Title: Environmental dynamics influence scale cortisol levels in Mexican cavefish

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Abstract:
Cortisol is a key hormone involved in stress responses, and its regulation can vary across populations experiencing different environmental pressures. While short term changes in certain environmental factors can result in immediate changes in cortisol levels, it remains unclear whether variable and fluctuating environments affect lifetime stress responses of individuals. In this study, we estimated lifetime-experienced stress in cave- and surface-dwelling populations of *Astyanax mexicanus*. The advantage of this model is that different populations are confronted with varied dynamics of environmental change, making it an ideal study system to investigate lifetime stress responses upon environmental fluctuations. To estimate lifetime-experienced stress, we measured cortisol levels in the scales of *A. mexicanus* for the first time using an ELISA assay. We studied six populations from six different habitats (four cave and two surface habitats) that vary in their dynamics of abiotic factors. We analysed scale cortisol levels across two years to examine temporal changes and individual variability within and between populations. Our results show that increased fluctuations in abiotic parameters are associated with an increased cortisol level in scales and, in addition, an increased inter-individual variability within a population irrespective of cave or surface habitat. These findings highlight the influence of varying ecological conditions on lifetime-experienced stress in *A. mexicanus*, providing insight into potential adaptive mechanisms underlying stress regulation in extreme environments.

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), pathogens (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 glands in fish) produce glucocorticoids, for example cortisol or corticosterone, which has downstream effects on metabolism (Brillon et al., 1995; Christiansen et al., 2007; Vijayan et al., 1997) and immunity (Webster Marketon and Glaser, 2008). Populations and individuals can vary in their sensitivity to stressors and their stress responses, thereby adapting to highly variable environments (DeKoning et al., 2004; Schulte et al., 2014). However, it remains unclear whether highly variable and fluctuating environments impose greater lifetime stress on individuals compared to more stable environments.

To investigate how fluctuations in abiotic parameters affect the cortisol levels, we use the Mexican Tetra *Astyanax mexicanus.* This model exhibits an extreme example of local adaptation to different habitats and environmental conditions. *A. mexicanus* consists of two ecotypes: surface-dwelling (surface fish) and cave-dwelling (cavefish), both distributed in north-eastern Mexico (Mitchell et al., 1977, see 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., 2025). Consequently, cavefish have evolved several adaptations 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). 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 erythrocyte production (van der Weele and Jeffery, 2022) as well as larger erythrocytes with higher hemoglobin content per cell (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 (Chin et al., 2018; Padmanaban et al., 2025) compared to surface fish.

While short-term elevations in blood-plasma cortisol occur in response to acute stress, scale cortisol levels provide a measure of chronic or lifetime-experienced stress, as cortisol accumulates in scales over time (Aerts et al., 2015). Consequently, scale cortisol levels have been widely used to assess lifetime stress across various fish species (Britton et al., 2023; Carbajal et al., 2019; Lebigre et al., 2022; Roque d'orbcastel et al., 2021; Vercauteren et al., 2022)

To examine differences in lifetime-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 stream population) and Presa El Oyul (a lake population) – as well as four cave populations: Pachón, Los Sabinos, Tinaja and Rio Subterráneo - which represent an isolation gradient from surface habitats (Mitchell et al., 1977; Moran et al., 2023; Simon et al., 2017; see Fig. 1A-D). Additionally, we measured several abiotic parameters in both years to assess fluctuation in abiotic parameters across habitats. To disentangle environmental and genetic effects on cortisol levels (Krishnan et al., 2020), we compared scale cortisol levels from wild individuals to those of laboratory-reared individuals from the same population. We hypothesized that scale cortisol levels, i.e. life-time experienced stress increases with a more dynamic and fluctuating environment. Therefore, we predicted lower scale cortisol levels and individual variation in cavefish compared to surface fish due to the less dynamic and fluctuating cave environment.

