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RESEARCH ARTICLE

# The lateral line confers evolutionarily derived sleep loss in the Mexican cavefish

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## ABSTRACT

Sleep is an essential behavior exhibited by nearly all animals, and disruption of this process is associated with an array of physiological and behavioral deficits. Sleep is defined by changes in sensory gating that reduce sensory input to the brain, but little is known about the neural basis for interactions between sleep and sensory processing. Blind Mexican cavefish comprise an extant surface dwelling form and 29 cave morphs that have independently evolved increased numbers of mechanoreceptive lateral line neuromasts and convergent evolution of sleep loss. Ablation of the lateral line enhanced sleep in the Pachón cavefish population, suggesting that heightened sensory input underlies evolutionarily derived sleep loss. Targeted lateral line ablation and behavioral analysis localized the wake-promoting neuromasts in Pachón cavefish to superficial neuromasts of the trunk and cranial regions. Strikingly, lateral line ablation did not affect sleep in four other cavefish populations, suggesting that distinct neural mechanisms regulate the evolution of sleep loss in independently derived cavefish populations. Cavefish are subject to seasonal changes in food availability, raising the possibility that sensory modulation of sleep is influenced by metabolic state. We found that starvation promotes sleep in Pachón cavefish, and is not enhanced by lateral line ablation, suggesting that functional interactions occur between sensory and metabolic regulation of sleep. Taken together, these findings support a model where sensory processing contributes to evolutionarily derived changes in sleep that are modulated in accordance with food availability.

**KEY WORDS: Sensory systems, Sleep, *Astyanax mexicanus*, Convergent evolution, Starvation response, Behavioral plasticity**

## INTRODUCTION

A defining characteristic of sleep is an elevated arousal threshold, where a greater sensory stimulus is required to induce a response during sleep as a result of the gating of incoming sensory information (Campbell and Tobler, 1984; Hartmann, 1973). In mammals, auditory, visual and olfactory input potently impact sleep–wake regulation, suggesting that sensory processing is an important regulator of sleep duration and architecture (Velluti, 1997). Furthermore, both thalamus-dependent and thalamus-independent gating of sensory information attenuates signals from diverse sensory modalities in the mammalian cortex during sleep (Edeline et al., 2000; Livingstone and Hubel, 1981). The modulation of sensory processing in accordance with the sleep–wake state is conserved across phyla, from the nematode *Caenorhabditis elegans* to humans, revealing that sensory gating is an important regulator of the sleep–wake transition (Cho and Sternberg, 2014; Kisley et al., 2001). Here, we investigate the relationship between sleep loss and sensory adaptations in the blind Mexican cavefish, *Astyanax mexicanus* (De Philippi 1853), as a model for understanding the evolutionary relationship between sleep and sensory processing.

*A. mexicanus* consists of eyed 'surface' populations that inhabit rivers in the Sierra del Abra region of Northeast Mexico and 29 geographically isolated populations of cave morphs (Gross, 2012; Jeffery, 2009; Mitchell et al., 1977). The defined ecological habitat and evolutionary history provide a powerful model for investigating the mechanisms that underlie changes in sleep and sensory processing (Bibliowicz et al., 2013; Duboué et al., 2011; Varatharasan et al., 2009; Yoshizawa et al., 2010). Within the past 2–5 million years, at least five independent colonization events by two different migration waves of eyed surface fish have established independent cavefish populations in northeastern Mexico (Bradic et al., 2012; Coghill et al., 2014; Ornelas-García et al., 2008; Strecker et al., 2012). Despite this isolation, *A. mexicanus* surface fish and cavefish are interfertile, providing the opportunity to generate surface×cave and cave×cave hybrids that enable investigation of the genetic relationship between behavioral and physiological traits (Borowsky, 2008a; Wilkins, 1971, 1988).

The cave environment differs dramatically from the rivers inhabited by surface fish, and cavefish have evolved robust differences in foraging and feeding behavior, raising the possibility that differences in nutrient availability contribute to the evolution of sleep loss in cave populations (Keene et al., 2015). Furthermore, multiple cave populations have evolved substantial reductions in sleep duration and enhanced sensory systems, suggesting that sleep loss is evolutionarily and functionally associated with sensory and metabolic changes (Aspiras et al., 2015; Duboué et al., 2011; Elipot et al., 2013; Kowalko et al., 2013; Yoshizawa et al., 2010).

The lateral line is composed of neuromasts, which contain mechanosensory hair cells that relay information from the body exterior to the brain (Baker and Montgomery, 1999; Bleckmann, 2008; Boord and Montgomery, 1989; Coombs et al.). Neuromasts are categorized into two classes: large linearly distributed canal neuromasts (CNs) that lie deep in tissue and widely distributed superficial neuromasts (SNs). In numerous cavefish populations, the number of SNs is increased compared with levels in surface fish, whereas no differences in CNs are detected between cave and surface populations (Schemmel, 1967; Yoshizawa et al., 2010). To better understand how sensory processing regulates sleep, we investigated the association between the evolution of enhanced

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**Received 27 June 2016; Accepted 23 October 2016**



Journal of Experimental Biology

Journal of Experimental Biology (2017) 220, 284-293 doi:10.1242/jeb.145128

lateral line sensitivity and sleep loss. Our findings reveal that the lateral line regulates sleep loss in Pachón cavefish and we show that distinct mechanisms underlie the convergent evolution of sleep loss in independently evolved cavefish populations. We also show that starvation dramatically increases sleep, and there is no additive effect of lateral line ablation and starvation in Pachón cavefish, suggesting that starvation and lateral line ablation promote sleep through a shared mechanism. Taken together, these findings provide insight into the evolutionary mechanisms regulating sleep loss in cavefish and offer a model to examine the relationship between sensory processing and sleep.

## MATERIALS AND METHODS

### Fish maintenance and rearing

Animal husbandry was carried out as previously described (Borowsky, 2008b) and all protocols were approved by the IACUC at the University of Nevada-Reno and Florida Atlantic University. Fish were housed in the University of Nevada-Reno or Florida Atlantic University core facilities at 21±1°C constant water temperature throughout rearing and for behavior experiments (Borowsky, 2008b). Lights were on a 14 h light:10 h dark cycle that remained constant throughout the animal's lifetime. Light intensity was maintained between 25 and 40 Lx for both rearing and behavior experiments. All fish used for experiments were raised to adulthood and housed in standard 18–37 liter tanks. Adult male and female fish aged 1–2 years were fed a diet of black worms (Aquatic Foods, Fresno, CA) to satiation twice daily at zeitgeber time (ZT) 2 and ZT12 and standard fish food (Tetramin Pro) during periods when fish were not being used for behavior experiments or breeding. There were no sex-specific effects on basal sleep or in any of the treatment groups. The surface, Pachón, Los Sabinos, Tinaja and Molino strains are progeny from wild-caught fish collected with permits supplied by Richard Borowsky (NYU) and Dr William Jeffery (University of Maryland). The commercial Chica fish are descendants of fish originally described (Hubbs and Innes, 1936) and purchased (aquariumfish.net) in 2012.

