Phylogenetic mapping of sleep loss in wild-caught cavefish

Owen North[1*], Lourdes Citlalli Maza-Castañeda[2*], Aubrey E. Manning[3*], Aakriti Rastogi[1], Andrew Gluesenkamp[4], Leah Gluesenkamp[4], Nathan Swanson[5,6], Jorge Hernández-Lozano[2], Marco Garduño-Sánchez[2], Jesús Alberto Díaz-Cruz, Johanna E. Kowalko[3], Suzanne E. McGaugh[5], Alex C. Keene[1], and Claudia Patricia Ornelas-García[2]

1. Department of Biology, Texas A&M University, College Station, TX 77840

2. Colección Nacional de Peces, Departamento de Zoología. Instituto de Biología, Universidad Nacional Autónoma de México, Mexico City, MX 04510

3. Department of Biology, Lehigh University, Bethlehem, PA 18015

4. Center for Conservation & Research, San Antonio Zoo, San Antonio, TX 78212

5. Department of Evolution, Ecology, and Behavior, University of Minnesota, St. Paul, MN 55108

6. Department of Ecology and Evolutionary Biology, University of California Irvine, Irvine, CA 92697

* Denotes equal contribution

Address correspondence to patricia.ornelas.g@ib.unam.mx and KeeneA@TAMU.edu

Abstract:

Sleep is an evolutionarily ancient and nearly universal behavior throughout the animal kingdom. Multiple cave-dwelling populations of the Mexican tetra, *Astyanax mexicanus*, have converged on sleep loss compared to river dwelling surface fish. However, the extent to which sleep has been lost across cave populations, and whether sleep loss occurs in wild fish, is poorly understood. We have measured sleep and locomotor activity in 15 distinct populations of *A. mexicanus* representing multiple lineages that are broadly representative of the 34 cavefish populations identified to date. Strikingly, sleep was largely lost in all cave and hybrid populations that were tested. Hybrid populations retained intermediate eye and pigmentation phenotypes, suggesting sleep loss precedes cave-associated morphological changes. Mapping behavioral changes onto the phylogeny of *A. mexicanus* populations revealed that independent loss of sleep and elevated locomotor activity has occurred at least three times. Analysis of sleep in the wild confirms that the sleep loss phenotype observed in lab-reared fish is also present in the natural environment. Together, these findings reveal deep evolutionary convergence on sleep loss in cavefish and provide evidence for sleep loss as a primary trait contributing to cave adaptation.

Correspondence

Sleep is an ancient and nearly universal behavior throughout the animal kingdom (Anafi et al., 2019; Keene and Duboue, 2018). While the duration and timing of sleep varies across species, sleep function appears to be highly conserved (Allada and Siegel, 2008). Despite the critical role of sleep for maintaining core biological functions, numerous species can forgo sleep for extended periods of time under ecologically advantageous conditions including extended foraging periods or mating seasons (Rattenborg and Ungurean, 2023). Together, these findings suggest sleep duration varies based on evolutionary history and ecological context.

The Mexican cavefish, *Astyanax mexicanus*, is a model for investigating the evolution of sleep (Keene and Appelbaum, 2019). *A. mexicanus* exists as eyed surface-dwelling populations and at least 34 blind cave-dwelling populations (Cobham and Rohner, 2024; McGaugh et al., 2020). To date, sleep loss has been reported across multiple cave populations of lab-reared and wild-caught cavefish compared to surface fish counterparts (Duboué et al., 2011; Moran et al., 2022; Yoshizawa et al., 2015). However, the extent to which sleep has been lost across cave populations, and whether this occurs broadly in wild fish, is not well understood. Examining the evolution of sleep in these species provide a unique opportunity to phylogenetically map changes in sleep patterns across evolutionarily history to reveal ecological factors that shape sleep regulation in the Mexican tetra.

To determine whether sleep loss identified in laboratory-raised cave populations is widespread, we measured sleep and locomotor activity in 15 distinct wild-caught populations of *A. mexicanus* from independent lineages. This group included fish from four surface populations (Bocatoma, Cueva del Rancho Viejo, Micos River, and Rascón), five hybrid cave populations (Arroyo, Caballo Moro, Chiquitita, Cave Río Subterraneo, and Toro), and six cave populations (Piedras, Tigre, Pachón, Japonés, Pichijumo, and Escondido Fig 1A). Phylogenomic reconstructions validated

that cave and hybrid populations derive from at least two distinct lineages, and four independent clades (Garduño-Sánchez et al., 2023; Moran et al., 2022), that are representative of all 34 cavefish populations known to date (Fig S1A). Wild-caught fish from the four surface populations slept 3-6 hours per day, roughly comparable to previous analyses of sleep in lab-reared surface fish (Jaggard et al., 2018; Yoshizawa et al., 2015). Conversely, sleep was nearly absent in fish from all hybrid and cave populations tested (Fig 1B). Clustering of surface, hybrid, and cave groups revealed the hybrid and cave populations slept significantly less than surface group during the day and night (Fig 1C, S1B). Taken together, these findings reveal a wide-spread evolutionary convergence on sleep loss in wild-caught populations of *A. mexican*us.

