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Evidence for rapid phenotypic and behavioural shifts in a recently established cavefish population

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Cave colonization offers a natural laboratory to study an extreme environmental shift, and diverse cave species from around the world often have converged on robust morphological, physiological and behavioural traits. The Mexican tetra (*Astyanax mexicanus*) has repeatedly colonized caves in the Sierra de El Abra and Sierra de Guatemala regions of north-east Mexico ~0.20–1 Mya, indicating an ability to adapt to the cave environment. The time frame for the evolution of these traits in any cave animal, however, is poorly understood. *Astyanax mexicanus* from the Río Grande in South Texas were brought to Central Texas beginning in the early 1900s and colonized underground environments. Here, we investigate whether phenotypic and behavioural differences have occurred rapidly between a surface population and a geographically proximate cave population, probably of recent origin. Fish from the cave and surface populations differ significantly in morphological traits, including coloration, lateral line expansion and dorsal fin placement. Striking behavioural shifts in aggression, feeding and wall-following have also occurred. Together, our results suggest that morphological and behavioural changes accompanying cave colonization can be established rapidly, and this system offers an exciting and unique opportunity for isolating the genetic and environmental contributions to colonization of extreme environments.

ADDITIONAL KEYWORDS: *Astyanax mexicanus* – cave tetra – Edwards–Trinity aquifer – rapid colonization.

INTRODUCTION

Colonization of new environments and other rapid changes to an organism's environment offer unique opportunities to gain insights into the role of genetics and plasticity in shaping phenotypes and behaviours (Kinnison & Hairston, 2007; Gordon *et al.*, 2009; Møller, 2010; Atwell *et al.*, 2012; Cornette *et al.*, 2012; Colautti & Lau, 2015). Behavioural plasticity, in particular, can promote colonization of new environments by reducing the strength of selection imposed by novel environments (Ghalambor *et al.*, 2007; West-Eberhard, 2005; Gimonneau *et al.*, 2010), as behavioural traits are often more environmentally labile than morphological traits (Wcislo, 1989; West-Eberhard, 1989; Foster, 2013; Zuk *et al.*, 2014; Baños-Villalba *et al.*, 2017). In addition, extensive evidence points to the capacity for evolutionary responses on contemporary timescales (Hendry & Kinnison, 1999; Reznick & Ghalambor, 2001; Gordon *et al.*, 2009; Messer & Petrov, 2013; Whitehead *et al.*, 2017; Dargent *et al.*, 2019).

One dramatic environmental comparison is between cave and surface habitats. Caves are challenging environments due to perpetual darkness, absence of important environmental cues, low nutrient levels and elevated levels of CO_2 (Poulson & White, 1969; Howarth, 1993). Cave environments may also provide a refuge from predation, competition and weather extremes (Culver & Pipan, 2009). Despite extreme differences in the selective landscape, cave-dwelling

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animals are diverse, and caves have been repeatedly colonized by diverse organisms (Culver & Pipan, 2009; Pipan & Culver, 2012; Howarth & Moldovan, 2018). Cave organisms typically display a suite of characters including reduction of eyes and pigmentation (Romero & Paulson, 2001; Culver & Pipan, 2009; Romero, 2009; Keene *et al.*, 2015), decreased metabolic rate (Hadley *et al.*, 1981; Huppop, 1986; Niemiller & Soares, 2015), increased starvation tolerance (Huppop, 1986; Hervant *et al.*, 1999, 2001), and enhanced non-visual senses and associated structures (Hüppop, 1987; Protas *et al.*, 2008; Yoshizawa *et al.*, 2010; Bibliowicz *et al.*, 2013). Many of these changes have evolved convergently in a diverse array of troglobites (Howarth, 1993; Juan *et al.*, 2010; Pipan & Culver, 2012; Protas & Jeffery, 2012; Niemiller & Soares, 2015), but we do not yet fully understand the rate and sequence of these changes, the consistency of changes across taxonomic groups, or the role of plasticity and genetics in shaping the first cave-derived traits.

The Mexican tetra, *Astyanax mexicanus* (Baird & Girard, 1854), is a commonly used model organism for the study of vertebrate development, for biomedical research and for the study of adaptation to cave environments (Jeffery, 2001; McGaugh *et al.*, 2014; O'Quin & McGaugh, 2015; Krishnan & Rohner, 2017). At least 30 populations of *A. mexicanus* have persisted in caves in the Sierra de El Abra and Sierra de Guatemala regions of north-east Mexico for hundreds of thousands of years and offer a natural laboratory for the study of evolution in cave environments (Mitchell *et al.*, 1977; Gross, 2012; Espinasa *et al.*, 2018; Herman *et al.*, 2018). *Astyanax mexicanus* individuals from cave populations display convergent phenotypes including a reduction or loss of eyes and pigmentation, more posterior dorsal fin placement, shorter body length and greater length-standardized body mass relative to surface fish (Protas *et al.*, 2008). Differences in behavioural traits between cave and surface populations suggest a cavefish behavioural syndrome that includes lack of schooling (Kowalko *et al.*, 2013), increased wall-following behaviour (Sharma *et al.*, 2009), reduced total sleep (Duboué *et al.*, 2011; Jaggard *et al.*, 2017, 2018), reduced stress (Chin *et al.*, 2018), increased or decreased food consumption compared to surface fish [Tinaja and Pachón populations, respectively (Aspiras *et al.*, 2015)], and reduced aggression (Burchards *et al.*, 1985; Langecker *et al.*, 1995; Espinasa *et al.*, 2005; Elipot *et al.*, 2013, 2014; Rétaux & Elipot, 2013; Hinaux *et al.*, 2015). Long-established cave populations of *A. mexicanus* have been extensively studied (Ornelas-García *et al.*, 2008; Gross, 2012; Ornelas-García & Pedraza-Lara, 2015; Herman *et al.*, 2018), but to our knowledge no work has been conducted on recently established cave populations.

Our study takes advantage of a recently discovered, non-native cave population of *A. mexicanus* in Honey Creek Cave, Comal County, Texas, which probably colonized the cave within the past century (Fig. 1A). Honey Creek Cave is the longest known cave in Texas (>30 km; Veni, 1994) and is part of the Edwards–Trinity aquifer system, which spans about 109 000 km^2 of Texas (Barker & Ardis, 1996). With at least 91 cave species and subspecies (Bowles & Arsuffi, 1993; Culver *et al.*, 2000), this aquifer system is one of the most biodiverse subterranean systems in the world (Longley, 1981). Honey Creek Cave is home to several troglobitic organisms, including the Comal blind salamander (*Eurycea tridentifera* = *E. latitans*), for which it is the type locality (Mitchell & Reddell, 1965). Although *A. mexicanus* is native to the Rio Grande, Nueces and Pecos rivers in South Texas (Mitchell *et al.*, 1977), it was probably introduced to Central Texas from South Texas in the early part of the last century. The earliest record of *A. mexicanus* in the Guadalupe River Basin, which includes Honey Creek, was in 1953 (Constable *et al.*, 2010). Biological investigations of the cave have been conducted since the early 1960s. The earliest observations of *A. mexicanus* in Honey Creek Cave were probably in the 1980s (A. Cobb and L. Palit, pers. comm), suggesting that fish were either not present before the 1980s or were missed by earlier explorations.

We assayed the Honey Creek Cave population for evidence of phenotypic and behavioural differences relative to a geographically proximate Honey Creek surface population. We compared whether differences between Honey Creek Cave and surface populations are congruent with those observed between surface fish and long-established cave populations of *A. mexicanus* in the Sierra de El Abra and Sierra de Guatemala in Mexico. Our study lays a foundation for future work to explore the genetics and phenotypic plasticity underpinning observed trait differentiation in the early stages of cave colonization.

MATERIAL AND METHODS

Honey Creek sampling and data collection

Individuals of *A. mexicanus* were collected from Honey Creek Cave and Honey Creek in the Guadalupe River Basin in Comal County, Texas, from 21 to 25 May 2018 and 29 June to 3 July 2018 (Fig. 1A).

Honey Creek Cave has two natural entrances. A third entrance, 3.5 km upstream, is an artificial shaft excavated by cavers (Elliott & Veni, 1994). The primary entrance (known as the 'spring' or 'wet' entrance) is a spring where the cave stream emanates ~2 m above the bed of Honey Creek. Honey Creek Spring is the

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primary source of water in Honey Creek. The second downstream entrance (known as the 'dry' entrance) is 4 m above the creek bed and 20 m upstream of the spring entrance and is dry except during flood events. The two entrances connect to the main stream passage within the cave. The creek is typically dry upstream of the cave; however, flooding is not uncommon, as evidenced by large amounts of woody debris high in the branches of bald cypress (*Taxodium distichum*) that line the creek and the occasional presence of other surface species (*Ameiurus natalis*, *Campostoma anomalum* and *Lepomis cyanellus*) in the cave. Sunlight penetrates ~20 m into the cave from either entrance. Due to passage morphology, areas >20 m from either entrance are perpetually dark, but some light, not detectable by the human eye, may penetrate deeper into the cave.

