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Towards a Better Understanding of Zebrafish Sleep Behavior

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TOWARDS A BETTER UNDERSTANDING OF ZEBRAFISH SLEEP BEHAVIOR

by

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ABSTRACT

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Sleep serves many vital functions in humans, ranging from energy restoration to memory consolidation and information integration. Sleep deprivation is linked to worsened physiological states and psychological conditions. Zebrafish are an emerging model in neurobehavioral research and have recently demonstrated great utility in the study of sleep. This teleost species possesses several of the same neurotransmitter and neuropeptide systems that are involved in the regulation of sleep and waking rhythms in higher order mammals. Previous study of these animals has revealed a differential gene and proteomic expression following sleep deprivation through changes in environmental stimuli. The present study sought to expand on the current understanding of sleep behavior in this animal. The behavior of adult zebrafish was evaluated as they were exposed to varying environmental and pharmacological interventions. Animals were exposed to one of three conditions: (1) sustained darkness, (2) sustained brightness, and (3) sustained bright conditions, paired with the administration of an adenosine antagonist. The presentation of bright lights was effective in disrupting sleep rhythms. The administration of caffeine paired with the presentation of bright lights was the most effective method of reducing sleep in the zebrafish. Following sleep disruption animals were tested in the novel tank test or the open field task to elucidate the effects of sleep rhythm disruption on anxiety. We expand on the current interaction of sleep and anxiety

and report no increases in anxiety-like behaviors in the zebrafish following any of the environmental or pharmacological interventions.

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Finally, I thank my fellow graduate students, Erika Caramillo and Jonathan Doyon. Your continued support, insights, and friendship have been truly instrumental in the success of this project.

DEDICATION

My deepest gratitude to my parents, Saleema and Tariq Khan, who have always encouraged me in my academic pursuits. Your love and guidance are with me in all that I pursue. And to my sister Sarah, who has always been my greatest cheerleader. Your endless love and encouragement have helped make this accomplishment possible. I will never be able to fully express how much I appreciate your love and support. Thank you.

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CHAPTER I - INTRODUCTION

Sleep is characterized as a state of immobility in which consciousness is suspended and sensitivity to external stimuli is greatly decreased (Cirelli & Tononi, 2008). Sleep is a widely conserved function; several species have demonstrated stereotypic sleep behaviors (Siegel, 2008). In humans, sleep is accompanied by a loss in skeletal muscle tone, increase in eye movements, and variations in hormonal secretions (Carskadon & Dement, 2011). Broadly, there two types of sleep: Rapid Eye Movement (REM) and Non-Rapid Eye Movement (NREM) sleep. At the beginning of a sleep cycle, the individual enters stage 1 of NREM sleep; in this phase, heart rate and breathing begin to slow. Progressing deeper into the sleep cycle, the person enters stage 2 of sleep which is marked by a loss in muscle tone, and the gradual loss of conscious awareness of environmental surroundings (Andrillon et al., 2011). Electroencephalograph readings are often used to discriminate the various sleep stages. Stages 1 and 2 are marked by a dominance of theta waves (4-8Hz). Stage 2 is distinguished by the onset of sleep spindles and k-complexes. A sleep spindle is a high-frequency burst of brain activity that lasts between 0.5-2 seconds (Andrillon et al., 2011). Sleep spindle density is associated with a greater capacity for consolidation of declarative or procedural memory (Gais, Mölle, Helms, & Born, 2002; Tamminen, Payne, Stickgold, Wamsley, & Gaskell, 2010). The last two stages of NREM are collectively referred to as Slow Wave Sleep. They are characterized by the onset of high-voltage, low-frequency brain waves (delta waves). As the person progresses from stage 3 to stage 4, the EEG readings reveal increased amounts of high-voltage slow wave activity (Carskadon & Dement, 2005).

The last stage of a sleep cycle, sometimes referred to as stage 5, is REM sleep. Aside from the rapid darting of the eyes underneath the eyelids, this stage is characterized by desynchronized brain wave activity (Siegel, 2008). Examination of an EEG of persons in REM sleep, versus those in a wakeful state would prove the two to be indistinguishable. Other defining characteristics are muscle atonia and the loss of reflexes (Carskadon & Dement, 2011), which likely serve the purpose of preventing individuals from acting out their dream. The exact purpose of REM sleep remains a mystery, but it has been implicated in the maturation of the developing brain, formation of new memories (Siegel, 2001), and the processing of emotional information (Walker & van Der Helm, 2009).

Sleep-Regulatory Systems

Arousal and rest are modulated by the interaction of several subcortical monoamine cell populations. The neuromodulatory systems that promote arousal are largely excitatory in nature and include noradrenergic, cholinergic, dopaminergic, serotonergic, and histaminergic neurons. A group of neuropeptides known as hypocretins (also referred to as orexins) also play a role in wakefulness (Chiu & Prober, 2013). Sleep-promoting systems are inhibitory to the wake system; modulators include GABA and galanin (Chiu & Prober, 2013; Saper, Scammel, & Lu, 2005). In non-diseased individuals, these two systems work together to consolidate wake and sleep periods. Two models of the interaction of the sleep-and-waking systems have been postulated; the flip-flop model proposes a mathematical explanation for sleep-wake transitions and suggests very sudden changes in conscious states. The two-process model provides a more fluid transition between the two states (Borbély, 1982).

The two-process model is modulated by homeostatic and circadian rhythms. In this model, arousal (process C) is regulated by circadian rhythms. This system is controlled by the suprachiasmatic nucleus (SCN), a small structure that runs on a near-24 hour transcriptional-translational loop (Saper et al., 2005). Sleep is said to be driven by homeostatic mechanisms and is commonly referred to as process S ('sleep-driving'). Galanin and GABA-ergic neurons in the ventrolateral preoptic nucleus (VLPO) exert an inhibitory influence on the hypothalamic and brainstem areas that participate in arousal (Saper et al., 2005). This process is hypothesized to be driven by the accumulation of molecules that build the need for sleep. Chief among such molecules is adenosine (Porkka-Heiskanen, Alanko, Kalinchuk, & Stenberg, 2002). By binding to receptors in the basal forebrain, adenosine blocks inhibitory outputs to the VLPO; the resulting decrease in inhibition to this area allows for its activation and subsequent suppression of cortical and subcortical regions involved in arousal (Saper, Chou, & Scammel, 2001; Saper et al., 2005).

