Title

Scale cortisol levels vary with habitat type and population in the Mexican tetra *Astyanax mexicanus*

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Abstract

Cortisol is a key hormone involved in stress responses, and its regulation reflects how organisms cope with environmental stressors. While acute stress responses are well studied, much less is known about how cumulative, long-term stress shapes the responses of populations adapted to different habitats. Here, we investigated scale cortisol – a biomarker of long-term stress exposure in fish – in wild and laboratory reared populations of *Astyanax mexicanus*, a species with surface and cave-dwelling ecotypes adapted to contrasting environments. We measured scale cortisol levels in six populations (four cave and two surface) over two consecutive years, alongside abiotic parameters in the respective habitats. Some cave populations exhibited lower scale cortisol levels than surface fish under both wild and laboratory conditions, suggesting a genetically based enhancement of stress-coping ability or altered cortisol-mediated metabolic and osmoregulatory processes. Moreover, populations experiencing more environmental variability – such as the lake surface habitat and hydrologically dynamic cave systems – showed higher scale cortisol levels and increased inter-individual variation. These results suggest that evolutionary adaptations to different habitats shape long-term stress physiology in *A. mexicanus*, and they highlight scale cortisol as a valuable tool for assessing how organisms physiologically cope with diverse and extreme environments.

Keywords: *Astyanax mexicanus*, environmental variability, scale cortisol, stress

Introduction

Stress is a response to environmental challenges or threats (stressors), triggering a complex physiological and behavioral reaction that enables individuals to cope with environmental changes (Schulte et al., 2014). Stress can be induced by changes in abiotic conditions such as temperature (Gates et al., 1992), oxygen levels (Van Raaij et al., 1996) and pH (Wang et al., 2009) or by biotic factors such as predators (Van Dievel et al., 2016), parasites (Huffman et al., 2004), aggressive interactions among conspecifics (Sachser and Lick, 1991), and food availability (Pravosudov et al., 2001). During the vertebrate stress response, the adrenal glands (or interrenal tissue in fish) release glucocorticoids, for example cortisol or corticosterone, which maintain homeostasis by regulating, among other things, metabolism (Brillon et al., 1995; Christiansen et al., 2007; Vijayan et al., 1997) and immunity (Webster Marketon and Glaser, 2008).

Since habitats differ widely in abiotic and biotic characteristics, glucocorticoid regulation can vary across individuals and populations, reflecting both genetic adaptations and plastic responses to environmental pressures (DeKoning et al., 2004; Schulte et al., 2014). However, whether contrasting habitat types – such as different surface *vs.* cave water bodies – exert long-term and cumulative effects on an organism's stress physiology remains unclear. To investigate this, we use the Mexican Tetra *Astyanax mexicanus* which depicts an extreme example of local adaptation to different habitats. *A. mexicanus* consists of two ecotypes: surface-dwelling

(surface fish) and cave-dwelling (cavefish), both distributed in various caves and surface water bodies across Northeastern Mexico (Mitchell et al., 1977) (Fig. 1A). Cave aquatic environments present stressors such as reduced and infrequent availability of nutritional resources due to the absence of primary energy production in total darkness (Poulson, 2001; Venarsky et al., 2014). However, they also provide more stable abiotic conditions (Krishnan et al., 2020; Poulson and White, 1969; Tabin et al., 2018) but with differences between caves and pools within caves depending on the hydrological regime (Legendre et al., 2026). Consequently, cavefish have evolved several adaptations, some of which are population dependent, that allow them to thrive in resource-limited conditions. These include metabolic adaptations such as elevated blood sugar levels and insulin resistance (Riddle et al., 2018; Shimobayashi et al., 2023), enhanced lipogenesis (Xiong et al., 2022) and increased fat accumulation (Xiong et al., 2018). Behavioral adaptations include binge eating (Aspiras et al., 2015), reduced sleep (Yoshizawa et al., 2015) and decreased aggression (Elipot et al., 2013; Rodriguez-Morales et al., 2022). Additionally, cavefish must cope with hypoxia (Krishnan et al., 2020; Ornelas-García et al., 2018; Rohner et al., 2013) which has led to an increase in embryonic erythrocyte production (van der Weele and Jeffery, 2022) as well as larger erythrocytes with higher hemoglobin content per cell in adult fish (Boggs et al., 2022).

Unlike cavefish, surface-dwelling *A. mexicanus* experience greater fluctuations in abiotic conditions, such as temperature variation (Tabin et al., 2018), and are exposed to more biotic stressors, including parasites (Peuß et al., 2020; Santacruz et al., 2023, 2020), predators (Chin et al., 2018; Mitchell et al., 1977; Yoshizawa and Library, 2015) and aggressive interactions among conspecifics (Elipot et al., 2013; Rodriguez-Morales et al., 2022).

Strong ecological differences between cave and surface habitats resulted in specific stress response adaptations of cave- and surface fish. For example, cavefish show lower plasma cortisol levels upon exposure to confined space (Gallo and Jeffery, 2012), lower whole-body cortisol in total darkness (Bilandžija et al., 2020), and reduced stress behaviors such as freezing, and prolonged bottom dwelling (Chin et al., 2018; Padmanaban et al., 2025) compared to surface fish.

In addition to their ecological divergence, cavefish differ from surface fish in key life-history traits–including slower growth rates (Simon et al., 2017), increased offspring investment under nutrient limitation (Xia et al., 2025), and year-round reproduction (Espinasa et al., 2023)–, traits known to interact with glucocorticoid release (Crespi et al., 2013).

While short-term elevations in blood-plasma cortisol occur in response to acute stress, scale cortisol levels provide a measure of long-term experienced stress, as cortisol accumulates in scales over time (Aerts et al., 2015). Consequently, scale cortisol levels have been widely used to assess long-term experienced stress across various fish species (Britton et al., 2023; Lebigre et al., 2022; Roque d'orbcastel et al., 2021; Vercauteren et al., 2022).

