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ABSTRACT

REGULATION OF ACTIVITY PATTERNS IN ASTYANAX MEXICANUS CAVEFISH

by
Udodirim Nwosu

Organisms commonly modulate their behavioral activity in relation to the 24-hour solar cycle. This modulation of behavior is driven by a combination of responses to external cues in the environment, such as light/dark visual signals, and internal pattern generators that can persist in the absence of external cues. However, there are animals that live in caves or in the deep sea that are isolated from solar circadian cues. How these animals regulate their behavioral activity in relation to external and internal rhythms is an open question in neuroscience that may help us to understand the mechanisms that other animals, including humans, use to regulate behavioral states including sleep. This thesis explores functional relations between external and internal mechanisms for the regulation of activity patterns in two groups of *Astyanax mexicanus*, cavefish and surface fish. We found that surface fish had profound circadian modulation of behavior that was absent in cavefish.

REGULATION OF ACTIVITY PATTERNS IN ASTYANAX MEXICANUS CAVEFISH

by
Udodirim Nwosu

A Thesis
Submitted to the Faculty of
New Jersey Institute of Technology
in Partial Fulfillment of the Requirements for the Degree of
Masters of Science in Biology

Federated Biological Sciences Department

May 2022

APPROVAL PAGE

REGULATION OF ACTIVITY PATTERNS IN ASTYANAX MEXICANUS CAVEFISH

Udodirim Nwosu

Dr. Eric Fortune, Thesis Advisor — Date
Associate Professor of Biological Sciences, New Jersey Institute of Technology

---

Dr. Daphne Soares, Committee Member — Date
Associate Professor of Biological Sciences, New Jersey Institute of Technology

---

Dr. Xiaonan Tai, Committee Member — Date
Associate Professor of Biological Sciences, New Jersey Institute of Technology

BIOGRAPHICAL SKETCH

Author: Udodirim Nwosu

Degree: Master of Science

Date: May 2022

Date of Birth:

Place of Birth:

Undergraduate and Graduate Education:

- Master of Science in Biology,
  New Jersey Institute of Technology, Newark, NJ, 2022

- Bachelor of Science in Biological Sciences,
  New Jersey Institute of Technology, Newark, NJ, 2021

Major: Biology

To my grandmother Janet Onouha, I want to dedicate this to you. Thank you for the love and care you've provided to me, my siblings, and the rest of your grandchildren. You've raised such amazing people. I thank you for your role in molding us into the people we are today. Rest easy.

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CHAPTER 1

INTRODUCTION

Animals change temporal patterns of behavioral activity in relation to environmental and internal physiological cues. Environmental cues can include solar signals - the light of day and dark of night - changes in temperature, and availability of food. Internal cues can include physiological signals, such as hunger or thirst, and internally generated rhythms and/or patterns. The timing of changes in an individual's behavioral activity is often critical for survival, as animals coordinate their behavior to the availability of prey and the absence of predators, for example.

In this thesis. I examine an important category of biological rhythms known as circadian rhythms - those that follow the 24-hr cycle imposed by the rotation of the Earth. Organisms across the spectrum of life on Earth, from microorganisms to the largest animals on the planet, respond to the dramatic physical changes that occur each day (Carlson et al, 2018). I am interested in how animals synchronize their internal rhythms, which are generated via neural and other clocks within the organism, to external rhythms, particularly the day/night circadian cycle.

I am interested in the evolution of mechanisms for the coordination of internal rhythms and cues that regulate behavioral activity with external cues that occur in the environment. In my thesis work, I used a comparative approach: I compared patterns of behavioral activity populations of fish that are endemic to the environments with different circadian cues. Specifically, I examined swimming behavior in two populations of

Astyanax, a population of cavefish and a population of their surface closest living ancestors. The cavefish, which lack eyes and pigmentation, sleep in bouts of about 4 hours in relation to the availability of food and do not follow a 24-hour circadian pattern (Carlson et al, 2018). Critically, these animals both do not experience environmental circadian cues as they live in isolated caves, and do not exhibit circadian modulations of activity in their natural habitat. In contrast, the surface fish exhibit normal diurnal patterns of activity in which they sleep at night and are active during daylight hours.

Cavefish respond to food cues with increased activity. A goal of these experiments is to evaluate whether food cues can be used to entrain the internal circadian clock of cavefish. I predicted that food delivered in 24-hour intervals might activate or synchronize internal circadian rhythms in these fish. If food cues can synchronize an internal circadian rhythm, then I predict that these cavefish will show increased activity around the normal circadian feeding time even in the absence of food or other circadian cues. However, if food cues do not activate or entrain internal circadian rhythms in cavefish, then I expect to see increased activity only due to feeding and not in spontaneous circadian intervals. In contrast, surface fish have a robust circadian rhythm that is entrained to light and feeding cues, which will be used as a comparison for the data obtained in the cavefish.

CHAPTER 2

BACKGROUND

2.1 Regulation of Sleep

Sleep is regulated in most animals due to the synchronization of external circadian cues with internally generated rhythms. External circadian cues, known as 'zeitgebers,' entrain the internal rhythms that control sleep/wake cycles (Beale et al, 2013). The most common zeitgeber is the solar cycle – which drives an approximately 24-hour cycle known as the circadian rhythm (Beale et al, 2013). Circadian rhythms in animals are entrained to solar cues which are detected by photoreceptors but may also be influenced by other physical changes, such as temperature, or by the changes in the behavior of other animals that respond to solar cues (Beale et al, 2013).

Blind cavefish are useful animals in the study of circadian rhythms because they live in habitats without solar cues (Beale et al, 2013). Rather than regulating their sleep/wake cycles and activity patterns to solar cues, blind *Astyanax* cavefish regulate their sleep/wake to food cues (Carlson et al, 2018). The cavefish do not entrain to solar cues, have relatively short bursts of activity and inactivity, and increase their activity in the presence of food (Duboué et al, 2011). Surface *Astyanax* fish, in contrast, show typical circadian rhythms that are entrained to the solar cycle (Duboué et al, 2011). Surface fish sleep over five hours each night and reduce their activity in the presence of food (Duboué et al, 2011). Interestingly, different evolutionary forms of cavefish show different patterns of activity and sleep (Carlson et al, 2018).

2.2 Sleep in Humans

Sleep in humans is characterized by decreased movement, reduced responses to external stimuli, and reversible unconsciousness (Chokroverty, 1994). Sleep is controlled by neural circuits in the central nervous system. Changes in neural activity associated with sleep can be detected as changes in electrical patterns recorded on the skin (Chokroverty, 1994) using surface electrodes (Vitaterna et al, 2001).

Electrodes placed on the scalp can be used to record an electroencephalogram or EEG, which reveals patterns of activity in the central nervous system (Vitaterna et al, 2001). Electrodes near the eyes are used to record an electrooculogram, which detects subtle movements of the eyes (Vitaterna et al, 2001). In electromyography, electrodes are placed near muscles to detect changes in muscular activation (Vitaterna et al, 2001). Measurements from each of these systems reveal patterns of changes in activity during sleep. These changes in activity are known as sleep stages, which include four levels of non-rapid eye movement (NREM), and REM sleep (Chokroverty, 1994).

In adults, sleep typically progresses through NREM and REM sleep states. However, 75-80% of their sleep time is spent in NREM sleep (Chokroverty, 1994). Each sleep state usually has a duration of 90 to 110 minutes (Figure1.1; Chokroverty, 1994). NREM sleep, which is itself divided into 4 stages, is categorized by slow wave EEG activity, K complexes, spindles, and decreased muscle tone (Chokroverty, 1994). K-complexes are spontaneous high-voltage biphasic waves that are produced in response to internal stimuli such as respiratory complications and external stimuli including sound and touch (Gandhi et al, 2021). ‘Sleep spindles’ are burst-like electrical signals that are generated in the thalamocortical networks and are correlated with memory consolidation

(Aeschbach et al, 2013). In REM sleep, EEG traces include fast rhythms and theta waves in addition to the rapid phasic eye movements for which this stage of sleep is named (Chokroverty, 1994).

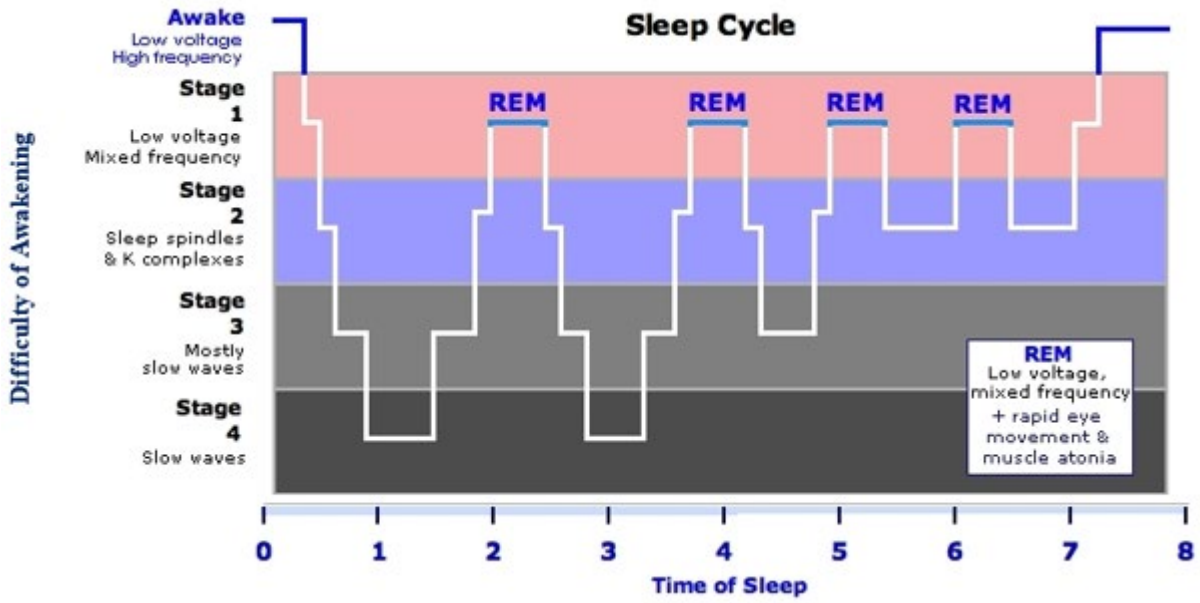

Figure 2.1 Progression of sleep stages in a typical 8-hour sleep cycle. Sleep is divided into stages based on behavioral and EEG features. Stages 1 and 2 are called "light sleep" as subjects awaken easily, whereas stages 3 and 4 are called "deep sleep" as subjects do not easily awaken. Stage 1 sleep is characterized by alpha (8-13 Hz) waves. REM sleep, which includes characteristic eye movements and dreaming, occurs during stage 1 sleep. Stage 2 is characterized by the appearance of theta waves (4-8 Hz), sleep spindles, and K-complexes. Stages 3 and 4 are characterized by the appearance of delta waves (0.5-4 Hz).