There is a small colony of cave myotis (*Myotis velifer*) that roosts in a dome ~100 m upstream from the spring entrance. The dome is situated over a deep (> 4 m) pool where salamanders (*Eurycea tridentifera* = *E. latitans*), and red swamp crayfish (*Procambarus clarkii*) congregate. However, *Astyanax* do not appear to congregate there, and no evidence of *Astyanax* guano-feeding in this cave has been observed to date. Other potential food resources in the cave include amphipods (*Stygobromus flagellatus* and *Hyallela azteca*), the isopod *Cirolanides texensis*, juvenile salamanders and crayfish, aquatic snails, cave crickets (*Ceuthophilus secretus*), and other aquatic and terrestrial invertebrates.

Most cave-dwelling fish and all surface fish were sampled with collapsible prawn traps baited with tinned cat food and/or sardines, but some cavefish were sampled using dip nets (1.6-mm mesh). All cavefish were collected within 100 m of the two cave entrances. We sampled surface fish using traps in two separate locations in Honey Creek. Creek distance (as opposed to straight-line distance) from Honey Creek Cave to the first surface sampling locality is 1464 m. The second surface sampling site was an additional 150–175 m further from the cave.

All fish were collected in accordance with UMN IACUC protocol 1705-34800A and were shipped to the University of Minnesota via DeltaDash Cargo Services. Upon arrival, fish were transferred to 37.9 and 75.7-L tanks and kept at a density of < 1 fish for every 6 L of water on a 10:14-h light cycle with lights on at 0800 h. Fish were fed frozen and dried bloodworms or brine shrimp *ad libitum* once or twice a day.

For all measurements, behaviour assays and quantification, the researcher was blind to ecotype identity. Sex of fish was difficult to discern, and assignments were inconsistent between researchers; therefore, we did not include it as a covariate in any tests. However, our preliminary sex assignments for the set of fish that started in the morphological and behavioural trials were roughly equal for both the cave (male = 11, female = 8) and surface (male = 23, female = 18, unknown = 1). We started all assays on the same set of 19 live cavefish and 42 live surface fish collected from the wild in 2018, although, due to attrition or unusable data (e.g. camera malfunction), this number varied slightly among assays. For each assay, the exact number of fish tested and analysed is provided.

Morphological analyses

Photographs were taken of each specimen using a Sony DSC-RX100 20.2 MP Digital Camera levelled on a tripod, with a colour standard and ruler in each photo. ImageJ v.1.46r (Schneider *et al.*, 2012) was used to record the following measurements (in mm): eye diameter, standard length (distance from the tip of the snout to the posterior end of the last vertebra) and distance to the dorsal fin (distance from the tip of the snout to the anterior insertion of the dorsal fin). Mass was taken with an AWS-100 balance (capacity 100 g, graduation 0.01 g).

Colour measurements were taken from photos of the right side of each fish. Using a similar procedure as in McGaugh (2008), photos were opened in Photoshop 2015.5, the image was flattened, and the eyedropper tool was used to sample 31 × 31 pixels for red, green, blue (RGB), and hue, saturation and lightness (HSL) values from the Color Picker window at four landmarks across the fish body. These landmarks included (1) the anterior insertion point of the dorsal fin on the body, (2) tail fin junction to the body with the landmark placed within the black stripe in the centre of the fin and body, (3) the anterior insertion point of the anal fin on the body, and (4) centrecentre of the body. To properly position the fourth landmark, we selected the rectangle tool and drew a rectangle between the tail fin and dorsal fin landmarks. The lower right corner of the rectangle was used as the centre body landmark and the rectangle was deleted before taking colour measurements. We avoided any water spots, glare or other abnormality on the fish or on the colour swatch and made notes of any abnormality. RGB and HSL values were also taken for a colour standard in each photo. Photographs and weights of each live fish were taken on the same day, directly after vibration attraction behaviour trials and feeding assays in lighted conditions (see below). In total, we analysed photographs and weights from 19 live cavefish and 37 live surface fish. We also analysed photographs from 18 dead cavefish and 13 dead surface fish, as physiological colour change can impact observed colour for live fish. The dead fish were frozen immediately upon death and were thawed for photographs.

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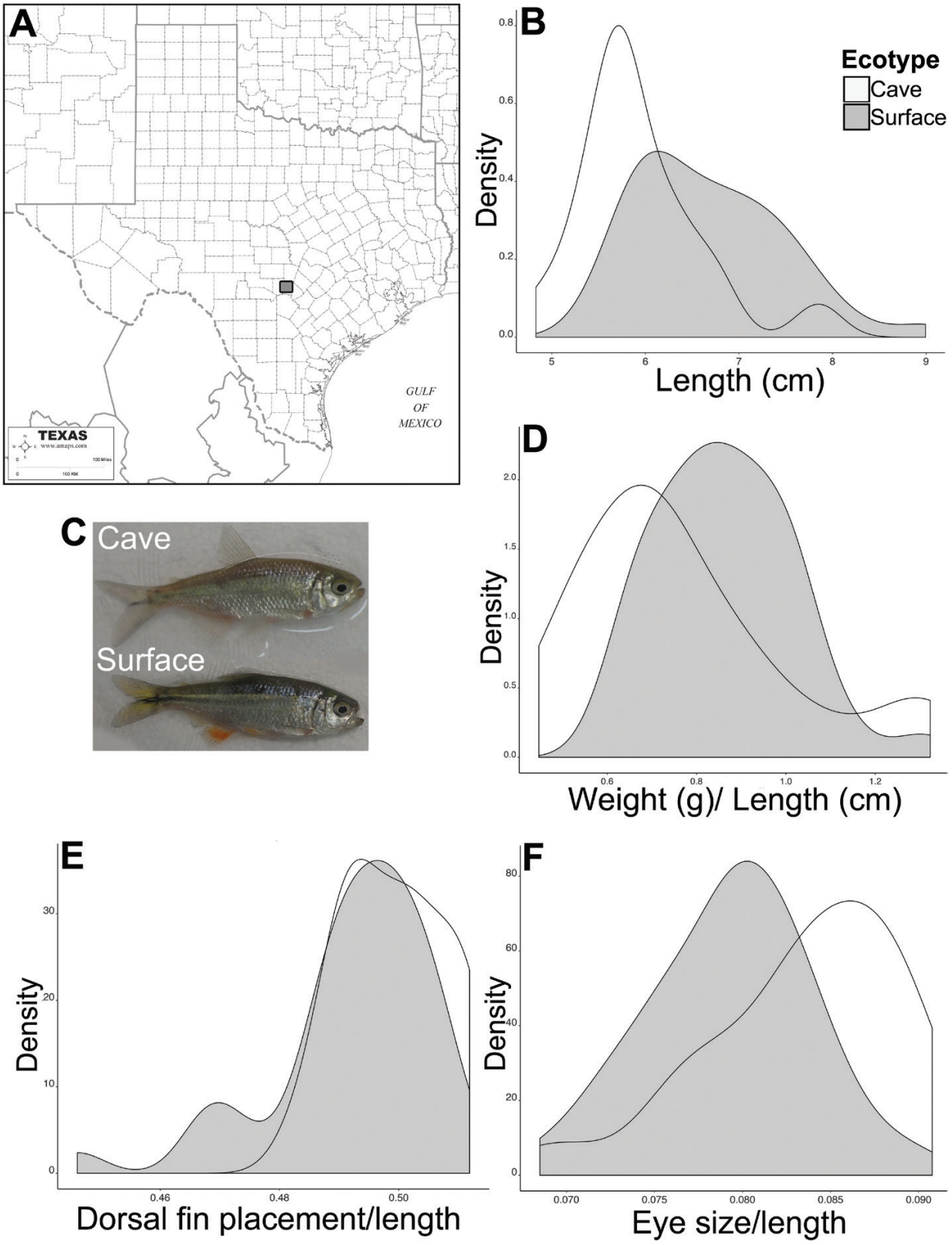

Figure 1. A, general location of Honey Creek Cave and surface sampling localities. B, length differences between cave and surface (mean = 5.93 and 6.68 cm, respectively). C, images of live cave and surface fish. D, weight standardized by length of

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Three months after these initial measures, neuromasts were stained using a procedure similar to previously published methods (Yoshizawa *et al.*, 2010; Jaggard *et al.*, 2017). Fish were submerged in conditioned, aerated water with 0.05% DASPEI (2-4-dimethylamino-*N*-ethylpyridinium iodide; Sigma Aldrich) to label superficial and canal neuromasts. After staining, fish were anaesthetized in an ice bath of conditioned water until immobilized. Ice-chilled water was required to immobilize surface fish, whereas cavefish required that water temperature be > 7 °C to survive. Images were acquired using a Nikon TE2000 inverted fluorescence microscope with a filter set for detection of green fluorescent protein (GFP; excitation 450–490 nm, 500 nm DM and 520/30 emission filter) through a Nikon 1, 0.04 NA objective, using a Hamamatsu Flash 4 V2 camera with a 500-ms exposure and 10× magnification, running Nikon Elements v.5.11 software. Just before image acquisition, fish gills were wetted with cool conditioned water via a bulb pipette. To ensure fish survival, only the right side of the body was imaged, and fish were immediately returned to aerated water from their home tank after being photographed.