To examine differences in long-term experienced stress between surface and cave-adapted *A. mexicanus*, we measured cortisol levels in scales of wild surface and cave individuals from different populations across two consecutive years. We sampled two distinct surface populations: Rio Choy (a river population; Fig. 1B, G) and Presa El Oyul (a lake population; Fig. 1C, H) – as well as four cave populations: Pachón (Fig. 1D, I), Los Sabinos (Fig. 1J), Tinaja (Fig. 1K) and Rio Subterráneo (Fig. 1L) – which represent an isolation gradient from surface habitats (Mitchell et al., 1977; Moran et al., 2023; Simon et al., 2017). Additionally, we

measured several abiotic parameters in both years to characterize the abiotic environment of each habitat type. To disentangle environmental and genetic effects on cortisol levels, we compared scale cortisol levels from wild individuals of Rio Choy surface and Pachón cavefish to those of laboratory-reared individuals from the same populations. We hypothesized that scale cortisol levels–reflecting long-term experienced stress–and individual variation therein would be lower in cavefish compared to surface fish, due to reduced stressors or stress responsiveness in cave-adapted populations. Furthermore, we expected this difference to persist under laboratory conditions, suggesting a genetic basis for stress-related adaptations to the cave environment. Our alternative prediction was that there are no differences in scale cortisol levels between laboratory-bred cave- and surface fish due to a plastic stress regulation.

Material & methods

Field sample collection

We collected wild individuals (males and females) of *A. mexicanus* in 2023 and 2024 under permit no. SPARN/DGVS/03371/23 granted by the Secretaría de Medio Ambiente y Recursos Naturales (SEMARNAT) to Ernesto Maldonado. In 2023, we sampled fish from Rio Subterráneo (Micos), Pachón and Los Sabinos caves as well as from Nacimiento Rio Choy (stream) and Presa El Oyul (lake) between April 5th and April 15th. Additionally, we collected fish from Tinaja cave between December 2nd and 3rd. In 2024, we resampled Rio Subterráneo, Tinaja, Rio Choy and Presa El Oyul (only females were found) between March 9th and March 19th.

The Pachón cave is the most isolated cave among the sampled caves due to its elevated cave entrance (210 m above sea level) (Mitchell et al., 1977) and hence has the most stable abiotic conditions, whereas the other caves are still regularly flooded during the rainy season (Legendre et al., 2026). In Rio Subterráneo, there is a regular introduction of surface fish due to a karst stream flooding into the cave during the rainy season which is thought to result in hybridization with the resident cavefish (Garduño-Sánchez et al., 2023; Moran et al., 2023). Consequently, Rio Subterráneo cavefish show variable and intermediate phenotypes such as reduced but not completely lost eyes and weakened pigmentation (see Fig. 1L).

For practical reasons, and to minimize impact on cave populations, capture techniques had to be adjusted to the local conditions. In cave environments, we used dip nets to capture fish, while in stream and lake habitats, we employed improvised fishing poles equipped with size 16 hooks (Gamakatsu, Japan) baited with tortilla or dried mango to catch surface fish. After capture, fish were kept alive until dissection and transported in plastic bags with oxygen supply by air bubblers to an improvised field lab. Here, we euthanized all fish with an overdose of MS-222 (0.5 g/L) and dissected them as quickly as possible, no longer than 24h post-capture (Supplemental Tab. 1). We weighed all fish to the nearest mg and took standardized pictures to determine the standard length of each fish to the nearest mm with ImageJ. Using a metal spatula, we carefully collected nearly all scales per individual and transferred them to pre-weighed 2 mL Eppendorf-tubes, which we immediately flash froze in liquid nitrogen. We stored the samples at -20 °C until further processing. The sex of each fish was determined by opening the body cavity and screening the reproductive organs.

Furthermore, we measured temperature [C°], pH (in 2023), dissolved oxygen [mg/L], conductivity [µS/cm], phosphate high range [mg/L], hardness ($CaCO_3$) high range [mg/L], iron [mg/L], copper [mg/L], and nitrite [mg/L] with a Hach SL 1000 multiparametric device and the respective Chemkeys and probes. Measurements were taken once per habitat and year (except Tinaja in 2023, see exact dates in Tab. 1). For pH measurements in 2024, we used an Akozon Smart Sensor Pen Type PH Meter Portable Water Quality Tester Acidimeter PH818.

Laboratory fish husbandry and sample collection

We obtained parental Pachón cavefish from the laboratory of Sylvie Retaux (Paris-Saclay Institute of Neuroscience) and Rio Choy surface fish from the laboratory of Horst Wilkens (University of Hamburg). These lines have been kept and bred in the lab for at least two decades (Pachón) (Bilandžija et al., 2020) or longer (Rio Choy) (Wilkens, pers. comm.). We grouped at least four males and two females together for breeding, following the methods described by Baumann and Ingalls (2022). We maintained offspring from both populations at 22°C and fed them daily with freshly hatched *Artemia* larvae until they reached approximately 10 mm in size. Between 10 and 30 mm, we provided food five times per week using Gemma Micro (Skretting, Norway). As they grew between 30 and 45 mm, we adjusted the feeding schedule to four times per week with Gemma Silk (Skretting, Norway) and once per week with frozen bloodworms. After reaching 45 mm, we fed them twice per week with Gemma Silk and once per week with frozen bloodworms. We housed adult fish (> 45 mm) in groups of at least six individuals in 14 L aquaria with recirculating tap water, temperature control, and mechanical, UV, and biological filtration (Vewa Tech, Hamm, Germany). We kept the fish at a photoperiod of 14/10 h light/dark and used Osram Lumilux Cool Daylight HO 39W/865 T5 lamps for tank illumination. We regularly monitored the water quality in the fish tanks by measuring pH, conductivity, nitrite, ammonium and phosphate (See Supplemental Tab. 2). To minimize aggression in surface fish (Baumann and Ingalls, 2022), we enriched Rio Choy surface fish tanks with hexagonal plastic structures. Since effects of environmental enrichment on cortisol release are related to reduced aggression and anxiety (Näslund et al., 2013; Zhang et al., 2020), enrichment was not needed for cavefish which lost these behaviors (Chin et al., 2018; Elipot et al., 2013; Padmanaban et al., 2025; Rodriguez-Morales et al., 2022). Due to slower growth rates of cavefish (Simon et al., 2017) and in order to obtain enough scale material from fish of approximately the same size, we euthanized fish at 10 months (Rio Choy) and 14 months (Pachón) of age using an overdose of MS-222 (0.5 g/L) in accordance with the permit T24.016UMS granted by the University of Münster. Replicating the field pipeline, we collected nearly all scales per individual with a metal spatula and transferred them to pre-weighed 2 mL Eppendorf tubes, storing them at -20°C until analysis.