Reduced sleep is hypothesized to have adaptive value by allowing for increased foraging time in a nutrient-poor environment (Horne, 2009). Indeed, previous studies in lab-reared fish revealed significantly greater locomotion and foraging-associated behaviors in cavefish (Duboué et al., 2011; Yoshizawa et al., 2015). To examine whether increased activity is present across cave and hybrid populations of *A. mexicanus*, we quantified waking activity, by normalizing the total activity to the wake duration. The average waking activity for surface populations was lower than the averages of hybrid and cavefish populations and the waking activity of hybrid populations was higher than cavefish (Fig S1C and S1D). These findings raise the possibility that increased activity in hybrid fish compensates for other cave-associated features that are yet to evolve. Together, these findings reveal that sleep loss is widespread across cavefish populations and reveal heterosis for waking activity across lineages of *A. mexicanus*.

The findings of near-complete sleep loss and elevated waking activity in hybrid populations raise the possibility that sleep loss is an early evolving cave adaptation. To determine whether sleep loss precedes other cave-associated traits we quantified eye size (orbit diameter), and pigment variation across previously identified hybrid populations. The eye-orbit diameter was significantly

greater in hybrid cavefish compared to cavefish (Fig S1E) and pigmentation in hybrid populations was intermediate between surface and cavefish (Fig 1D). The finding that sleep is lost in hybrid populations that retain several surface fish associated anatomical traits is particularly striking for Cave Rio Subterraneo, and Micos river, which are closely related and inhabit connected waterways (Garduño-Sánchez et al., 2023; Moran et al., 2023). These findings indicate that loss of sleep occurs prior to reduced pigment or eye-degeneration which are hallmarks of these cavefish populations.

Variation in sleep across lineages, combined with detailed knowledge of the phylogenetic history of *A. mexicanus*, offers the opportunity to trace the evolution of these traits. To this end, we mapped sleep duration and waking activity onto a phylogenetic tree based on the most recent phylogenetic reconstructions (Garduño-Sánchez et al., 2023; Moran et al., 2023). This mapping revealed a clear pattern of repeated evolution in sleep loss and waking activity for all cave and hybrid populations, including at least three independent origins for sleep loss (Fig 1E) (Garduño-Sánchez et al., 2023; Moran et al., 2023).

To date, the evolution of cavefish behavior has been studied extensively in the laboratory, but little is known about their behavior under natural conditions (McGaugh et al., 2020). To validate the ecological relevance of lab-based findings, we confined wild Rascón surface fish and Pachón cavefish to individual netting enclosures and recorded activity. Inactivity was manually scored in individually housed fish for 24-hours. Similar to results from lab-raised fish, sleep is significantly reduced in cavefish compared to surface fish (Fig 1F, Fig S1F-G). These findings provide the first confirmation of sleep loss in any wild fish species and validate laboratory recordings from lab-reared and wild-caught fish.

Our findings reveal repeated sleep loss in *A. mexicanus* cavefish lineages. Several species have been identified that forgo sleep under specific contexts. For example, arctic sandpipers suppress sleep seasonally during mating season, while cetacean calves and their mothers do not sleep for a month after birth (Lesku et al., 2012; Lyamin et al., 2005, 2018). Other animals including fur seals and frigate birds have developed unihemispheric sleep that allows for continuous activity during times when sleep may be unfavorable (Aulsebrook et al., 2016; Rattenborg et al., 2016). While these findings suggest numerous species possess the capability to forgo sleep, *A. mexicanus* are unique since sleep loss occurs both across development and context (Duboué et al., 2011; Yoshizawa et al., 2015).

