Video Article

Automated Measurements of Sleep and Locomotor Activity in Mexican Cavefish

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Abstract

Across phyla, sleep is characterized by highly conserved behavioral characteristics that include elevated arousal threshold, rebound following sleep deprivation, and consolidated periods of behavioral immobility. The Mexican cavefish, *Astyanax mexicanus* (*A. mexicanus*), is a model for studying trait evolution in response to environmental perturbation. *A. mexicanus* exist as in eyed surface-dwelling forms and multiple blind cave-dwelling populations that have robust morphological and behavioral differences. Sleep loss has occurred in multiple, independently-evolved cavefish populations. This protocol describes a methodology for quantifying sleep and locomotor activity in *A. mexicanus* cave and surface fish. A cost-effective video monitoring system allows for behavioral imaging of individually-housed larval or adult fish for periods of a week or longer. The system can be applied to fish aged 4 days post fertilization through adulthood. The approach can also be adapted for measuring the effects of social interactions on sleep by recording multiple fish in a single arena. Following behavioral recordings, data is analyzed using automated tracking software and sleep analysis is processed using customized scripts that quantify multiple sleep variables including duration, bout length, and bout number. This system can be applied to measure sleep, circadian behavior, and locomotor activity in almost any fish species including zebrafish and sticklebacks.

Video Link

The video component of this article can be found at https://www.jove.com/video/59198/

Introduction

Sleep is highly conserved throughout the animal kingdom at the physiological, functional, and behavioral levels[1,2,3]. While sleep in mammalian laboratory animals is typically assessed using electroencephalograms, electrophysiological recordings are less practical in small genetically amenable model systems and thus sleep is typically measured based on behavior[3,4]. Behavioral characteristics associated with sleep are highly conserved throughout the animal kingdom and include increased arousal threshold, reversibility with stimulation, and prolonged behavioral quiescence[5]. These measures can be used to characterize sleep in animals ranging from the nematode worm, *C. elegans*, through humans[6].

The use of behavioral quiescence to characterize sleep requires automated tracking software. With tracking software, periods of activity and immobility are determined over a number of days, and long periods of inactivity are classified as sleep[7,8]. In recent years, multiple tracking systems have been developed for acquiring activity data among a diversity of small genetically-amenable model systems; including worms, fruit flies and fish[9,10,11]. These programs are accompanied by software that allows for automated tracking of animal behavior, including both open source freeware and commercially available software[7,12,13,14]. These systems differ in their flexibility and allow for efficient screening and characterization of sleep phenotypes in numerous genetically amendable models.

Genetic investigation of sleep in the zebrafish, *Danio rerio*, has led to the identification of numerous genes and neural circuits that regulate sleep[15,16]. While this has provided a powerful system for investigating the neural basis of sleep in a vertebrate laboratory animal, much less is known about how sleep evolves and how natural variation contributes to sleep regulation. The Mexican cavefish, *Astyanax mexicanus* (*A. mexicanus*), have evolved dramatic differences in sleep, locomotor activity and circadian rhythms[17,18]. These fish exist as eyed surface fish that inhabit the rivers of Mexico and Southern Texas and at least 29 cave populations around the Sierra Del Abra region of Northeast Mexico[19,20,21]. Remarkably, many behavioral differences, including sleep loss, appear to have emerged independently in multiple cavefish populations[14,22]. Therefore, cavefish provide a model for investigating the convergent evolution of sleep, circadian, and social behaviors.

This protocol describes a system for measuring sleep and locomotor behavior in *A. mexicanus* larvae and adults. A custom-built infrared-based recording system allows for video recording of animals under light and dark conditions. Commercially available software can be used to measure activity and custom macros are used to quantify several aspects of inactivity and determine periods of sleep. This protocol also describes experimental modifications for tracking the activity of multiple animals within a tank, providing the ability to examine interactions between sleep

and social behaviors. These systems can be applied to measure sleep, circadian behavior, and locomotor activity in additional fish species including zebrafish and sticklebacks.

Protocol

NOTE: Set up systems for behavioral tracking in larvae and adults.

