught subterranean-surface pairs and lab-born subterranean surface pairs are indicated by the brackets (ns: $p>0.05$, *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$). For p-values, ranges, and medians see Tables S1 and S2 for Honey Creek and San Antonio River drainages, respectively.

Stress Light Trial

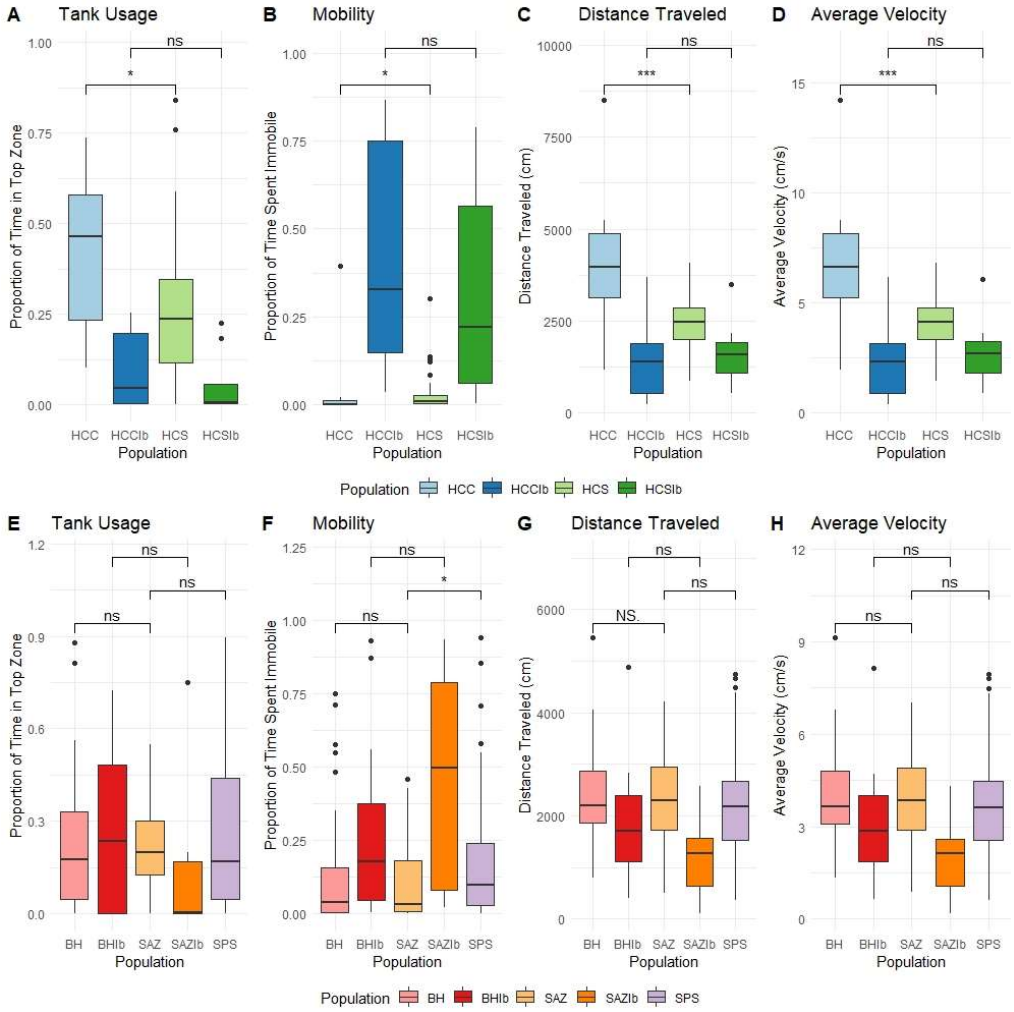

Figure 2: Box and whisker plot of stress assay, dark trial results for the Honey Creek (top row) and San Antonio River (bottom row) drainages. Each column of plots represents the results for a different behavioral indicator of stress. Elevated stress is indicated by a reduction in the proportion of time spent in the top half of the tank (plots A and E), increased proportion of time spent immobile (plots B and F), decreased distance traveled (plots C and G), and decreased average velocity (plots D and H). Significance codes for wild caught subterranean-surface pairs and lab-born subterranean-surface pairs are indicated by the brackets (ns: $p>0.05$, *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$). For p-values, ranges, and medians see Tables S1 and S2 for Honey Creek and San Antonio River drainages, respectively.