Superficial neuromast size, number, and size of the cranial third suborbital bone (SO-3) were counted using a custom macro written in Fiji v.2.0.0-rc-69/1.52n (Supporting Information). The macro counted neuromasts by delineating light intensity and absolute size thresholds. The steps of the macro include: (1) taking a duplicate of the image, smoothing out the duplicate using the median value with radius of 15 pixels, then subtracting the original image from the smoothed-out duplicate to remove noise; (2) drawing the region of interest; and (3) adjusting the threshold for size to be 2000–15 600 μm^2 and circularity of the neuromast to be 0.6–1. Size and circularity were empirically determined to include as many true positives as possible while excluding false positives. Each macro-processed image was visually inspected and corrected, if needed. To account for size differences between cave and surface fish, neuromast size (in pixels) and number were each divided by the area of the polygon (in pixels) of the cranial third suborbital bone as a standardization before statistical analysis. We tested 14 cavefish and 29 surface fish.

Collection and analysis of behavioural data

We quantified behavioural differences between cave and surface populations for selected assays previously tested in the long-established *A. mexicanus* populations in north-east Mexico. These assays included: (1) vibration attraction behaviour (VAB), which is a proxy for sensing moving food objects (Yoshizawa *et al.*, 2010); (2) amount of food consumed under both light and dark conditions; (3) fish movement and spatial tank usage (i.e. a proxy for stress levels; Chin *et al.*, 2018); and (4) aggression in response to a mirror.

Room temperature was 20–21 °C for all behaviour assays, with no additional heat provided to tank water. Wyze Cams (v.2) were used to record fish behaviour in all trials across all lighting conditions. These cameras provide clear, infrared recording and wireless connection. One camera per fish was used, and we controlled the cameras wirelessly while in a separate room from the behaviour trials.

The VAB assays were conducted in the dark and were followed by the feeding assays in the light on the same day. About 5 weeks later, feeding in the dark was conducted. Approximately 2 weeks after the dark feeding trials, stress assays were conducted in both the dark and the light and were immediately followed by the mirror-elicited aggression assays in the light. On trial days, the light cycle did not resume automatically at 0800 h and the dark period was extended into the morning.

Vibration attraction behaviour assays

Fish were fasted for 48 h before the trials in individual 2.8-L Aquaneering system tanks with aeration and Stress Coat-treated (API) fresh tap water, as in their home tanks. After ~24 h a 20% water change was performed to prevent the buildup of ammonia.

For VAB trials, conducted with lights off and infrared recording, each fish was transferred and allowed to acclimatize for 1 h into a 22-cm circular arena with 10 cm depth of water (following Yoshizawa *et al.*, 2010). Three consecutive 3-min trials were conducted for each fish in the following order: (1) no rod, (2) rod with no vibration and (3) rod with ~35-Hz vibration. The researcher briefly entered the room between each trial to either place the rod or turn on the vibration and left the room for the recorded trials. Six fish were tested per batch, and we conducted trials on three batches per day, resulting in a total of 18 fish tested per day. Trial arenas were rinsed with fresh tap water and refilled with conditioned water between batches of fish.

the fish (mean = 0.77 and 0.86 g/cm, respectively). E, location of anterior insertion of the dorsal fin standardized by length of the fish (mean = 0.499 and 0.492, respectively). F, eye diameter standardized by length of the fish (mean cave = 0.0834 and surface 0.0793).

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The level of vibration we targeted elicited the peak response from cavefish found by Yoshizawa *et al.* (2010). Vibration apparatuses were fashioned with Yootop 1-kΩ 0.5-W Trimming Variable Resistors Potentiometers, BestTong 14 000 r.p.m.2 Wires Miniature Micro Vibrating Vibration Vibrator Motor DC 3 V, and USB A Male Adapter Cables for power (Supporting Information, Fig. S1). These were mounted on standard ring stands with clamp holders, and a thin metal wire was used to transmit the vibration into the water (Fig. S1). The iPhone iOS 11.4 application Tuner T1 v.3.4 was used before each vibration trial to ensure the rod was vibrating at ~35 Hz. The frequency was tuned initially in the lab by using a pair of aluminium foil contacts connected to a 9-V battery and a handheld multimeter (Model 173, Fluke Manufacturing) set to frequency mode.

Vibration attraction behaviour was quantified from the video. Within the arena, we drew an 8.9-cm circle centred on the location of the vibrating rod. An approach was defined as when a fish changed direction to swim towards the wire and reached within the radius of the inner circle to the wire. We recorded the number of approaches manually and recorded the time within the inner circle, transitions in and out of the inner circle, total distance travelled, and velocity with Ethovision XT 14 (Noldus). In total, we tested 18 cavefish and 39 surface fish for VAB. For four cavefish and five surface fish, the metal rod was not placed perfectly over the centre of the inner circle. We were able to quantify the number of approaches to the rod for these trials, but did not include them in the automated Ethovision analysis, resulting in a total of 14 cavefish and 34 surface fish for which the Ethovision-measured parameters were included in statistical analyses.

Feeding assays

Directly after the VAB trials, fish were given at least 1 h to acclimatize into their original 2.8-L Aquaneering fasting tanks, with opaque separators between tanks to prevent fish from seeing and interacting with each other. If repositioning tanks was needed for better observation, fish were given another 30 min of acclimatization. Fish were given an additional 10 min of acclimatization time if observers were added to the room. Frozen bloodworms (San Francisco Bay Brand, The Lunchbox) were weighed to the nearest 0.01 g. Researchers used tweezers to feed individual bloodworms to individual fish for 10 min, feeding an additional bloodworm when one was consumed. We recorded (1) the weight of the remaining bloodworms by reweighing the weighboat to the nearest 0.01 g, *2) the time to first feeding (latency) and (3) how many bloodworms were consumed over a 10-min period. We found that evaporation led to unreliable weights of the bloodworms, so we analysed data for the number of bloodworms consumed. We tested 19 cavefish and 37 surface fish.

Because the first feeding trial was conducted under conditions which might have inhibited a feeding response from cavefish (e.g. researchers were present for the first feeding trial, and it was conducted in the light), we conducted a second feeding trial several weeks later in the dark without researchers present. The same fish were used as before, but because fish were returned to their communal tanks between trials, we did not match fish identity between the first and second feeding trials. Trials were conducted approximately at the normal 'lights on' time of 0800 h, but the lights remained off. As Mexican cavefish are known to be more resilient to starvation (Aspiras *et al.*, 2015), fish were fasted for ~120 h before this trial (3 days longer than for the first feeding trial) to ensure we fasted the cavefish for long enough to elicit a feeding response. Each fish was supplied with 50 bloodworms in total, precounted and stored in an Eppendorf tube. The researcher emptied the Eppendorf tube into the tank water under very low light conditions, then left the room. After 10 min, fish were removed from the tank, and the remaining bloodworms were counted. Fish were weighed after the trial to account for fish size differences in the analysis. Over the course of 3 weeks, we tested 17 cavefish and 17 surface fish.

Stress assays

Cavefish experience a reduced predation risk compared to surface fish, and thus cavefish may demonstrate decreased behavioural responses to stressful stimuli (Chin *et al.*, 2018). Indicators of stress rely on reduced exploratory behaviour of a new environment, which includes: shorter distances travelled, longer durations of time spent in the bottom half of the tank, lower velocity and longer periods of 'freezing' immobility (Chin *et al.*, 2018). Thus, we measured four stress behaviours: total distance travelled, velocity, duration of time spent in the bottom half of the tank, and duration of time spent immobile (using Ethovision(. Immobility state threshold was set as ≤ 10.00% change in the complete area of the subject. We tested 15 cavefish and 37 surface fish.