Alterations to Sleep and Waking

Under normal conditions, the homeostatic and circadian mechanisms work in opposition to each other. Though the SCN is internally regulated, it remains very sensitive to external stimuli, especially ambient light. This small structure is innervated by retinal ganglion cells (Schmidt, Chen, & Hattar, 2011) and is activated when the individual is exposed to light. Subtle changes in light intensity may have great impact on arousal and waking and have the potential to alter entrainment to the environment (Zietzer, Dijk, Kronauer, Brown, & Czeisler, 2000). Such sensitivity can prevent an individual from entering a sleep cycle; this is of particular concern for individuals who

are constantly surrounded by even low-level lights, such as those from a cell phone, tablet, or laptop.

Damage to various neural pathways may also impact the sleep & wake cycles. Lesions to orexin/hypocretin pathways disrupt the circadian rhythms and leave the individual in a narcoleptic-like state (Nishino, Ripley, Overeem, Lammers, & Mignot, 2000). Persons with this condition experience periods of extreme drowsiness during the day and are subject to sudden bouts of sleep (Nishino et al., 2000). On the other hand, lesions to the lateral hypothalamus or malfunctioning GABA receptors within the lateral hypothalamus, prevent the VLPO from exerting its inhibitory effect on the circadian rhythms. This leaves the individual vulnerable to insomnia (Moehler, 2006). Malfunctioning GABA receptors have also been implicated in other neurological and psychological disorders including epilepsy, anxiety disorders and schizophrenia (Moehler, 2006).

Drugs also have the ability to alter sleep and waking levels. In particular, caffeine is very effective in maintaining arousal states. This drug is a natural antagonist of adenosine and blocks the binding of a normal metabolic product to receptors in the basal forebrain. This action inhibits the progression of homeostatic mechanisms, thereby allowing an individual to remain alert for an extended period of time (Saper et al., 2005; Zietzer et al., 2000).

Sleep serves many vital functions, ranging from energy and tissue restoration in slow wave sleep to memory consolidation in stage 2 of NREM (Gais et al., 2002; Tamminen et al., 2010). Sleep deprivation, or disruptions in the sleep pathways, result in the detriment of immune and cognitive functioning in the individual. It is recommended

that adult humans get 7-9 hours of sleep per night (Hirshkowitz et al., 2015). However, estimates from the National Health Interview Survey reveal that roughly 1 in 3 American adults get less than 6 hours of sleep per night (Centers for Disease Control and Prevention, 2011). In the short term, sleep deprived individuals suffer cognitive and attentional deficits. Brain imaging studies reveal a preferential increase in blood flow to the prefrontal cortex during a cognitive task, as compared to non-sleep deprived controls (Drummond & Brown, 2001). This preferential increase in blood flow suggests a greater need for cognitive resources following sleep loss. Chronic partial sleep deprivation in humans has been linked to physiological and psychological disorders. Extending wakefulness by a mere 8 hours increases total body energy expenditure by roughly 7%; this is equivalent to the amount of energy expended if one were to walk a distance of one mile at a moderate pace (Jung et al., 2011). Extended partial sleep deprivation and subsequent energy expenditure may explain the weight gain that is often seen in this population.

The cognitive and physiological deficits are met with worsened psychological states as well. In humans, persistent sleep disturbances are also associated with clinical depression and anxiety (Moehler, 2006; Riemann, 2007). However, the directional relationship between sleep disturbance and resultant psychological disturbances is not well understood. In such cases, work with animal models proves invaluable to increasing our understanding of human conditions and the ontogeny of disease states (Driscoll, Fernández-Teruel, Corda, Giorgi, & Steimer, 2009; Fullerton et al., 2002). Traditionally, the rodent model has been the standard laboratory model and has provided invaluable insight to the mechanism of action for sleep-associated disorders (Everson, 1993, 1995;

Everson, Bergmann, & Rechtschaffen, 1989; Pires, Tufik, & Andersen, 2015; Rechtschaffen, Bergmann, & Everson, 2002). Total sleep deprivation in rats weakens heat retention mechanisms, results in an increase in food intake, and impairs immune response, leaving the animal susceptible to opportunistic pathogens (Everson, 1993). Recently, the zebrafish (Danio rerio) has gained tremendous popularity as a neurobehavioral model of translation. Zebrafish produce robust phenotypes and provide a valuable model for the study of behavior, cognition, and anxiety.

The Zebrafish Model

Zebrafish are a small teleost animal, native to freshwater streams and rivers in Southeast Asia (Bhat, 2004). Their utility in research was first established in the 1970s and since then zebrafish have been widely used in behavioral, genetic, and developmental research (Collier & Echevarria, 2013; Gerlai, 2003; Stewart, Braubach, Spitsbergen, Gerlai, & Kalueff, 2014). Full genome sequencing has revealed a 70% homology to humans (Howe et al., 2013). This homology extends to the functional and structural conservation of brain regions, such as the hypothalamus, between zebrafish and mammals (Alsop & Vijayan, 2009; Howe et al., 2013; Renier et al., 2007). Further, the homology of neurotransmitter and neuropeptide systems allows for the study of psychoactive compounds that act on such pathways (Alsop & Vijayan, 2009; Schweitzer & Driever, 2009).

Zebrafish have demonstrated the ability to discriminate between visual stimuli (Saverino & Gerlai, 2008), have demonstrated a capacity for learning (Collier, Khan, Caramillo, Mohn, & Echevarria, 2014), and exhibit robust phenotypes in measures of anxiety (Egan et al., 2009). Zebrafish are also sensitive to and respond in similar way to

many of the psychoactive drugs that are tested in rodent and human models. Such a high degree of homology lends credence to their participation in comparative studies, and within the past four decades, zebrafish have provided valuable insight in the fields of genetics, neuroscience, pharmacology, and toxicology (Levin & Cerutti, 2009).