Aggression Response

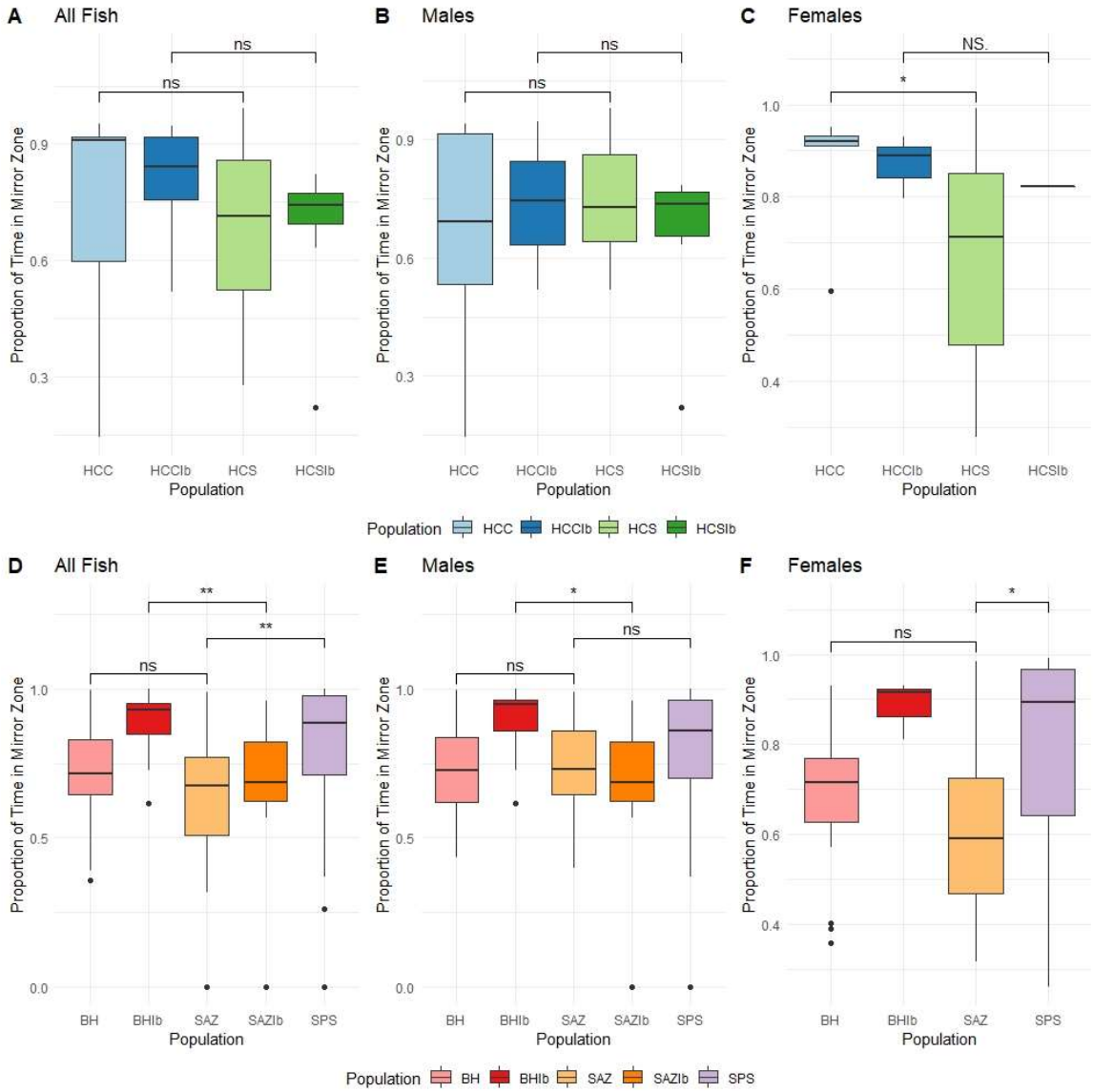

Figure 3: Box and whisker plot for aggression assay results for the Honey Creek (top row) and San Antonio River (bottom row) drainages. Plots represent the proportion of time near the mirror (time in the mirror zone divided by total tracked time) for all fish (A and D), male fish (B and E), and female fish (C and D). Significance codes for wild caught subterranean-surface pairs and lab-born subterranean-surface pairs are indicated by the brackets (ns: $p>0.05$, *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$). For p-values, ranges, and medians see Tables S1 and S2 for Honey Creek and San Antonio River drainages, respectively.