### Sleep behavior

Fish were recorded in standard conditions in 10 liter tanks with custom-designed partitions that allowed 5 fish (2 liters per fish) to be individually housed in each tank as previously described (Yoshizawa et al., 2015). Recording chambers were illuminated with custom-designed IR LED source (Infrared 850 nm 5050 LED Strip Light, Environmental Lights). After a 4–5 day acclimation period, behavior was recorded for 24 h from ZT0–ZT2. Videos were recorded at 15 frames $s^{-1}$ using a USB webcam (LifeCam Studio 1080p HD Webcam, Microsoft) fitted with a zoom lens (Zoom 7000, Navitar). An IR high-pass filter (Edmund Optics Worldwide) was placed between the camera and the lens to block visible light. Videos were recorded using Virtualdub, a video-capturing software (v.1.10.4) and were subsequently processed using Ethovision XT 9.0 (Noldus, IT). Water temperature and chemistry were monitored throughout recordings, and maintained at standard conditions in all experiments. Ethovision tracking was set up as previously described (Yoshizawa et al., 2015). Data were then processed using Perl scripts (v.5.22.0, developed on-site) and Excel (Microsoft) (Yoshizawa et al., 2015). These data were used to calculate sleep information by finding bouts of immobility of 60 s and greater, which are highly correlated with increased arousal threshold – one of the hallmarks of sleep (Yoshizawa et al., 2015). Zone analysis was carried out using Ethovision XT 9.0 by designing three equal zones (top, middle and bottom) for each individual within their respective arena setup. Raw data were exported to Excel and then matched with sleep and activity data in 10 min bins. To characterize total time in each zone, the sum of periods of activity was calculated over the 24 h testing period. To determine waking and sleeping zone preference, time within each zone was matched to waking or sleeping activity to segregate each behavior to zone preference.

### Starvation experiments

Fish were housed individually in testing tanks throughout the duration of the food deprivation experiments. Sleep was measured prior to the onset of starvation, and on days 0, 5, 10, 20 and 30. To account for housing effects, control fish from each population were maintained in testing tanks and fed black worms daily using a standard protocol. The effects of starvation were determined by comparing starved with fed controls of the same genotype by repeated measure analysis of starvation days 0, 5, 10, 20 and 30.

### Vital dye labeling

Fish were treated for 1 h with 0.05% DASPEI (2-4-dimethylaminostyryl-N-ethylpyridinium iodide) solution (Sigma Aldrich), a dye that specifically labels both SNs and CNs (Van Trump et al., 2010). Although the exact mechanism of DASPEI labeling is unknown, the dye is thought to enter cells through transduction channels and apical endocytosis, allowing its uptake by active hair cells via transduction-dependent mechanisms and making it highly specific for labeling intact neuromasts of the lateral line (Van Trump et al., 2010). After staining, fish were anesthetized for 30 s in an ice bath. Neuromasts were observed using a microscope (Leica M205 FA) set to 40× magnification, 5.17 mm FOV, with a GFP filter set (excitation 450–490 nm). Photographs were captured with a high-resolution CCD camera (ProgRes C14) with ImagePro software (v.9.1). All images were acquired within ∼4 h of the end of baseline behavior recordings. All experimental fish were placed back in their recording tanks and given approximately 24 h to recover before any further testing was done. Whole-body images represent tiled images merged in Photoshop CS6 (Adobe).

### Analysis of neuromast number

Images acquired by DASPEI staining were analyzed to determine the number and diameter of neuromasts of each fish. Each individual was imaged on both sides of their body, producing two cranial and trunk images. The average of these two images was used as the final reported value for the cranial and trunk neuromast counts. These images were processed using ImageJ (1.48v) paired with a custom analysis macro. This program allowed for accurate counting by setting a threshold for light intensity, as well as absolute size, thereby allowing only actual neuromasts to be counted, thus eliminating any background noise. The raw individual data was exported to the Excel analysis macro (v.1.2, developed on-site) to extract all individual data and compile it into one file.

### Lateral line ablation

To measure the effects of lateral line ablation on sleep in fed and starved animals, 28 day starved fish or fed controls were treated with 0.002% gentamicin sulfate (Sigma Aldrich, 1405-41-0) (Van Trump et al., 2010). Following 24 h of recovery, fish were tested for sleep at day 30 of starvation along with fed controls. Because the lateral line regenerates within a few days of gentamicin treatment (M.Y., unpublished results), it was not possible to measure the effects of treatment over an entire 30 day period.

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Selective SN ablation was achieved by treating with 0.5 µl Vetbond™ superglue (3M) allowing for localized ablations to the cranial and trunk of the lateral line. Fish treated with Vetbond™ or gentamicin were housed in separate tanks for at least 1 month after treatment in order to avoid contamination. Lateral line re-growth was confirmed with DASPEI staining to confirm that there were no long-term effects from the ablation treatments.

### Statistics

Repeated measure two-way ANOVA tests were carried out to test the effects of gentamicin and Vetbond™ among different groups and populations on behavior. Significance for all tests was set at $P<0.05$. No fish were excluded from analysis and the experimenter was blinded to the treatment group in all cases. When the ANOVA test detected significance, the Holm–Šídák multiple comparisons post-test was carried out to correct for the number of comparisons. For backcross hybrid grouping by neuromast number, individual hybrids were compared with the mean values of pure Pachón and surface fish. Each individual hybrid was compared by non-parametric *t*-test and grouped according to significance. If fish were not significantly different from the interaction they were being tested against, they would be grouped as being 'like' that group. If individuals were statistically different from both Pachón and surface fish, they were labeled as intermediates. All statistics were carried out using inStat software (GraphPad Software 6.0) or SPSS 22.0 software (IBM).

## RESULTS

The number and size of lateral line neuromasts are increased in multiple cavefish populations compared with surface fish (Yoshizawa et al., 2010, 2012), raising the possibility that sleep loss is related to enhanced mechanosensation. To directly assess the functional relationship between sleep and the lateral line, we examined the effect of lateral line ablation on sleep in Pachón cavefish. In agreement with previous findings, staining with DASPEI revealed greater numbers of superficial neuromasts in the cranial and trunk regions of Pachón cavefish compared with surface fish (Fig. S1; Yoshizawa et al., 2010). To test the hypothesis that sensory input from the lateral line suppresses sleep, we chemically ablated the entire lateral line by bathing fish in the ototoxic antibiotic gentamicin (Van Trump et al., 2010). Gentamicin disrupts the hair cells in lateral line neuromasts (Van Trump et al., 2010) and treatment resulted in complete ablation of the lateral line in both surface fish and Pachón cavefish. To allow for comparison of treatment effect within individual fish, sleep and locomotor activity were measured for 24 h prior to gentamicin treatment (−gentamicin group) to establish baseline behavior. After treatment, fish were given 48 h to recover and acclimate to the testing chamber, followed by 24 h of sleep and locomotor measurements (+gentamicin group). For all experiments, sleep was measured as previously described (Yoshizawa et al., 2015). The effect of gentamicin treatment was validated by DASPEI staining following the completion of behavioral testing (Fig. 1A–D). Sleep was significantly increased in Pachón cavefish following gentamicin treatment, whereas sleep was not affected in surface fish (Fig. 1E and Fig. S1). The total sleep duration of gentamicin-treated Pachón cavefish did not differ from that of treated or untreated surface fish (Fig. 1E), suggesting that enhanced sensory input underlies the evolutionarily derived sleep loss in Pachón cavefish.