Sleep Research in the Zebrafish

Sleep and waking rhythms are developed and fully functional within four days of development in the larval zebrafish (Pando & Sassone-Corsi, 2002). Similar to the circadian oscillator in humans, zebrafish possess a photosensitive structure located in the epithalamus of the retina. This structure, composed of the pineal and parapineal organs, is termed the pineal complex; together with the retina, the pineal complex forms the circadian clock and evidence suggests functional similarity to the mammalian SCN (Ben-Moshe, Foulkes, & Gothilf, 2014; Cahill, 1996; Hirayama, Kaneko, Cardone, Cahill, & Sassone-Corsi, 2005).

Organ and tissue culture experiments have revealed the presence of peripheral circadian oscillators in the organs and various tissues of the zebrafish (Cahill, 2002; Pando & Sassone-Corsi, 2002; Whitmore, Foulkes, Strähle, & Sassone-Corsi, 1998). Circadian rhythms are maintained by the oscillating rhythms of clock genes (Pando & Sassone-Corsi, 2002), many of which are orthologues of the mammalian clock (Pando & Sassone-Corsi, 2002). Oscillating rhythms are responsible for the fluctuations in hormone levels, as well as maintaining the rise and fall of neurotransmitters involved in rest onset and maintenance (Alsop & Vijayan, 2008; Babin et al., 1997; Falcon, Miguad, Munoz-Cueto, & Carrillo, 2010; Faraco et al., 2006; Kim, Nam, Yoo, & Lee, 2004; Rinkwitz, Mourrain, & Becker, 2011).

In larval animals, sleep is behaviorally defined as a state of immobility that lasts longer than 1 minute and is correlated with an increased arousal threshold (Prober, Rihel, Onah, Sung, & Schier, 2006). This is accompanied by a slowing of breathing and heart rate (Zhdanova, 2006). In the young zebrafish, an overexpression of orexins greatly reduces the amount of sleep (Prober et al., 2006), producing a state similar to insomnia in humans. On the other end, exogenous administration of melatonin greatly increases the amount of time spent in sleep like states (Zhdanova, Wang, Leclair, & Danilova, 2001). Criteria for sleep are slightly modified for the adult animals; a sleep-like state is defined as a period of immobility lasting longer than 6 seconds in the presence of an aversive or disruptive stimulus (Elbaz, Foulkes, Gothilf, & Appelbaum, 2013; Singh, Subhashini, Sharma, & Mallick, 2013; Yokogawa et al., 2007). Disruptive stimuli can take the form of bright lights presented during the night phase, sounds in the environment (causing vibrations within the water), or administration of electricity to the tank environment (Sigurgeirsson et al., 2013; Singh et al., 2013; Yokogawa et al., 2007).

Manipulating Sleep Cycles

Zebrafish are typically maintained under a 14-hour day and 10-hour night cycle. Manipulating the amount of time that animals are exposed to light conditions or dark conditions results in subsequent variation of time spent in a sleep like state and the number of sleep-wake transitions that each animal experiences throughout the night (Sigurgeirsson et al., 2013; Singh et al., 2013; Yokogawa et al., 2007).

The presentation of bright lights (>150 lux) during the night phase greatly reduces the number of sleep-wake transitions as well as total amount of sleep experienced by the animal (Sigurgeirsson et al., 2013; Yokogawa et al., 2007). Change in environment

luminosity and sleep duration, in turn, impact the regulation of several housekeeping genes, including those associated with adiposity, growth, morphogenesis and energy balance (Sigurgeirsson et al., 2013). Keeping animals under extended light conditions (>14 hours) causes the up-regulation of roughly 279 transcripts (after controlling for ambient light effects) while keeping animals under extended dark conditions results in the up-regulation of just one gene transcript (Sigurgeirsson et al., 2013). A differential protein expression is also found following extended light and extended dark conditions (Purushothaman et al., 2015). Manipulation of environmental conditions results in a 1.5-fold increase in the expression of proteins related to the circadian pathways, and have implicated roles in the regulation of light/dark-induced stress in humans (Purushothaman et al., 2015). The effect of sleep disruption on the swim and sleeping patterns produces age-dependent effects in the zebrafish. Larval animals will typically display a sleep rebound when they are exposed to constant light conditions (Zhdanova et al., 2008). Following several hours of sleep deprivation, adult animals will reduce their level of activity, and display greater arousal thresholds (Yokogawa et al., 2007).

Sleep disruption is also attained through the administration of mild electric shocks (2-6V) to the tank environment (Sigurgeirsson et al., 2013; Yokogawa et al., 2007). This environmental manipulation effectively reduces the number of sleep-wake transitions as well as total duration of sleep (Yokogawa et al., 2007). However, these low voltage shocks do not impact gene regulation, suggesting a preferential effect of ambient lighting (Sigurgeirsson et al., 2013).

Susceptibility of the System to Drug Manipulations

While the zebrafish pineal organ is internally regulated, it remains susceptible to the influences of environmental conditions (Appelbaum et al., 2009; Purushothaman et al., 2015; Sigurgeirsson et al., 2013; Yokogawa et al., 2007). In addition to this, sleep and arousal rhythms are susceptible to the influences of pharmacological intervention; the homology between zebrafish and mammals allows for the translation of findings between the two models. For example, GABA, an inhibitory neurotransmitter, plays an important role in the regulation of sleep. In low doses, GABA-ergic drugs produce an anesthetic effect on the individual. Similarly, in the zebrafish, an administration of sedative-hypnotics (e.g., barbiturates) reduce locomotor activity and increase arousal threshold (Zhdanova et al., 2001). Further, the exogenous administration of melatonin promotes sleep in the zebrafish, as in humans, evidenced by an increase in arousal threshold and a decrease in locomotion (Zhdanova et al., 2001).