Wall Following

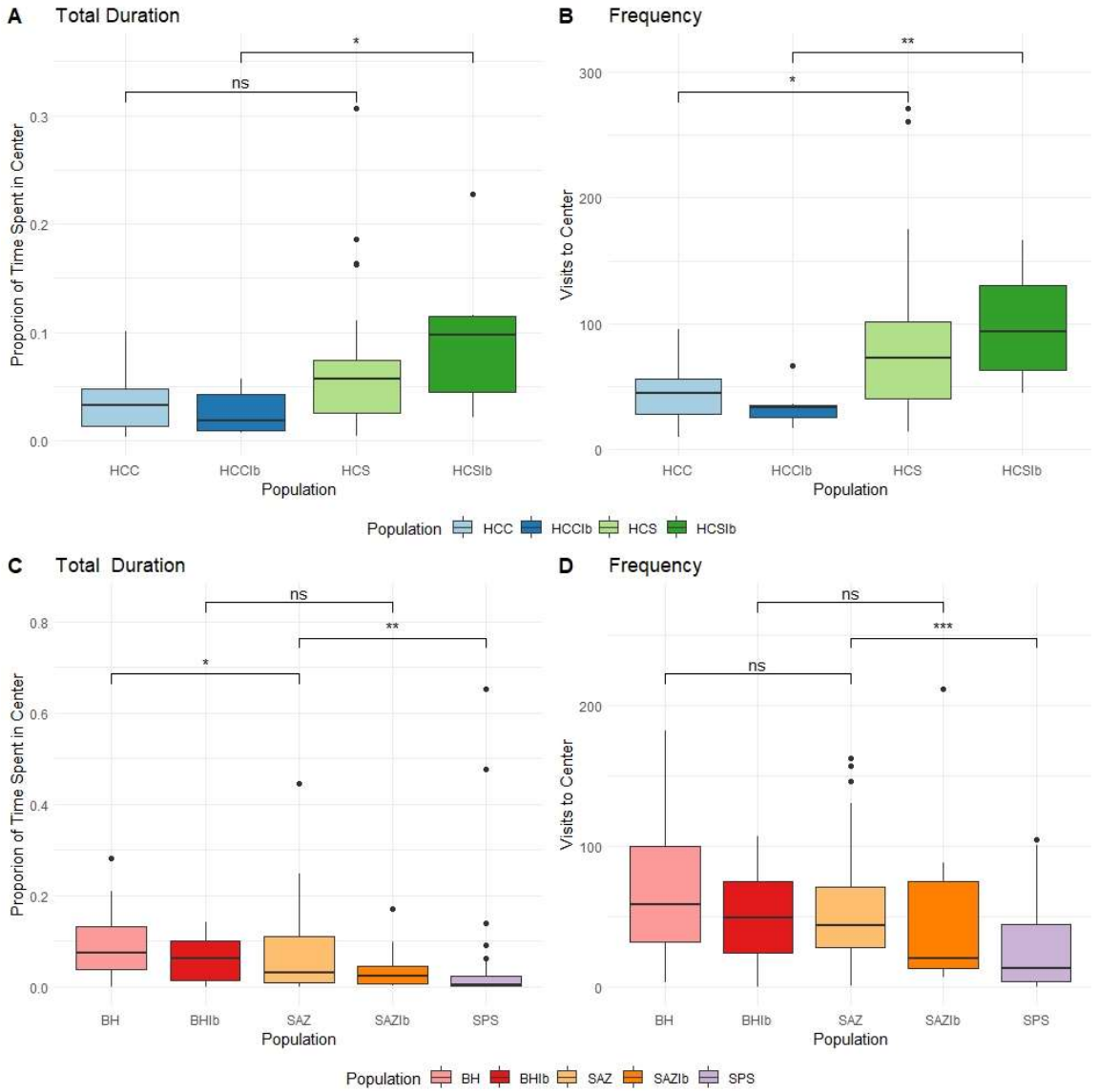

Figure 4: Box and whisker plots of wall following assay results for the Honey Creek (top row) and San Antonio River (bottom row) drainages. Plots A and C indicate the proportion of time spent in the center zone, while plots B and D represent the frequency of visits to the center zone. Significance codes for wild caught subterranean-surface pairs and lab-born subterranean-surface pairs are indicated by the brackets (ns: $p>0.05$, *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$). For p-values, ranges, and medians see Tables S1 and S2 for Honey Creek and San Antonio River drainages, respectively.

Post-Fast Feeding

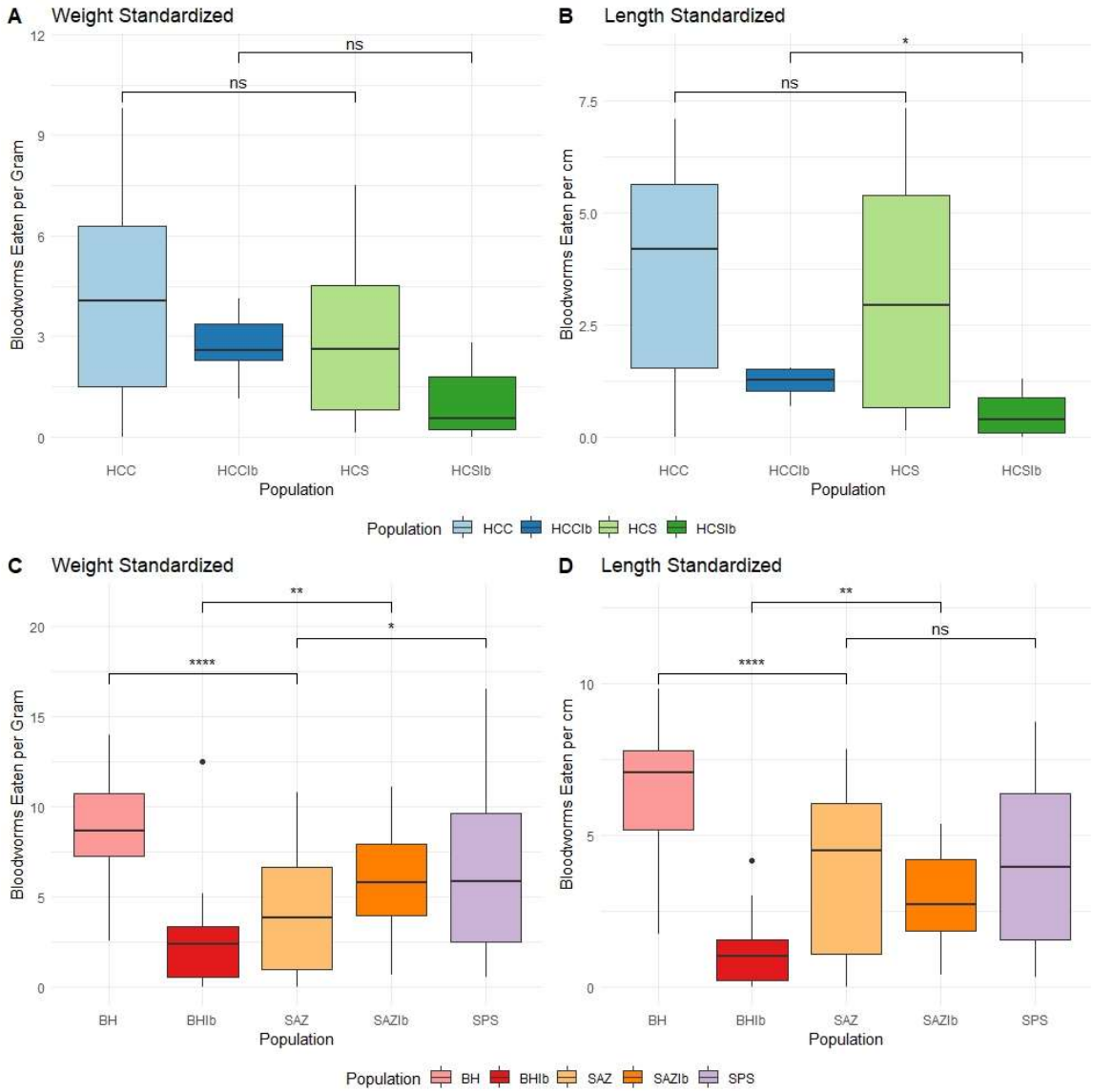

Figure 5: Box and whisker plots of feeding assay results for the Honey Creek (top row) and San Antonio Zoo (bottom row) drainages. Results were standardized by both fish weight (bloodworms/g) (A and C) and fish length (bloodworms/cm) (B and D). Significance codes for wild caught subterranean-surface pairs and lab-born subterranean-surface pairs are indicated by the brackets (ns: $p>0.05$, *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$). For p-values, ranges, and medians see Tables S1 and S2 for Honey Creek and San Antonio River drainages, respectively.