Fish were allowed to acclimatize in 18.9-L tanks at room temperature (20–21 °C) for 1 h without aeration. After 1 h of acclimatization, we conducted 5 min of recordings and analysis in the dark, and 5 min in the light, waiting for a full minute after lights were turned on to begin quantification of behaviours. To enhance the ability of Ethovision to recognize the fish, we used the differencing function under advanced detection settings to compare the video to a reference image without the subject. Acquisition resulted in < 9.2%

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'subject not found' data for each video (median = 1.1%) in the dark and < 32.6% in the light (median = 5.25%), and we interpolated missing data using the Track Editor function. Incorrect subject tracking and interpolation data were also manually corrected using the Track Editor function so that the final measures contained no missing data.

Aggression assays

Directly after the stress assay, we conducted mirror-elicited aggression assays. A researcher entered the room and placed a mirror in the tank, and this mirror covered the entire short side of the aquarium except for a few centimetres at the top of the tank. The proportion of time spent within 15 cm of the mirror was quantified with Ethovision over the course of an hour-long trial. Similar methods for video acquisition and interpolation were used as in the stress assay, except the differencing function and reference videos were not used for all videos. We tested 14 cavefish and 35 surface fish for aggression because we excluded three trials with > 20% missing data before interpolation (maximum in retained trials = 19.7%, median = 10.9%).

All statistical analyses were performed in R Studio v.1.1.463 (RStudio Team, 2015).

RESULTS

MORPHOLOGICAL ANALYSIS

Cavefish are shorter and weigh less than surface fish

Honey Creek Cave fish were significantly shorter than Honey Creek surface fish (mean = 5.93 and 6.68 cm, respectively; Wilcoxon $W = 145.5$, $P = 0.0004$, Fig. 1B). This is similar to that documented by Protas *et al.* (2008), although length distributions of wild collections can vary substantially when not age-matched. Examples of collected fish are given in Figure 1C. After being kept in a common environment with a similar diet for 2 months, Honey Creek Cave fish weigh less per unit length than surface fish (mean = 0.77 and 0.86 g/cm, respectively; Wilcoxon $W = 220$, $P = 0.0224$, Fig. 1 D). This is not consistent with previous findings in Mexican cavefish, which typically weigh more than their surface counterparts on a laboratory diet (Protas *et al.*, 2008; Aspiras *et al.*, 2015; Riddle *et al.*, 2018). Measurements were taken within 2 months of capture in the field, and thus these body conditions could potentially reflect lower resource availability in the caves relative to the surface populations.

Dorsal fin placement is more posterior in Honey Creek Cave fish

Previous work has documented that the anterior insertion of the dorsal fin is more posterior in cave populations of *A. mexicanus* than in surface populations, when standardized for the length of the fish (Protas *et al.*, 2008). We standardized dorsal fin placement by dividing by the standard length of each specimen, as done previously (Protas *et al.*, 2008). We found that the insertion of the dorsal fin was more posterior in cave individuals than in surface individuals, although the effect size was not large and not statistically significant with a non-parametric test (mean = 0.499 and 0.492, respectively; Wilcoxon $W = 451$, $P = 0.087$; Welch's two-sample t-test $t = 2.28$, d.f. = 50.887, $P = 0.027$) (Fig. 1 E).

Eye diameter is larger in Honey Creek Cave fish

Comparisons of length-standardized eye diameter revealed significant differences between cave and surface individuals. Individuals from Honey Creek Cave exhibited larger standardized eye diameter than surface individuals (mean = 0.0834 and 0.0793, respectively; Wilcoxon W = 520, $P = 0.0031$, Fig. 1 F). Notably, the difference in eye diameter between ecotypes is not present when the data are analysed with a linear model with eye diameter as the response variable, standard length as a covariate and ecotype as the factor. We suspect this is because fish length distributions are substantially different for ecotypes, so standardizing eye diameter for each fish by their length is more appropriate. Also of note, when standardizing eye diameter with head height of the fish (e.g. by drawing a dorsal–ventral line that intersected with the posterior-most edge of the fish eye) we found no difference in eye diameter between cave and surface fish ($W = 391$, $P = 0.50$).

Honey Creek Cave fish exhibit higher saturation coloration than surface fish

Mexican cavefish are albino or exhibit a reduced number and size of melanophores (Protas *et al.*, 2006; Gross *et al.*, 2009; Stahl & Gross, 2015). We used a cursory fish coloration assay. For four landmarks on each fish, we quantified HSL and RGB values from photographs of live fish (McGaugh, 2008; Sacchi *et al.*, 2013). We analysed all colour data for all landmarks using a principal components analysis (PCA) and determined that cave and surface fish were separated mainly by PC2 (Fig. 2A). Next, we analysed each metric for all landmarks using PCAs and determined that saturation was probably driving the majority of the signal from the PCA of all colour components (Fig. 2B). Higher saturation values represent colours with

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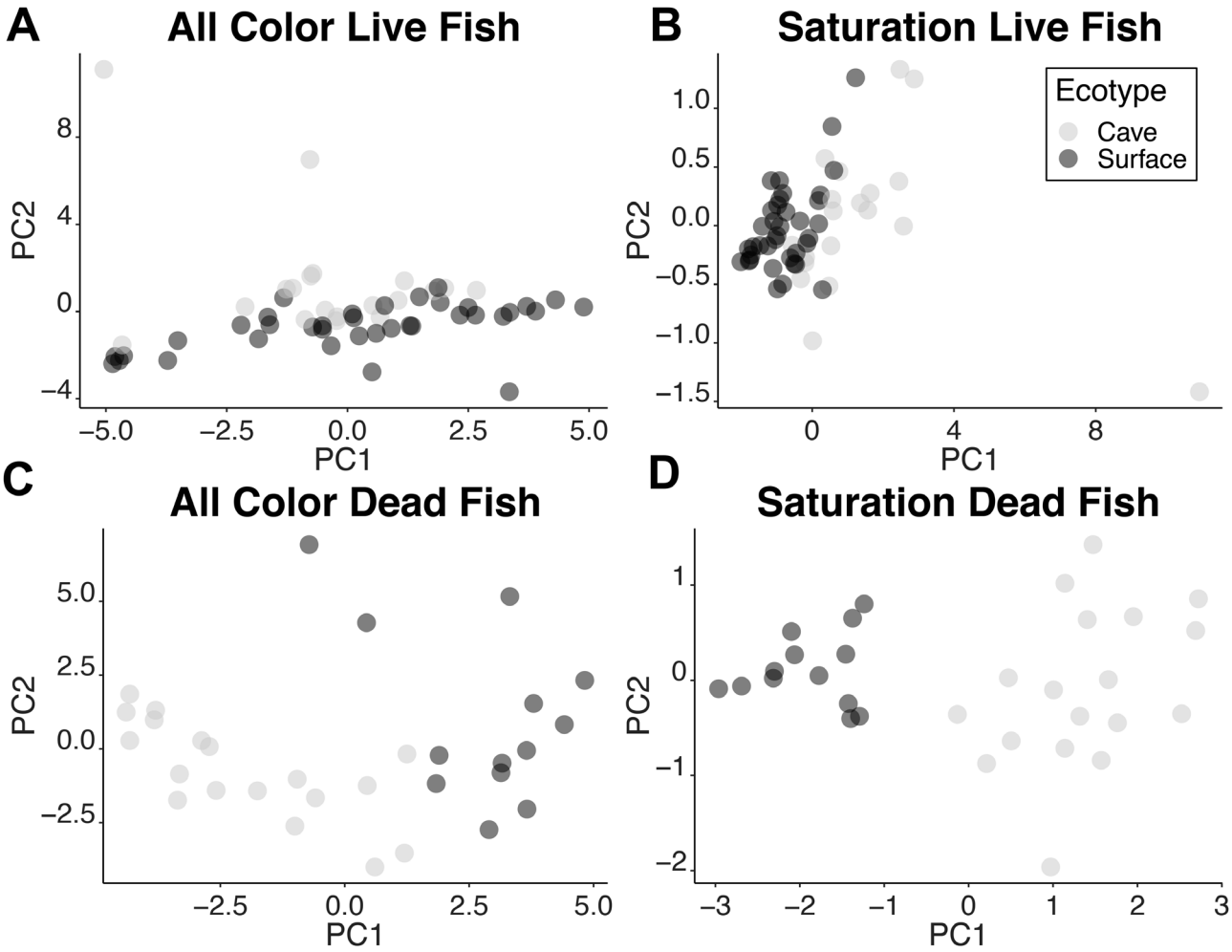

Figure 2. A, principal components analysis (PCA) of all colour components of live fish (N = 19 cave, 37 surface). B, saturation colour component of live fish (N = 19 cave, 37 surface). C, PCA of all colour components of dead fish (N = 18 cave, 13 surface). D, saturation of dead fish (N = 18 cave, 13 surface).

fewer grey components, which could be interpreted as less dark pigmentation.