Source: Cooper, T. (2014, April 14). *Understanding sleep for optimal recovery & productivity*. Olympic Weightlifting: Catalyst Athletics. Retrieved April 7, 2022, from https://www.catalystathletics.com/article/1845/Understanding-Sleep-for-Optimal-Recovery-Productivity/

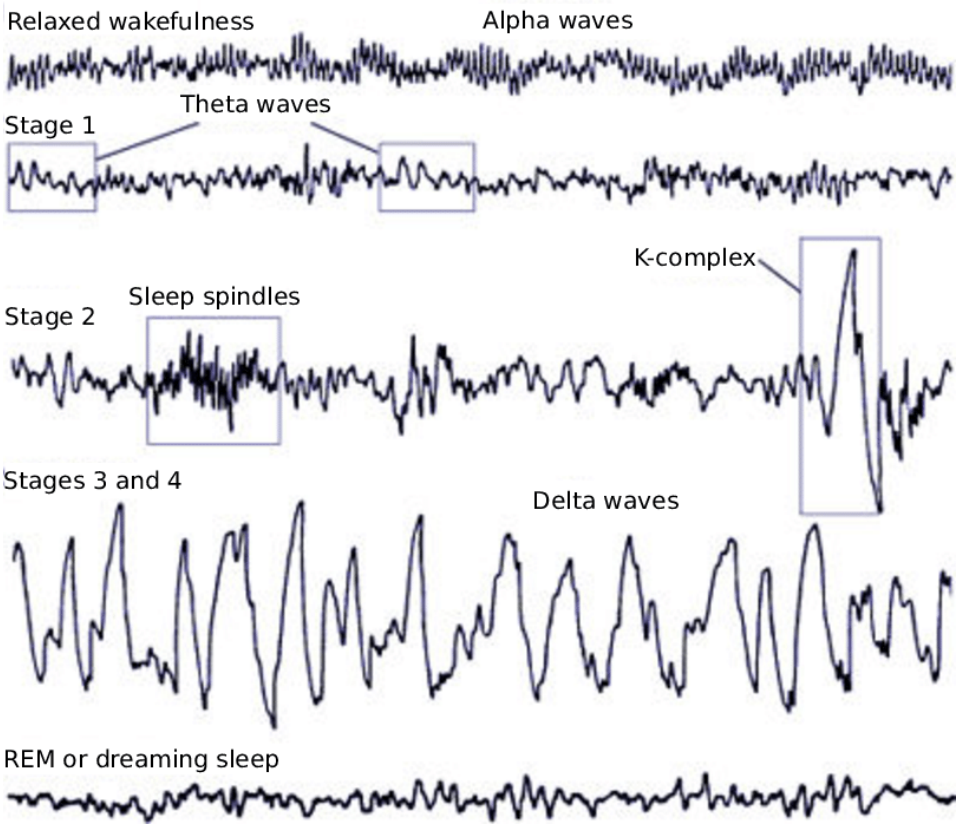

Figure 2.2 EEG recordings of the different sleep stages. Alpha waves predominate in relaxed wakefulness (drowsiness). During light sleep (stage 1) alpha waves are replaced by lower frequency theta waves. Theta waves are also present at the onset of sleep and right before waking. Sleep spindles and K-complexes appear in the second stage of sleep during the transition into deep sleep. Deepest sleep is divided into stages 3 and 4 during which delta waves – low frequency, high amplitude waves, predominate. REM sleep is associated with dreaming and resembles EEG readings that are similar to wake states.

Source: Hitziger, S. (2015). Modeling the variability of electrical activity in the brain.

Sleep is not only regulated in by external cues but also by internal clocks (Vitaterna, et al, 2001). The entrainment of internal cues by zeitgeber has been studied extensively and is described below in section 2.3.

Finally, there are dramatic changes in sleep over the lifetime of a human (Chokroverty, 1994). These changes are due to genetic and environmental factors. Newborns sleep for about 16 hours a day (Chokroverty, 1994). Their sleep is split into

more than two epochs, which vary in duration (Chokroverty, 1994). Children from ages 3-to-5 have a biphasic sleeping pattern and sleep for about 11 hours (Chrokroverty, 1994). Biphasic sleep, also known as bimodal sleep, includes two main sleep epochs each day. Adolescents (ages 9-12) switch to a monophasic sleep pattern, with a longer 10-hour sleep period (Chroverty, 1994). As a person matures, the duration of their sleep shortens to 7-8 hours (Chroverty, 1994).

2.3 Circadian Rhythms

Internal circadian rhythms include physical, mental, and behavioral changes that occur in organisms over a 24-hour cycle. Although most circadian rhythms are synchronized by light zeitgeber (Blume et al, 2019; Vitaterna et al, 2001), experiments have demonstrated that these rhythms can be driven solely by internal pattern generators in humans and some other animals.

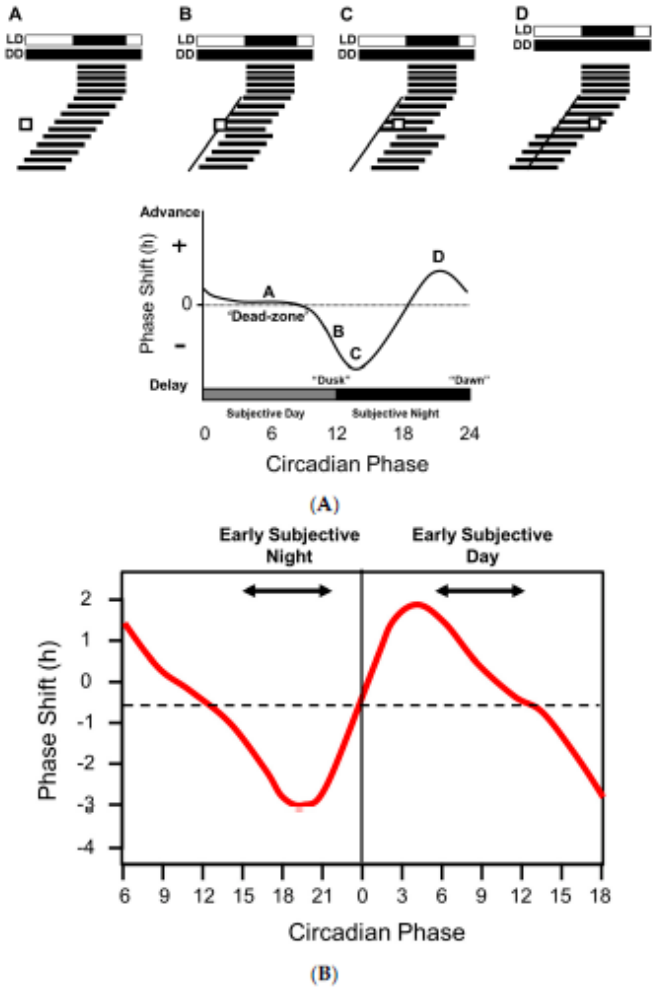

Figure 2.3 Phase-dependent effects of light on circadian entrainment. (A): Represents the phase response curve of a nocturnal animal (mouse). The light/dark cycle is shown in portions (A–D). The dark line represents the duration of activity. For the first four days, animals are under a 12-hr light and 12-hr dark (L:D 12:12) light/dark cycle. On day five, the animal was kept under constant darkness. The onset of activity in nocturnal animals is termed circadian time 12 (CT 12). Subjective day = CT 0–12; subjective night CT 12–24. During portion A, the animal is exposed to a one-hour light pulse during its subjective circadian day and shows little to no phase shift during free-running rhythm. Portion B represents an early light pulse during the subjective night, and this resulted in activity starting slightly later the next day (delayed phase shift). In portion C, the light pulse is exposed later in the night and an increased delay is exhibited the next day. Portion D shows light exposure in the second half of the night and the free-running rhythm is advanced. The following phase response curve (PRC) represents the phase shifts (A-D) plotted against circadian time. (B)Human subjects in darkness were exposed to a 10-minute duration pulse of light relative to their circadian rhythm. Circadian rhythms were measured as changes in circulating levels of melatonin. The plot shows the change in timing (hours) of the subsequent rhythm to the phase of the light pulse. Phase advances (positive values) and delays (negative values) varied quasi-sinusoidally over the circadian

period with the greatest shifts occurring a few hours before and after the night-to-day transition.

Source: Foster, R. G., Hughes, S., Peirson, S. N. (2020). Circadian photoentrainment in mice and humans. Biology, 9(7), 180. https://doi.org/10.3390/biology9070180

In the absence of light cues, many species including humans exhibit cycles of sleep and wake states that are regulated by internal rhythms. In a classic experiment, scientists measured their sleep/wake cycles while living in Mammoth cave. While in this cave, the scientists were deprived of external solar circadian cues. Despite the lack of external cues, they nevertheless exhibited a daily pattern of waking and sleeping that was slightly longer than 24 hours (Kleitman, 1939). The appearance of these sleep-wake cycles in the absence of external temporal cues demonstrated that humans have an internal circadian clock that does not depend on external cues (Kleitman, 1939).

Subsequent to this experiment, many of the neuronal and molecular mechanisms that give rise to endogenous circadian rhythms have been revealed (Kleitman, 1939). Circadian rhythms are often generated by molecular oscillators that rely on feedback loops and the dynamics of clock-gene expression (Blume, 2019). In humans and other mammals, a molecular circadian clock is found in the suprachiasmatic nucleus (SCN) of the hypothalamus (Vitaterna, 2001).

2.3.1 Impacts of Circadian Disruption

Proper coordination between internal clocks and environmental rhythms can be maintained by exposure to light cues. These cause phase changes in the timing of the internal clock, which lead to the entrainment of the internal clock to the external cycle (Walker, 2020).

Non-natural exposure to light can cause abnormal phase shifts in internal clocks, degrading synchronization to the solar circadian cycle (Walker et al, 2020). Such mistimed light exposure has been shown to have impacts on health (Walker et al, 2020). Even naturally occurring circannual changes in daylight length can affect circadian entrainment and health – including a syndrome known as seasonal affective disorder (Walker et al, 2020). Seasonal affective disorder is a form of depression believed to be due to a lack of light exposure (Walker et al, 2020). During the short days of winter, people experience dysthymia, also known as persistent depressive disorder (Walker et al, 2020). Dysthymia is a very common form of depression diagnosed by the long-term occurrence of any three symptoms of depression (Walker et al, 2020). During the long days of summer, people experience euthymia – a stable mood that is neither manic nor depressive (Walker et al, 2020).

Variation in light exposure can cause several mood disorders via modulation of melatonin and serotonin (Blume et al, 2019). Melatonin is a hormone that is secreted by the pineal gland in response to darkness and aids in the timing of the circadian rhythm. Serotonin is a neurotransmitter that at normal levels, acts as a mood stabilizer, and aids in healthy sleep patterns. Disruption in the regulation of melatonin in the body can lead to anxiety, depression, and difficulty sleeping (Blume et al, 2019). Irregular levels of serotonin in the human body have been linked to depression and sleep disruptions/disorders (Blume et al, 2019).

2.3.2 Circadian Clocks/Rhythms in Cavefish

Cavefish and deep-sea organisms – those that live at depths beyond where solar cues penetrate – do not exhibit internal circadian rhythms entrained to light cues (Beale et al,

2013). Instead, cavefish circadian rhythms are entrained by food cues (Beale et al, 2013). Cavefish respond to food cues by prolonging their wake state to increase foraging duration (Duboué et al, 2011).