Neuromast Density

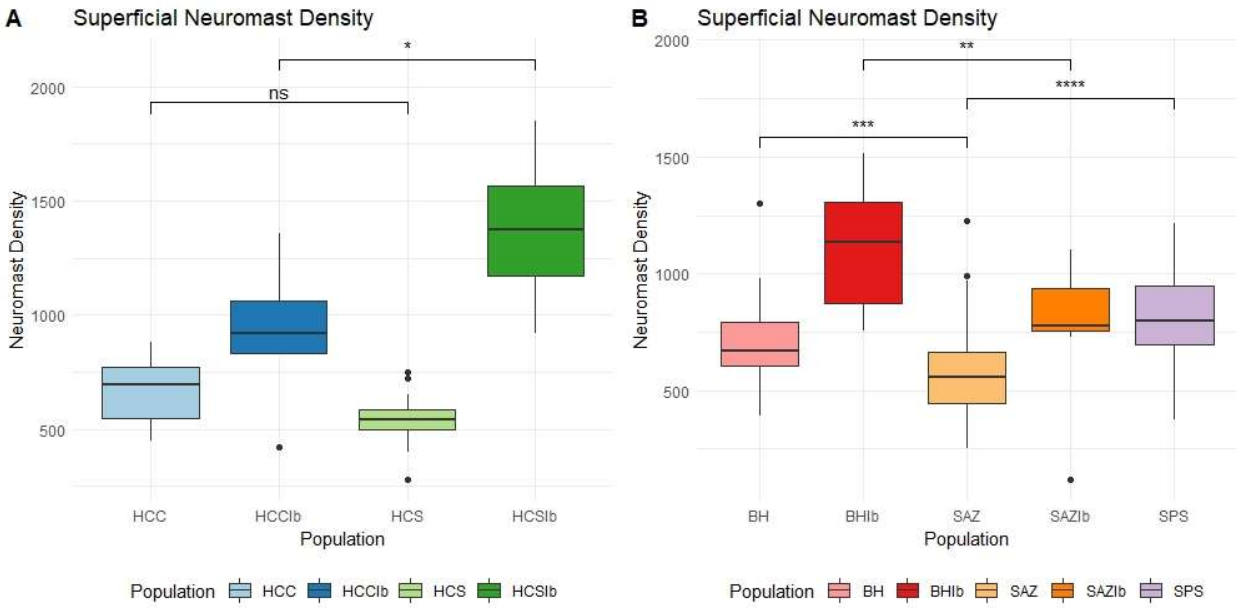

Figure 6: Box and whisker plots of suborbital superficial neuromast densities for the Honey Creek (A) and San Antonio River (B) drainages. Density is given as the total number of neuromasts counted on the SO-3 bone divided by the bones surface area (mm^2). Significance codes for wild caught subterranean-surface pairs and lab-born subterranean-surface pairs are indicated by the brackets (ns: $p>0.05$, *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$, ****: $p<0.0001$). For p-values, ranges, and medians see Tables S1 and S2 for Honey Creek and San Antonio River drainages, respectively.

Linear Regressions of Neuromast Density for Wild-caught Fish

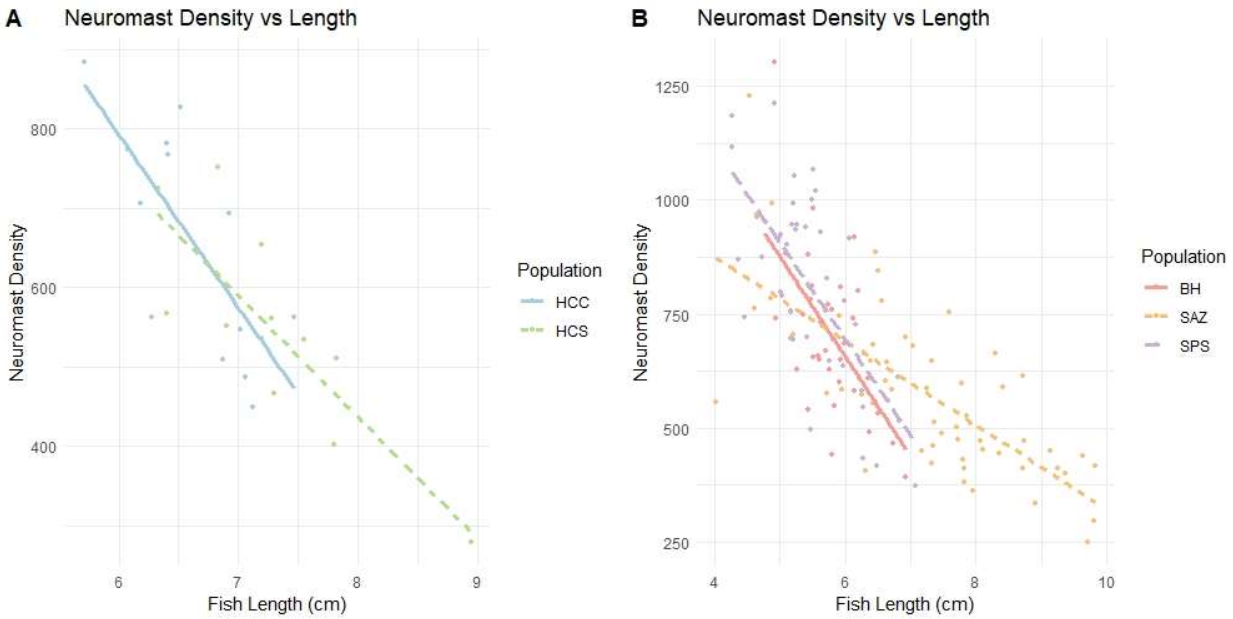

Figure 7: Linear regressions for neuromast density versus standard length for wild-caught populations. Wild-caught Honey Creek Cave fish had significantly higher neuromast density than Wild-caught Honey Creek Surface, and the slope of the regressions were not significantly different. Wild-caught San Pedro Springs and Blue Hole both exhibited increased neuromast density than wild-caught San Antonio Zoo fish. Linear regressions for the two subterranean populations also showed a greater decrease in density with increasing length than wild-caught San Antonio Zoo.