1. Constructing a sleep system for larvae

NOTE: The monitoring system for tracking larval through juvenile fish aged 4 days post fertilization (dpf) through 30 dpf *A. mexicanus* requires multiple pieces of equipment including infrared (IR) lighting, acrylic IR light diffusers, automated light controls (timers), computers, cameras, and secondary materials such as wiring and power controllers (**Figure 1A**). The following instructions will inform how to build a system to accurately track locomotor behavior to study sleep and circadian rhythms in larval *A. mexicanus.*

1. Construct a lighting system consisting of IR and white light emitting diodes (LED): Place three IR lights in a triangle approximately 7.62 cm from each other on a 30.5 cm x 30.5 cm thin metal platform heatsink. Wire lights in series with electrical wire and connect to a power source.
2. Place a single white light LED in the center of the three IR lights and attach them to the power source.
3. Connect the power source for the LED to a light timer set to a standard circadian time.
4. Construct a platform for larval tracking system. Use 0.33 cm thick white sign acrylic for all of the components of the platform.
5. Place the recording platform on top of the lighted square heatsink on which arenas containing fish will reside during behavioral tracking.
6. Place a second acrylic inside the box between the lights and the animals to diffuse the IR for optimal lighting and contrast.
   NOTE: The dimensions for the larval light box is as follows: Two 18 cm x 8.5 cm and two 17 cm x 8.5 cm LEDs that are chemically bonded together to form an 18 cm x 18 cm square that is 8.5 cm tall. Additionally, acrylic may be easily cut or drilled using the proper tools for customized sizing.
   1. Place the entire larval tracking stage and lighting setup within an enclosed plastic tube, and then position the camera on top of the tube.
      NOTE: It is important to keep lighting reflections from appearing on the tracking video, as this will impair tracking accuracy. The placement of cameras on top of a tube that surrounds platform improves lighting and sharpness for the cameras that are used for these experiments.
7. Manipulate a webcam (see **Table of Materials**) for IR-based recording. Remove the manufacturer lens using a rotary tool (see **Table of Materials**).
8. Remove the small silver screws on the back sides of the camera to remove the inner housing.
9. Remove the small black screws inside the body of the camera to loosen the remainder of the lens. Use a small screwdriver to remove any portions of the lens housing that is left after cutting the lens.
10. Remove the blue LED on the top part of the charged coupled device (CCD) housing.
11. Put the camera back together by placing the inner housing back to its original orientation and screwing the two silver screws back to their original position.
12. Route the inside of the camera using a small saw to fit with a rounded plastic routing bit. Smooth down the extra plastic until it can fit a lens adapter.
13. Install an IR-pass filter inside the camera as close to the CCD as possible without making direct contact with the camera.
    NOTE: Take care not to damage the CCD chip within the housing of the camera. Be sure to keep the cut as level as possible. Seal any open space between the outside of the IR filter and the body of the camera to keep light from reaching the CCD without being filtered.
14. Attach the camera to a 35 mm fixed lens (see **Table of Materials**) by screwing the adaptor in the front of the camera into the back of the lens.
15. Place the camera and lens in the hole drilled into the lid on top of the tube that houses the stage and lights and attach the USB to the computer the animals will be recorded from.
    NOTE: Place fish in a behavior room that is separate from where stock fish are housed to ensure minimal disruptions during behavioral recordings. Take care to minimize fluctuations in temperature and ventilation which can confound behavioral experiments.

2. Sleep system for adults

1. Construct an IR lighting system for tracking adult fish by cutting IR strips to approximately 46 cm intervals. One 46 cm strip is sufficient for each 10 L behavior tank.
2. Wire each strip together in a series, soldering each strip to DC electrical wire and attach to a 9 V power source.
3. Attach each IR strip to a 51 cm x 5.1 cm piece of aluminum that will act as a heat sink.
4. Place a 46 cm x 5 cm, 0.32 cm thick 9% light-pass white sign acrylic sheet directly in front of each IR light strip to diffuse the IR light.
5. Place all tanks on a rack that supports rear-mounted IR lighting.
6. Use opaque plastic dividers in 10 L glass tanks to create individual arenas.
   NOTE: The arena size can be varied based on the number of dividers used and the size of the tank. Arena size impacts locomotor activity and sleep in both cave and surface fish[23].
7. Mount cameras approximately 4-6 m away from tanks. Each camera can typically record from 3 tanks at a time to provide sufficient resolution for tracking.
   NOTE: Adult behavioral recordings generally do not require a separate white lighting system to control day-night changes. Simply utilizing standard overhead lights in a behavioral room connected to a timer is likely to be sufficient.

3. Recording locomotor activity

NOTE: All behavioral recordings are made using a standard laptop computer or desktop with a backup battery source. Due to the large file size of a 24 h recording (60-100 GB), save all recordings on external hard drives.