Rapid physiological colour change occurs in fish and can potentially interfere with colour analyses of live fish (Sköld *et al.*, 2013). Overall, the dead fish (18 cave, 13 surface) corroborate colour shifts between the two ecotypes. Separation with PCAs based on all colour components and saturation alone was evident for dead cave and surface fish (Fig. 2C, D).

Cavefish consistently exhibit higher saturation values than surface fish (P < 0.001, for all four landmarks) for both live and dead fish (Supporting Information, Tables S1 and S2). No metrics apart from saturation were significant across all four landmarks. Dead cavefish exhibited significantly greater lightness values for anal and tail landmarks than dead surface fish, but the reverse was true for the middle body landmark (Table S2). Overall, our analysis suggests that Honey Creek Cave fish exhibit coloration shifts toward less dark pigmentation, similar to Mexican cavefish (Protas *et al.*, 2006, 2007; Gross *et al.*, 2009; Kronforst *et al.*, 2012; Pipan & Culver, 2012; Culver & Pipan, 2016; Howarth & Moldovan, 2018).

Cavefish have more suborbital superficial neuromasts

We observed that Honey Creek Cave fish (N = 14) possessed about 1.3-fold the number of suborbital superficial neuromasts than surface fish (N = 29), after dividing the neuromast count by the size of the cranial third suborbital bone (Fig. 3A, Wilcoxon W = 330, P = 0.0007). In absolute numbers, cavefish had about 1.05-fold the number of neuromasts (mean: cave = 136, surface = 131, median: cave = 135, surface = 126), but the area of the suborbital bone of cavefish was 77%

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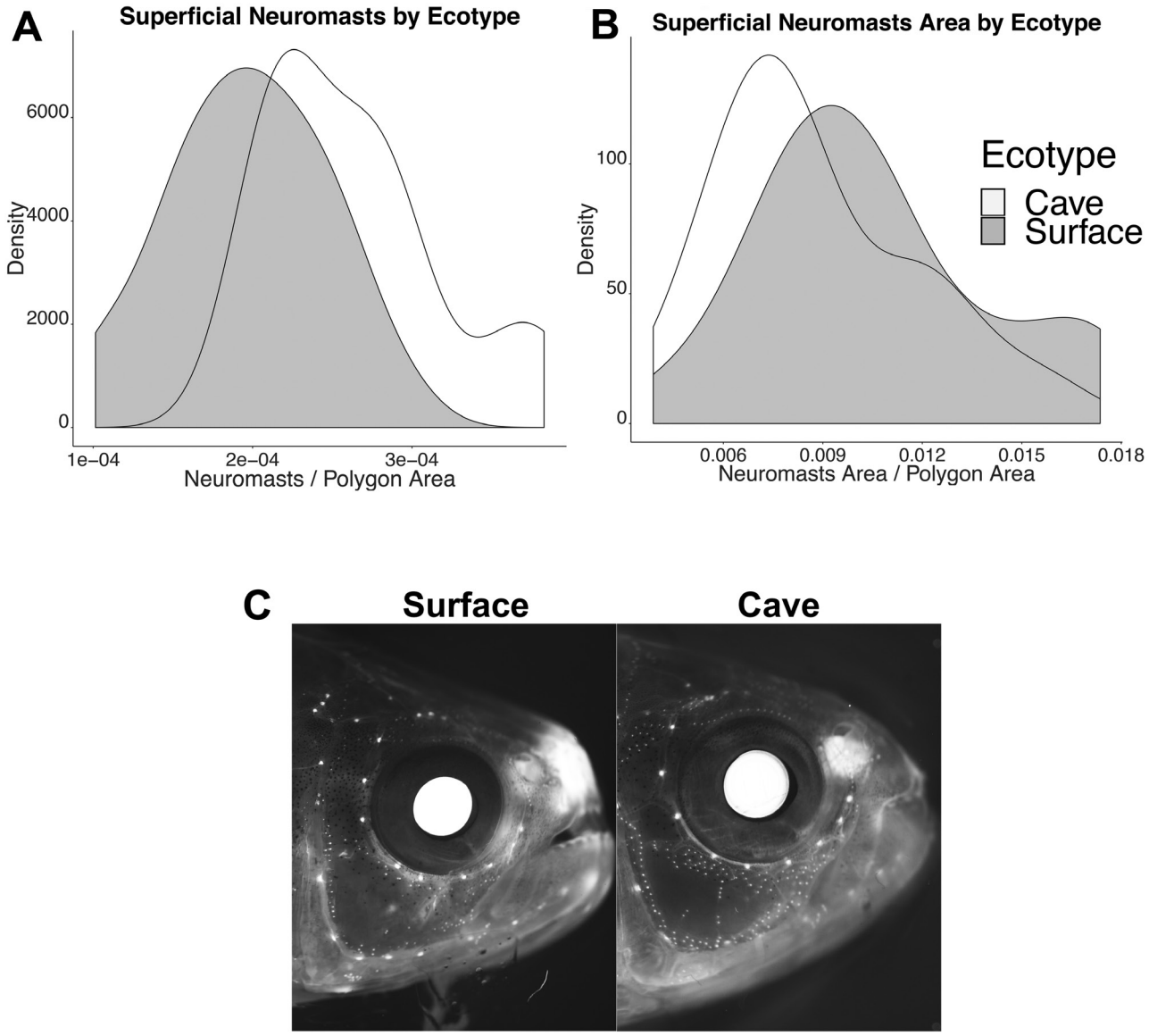

Figure 3. A, number of superficial neuromasts standardized by the size of the suborbital bone. B, mean area of superficial neuromasts standardized by the size of the suborbital bone. C, images picked from the ends of the distribution for number of neuromasts. Original images were adjusted image-wide for brightness and contrast only. N = 14 cavefish, N = 23 surface fish.

the size of the surface fish (cave = 532 312.3 pixels, surface = 684 035.0 pixels). Thus, after accounting for the size of the focal area, cavefish exhibited a substantial and significant increase in neuromast number relative to surface fish.

We observed a qualitative trend of smaller neuromasts in cavefish relative to surface fish when comparing mean or median neuromast size per fish (divided by the size of the cranial third suborbital bone: cave mean = 0.009, SD = 0.003; surface mean = 0.011, SD = 0.003), but this was not significant (Fig. 3B, mean: Wilcoxon W = 124, P = 0.257; median: W = 113, P = 0.138).

Our observations (Fig. 3C) are concordant with observations in Mexican cavefish, which have more numerous neuromasts than surface fish in the area delineated by cranial third suborbital bone. In contrast to our observations here, Mexican cavefish exhibit larger neuromasts than Mexican surface fish. We did not observe fragmentation of the cranial third suborbital bone, which is associated with superficial neuromast distribution, or fusion of additional facial bones, which is associated with canal neuromast spacing in Mexican cavefish (Gross *et al.*, 2016; Powers *et al.*, 2018).

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Behavioural analysis

Cavefish and surface fish do not display VAB; cavefish prefer the edges of the arena and do not avoid novel objects

To assess VAB, we analysed the number of approaches to a rod and the proportion of the trial the fish spent in the inner circle of the arena for 18 cavefish and 39 surface fish. For the first measure of VAB, the number of approaches was qualitatively larger for cavefish in trials with the plastic rod without vibration (Trial 2: cave mean = 5, surface mean = 4.2; cave median = 5, surface median = 3, Fig. 4A) and in trials with the rod vibrating at 35 Hz (Trial 3: cave mean = 4.7, surface mean = 4; cave median = 4.5, surface median = 4, Fig. 4B). However, the larger number of approaches for cavefish was not statistically significant (Trial 2: Wilcoxon $W = 391.5$, $P = 0.489$; Trial 3: Wilcoxon $W = 373.5$, $P = 0.704$). We also observed no difference between the number of approaches between Trial 2 (rod only) and Trial 3 (rod + vibration) for either ecotype (paired one-tailed Wilcox tests, $P > 0.57$, both cases, Fig. 4). Thus, fish did not increase the number of approaches once vibration was added, regardless of ecotype (Supporting Information, Table S3).