Eye Size

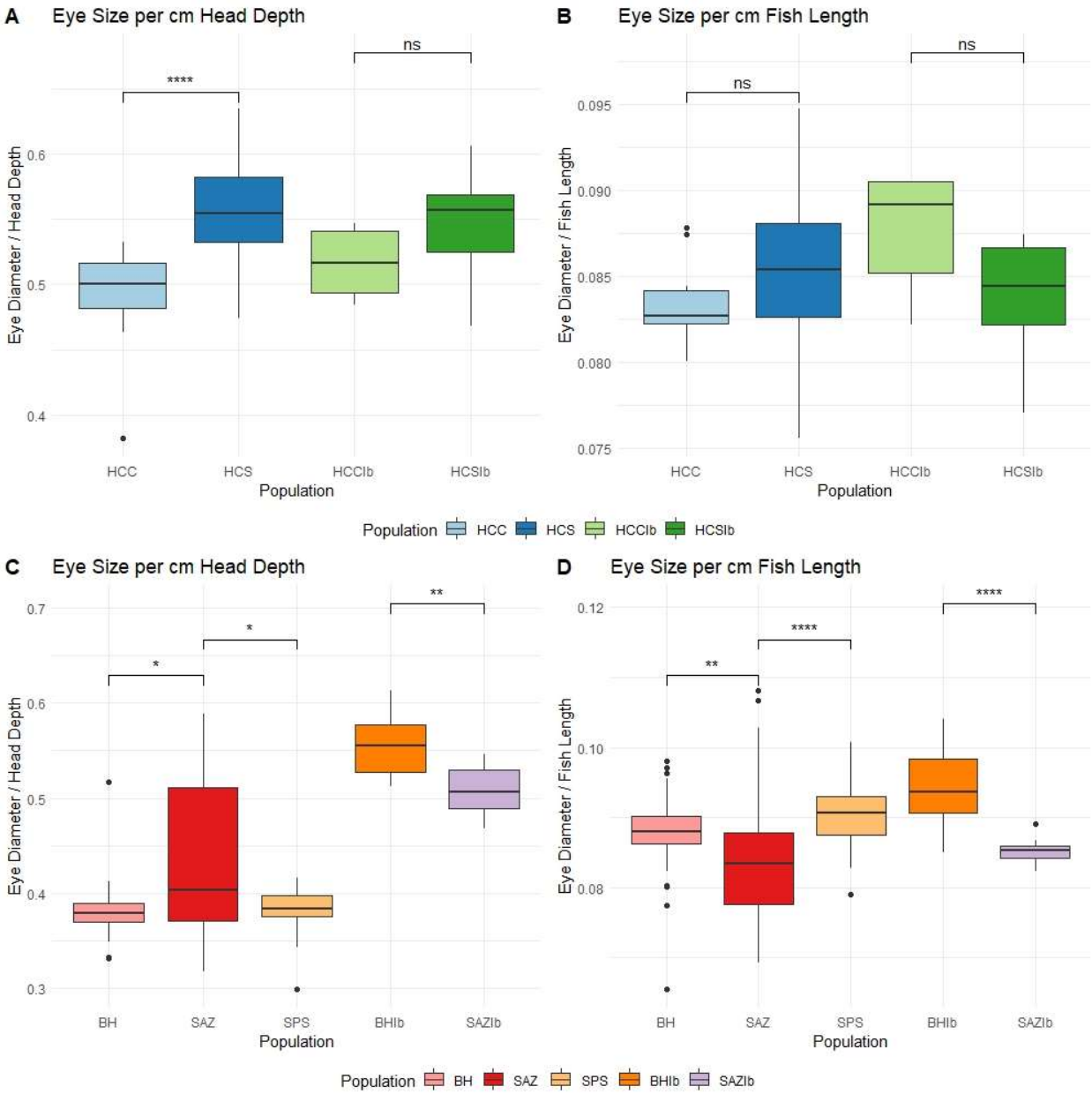

Figure 8: Box and whisker plot of eye size per cm head depth (A and C), eye size per cm fish length (B and D) for the Honey Creek (top row) and San Antonio River (bottom row) drainages. Significance codes for wild caught subterranean-surface pairs and lab-born subterranean-surface pairs are indicated by the brackets (ns: $p>0.05$, *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$, ****: $p<0.0001$). For p-values, ranges, and medians see Tables S1 and S2 for Honey Creek and San Antonio River drainages, respectively.