1. Acclimate fish ages 4-30 dpf for 18-24 h prior to initiating recordings. Feed larval fish with live brine shrimp when first placed in the recording chamber, and 1 h prior to beginning the recording. Acclimate adult fish 4-5 days before recording behavior and feed once daily with flake food or with live blackworms.
   NOTE: Be sure to place larval fish in fresh water prior to recording, as leftover brine shrimp will cause tracking issues during later analysis. Co-culturing with rotifers provides an alternative option, as their small size does not interfere with tracking.
2. Place fish aged 4-6 dpf in 24-well tissue culture plates. House fish aged 20-30 dpf in 12-well tissue culture plates for recording.
3. Record adults in 10 L tanks fit with dividers to accommodate five individually housed fish, or without dividers to record sleep and activity in a social setting.
   NOTE: Take care to focus the camera prior to initiating the recording in order to maximize tracking accuracy. Do not open the iris of the camera lens too far, as this will drastically reduce sharpness of the image. There is a balance, however; if the iris is closed too far, the frame rate of the video will fall below 15.00 fps. It is essential for later analysis that the frame rate remains 15.00 frames per s for use with some custom-written sleep script[23,24].
4. Set lighting/brightness background illuminations.
5. Optimize lighting prior to the start of recording. Always keep the contrast at the highest level possible and use the **Brightness** and **Background Illumination** to adjust the brightness till the animals are most clear.
6. Record fish for 24 or 48 h.
   NOTE: Emergency backup battery packs that power all the lighting, computers, and cameras should be purchased in case of power outages. All battery packs should also be plugged into emergency power outlets, if possible. Battery packs will generally not power the equipment for more than a few minutes to an hour at most and serve to bridge between losing main power and transferring to the emergency power system.

4. Analysis of locomotor activity in individually housed fish using automated tracking software

1. To begin analysis of behavior, open tracking software, select **New Experiment from Template**, then select **Apply a Pre-Defined Template**.
2. As the program will now ask what species to track, select **Fish**. Use the dropdown box to select either **Zebrafish Larvae** of **Zebrafish Adult** depending the experimental paradigm.
3. Set up the arenas in which each animal will be tracked. For larvae, select **Well Plate, Round Well** and **No Zone Template**. For adults, use **Open Field, Square** with **No Zone Template**. Then determine the proper number of arenas, one for each animal being tracked in the video.
4. Select the model for optimal tracking, click on track **Center-point,** and be sure that the animal color is selected to be darker than the background. Apply the frame rate at which the video was acquired. The tracking software should automatically detect this.
5. Draw a scale to calibrate the real-world distance of an object to accurately determine the locomotor behavior of the fish using **Arena Settings**.
6. Edit the arenas to make sure that the entire area that the fish are in will be tracked; otherwise samples will be lost during acquisition.
   NOTE: Use care when setting up areas. Larval fish especially are sensitive to tracking errors if the arenas are either too big or small. Bad lighting in the experiment can also create shadows on the walls of well plates, which the program may think is an animal, creating a false positive.
7. Click on **Advanced**. Under **Method** in **Detection Setting**, select **Dynamic Subtraction**, then click on **Background** and select **Start Learning**. Adjust the dark contrast signal/noise ratio till the animals are being tracked well, and the background is not causing the tracking to jump.
   NOTE: Video quality can vary between experiments, so each trial may need to use different settings, accordingly. Using the **Subject Contour** and **Subject Size** features can significantly improve tracking results.
8. Select Trial list and load the proper parameters before starting to record the data into the program.
9. Click into the **Acquisition** tab, select **Track All Planned Trials** and click on the record button.
10. Under **Analysis Profile**, make sure **Distance and Time & Movement** are selected.
    NOTE: In order for later sleep analysis, it is critical that these settings are correct, as the .perl file must read these data in the correct order to calculate sleep.
11. Under Export, select **Raw Data**, export the data as Unicode text.

5. Tracking socially housed fish

1. Follow steps 4.1-4.4 to set up the experiment in the tracking software.
2. In the dropdown menu, select how many animals to track for the experiment.
3. In **Arena** settings, draw the correct scale to calibrate the real-world distance.
4. In **Detection** settings, use **Dynamic Subtraction**, and adjust the dark contrast to best track the animal.
5. Adjust the subject size under the detection settings, so that only a very small portion of the animal is tracked.
   NOTE: By tracking only a small part of the animal, this will reduce the amount of switching between animals when they cross paths during acquisition.
6. Once the tracks are acquired, use the **Track Editor** to manually fix times where fish may cross paths.