For a second measure of VAB, we recorded the proportion of the trial the fish was in the inner circle for 14 cavefish and 34 surface fish. Cavefish spent considerably less of their time in the inner circle compared to surface fish for all three trials. This difference was statistically significant for Trial 1 (no rod: Wilcoxon $W = 83.5$, $P < 0.001$) and Trial 3 (rod with vibration: Wilcoxon $W = 143.5$, $P = 0.033$). The lack of statistical significance in Trial 2 was probably due to a lack of statistical power, as cavefish were located in the centre of the arena only about 60% as often as surface fish. We interpret these results as stronger wall-following behaviour in cavefish relative to surface fish, which has been documented previously (Sharma *et al.*, 2009). This is further supported by our observation that cavefish exhibit significantly fewer transitions in and out of the centre circle than surface fish for Trial 1 (no rod: Wilcoxon $W = 138.5$, $P = 0.024$; cave median = 6.5, surface median = 11; Fig. 4C) and qualitatively fewer transitions for Trial 3 (rod with vibration: Wilcoxon $W = 181.5$, $P = 0.203$; cave median = 4.5, surface median = 7.5).

This difference between cave and surface fish behaviour appears to be due mainly to a decrease in

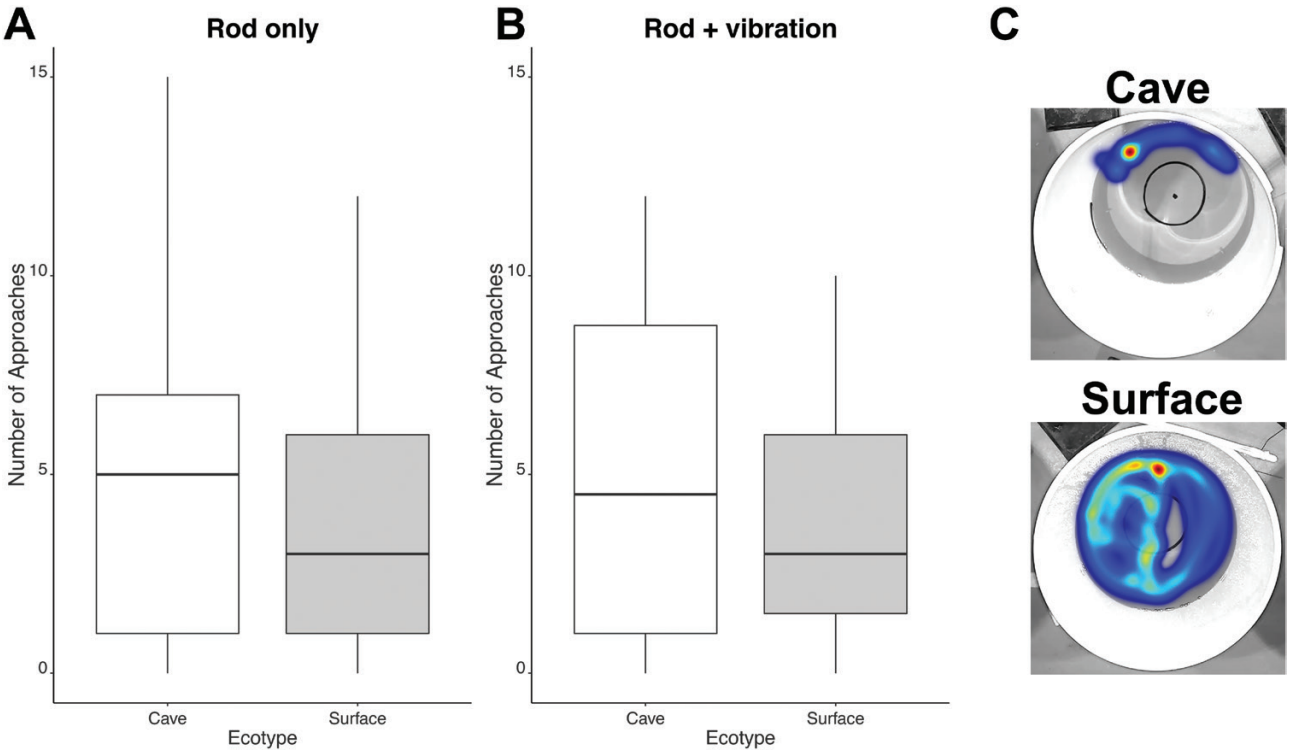

Figure 4. A, number of approaches to a non-vibrating rod across a 3-min trial period. B, number of approaches to a rod vibrating at 35 Hz across a 3-min trial period. Both trials were conducted in the dark with the vibrating rod trial occurring immediately after the non-vibrating rod trial ($N = 14$ cave, 34 surface scored for manual approaches). C, heatmaps from a trial with no rod (Trial 1) from cave and surface fish. Images were picked from the opposite ends of the distribution for number of crossings of the inner circle ($N = 18$ cave, 39 surface scored for spatial tank usage).

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surface fish transitions and a decrease in the amount of time surface fish spent in the centre of the arena once the rod was added, rather than a change in cavefish behaviour (paired one-tailed Wilcoxon tests, Supporting Information, Table S3). Together, these results suggest that cavefish prefer to be located near the margins of the arena [similar to Sharma *et al.* (2009) and Patton *et al.* (2010)], but are qualitatively less likely than surface fish to avoid novel objects [similar to Yoshizawa *et al.* (2010), who observed a shorter latency to approach the non-vibrating rod].

Cavefish eat less than surface fish

Populations of Mexican cavefish vary in their feeding behaviour, as they have been documented to eat significantly more (e.g. Tinaja population) and significantly less (e.g. Pachón population) than surface fish (Aspiras *et al.*, 2015). We conducted two rounds of feeding trials with Honey Creek cave and surface fish. First, after fasting the fish for 48 h, we placed a bloodworm with individually housed fish (19 cavefish, 37 surface fish) and waited for the fish to consume the worm before adding another. This was performed in a lighted room. We found cavefish ate significantly fewer worms than surface fish over a 10-min period after dividing the number of worms eaten by mass of the specific fish [mean cavefish = 4.63 total worms (1.21 worms/g of fish), surface fish = 26.81 total worms (4.70 worms/g of fish); Wilcoxon $W = 122$, $P < 0.0001$, Fig. 5A].

Second, we repeated feeding trials with a longer fasting time before the trial (120 h) and conducted the trials in the dark without a researcher present using 17 cavefish and 17 surface fish. As cavefish may be less likely than surface fish to eat when researchers were present and could be less affected by fasting conditions than surface fish (Aspiras *et al.*, 2015), our goal with the second round of feeding trials was to reduce the impact of these variables. Cavefish ate significantly fewer worms than surface fish over a 10-min period after dividing the number of worms eaten by the mass of the specific fish (mean cavefish = 14.94 worms, surface fish = 31.12 worms; Wilcoxon $W = 72$, $P < 0.012$, Fig. 5B). Thus, it appears lower feeding rates are a consistent trait of Honey Creek Cave fish relative to surface fish.

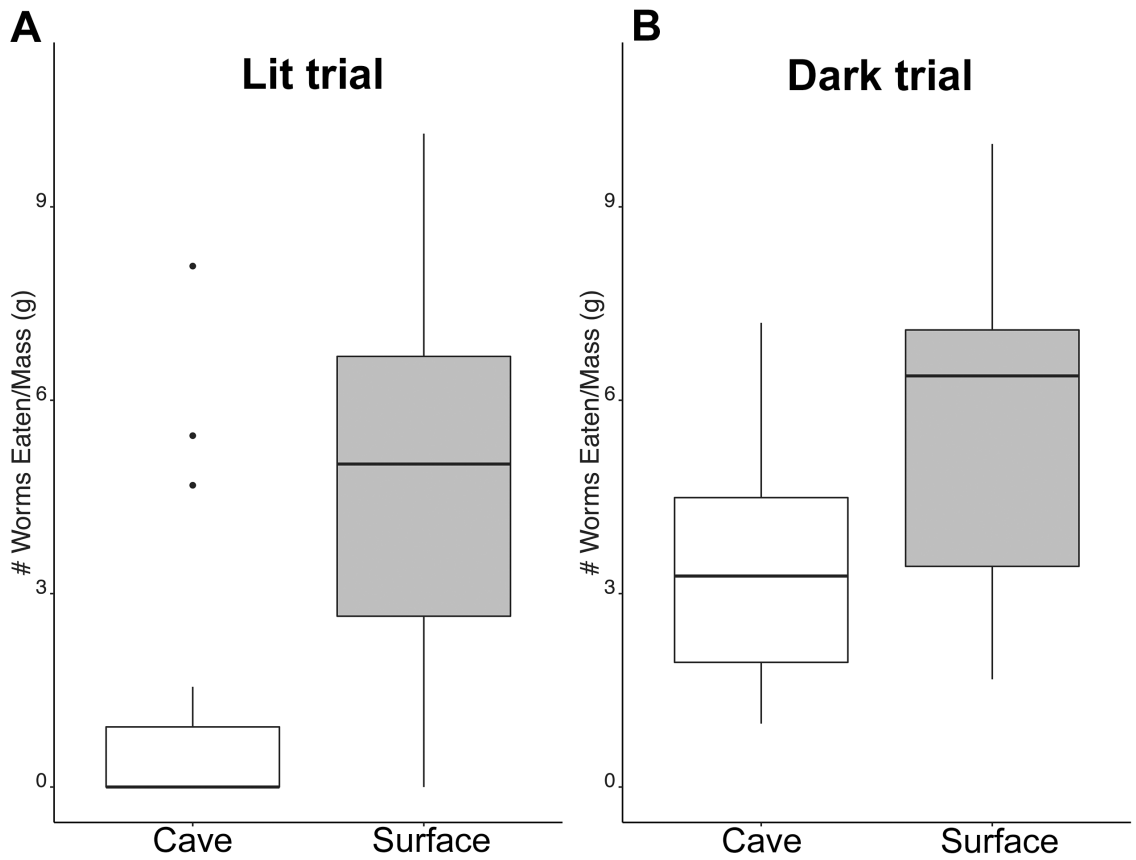

Figure 5. A, number of worms consumed over a 10-min period after a 48-h fasting period conducted in a lighted room with researcher present ($N = 19$ cave, 37 surface). B, number of worms consumed over a 10-min period after a 120-h fasting period conducted in the dark with no researcher present ($N = 17$ cave, $N = 17$ surface).