Melanophore Density

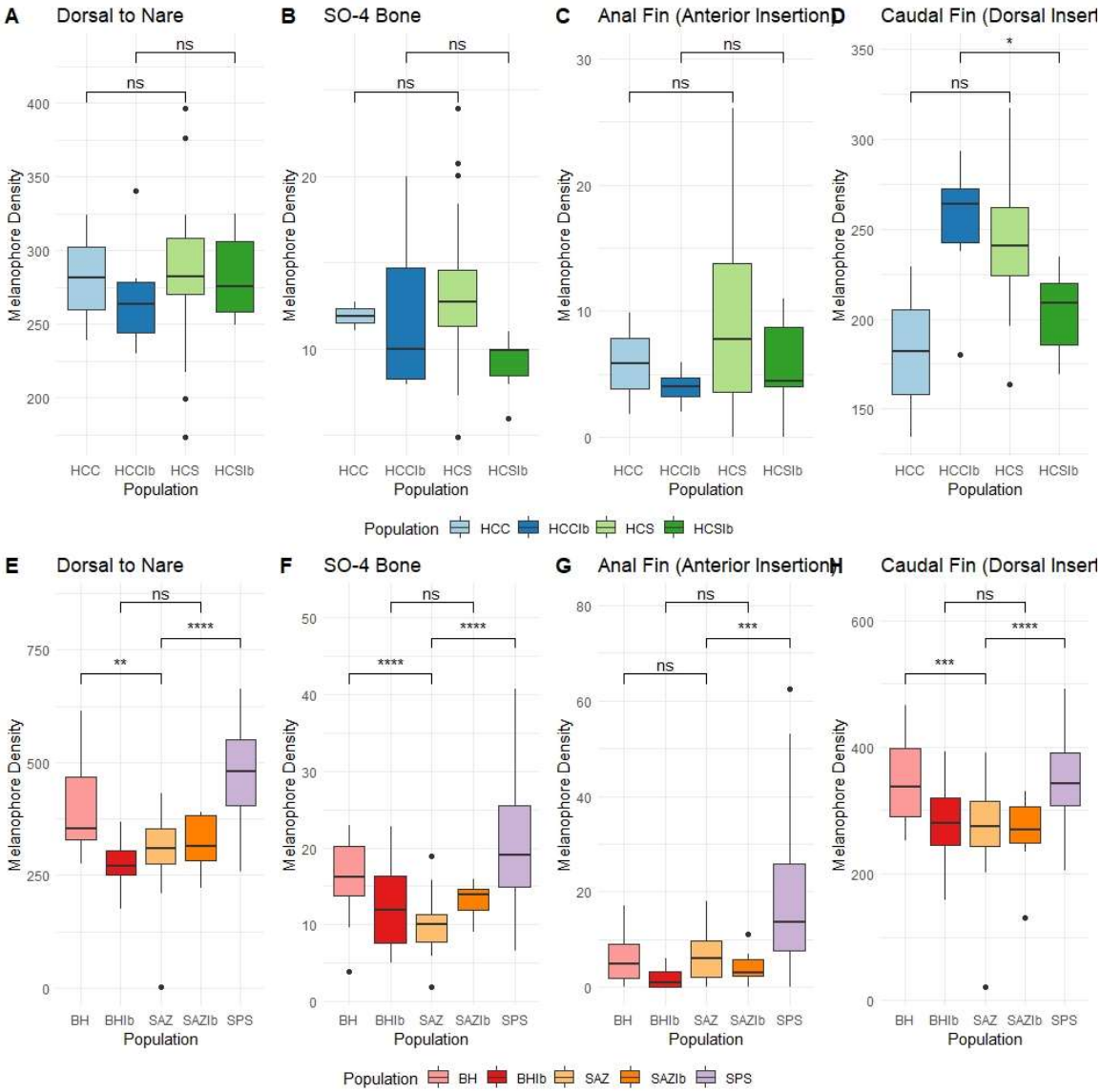

Figure 9: Box and whisker plot of melanophore density for the Honey Creek (top row) and San Antonio River (bottom row) drainages. Imaged areas included: dorsal to the nare (A and E), on the fourth suborbital bone (B and F), at the anterior insertion point of the anal fin (C and G), and the dorsal insertion point of caudal fin (D and F). Significance codes for wild caught subterranean-surface pairs and lab-born subterranean-surface pairs are indicated by the brackets (ns: $p>0.05$, *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$, ****: $p<0.0001$). For p-values, ranges, and medians see Tables S1 and S2 for Honey Creek and San Antonio River drainages, respectively.

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Appendix

Methods

Video acquisition

Videos for each assay were recorded using Wyze Cam v2's. The videos are broken into one minute segments in order to be saved to the internal microSD cards. Clips from each trial were then compiled into one continuous video to be used for analysis. Due to a video compression error that occurred during recording, each clip was compressed to differing lengths ranging from 55-62 seconds. Luckily, no video data was lost due to the error, so during video compilation the clips were adjusted to the proper length.

Sleep assay (methods)

Immediately upon completion of behavioral trials described above, fish were transferred to individual circular arenas for a sleep and wall-following assay. *Astyanax* can jump between containers, and the previous trial tanks would not allow observation of the fish from an aerial view needed to observe sleep without the risk of fish escaping. This separate arena prevented fish from escaping their respective arenas for the duration of the assay. Sleep arenas consisted of individual white 20L buckets, filled with cool, conditioned water to a height of 12.7cm, and aerated with a single air stone. A second white 20L bucket was inverted and secured over the top of the sleep arena, so that the top rims of the buckets were touching. Fish were allowed to acclimate to their arena for 48 hrs. During this time, fish were kept on a 14:10 dark-light cycle and fed at approximately 9:00 CST and 17:00 CST, though leftover food was available in the buckets throughout the day. After 48hrs of acclimation, the air stone was removed and a Wyze Cam v2 was placed over a pre-drilled hole in the top bucket of each arena. Fish were recorded continuously for 24hrs (from 5:00pm Thursday to 5:00pm Friday), during which they were kept on the same light and feeding schedule as the acclimation period. Sleep was measured as time spent immobile for more than 60 seconds. Due to a high amount of glare during dark hours, and the sheer quantity of video, we were only able to analyze 10 fish from each wild population for the 10 hours of daylight. After removing individuals

which exceeded the threshold of missing data from Ethovision processing, our final sample sizes included 50 individuals (Blue Hole, N = 10; San Pedro Springs N = 10, San Antonio Zoo, N = 10, Honey Creek Cave N = 10, and Honey Creek Surface N = 10).