6. Extraction of sleep data from locomotor activity

NOTE: The behavioral definition of sleep in both larval and adult *A. mexicanus* is 1 min or more of quiescence. This definition was determined using arousal threshold experiments, where a greater sensory stimulus is required to initiate a behavioral response in a sleeping state (>60 s) compared to waking[14,17]. To account for small movements and drifting common to fish species, there are velocity thresholds applied to segregate real movement from noise or drift. These thresholds are computationally-derived by comparing correlations between distance and sleep duration to find the highest R-squared values; thereby determining the most accurate velocity for movement and sleep. For larval fish, the upper and lower limits are both 12 mm/s as there is little to no drift. For adult fish the lower limit is 2 cm/s with an upper limit of 4 cm/s to account for drift.

1. Install Cygwin to the analysis computer to carry out the execution of the custom-written scripts to extract locomotor and sleep behavior.
2. Create a new experiment folder within the Cygwin home directory.
3. Import the raw Unicode data from tracking software and execute the .sh file in Cygwin to convert the encoding from UTF-16 to UTF-8.
4. Execute the .perl file in Cygwin to extract the sleep data.
5. Open the macro file and follow the instructions within the spreadsheet to finalize the data analysis as desired.
   NOTE: A basic level of command-line coding will be necessary to carry out this portion of analysis. General Linux commands will suffice. If there are issues with the programs reading the data properly, check the data from tracking software in any freely available text editor to be sure the encoding and order is correct for the executables to write properly.

Representative Results

Larvae ages 4-30 dpf can be reliably recorded in the custom-build closed system described in **Figure 1**. The system includes both IR and visible lighting to allow for recordings under light and dark conditions, under various visible light conditions (**Figure 1A**). The videos are then analyzed using tracking software (**Figure 1B,C**) and post-processed using a custom sleep macro (See **Supplemental Download**). Larval fish from three independent cavefish populations display a significant reduction in sleep compared to surface fish (**Figure 1D**) and 20 dpf, and this sleep loss is consistent across developmental stages. The age of fish analyzed for sleep is often dependent on the experimental manipulation. For example, *A. mexicanus* do not consume food at 4 days, so experiments examining interactions between sleep and feeding would typically occur in older larvae[25]. Conversely, morpholinos are only effective in early fry (typically younger than 4 dpf) so this age is used to asses sleep[24,26,27].

A. mexicanus can live for up to 30 years in the laboratory, but experiments using adults are typically performed in fish aged 6 months to 3 years. Fish can be recorded in a variety of tank sizes depending on the experiment and IR lighting allows for recordings during light and dark periods (**Figure 2A**). Individual arenas are labeled in tracking to allow for tracking of fish, and post-processing using a custom macro provides a readout of sleep (**Figure 2B,C**). Sleep is significantly reduced in Pachón, Molino, and Tinaja cavefish, compared to surface fish (**Figure 2D**). In addition, this system allows for recording multiple fish in a single arena (typically 10 gallon tanks), allowing for analysis of how social interactions affect sleep (**Figure 2E,F**). Social housing robustly reduces sleep in surface fish, without affecting sleep in Pachón cavefish (**Figure 2G**). The lack of effect in cavefish is likely due to a basement effect, where cavefish sleep little, particularly in the larger arenas used to examine social behavior.