There are differences in clock genes between surface and cavefish forms morphs of *Astyanax mexicanus* that underlie the differences in internal circadian rhythms (Beale et al, 2013). Differences in three clock genes in (per1, cryptochrome1a (cry1a), and per2) in Pachón, Chica, and surface fish give rise to changes in the regulation of circadian cycling in these populations (Beale et al, 2013). Clock genes are components of the internal circadian clock that interact with each other producing oscillations of gene expression (Beale et al, 2013). They generate a feedback loop by successive cyclic gene activation (Beale et al, 2013).

Per1 contributes to the maintenance of circadian rhythms and is a marker of clock function (Beale et al, 2013). Oscillations in per1 levels have been found in all three *Astyanax* populations (Beale et al, 2013). This suggests that, despite their loss of eyes, both cave populations retain their ability to detect light and discern light from darkness (Beale et al, 2013). However, circadian oscillation in per1 gene expression differs between the morphs. Expression in surface fish is more robust and increases in activity during dark and decreases in activity in the light. In the two blind cavefish, expression levels weren't as high as the surface fish and increased in activity in light and decreased in the dark (Beale et al, 2013).

The differences between populations of *Astyanax* are mediated by a molecular pathway that is regulated by cry1a and per2 (Beale et al, 2013). Surface fish exhibit a high expression of cry1a and per2, while the two cavefish light responses are reduced

(Beale et al, 2013). The reduction of responses in the cavefish populations is due to the increased expression of clock genes during the dark phases. These proteins act as repressors to the transcriptional activity of CLOCK-BMAL (Beale et al, 2013). This leads to a decrease in per1 expression and stops the circadian clock when experiencing either overexpression of per1 or light exposure (Beale et al, 2013).

These data suggest that the light input pathway in blind cavefish is in a constantly activated state, and the internal state of cavefish is more similar to a constant light exposure rather than constant darkness. The constantly-activated pathway keeps blind cavefish in an awake state (Beale et al, 2013).

2.4 Neurotransmitter Systems (β-Adrenergic Signaling)

In cavefish, hyperactivity in the β-adrenergic signaling pathway is a mechanism that contributes to a reduction in sleep (Duboué et al, 2012). Cavefish treated with inhibitors of β-adrenergic signaling show a significant increase in sleep (Duboué et al, 2012). The application of α-adrenergic inhibitors, however, did not affect sleep suggesting that β1 receptors mediate this effect (Duboué et al, 2012).

β-Adrenergic receptors transduce signals via catecholaminergic signaling and hormones (Duboué et al, 2012). Whether β-adrenergic signaling regulates feeding-associated behaviors or directly impacts sleep regulations is still not known. Neuroanatomical analysis revealed that catecholamine neuron morphology in cavefish is similar to surface fish (Duboué et al, 2012). This indicates that signals transmitted by the β-Adrenergic receptors are likely due to changes in neural activity rather than features related to cell morphology (Duboué et al, 2012). On a molecular level, elevated levels of catecholamines due to hyper β-adrenergic signaling decreases sleep duration and

increases foraging time for cavefish in their poor resource environment (Duboué et al, 2012). Blockades of β-adrenergic receptors restore sleep in cavefish (Duboué et al, 2012).

2.5 Neurotransmitter Systems (Hypocretin)

Another neurotransmitter believed to be involved in the regulation of sleep in blind cavefish is hypocretin. Hypocretin (also known as orexin) is a neuropeptide hormone produced in the hypothalamus that greatly influences factors such as sleep, energy expenditure, and arousal (Jaggard et al, 2018). Abnormalities in the functioning of hypocretin signaling result in fragmentation of both the wake and sleep states, commonly seen in sleep disorders like narcolepsy in humans (Leung et al, 2018).

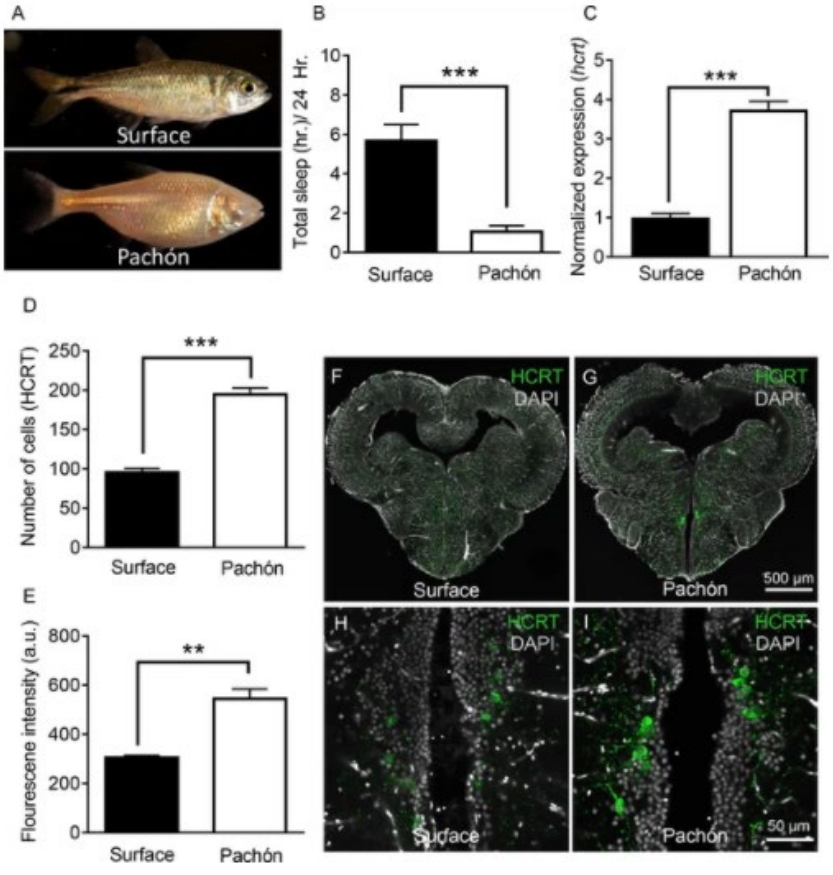

Figure 2.4 Expression levels of hypocretin receptors are elevated in Pachón cavefish and surface fish. (A) Image comparisons of Pachón cavefish and surface fish. (B) Sleep

duration in Pachón cavefish is significantly reduced compared to surface fish. (C) HCRT expression in Pachón cavefish's brain extracts is significantly enhanced compared to surface fish. (D) HCRT neuropeptide signal in Pachón cavefish is significantly elevated compared to surface fish. (E) Pachón cavefish have an increased number of HCRT-positive cells in their hypothalamus compared to surface fish. (F & G) Whole brain coronal slice of surface fish and cavefish respectively. (H & I) The dorsal hypothalamus of surface fish and Pachón cavefish respectively, containing HCRT neurons.

Figure Source: Jaggard, J. B., B. A. Stahl, E. Lloyd, D. A. Prober, E. R. Duboue, and A. C. Keene. 2018. Hypocretin underlies the evolution of sleep loss in the Mexican cavefish. eLife 7. PMID: 29405117.

In blind cavefish, there are increased levels of hypocretin in neurons that result in the saturation of hypocretin receptors, which, in turn, prolongs their wake state (Jaggard et al, 2018).

Compared to their surface counterparts, blind cavefish have an enlarged hypothalamus with twice as many orexin neurons (Jaggard et al, 2018). Each orexin neuron in the hypothalamus of cavefish also expresses greater levels of hypocretin than surface fish, which increases orexin signaling (Leung et al, 2018). These increases in orexin signaling increase overall activity and the duration of periods of activity, resulting in less time sleeping (Jaggard et al, 2018).

Sleep patterns of cavefish can be changed by pharmacologically disrupting hypocretin receptors, leading to significant increases in sleep duration (Leung et al, 2018). There are two known HCRT receptors, HCRTR1 and HCRTR2 (Jaggard et al, 2018). In *Astyanax*, the more evolutionary ancient receptor, HCRTR2, is the only receptor encoded in both surface and cavefish populations (Jaggard et al, 2018).

To examine the role of hypocretin, surface fish and Pachón cavefish were soaked in TCSOX299, an HCRTR2 pharmacological inhibitor. Surface fish showed no difference in sleep duration or bout sleep (Figure 2.5). Cavefish experienced a four-hour

increase in sleep, as shown in Figure 2.5 (Jaggard et al, 2018). There is still a possibility that sleep in surface fish is regulated by hypocretin receptors, however, these results highlight the fact that cavefish are particularly sensitive to changes in hypocretin signaling (Jaggard et al, 2018).

When hypocretin neurons in cavefish are genetically silenced to mimic neuron concentration in surface fish, there is a reduction in locomotor activity and a drastic increase in sleep levels, very similar to levels documented in surface fish (Leung et al, 2018). This again demonstrates the significance of hypocretin signaling in the regulation of hyperactivity and sleep loss in cavefish (Leung et al, 2018).

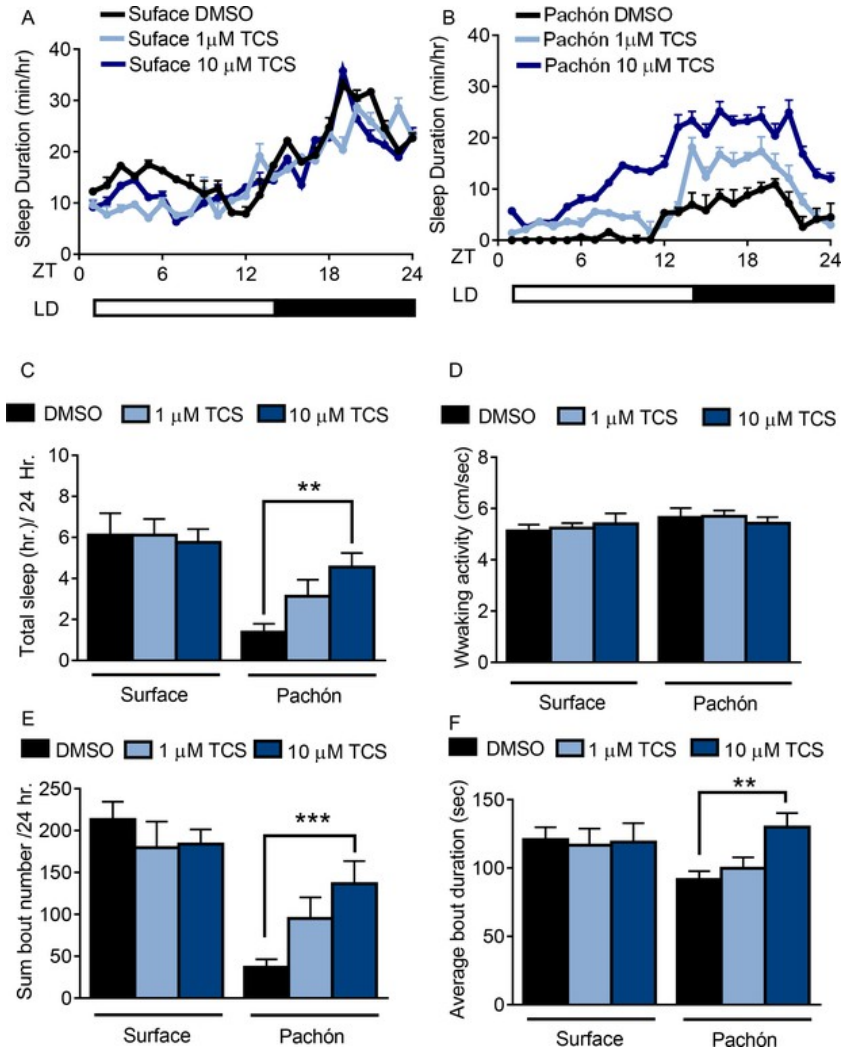

Figure 2.5 Sleep promotion in Pachón cavefish after pharmacological inhibition of HCRTR2. Twenty-four-hour sleep profile for both surface and cave fish, treated with DMSO (black), 1 μm TCS (light blue), or 10 μm TCS (dark blue). DMSO is the control. Total sleep and average sleep in surface fish are not affected by TCS treatment. TCS treatment in Pachón cavefish increases sleep. As TCS treatment increased, sleep and sleep duration in cavefish increased. No significance in waking activity or sleep bout in both surface fish and cavefish under treatment.