Waking activity, defined as the amount of activity while the animal is awake, corrects for differences in sleep and can be used to differentiate between lethargy or hyperactivity and changes in sleep. It is unlikely that the sleep-promoting effect of gentamicin is due to toxicity or lethargy induced by drug treatment because treatment did not affect waking activity in Pachón cavefish or surface fish (Fig. 1F). Taken together, these findings reveal a wake-promoting role for the lateral line in Pachón cavefish.

Confinement in small arenas induces stress in numerous fish species (Gallo and Jeffery, 2012; Ramsay et al., 2009; Rey et al.,

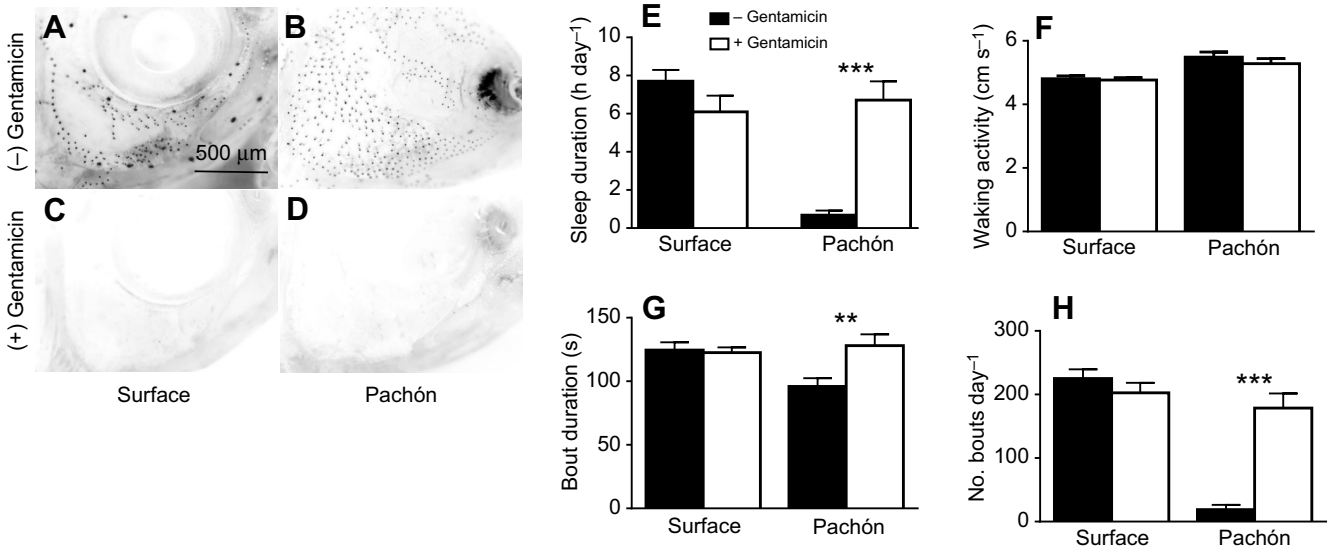


**Fig. 1. Sensory neuromasts are wake-promoting in Pachón cavefish.** (A-D) Micrographs of surface fish and Pachón cavefish before and after gentamicin treatment. Surface fish (A) harbor significantly fewer SNs (small stained dots) compared with Pachón fish (B) in the cranial region. No differences were detected in CNs (large stained dots). (C,D) Gentamicin treatment fully ablates both SNs and CNs in both surface and Pachón cavefish. (E) Sleep duration in Pachón cavefish and surface fish before and after gentamicin treatment. (F) Waking activity in surface and Pachón cavefish before and after gentamicin treatment. (G) Bout duration in Pachón cavefish and surface fish before and after gentamicin treatment. (H) Number of bouts per day in Pachón cavefish and surface fish before and after gentamicin treatment. Data are means±s.e.m. $^{**}P<0.01$, $^{***}P<0.001$ (two-way ANOVA). Pachón cavefish, $N=30$; surface fish, $N=29$.

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2015), raising the possibility that the lateral line suppresses sleep through activation of a stress response in the context of the arenas used to measure sleep. To determine whether the effect of gentamicin on sleep results from the confinement stress experienced in the standard 2 liter testing arena, we measured sleep of individual fish in a 10 liter arena. In the larger arena, sleep was reduced, and waking activity was increased in both surface fish and Pachón cavefish, which is likely to reflect increased foraging behavior in a large arena (Fig. S2A,B). Treatment with gentamicin significantly increased sleep in Pachón cavefish, without affecting sleep in surface fish (Fig. S2C), phenocopying the effect of treatment observed in 2 liter arenas. Again, there was no effect of gentamicin treatment on waking activity in surface fish or Pachón cavefish (Fig. S2D), supporting the notion that the sleep increases in Pachón cavefish are not due to lethargy. Therefore, the effect of gentamicin on sleep is not dependent on arena size, revealing that the wake-promoting role of the lateral line in Pachón is not due to confinement-induced stress.

In diverse species including those from *Aplysia*, *Drosophila* and *Danio* genera, sleep is associated with a preferred arena location, and the majority of sleep episodes in adult zebrafish occur at the bottom or top of a tank (Donelson et al., 2012; Vorster et al., 2014; Yokogawa et al., 2007). To confirm that the increase in sleep induced by lateral line ablation does not result from a generalized disruption of locomotion, we investigated whether sleep location is affected by gentamicin treatment. Surface and Pachón cavefish were bathed in gentamicin and their location was measured over a 24 h period in standard 2 liter tanks. Each arena was divided into three zones (top, middle and bottom) and the time spent in each zone was measured. While surface fish spent more time in the bottom portion of the tank, Pachón cavefish spent equal time in all areas (Fig. S2E). Gentamicin treatment had no location effect on surface fish, whereas it increased the length of time cavefish spent in the bottom portion of the testing arena, suggesting that the change in tank location is due to increased sleep duration (Fig. S2E). To determine tank position during sleep, we selectively quantified location during sleep periods in gentamicin-treated and control fish. In both cases, surface and Pachón cavefish spent more time in the bottom area of the tank and there was no effect of gentamicin treatment on arena location in treated or untreated Pachón cavefish (Fig. S2F). These findings support the notion that gentamicin treatment does not generally disrupt swimming behavior and that the increased sleep observed following treatment does not result from general perturbation of locomotor systems.

The number of sleep bouts and length of individual bouts provide a measure of sleep consolidation (Duboué et al., 2011). Sleep loss in adult Pachón cavefish results primarily from a reduction in the number of total sleep bouts compared with that in surface fish, suggesting that sleep initiation, but not maintenance, is impaired in cavefish (Yoshizawa et al., 2015). Analysis of sleep architecture revealed treatment with gentamicin significantly enhanced bout number in Pachón cavefish, without impacting sleep in surface fish (Fig. 1H). Gentamicin treatment also significantly increased bout duration in Pachón cavefish (Fig. 1G), indicating that ablation of the lateral line promotes sleep by increasing bout number and bout duration. No differences in bout duration were detected between gentamicin-treated Pachón cavefish and treated or untreated surface fish, supporting the notion that gentamicin restores surface-fish-like sleep duration and architecture in Pachón cavefish. These findings indicate that the evolutionarily derived reduction in sleep duration is dependent on gentamicin-sensitive lateral line neuromasts.