Extraction of cortisol from scales and determination of cortisol levels

Before processing, we thawed the scales and washed them three times by adding 1.2 mL of isopropanol to each tube, shaking them at 20 Hz for 3 minutes in a ball mill (MM 400, Retsch GmbH, Germany). We discarded the supernatant and allowed the scales to dry overnight in a fume cupboard before recording their dry weights. Next, we placed two 5 mm steel balls into each tube and pulverized the scales for 3 minutes at 30 Hz in the ball mill. We weighed up to 13 mg of powdered scales and added 1.3 mL of 100% methanol, maintaining a final

concentration of 10 mg powdered scales/mL methanol. We incubated the samples overnight in methanol on an overhead shaker. The next day, we centrifuged the samples at 1,000 × *g* for 1 minute, transferred the supernatant to a new tube, and centrifuged it again at 20,000 × *g* for 10 minutes. We transferred 1 mL of the supernatant to a new vial and evaporated it in a vacuum concentrator. We then resuspended the cortisol residue in 250 µL of ELISA buffer provided by the assay kit (Neogen, product no. #402710, USA). We selected this assay kit based on previous validations in other fish species (Britton et al., 2023; Carbajal et al., 2018; Ding et al., 2024) and because it showed the best performance during our own validation trials with an average recovery rate of approximately 90% (for details about the assay validation see Supplemental Methods). We stored the samples at −20°C until performing the ELISA. Before measurement, we thawed and vortexed the samples. We determined cortisol concentrations in duplicate following the manufacturer's instructions. To minimize potential batch effects, we randomly loaded each ELISA plate with samples from different populations.

Statistical analysis

All statistical analyses were conducted in R (version 4.3.2). First, we performed a principal component analysis (PCA) on abiotic parameters that were measured during the respective sampling day to characterize the abiotic environment of cave and surface habitats. Zero values were imputed to 1E-10.

For statistical analysis of scale cortisol levels, after excluding some individuals (Rio Choy 2023=4, Rio Choy 2024=2, Presa El Oyul 2023=2, Presa El Oyul 2024=1, Pachón 2023=13, Pachón lab=1, Los Sabinos 2023=2, Tinaja 2023=15, Tinaja 2024=1, Rio Subterráneo 2023=4, Rio Subterráneo 2024=4) due to non-sufficient scale material and a technical problem in the laboratory, we obtained the following sample sizes per population: Rio Choy 2023: N=21, Rio Choy 2024: N=23, Rio Choy lab: N=10, Presa El Oyul 2023: N=23, Presa El Oyul 2024: N=24, Pachón 2023: N=7, Pachon lab: N=9, Los Sabinos 2023: N=23, Tinaja 2023: N=8, Tinaja 2024: N=10, Rio Subterráneo 2023: N=21, Rio Subterráneo 2024: N=16. We first assessed normality using Shapiro-Wilk tests and visualized distributions with histograms and density plots. Since cortisol levels were not normally distributed but strictly positive, and right-skewed, we fitted generalized linear models (GLMs) with a Gamma distribution and log-link function. Model selection was based on Akaike Information Criterion (AIC) values, model diagnostics (residual plots from the car package) (see Supplemental Fig. 1-3), and the biological meaningfulness of fixed effects. We analyzed habitat and population effects in separate models because populations are fully nested within habitats, and not all populations were sampled in both years. Using different models allowed us to test for an overall habitat difference and assess within and among population × year variation. Model 1 tested for an overall habitat effect on cortisol levels including habitat (river, lake and cave), Fulton's *k*-index as a measure for body condition (Barton et al., 2002), which controls for the length of an individual, and sex as fixed effects. Model 2 tested for population differences in scale cortisol levels including population × year together with Fulton's *k*-index and sex as fixed effects. With Model 3, we tested for interaction effects of origin (wild *vs.* laboratory) × population for Rio Choy surface and Pachón cavefish, again including *k*-index and sex as fixed effects. Model residuals were checked for normality and homoscedasticity. We conducted pairwise comparisons of estimated marginal means

(EMMs) using the emmeans package and applied compact letter display (CLD) to summarize significant differences among habitats and populations. Additionally, we used bootstrap resampling (1,000 iterations) to compare individual cortisol variability across populations and years. All statistical tests were two-tailed, and significance was set at $p < 0.05$.

Results

Abiotic parameters

A principal component analysis (PCA) based on abiotic parameters (Tab. 1) revealed notable differences not only between cave and surface habitats but also between the river (Rio Choy) and lake (Presa El Oyul) surface habitats (Fig. 1E). Additionally, Presa El Oyul exhibited significant differences in some parameters between 2023 and 2024, primarily reflected in principal component (PC) 1 (year 2023: 2.257, year 2024: 5.416) (Fig. 1E). PCA loading factors indicated that the variation in PC 1 was primarily driven by temperature (0.455), pH (0.470), dissolved oxygen (0.396), and iron (0.498) while variation in PC2 was mostly determined by conductivity (-0.556) and hardness (-0.565) Fig. 1F).

Table 1. Abiotic water parameters in surface (Rio Choy, Presa El Oyul) and cave (Pachón, Los Sabinos, Rio Subterráneo, Tinaja) habitats measured in spring 2023 and 2024.