Source: Jaggard, J. B., B. A. Stahl, E. Lloyd, D. A. Prober, E. R. Duboue, and A. C. Keene. 2018. Hypocretin underlies the evolution of sleep loss in the Mexican cavefish. eLife 7. PMID: 29405117

Inhibition of orexin molecules can also be accomplished by ablation of the lateral line (Jaggard et al, 2017). The lateral line is a major sensory organ that allows fish and some other species of vertebrates to sense vibrations through changes in the movement of

the water (Leung et al, 2018). In cavefish, individuals rely more heavily on the lateral line to locate food as visual information is absent. In cavefish, foraging maintains the arousal of the fish, resulting in them sleeping less (Leung et al, 2018). Pharmacological ablation of the lateral line using ototoxic antibiotic gentamicin, generated increases in bouts of sleep and sleep duration (Leung et al, 2018). Ablation of the lateral line appears to remove self-stimulation (feedback), thereby increasing sleep in cavefish (Jaggard et al, 2017).

Starvation in cavefish is another mechanism that promotes sleep. Cavefish suppress sleep during periods/seasons in which food is abundant in order to forage, and they increase sleep during seasons where food is sparse to conserve energy (Jaggard et al, 2017). Starvation increases sleep in cavefish by suppressing HCRT indirectly reducing the expression of hypocretin (Jaggard et al, 2017).

2.6 Oca2 Gene

Oca2 is a gene that is part of the "oculocutaneous albinism" group of rare inherited disorders that produces melanin pigment in the hair, skin, and eyes (Figure 2.6) (Bilandžija et al, 2013). Albinism in cavefish is caused by a mutation in the protein that encodes for the oca2 gene. This protein functions during the initial step of the melanin synthesis pathway, converting L-tyrosin to L-DOPA (Bilandžija et al, 2013).

Mutations in the oca2 gene have been linked to irregularities in catecholamine levels (Bilandžija et al, 2013). For example, albino cavefish have higher levels of dopamine and norepinephrine (Bilandžija et al, 2013). Similar increases in dopamine are present in the oca2 knockout of morpholino surface fish larvae (Bilandžija et al, 2013). Oca2 can modulate behavior through the regulation of catecholamines –

neurotransmitters that regulate feeding, sleeping, and social behaviors (Bilandžija et al, 2013).

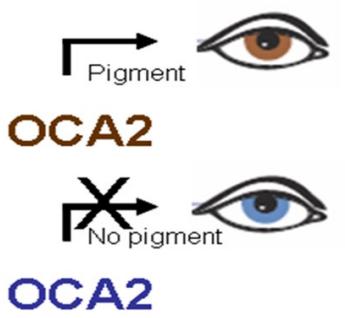

Figure 2.6 Effect of oca2 gene on pigment production. Eye color is the result of the inheritance of different alleles of the oca2 gene. In the absence of the oca2 gene, there is no pigmentation and eye color changes.

Source: Starr, D. B. (2022, February 12). If both parents have blue eyes, how could they have a child with brown eyes? The Tech Interactive. Retrieved April 9, 2022, from https://www.thetech.org/ask-a-geneticist/ask424

Further research has been conducted on the relationship between sleep and oca2-mediated albinism. In a study conducted by (O'Gorman et al), comparisons in sleep duration, bout duration, and bout number were made between surface fish and oca2 knockout surface fish (Figure 2.8). The oca2 knockout gene is an engineered mutation by the CRISPR/cas9 deletion of exon 21 in the gene. Complete loss of pigmentation was evident in surface fish homozygous for this mutation.

Sleep duration and bout duration in knockout surface fish were significantly less compared to the control (O'Gorman et al, 2021). Similar results were gathered from comparisons between pigmented and albino F2 cave-surface hybrids (Figure 2.7) (O'Gorman et al, 2021). In the absence of a working oca2 gene, albino F2 cave-surface hybrids sleep less than pigmented F2 cave-surface hybrids (O'Gorman et al, 2021).

Differences in sleep duration are also present when comparing cave-surface crossings heterozygous for a silenced oca2 and cave-surface crossings homozygous for a silenced oca2 (O'Gorman et al, 2021). Cave-surface crossings heterozygous for the oca2 are still pigmented and sleep longer than homozygous albino crossings (Figure 2.9; O'Gorman et al, 2021). These results are consistent with the hypothesis that oca2 creates a pleiotropic relationship between sleep and pigmentation (O'Gorman et al, 2021).

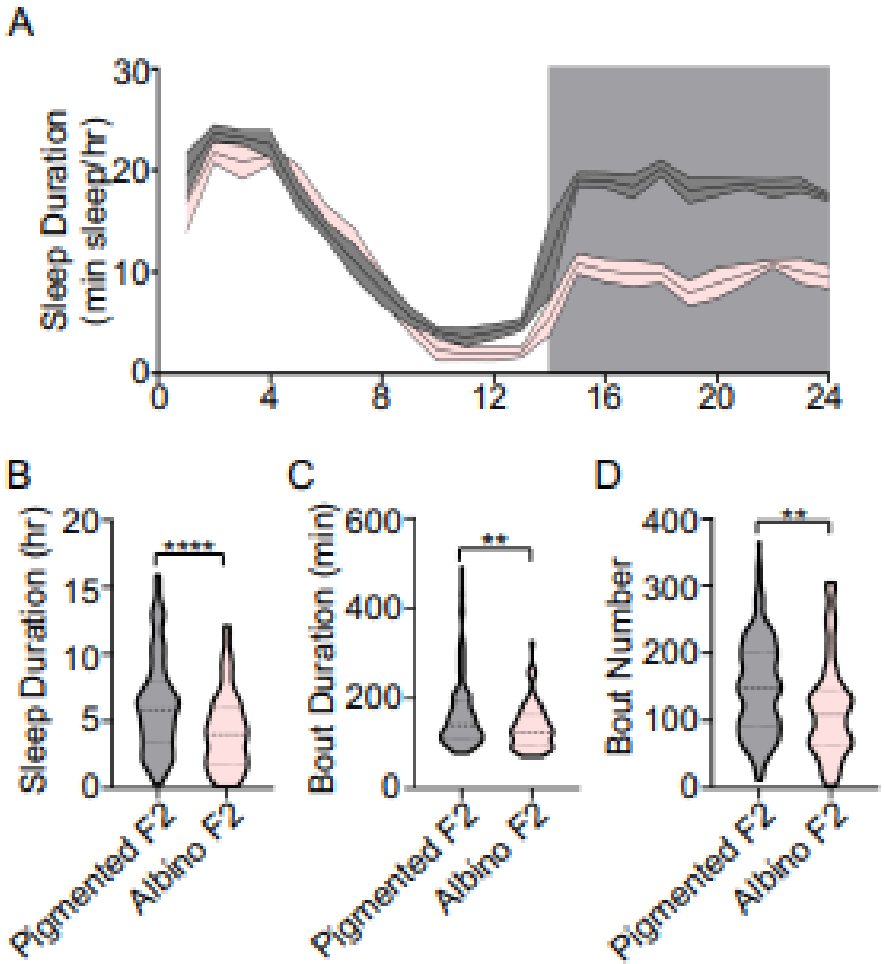

Figure 2.7 Correlation between pigmentation and sleep in pigmented and albino surface-cave F2 hybrid fish. F2 hybrids are obtained by the crossing of a female surface fish with a male Pachón cavefish to produce cave-surface F1 hybrids. Crossing of cave-surface F1 hybrids produces cave-surface F2 hybrids. (A) Sleep profile of cave-surface F2 hybrids depicting sleep duration over 1-hour intervals for 24 hours. Gray line = pigmented F2

hybrids; Pink line = albino F2 hybrids. The gray region of the graph represents night (dark) and the white portion of the graph represents day (light). Comparisons in (B) total duration of sleep (in hours), (C) bout duration (in minutes), and (D) bout number in pigmented versus albino F2 hybrids. Albino cave-surface F2 hybrids slept significantly less than pigmented cave-surface F2 hybrids. Bout duration and bout number in albino cave-surface F2 hybrids are significantly less than in pigmented cave-surface F2 hybrids, proving that the evolution of albinism and sleep is the result of shared genetic factors (pleiotropy).

Source: O'Gorman, M., S. Thakur, G. Imrie, R. L. Moran, E. Duboue, N. Rohner, S. E. McGaugh, A. C. Keene, and J. E. Kowalko. 2021. Pleiotropic function of the OCA2 gene underlies the evolution of sleep loss and albinism in Cavefish. Current Biology 31:3694–3701. PMID: 34293332