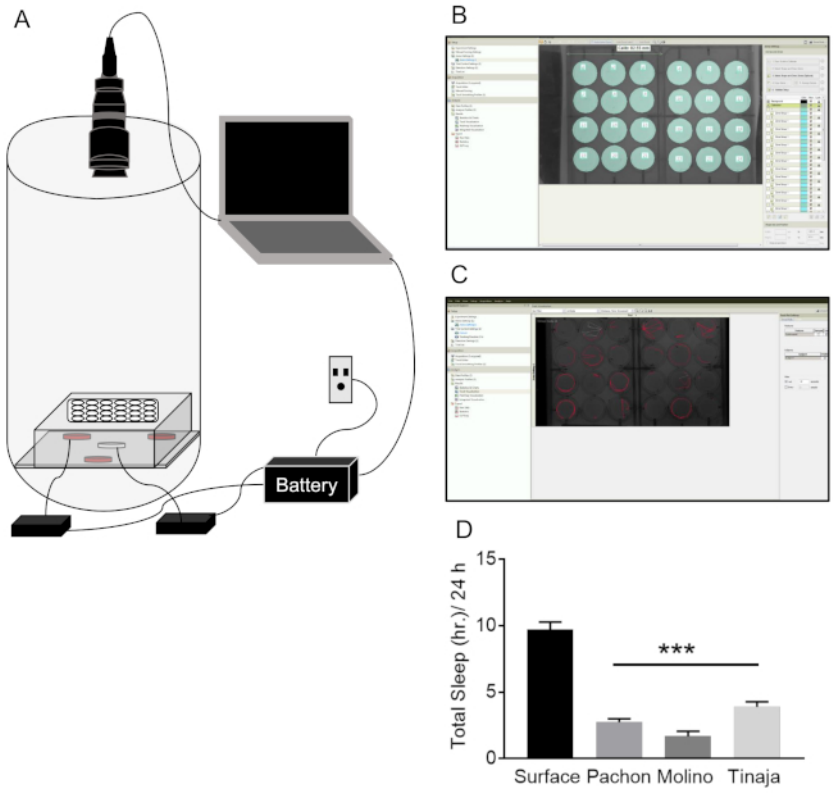

Figure 1: Recording sleep behavior in larval and juvenile *A. mexicanus.* (**A**) Schematic of larval sleep behavior setup: Larva are placed on a platform within a light-controlled tube. Infrared and white lighting systems sit below the fish at the bottom of the tube. An IR-pass camera sits at the top of the tube and is connected to a laptop on which the video is recorded. All powered systems (lighting and laptop) are plugged in to backup power. (**B**) Arena settings in tracking software. Individual larvae are kept in wells in a tissue culture plate, and arenas (cyan) are made for each animal. (**C**) Locomotor traces of fish locomotor behavior after acquiring the data in tracking software. Red traces represent 10 s of activity in 20-day old fish. (**D**) Resulting sleep data from tracking software. Juvenile cavefish converge upon reduced sleep behavior compared to surface fish morphs (One-way ANOVA $F(3, 116) = 76.12$; Dunnett post-hoc analysis was applied to compare each cavefish population with surface fish, $P < 0.001$). Please click here to view a larger version of this figure.