Multiple populations of independently evolved *A. mexicanus* cavefish were found to sleep less than surface fish (Fig. 2A,B), revealing the convergent evolution of sleep loss in cave populations (Duboué et al., 2011; Yoshizawa et al., 2015). Furthermore, the convergent evolution of enhanced lateral line function or increased neuromast morphology has been reported in Pachón, Los Sabinos, Piederas, Tinaja and Molino populations of cavefish (Kowalko et al., 2013; Yoshizawa et al., 2010). Genomic and geological evidence suggest Pachón, Los Sabinos, Tinaja and Chica cavefish are more ancient lineages that evolved independently from Molino cavefish (Borowsky and Cohen, 2013; Dowling et al., 2002; Ornelas-García et al., 2008; Wilkins, 1988). One possibility is that increased sensory input from lateral line neuromasts contributes to sleep loss across cavefish populations. This notion predicts that ablation of the lateral line would enhance sleep in all cavefish populations. Alternatively, sleep loss may have evolved through different mechanisms in independent cavefish populations. To distinguish between these possibilities, sleep was analyzed in gentamicin-treated and untreated controls in Molino, Tinaja, Los Sabinos, and a commercial Chica cavefish line (Fig. 2A). In cave populations pretreatment, total sleep was moderately reduced in the Tinaja, Los Sabinos and Chica cavefish compared with surface fish, while a greater reduction was observed in Molino and Pachón cavefish (Fig. 2B, black bars). In contrast to the striking effects of gentamicin treatment on Pachón cavefish sleep, there was no effect of gentamicin treatment on sleep duration in any of the other cavefish, indicating that the sleep-promoting effects of lateral line ablation are not generalizable across cavefish populations (Fig. 2B). In addition, we observed no effects of gentamicin treatment on either sleep duration or bout number in Molino, Tinaja, Los Sabinos or Chica cavefish (Fig. 2C,D). Together, these findings indicate that while lateral line sensory neuromasts are required for sleep loss in the Pachón population, the sleep loss observed in Molino, Tinaja, Los Sabinos, and Chica populations is independent of the lateral line. Therefore, distinct mechanisms regulate evolutionarily derived sleep loss in different cavefish populations.

The lateral line is composed of the superficial neuromasts (SNs) and the canal neuromasts (CNs), both of which are ablated by gentamicin treatment. Therefore, the sleep-promoting effects of gentamicin exposure could be explained by ablation of the SNs, CNs, or both. Moreover, chemical ablation of the lateral line using gentamicin indiscriminately ablates neuromasts along the entire length of the animal, without providing anatomical specificity (Van Trump and McHenry, 2013; Van Trump et al., 2010). Application of Vetbond tissue adhesive to the body surface of the fish selectively ablates SNs without disrupting CN function (Van Trump et al., 2010; Yoshizawa et al., 2010). To localize the wake-promoting neuromasts, Vetbond was applied to the trunk, cranial region or belly (Fig. 3A). In agreement with previous findings, Vetbond efficiently ablated SNs, leaving CNs intact in both surface and Pachón populations (Van Trump et al., 2010; Yoshizawa et al., 2010). Sleep was measured prior to Vetbond application and animals were then allowed a 48 h recovery and acclimation period, followed by 24 h of recording post-treatment. The region and effectiveness of Vetbond treatment was confirmed by DASPEI staining in all fish following behavioral testing. As predicted, there was no effect of Vetbond application to the belly, trunk or cranial regions on sleep duration in surface fish (Fig. 3B). Conversely, in the Pachón cave population, sleep was significantly increased following application to the cranial or the trunk regions, while Vetbond application to the belly, where there are few SNs, did not increase sleep (Fig. 3B). Consistent with gentamicin treatment,

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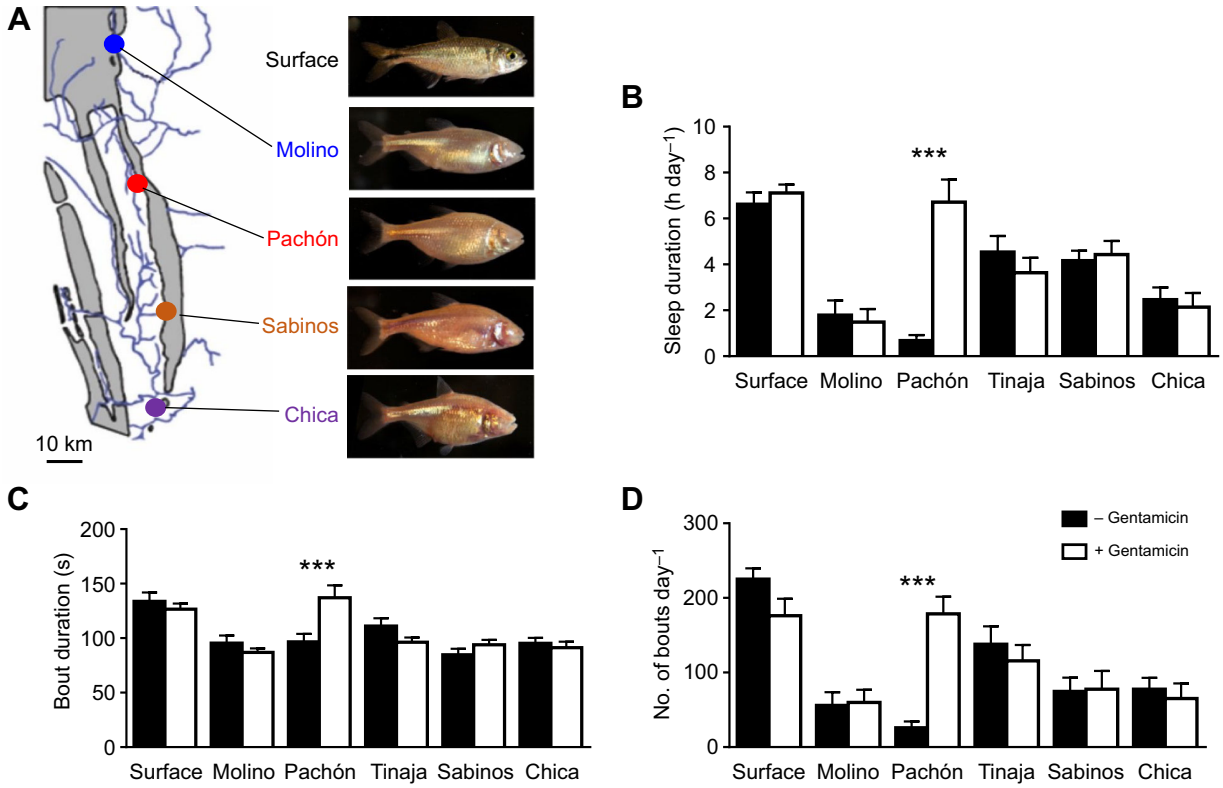


**Fig. 2. Lateral line-dependent sleep loss is unique to the Pachón population.** (A) Geographic map of the Sierra del Abra region of Northeast Mexico (left) and image (right) of each *A. mexicanus* population used in this study. The Pachón, Tinaja, Los Sabinos and Chica populations are derived from an older surface fish stock while the Molino population is suggested to have arisen from a more recent invasion and newer surface population. (B) In pre-ablation fish (black), sleep is reduced in all five cave populations compared with surface fish. There was no effect of gentamicin treatment on sleep but sleep was significantly increased in the Pachón population following gentamicin treatment. (C,D) Bout duration (C) and bout number (D) increased only in Pachón cavefish following gentamicin treatment. Data are means±s.e.m. ***$P$<0.001 (two-way ANOVA). Surface, $N$=54; Molino, $N$=12; Pachón, $N$=30; Tinaja, $N$=24; Los Sabinos, $N$=23; Chica, $N$=14.