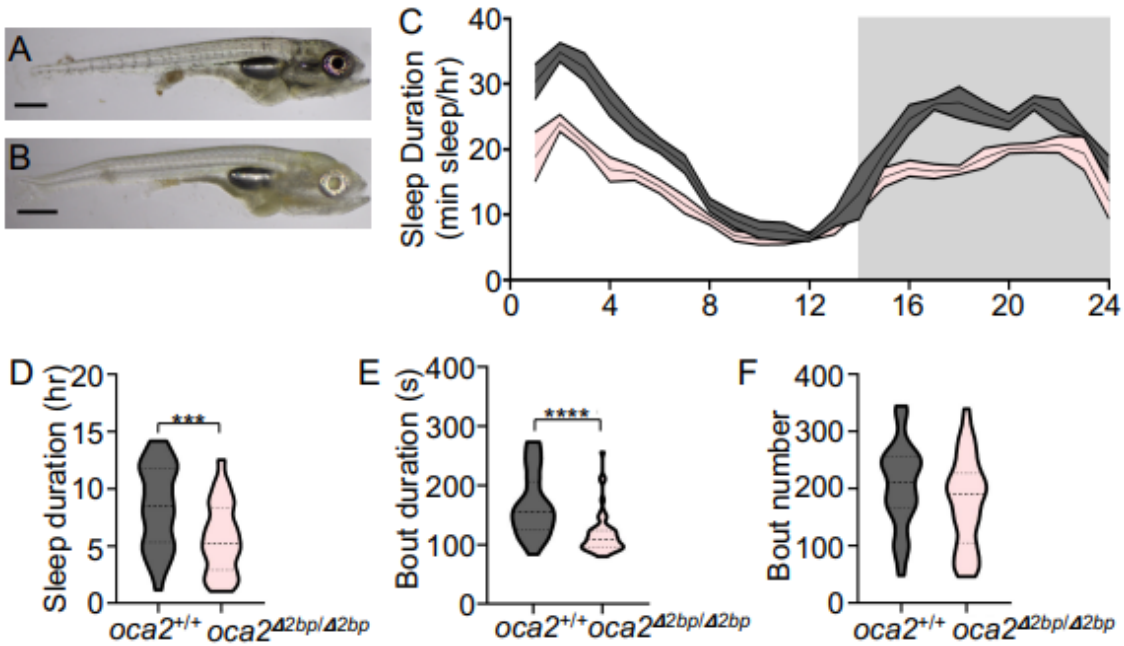

Figure 2.8 Reduction in sleep duration and bout duration in oca2 mutant surface fish. Comparison between (A) pigmented wild-type ($oca2^{+/+}$) and CRISPR/cas9 engineered deletion in exon 21 of oca2 ($oca^{2\Delta bp/2\Delta bp}$). (C) Amount slept at 10-minute intervals by $oca2^{+/+}$ and $oca^{2\Delta bp/2\Delta bp}$ over a 24 sleep profile. Significant reduction in sleep duration during the day and night in $oca^{2\Delta bp/2\Delta bp}$. Comparison of (D) sleep duration (in hours), (E) bout duration (in seconds), and (F) bout number in $oca2^{+/+}$ and $oca^{2\Delta bp/2\Delta bp}$. Significant reduction in bout duration in $oca^{2\Delta bp/2\Delta bp}$ resulted in a significant reduction in sleep duration in $oca^{2\Delta bp/2\Delta bp}$. Bout number has no significant difference between $oca2^{+/+}$ and $oca^{2\Delta bp/2\Delta bp}$.

Source: O'Gorman, M., S. Thakur, G. Imrie, R. L. Moran, E. Duboue, N. Rohner, S. E. McGaugh, A. C. Keene, and J. E. Kowalko. 2021. Pleiotropic function of the OCA2 gene underlies the evolution of sleep loss and albinism in Cavefish. Current Biology 31:3694–3701. PMID: 34293332

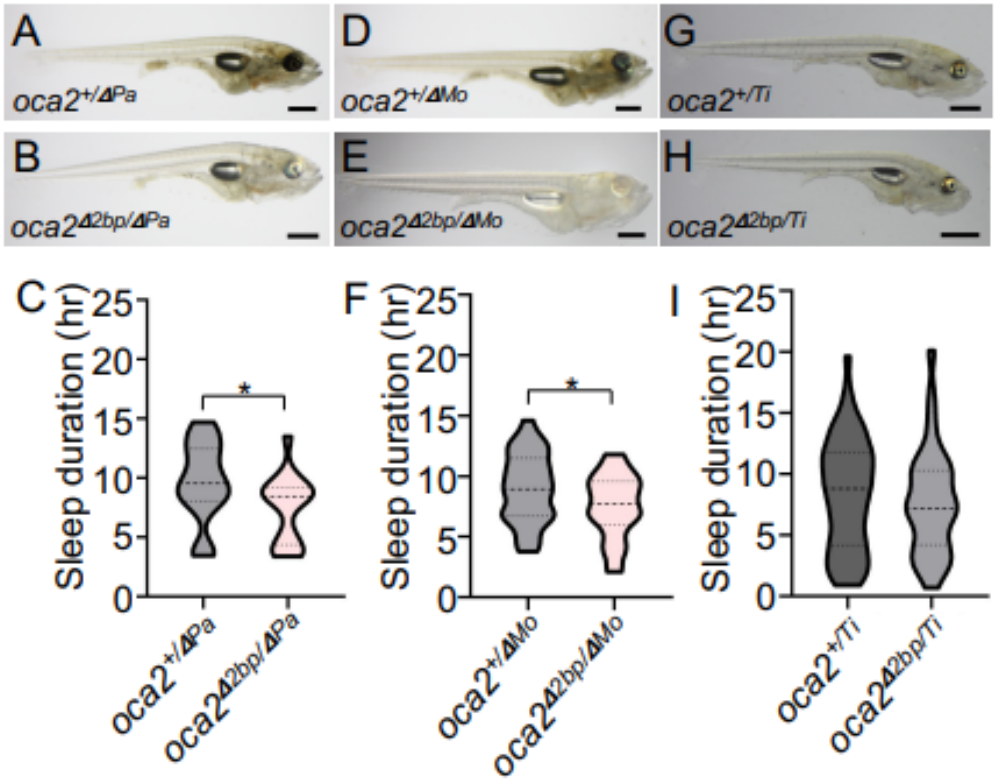

Figure 2.9 Reduction in sleep duration in cave-surface crossings with homozygosity in mutated oca2 gene. Pigmented heterozygous hybrids of (A) surface- Pachón cavefish crossings ($oca2^{+/\Delta Pa}$) and (D) surface-Molino cavefish crossings ($oca2^{+/\Delta Mo}$). Albino hybrids of (B) surface- Pachón cavefish crossings ($oca2^{2\Delta bp/\Delta Pa}$) and (E) surface-Molino cavefish crossings ($oca2^{2\Delta bp/\Delta Mo}$). Both (G) heterozygous ($oca2^{+/\Delta Ti}$) and (H) albino ($oca2^{2\Delta bp/\Delta Ti}$) hybrids of surface-Tinaja cavefish are pigmented. Sleep duration in albino $oca2^{2\Delta bp/\Delta Pa}$ and $oca2^{2\Delta bp/\Delta Mo}$ hybrids are significantly less than their pigmented siblings $oca2^{+/\Delta Pa}$ and $oca2^{+/\Delta Mo}$. No significance in sleep duration in heterozygous ($oca2^{+/\Delta Ti}$) and albino ($oca2^{2\Delta bp/\Delta Ti}$) hybrids.

Source: O'Gorman, M., S. Thakur, G. Imrie, R. L. Moran, E. Duboue, N. Rohner, S. E. McGaugh, A. C. Keene, and J. E. Kowalko. 2021. Pleiotropic function of the OCA2 gene underlies the evolution of sleep loss and albinism in Cavefish. Current Biology 31:3694–3701. PMID: 34293332

CHAPTER 3

MATERIALS AND METHODS

Here I describe the fish and procedures for animal care used in this study, the experimental design, and data collection and analysis. I used DeepLabCut and Matlab for analysis, plots were generated using Matlab, and drawings were made using GoodNotes 5.

3.1 Fish Care

All the organisms in this study were adult surface or cavefish forms of the species *Astyanax mexicanus* that were bred at NJIT. All the cavefish belong to the Pachón cave in the Sierra de el abra region in Mexico.

Fish were maintained in the aquatic animal facility under a 12-hour light/ 12-hour dark cycle. The fish were fed fish flakes daily. Water temperature was maintained at approximately 21 °C.

3.2 Experimental Design

For experiments, each fish was taken from the colony and housed in a plastic tank (33.02 cm L x 19.685 cm W x 21.6 cm H) filled with 10 liters of water. Four of these tanks were positioned in front of a Thorlabs DCC3240N CMOS camera on a tripod at approximately 1m distance. This set-up allowed for data collection from 4 fish at the same time during each experiment. Video recordings, 60 seconds in duration, were made every 10 minutes.

Each 6-day duration experiment involved four tanks that each housed a single fish – each of these fish is an individual trial. In each experiment, three of the tanks contained one morph of *Astyanax mexicanus*, while the remaining housed the other morph (Figure 3.1). This arrangement is a form of control – we always mixed morphs in experiments to control for variations within experiments.

There were four types of experiments. All four experiments began with a 12-hr light/12-hr dark cycle for 72 hours in the presence of food. In the first experiment, the fish were fed Tetra Cichlid flakes at 10 am during the subjective day for the first 72 hours. After the first three days, the light regime was maintained for another three days in the absence of food. This experiment was performed twice. The fish in the second experiment were fed flakes during the day. After the first three days, they were no longer given food or light cues

The fish in the third experiment were fed worms ad libitum throughout the 6-day experiment. After the first three days, the light regime switched to a 72-hr dark schedule. This experiment was performed twice. In the last experiment, the fish were fed worms ad libitum. The 12-hr light/12-hr dark light regime was maintained throughout the 6-day experiment. This experiment was also performed twice.

We used DeepLabCut to track the position of each fish in the video recordings. We calculated the instantaneous velocity of each fish by measuring the distance the fish moved between each frame of the video. We looked for changes in the average velocity, the standard deviation of the velocity, and the number of crossings a fish made during the experiment (Figure 4.1 B, C, D). Specifically, we looked for circadian modulations in behavior over the 6 days of each experiment.

The purpose of this experimental design was to compare the difference in activity patterns between surface fish and cavefish and to compare the modulation of activity by light and food cues.