Sleep rhythms are also influenced by non-neurotransmitter and non-neuropeptidergic compounds. Adenosine is a natural byproduct of metabolism and is produced from the consumption of ATP (adenosine tri-phosphate) in the brain. The accumulation of adenosine molecules has been proposed to facilitate the homeostatic drive for sleep (Bjorness & Greene, 2009). Caffeine is a very effective antagonist to adenosine and prevents the binding of such molecules to their receptors. Zebrafish express adenosine receptors in the central nervous system (Boehmler et al., 2009), and the administration of caffeine has been shown to reduce the homeostatic drive for sleep (Porkka-Heiskanen et al., 2002). However, this effect has not been tested in the zebrafish as of yet. Caffeine exposure is linked to anxiogenic states in the zebrafish, and an

increase in total locomotion (Egan et al., 2009; Wong et al., 2010). It would follow then, that an exposure to adenosine antagonists would reduce the total sleep time in adult zebrafish by increasing total swim activity, and by reducing the homeostatic drive for sleep.

Anxiety in Zebrafish

The zebrafish stress response is modulated through the hypothalamus-pituitary-interrenal (HPI) axis, which is comparable to the human hypothalamus-pituitary-adrenal (HPA) axis (Alsop & Vijayan, 2008). Following exposure to a stressful stimulus, zebrafish will produce greater amounts of the steroid hormone cortisol, which is easily quantifiable (Canavello et al., 2011). In addition to physiological markers, researchers may also look to the behavioral phenotypes that are produced following exposure to stressful stimuli. Many of the behaviors that the zebrafish produce are environmentally stimulated, and evolutionarily driven (Egan et al., 2009). As such, they have a very robust and dependable phenotype. There are many modes to measure anxiety-like behaviors in the zebrafish, and these rely largely on the natural instinct of the fish.

The novel tank test operates on the natural tendency of the zebrafish to dive to deeper regions of its environment following exposure to a stressful stimulus (Egan et al., 2009). In this task, the animal is placed in a narrow 1.5L maximally filled trapezoidal tank, largely limiting exploration to the vertical axis (Figure 1). A horizontal line demarcates the top half of the tank from the bottom. Following exposure to a stressor, the animal swims around the tank for roughly 6 minutes. Behavioral endpoints measured include (a) amount of time spent in the top half of the tank, (b) the number of transitions made to the top half of the tank, (c) distance traveled, (d) angular velocity, (e) freezing

behavior, and (f) erratic swimming. Animals exhibiting a greater anxiety-like response typically spend a greater amount of their time in the bottom half of the tank (Egan et al., 2009). This is also accompanied by fewer transitions from the bottom to the top half of the tank (Egan et al., 2009).

In the light-dark preference task, the focal animal, following exposure to a stressing stimulus, is introduced into the center of a rectangular tank. The zebrafish is freely able to swim between two sides of the tank, each of which has a different lighting condition. Typically, the whole tank is under standard illumination (e.g., 150-250 lux); one-half of the tank being covered in a white liner, while the other is covered with a black or dark liner, affording an opportunity for hiding. A tendency to remain in the darker regions (scototaxis) along with differences in distance, velocity, and erratic swimming indicate anxiety-like states in the animal (Champagne, Hoefnagels, de Kloet, & Richardson, 2010; Egan et al., 2009; Stewart et al., 2012).

In the open field task, animals are placed into an open arena which they are allowed to swim freely (Figure 2). This task is a measure of the exploratory tendencies of the animals following exposure to a stressful stimulus. Behavioral endpoints include (a) distance traveled, (b) time spent in the periphery of the tank, versus the middle regions, (c) erratic swimming behavior. Stressed animals will tend not to explore their environment, and as such will spend a greater portion of their time in the periphery (Egan et al., 2009; Kalueff et al., 2013).

Following sleep disruption procedures, zebrafish exhibit varied anxiety profiles. In the light-dark task, sleep deprived zebrafish prefer to spend a greater portion of their time in the dark regions of the tank (Singh et al., 2013), which typically indicates anxiety-

like responses. However, because of the nature of the stressing stimulus (sleep deprivation), the heightened preference for dark environments could be motivated by a drive for sleep. In the quest to evaluate the behavioral effects of sleep disruption on anxiety, the novel tank test, and the open field task would be better suited to evaluate the anxiety-like responses following sleep disruption.

Because of the high degree of homology between humans and zebrafish, and in light of the deleterious effects of sleep disruption on health and anxiety, we sought to evaluate the swim patterns and activity levels of adult zebrafish as they are exposed to various environmental and pharmacological interventions. Swim and activity levels were assessed over a 24-hour period as zebrafish were exposed to sustained darkness (lux = 0), sustained bright conditions (lux > 200), and the long-term administration of caffeine. Fish exposed to sustained light conditions were expected to exhibit a reduction in total sleep frequency during the night phase relative to the control group and maintain greater activity levels. Relative to the control group, fish exposed to sustained darkness were expected to show an increase in sleep frequency during the day, but not the night phase. Fish in the sustained light + caffeine condition were expected to have a lower sleep frequency during the night phase, relative to the control group. This was expected to be met with an increase in locomotion (distance traveled, and average velocity). Resulting anxiety profiles were also evaluated; immediately following each environmental/pharmacological intervention, individual zebrafish were tested on either the open field or the novel tank tests. Two anxiety tests were performed, as each task evaluates a different type of behavior: the novel tank affords vertical swimming and measures vertical exploration, whereas the open field task assesses horizontal swimming.

CHAPTER II – MATERIALS AND METHODS

Animal Maintenance

All fish were maintained, and protocols were carried out in accordance with the Institutional Animal Care and Use Committee of the University of Southern Mississippi, Hattiesburg MS, USA. A total of 180 adult wild-type zebrafish were used for the study. Adult zebrafish were randomly assigned to one of four environmental conditions; fish were exposed to either typical light-dark cycle, sustained darkness sustained bright conditions or sustained brightness that was paired with caffeine exposure. Immediately following environmental manipulation, 120 animals were randomly assigned to one of two anxiety measures: the open field task (n=15 per condition), or the novel tank test (n=15 per condition; two fish were excluded from the brightness and caffeine condition due to hard-drive malfunction). An additional 60 fish were tested to evaluate daily swim and activity patterns during varied environmental and pharmacological intervention.