Results

Morphology: Nare size

We found no difference in nare size for any pairwise population comparison, except nare size was substantially larger in Honey Creek Surface than Honey Creek Cave fish ($W = 1$, $p = 0.007$), when correcting for standard length of the fish. Males exhibited significantly larger nare size (standardized by length) than females in San Antonio Zoo and San Pedro Springs ($W= 6$, $p = 0.006$; $W = 118$, $p = 0.002$), and qualitatively so in Honey Creek Surface and Blue Hole populations, suggesting males generally tend to have larger standardized nare size than females. Notably, we only had two Honey Creek Cave males included in this analysis and so could not test for a relationship between nare sized and sex. Overall, it appears that for most populations, nare size is not impacted by population of origin.

Sleep assay

Cave morphs of *A. mexicanus* exhibit shorter sleep duration than their surface conspecifics (Duboué, Keene, and Borowsky 2011), thus, we sought to assay any sleep differences between populations. Due to poor video quality during dark hours and sheer quantity of video data, we assayed the 10 hours of lighted recordings for 10 fish from each wild population. No significant differences were found in total sleep, number of bouts or average bout length between cave and surface fish from either drainage.

Melanophore Sample Locations

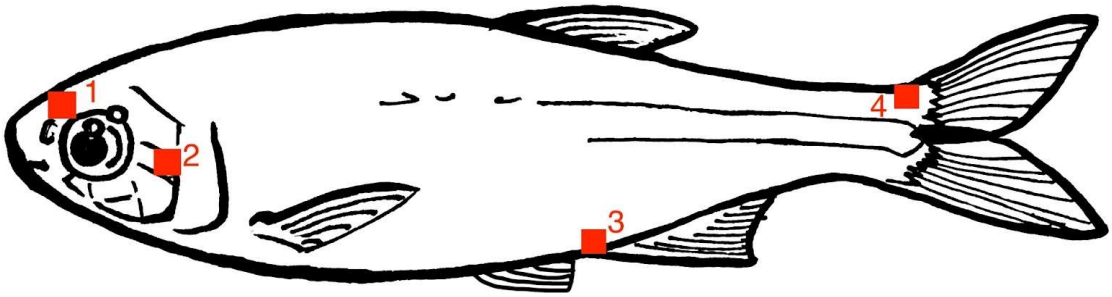

Figure S1: Image denoting melanophore collection areas. Box 1: Nare region. Box 2: SO4 region. Box 3: Anal fin region. Box 4: Tail fin area.

Exploratory ANOVAs of Neuromasts

Table S3: Statistical results from exploratory ANOVAs of neuromast density. Statistical output for each interaction term is given in each row. Significant results are listed in bold. Populations are separated by bold headings.

Blue Hole versus San Antonio Zoo

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Fish.length	**1**	**1743060**	**1743060**	**112.4155**	**< 2.2e-16**
Sex	**1**	**94058**	**94058**	**6.0661**	**0.015873**
Population	1	2967	2967	0.1913	0.662948
Fish.length*Sex	**1**	**127742**	**127742**	**8.2385**	**0.005216**
Fish.length*Population	**1**	**69437**	**69437**	**4.4782**	**0.037363**
Sex*Population	1	1769	1769	0.1141	0.736401
Fish.length*Sex*Population	1	5793	5793	0.3736	0.542737
Residuals	82	1271452	15506		

San Pedro Springs versus San Antonio Zoo

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Fish.length	**1**	**3147761**	**3147761**	**178.5373**	**< 2e-16**
Sex	1	22867	22867	1.2970	0.25772
Population	1	2504	2504	0.1420	0.70713
Fish.length*Sex	**1**	**78263**	**78263**	**4.4390**	**0.03785**
Fish.length*Population	**1**	**121111**	**121111**	**6.8693**	**0.01026**
Sex*Population	1	4201	4201	0.2383	0.62663
Fish.length*Sex*Population	1	38485	38485	2.1828	0.14297
Residuals	92	1622037	17631		

Honey Creek Surface versus San Antonio Zoo

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Fish.length	**1**	**1201280**	**1201280**	**89.3328**	**2.036e-13**
Sex	1	3516	3516	0.2615	0.6110
Population	1	8905	8905	0.6623	0.4190
Fish.length*Sex	1	30640	30640	2.2785	0.1365
Fish.length*Population	1	20659	20659	1.5363	0.2201
Sex*Population	1	553	553	0.0411	0.8400
Fish.length*Sex*Population	1	3884	3884	0.2888	0.5930
Residuals	59	793387	13447		

Exploratory ANOVAs of Melanophores

Table S4: Statistical results from exploratory ANOVAs for melanophore density. Statistical output for each interaction term is given in each row. Significant results are listed in bold. Populations are separated by bolded titles and body regions are separated by italicized headings.

Blue Hole compared to San Antonio Zoo

Response: SO-4 Melanophore density

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Sex	**1**	**356.78**	**356.78**	**22.8615**	**<0.001**
Fish.length	1	34.66	34.66	2.2206	0.1440
Population	**1**	**75.36**	**75.36**	**4.8286**	**0.0338**
Sex*Fish.length	1	0.02	0.02	0.0011	0.9733
Sex*Population	1	0.01	0.01	0.0007	0.9791
Fish.length*Population	1	6.47	6.47	0.4144	0.5234
Sex*Fish.length*Population	1	5.19	5.19	0.3328	0.5672
Residuals	40	624.25	15.61		

Response: Eye melanophore density

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Sex	**1**	**36786**	**36786**	**4.7810**	**0.0348**
Fish.length	1	14126	14126	1.8359	0.1832
Population	**1**	**50047**	**50047**	**6.5046**	**0.0148**
Sex*Fish.length	1	31100	31100	4.0420	0.0513
Sex*Population	1	5	5	0.0006	0.9807
Fish.length*Population	1	5881	5881	0.7644	0.3873
Sex*Fish.length*Population	1	8879	8879	1.1540	0.2893
Residuals	39	300070	7694		