Vetbond application to the trunk and cranial regions significantly increased bout duration and bout number in Pachón cavefish, while no effect on sleep architecture was observed in surface fish or belly application in Pachón cavefish (Fig. 3C,D). These findings indicate that the SNs in the cranial and trunk regions are wake promoting and are required for evolutionarily derived sleep loss in Pachón cavefish.

The wake-promoting function of the lateral line in Pachón cavefish could be due to an increased number of neuromasts in populations of Pachón cavefish, or altered processing of sensory information within the brain. To address this question, we investigated the functional relationship between sleep and neuromast number by analyzing these parameters in individual fish (Fig. 4A and Fig. S3A). There is significant within-population variation in sleep duration and neuromast number in surface fish and cavefish, providing the opportunity to examine the relationship between these traits. There was no correlation between cranial or trunk neuromast number and sleep duration in parental surface fish, whereas the number of neuromasts in the cranial and trunk regions of Pachón cavefish were correlated with reduced sleep (Fig. 4A,B, Fig. S3A,B, Fig. S4). To increase the variability in both sleep and neuromast number, we generated surface×cave hybrid fish by backcrossing F1 Pachón×surface hybrids to surface fish. Labeling neuromasts with DASPEI revealed high variability in the numbers of cranial and trunk neuromasts of backcross hybrids compared with pure Pachón or pure surface fish (Fig. 4C,D, Fig. S3C,D, Fig. S4). In backcross hybrids, reduced sleep duration correlated with cranial and trunk SN number, providing further evidence that the lateral line SNs are wake promoting (Fig. 4E, Fig. S3E,F, Fig. S4). The ability to generate hybrids with variable morphology and sleep patterns allows us to segregate how traits drive behavior. Binning hybrid fish by neuromast morphology into surface-like, intermediate or Pachón-like (see Fig. 4D) revealed that significantly shorter sleep was found in the fish with Pachón-like neuromasts compared with surface-like neuromasts (Fig. S4). These findings add support to the notion that enhanced sensory input from the lateral line underlies evolutionarily derived sleep loss.

While the adaptive value of sleep loss remains unclear, it has been hypothesized that sleep loss represents a mechanism to increase foraging, and an abundance of evidence suggests sleep is acutely regulated by food availability and metabolic state (Capellini et al., 2008; Danguir and Nicolaidis, 1979; Horne, 2009; Yurgel et al., 2015). We hypothesized that sleep in cavefish would vary in accordance with nutrient availability. To test this assertion, we measured the effects of starvation in surface, Pachón and Molino cavefish over a 30 day period in an effort to model the nutrient paucity that occurs during the dry season from approximately May to January. We directly compared each population with fish housed under similar conditions fed daily, which is likely to mimic the wet season when food is more plentiful (Fig. 5A). While starvation did not significantly affect sleep duration in surface fish, it increased sleep in Pachòn and Molino cavefish at 30 days of starvation (Fig. 5B). These findings provide evidence for evolved differences in sleep–metabolism interactions in the cavefish, and indicate that sleep loss provides a mechanism for increasing foraging when food is available.

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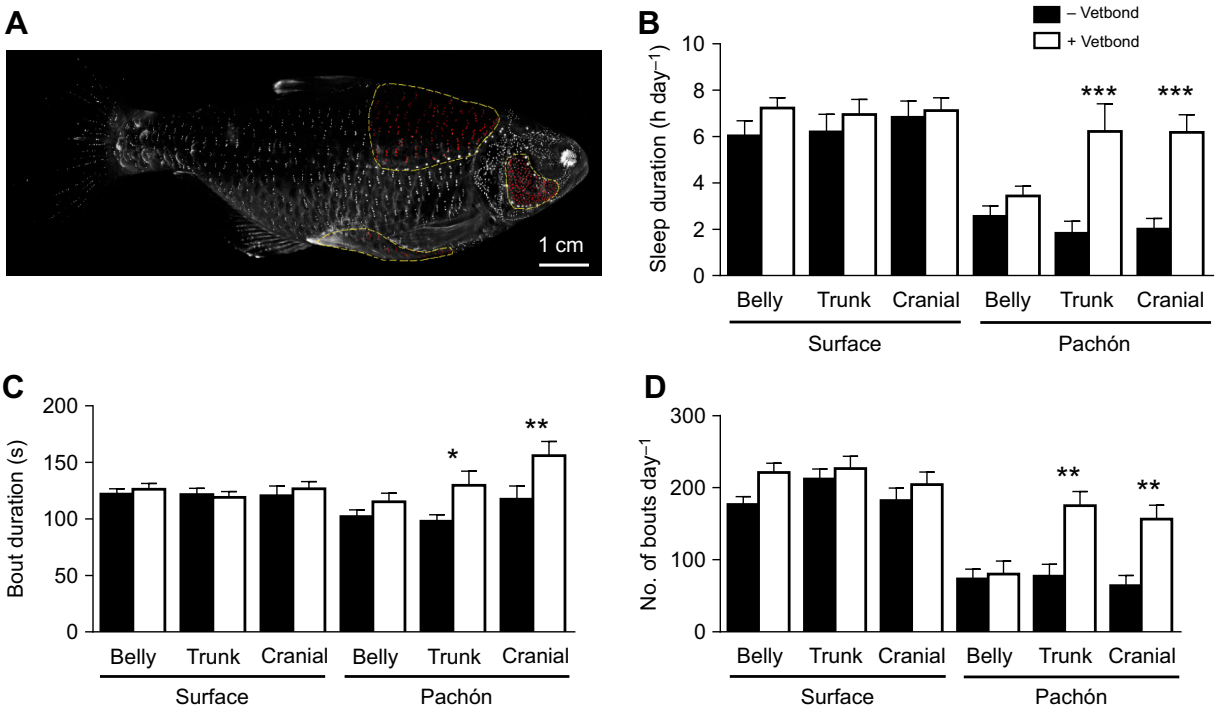


**Fig. 3. Superficial neuromasts in the trunk and cranial regions promote sleep loss in Pachón cavefish.** (A) Representative cavefish after ablation of superficial neuromasts. DASPEI labels SNs (white) and ablated SNs are pseudocolored (red). Dashed lines represent the region of Vetbond application. (B) The effects of Vetbond treatment on sleep duration in surface and Pachón fish. (C,D) Effects of Vetbond application on bout duration (C) and number of bouts (D). Data are means±s.e.m. **P*<0.05, ***P*<0.01, ****P*<0.001 (two-way ANOVA). Surface fish: belly, *N*=18; trunk, *N*=17; cranial region, *N*=19. Pachón cavefish: belly, *N*=32; trunk, *N*=18; cranial region, *N*=26.