Taken together, these findings reveal sleep loss is broadly conserved across cave and hybrid populations. This study also represents the first study to compare sleep in wild-caught fish from cave populations to fish from multiple independent populations of surface fish, allowing for the evolutionary origins of sleep loss to be determined. Our findings demonstrate that sleep loss has arisen at least three times across Mexican cavefish evolution. To our knowledge, *A. mexicanus* cavefish remains the only species identified to date with nearly complete sleep loss across its life cycle (Keene and Duboue, 2018). Since these populations of cave morphs arose from cave invasions by independent surface stocks, and have evolved over different periods of time, it is likely that sleep loss is critical for fitness in the cave system. The identification of sleep loss in these populations provides the opportunity to test the physiological and functional trade-offs between sleeping and non-sleeping populations of *A. mexicanus*, with potential to provide insights into the long-elusive evolutionary function of sleep.

Acknowledgements

This work was supported by the National Institute of Health R24OB030214 to ACK and R21 NS122166 to ACK and US-Israel Binational Science Foundation Award 2021177 to ACK and NSF IOS 2202359 to JEK. This research was also supported by the PAPIIT UNAM No. IN214624 to CPOG.

Supplemental Methods

Husbandry and Video Recordings

We included *A. mexicanus* specimens from a total of 15 different localities from the Sierra de El Abra (i.e, Pachón, Japonés, Piedras, Tigre, Arroyo, Pichijumo, Chiquitita and Toro), Sierra de Guatemala (i.e., Caballo Moro and Escondido), and Sierra de La Colmena (i.e, Micos) (Fig. S1A) regions, and four surface populations: Bocatoma, Rancho Viejo, Subterráneo and Rascón from regions in the states of San Luis Potosí and Tamaulipas in Mexico. These specimens were collected during various field trips conducted from 2015 to 2020. Collection permits were obtained from the relevant authorities (SEMARNAT SGPA/DGVS/2438/15-16, SGPA/DGVS/05389/17, and SGPA/DGVS/1893/19). The fish were kept alive and maintained in our facility at the Institute of Biology, UNAM (CNPE, IB-UNAM, Mexico City). All animals were housed at the fish facility at 22°C ± 1°C. Two weeks prior to the experiments, animals were fed a mixture of dry and live food diets (i.e. commercial flakes and blood worms). The protocol was approved by the Comisión de ética académica y responsabilidad científica, Science Faculty, UNAM.

For behavioral assays, we established a tracking system based on previously described methods (Moran et al., 2022). Individual fish were placed in 20 L tanks fitted with custom-cut white corrugated plastic (3 mm thick) illuminated with constant infrared light, and 12:12 LD white light. Following a 24 hrs acclimation period, fish were recorded for a period of 24 hrs using a Microsoft

LifeCam Studio 1080p HD Webcam - Gray, fitted with an IR lens (Quanmin 9.6mm×1.0 mm Slim Optical 780nm Filter Infrared Cold Mirror). Locomotor behavior was tracked in Ethovision XT 17.0 (Noldus), and frame-by-frame velocity data was exported and analyzed using a custom MATLAB script, with sleep defined as 60 seconds or more of consolidated immobility. A velocity cut-off of 4 cm/s was used to distinguish active swimming from passive drift. Data was processed using custom-written MATLAB scripts as previously described (Moran et al., 2022).

Morphological Phenotyping

After the behavior recordings, fish were anesthetized in cold water for 30 sec. The fish were photographed from the left side with a reference scale. Subsequently, using a stereomicroscope (Stemi 305, ZEISS ZEN), a photograph was taken of the left side of the head of the specimen with a size scale. Using ImageJ v.1.54r (Schindelin et al., 2012), the following measurements were taken: 1) orbit diameter (mm), and 2) head length (mm) from the tip of the mouth to the posterior edge of the pre-operculum. The orbit diameter dimensions were corrected using the head length (mm). A variance analysis was performed to compare the mean differences between the ecotypes (i.e., caves, surface, and hybrids). Kruskal-Wallis and a pairwise Wilcoxon rank test were used to compare orbit diameter across ecotypes.

Pigmentation measurements

Color measurements of RGB, HSL, and K values of melanocytes were obtained using Adobe Photoshop 2017, 18.0 Portable 64-bit (Adobe Systems Incorporated), from photographs of the left side of each fish. The reference area comprises a rectangle including the base of the dorsal fin to the lateral line of the fish. A grid was used to divide the image so that the section could be located for analysis. Together with the eyedropper tool, samples of 31 × 31 pixels were selected for red, green, blue (RGB); -Hue, saturation and lightness (HSL), and within the Cyan, Magenta, Yellow, Key/Black (CMYK) scale, we only got the K-value (i.e., the black component, which is