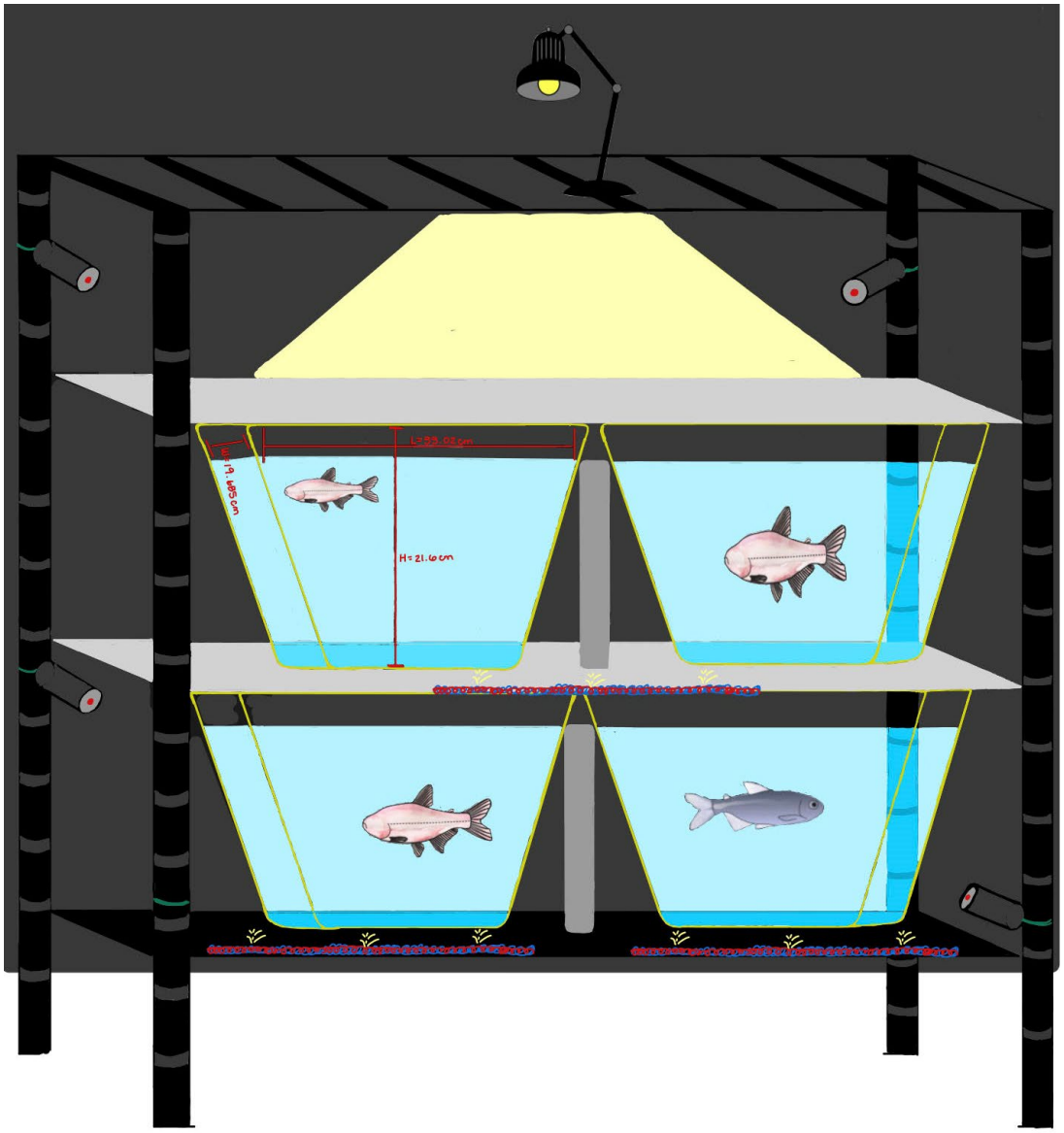

Figure 3.1 Four tanks recorded simultaneously. Each trial included both surface and cavefish. LED lights (white and infrared) were arranged around the tanks. The camera was positioned about 1m in front of the rack so that all 4 fish were visible in the frame. Tank water is at room temperature, approximately 21°C.

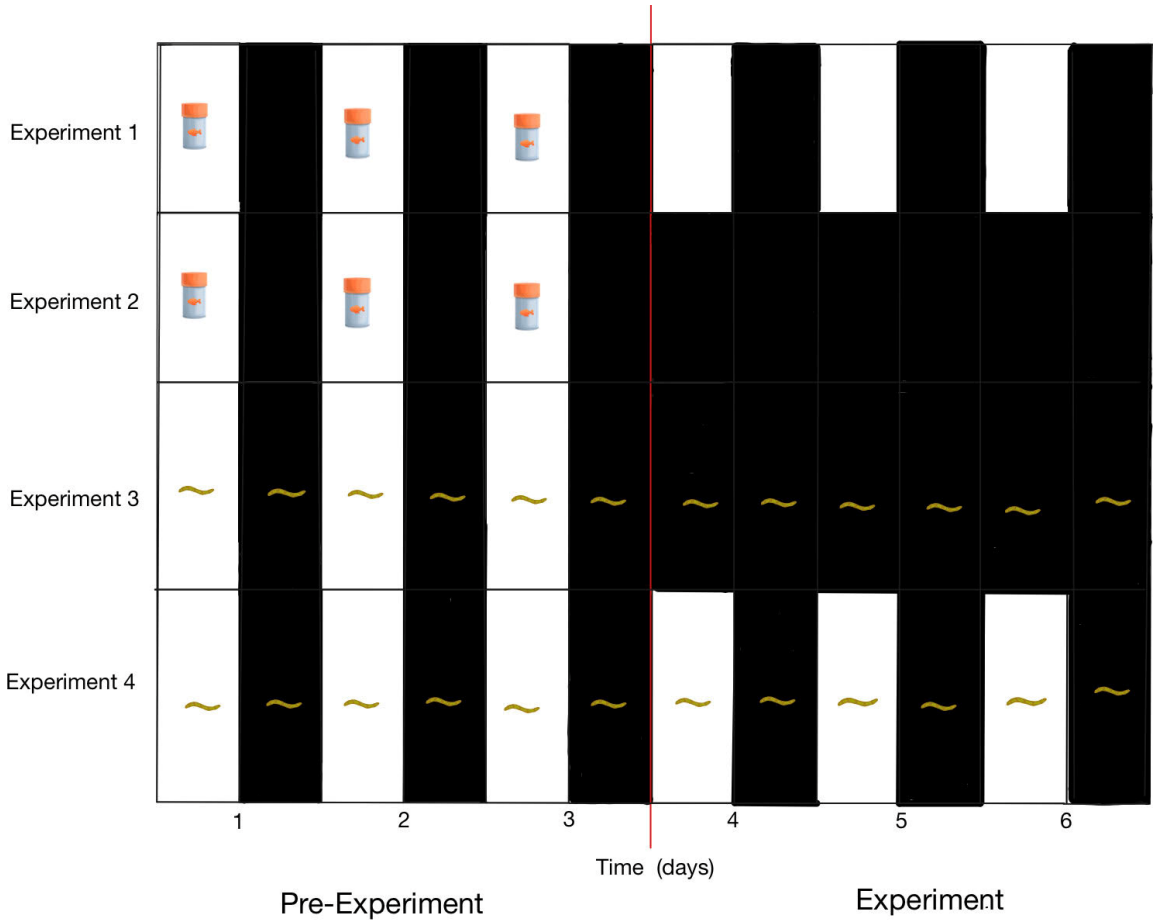

Figure 3.2 Feeding and light regime of the four experimental conditions. Each fish was fed flakes or worms daily. Experiments 1 and 2 were fed during the day. Experiments 3 and 4 had worms always available to them. The pre-treatment for all 4 experiments began on a l2-hr light/ 12-hr dark cycle. l2-hr light/ 12-hr dark cycle was maintained for experiments 1 and 4. In experiments 2 and 3, the light regime was switched to 72-hr continuous darkness.

CHAPTER 4

RESULTS

I examined how sensory cues interact with internal circadian control systems for the control of behavioral activity in *Astyanax*. I exposed individual fish to normal 24-hour days and feedings for 3 days and then exposed them to a test condition. The test conditions were 1) 12/12 light/dark cycle / no feeding, 2) continuous darkness/ no feeding 3) continuous darkness, ad libitum food, and 4) 12/12 light/dark cycle with ad libitum food.

I performed two experimental runs of 4 tanks each (eight trials total) for each experimental condition. The position of each fish over a 60-second sample (1000 video frames) was plotted (surface fish: Figure 4.1A; cavefish: Figure 4.4A). Measurements from each video, including the mean velocity, the standard deviation of velocity, and the number of tank crossings were calculated and plotted over time (surface fish: Figures 4.3 B, C, D; cavefish: Figure 4.6 B, C, D). I expected that these different measurements, mean velocity, standard deviation of velocity, and tank crossings would be similar – I tried all three measures to determine which might be most reliable and/or provide the best insight into circadian control.

In the presence of light cues and absence of food cues, surface fish maintained an oscillatory rhythm (Figure 4.1). Similarly, surface fish in the absence of food and light cues and surface under continuous darkness but with ad libitum food also exhibited circadian oscillations in swimming (Figure 4.2, Figure 4.3). Finally, the magnitude of the behavioral oscillations in the light/dark experiment was higher than in either experiment in which the fish was in continuous darkness (Figure 4.3).

I found that light cues are more important in the regulation of activity patterns in surface fish than food cues. The rhythm seen in Figure 4.2 and the DD experiment in Figure 4.3 have similar rhythms which are consistent with the idea that food isn't the major factor involved in the regulation of activity patterns in surface fish.

Cavefish showed oscillating rhythms under the light/dark/ no feeding experimental condition (Figure 4.4C). Increased activity in cavefish was seen during the light pulses, which is expected if they have a circadian rhythm. However, it can also be an indication that the cavefish have associated food with light cues.

In the 72-hr DD experiment without either food or light cues, I did not find a significant circadian rhythm in cavefish (Figure 4.5). In figure 4.5, increased activity in cavefish was seen at the beginning and end of the experiment. Increased activity at the beginning of the experiment is expected as the fish were fed before being recorded for the experiment. Eventually, cavefish activity declined over days without food, presumably as part of their natural starvation adaptation strategy to reduce energy expenditure in the absence of food. Under the other two experimental conditions, LD worms ad libitum and DD worms ad libitum, the activity patterns in both conditions did not include circadian oscillations. The behavioral patterns in cavefish during both experimental conditions exhibited the same behavioral patterns.

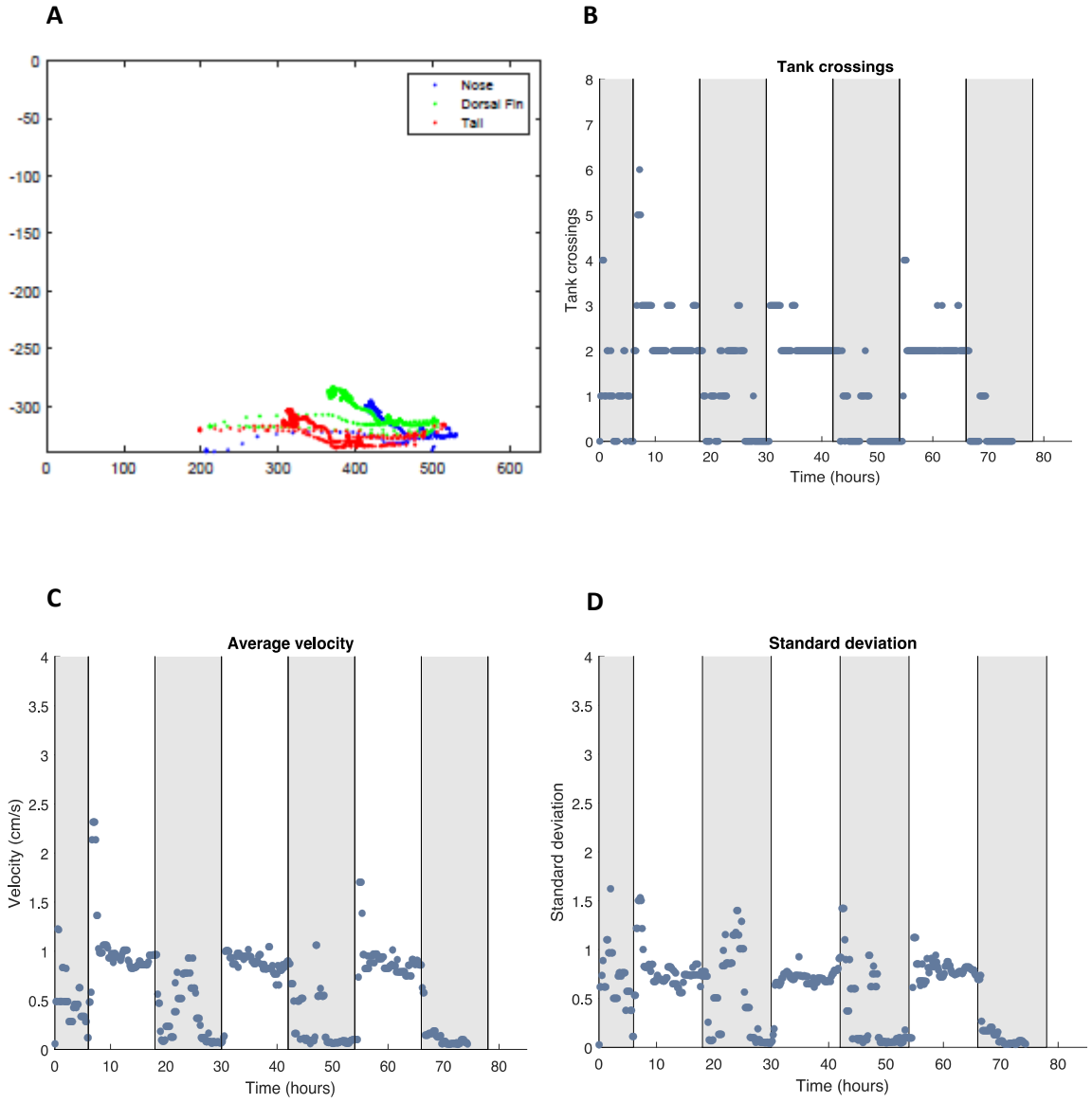

Figure 4.1 Circadian rhythm in surface fish. **(**A) X/Y position of surface fish fed during the day. Each dot in the plot consists of one 1,000 frame video. Each X/Y plot is a singular dot in the B, C, and D graphs. This position graph is dot 12 in the following graphs. (B) The velocity of surface fish fed during the day. (C) Mean velocity of all trials of surface fish fed during the day. (D) The standard deviation of surface fish fed during the day. (B, C, D) each dot consists of one position graph (A).