Site	Date	Temperature [C°]	pH	Dissolved Oxygen [mg/L]	Conductivity [µs/cm]	Phosphate [mg/L]	Hardness (CaCO3) [mg/L]	Iron [mg/L]	Copper [mg/L]	Nitrite [mg/L]
Rio Choy	09.04.2023	25.9	7.0	7.36	1164	0.75	710.5	0	0	0.004
Rio Choy	11.03.2024	25.8	7.2	6.80	1123	0.06	560	0	0.02	0.005
Presa El Oyul	14.04.2023	26.0	8.4	7.59	428	1.00	214	0.05	0.04	0.002
Presa El Oyul	17.03.2024	30.5	9.5	9.40	316	0.60	64	0.13	0	1E-10
Pachón	06.04.2023	24.3	7.3	5.34	568	0.45	307	0.005	0.005	0.013
Pachón	17.03.2024	25.7	7.5	5.80	490	0.60	208	0	0.01	0.027
Los Sabinos (pool 1)	12.04.2023	22.8	7.4	1.97	484	1.30	251	0	0	0.065
Los Sabinos (pool 1)	21.03.2024	23.1	7.8	1.73	534	0.60	239	0	0.03	0.177
Tinaja (pool 2)	19.03.2024	23.3	8.1	6.29	667	2.40	346	0	0	0.009
Rio Subterráneo (pool 1)	07.04.2023	23.7	7.2	1.76	470	1.30	232	0	0	0.229
Rio Subterráneo (pool 2)	07.04.2023	23.8	7.3	5.77	481	1.40	228	0	0	0.048
Rio Subterráneo (pool 1)	14.03.2024	25.0	7.5	3.37	524	1.90	234	0	0	0.007
Rio Subterráneo (pool 2)	14.03.2024	25.1	7.2	3.38	519	4.70	212	0	0	0.063

Habitat and population differences in scale cortisol levels

We fitted two generalized linear models (GLMs) with scale cortisol level as response, habitat (model 1) or population × year (model 2) as the predictor and body condition (Fulton's K-

index) and sex as covariates. We found significantly higher scale cortisol levels in lake surface fish compared to river surface fish and cavefish while there was no difference between river surface and cavefish (Fig. 2A, see Supplemental Tab. 3, 4 for EMMs and pairwise comparisons).

Comparing populations and years with model 2 revealed that Pachón cavefish in 2023 had the lowest scale cortisol levels (Fig. 2B, see Supplemental Tab. 5 for EMMs), followed by Los Sabinos cavefish. Tinaja cavefish exhibited intermediate cortisol levels in both 2023 and 2024 (Fig. 2B). Interestingly, Rio Choy surface fish showed similar cortisol levels in both wild and laboratory conditions (Fig. 2B). We observed the highest cortisol levels in Presa El Oyul surface fish (Fig. 2B). Rio Subterráneo cavefish exhibited intermediate cortisol levels in 2023, but levels increased in 2024 (Fig. 2B).

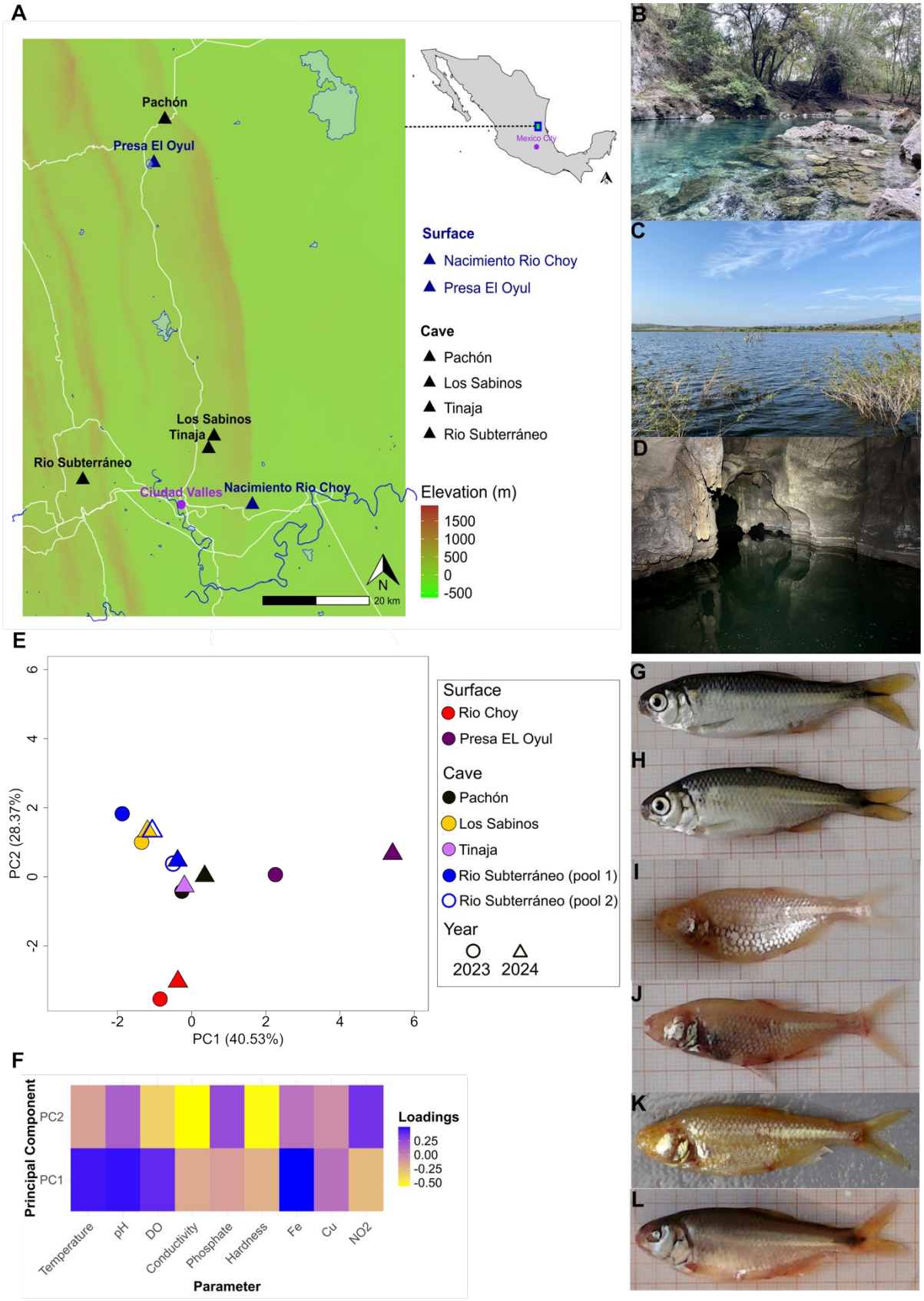

Figure 1. Sampling habitats. (A) Map of sampling area in the Sierra de El Abra Tanchipa and Sierra Micos region in North-eastern Mexico with sampling locations of respective populations. (B) Stream surface fish habitat (Nacimiento Rio Choy). (C) Lake surface fish habitat (Presa El Oyul). (D) Cavefish habitat (Pachón) cave. (E) Principal component analysis (PCA) plot based on abiotic parameters from Table 1 showing scores of principal components (PC) 1 (x-axis) and 2 (y-axis) per habitat and year. The percentage of explained variation per PC is

displayed in brackets. (F) Heatmap showing PC loading factors per measured parameter. Representative images of (G) Rio Choy and (H) Presa El Oyul surface- and (I) Pachón, (J) Los Sabinos, (K) Tinaja and (L) Rio Subterráneo cavefish.