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Surface fish exhibit more stress than cavefish

We examined the average distance, average velocity, proportion of time in the lower half of the tank and proportion of time spent immobile for 15 cavefish and 37 surface fish for 5 min under dark conditions. We switched the lights on and recorded for another 5 min under lighted conditions. We found no difference in any of these metrics under dark conditions (Wilcoxon rank sum tests, distance: $W = 250$, $P = 0.589$; velocity: $W = 253$, $P = 0.631$; lower half of tank: $W = 213$, $P = 0.198$; immobile: $W = 274$, $P = 0.952$; results consistent if tested with a parametric *t*-test) or under lighted conditions (Wilcoxon rank sum tests, distance: $W = 317$, $P = 0.435$; velocity: $W = 340$, $P = 0.213$; lower half of tank: $W = 299$, $P = 0.675$; immobile: $W = 225.5$, $P = 0.298$; results consistent if tested with a parametric *t*-test).

However, when the dark and light trials were analysed by a paired Wilcoxon test, some interesting differences emerged. Notably, when lights were turned on, surface fish significantly slowed their velocity ($V = 501$, $P = 0.023$) and reduced their distance travelled ($V = 509$, $P = 0.017$) by about 85%. In contrast, cavefish did not change their velocity or distance travelled once the lights were turned on ($V = 56$, $P = 0.847$; $V = 66$, $P = 0.762$, respectively). Both cave and surface fish spent significantly more time at the bottom of the tank once lights were turned on (Supporting Information, Table S4; $P < 0.0001$ in both cases). These results suggest that cavefish exhibit fewer stress behaviours than surface fish.

Cavefish are more aggressive than surface fish

In the long-established Mexican populations, surface fish are more aggressive than cavefish (Elipot *et al.*, 2013; Rétaux & Elipot, 2013). In contrast, Honey Creek Cave fish spent 85.1% of their time in the 15-cm zone closest to the mirror, whereas Honey Creek surface fish spent 64.8% of their time in the same zone ($W = 349$, $P = 0.021$; 14 cavefish and 35 surface fish). Fish generally appeared to pace vertically on the side of the tank with the mirror. We observed very few ramming motions that would be typical of two fish interacting in a tank.

DISCUSSION

In this study, we found evidence for shifts in morphological and behavioural traits in a recently established cave population of *Astyanax mexicanus* relative to a geographically proximate, surface population. Our study is the first to examine phenotypes of a recently established *A. mexicanus* cave population. Repeated evolution can tell us much about the evolutionary process, but complicating factors such as gene flow between populations can limit the strength of inference about the deterministic and predictable nature of evolution and natural selection (Stern, 2013; Rosenblum *et al.*, 2014). One advantage of studying the Honey Creek Cave population is that it is unlikely that gene flow is transporting cave-adapted alleles from Mexican caves to Texas caves. Thus, if the morphological and behavioural differences described in this work are maintained after breeding in the laboratory in a common garden experiment, this population may provide a potentially novel origin of troglomorphy and an opportunity to explore selection and plasticity in the early stages of cave colonization.

The earliest records for *A. mexicanus* in Central Texas come from fish hatcheries in Kerrville and San Marcos, and *A. mexicanus* may have been inadvertently collected along with gamefish stock sourced from within the native, South Texas range of *A. mexicanus*. Brown (1953) reported deliberate introductions in San Pedro Springs (San Antonio River system) and the San Marcos River in 1908 and 1928–1930, respectively. Later, range expansion of this species into other local rivers was facilitated by people who collected *A. mexicanus* from the Rio Grande for sale as bait, beginning in the 1950s (C. Critchfield, pers. comm.). Routine surveys reported no *Astyanax* present in Comal Springs and the Comal River, a tributary of the Guadalupe River in the early 1950s (Ball *et al.*, 1952), and 1953 was the earliest museum record of *A. mexicanus* in the Guadalupe River (VertNet, Constable *et al.*, 2010). *Astyanax mexicanus* is currently abundant at those sites, suggesting it is unlikely that *A. mexicanus* was present in the Guadalupe River Basin before the 1950s.

While *A. mexicanus* may have invaded Honey Creek Cave soon after colonizing the Guadalupe River Basin, the earliest observations of *A. mexicanus* in Honey Creek Cave were in the 1980s (A. Cobb and L. Palit, pers. comm.). James Reddell, who collected and described the Comal blind salamander in the 1960s, did not recall observing *A. mexicanus* in the cave during that time (J. Reddell, pers. comm.). Although speculative, it is possible that extreme flooding of the Guadalupe River Basin from tropical storm Amelia (1978) catalysed *A. mexicanus* colonization of Honey Creek Cave. Since the 1980s, casual observations have documented the persistence of *A. mexicanus* in Honey Creek Cave. While historical records suggest a compelling case for a recent invasion, without genetic data we cannot fully rule out a Pleistocene refugial origin for this population. Characiforms ranged into Canada and Utah during substantially warmer periods in the late Cretaceous, although no additional fossil data for tetras exist in North America (Newbrey

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et al., 2009; Brinkman *et al.*, 2013), which could shed light on this hypothesis.

Some of the observed shifts in Honey Creek Cave fish parallel those found in long-established Mexican cave populations (Table 1). First, Honey Creek Cave individuals have a qualitatively more posterior dorsal fin location than their surface-dwelling counterparts. The dorsal fin serves as an important stabilizer and force generator for fish locomotion (Drucker & Lauder, 2001, 2005; Standen & Lauder, 2005, 2007; Liao, 2007); thus, the shift towards a more posterior dorsal fin is consistent with long-established Mexican cave populations (Protas *et al.*, 2008) and may be in response to factors in the cave environment (e.g. reduced current and predation pressure).

Second, individuals from Honey Creek Cave exhibit lighter coloration than surface fish when both alive and dead, suggesting that this observation is not driven simply by physiological colour responses. Reduced pigmentation is one of the most commonly observed troglomorphic traits across cave-dwelling taxa (Protas *et al.*, 2006, 2007; Gross *et al.*, 2009; Kronforst *et al.*, 2012; Pipan & Culver, 2012; Culver & Pipan, 2016; Howarth & Moldovan, 2018). Lighter coloration in the cave environment may be shaped predominantly by drift (Borowsky, 2015). Yet, other work suggests that coloration can pleiotropically impact basic physiological processes (Ducrest *et al.*, 2008; Roulin & Ducrest, 2011) and may be advantageous in the cave environment (Bilandžija *et al.*, 2018, 2013). Future work will assess the proximate mechanisms (e.g. number of melanophores) that may be responsible for the colour shift in Honey Creek Cave fish.

As in Mexican cavefish (Gertychowa, 1970; Sharma *et al.*, 2009; Patton *et al.*, 2010), Honey Creek Cave fish exhibit a stronger preference for the outer edge of the arena than surface fish, as shown by our VAB trials. Despite spending less time in the centre of the arena than surface fish, cavefish approached the rod at equal or higher rates, in part because surface fish reduced their time in the centre of the arena after addition of a novel object. These two observations suggest that Honey Creek Cave fish may exhibit more exploratory behaviour than surface fish (Sharma *et al.*, 2009), which is similar to observations in colonizers of other species (Candler & Bernal, 2014). Increased exploratory behaviour in Honey Creek Cave fish also seems to be supported by the tendency of cavefishes to adhere to and pace along the mirror during aggression trials. This increased exploratory behaviour may be influenced by reduced predation in the cave environment, and reduced stress-related behaviours in Honey Creek Cave fish during lighted trials support this notion (Chin *et al.*, 2018).