These findings suggest that both starvation and lateral line ablation increase sleep duration, raising the possibility that sensory-dependent sleep is influenced by metabolic state. To determine whether similar processes increase underline starvation- and lateral line ablation-induced sleep, we measured the effects of gentamicin treatment on sleep in fed fish and in fish starved for 30 days (Fig. 5C). Consistent with previous findings, gentamicin treatment did not affect sleep in starved surface fish, whereas sleep was increased in both starved or gentamicin-treated Pachón cavefish (Fig. 5C). In Pachón cavefish, there was no additive effect of starvation and gentamicin treatment, supporting the notion that conserved mechanisms underlie sensory and metabolism-induced increases in sleep (Fig. 5C). We bolster support for previous findings, indicating that starvation, lateral line ablation or a combination of the two does not alter waking activity in surface or Pachón cavefish, supporting the notion that changes in sleep are not due to generalized deficits in locomotor activity or lethargy (Fig. 5D). Taken together, these findings suggest that the evolution of sleep loss in cavefish may be associated with metabolic state and food availability.

We have demonstrated that ablation of the lateral line restores sleep in Pachón cavefish and that sleep loss is negatively correlated with the number of neuromasts, suggesting that the evolution of enhanced lateral line sensitivity underlies sleep loss. Ablation of the lateral line does not promote sleep in several other cave populations, suggesting that multiple mechanisms underlie the convergent evolution of sleep loss in the Mexican cavefish. Furthermore, our findings indicate that sleep loss is associated with food availability, and provide evidence for functional interactions between lateral-line-dependent regulation of sleep and metabolic state.

## DISCUSSION

Across phyla, an elevated arousal threshold is a defining hallmark of sleep, implicating the processing of sensory information in the maintenance and initiation of sleep (Hendricks et al., 2000; Prober et al., 2006; Raizen et al., 2008). Our findings provide multiple lines of evidence that enhanced sensory input from the lateral line underlies sleep loss in Pachón cavefish. Ablation of all sensory neuromasts with the ototoxic antibiotic gentamicin and selective ablation of superficial neuromasts with Vetbond restored sleep duration and architecture in Pachón cavefish to levels similar to that seen in surface fish. Furthermore, the number of sensory neuromasts in the trunk and cranial regions was increased in Pachón cavefish and was correlated with sleep duration in cave-like hybrid fish. No effect of lateral line ablation on sleep was observed in surface fish, suggesting that the role of the lateral line in sleep regulation is a gain-of-function phenotype in Pachón cavefish and is independent of mechanisms regulating sleep in surface fish. These findings suggest that evolutionarily derived enhancements in sensory processing can result in sleep loss.

### Mechanistic differences in sleep loss between cavefish populations

Our findings indicate that the biological basis for sleep loss in Pachón cavefish differs from other populations tested. Morphological and behavioral analyses have identified dimorphisms associated with parallel evolution in different cavefish populations (Gross and Wilkens, 2013; Jeffery, 2009). Of the five cavefish populations assayed for sleep, all exhibited reduced sleep compared with surface fish. However, the Molino, Tinaja, Los Sabinos and commercial Chica cavefish populations were not sensitive to lateral line ablation, highlighting mechanistic

Journal of Experimental Biology (2017) 220, 284-293 doi:10.1242/jeb.145128

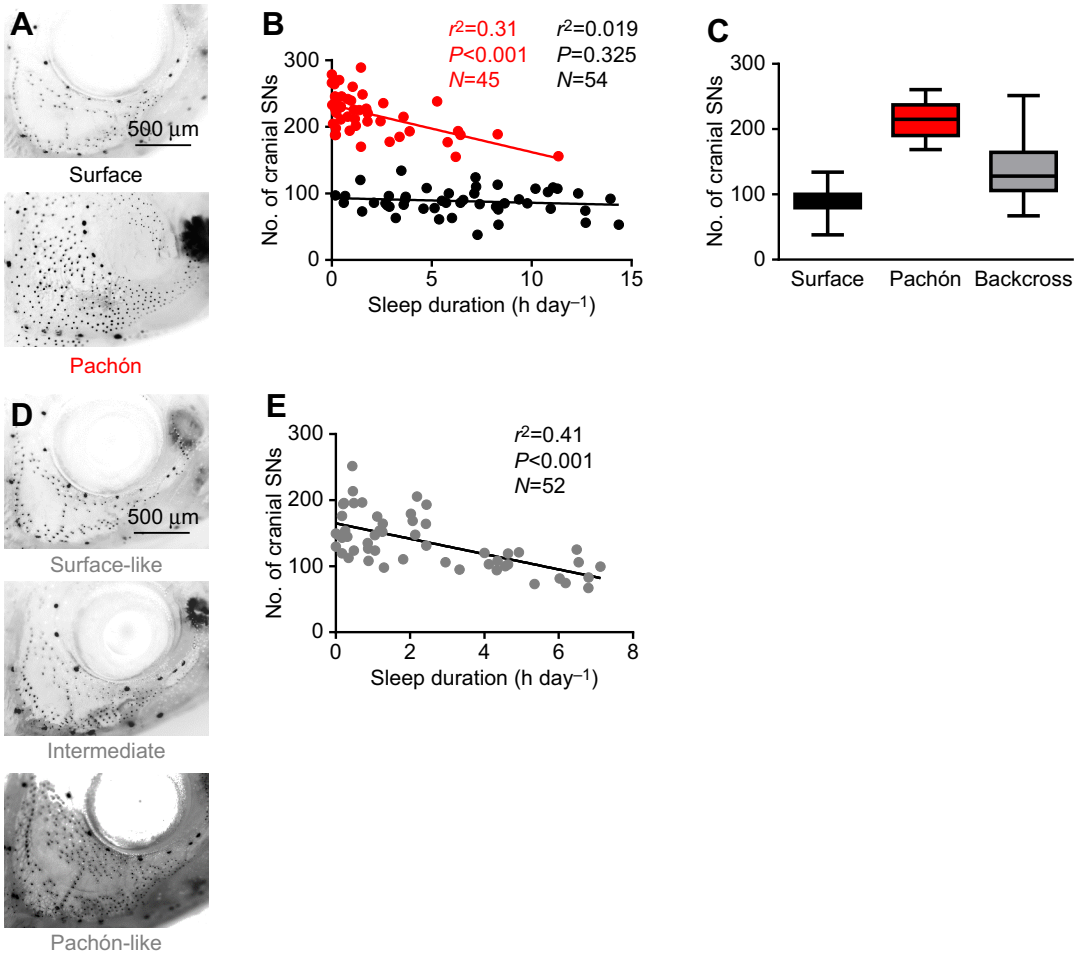


**Fig. 4. Superficial neuromasts in the cranial region correlate with sleep in Pachón cavefish and hybrid populations.** (A) SNs in the cranial region of surface fish and Pachón cavefish, labeled with DASPEI. (B) Regression analysis shows a correlation between sleep duration and cranial SN number in Pachón fish (red) but not surface fish (black). (C) Grouped analysis of lateral line morphology (number of cranial SNs) among surface, Pachón and backcross hybrids. Boxes and whiskers represent minimum, maximum, median, and upper and lower quartiles. Surface *N*=32; Pachón *N*=30; Backcross *N*=39. (D) Images representing different phenotypes in the cranial region in Pachón×surface backcross fish where the SN morphology was highly variable. (E) Increased cranial SN number is associated with reduced sleep in Pachón×surface backcross fish.

differences in the evolution of sleep loss between cavefish populations. Of the lineages tested for sleep, the Molino population is most distantly related to Pachón, originating from a geographically distinct region of Mexico and a more recent surface fish invasion compared with the older stocks of the Sierra del Abra region that include Pachón, Tinaja, Los Sabinos and Chica (Bradic et al., 2012; Coghill et al., 2014; Strecker et al., 2012).