Pairwise comparisons between populations

To assess statistical differences between populations and across years within populations, we conducted pairwise comparisons based on EMMs (see Supplemental Tab. 6 for pairwise comparisons). Pachón and Los Sabinos cavefish exhibited significantly lower scale cortisol levels compared to Rio Choy surface fish in 2024, whereas no significant difference was observed in 2023. However, Pachón cavefish still showed a trend toward lower cortisol levels (Fig. 2B). We observed the largest differences in cortisol levels between Pachón and Los Sabinos cavefish and Presa El Oyul surface fish in both years (Fig. 2B). Comparisons between Tinaja cavefish and both Rio Choy and Presa El Oyul surface fish did not yield any significant differences (Fig.2B). However, Tinaja and Rio Subterráneo cavefish in 2024 exhibited higher cortisol levels than Pachón cavefish (Fig. 2B). We found a notable within-population difference in Rio Subterráneo cavefish, where individuals caught in 2024 had significantly higher cortisol levels than those caught in 2023 (Fig. 2B). Finally, Presa El Oyul surface fish caught in 2023 had significantly higher cortisol levels than Rio Choy surface fish in both years whereas no such difference was observed for Presa El Oyul surface fish in 2024 (Fig. 2B).

Comparison between laboratory and wild surface and cavefish

In model 3, we tested for differences between wild and laboratory Rio Choy surface and Pachón cavefish with body condition (Fulton's K-index) and sex as fixed effects. Pairwise comparisons based on EMMs (see Supplemental Tab. 7, 8) revealed that there was neither a significant within ecotype difference in scale cortisol levels between wild and laboratory Rio choy surface fish, nor between wild and laboratory Pachón cavefish (Fig. 2C). However, among ecotypes, Rio Choy surface- and Pachón cavefish differed significantly both between wild and laboratory populations (Fig. 2C).

Covariate effects on scale cortisol levels

Overall, body condition (Fulton's k-index) was a significant predictor of cortisol levels in models 1 and 2 and still showed a positive trend in model 3 (Supplemental Tab. 9-11, Supplemental Fig. 4-6), indicating that individuals with better body condition exhibited higher cortisol levels. In contrast, sex did not significantly influence cortisol levels (Supplemental Tab. 9-11, Supplemental Fig. 7-9). In model 2, year had no overall influence on cortisol levels (Supplemental Tab. 10) as well as origin in model 3 (Supplemental Tab. 11).