Results

Scale cortisol levels

To compare lifetime-experienced stress across wild and laboratory populations of cave and surface fish, we measured scale cortisol levels using an ELISA assay. We fitted a generalized linear model (GLM) with scale cortisol level as response, population as the predictor and body condition (Fulton's K-index), standard length (as a proxy for age), and sex as covariates. Our results indicate that wild Pachón cavefish (Fig. 1H) in 2023 had the lowest scale cortisol levels (EMM: 8.74 pg/mg, 95% CI: 5.65–13.5; Fig. 1E, see Supplemental Table 1 for standard errors), followed by laboratory-raised Pachón cavefish (EMM: 9.24, 95% CI: 6.09–14.01; Fig. 1E) and wild Los Sabinos cavefish (Fig. 1I) (EMM: 12.9, 95% CI: 10.11–16.46; Fig. 1E). Wild Tinaja cavefish (Fig. 1J) exhibited intermediate cortisol levels in both 2023 (EMM: 22.51, 95% CI: 14.71–34.44; Fig. 1E) and 2024 (EMM: 23.87, 95% CI: 16.76–34.0; Fig. 1E). Interestingly, Rio Choy surface fish (Fig. 1F) showed similar cortisol levels in both wild and laboratory conditions: EMMs were 23.08 (95% CI: 17.98–29.62) in 2023, 25.35 (95% CI: 19.67–32.67) in 2024, and 21.17 (95% CI: 14.95–29.98) in the lab (Fig. 1E). The highest cortisol levels were observed in wild Presa El Oyul surface fish (Fig. 1G), with an EMM of 43.87 (95% CI: 35.24–54.61) in 2023 and 30.15 (95% CI: 24.01–37.87) in 2024 (Fig. 1E). Rio Subterráneo cavefish (Fig. 1K) exhibited intermediate cortisol levels in 2023 (EMM: 19.38, 95% CI: 15.27–24.6), but levels increased in 2024 (EMM: 37.32, 95% CI: 28.4–49.05) (Fig. 1E).