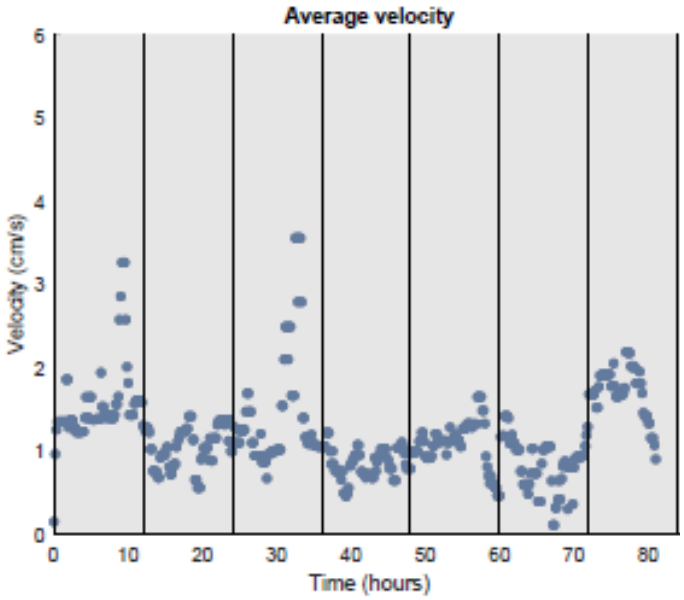

Figure 4.2 Surface fish subjected to 72-hr continuous darkness. The surface fish circadian rhythm was maintained in the absence of light and food cues.

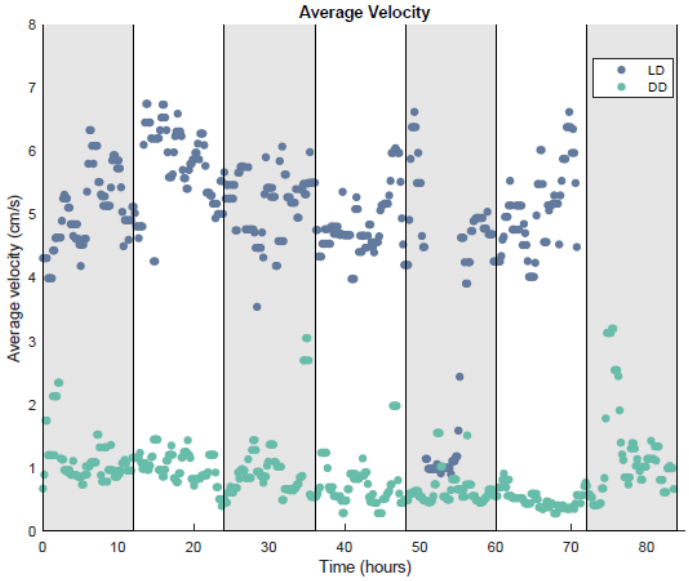

Figure 4.3 Comparison of light regimes on surface fish fed worms ad libitum. Circadian rhythms are conserved in both light/dark (LD) and dark/dark (DD) regimes. During LD cycles, activity is increased, while during DD cycles activity is reduced.

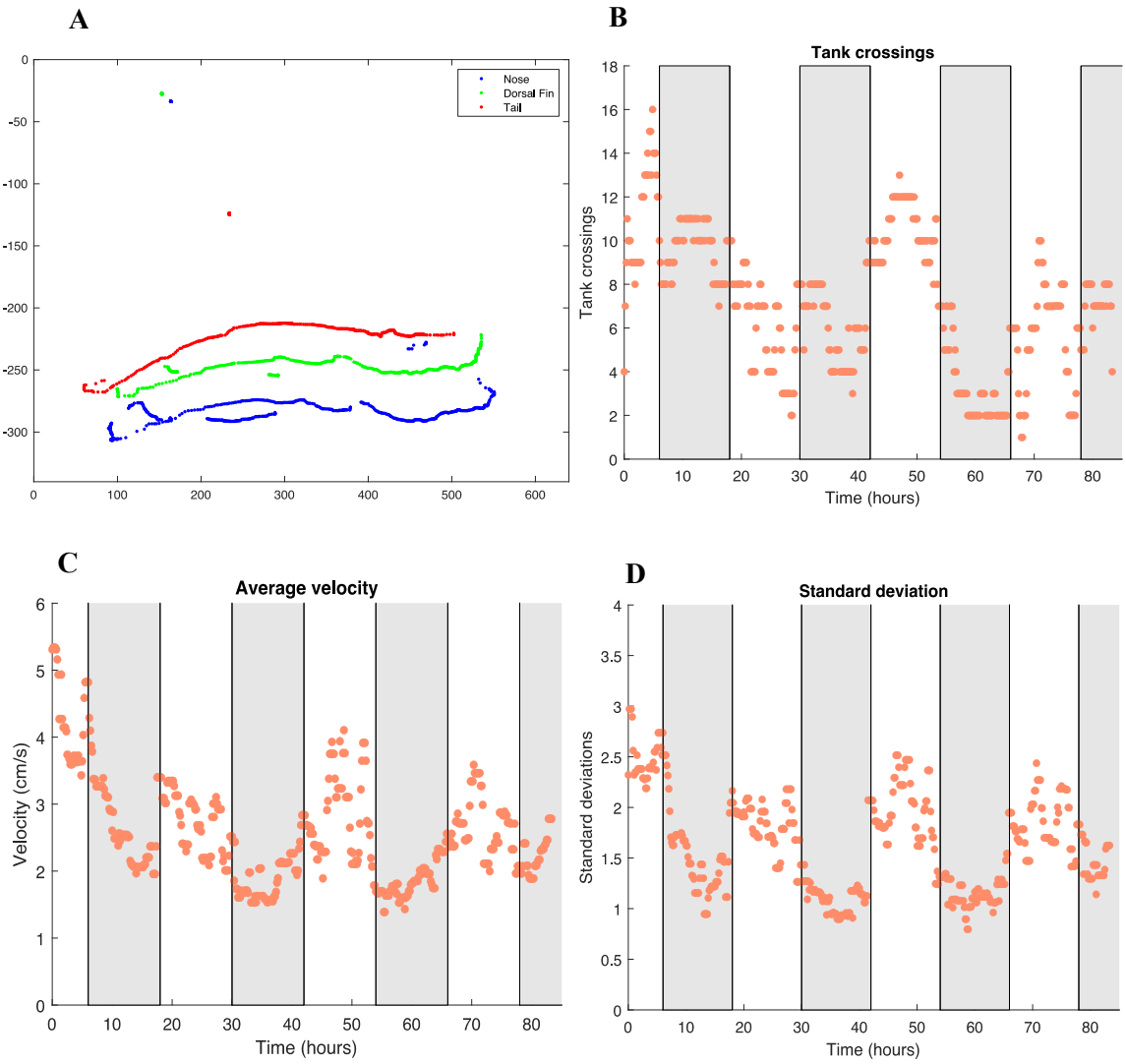

Figure 4.4 Associative behavior in cavefish. (A) X/Y position of Pachón cavefish fed during the day. This position graph is dot 3 in the B, C, and D. (B) Velocity of Pachón cavefish fed during the day. (C) The mean velocity of all trials of Pachón cavefish fed during the day. (D) The standard deviation of Pachón cavefish fed during the day.

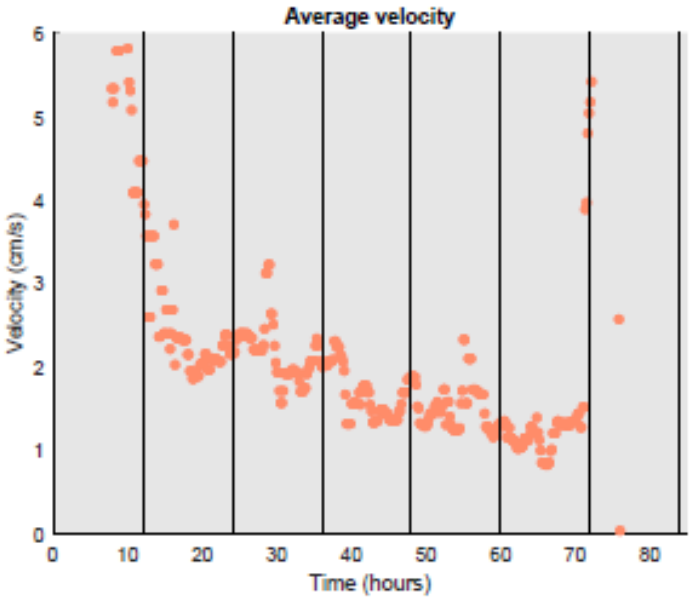

Figure 4.5 Cavefish subjected to 72-hr continuous darkness. Cavefish did not maintain a circadian rhythm in the absence of light cues.

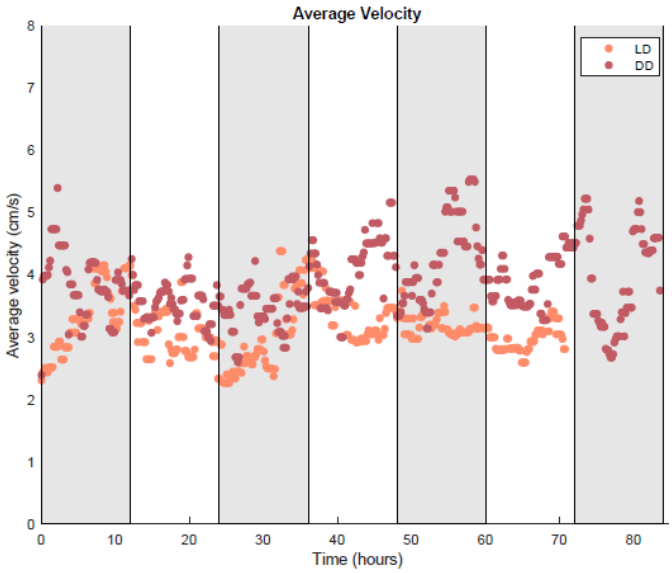

Figure 4.6 Comparison of light regimes on cavefish fed worms ad libitum. Circadian rhythms are not conserved in either light/dark (LD) or dark/dark (DD) regimes. Activity levels are relatively the same during both LD cycles and DD cycles.