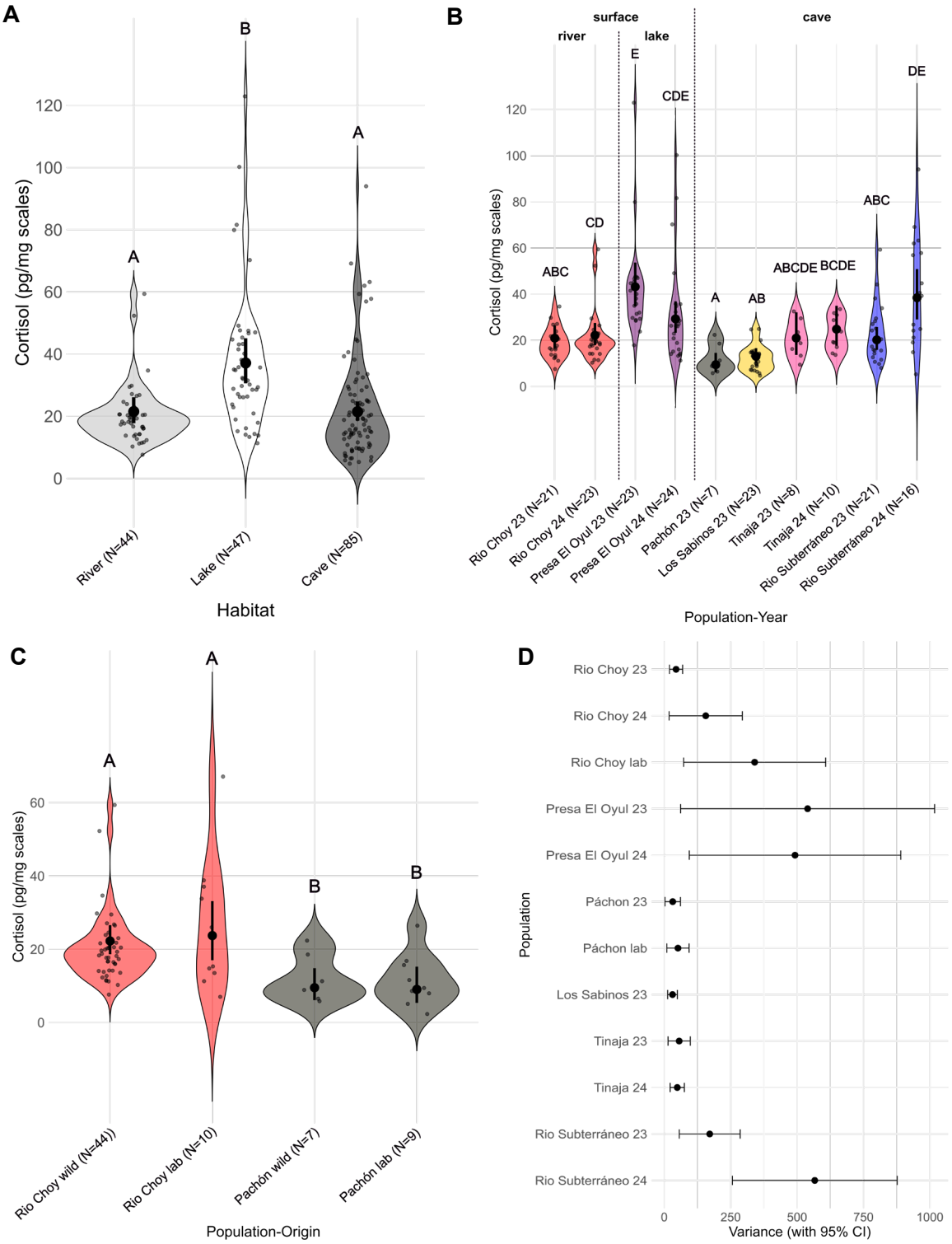

Figure 2. Scale cortisol levels across different habitats and wild and laboratory populations of *A. mexicanus*(A) Scale cortisol levels in pg/mg scales of wild river (Rio Choy) and lake (Presa El Oyul) surface fish *vs.* cavefish (Pachón, Los Sabinos, Tinaja, Rio Subterráneo) with respective sample size (N) in brackets. The small points and violins show the distribution of the raw data. Large points with error bars show the back-transformed (from log to normal) estimated marginal means (EMMs) with 95% confidence intervals (CIs) under model 1 with scale cortisol as response variable, habitat (lake, river and cave) as predictor, and body condition (K-index) and sex as fixed effects. Significant differences (adjusted p-value <0.05) are indicated by the compact letter design

with two habitats being significantly different when no letter overlaps. (B) Scale cortisol levels in pg/mg scales of wild surface and cave populations with respective sample size (N) in brackets. The small points and violins show the distribution of the raw data. Large points with error bars show the back-transformed EMMs with 95% CIs under model 2 with scale cortisol as response variable, population × year as predictor, and body condition (K-index) and sex as fixed effects. Significant differences (adjusted p-value <0.05) are indicated by the compact letter design with two populations × years being significantly different when no letter overlaps. (C) Scale cortisol levels in pg/mg scales of wild *vs.* laboratory Rio Choy surface and Pachón cavefish with respective sample size (N) in brackets. The small points and violins show the distribution of the raw data. Large points with error bars show the back-transformed EMMs with 95% CIs under model 3 with scale cortisol as response variable, population × origin (laboratory vs. wild) as predictor, and body condition (K-index) and sex as fixed effects. Significant differences (adjusted p-value <0.05) are indicated by the compact letter design with two populations × years being significantly different when no letter overlaps. (D) Individual variation in scale cortisol levels displayed by 95% confidence intervals (error bars) with means (points) based on bootstrap resampling (1000 iterations) per population and year/origin (laboratory *vs.* wild).

Individual variation in scale cortisol levels

To examine individual variation in scale cortisol levels across wild and laboratory populations, we performed bootstrap resampling with 1000 iterations and reported 95% confidence intervals (see Supplemental Tab. 12) for each population × year and population × origin pair. We found that Los Sabinos cavefish in 2023 exhibited the lowest degree of individual variation, followed by wild Rio Choy surface fish in 2023, Tinaja cavefish in 2024, wild Pachón cavefish in 2023, Tinaja cavefish in 2023, and laboratory Pachón cavefish (Fig. 2D). We observed more individual variation in Rio Subterráneo cavefish in 2023, wild Rio Choy surface fish in 2024, laboratory Rio Choy surface fish, and Presa El Oyul surface fish in 2024 (Fig. 2D). Presa El Oyul surface fish in 2023 and Rio Subterráneo cavefish in 2024 (Fig. 2D) exhibited the highest degree of individual variation in scale cortisol levels.

We observed significant differences in individual variation (non-overlap in CIs) between laboratory Rio Choy surface fish and wild Pachón, Los Sabinos, and Tinaja cavefish in 2024 (Fig. 2D). Additionally, Rio Subterráneo cavefish in 2023 and Presa El Oyul surface fish in 2023 showed significantly higher individual variation compared to Los Sabinos cavefish (Fig. 2D). In 2024, both Presa El Oyul surface fish and Rio Subterráneo cavefish exhibited consistently higher individual variation compared to all other cave populations and wild Rio Choy surface fish in 2023 (Fig. 2D).

Discussion

Lake habitat effects on scale cortisol levels

Our study provides evidence that long-term experienced stress in *A. mexicanus* varies with habitat type, but not generally between cave and surface ecotypes. Lower scale cortisol levels in cavefish and river surface fish compared to lake surface fish suggest that adaptations to specific habitat characteristics explain variation in scale cortisol levels. Our abiotic measurements indicate that the lake (Presa El Oyul) habitat differed from river surface (Rio Choy) and cave habitats, particularly in temperature, pH, conductivity and iron content. These parameters are known to influence cortisol levels and stress in fish (Choi and An, 2008; Cockrem et al., 2019; Ghazal et al., 2025; Hanke et al., 2019; Lappivaara et al., 1999;

Lappivaara and Marttinen, 2005; Mota et al., 2017; O'Toole et al., 2023; Rohner et al., 2013; Samaras et al., 2022; Wagner et al., 1997). However, although differences in these parameters between surface habitats were greater in 2024, we observed no significant difference in scale cortisol levels, suggesting that other environmental factors likely influenced the differences in scale cortisol levels in 2023. Since our abiotic measurements only comprised nine different parameters that were measured once per sampling (and year), they do not represent a dynamic but a snapshot of conditions on the respective day. Hence, more detailed habitat characterizations and long-term tracking of abiotic parameters are needed to better understand the effect of abiotic dynamics on scale cortisol levels in *A. mexicanus*.