Fish were purchased from a local pet store (Pet Palace, Hattiesburg, MS) and allowed to acclimate to the aquatic environment for a minimum of ten days. During their acclimation to the aquatic environment, animals were group housed in a 10 L tank (n = 20 maximum per tank) within a water recirculating system. Water temperature was maintained at 26-280C and had four levels of filtration: mechanical, chemical, biological, and sterilization by UV light. Following an acclimation period of at least 10 days, individual fish were transferred to a separate, sleep behavior tank for experimentation and assessment.

Sleep Deprivation Paradigm

Fish were housed individually within a 2.5-gallon tank (dimensions: 309.9 x 154.9 x 205.74 millimeters). Each fish was allowed to acclimate to individual housing for one day and then were transferred to a separate room where the tank was kept within a sound-attenuating chamber (dimensions: 635 x 584.2 x 381 mm). The chamber was fitted with a pair of fluorescent lights (>200 lux), allowing for the artificial manipulation of light-and-dark cycles. Chambers were also equipped with two infrared (IR) lights; the illumination from the IR lights were non-visible to the fish and provided illumination for the camera which was necessary for recording during darkness. Animals were allowed to acclimate to the sound attenuating chamber for one day. During both acclimation days, animals were maintained on a normal light-dark cycle: 14 hours in light (lights on at 8 am), and 10 hours of darkness (lights off at 10 pm). On day 3, disruption day, animals were exposed to one of four conditions: (1) control (LD), (2) twenty-four hours of constant light (LL), (3) twenty-four hours of constant darkness (DD), and (4) twenty-four hours of constant light along with the gradual administration of caffeine (LLC).

On disruption day, the lights were switched on at 8 am for all conditions. In the control group, the lights were switched off at 10 pm, signaling the beginning of the night phase. In the 24L group, lights remained on until 8 am the following morning. In the 24D group, lights were turned off at 9 am (to allow 1 hour for washout effects of darkness). In the 24L+Caffeiene condition, the lights remained on until 8 am the following morning. Caffeine administration began at 10 pm on disruption day. Drug was administered directly into the tank water via a small plastic tube. Drug administration occurred five times throughout the night (10 pm, midnight, 2 am, 4 am, and 6 am),

resulting in an ending concentration of 100mg/L. At the end of the disruption day, animals were transferred to one of two anxiety testing apparatuses. Behavioral endpoints were daily swim activity (distance traveled and average velocity), the number of sleep bouts the animals entered, and the length of an each sleep bout during the disruption day.

In accordance with previous work in zebrafish sleeping behavior, a period of sleep was defined as any period of immobility lasting longer than 6 seconds (Yokogawa et al., 2007). Following this definition, a period of immobility lasting 8 seconds will be considered 2 seconds of sleep. Behaviors were coded via tracking software, idTracker (Madrid, Spain).

Sleep Recording & Behavior Tracking

Each sound-attenuating chamber was fitted with one of two USB cameras. A 29.9 fps USB camera was used to record swim activity for the LL and LLC groups. A modified 24.7 fps USB camera was used for the DD and LD groups. The 24.7 fps was modified such that the IR filter was removed, and a Kodak Wratten 2 No. 87C filter was placed over the camera lens to block out natural light. These modifications made it possible to record swim behavior during the dark phase. Animals were continuously recorded during the disruption day. Each recording was divided into twenty-four 5-minute segments, through Windows Movie Maker, then processed through idTracker, yielding x- and y- coordinates of the animal at each frame for the duration of the video. Coordinates were processed through Matlab to extract behavioral endpoints (total distance traveled, average swim velocity, sleep activity).

Anxiety Testing

Immediately following each sleep and waking cycle manipulation, fish were transferred to either the open field box or the novel tank apparatus for anxiety testing. Animals were allowed to acclimate to the new environment for a period of 60 seconds, after which, behavior was recorded and analyzed. All behaviors were recorded via a 29.9 fps USB camera and recorded as a QuickTime movie. Videos were prepared through Matlab and ImageJ, then processed through IdTracker. The resultant x- and y-coordinates were processed through Matlab to extract desired behavioral endpoints.

Novel Tank Test

The novel tank arena was a narrow 1.5L trapezoidal tank that afforded vertical swimming (Figure 1). Testing took place under similar lighting conditions as those presented during the disruption day for the 24L group (>200 lux). Swim behavior was recorded for a period of 6 minutes and the following behaviors were measured: (1) amount of time spent in the top half of the tank, (2) the number of times the animal transitioned to the top of the tank, (3) the number of immobile bouts, (5) distance traveled, and (6) average velocity. Freezing was operationally defined as a bout of immobility, where speed was less than 0.5cm/s that lasts for at least one second, and that occurred in the bottom of the tank (Gerlai, Lahav, Guo, & Rosenthal, 2000; Kalueff et al., 2013). This behavioral task allows the researcher to assess the amount of geotaxic behaviors exhibited following sleep cycle manipulation. An increase in freezing swimming is also indicative of stress-related behaviors (Egan et al., 2009; Kalueff et al., 2013). Following a disruption in sleep rhythms, it was expected that animals would

exhibit an increase in geotaxic behavior, make fewer transitions to the top regions of their environment, and exhibit an increase in freezing behavior.

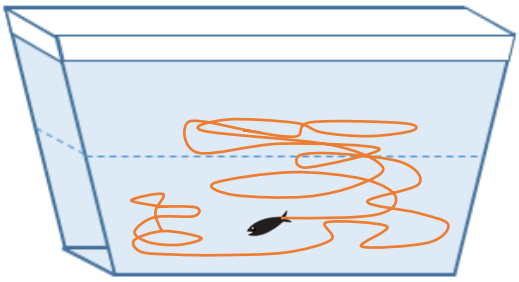

Figure 1. Tank used in a novel tank test.

Note: The top half of the tank is virtually demarcated from the bottom allowing for the analysis of top versus bottom swim behavior. Image used with permission from Khan et al. (2017).