Despite close evolutionary relationships between older populations of Sierra del Abra cavefish, many ecological factors differ between subterranean populations. For example, food availability differs between caves, depending on a number of ecological factors, including the presence of bat populations and seasonal flooding (Mitchell et al., 1977). Furthermore, early reports suggested that there is less seasonal flooding in the Pachón cave compared with other caves, resulting in lower food availability (Hüppop, 1986; Mitchell et al., 1977; Wilkens and Hüppop, 1986). This probably resulted in broader metabolic changes that may have contributed to the mechanisms underlying sleep loss. For example, metabolic rate is lower in Pachón cavefish than in other known cavefish populations, indicating distinct or more significant metabolic adaptations in Pachón cavefish (Hüppop, 1986). Indeed, our findings indicate that Pachón cavefish sleep less than closely related Tinaja and Los Sabinos cavefish populations. Understanding the ecological differences between these caves may be informative in understanding the mechanistic differences underlying sleep loss in their cavefish populations.

A number of factors indicate the geological uniqueness of the Pachón cave that may provide insight into the mechanistic differences observed in sleep loss. The Pachón cave is geologically isolated from other caves in the Sierra del Abra, making introgression with other cavefish populations unlikely (Bradic et al., 2012; Mitchell et al., 1977). The Pachón cave was probably formed from a perched pool or lake (Mitchell et al., 1977) and it has been suggested that its relatively high elevation is indicative that it is more ancient than other fish caves in the region (Mitchell et al., 1977). Unlike most other caves in the region, the Pachón cave has a remarkably shallow depth of only 8 m, compared with Molino (138 m), Tinaja (82 m) and Los Sabinos (96 m). Geological evidence indicating that the Pachón cave is unique is further supported by more recent genomic studies. Analysis of microsatellites from 12 cavefish populations revealed significant differences between Pachón and all other cavefish populations tested, suggesting isolation and a possible

Journal of Experimental Biology (2017) 220, 284-293 doi:10.1242/jeb.145128

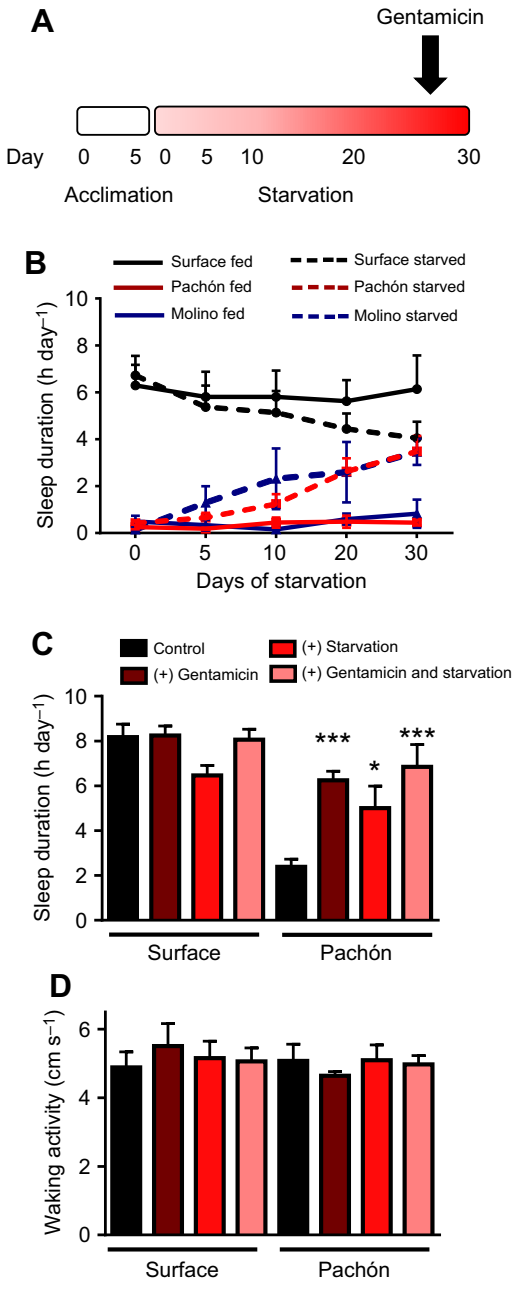


**Fig. 5. Starvation-induced sleep underlies sensory regulation of sleep loss in Pachón cavefish.** (A) Schematic diagram of experimental design for starvation experiments. After acclimation, fish were separated into fed and starved groups for 30 days and regular behavior recorded. For gentamicin experiments, the drug was treated at day 28 in order to measure the day 30 response. (B) The effects of starvation on sleep for surface fish, and Molino and Pachón cavefish (two-way ANOVA, $F_{(5,73)}$=24.04, $P$<0.001). Beginning at day 30, sleep significantly increased in both starved Molino ($P$=0.036, $N$=10) and Pachón ($P$=0.025, $N$=18) cavefish compared with fed controls ($N$=20). (C,D) Total sleep duration (C) and waking activity (D) in response to starvation and gentamicin treatment in surface fish and Pachón cavefish. $N$=10 fish for each group. Data are means±s.e.m. *$P$<0.05, ***$P$<0.001 (two-way ANOVA).

independent origin of this population (Bradic et al., 2012). Therefore, differences in ecology, geology and evolution are likely to be contributing factors to the wake-promoting role of the lateral line in the Pachón cavefish population that is not present in other cave populations tested.

### Localization of wake-promoting neuromasts

Our findings provide evidence that lateral line neuromasts in the trunk and cranial regions are wake-promoting in Pachón cavefish and probably result in enhanced lateral line sensitivity to water flow (Elder and Coombs, 2015; Tan et al., 2011; Yoshizawa et al., 2010). Ablating either the trunk or cranial populations of SNs in Pachón cavefish restores sleep to surface fish levels, suggesting input from both populations is required to inhibit sleep. Individual ablation of the trunk and cranial regions in Pachón cavefish results in similar sleep duration to that observed in gentamicin-treated fish with both regions ablated, indicating that the effects of ablating both regions are not cumulative.

The lateral line modulates numerous sensory behaviors, including space recognition, schooling and prey detection, and therefore, it is possible that enhanced functionality of this organ provides critical adaptation for many aspects of the cave environment (Baker and Montgomery, 1999; Kowalko et al., 2013; Yoshizawa et al., 2010). In *A. mexicanus*, the lateral line is composed of small dorsoventrally aligned SNs dispersed over the entire body, as well as large CNs associated with skeletal pores that run linearly from the tail to the head and innervate the suborbital bone of the cranial region (Schemmel, 1967). Across numerous fish species, including *A. mexicanus*, SNs detect direct-current, low-frequency stimuli (<30 Hz) allowing the fish to process the net velocity between itself and its environment (Coombs and Janssen, 1990). Conversely, CNs detect higher frequency stimuli (30–150 Hz) and appear to be important for determining acceleration (Coombs and Janssen, 1990). Our findings demonstrate that the SNs are required for sleep loss and suggest a link between active flow-sensing and sleep regulation in Pachón cavefish.