Another important aspect to better understand habitat level differences in scale cortisol levels is the time populations could adapt to different habitat types. While river surface and cave populations naturally colonized and adapted to their respective habitat for at least 30,000 to 191,000 years (Fumey et al., 2018; Garduño-Sánchez et al., 2023; Herman et al., 2018; Moran et al., 2023), fish were introduced into the lake Presa El Oyul due to its artificial origin no longer than approximately 45 years ago ("Portal Gob MX,"n.d.). Stressors present in *A. mexicanus* such as predation risk (Chin et al., 2018; Mitchell et al., 1977; Yoshizawa and Library, 2015), aggression (Elipot et al., 2013; Rodriguez-Morales et al., 2022) and parasites (Peuß et al., 2020; Santacruz et al., 2023, 2020) might be different in the natural river and cave vs. artificial lake habitat, which could potentially explain the observed differences in scale cortisol levels between lake and river surface fish and cavefish (Allan et al., 2020; Chin et al., 2018; O'Dwyer et al., 2020; Padmanaban et al., 2025; De Marco et al., 2013; Winberg and Lepage, 1998; Ziv et al., 2012).

Population differences in scale cortisol levels

Even though we could not show an overall difference in scale cortisol levels between cave and surface fish, we found differences among and within specific cave and surface populations. In 2024, Rio Subterráneo cavefish showed higher scale cortisol levels than Pachón, Los Sabinos and their own population in 2023. Similarly, Tinaja cavefish in 2024 exhibited higher scale cortisol levels than Pachón cavefish. Although our single time-point abiotic measurements showed little variation among these habitats, previous studies have documented seasonal hydrological differences. For instance, unlike Los Sabinos, Tinaja, and Rio Subterráneo, the Pachón cave is not affected by seasonal epigean water inflow due to its elevated entrance (210 m above sea level) (Mitchell et al., 1977). Long-term monitoring confirms that Pachón cave exhibits more stable physico-chemical water parameters, whereas Los Sabinos and Rio Subterráneo experience seasonal fluctuations in temperature, pH, and conductivity (Legendre et al., 2026; Tabin et al., 2018). Thus, combining long-term abiotic data with subsequent scale cortisol sampling could reveal a link between environmental variability and long-term experienced stress.

Differences in the degree of isolation from surface water bodies may also explain within-ecotype and population variation, particularly in Rio Subterráneo. Periodic floodings in this cave increase the influence of seasonal variation, which was shown to impact scale cortisol levels in other fish (Lebigre et al., 2022). In addition, such floodings also introduce surface fish which hybridize with cavefish (Garduño-Sánchez et al., 2023; Moran et al., 2023). Therefore,

yearly differences in scale cortisol levels in this population could also result from different sub-samples drawn from a genetically heterogenous cave population (Bradic et al., 2012).

Genetic determination of scale cortisol levels

We could show that wild Rio Choy surface fish had higher scale cortisol levels than Pachón cavefish. There is direct evidence that Pachón cavefish exhibit altered cortisol regulation compared to Rio Choy surface fish: they show reduced plasma cortisol when exposed to confined space (Gallo and Jeffery, 2012) and lower whole-body cortisol under constant darkness (Bilandžija et al., 2020). Since we did not find significant differences in scale cortisol levels between wild and laboratory-reared Pachón cavefish and Rio Choy surface fish, this suggests that genetic adaptations, rather than plastic responses to short-term environmental conditions, primarily determine long-term stress levels. Furthermore, as the abiotic conditions were different between wild and laboratory populations, this indicates that other genetic adaptations than potential adaptations to the measured parameters might be at play. While cavefish have evolved adaptations that enable them to persist in resource-limited and hypoxic environments over extended periods (Aspiras et al., 2015; Boggs et al., 2022; Olsen et al., 2023; Riddle et al., 2018; Rohner et al., 2013; Shimobayashi et al., 2023; van der Weele and Jeffery, 2022; Xiong et al., 2022, 2018), cortisol responses to food restriction or hypoxia are inconsistent across fish species, being sometimes elevated, reduced or unaffected (Arslan et al., 2016; Caruso et al., 2010; Poursaeid and Falahatkar, 2022; Samaras et al. 2023; Sumpter et al., 1991; White and Fletcher, 1986; Léger et al., 2021; O'Connor et al., 2011). In case of hypoxia, we also observed similarly low scale cortisol levels in Pachón and Los Sabinos cavefish despite strikingly different dissolved oxygen concentrations. Together, this suggests that lower scale cortisol levels in some cave populations might rather be associated with basal metabolic and osmoregulatory processes (Laiz-Carrión et al., 2003; Lawrence et al., 2019) upon food restriction. Therefore, previously discussed stressors as well as basal cortisol regulation in cavefish could be systematically tested in laboratory settings to assess their causal roles in cortisol accumulation in scales. Additionally, genetic mapping (Jeffery, 2020) of scale cortisol levels, through crossbreeding and quantitative trait loci analysis, could help identify underlying genetic loci involved in cortisol regulation. Including more cave populations such as Tinaja and Rio Subterráneo could clarify whether cortisol regulation and potential stress adaptations are convergent or population specific. Furthermore, the functional consequences of reduced cortisol in Pachón cavefish remain complex. While lower cortisol may promote energy allocation to somatic growth (Mommsen et al., 1999), support year-round reproduction (Schreck, 2010; Espinasa et al., 2023; Xia et al., 2025), shift immune investment (Peuß et al., 2020) and contribute to longevity (Cobham et al., 2024; Medley et al., 2022) due to reduced immunosuppression and oxidative stress (Sapolsky et al., 2000), glucocorticoid interactions with life-history traits are multifaceted (Crespi et al., 2013). Sustained cortisol reduction may thus provide important advantages in some cave habitats, but further research is required to clarify these links.

Individual variation in scale cortisol levels

We observed population-level differences in individual variability of scale cortisol levels. Low individual variation in Los Sabinos, Pachón, and Tinaja cavefish suggests that these cave

habitats are characterised by low fluctuations in stressors, leading individuals to occupy similar niches within the given habitat. Adaptations to strong and consistent selective pressures (Poulson, 2001; Venarsky et al., 2014), such as limited food availability and stable, yet sometimes extreme abiotic conditions (Legendre et al., 2025; Poulson and White, 1969; Tabin et al., 2018), may contribute to reduced individual variation in stress responses and long-term stress levels. This reduced variability could, in turn, limit the ability to conform to niche changes through glucocorticoid mediated stress responses. Presa El Oyul Lake potentially exhibits greater environmental heterogeneity and dynamic stressors, including fluctuating abiotic parameters, and potentially, predation, and aggressive interactions among conspecifics, which may explain the higher individual variation in scale cortisol levels. In Rio Subterráneo, periodic flooding from nearby surface streams, along with the introduction and hybridization of surface individuals (Garduño-Sánchez et al., 2023; Moran et al., 2023) together with invasive parasites (Santacruz et al., 2023), could have contributed to greater individual variation in scale cortisol levels. This variation may stem from gradients of cave-adapted traits across the sampled individuals leading to differences in stress responses.