Honey Creek Cave fish eat significantly less than surface fish under multiple conditions, similar to comparisons of Pachón cavefish to Mexican surface fish (Aspiras *et al.*, 2015). Reduced food consumption and reduced body condition (mass per unit length), despite being on a common diet to their surface counterparts, suggest that metabolic shifts in Honey Creek Cave fish should be investigated in future work, as low and unpredictable food availability is a major selection pressure in many caves (Huppop, 2000; Niemiller & Soares, 2015).

Finally, neuromasts in Honey Creek Cave fish are more numerous than in surface fish, concordant with observations in Mexican cavefish where expansion of neuromasts provides critical spatial and environmental information in dark environments (Yoshizawa *et al.*, 2010). In sum, we observe many shifts in behaviour and several changes in morphology between surface fish and the recently established cavefish population that parallel those observed in the long-established Mexican tetras.

Table 1. Traits for *Astyanax mexicanus* inhabiting Sierra de El Abra and Guatemala regions of north-east Mexico and comparisons to Texas Honey Creek Cave and surface fish; traits in bold are concordant between the two comparisons

Trait	Mexican cavefish vs. surface	Texas cavefish vs. surface
Length	**Surface > Cave** (Protas *et al.*, 2008)	**Surface > Cave**
Coloration	**Surface > Cave** (Protas *et al.*, 2008)	**Surface > Cave**
Feeding	**Cave-specific** (Aspiras *et al.*, 2015)	**Surface > Cave**
Stress	**Surface > Cave** (Chin *et al.*, 2018)	**Surface > Cave**
Wall-following	**Surface < Cave** (Sharma *et al.*, 2009)	**Surface < Cave**
Neuromast number	**Surface < Cave** (Yoshizawa *et al.*, 2010)	**Surface < Cave**
Dorsal fin	**Surface < Cave** (Protas *et al.*, 2008)	**Surface < Cave**
Mass/Length	Surface < Cave (Protas *et al.*, 2008)	Surface > Cave
Eye diameter/Length	Surface > Cave (Protas *et al.*, 2008)	Surface < Cave
Aggression	Surface > Cave (Rétaux & Elipot, 2013)	Surface < Cave
Vibration attraction behaviour	Surface < Cave (Yoshizawa *et al.*, 2010)	Surface = Cave
Neuromast size	Surface < Cave (Yoshizawa *et al.*, 2010)	Surface = Cave

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In contrast, several traits in Honey Creek Cave fish were notably discordant with those observed in Mexican cavefish populations (Table 1). First, we found that eye diameter was larger in Honey Creek Cave individuals relative to surface fish when standardized by length of the fish, whereas in most Mexican *Astyanax* cavefish eye size is smaller or eyes are almost completely degenerated. Fish in our study were all collected within 100 m of the cave entrance, as fish are not abundant farther into the cave and primarily occur near the twilight zone of cave entrances. While our observation is contrary to most other studies in cavefish (Borowsky, 2015), it may reflect selection for improved vision in low light conditions, as documented in nocturnal fish (Schmitz & Wainwright, 2011) and organisms that live in the twilight zone (Camp & Jensen, 2007; but see Iglesias *et al.*, 2018). Additional investigations of eye size in fish inhabiting Mexican caves with a karst window, like Caballo Moro, would be valuable (Espinasa & Borowsky, 2000; Elliott, 2018). Importantly, our observations were sensitive to the method of size standardization (e.g. body length or head height).

Second, contrary to established Mexican cavefish populations that are less aggressive than surface fish (Burchards *et al.*, 1985; Langecker *et al.*, 1995; Espinasa *et al.*, 2005; Elipot et al., 2013, 2014; Rétaux & Elipot, 2013; Hinaux *et al.*, 2015), Honey Creek Cave fish adhered to the mirror (a proxy for aggression) for a higher proportion of time than surface fish. The mirror test is not possible with blind Mexican cavefish, because they cannot see the mirror, so aggression must be measured by intruder assays (Elipot *et al.*, 2013). We chose the mirror aggression assay rather than an intruder assay to avoid the need to match fish by size and sex. The use of different test types may have influenced the results (Oliveira & Canário, 2011), but a comparison of the mirror assay and intruder assay in zebrafish revealed high concordance (Way *et al.*, 2015), and increased aggression is seen in low-resource caves in other species (Melotto *et al.*, 2019). Part of the increased aggression we observed may simply be a response to a novel object, as cavefish demonstrated qualitatively less avoidance of novel objects in VAB trials. Future investigations will work to tease apart these two explanations.

Long-established populations of Mexican cavefish possess neuroanatomical and neurochemical differences suggested to shift cavefish behaviour from fighting to feeding, including: larger anterior paraventricular hypothalamic nucleus that contains more neurons, higher serotonin (5-HT), dopamine and noradrenaline in the forebrain, and about half of the monoamine oxidase (MAO) activity seen in surface fish (Elipot *et al.*, 2013, 2014). Intriguingly, we see shifts toward aggression and away from feeding in Honey Creek Cave fish, which is opposite to the pattern observed in Mexican cavefish.

Notably, our work suggests additional phenotypes to examine in future studies. While anecdotal, in the process of anaesthetizing fish for neuromast staining, we observed that ice-bath chilled water was required to immobilize surface fish, whereas cavefish did not survive in water temperature < 7 °C. While this is a qualitative observation, surface fish appear to withstand a cold shock substantially better than cavefish. Stenothermy is common among troglobitic organisms, and reduced cold tolerance in Honey Creek Cave fish may represent an additional troglomorphic trait (Barr, 1967; Mermillod-Blondin *et al.*, 2013). Future work will include assaying additional traits that are increased or enhanced in Mexican cavefish, such as the number of taste buds (Varatharasan *et al.*, 2009; Yamamoto *et al.*, 2009), number of teeth (Yamamoto *et al.*, 2003; Atukorala *et al.*, 2013), odorant detection ability (Protas *et al.*, 2008; Bibliowicz *et al.*, 2013) and prey capture skills (Espinasa *et al.*, 2014).

In addition to increasing the number of traits characterized, there is ample opportunity to expand this work to other populations. We have observed *A. mexicanus* in other caves and wells in Central Texas that lack direct connection to surface populations or are only ephemerally connected. A better understanding of the distribution, life history and ecology of subterranean *A. mexicanus* populations of recent origin is important from a conservation context. Many stygobitic organisms in the Edwards Aquifer (including federally listed invertebrates, fish and salamanders) are threatened by anthropogenic factors, primarily alterations to the quality and quantity of groundwater in the Edwards Aquifer (Bendik *et al.*, 2014; Devitt *et al.*, 2019). *Astyanax mexicanus* has been documented at several sites where federally listed species occur (Gluesenkamp *et al.*, 2018) and has been observed consuming state-listed groundwater species (A.G.G., pers. obs.). The invasion of a new potential predator in *A. mexicanus* could have a pronounced, negative effect on sensitive cave and aquifer ecosystems.

In conclusion, we have documented morphological and behavioural trait differences between Honey Creek surface and cave populations of *A. mexicanus* in Central Texas, despite the potential recent origin of the cave population. Shifts in several of these traits (e.g. coloration, dorsal fin placement, feeding, wall-following and neuromast number) are concordant with changes in traits observed in the long-established cave populations in the Sierra de El Abra and Sierra de Guatemala regions in Mexico. Interestingly, we found that some trait shifts are in the opposite direction to those observed in Mexican cavefish populations (e.g. neuromast size, aggression and body condition). While additional studies of the underlying processes

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shaping these phenotypes are needed, this population offers a promising and unique opportunity to study the first stages in the colonization of a subterranean environment by a surface organism.

Data Accessibility

All raw data are available as supplementary files associated with this manuscript.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's website.

Table S1. Wilcoxon test *W* and *P*-values for all tests for live fish to determine which colour components are different between cave and surface fish. Significant values are in bold.
Table S2. Wilcoxon test *W* and *P*-values for all tests for dead fish to determine which colour components are different between cave and surface fish. Significant values are in bold.
Table S3. Paired one-tailed Wilcoxon test results for VAB trials. Numbers are mean with median in parentheses.
Table S4. Mean and medians (in parentheses) for values tracked by Ethovision. For dark trials and light trials, no variables exhibited a significant difference between cave and surface fish. However, in a paired analysis both cave and surface fish spent more time at the bottom of the tank in the light trials. Surface fish significantly slowed their velocity and reduced their distance travelled. We analysed 15 cavefish and 37 surface fish. Asterisks represent values that were different bewteen light and dark trials.
Figure S1. A, circuit diagram of the excitation mechanism connected to the plastic rods. B, set-up for the VAB trials.

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