used to increase the depth and contrast of the color). A mean value for each individual was obtained for the 10 measurements and a principal component analysis (PCA) was carried out to compare the distribution of individual specimens, using the prcomp() function in R (Team R Core, 2019). Data were zero-centered and scaled to have unit variances prior to analysis. The contributions of each variable, as well as of the principal components, were visualized using the functions fviz_pca_var() and fviz_contrib() of the factoextra package. The first two principal components (PC1 = 71.43%, PC2 = 18.6%), were used to perform a linear discriminant analysis. For the fitting of the discriminant model, the principal component data set was divided into a training set (70% of the data) and a test set (30% of the data). The lda() function of the MASS library (Venables & Ripley, 2002), was employed using phenotype as the categorical variable (i.e., 'cave', 'hybrid' or 'surface') and principal components. The accuracy of the model (86%) was estimated by comparing the a priori classification with that proposed by the model, using the accuracy() function of the generics library. All data manipulation in R was performed using the tidyverse and reshape2 libraries. Both the assignment probabilities and the distribution in the discriminant space were visualized using ggplot2 in R. We evaluate the correlation between the sleep duration and the residuals of the correlation between the orbit diameter and the head length.

Phylogenetic Analysis

Based on current phylogenetic evidence (Garduño-Sánchez et al., 2023), we edited the topology pruning the populations that were not included in our behavioral study (Fig. S1A) using ape version 5.7.-1. The final topology was edited by hand to include 136 individuals corresponding to the 15 populations analyzed (Fig S1A). To visualize the evolutionary patterns, we mapped on the phylogeny the following data: 1) raw sleep data, 2) walking activity, 3) corrected orbit diameter by head length, and 4) PC1 of the color measurements analysis. Ancestral character estimation was conducted using the anc.ML function following the Brownian Motion model and character mapping using contMap function, both included in phytools version 2.1-1in R-Studio v. 4.02.

Analysis of fish in the natural environment

Surface fish were assayed from the Río Frio near Rascón, Mexico and cavefish were assayed from Pachón cave in Mexico between March 18 - March 26 2022. Fish were caught using collapsible bait traps and sardines as bait. Each fish was transferred to its individual breeder box (Nylon Mesh Fish Fry Hatchery Breeder Box Separation; 26.5 x 15 x 15.5cm, L x W x H) which was modified to have a white plastic poster board bottom to better visualize the fish, and which had the top replaced with screen door mesh to avoid fish escapes. Breeder boxes were mounted to garden stakes and submerged so the top of the breeder box was just below the water surface. Outdoor Wyze Cam v1 cameras were mounted above the breeder boxes, using one camera per fish/arena and visualized to ensure the breeder box was encompassed within the camera frame. Outdoor Wyze Cams were set to auto mode to turn on infrared and infrared filters to record in night vision mode once the camera detected sundown. Cameras continuously recorded in 1080p resolution to a 32Gb microSD card. Renogy 72000mAh Laptop Power Banks were used to power the cameras beyond the camera's battery life. The power banks required a small load to be pulled to stay in charging mode until the cameras' batteries were exhausted and then the camera started to pull the load. A FINENIC 6-Black Mini USB LED Light Lamp wrapped in aluminum tape to completely block out any light coming from the lamp was plugged into a VENTION 4-Port USB 3.0 Hub Ultra-Slim Data USB Splitter to pull this small load and keep the Power Bank from turning off. After recording concluded, fish were photographed with a ruler in frame. Methods were approved by University of Minnesota.

We analyzed the behavior of fish for a continuous 24-hour period beginning at 6:40am local time, coinciding with the return of day mode recording settings, and ending at 6:40am the following day. The beginning of the analysis period corresponds to local sunrise, which occurred f 6:35am - 6:41am, depending on location and date of recording. Fish were acclimated within the arena for

at least 14 hours before analysis began. Videos were annotated for when the fish was performing non-active swimming and when the fish was obscured using the open-source software BORIS (v.8.27.1) (Friard and Gamba, 2016).

Non-active swimming was scored as the fish not actively making body movements for a period longer than 20 seconds. This metric included periods of drift due to river flow during which the fish slowly drifted but did not actively engage in swimming movements. Bouts of non-active swimming conclude with a sharp, distinct change in orientation or a sudden increase in velocity. Small adjustments to the fish's positioning did not end a bout of non-active swimming. Due to the unique challenges of filming in the field, fish were difficult to observe for portions of the 24-hour period. Fish behavior was scored as obscured when the fish was not sufficiently visible to make an accurate assessment of the positioning of the fish for a period of 20 seconds or longer. For example, if a fish was observed in active swimming at t=1 sec and t=19 sec, but obscured in between, the fish was scored as active swimming rather than obscured.