CHAPTER 5

DISCUSSION

5.1 General Discussion

I investigated whether two populations of *Astyanax* had a circadian clock that could be entrained by food or light cues. Surface fish maintained a circadian rhythm in absence of light or food cues. I also found that food cues alone were a weak modulator of their behavior. Surface fish showed increased activity during the LD experimental condition and decreased activity in both DD experimental conditions.

Cavefish did not maintain a circadian rhythm. There were oscillations in their activity levels during 12/12 light/dark/ no feeding, but these oscillations are due to associative behavior. In anticipation of a light cue, activity in cavefish increases presumably because they associate food with light.

Cavefish exhibited relatively low activity levels compared to surface fish. During the D/D worm ad libitum and L/D worm ad libitum experimental conditions, activity levels were the same (Figure 4.6), but lower in activity compared to L/D worm ad libitum experimental condition in surface fish (Figure 4.3). In cavefish, activity levels during the LD and DD ad libitum experimental conditions were higher than in experimental conditions without food. I never observed spontaneous circadian oscillations in behavior. Further, food cues were insufficient to induce circadian oscillations in the cavefish.

5.2 Future Studies

Based on the results from this study, I am unclear whether cavefish have an internal circadian rhythm. Cavefish showed oscillations in behavior in the absence of food during the L/D experimental condition. During the D/D experiment in the absence of food, no rhythm was present and cavefish maintained low levels of activity. These results show that these fish have learned to associate food cues with the presence of light. They maintained low activity levels during the DD experiment in anticipation of a light cue.

Another reason for their low activity levels could be due to cavefish's olfactory senses. As these fish lost their ability to see over generations, their olfactory sense became more sensitive to the presence of food. It is possible that cavefish were unable to sense the presence of food, so they operated at low activity states to conserve energy in anticipation of a food cue.

Performing these sets of experiments on other cavefish populations and comparing them to my Pachón data, we can observe whether there is a standard level of activity of cavefish as a whole. It would be beneficial to observe - whether other populations of cavefish have an internal clock. If so, these results would highlight some possible evolutionary differences that may have occurred among cavefish populations. If none of the cavefish show circadian rhythms, then all cavefish may rely solely on their olfactory senses.

Altering the perception of the cavefish's subjective day (12-hrs of light) and subjective night (12-hrs of dark) may also help determine whether cavefish have circadian rhythms or are performing associative behaviors. My next step would be to experimentally manipulate their perception of subjective day or night by feeding the fish

at the same time every day but switching what light cue they are receiving at that time. For example, the fish are fed at 12 pm each day on a 12-hr light (6 am - 6 pm) and 12-hr dark (6 pm - 6 am) cycle. Swapping the time in which they receive the light cue (receiving cue from 6 pm – 6 am instead of 6 am – 6 pm) and then removing food cues will allow us to compare activity levels before and after the change to see whether they differ.

If they have an internal circadian rhythm, then I would expect to see high activity around 12 pm even though it is now dark because they have been entrained to eat at this time. However, in the absence of a circadian cue cavefish will perform at low activity. In this scenario, they may become highly active towards the end of the day once the lights turn on if they are associating light cues with food. However, if they remain at low activity throughout the day, cavefish may be operating on other sensory cues and are using these cues to regulate their behavior.

REFERENCES

[1] Alié, A., L. Devos, J. Torres-Paz, L. Prunier, F. Boulet, M. Blin, Y. Elipot, and S. Retaux. 2018. Developmental evolution of the forebrain in cavefish, from natural variations in neuropeptides to behavior. eLife 7. PMID: 29405116

[2] Beale, A., C. Guibal, T. K. Tamai, L. Klotz, S. Cowen, E. Peyric, V. H. Reynoso, Y. Yamamoto, and D. Whitmore. 2013. Circadian rhythms in Mexican blind cavefish astyanax mexicanus in the lab and in the field. Nature Communications 4:2769. PMID: 24225650

[3] Bilandžija, H., L. Ma, A. Parkhurst, and W. R. Jeffery. 2013. A potential benefit of albinism in astyanax cavefish: Downregulation of the OCA2 gene increases tyrosine and catecholamine levels as an alternative to melanin synthesis. PLoS ONE 8. PMID:24282555

[4] Carlson, B. M., and J. B. Gross. 2018. Characterization and comparison of activity profiles exhibited by the cave and surface morphotypes of the blind Mexican tetra, Astyanax mexicanus. Comparative Biochemistry and Physiology Part C: Toxicology and Pharmacology 208:114–129. PMID: 28823830

[5] Duboué, E. R., A. C. Keene, and R. L. Borowsky. 2011. Evolutionary convergence on sleep loss in cavefish populations. Current Biology 21:671–676. PMID: 21474315

[6] Duboué, E. R., R. L. Borowsky, and A. C. Keene. 2012. B-adrenergic signaling regulates evolutionarily derived sleep loss in the Mexican cavefish. Brain, Behavior and Evolution 80:233–243. PMID: 22922609

[7] Jaggard, J. B., B. A. Stahl, E. Lloyd, D. A. Prober, E. R. Duboue, and A. C. Keene. 2018. Hypocretin underlies the evolution of sleep loss in the Mexican cavefish. eLife 7. PMID: 29405117

[8] Jaggard, J. B., E. Lloyd, A. Yuiska, A. Patch, Y. Fily, J. E. Kowalko, L. Appelbaum, E. R. Duboue, and A. C. Keene. 2020. Cavefish brain atlases reveal functional and anatomical convergence across independently evolved populations. Science Advances 6. PMID: 32938683

[9] Jaggard, J., B. G. Robinson, B. A. Stahl, I. Oh, P. Masek, M. Yoshizawa, and A. C. Keene. 2017. The lateral line confers evolutionarily derived sleep loss in the Mexican cavefish. Journal of Experimental Biology 220:284–293. PMID: 28100806

[10] Leung, L. C., and P. Mourrain. 2018. Sleep: Short sleepers should keep count of their hypocretin neurons. Current Biology 28. PMID: 29738730

[11] Mack, K. L., J. B. Jaggard, J. L. Persons, C. N. Passow, B. A. Stanhope, E. Ferrufino, D. Tsuchiya, S. E. Smith, B. D. Slaughter, J. Kowalko, N. Rohner, A. C. Keene, and S. E. McGaugh. 2020. Repeated evolution of circadian clock dysregulation in cavefish populations. PLOS Genetics. PMID: 34252077

[12] McGaugh, S. E., C. N. Passow, J. B. Jaggard, B. A. Stahl, and A. C. Keene. 2020. Review for "unique transcriptional signatures of sleep loss across independently evolved cavefish populations". Journal of Experimental Zoology Part B: Molecular and Developmental Evolution 334:497–510. PMID: 32351033

[13] O'Gorman, M., S. Thakur, G. Imrie, R. L. Moran, E. Duboue, N. Rohner, S. E. McGaugh, A. C. Keene, and J. E. Kowalko. 2021. Pleiotropic function of the OCA2 gene underlies the evolution of sleep loss and albinism in Cavefish. Current Biology 31:3694–3701. PMID: 34293332

[14] Stahl, B. A., J. B. Jaggard, J. S. R. Chin, J. E. Kowalko, A. C. Keene, and E. R. Duboué. 2019. Manipulation of gene function in Mexican cavefish. Journal of Visualized Experiments. PMID: 31058898

[15] Yoshizawa, M., B. G. Robinson, E. R. Duboué, P. Masek, J. B. Jaggard, K. E. O'Quin, R. L. Borowsky, W. R. Jeffery, and A. C. Keene. 2015. Distinct genetic architecture underlies the emergence of sleep loss and prey-seeking behavior in the Mexican cavefish. BMC Biology 13. PMID: 25761998

[16] Blume, C., Garbazza, C., and Spitschan, M. (2019). Effects of light on human circadian rhythms, sleep and mood. Somnologie, 23(3), 147–156. https://doi.org/10.1007/s11818-019-00215-x

[17] Chokroverty, S. (1994). An overview of sleep. Sleep Disorders Medicine, 7–16. https://doi.org/10.1016/b978-0-7506-9002-7.50007-0

[18] Ferrara, M., and De Gennaro, L. (2001). How much sleep do we need? Sleep Medicine Reviews, 5(2), 155–179. https://doi.org/10.1053/smrv.2000.0138

[19] Mistlberger, R. E., and Antle, M. C. (2011). Entrainment of circadian clocks in mammals by arousal and food. Essays in Biochemistry, 49, 119–136. https://doi.org/10.1042/bse0490119

[20] Vitaterna, M. H., Takahashi, J. S., & Turek, F. W. (2001). Overview of circadian rhythms. Alcohol research & health: the journal of the National Institute on Alcohol Abuse and Alcoholism, 25(2), 85–93.

[21] Cooper, T. (2014, April 14). *Understanding sleep for optimal recovery & productivity*. Olympic Weightlifting: Catalyst Athletics. Retrieved April 7, 2022, from https://www.catalystathletics.com/article/1845/Understanding-Sleep-for-Optimal-Recovery-Productivity/

[22] Foster, R. G., Hughes, S., and Peirson, S. N. (2020). Circadian photoentrainment in mice and humans. Biology, 9(7), 180. https://doi.org/10.3390/biology9070180

[23] Starr, B. (2022, February 12). If both parents have blue eyes, how could they have a child with brown eyes? The Tech Interactive. Retrieved April 9, 2022, from https://www.thetech.org/ask-a-geneticist/ask424

[24] Ward, V. (n.d.). What are circadian rhythms? What are Circadian Rhythms? The University of Auckland. Retrieved April 7, 2022, from https://www.lenscience.auckland.ac.nz/en/about/teaching-and-learning-resources/senior-biology-learning-resources/circadian-rhythms-keeping-time/what-are-circadian-rhythms.html

[25] Walker, W. H., Walton, J. C., DeVries, A. C., and Nelson, R. J. (2020). Circadian rhythm disruption and mental health. Translational Psychiatry, 10(1). https://doi.org/10.1038/s41398-020-0694-0

[26] Beck, J. (2015, November 16). That time when people lived alone in caves for science. The Atlantic. Retrieved April 11, 2022, from https://www.theatlantic.com/health/archive/2015/11/the-caves-of-forgotten-time/414894/

[27] Gandhi MH, Emmady PD. Physiology, K Complex. [Updated 2021 May 9].

In: StatPearls [Internet]. Treasure Island (FL): StatPearls Publishing; 2022 Jan-. Available from: https://www.ncbi.nlm.nih.gov/books/NBK557469/

[28] Aeschbach, D., and Santhi, N. (2013). Attention and memory changes. Encyclopedia of Sleep, 217–224. https://doi.org/10.1016/b978-0-12-378610-4.00043-7

[29] Kleitman, N. (1939). Sleep and wakefulness as alternating phases in the cycle of existence. Univ. of Chicago Press.