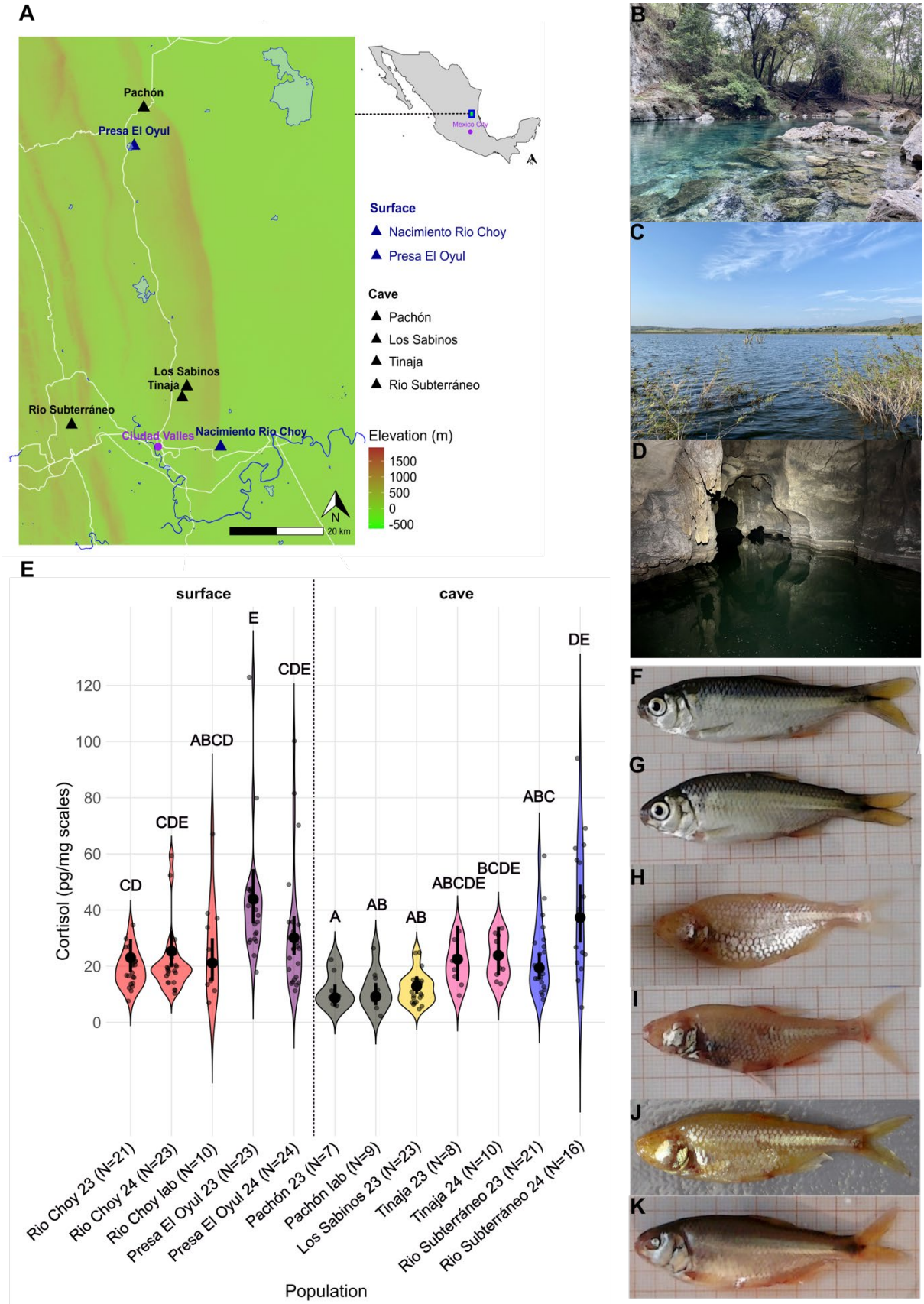

Figure 1. Scale cortisol levels of wild and laboratory *A. mexicanus* populations. (A) Map of sampling area in the Sierra de El Abra Tanchipa and Sierra Micos region in north-eastern Mexico with sampling locations of respective populations. (B) Stream surface fish habitat (Nacimiento Rio Choy). (C) Lake surface fish habitat (Presa El Oyul). (D) Cavefish habitat (Pachón) cave. (E) Scale cortisol levels in pg/mg scales per population and sampling year

with respective sample size (N) in brackets. The raw data points are plotted together with violins to show the distribution per population. Large points with error bars show the estimated marginal means with 95% confidence intervals under the fitted generalized linear model with scale cortisol as response, population as predictor and body condition (K-index), mean-centred standard length and sex as covariates. Significant differences (adjusted p-value <0.05) between populations are indicated by the compact letter design with two populations being significantly different when no letter overlaps. Representative images of (F) Rio Choy and (G) Presa El Oyul surface- and (H) Pachón, (I) Los Sabinos, (J) Tinaja and (K) 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 Table 2 for all pairwise comparisons). Wild and laboratory Pachón cavefish and wild Los Sabinos cavefish had significantly lower scale cortisol levels than wild Rio Choy surface fish in both years (Fig. 1E, Supplemental Table 2). The largest differences in cortisol levels were observed between wild and lab Pachón and Los Sabinos cavefish and wild Presa El Oyul surface fish in both years (Fig. 1E, Supplemental Table 2). Comparisons between wild Tinaja cavefish and both wild Rio Choy and Presa El Oyul surface fish did not yield any significant differences (Fig. 1E, Supplemental Table 2). However, wild Tinaja cavefish in 2024 exhibited higher cortisol levels than wild Pachón cavefish and showed a tendency toward higher levels compared to laboratory Pachón cavefish (Fig. 1E, Supplemental Table 2). A notable within-population difference was found in Rio Subterráneo cavefish, where individuals caught in 2024 had significantly higher cortisol levels than those caught in 2023 (Fig. 1E, Supplemental Table 2). Finally, Presa El Oyul surface fish caught in 2023 had significantly higher cortisol levels than both laboratory and wild Rio Choy surface fish caught in the same year and showed a trend toward higher levels compared to Rio Choy surface fish in 2024 (Fig. 1E, Supplemental Table 2).

Covariate effects on scale cortisol levels

Overall, body condition (Fulton's k-index) was a significant predictor of cortisol levels (β=0.509, SE=0.178, p=0.0047, see Supplemental Figure 3, Model Summary 1), indicating that individuals with better body condition exhibited higher cortisol levels. The effect of mean-centred standard length on cortisol was negative but not statistically significant ($\beta = -0.084$, $SE = 0.048$, $p = 0.085$, see Supplemental Figure 4, Model Summary 1), suggesting a trend where larger individuals might have lower cortisol levels. Similarly, sex did not significantly influence cortisol levels ($\beta = -0.104$, $SE = 0.097$, $p = 0.288$, see Supplemental Information, Model Summary 1).