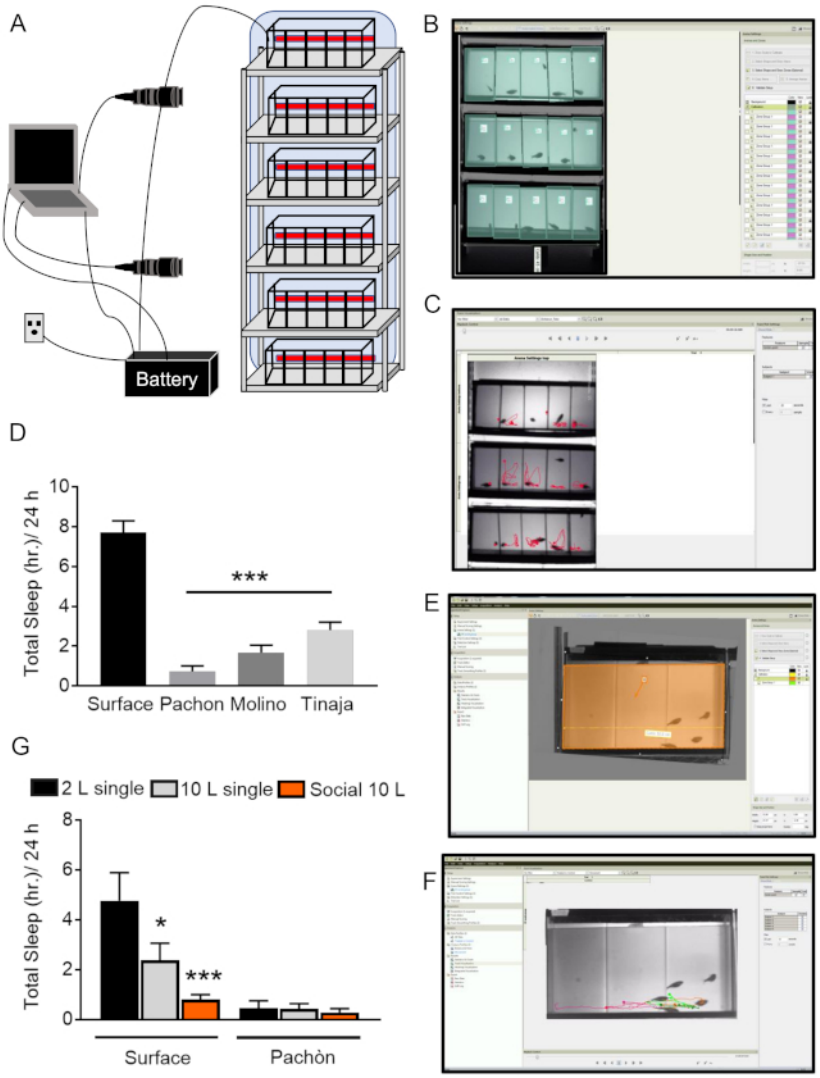

Figure 2: Adult *A. mexicanus* system for sleep and circadian behavior. (**A**) Diagram of sleep-recording system: Fish are held in tanks on a rack opposite of tracking cameras. An infrared lighting system is placed behind the fish tanks, while IR-pass cameras are attached to a laptop for recording behavior. All powered systems are plugged in to a backup battery system in case of power fluctuations. (**B**) Arena setup in tracking software. Individual fish are marked by creating separate arenas (cyan) to track locomotor behavior. (**C**) Representative locomotor tracks (red lines) of individual fish after acquiring a behavioral recording on tracking software. Traces represent 20 s of activity. (**D**) Total sleep duration over 24 h is significantly reduced in three distinct populations of cavefish compared to surface fish (One-way ANOVA $F(3, 106) = 52.66$; Dunnett post-hoc tests were applied between surface fish and each cave population, $P < 0.001$). (**E**) A single tank containing multiple fish in which one arena (orange) is made to track social interactions and sleep. (**F**) Locomotor traces of multiple fish after data acquisition in tracking software (each line color represents an individual fish). (**G**) Representative data of surface and Pachón cavefish in social sleep tracking. Surface fish significantly reduce sleep in 10 L tanks compared to 2 L arenas; Surface fish sleep is further reduced when fish are socially housed. Cavefish sleep is not significantly altered in any condition (Two-way ANOVA $F(2,46) = 4.545$; post hoc analysis was performed within each population to test the effect of the tank size and social state on total sleep 10 L single, $P = 0.013$; 10 L Social, $P = 0.0003$). Please click here to view a larger version of this figure.

Discussion

This protocol describes a custom system for quantifying sleep and locomotor activity in larval and adult cavefish. Cavefish have emerged as a leading model for studying the evolution of sleep that can be used to investigate the genetic and neural basis of sleep regulation[1]. The critical steps in this protocol include optimization of lighting and video quality in order to assure accurate tracking that is necessary to quantify sleep. The system for acquisition and analysis described here are fully functional, as are many other systems, both commercial and custom-built, to quantify locomotion and behavior[28,29,30]. The previous assay examining sleep in single fish can be extended to allow for analysis of group-housed fish. A significant consideration when trouble-shooting or designing assays is the confounds social behavior may have on the sleep of an individual. For

example, aggression is common in *Astyanax*, and aggression levels differ between surface fish and cavefish[31]. Optimizing the number of fish, size of the arena, and sex ratio, in order to minimize aggression will allow for reproducible measurements of sleep regulation.

A limitation of the technique, as described, is a lack of reliability following individual fish throughout the assay. Automated animal tracking will often switch animals when they come into close contact. This can be addressed by careful optimization of thresholds, or by manually correcting any switches. In addition, the system described is not a flow-through system, and therefore, water quality can become an issue after recordings lasting more than a few days. Other flow through systems have been described in zebrafish[13] and these could be readily applied to the sty of Mexican cavefish.

The methodology described is significant because of its broad applicability to measure behavior in diverse fish species. Sleep has yet to be characterized in almost any marine or fresh water fish including Sticklebacks, Cichlids, and swordtails[32,33,34]. The versatility of this system to measure sleep in *A. mexicanus* and other fish models may address diverse questions about the evolution and genetic underpinnings of sleep. The hardware associated with this system is highly cost-effective, making it highly accessible and providing potential for high throughput analysis of pharmacological and ecotoxilogical analysis of sleep and locomotor activity.

Disclosures

The authors declare they have no competing interests.

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