Open Field Task

The open field arena was a 2.5L square tank (dimensions: 20cm height x 20cm width x 6cm height; Figure 2). Testing took place under similar lighting conditions as those presented during the disruption day for the 24L group (>200 lux). Behavioral endpoints included (1) thigmotaxic behavior (i.e. remaining towards the edges of the tank), (2) distance traveled, and (3) average velocity. The open field task allows the researcher to evaluate the tendency of the animals to either explore their environment or seek out opportunities for escape. Following a disruption in sleep rhythms, it was expected that animals would exhibit an increase in thigmotaxic behavior as well as increases in freezing swimming behavior. Freezing was defined as a bout of swimming where the swim speed is less than 0.5cm/s, that lasts for at least one second (Gerlai et al., 2000).

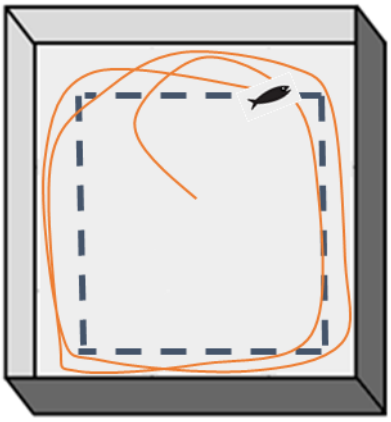

Figure 2. Open field tank.

Note: The middle arena was virtually demarcated from the peripheral 1/5th of the tank allowing for the collection of thigmotaxis behavior. Image used with permission from Khan et al. (2017).

CHAPTER III – RESULTS

Swim activity and effect of environmental/pharmacological condition were evaluated by measuring total activity (distance swum, and average velocity) and total number of rest bouts. Swim activity was evaluated across groups, and within conditions, measuring the differences between swim activity during the relative day and during the relative night. A 2 (time of day) x 4 (condition) repeated measure ANOVA was performed for each of the dependent measures: distance traveled, average velocity, frequency of sleep bouts, and average length of a sleep bout. This was followed up by (1) a paired samples t-test, to observe differences in means between the relative day and night for each experimental condition, and (2) a one-way ANOVA to observe differences between experimental conditions at each time point.

Anxiety tests were analyzed with a multivariate regression model with condition as the predicting factor. This was followed up by univariate ANOVAs to observe differences across groups for each of the dependent measures.

Swim Activity

In general, fish maintained a greater level of activity during the relative day (Tables 1-2). The repeated measures ANOVA revealed a significant main effect of the time of day on the distance traveled, $F(3,56) = 31.20$, $p<0.001$. There was also a significant interaction of time of day with experimental condition $F(3,56)=33.10$, $p<0.001$.

Table 1

Average distance traveled (mm) during the 5minute probing period

	Relative day	Relative night
LD	9060.18 ± 2759.90	2841.09 ± 1201.31
LLC	5520.68 ± 1611.11	7718.64 ± 2693.17
LL	6292.84 ± 2579.08	5464.77 ± 2528.52
DD	6235.73 ± 1358.96	4318.61 ± 1535.74

Under typical light-dark conditions, fish exhibit greater activity during the day; distance traveled for the LD fish is greater during the day (9060.18mm ± 2756.90), relative to the night (2841.09 ± 1201.31), $t(14) = 10.60$, $p<0.001$. These fish also maintain higher swim velocity during the day (30.14 mm/s ± 9.17), compared with the night (9.97 ± 4.03); $t(14)=9.86$, $p<0.001$. A similar pattern was observed in the DD condition. There was greater distance covered during the day (6235.73 ± 1358.96), relative to the night (4318.61 ± 1535.74), $t(14)=4.19$, $p=0.001$. Similarly, average velocity was greater during the relative day (20.56 ± 4.54), versus relative night (14.34 ± 5.10), $t(14)=4.11$, $p=0.001$ (Table 2). The DD fish traveled a lesser distance relative to the LD during the day; $p = 0.010$. During the night phase, the DD fish traveled a greater distance than the LD group; $p=0.032$. We are not aware of any work that directly evaluates the effect of extended dark conditions on locomotion. Sigurgeirsson et al. (2013) report an increase in the sleep bout frequency, though they do not discuss trends in distance in velocity. It should be noted that though differences exist between the LD and DD conditions in levels of activity, that these conditions exhibit similar patterns over the course of the day. Though the DD fish were deprived of a light to dark luminous transition, these fish exhibit a natural reduction in activity, which lends support to the presence of homeostatic drives for sleep in adult zebrafish.

Table 2

Average velocity (mm/s) during the 5-minute probing period

	Relative day	Relative night
LD	30.14 ± 9.17	9.97 ± 4.03
LLC	18.36 ± 5.36	25.62 ± 8.85
LL	21.11 ± 8.49	18.18 ± 8.41
DD	20.56 ± 4.54	14.34 ± 5.10

Fish in the LL condition were exposed to continuous bright conditions and did not exhibit any changes in locomotion between the day and night phases, $t(14)=1.35$, $p=0.20$. Interestingly, LL (6292.84 ± 2579.08) fish traveled a lesser distance than the LD fish during the day, $p=0.039$. This effect is unexpected as the animals in each group receive similar treatment until the beginning of the night phase. In a similar fashion, the LLC fish traveled less (5520.68 ± 1611.11), relative to the LD group during the day, $p=0.001$. At night, however, LLC fish maintain a greater level of activity (7718.64 ± 2693.17) relative to the LD group, $p<0.001$, which is likely the result of caffeine exposure during the night phase. In support of this, the LLC fish exhibited a greater level of activity in terms of distance traveled during the night relative to the day phase, $t(14) = -3.00$, $p=0.009$. However, there are no observed differences in distance swum between the LL and the LLC condition during the night phase, $p=0.108$, which suggests that caffeine (in the doses administered), was not more effective in increasing activity than mere exposure to bright lights.