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	Year	Temp. [C°]	pH	LDO [mg/L]	Cond. [µs/cm]	PO4 [mg/L]	CaCO3 [mg/L]	Fe [mg/L]	Cu [mg/L]	NO2 [mg/L]
Rio Choy	2023	25.9	7	7.36	1164.5	0.75	710.5	0	0	0.004
Rio Choy	2024	25.8	7.26	6.8	1123	0.06	560	0	0.02	0.005
Presa El Oyul	2023	26	8.47	7.59	428	1	214	0.05	0.04	0.002
Presa El Oyul	2024	30.5	9.5	9.4	316	0.6	64	0.13	0	1E-10
Pachón	2023	24.35	7.35	5.345	568	0.45	307	0.005	0.005	0.013
Pachón	2024	25.7	7.58	5.8	490	0.6	208	0	0.01	0.027
Los Sabinos (pool 1)	2023	22.8	7.43	1.97	484	1.3	251	0	0	0.065
Los Sabinos (pool 1)	2024	23.1	7.84	1.73	534	0.6	239	0	0.03	0.177
Tinaja (pool 2)	2024	23.3	8.17	6.29	667	2.4	346	0	0	0.009
Rio Subterráneo (pool 1)	2023	23.7	7.22	1.76	470	1.3	232	0	0	0.229
Rio Subterráneo (pool 2)	2023	23.8	7.33	5.77	481	1.4	228	0	0	0.048
Rio Subterráneo (pool 1)	2024	25	7.59	3.37	524	1.9	234	0	0	0.007
Rio Subterráneo (pool 2)	2024	25.1	7.25	3.38	519	4.7	212	0	0	0.063

Abiotic parameters

To explore the impact of abiotic factors on scale cortisol levels, we measured temperature (°C), pH, liquid dissolved oxygen (mg/L), conductivity (µs/cm), phosphate (mg/L), hardness ($CaCO_3$) (mg/L), iron (mg/L), copper (mg/L), nitrite (mg/L), and ammonium (mg/L) in each habitat (Table 1). A principal component analysis (PCA) based on these parameters revealed notable differences not only between cave and surface habitats but also between the stream (Rio Choy) and lake (Presa El Oyul) surface habitats (Fig. 2A). Additionally, Presa El Oyul exhibited significant differences in some parameters between 2023 and 2024, primarily reflected in principal component (PC) 1 (2023: 2.257, 2024: 5.416) (Fig. 2A). PCA loading factors indicated that the variation in PC 1 was primarily driven by temperature (0.455), pH (0.47), dissolved oxygen (0.396), and iron (0.498) (Fig. 2B). We correlated scale cortisol levels with PC 1 and fitted a simple GLM with cortisol as the response variable and abiotic PC 1 as the predictor. We found that abiotic PC 1 had a significantly positive effect on cortisol (β=0.095, SE=0.022, p<0.0001), indicating that higher PC 1 values correspond to elevated scale cortisol levels (Fig. 2C).