The behavior of 11 Rascón surface fish and 11 Pachón cavefish was analyzed for 24 hours. Following analysis, observation time stamps were exported to a .csv in BORIS. Even though behaviors were annotated after 20 seconds, only observations longer than 60 seconds were considered for further analysis.

Statistics

All statistical tests of behavior were conducted using InStat software (GraphPad Prism 10). An ordinary one-way ANOVA test followed by a Tukey's multiple comparison was used to test for significant differences in sleep behaviors between the 15 independent populations of wild-caught surface, hybrid, and cave fish (Fig 1A). To test for differences in total sleep between grouped surface, hybrid, and cave populations, an ordinary one-way ANOVA test followed by a Tukey's

multiple comparison was conducted (Fig S1B). To test for differences in waking velocity, an ordinary one-way ANOVA test followed by a Tukey's multiple comparison was conducted (Fig S1C). To test for differences between velocity in grouped surface, hybrid, and cave populations, an ordinary one-way ANOVA test followed by a Tukey's multiple comparison was conducted (Fig S1D). To analyze differences and variability in eye size, the average eye orbit diameter of each individual (mm) was standardized by the length of their head (mm), defined as "corrected eye orbit diameter". To test for significant differences in corrected eye orbit diameter, an ordinary one-way ANOVA was performed followed by Tukey's multiple comparison (Fig S1E). To test for significant differences in sleep durationbetween wild individuals recorded in the Río Frio near Rascón, Mexico and the Pachon cave , a Mann-Whitney T-test was used (Fig S1G).

References

Allada R, Siegel JM. 2008. Unearthing the phylogenetic roots of sleep. *Curr Biol* 18:R670–R679. doi:10.1016/j.cub.2008.06.033

Anafi RC, Kayser MS, Raizen DM. 2019. Exploring phylogeny to find the function of sleep. *Nat Rev Neurosci* 20:109–116. doi:10.1038/s41583-018-0098-9

Aulsebrook AE, Jones TM, Rattenborg NC, Roth TC, Lesku JA. 2016. Sleep Ecophysiology: Integrating Neuroscience and Ecology. *Trends Ecol Evol* 31:590–599. doi:10.1016/j.tree.2016.05.004

Cobham AE, Rohner N. 2024. Unraveling stress resilience: Insights from adaptations to extreme environments by Astyanax mexicanus cavefish. *J Exp Zool B Mol Dev Evol* 342:178–188. doi:10.1002/jez.b.23238

Duboué ER, Keene AC, Borowsky RL. 2011. Evolutionary convergence on sleep loss in cavefish populations. *Current Biology* 21:671–676. doi:10.1016/j.cub.2011.03.020

Friard O, Gamba M. 2016. BORIS: A free, versatile open-source event-logging software for video/audio coding and live observations. *Methods Ecol Evol* 7. doi:10.1111/2041-210X.12584

Garduño-Sánchez M, Hernández-Lozano J, Moran RL, Miranda-Gamboa R, Gross JB, Rohner N, Elliott WR, Miller J, Lozano-Vilano L, McGaugh SE, Ornelas-García CP. 2023. Phylogeographic relationships and morphological evolution between cave and surface Astyanax mexicanus populations (De Filippi 1853) (Actinopterygii, Characidae). *Mol Ecol* 32:5626–5644. doi:10.1111/mec.17128

Horne J. 2009. REM sleep, energy balance and “optimal foraging”. *Neurosci Biobehav Rev* 33:466–474. doi:10.1016/j.neubiorev.2008.12.002

Jaggard JB, Stahl BA, Lloyd E, Prober DA, Duboue ER, Keene AC. 2018. Hypocretin underlies the evolution of sleep loss in the Mexican cavefish. *Elife* 7. doi:10.7554/eLife.32637

Keene AC, Appelbaum L. 2019. Sleep in Fish Models, Handbook of Behavioral Neuroscience. doi:10.1016/B978-0-12-813743-7.00024-4

Keene AC, Duboue ER. 2018. The origins and evolution of sleep. *J Exp Biol*. doi:10.1242/jeb.159533

Lesku JA, Rattenborg NC, Valcu M, Vyssotski AL, Kuhn S, Kuemmeth F, Heidrich W, Kempenaers B. 2012. Adaptive sleep loss in polygynous pectoral sandpipers. *Science* 337:1654–8. doi:10.1126/science.1220939