In general, there might also be differences in the inter-individual cortisol responsiveness among populations. A recent study found that individuals responding to stress with either high or low cortisol levels can also be identified based on their scale cortisol levels (Samaras et al., 2021) which could explain the observed differences in individual variation.

Scale cortisol as a measure for long-term stress

Surprisingly, we found a significant positive relationship between body condition (K-index) and scale cortisol levels, contrasting with previous studies in other fish species, where higher scale cortisol levels were either associated with reduced body condition (Brosset et al., 2023) or showed no correlation (Carbajal et al., 2019). One possible explanation is that cavefish exhibit increased fat accumulation (Xiong et al., 2022, 2018), which elevates Fulton's K-index through increased body mass. In humans, obesity and metabolic syndrome are linked to increased cortisol levels and hyperactivity of the hypothalamic-pituitary-adrenal axis (Anagnostis et al., 2009). A similar mechanism may influence cortisol regulation in *A. mexicanus*, warranting further research into interactions between cortisol, fat storage, and body condition in this species.

Even though scales have been shown to be capable of releasing cortisol in cell culture medium upon stimulation (Samaras and Pavlidis, 2022), it is unlikely that potential short-term released cortisol due to capture and handling of fish accumulated in scale tissue in such a short amount of time, as it usually takes longer than 72h after an acute stressor to elevate scale cortisol levels (Laberge et al., 2019). Therefore, our scale cortisol levels likely reflect long-term cortisol accumulation. Nonetheless, future studies should aim to integrate precise age estimates, such as counting annual growth increments on scales or otoliths (Simon et al., 2017), into scale cortisol analyses of wild populations and directly test whether cortisol accumulates in scales over time in *A. mexicanus* to support its suitability as a measure for long-term-experienced stress.

While we also cannot rule out a possible bias introduced by differential catching methods (dip-netting vs. angling), the use of both methods was necessary to ensure adequate sampling across

habitats. Both angling and dip-netting can produce non-random samples of fish populations such as selecting for bolder or larger/smaller individuals (Ghazal et al., 2025; Thorburn, 1992) with potential consequences for scale cortisol levels. Therefore, both methods should be further tested with respect to scale cortisol sampling in *A. mexicanus*.

Furthermore, the actual responsiveness to cortisol needs to be investigated in this system since there could be individual or population differences due to varying glucocorticoid receptor expression, as shown for example in arctic charr and trout (Johansen et al., 2011; Vindas et al., 2017), which can be organ and time-specific (Teles et al., 2013). Teleost fish also have different glucocorticoid receptor variants with different sensitivities and metabolic functions (Stolte et al., 2006). It is also important to consider that an individual's stress phenotype cannot be determined solely by cortisol levels, as both high and low levels may be either pathological or adaptive (Romero and Beattie, 2022). Therefore, it is necessary to include other measures of stress such as catecholamines (Reid et al., 1998) and stress-induced behaviours (Balasch and Tort, 2019) into an integrated view on the stress response in *A. mexicanus.*

Conclusion

Our findings suggest that long-term stress, as indicated by scale cortisol levels, is higher in lake surface fish compared to river surface and cavefish, potentially due to specific habitat characteristics and adaptations. Furthermore, we could show that there are differences in scale cortisol levels among cave populations and between river surface fish and the most isolated cave population. Genetic adaptations to habitat specific stressors appear to shape long-term stress responses across populations, emphasizing the role of stress physiology in colonizing diverse and extreme habitat types. Future research should experimentally test the effects of specific stressors, such as hypoxia, food restriction, abiotic parameters, predation, parasites and aggression on cortisol regulation and explore the genetic basis of stress-related differences between ecotypes. Our study highlights the importance of scale cortisol as a biomarker for long-term stress and provides insights into how habitat and population specific adaptations shape long-term stress.

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Competing interests

The authors declare no competing interests.

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Conflict of Interest

The authors have no conflicts of interest.

Author contributions

Marc Bauhus: investigation (lead); formal analysis (lead); project administration (lead); writing – original draft (lead); visualization (lead); conceptualization (support). **Luise Wander**: investigation (support); formal analysis (support); writing – review & editing (equal). **David Boy:** investigation (support); formal analysis (support); writing – review & editing (equal). **Ana Santacruz:** investigation (support); writing – review & editing (equal). **Ernesto Maldonado:** investigation (support); writing – review & editing (equal). **Joachim Kurtz:** resources (support); supervision (support); writing – original draft (support); writing – review & editing (equal). **Sylvia Kaiser:** methodology (lead); resources (support); funding acquisition (support); writing – review & editing (equal). **Robert Peuß:** conceptualization (lead); funding acquisition (lead); project administration (support); supervision (lead); resources (lead); visualization (support); writing – original draft (support); writing – review & editing (equal).

Statement on inclusion

Our study brings together authors from a number of different countries, including scientists based in the country where the study was carried out. All authors were engaged early on with the research and study design to ensure that the diverse sets of perspectives they represent was considered from the onset. Whenever possible, our research was discussed with the local community to inform on our science and the consequences that our field work potentially has on the local ecosystem.

Ethics statement

All wild animals were collected under permit no. SPARN/DGVS/03371/23 granted by the Secretaría de Medio Ambiente y Recursos Naturales (SEMARNAT) to Ernesto Maldonado. All animals were treated in accordance with local veterinary and animal welfare authorities and their respective guidelines. Scales from laboratory fish were used under the permit T24.016UMS granted by the University of Münster, Germany in accordance with local veterinary and animal welfare authorities.

Data availability statement

Data and code can be found under https://github.com/Marc9704/Amex_scale_cortisolG