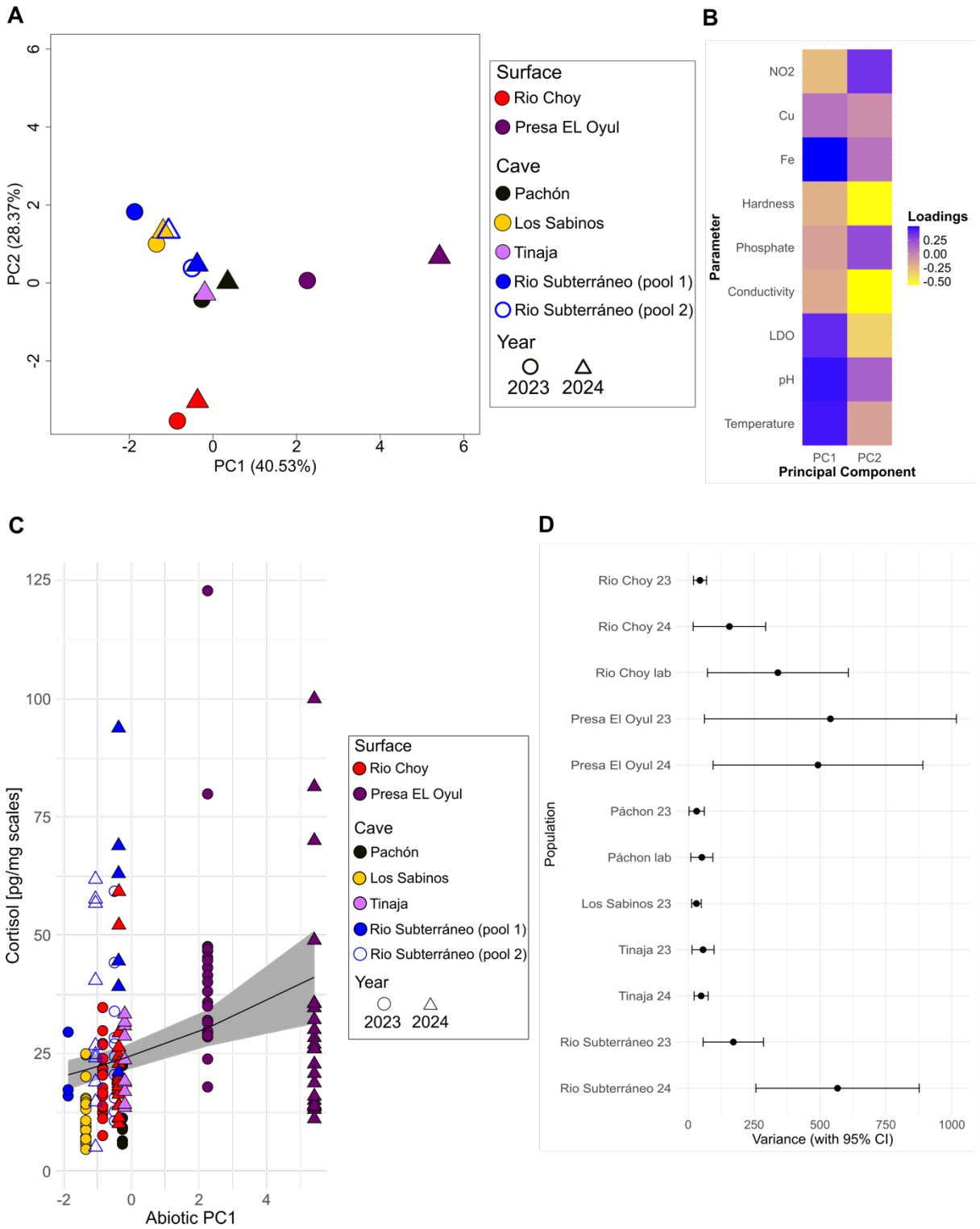

Figure 2. Dynamics of abiotic parameters influencing scale cortisol levels and individual variation in *A. mexicanus*. (A) Principal component analysis (PCA) plot based on parameters from Table 1 showing scores of principal components (PC) 1 (x-axis) and 2 (y-axis) per habitat and year. The percentage of explained variation per PC is displayed in brackets. (B) Heatmap showing PC loading factors per measured parameter. (C) Correlation of individual scale cortisol levels with PC 1 scores for each population and sampling year. The regression line represents the predicted regression with 95% confidence intervals (shaded area) of a generalized linear model with scale cortisol levels as response and PC 1 as predicting variables. (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.