Lyamin O, Pryaslova J, Lance V, Siegel J. 2005. Animal behaviour: continuous activity in cetaceans after birth. *Nature* 435:1177. doi:10.1038/4351177a

Lyamin OI, Kosenko PO, Korneva SM, Vyssotski AL, Mukhametov LM, Siegel JM. 2018. Fur Seals Suppress REM Sleep for Very Long Periods without Subsequent Rebound. *Curr Biol* 28:2000-2005.e2. doi:10.1016/j.cub.2018.05.022

McGaugh SE, Kowalko JE, Duboué E, Lewis P, Franz-Odendaal TA, Rohner N, Gross JB, Keene AC. 2020. Dark world rises: The emergence of cavefish as a model for the study of evolution, development, behavior, and disease. *J Exp Zool B Mol Dev Evol* 334:397–404. doi:10.1002/jez.b.22978

Moran RL, Jaggard JB, Roback EY, Kenzior A, Rohner N, Kowalko JE, Ornelas-García CP, McGaugh SE, Keene AC. 2022. Hybridization underlies localized trait evolution in cavefish. *iScience* 25:103778. doi:10.1016/j.isci.2022.103778

Moran RL, Richards EJ, Ornelas-García CP, Gross JB, Donny A, Wiese J, Keene AC, Kowalko JE, Rohner N, McGaugh SE. 2023. Selection-driven trait loss in independently evolved cavefish populations. *Nat Commun* 14:2557. doi:10.1038/s41467-023-37909-8

Rattenborg NC, Ungurean G. 2023. The evolution and diversification of sleep. *Trends Ecol Evol* 38:156–170. doi:10.1016/j.tree.2022.10.004

Rattenborg NC, Voirin B, Cruz SM, Tisdale R, Dell'Omo G, Lipp H-P, Wikelski M, Vyssotski AL. 2016. Evidence that birds sleep in mid-flight. *Nat Commun* 7:12468. doi:10.1038/ncomms12468

Schindelin J, Arganda-Carreras I, Frise E, Kaynig V, Longair M, Pietzsch T, Preibisch S, Rueden C, Saalfeld S, Schmid B, Tinevez JY, White DJ, Hartenstein V, Eliceiri K, Tomancak P, Cardona A. 2012. Fiji: an open-source platform for biological-image analysis. *Nat Methods* 9:676–682. doi:10.1038/nmeth.2019

Yoshizawa M, Robinson BG, Duboué ER, Masek P, Jaggard JBJ, O'Quin KEK, Borowsky RLR, Jeffery WRW, Keene ACA, Duboue E, Masek P, Jaggard JBJ, O'Quin KEK, Borowsky RLR, Jeffery WRW, Keene ACA. 2015. Distinct genetic architecture underlies the emergence of sleep loss and prey-seeking behavior in the Mexican cavefish. *BMC Biol* 20:15. doi:10.1186/s12915-015-0119-3