In multiple populations of *A. mexicanus*, including Pachón cavefish, the enhanced sensitivity in the lateral line provides an adaptive advantage by enhancing vibration attractive behavior (VAB) and prey-seeking (Yoshizawa et al., 2010). A population of SNs that surround the eye orbit is required for VAB, revealing spatially localized functional specialization of SNs (Yoshizawa et al., 2010). While our results indicate that the cranial neuromasts suppress sleep in Pachón cavefish, this is likely to be a distinct population from those that regulate VAB. We have previously reported a lack of correlation between sleep and the number of eye orbit SNs, or VAB itself in Pachón×surface hybrids (Yoshizawa et al., 2015). Therefore, the wake-promoting cranial neuromasts are probably independent of those regulating VAB. Subsequent studies refining the regions ablated with Vetbond or a fine-scale examination of morphological SN differences may allow for the identification of the specific wake-promoting SNs in Pachón cavefish.

Identifying how the lateral line regulates sleep will likely require localization of the wake-promoting lateral line neurons in Pachón cavefish. In goldfish and zebrafish, lateral line sensory information from the trunk is transmitted by the peripheral lateral line nerve (PLLN) whereas information from the cranial region is transmitted by the anterior lateral line nerve (ALLN) (Fame et al., 2006; Northcutt, 2006; Puzdrowski, 1989). Both of these nerves are integrated at the medial octavolateralis nucleus (MON) (McCormick and Braford, 1994; Puzdrowski, 1989). Several species of fish have topographically organized inputs from the lateral line. In the Bowfin *Amia calva* ALLN inputs terminate in the medial and ventromedial regions of the nucleus medialis of the MON, while PLLN fibers project within the dorsal zones of the nucleus medialis (McCormick, 1997). In the Sleeper Goby *Dormitator latifrons*, both ALLN and PLLN inputs are generated

within the center of the MON, with ALLN fibers projecting ventrally to the dorsal projections of PLLN fibers (Tomchik and Lu, 2005). Inputs from the octavolateralis nucleus make secondary and tertiary projections to multiple areas of the midbrain and forebrain, including the hypothalamus (Fame et al., 2006; Northcutt, 2006; Puzdrowski, 1989). These findings raise the possibility that while inputs to the octavolateralis nucleus are topographically organized, they may be structurally dimorphic between surface fish and cavefish owing to innervation differences from the PLLN and ALLN. Further examination of the connectivity between lateral line circuitry and the hypothalamus may provide insight into the novel wake-promoting function of the lateral line in Pachón cavefish.

### Interactions between sleep and locomotor behavior

In cavefish, the lateral line is crucial for sensing water flow and directing locomotor behavior in the absence of visual cues (Kulpa et al., 2015; Yoshizawa et al., 2010). The lateral line contributes to rheotaxis (orientation towards a current) and wall-following behavior in cavefish, revealing an important role in locomotor regulation (Baker and Montgomery, 1999; Patton et al., 2010). A number of lines of evidence suggest that the effect of the lateral line on sleep is not due to general defects in locomotor activity. There is limited water flow in the arenas used for sleep analysis and rheotaxis is therefore not expected to impact locomotor behavior. Moreover, gentamicin treatment did not affect the preference of surface or cavefish for the bottom portion of the arena, suggesting location preference was intact following lateral line ablation. In addition, gentamicin treatment also increases sleep in Pachón cavefish, without changing waking activity. Taken together, these findings suggest that under the testing conditions used in this study, the effect of lateral line ablation on sleep is not due to general defects in locomotor activity.

### Metabolic regulation of sleep and circadian function

While the function of sleep loss in cavefish remains poorly understood, it may be related to differences in food availability and metabolism in the cave environment. To date, studies investigating cavefish sleep and feeding behaviors have predominantly taken place in the laboratory, under standard rearing conditions, where food is plentiful (Keene et al., 2015). In the field, there are dramatic seasonal differences in food availability that may have contributed to the evolved differences in sleep and feeding behavior. While food is typically less abundant in caves compared with rivers, field studies indicate greater food availability in the caves during the wet season (Wilkens, 1988). These environmental differences raise the possibility that cavefish are adapted to intensely forage and forego sleep during the wet season when food is available and conserve energy by increasing sleep during the dry season when food is sparse. It is possible that observed sleep reductions in the laboratory setting may relate to constant food availability, mimicking the wet season.

Sleep is also regulated by circadian rhythms, and both processes are regulated by metabolic state. Cavefish from the Pachón and Chica caves are behaviorally arrhythmic and light-inducible genes are constitutively elevated (Beale et al., 2013). Further circadian regulation of metabolic rate is dramatically reduced in cavefish, resulting in a significant decrease in total oxygen consumption, suggesting that loss of molecular, metabolic and behavioral rhythms provide a mechanism of energy conservation in cavefish (Moran et al., 2014). It is possible that the metabolic regulation of sleep observed in Pachón and Molino cavefish provide flexibility in foraging behavior and energy expenditure. Moreover, reduced sleep during times of food availability maximizes foraging opportunity, while increasing sleep in the absence of food allows for energy conservation. Although our findings demonstrate a role for sensory neurons in sleep regulation, it is not clear whether the lateral line contributes to the regulation of metabolic rate. Investigating the effects of lateral line ablation and starvation on metabolic rate will provide insight into the complex relationship between sleep, circadian processes and metabolic rate.

## Conclusions

In conclusion, we demonstrate that the evolution of sleep loss in Mexican cavefish results from both sensory-dependent and -independent mechanisms in independently evolved cavefish populations. The wake-promoting role for the lateral line in Pachón cavefish can be localized to the SNs in the cranial and trunk regions. In four other populations of cavefish, sleep loss was independent of these SNs, suggesting that alternative mechanisms underlie the evolution of sleep loss. These findings provide a model for understanding how the brain sensory systems modulate sleep and provide insight into the evolution of dramatic differences in sleep duration observed throughout the animal kingdom.

#### Acknowledgements

The authors are grateful to David Raizen (University of Pennsylvania) for advice and the initial suggestion that inspired this line of investigation. The authors thank Richard Borowsky (New York University) and Erik Duboúe (Carnegie Institution) for support and valuable feedback. The authors also thank Alexander van der Linden (University of Nevada) for generously providing access to equipment used in this manuscript. Diane Baronas-Lowell (FAU) provided feedback on this manuscript.

#### Competing interests

The authors declare no competing or financial interests.

#### Author contributions

Experiments were designed by J.J., B.A.S., M.Y. and A.C.K. Data collection was performed by J.J., B.G.R., I.O. and M.Y. Data analysis was performed by J.J., B.A.S., M.Y., I.O. and P.M. The paper was written by J.J., B.A.S., M.Y. and A.C.K.

#### Funding

This work was supported by a National Science Foundation IOS 125762 award to A.C.K. and an undergraduate Evo-Devo-Eco Network (EDEN) award to B.G.R.

#### Supplementary information

Supplementary information available online at
http://jeb.biologists.org/lookup/doi/10.1242/jeb.145128.supplemental

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