Individual variation in scale cortisol levels

To examine individual variation in scale cortisol levels across populations, we performed bootstrap resampling with 1000 iterations and reported 95% confidence intervals (CIs). We found that Los Sabinos cavefish in 2023 exhibited the lowest degree of individual variation (95% CI: 12.56–48.83), followed by wild Rio Choy surface fish in 2023 (95% CI: 19.3–68.63), Tinaja cavefish in 2024 (95% CI: 21.12–75.14), wild Pachón cavefish in 2023 (95% CI: 3.14–59.77), Tinaja cavefish in 2023 (95% CI: 15.55–94.94), and laboratory Pachón cavefish (95% CI: 10.25–93.98) (Fig. 2D). More individual variation was observed in Rio Subterráneo cavefish in 2023 (95% CI: 55.95–282.39), wild Rio Choy surface fish in 2024 (95% CI: 16.28–271.85), laboratory Rio Choy surface fish (95% CI: 79.49–612.57), and Presa El Oyul surface fish in 2024 (95% CI: 104.87–954.53) (Fig. 2D). The highest degree of individual variation in scale cortisol levels was found in Presa El Oyul surface fish in 2023 (95% CI: 58.54–1018.52) and Rio Subterráneo cavefish in 2024 (95% CI: 267.53–908.98) (Fig. 2D). Significant differences in individual variation (non-overlap in CIs) were observed 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

Cave stressors

We found substantial evidence that life-time stress in *A. mexicanus* is connected to variability of specific abiotic factors. Our results show that increased fluctuations in abiotic parameters are associated with increased cortisol levels in both cave and surface habitats. Furthermore, low scale cortisol levels of Pachón and Los Sabinos cavefish suggest that those fish are adapted to extreme environmental conditions such as food scarcity. Specific metabolic and behavioural adaptations (Aspiras et al., 2015; Olsen et al., 2023; Riddle et al., 2018; Shimobayashi et al., 2023; Xiong et al., 2022, 2018; Yoshizawa et al., 2015) enable cavefish to thrive upon resource-limited conditions in the cave environment over long periods. However, cortisol showed an ambiguous role in response to starvation in other fish, being sometimes elevated (Arslan et al., 2016; Caruso et al., 2010) and sometimes reduced or unaffected (Poursaeid and Falahatkar, 2022; Sumpter et al., 1991; White and Fletcher, 1986).

Another stressor in caves is hypoxia (Krishnan et al., 2020; Legendre et al., 2025; Ornelas-García et al., 2018). Our measurements confirmed lower liquid dissolved oxygen levels in all cave habitats compared to surface habitats, with particularly low levels in Los Sabinos cave. Low scale cortisol levels in Pachón and Los Sabinos cavefish (Fig. 1E) may indicate that hypoxia adaptations (Boggs et al., 2022; van der Weele and Jeffery, 2022) contribute to a reduced life-time stress response. However, the effects of hypoxia on cortisol release in other fish species remain inconsistent (Léger et al., 2021; O'Connor et al., 2011).

Dynamics in abiotic parameters

While Tinaja and Rio Subterráneo cavefish experience similar hypoxic conditions and show similar starvation adaptations as Pachón and Los Sabinos fish (Aspiras et al., 2015; Boggs et al., 2022; Legendre et al., 2025; Ornelas-García et al., 2018; Riddle et al., 2018; Wilkens and Hüppop, 1986), their higher scale cortisol levels in 2024 suggest the presence of additional stressors. Hydrological regimes influence the dynamics of physico-chemical water parameters in cave habitats (Legendre et al., 2025). Unlike Los Sabinos, Tinaja, and Rio Subterráneo caves, Pachón cave is not affected by seasonal epigean water inflow due to its elevated entrance (210 m asl) (Mitchell et al., 1977). Our PCA analysis showed minimal annual variation in measured water parameters for Pachón, Los Sabinos, and Rio Subterráneo caves. However, long-term data revealed that Pachón cave exhibits the most stable conditions, whereas Los Sabinos and Rio Subterráneo experience seasonal fluctuations in temperature, pH, and conductivity due to epigean water inflow (Legendre et al., 2025; Tabin et al., 2018). Such fluctuations are known to elevate cortisol and stress responses in fish (Choi and An, 2008; Cockrem et al., 2019; Mota et al., 2017; Rohner et al., 2013; Wagner et al., 1997), potentially explaining higher scale cortisol levels in Tinaja and Rio Subterráneo cavefish in 2024. Higher scale cortisol levels in Rio Subterráneo in 2024 compared to 2023 suggest differences in experienced stress across years in this cave. We observed drastically lower water levels and even the disappearance of smaller pools in this cave in 2024 compared to 2023 (personal observation, Marc Bauhus), potentially due to lower precipitation in the area ("Servicio Meteorológico Nacional," n.d.). Alongside potential variations in abiotic dynamics, this could suggest heightened stress due to increased population density and resource competition, both of which can elevate cortisol levels (Bolasina et al., 2006; Ramsay et al., 2006; Ruane and Komen, 2003).