Figure 1

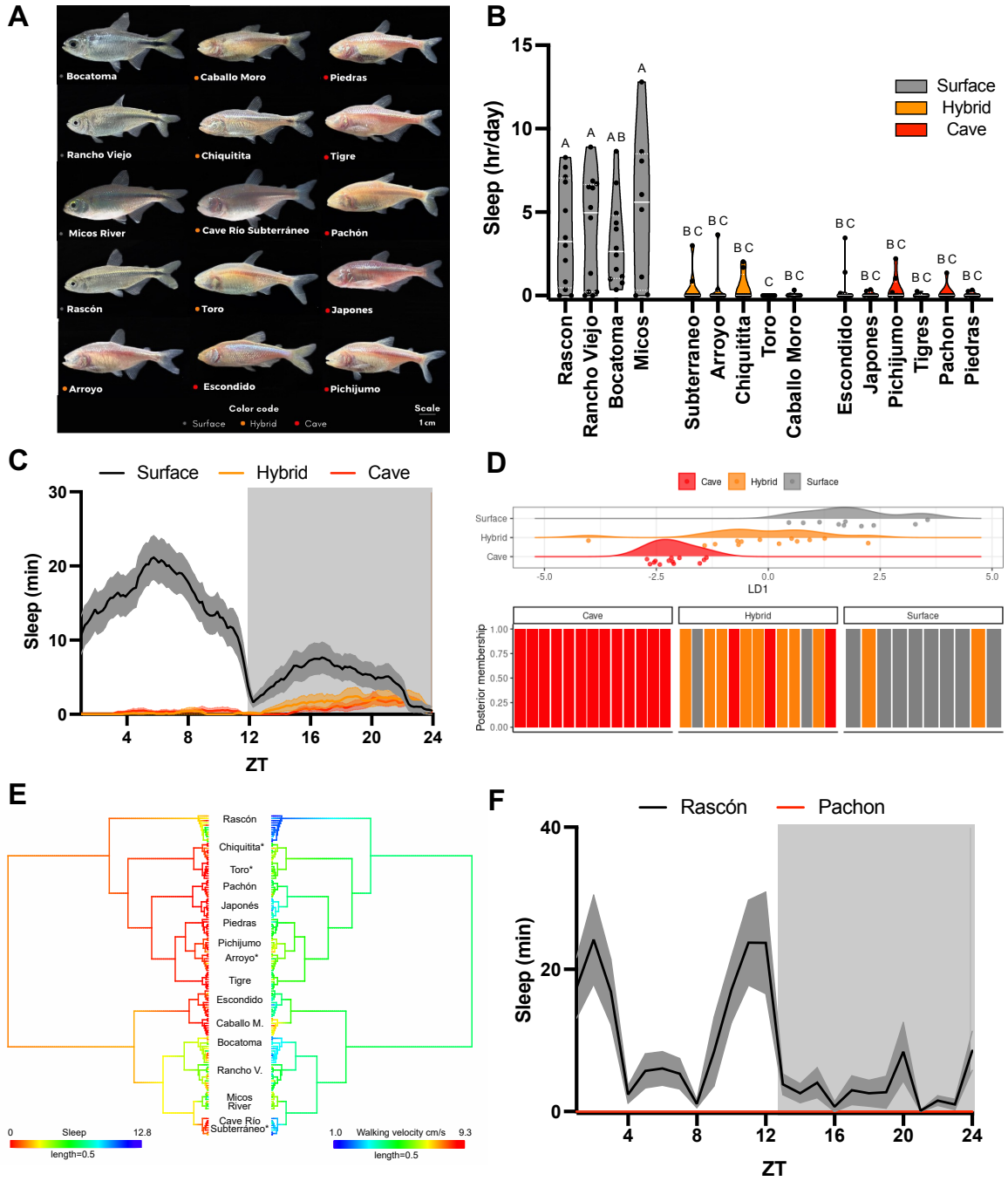
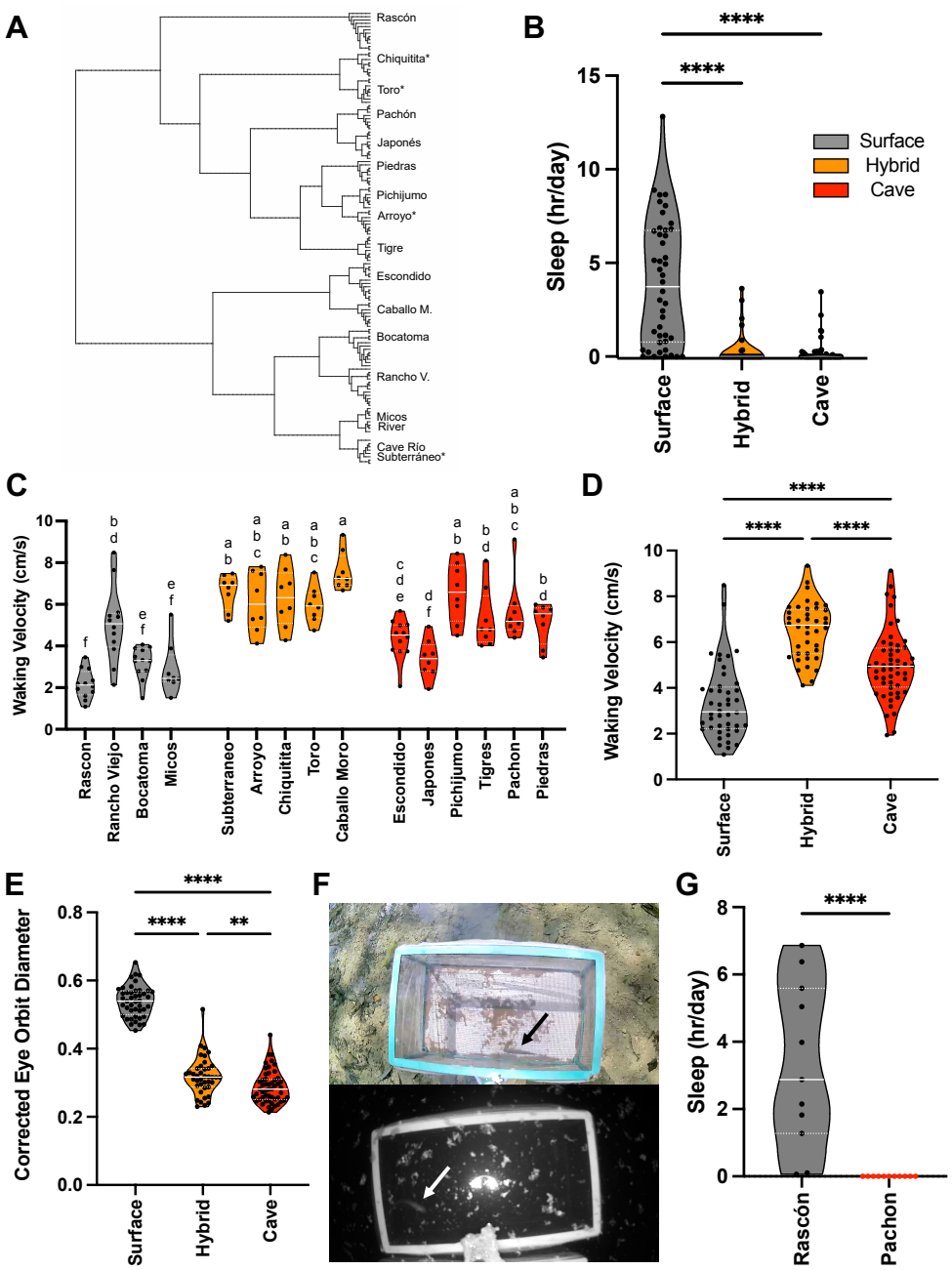