Sleep Activity

The repeated measures ANOVA revealed a significant interaction effect of the time of day and condition on the number of sleep bouts entered, $F(3,56) = 12.80$ $p<0.001$. All groups entered an equivalent number of sleep bouts during the day phase,

F(3,56)=1.34, p=0.272. However, there were some effects of time of day on sleep frequency. LD fish enter sleep bouts more frequently during the night phase (6.24 ± 431) relative to the day phase (1.31 ± 1.33), t(14)=-5.16, p<0.001. A similar pattern is observed in the DD fish; sleep at night is more frequent (2.86 ± 2.45), relative to the day phase (1.20 ± 0.94), t(14)=-.3.36, p=0.005. This trend in the DD condition provides further evidence for a homeostatic drive for sleep; though the DD fish are without a change in environmental luminosity, sleep onset follows similar patters as LD fish.

There are several differences in the onset of sleep during the night phase, F(3,56)=7.9, p<0.001 (Table 3). The LD entered sleep more frequently than any other condition. There were significant differences observed in comparison to the LLC (1.46 ± 1.16; p = 0.004), and moderate differences against DD (2.84 ± 2.45; p=0.066). Relative to the LD condition, fish exposed to the sustained presentation of lights tended to enter in fewer sleep bouts (2.74 ± 2.40; p = 0.053, Cohen's d = 1.24). In comparing sleep frequency during the day versus that during the night phase, the sustained presentation of lights was effective in preventing the onset of sleep; there were no observed differences in sleep frequency between the day and night phase for the LL and LLC groups. Further, the administration of caffeine (in the doses administered) was no more effective than the presentation of lights in preventing the onset of sleep, p=0.275.

Table 3

Average number of sleep bouts entered during the 5-minute probing period

	Relative day	Relative night
LD	1.31 ± 1.33	6.24 ± 4.31
LLC	2.36 ± 2.54	1.46 ± 1.16
LL	1.80 ± 1.86	2.74 ± 2.40
DD	1.20 ± 0.94	2.86 ± 2.45

There was also an effect of condition on the average length of a sleep bout, $F(3,56)=2.76$, $p=0.05$ (Table 4). The main effect of the time of day was not significant, $F(1,56)=0.44$, $p=0.51$. Post-hoc analyses of the effect of condition on sleep bout length were analyzed for the entire 24-hour period as a whole. The LL (8.09 sec ± 1.76) had longer sleep duration relative to the LD (2.91 ± 1.76; $p=0.04$), and the DD (1.836 ± 1.76; $p=0.015$). Thus, while the LL fish entered fewer sleep bouts, they tended to remain asleep for a longer duration. There were no differences for the LLC against all experimental conditions, suggesting the reduction in homeostatic drive for sleep, via adenosine receptor antagonism.

Table 4

Average length of a sleep bout (sec)

	Sleep bout length
LD	2.91 ± 1.76
LLC	6.37 ± 1.76
LL	8.09 ± 1.76
DD	1.83 ± 1.76

Novel Tank Test

There were statistically significant effects of condition on anxiety behavior, $F(15,138.43) = 3.175$, $p < 0.001$; Wilk's $\Lambda = 0.442$, partial $\eta 2 = 0.238$ (Table 5). There was a statistically significant effect of condition on the number of transitions to the top half of the tank, $F(3,54)=8.00$, $p<0.001$. The LLC made fewer transitions (35.69 ± 31.39) relative to the LD (93.33 ± 37.24) group, $p=0.005$. This was met with an overall increase in the amount of time that LLC fish spent in the top of the tank (234.11 sec ± 114.53) compared with LD (68.34 ± 68.34). Thus, though there were fewer transitions to the top regions of the environment, which would typically suggest an anxiety-like behavior, the

fish spent a greater portion of the time exploring the top regions of their environment. This might suggest that a gradual administration of low doses of caffeine is not anxiogenic to the animal.

Table 5

Novel tank test; descriptive statistics.

Group	Distance (mm)	Velocity (mm/s)	Transitions to the top	Top time (sec)	Immobility bouts
LD	13589.65 ± 4072.71	37.74 ± 11.31	93.23 ± 40.21	122.93 ± 71.57	0.23 ± 0.44
DD	13730 ± 5505.15	40.80 ± 12.14	126.67 ± 62.84	199.59 ± 51.16	0.00 ± 0.00
LL	16722.39 ± 4088.92	46.37 ± 11.39	113.33 ± 66.33	151.90 ± 71.85	0.07 ± 0.26
LLC	7887.86 ± 3670.97	21.91 ± 10.20	30.67 ± 26.77	245.22 ± 112.07	0.17 ± 0.39

There was a statistically significant effect of condition on distance traveled, $F(3,54)=8.60$, $p<0.001$, though no differences were observed between the LD, LL, and DD groups on distance traveled in the novel tank test. In the novel tank test, the LLC swam (8265.14 ± 3768.75) a significantly smaller distance relative to the LD (13631.37 ± 3948.95; $p=0.002$), LL (16722.39 ± 4088.92; $p<0.001$), and DD (13730 ± 5505.15; $p=0.002$) conditions. There was also a statistically significant effect of condition on velocity, $F(3,54)=10.71$, $p<0.001$. The LLC fish maintained a lower velocity relative to the remaining experimental groups. This decrease in total activity in the novel tank test could be the result of increased whole body energy expenditure in the 10 hours prior. The LL fish did exhibit greater velocity (46.37 ± 11.39) relative to the LD (37.85 ± 10.97) condition, $p=0.044$. This difference is not associated with any other behavioral changes, and we cannot draw meaningful conclusions from this difference. Lastly, there were no differences across any conditions on the frequency of immobility behaviors $F(3,54)=1.267$, $p=0.295$. Paired with little evidence for the existence of anxiety-like behaviors, we fail to reject the hypothesis that sleep disruption produces a heightened anxiety-like response.

Open Field Task

Following sleep disruption, fish were introduced to the open field arena, where they were allowed to explore their environment freely. There were statistically significant effects on anxiety behavior based on experimental condition $F(9,131.57) = 3.07$, $p = 0.002$; Wilk's $\Lambda = 0.629$, partial $\eta 2 = 0.143$ (Table 6). There was a statistically significant effect of condition on thigmotaxis behavior, $F(3,56)=5.29$, $p=0.003$. LLC fish

spent a greater amount of time (362.11 ± 206.65) exploring the middle regions of the tank relative to the LD (161.23 ± 95.29; p=0.001), the DD (177.06 ± 176.76), and LL (169.45 ± 151.24) groups. This was met with an overall decrease in LLC distance swum over the course of the behavioral test. Fish in LLC traversed a lesser distance (29139.84 ± 11155.47) relative to the LD (36969.51 ± 11384.20), the DD (38688.46 ± 12598.46) and LL (42693.42 ± 9186.54) conditions. There were no differences observed between the LD, DD, or LL on thigmotaxis, distance traveled or velocity.