Rio Choy surface fish had similar cortisol levels to Tinaja and Rio Subterráneo cavefish, despite differing abiotic conditions. Our data showed no significant annual variation in abiotic parameters in Rio Choy, but seasonal fluctuations—undetected due to our single annual measurements—may still impact cortisol levels. Long-term monitoring of stream habitats is necessary to fully understand the environmental dynamics affecting surface fish stress levels.

In contrast to stream and cave habitats, we observed a much greater dynamic in abiotic parameters in the lake habitat Presa El Oyul. The difference in abiotic conditions in the lake between spring 2023 and 2024 was similarly high as the difference to other habitats such as Rio Choy and the caves, with parameters being the most extreme in 2024. This was mostly driven by higher temperature, pH, oxygen and iron levels. In addition to the beforementioned potential effects of temperature, pH and oxygen fluctuations on cortisol levels, increased iron concentrations were shown to affect cortisol and physiological stress responses in whitefish (Lappivaara et al., 1999; Lappivaara and Marttinen, 2005). We could show that scale cortisol levels were positively correlated with increased abiotic PC 1 values, suggesting an association of temperature, pH, oxygen and iron with lifetime stress levels across populations

Biotic stressors

Biotic stressors, such as increased predation risk in the lake habitat, could likewise explain increased scale cortisol levels and lifetime stress. During fieldwork, we observed piscivorous birds and predatory fish in Presa El Oyul Lake (personal observation, Marc Bauhus). Studies in other fish report mixed effects of predation risk on cortisol levels (Fischer et al., 2014;

Humphrey et al., 2020). In *A. mexicanus*, surface fish show more predation associated stress behaviors, such as prolonged bottom-dwelling and freezing, compared to cavefish (Chin et al., 2018; Padmanaban et al., 2025). Given that these behaviors are mediated by glucocorticoid hormones (De Marco et al., 2013; Ziv et al., 2012) surface *A. mexicanus* likely experience greater predation-induced cortisol responses. However, differences in scale cortisol levels between Presa El Oyul and Rio Choy in 2023 could suggest that predation pressures vary among surface populations. Further studies are needed to clarify this relationship.

Parasite infections can also trigger elevated glucocorticoid hormone responses in fish (Allan et al., 2020; O'Dwyer et al., 2020). Therefore, variations in parasite abundance and diversity between and among cave and surface populations may account for the higher stress levels observed in Rio Choy and Presa El Oyul surface fish, as well as Tinaja and Rio Subterráneo cavefish in 2024, compared to Pachón cavefish. This difference could be due to the reduced parasite diversity in the Pachón cave population (Peuß et al., 2020; Santacruz et al., 2023, 2020).

Another potential stressor is intraspecific aggression. Cavefish display reduced aggressive behaviours compared to surface fish (Elipot et al., 2013; Rodriguez-Morales et al., 2022). Aggressive interactions among conspecifics cause stress in fish (Winberg and Lepage, 1998), suggesting that reduced aggression in cavefish could contribute to their lower scale cortisol levels.

A comparison of wild and lab populations of Pachón cavefish and Rio Choy surface fish, respectively, revealed no significant differences in scale cortisol levels between lab-reared and wild-caught fish. However, a trend toward higher cortisol levels in lab surface fish compared to lab cavefish suggests a potential genetic basis for stress differences. Given that environmental conditions were standardized in the lab, differences in aggression behaviour could be a contributing factor. Genetic mapping of scale cortisol levels through crossbreeding experiments could help identify underlying genetic determinants.

Individual variation in scale cortisol levels

We found evidence that individual variation in scale cortisol levels varies across populations. 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 (Krishnan et al., 2020; Legendre et al., 2025; Poulson and White, 1969; Tabin et al., 2018), may contribute to reduced individual variation in stress responses and lifetime stress levels. This reduced variability could, in turn, limit the ability to conform to niche changes through stress-related mechanisms. Interestingly, Rio Choy surface fish did not exhibit greater individual variation in scale cortisol levels than the mentioned cave populations. This could be due to adaptations to different but equally stable selective pressures. In contrast, Presa El Oyul Lake 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 abiotic fluctuations or from gradients of cave-adapted traits across the sampled individuals leading to differences in stress responses.