Figure 1. Convergence on sleep loss in wild-caught populations: A) Images of an individual from each independent population tested. Populations were categorized as surface (gray), hybrid (orange), or cave (red). Scale bar denotes 1cm. B) Totals sleep (hrs/day) across each independent population of *Astyanax mexicanus*. Sleep in Rascon, Rancho Viejo, and Micos was greater than all hybrid and cave populations (P<0.05; P<0.03; P<0.001). C) Grouped sleep profile (mins) over a 24-hour period in a 12:12 light-dark cycle. Sleep duration over time from all individuals of surface, hybrid, and cave populations were averaged together. D) Linear discriminant function analysis (LDA) of color measurements across the populations; the top graph shows the density for each category obtained by the model (Cave, Hybrid, and Surface), and the bottom graph shows the probability of individual assignment to each group. E) Sleep and waking activity mapped onto a reconstruction of the *A. mexcianus* phylogeny. Branch colors depict the distribution of the observed values for each trait mapped, if they are red they showed the lowest values in both sleep or locomotion, while the blue values corresponded to the highest values. F) Sleep profile over a 24-hour period captured in the wild reveals reduced sleep in Rascon surface fish compared to Pachón cavefish.



Supplemental Figure 1 related to Figure 1) Phylogenic tree based on current molecular phylogenetic analysis of cavefish populations tested in this manuscript. B) Total sleep (hrs/day) of all independent surface, hybrid, and cave populations grouped to their respective categories. Grouped surface populations slept significantly more than both hybrid ($p < 0.0001$) and cave ($p < 0.0001$) populations, while cave and hybrid groups slept similarly ($p = 0.9810$). C) Total waking activity (cm/s) across each independent population. Waking activity in Rascon, Bocatoma, and Micos was lower than all hybrid populations ($P<0.001$) as well as all cave populations except for Japones ($P<0.05$; $P<0.005$). D) Grouped population average waking activity (cm/s) of surface, hybrid, and cave categories. Grouped hybrid populations had significantly higher waking velocity than grouped surface ($p < 0.0001$) and cave ($p < 0.0001$) populations. Grouped cave populations had significantly higher waking velocity than grouped surface populations ($p < 0.0001$). E) Grouped populations average eye orbit diameter (mm) standardized by head length (mm). Grouped surface populations have significantly larger eye orbit diameters than grouped hybrid ($p <0.0001$) and cave populations ($p < 0.0001$). Grouped hybrid populations have significantly larger eye orbit diameters than grouped cave populations ($p< 0.01$). F) Images depicting a wild fish sleep recording setup from the Río Frio near Rascón, Mexico (top) and Pachón cave (bottom). G)Average total sleep (hrs/day) of wild fish from the Río Frio near Rascón, Mexico and Pachón cave. Wild Rascón individuals slept significantly more than wild Pachón cave individuals ($p < 0.0001$).