Response: Base of anal fin melanophore density

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Sex	1	16.65	16.654	0.5674	0.4558
Fish.length	1	32.85	32.848	1.1191	0.2966
Population	1	3.73	3.731	0.1271	0.7234
Sex*Fish.length	1	58.64	58.644	1.9979	0.1654
Sex*Population	1	32.38	32.381	1.1032	0.3000
Fish.length*Population	1	63.91	63.913	2.1774	0.1481
Sex*Fish.length*Population	1	11.03	11.027	0.3757	0.5435
Residuals	39	1144.75	29.353		

Response: Base of caudal fin melanophore density

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Sex	1	2230	2230	0.6889	0.4116
Fish.length	1	7637	7637	2.3592	0.1326
Population	**1**	**46586**	**46586**	**14.3917**	**0.0005**
Sex*Fish.length	1	1167	1167	0.3607	0.5516
Sex*Population	1	1384	1384	0.4276	0.5170
Fish.length*Population	1	12272	12272	3.7911	0.0588
Sex*Fish.length*Population	1	37	37	0.0113	0.9158
Residuals	139	126243	3237		

San Pedro Springs compared to San Antonio Zoo

Response: SO-4 Melanophore density

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Sex	**1**	**351.46**	**351.46**	**8.1706**	**0.0058**
Fish.length	1	43.51	43.51	1.0116	0.3184
Population	**1**	**1483.22**	**1483.22**	**34.4815**	**<0.001**
Sex*Fish.length	1	75.85	75.85	1.7634	0.1890
Sex*Population	1	7.69	7.69	0.1789	0.6738
Fish.length*Population	1	134.74	134.74	3.1325	0.0816
Sex*Fish.length*Population	1	1.15	1.15	0.0267	0.8708
Residuals	63	2709.93	43.01		

Response: Eye melanophore density

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Sex	**1**	**51245**	**51245**	**5.4956**	**0.0222**
Fish.length	**1**	**110174**	**110174**	**11.8153**	**0.0011**
Population	**1**	**381063**	**381063**	**40.8660**	**<0.001**
Sex*Fish.length	**1**	**48486**	**48486**	**5.1998**	**0.0260**
Sex*Population	1	1295	1295	0.1389	0.7107
Fish.length*Population	1	57	57	0.0061	0.9379
Sex*Fish.length*Population	1	728	728	0.0781	0.7809
Residuals	63	587456	9325		

Response: Base of anal fin melanophore density

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Sex	1	415.2	415.17	3.1502	0.0808

Fish.length	1	182.5	182.53	1.3849	0.2437
Population	**1**	**2251.5**	**2251.45**	**17.0831**	**0.0001**
Sex*Fish.length	1	1.4	1.39	0.0106	0.9185
Sex*Population	1	91.8	91.78	0.6964	0.4072
Fish.length*Population	**1**	**653.0**	**653.03**	**4.9549**	**0.0296**
Sex*Fish.length*Population	1	28.9	28.85	0.2189	0.6415
Residuals	63	8303.0	131.79		

Response: Base of caudal fin melanophore density

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Sex	1	978	978	0.2536	0.6163
Fish.length	**1**	**29144**	**29144**	**7.5573**	**0.0078**
Population	**1**	**42959**	**42959**	**11.1396**	**0.0014**
Sex*Fish.length	1	4	4	0.0011	0.9742
Sex*Population	1	820	820	0.2128	0.6462
Fish.length*Population	1	34	34	0.0088	0.9254
Sex*Fish.length*Population	1	1071	1071	0.2777	0.6001
Residuals	62	239098	3856		

Honey Creek Surface compared to San Antonio Zoo

Response: SO-4 Melanophore density

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Sex	1	37.41	37.415	2.7932	0.1018
Fish.length	1	6.93	6.933	0.5176	0.4757
Population	1	37.39	37.387	2.7911	0.1019
Sex*Fish.length	1	2.79	2.789	0.2082	0.6504
Sex*Population	1	22.79	22.791	1.7014	0.1989
Fish.length*Population	1	7.80	7.799	0.5822	0.4495
Sex*Fish.length*Population	1	0.43	0.430	0.0321	0.8586
Residuals	44	589.38	13.395		

Response: Eye melanophore density

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Sex	1	441	441.1	0.0973	0.7566
Fish.length	1	8984	8984.4	1.9821	0.1664
Population	1	16	16.3	0.0036	0.9524
Sex*Fish.length	1	648	647.7	0.1429	0.7073

Sex*Population	1	9629	9629.0	2.1244	0.1522
Fish.length*Population	1	249	249.1	0.0550	0.8157
Sex*Fish.length*Population	1	11029	11028.6	2.4331	0.1261
Residuals	43	194906	4532.7		

Response: Base of anal fin melanophore density

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Sex	1	7.66	7.655	0.1973	0.6591
Fish.length	1	0.00	0.000	0.0000	0.9997
Population	1	25.58	25.579	0.6591	0.4212
Sex*Fish.length	1	7.01	7.006	0.1805	0.6730
Sex*Population	1	6.24	6.243	0.1609	0.6903
Fish.length*Population	1	82.22	82.215	2.1186	0.1526
Sex*Fish.length*Population	1	130.50	130.499	3.3628	0.0735
Residuals	44	1707.48	38.806		

Response: Base of caudal fin melanophore density

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Sex	1	1280	1280.5	0.7621	0.3877
Fish.length	1	2310	2309.7	1.3746	0.2476
Population	**1**	**12995**	**12995.1**	**7.7340**	**0.0081**
Sex*Fish.length	1	1647	1646.8	0.9801	0.3278
Sex*Population	1	4241	4241.5	2.5243	0.1196
Fish.length*Population	1	1204	1204.0	0.7165	0.4021
Sex*Fish.length*Population	1	1051	1050.9	0.6254	0.4335
Residuals	42	70571	1680.3		