Scale cortisol as a measure for lifetime 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.

Additionally, we observed a tendency toward a negative relationship between standard length and scale cortisol levels (see Supplemental Information). Standard length was included as a proxy for age in the model, given its positive correlation with age despite differing growth rates between cave and surface fish (Simon et al., 2017). However, previous studies have shown that scale cortisol levels tend to increase with age (Lebigre et al., 2022), raising the possibility that standard length is an inadequate proxy for age in *A. mexicanus*. Alternatively, our results could reflect a selection bias, where individuals with chronically high cortisol levels experience higher mortality (Pickering and Pottinger, 1989), leading to the selective survival of individuals with lower cortisol levels in older (larger) age classes. In addition, exclusion of some small individuals because of insufficient scale material could have further exacerbated this potential source of error. 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 lifetime-experienced stress. Furthermore, the actual responsiveness to cortisol needs to be further investigated in this system since there could be individual or population differences due to varying glucocorticoid receptor expression in the brain, as shown for example in arctic charr (Vindas et al., 2017).

Conclusion

Our findings suggest that lifetime stress, as indicated by scale cortisol levels, is lower in isolated cave populations of *A. mexicanus*, likely due to their stable, yet extreme environments, and associated adaptations. In contrast, surface fish experience greater environmental fluctuations and biotic stressors, leading to higher and more variable cortisol levels. Environmental dynamics, particularly hydrological fluctuations and associated changes in abiotic parameters, appear to shape life-time stress responses across populations, emphasizing the complex interplay between abiotic and biotic factors in shaping stress physiology. Future research should experimentally test the effects of specific stressors, such as hypoxia, starvation,

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 the role of environmental stability in shaping stress adaptations.

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, Nacimiento Rio Choy and Presa El Oyul (only females) between March 9th and March 19th.

In cave environments, we used hand 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. We euthanized all fish with an overdose of MS-222 (0.5 g/L) and dissected them within 24h post capture. 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 scales 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.

Furthermore, we measured temperature [C°], pH (in 2023), liquid 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], nitrite [mg/L] and ammonium [mg/L] with a Hach SL 1000 multiparametric device and the respective chemkeys and probes at each sampling except Tinaja in 2023. Additionally, we measured these parameters in Pachón and Los Sabinos in 2024 despite not sampling fish. 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

To generate F1 offspring, we obtained parental Pachón cavefish from the Retaux laboratory (Paris) and Rio Choy surface fish from the Wilkens laboratory (Hamburg). We grouped at least four males and two females together for breeding, following the methods described by (Baumann and Ingalls, 2022). We maintained F1 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. As they grew between 30 and 45 mm, we adjusted the feeding schedule to four times per week with Gemma Silk 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). To minimize aggression, we enriched Rio Choy surface fish tanks with hexagonal plastic structures. 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. We collected scales 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 corticosterone 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. 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

After excluding some individuals due to non-sufficient scale material and a technical problem in the laboratory, we obtained the following sample sizes per population for statistical analysis: 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. All statistical analyses were conducted in R (version 4.3.2). To test for differences in scale cortisol levels between populations, 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), and the biological meaningfulness of fixed effects. The final model included population, Fulton's *k*-index as a measure for body condition (Barton et al., 2002), mean-centred standard length and sex as fixed effects. To evaluate the relationship between cortisol and environmental variation, we performed a principal component analysis (PCA) on abiotic parameters where we imputed zero values to 1E-10. We then incorporated the first principal component (PC1) into an additional GLM to assess its effect on cortisol

levels. 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 populations. Additionally, we used bootstrap resampling (1,000 iterations) to compare individual cortisol variability across populations. All statistical tests were two-tailed, and significance was set at $p < 0.05$.

Data availability

Data and code will be made available upon successful publication in an online repository.

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Competing interests

The authors declare no competing interests.

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.