Table 6

Open field task; descriptive statistics.

Group	Distance (mm)	Velocity (mm/s)	Time spent in the center of the tank (sec)
LD	36969.51 ± 11384.20	40.95 ± 12.59	161.23 ± 95.29
DD	38688.46 ± 12598.46	42.84 ± 13.60	177.06 ± 176.76
LL	42693.42 ± 9186.54	47.20 ± 10.25	169.45 ± 151.24
LLC	29139.84 ± 11155.47	32.31 ± 12.41	362.11 ± 206.65

Note: the periphery of the tank was defined as the outer 1/5th of the arena.

Stressed animals will tend to remain in the periphery and swim close to the walls, as this behavior may afford an opportunity for escape or hiding. In this experiment, the animals who were exposed to sustained bright conditions, and who had also received a prolonged administration of an adenosine antagonist demonstrated a reduction in anxiety-like behaviors. This could be due to the fact that the gradual administration of caffeine did not produce anxiogenic behaviors, as fish were administered 20mg/L doses of caffeine every 2 hours. The reduction in locomotion could be in part due to the increase in energy expenditure during the night phase of the previous night; Tables 1-2).

CHAPTER IV – DISCUSSION

Sleep is an evolutionary and biological enigma. Many of the species tested to date have demonstrated sleep and rest behaviors (Siegel, 2008). The current project sought to explore the effects of environmental manipulation, as well as pharmacological manipulation on daily activity, and sleep rhythms. It also sought to gain a deeper understanding of the effect of sleep rhythm disruption on anxiety-like behaviors in adult zebrafish.

Previous work in adult zebrafish have demonstrated the ability of environmental lighting and various other stimuli to disrupt normal sleep rhythms (Sigurgeirsson et al., 2013; Singh et al., 2013; Yokogawa et al., 2007). This is associated with differential gene and protein expression, and altered anxiety profiles. However, there is some discordance between anxiety tasks following sleep deprivation, which may in part be due to the mode of sleep disruption (e.g., presentation of bright lights versus presentation of electrical stimulus). The present study sought to evaluate the anxiety profiles of adult zebrafish following a disruption to day and night phases and to evaluate this in light of the respective activity and sleep profiles. We measured anxiety in two tanks that afford swimming in different dimensions. The novel tank test limits swimming and exploration to the vertical axis, and operates on the natural tendency of the zebrafish to remain in the deeper regions of its environment when threatened (Egan et al., 2009; Stewart et al., 2012). The open field task affords horizontal swimming and exploits the tendency of the stressed zebrafish to remain near the periphery of the environments walls, as if it were searching for an opportunity for escape or hiding (Stewart et al., 2012).

In the endeavor to manipulate the activity and sleep profiles, we successfully replicated the disruption of sleep via presentation of bright lights. Keeping zebrafish under extended light conditions (LL) effectively reduced the frequency of sleep bouts that it enters over the course of the 24-hour day (Chiu & Prober, 2013; Yokogawa et al., 2007; Zhdanova et al., 2001). We successfully replicated the prevention of sleep by ambient lighting (Sigurgeirsson et al., 2013). The presentation of bright environmental lights disrupted the onset of sleep, and LL fish entered an equivalent number of sleep bouts during the day and night phase; further, the LL fish entered fewer sleep bouts relative to the LD fish during the night phase. However, the fish in this group were more likely to engage in longer sleep bouts. Thus though the fish slept fewer times, when it did engage in sleep behavior, it slept longer. The presentation of bright environmental lighting is well known to disrupt sleep in adult zebrafish (Sigurgeirsson et al., 2013; Singh et al., 2013), however, we are unaware of any evaluation of the length of the sleep bout as a function of sleep disruption. The results suggest the presence of a homeostatic drive for sleep in the zebrafish; because there is a natural drive for sleep that builds during the night phase (Borbély, 1982; Landolt, 2008), and the environment does not afford rest, the propensity for rest accumulates, resulting in longer sleep durations. Compared with this, the LLC condition received small doses of caffeine at several points during the night phase. Fish in this group entered an equivalent number of sleep bouts as the LL group which suggests that the given dose of caffeine is no more effective than the presentation of bright lights in disrupting the onset of sleep. However, the average length of each sleep bout in the LLC condition was not different from the average LD sleep bout

length. Thus, while the given doses of caffeine is equivalent to the presentation of bright lights in the disruption of sleep onset, it may attenuate some of the homeostatic drive for rest.

It was hypothesized that caffeine administration would increase the number of anxiety-like behaviors in the novel tank and the open field test. We observed that the gradual administration of caffeine did not produce an anxiety profile in the zebrafish, relative to the control group. In both the novel tank and open field tasks, zebrafish maintained a lesser swim speed and traversed a smaller distance (Tables 5-6). This was met by an increase in total exploration of the tanks; in the novel tank test the zebrafish made fewer transitions to the top regions of the tank and remained in the top region for a greater amount of time. In the open field task, the LLC group explored the center of the tank to a greater degree than the remaining experimental conditions. In the open field task, the center of the tank was virtually separated from the periphery, and time spent in either arena was measured using code written in Matlab. We evaluated the amount of time spent in the center versus the outer 1/5 of the tank environment (Figure 2). Previous study of the effects of caffeine on behavior demonstrated an anxiogenic effect of the drug (Egan et al., 2009). In that study the dose tested was 100mg/L, which was our ending concentration in the tank after the last dose. We did not detect any anxiety-like behaviors either with the novel tank nor with the open field task, which may in part be due to the gradual administration of the drug. Zebrafish were dosed in small increments; each dose increased the tank caffeine concentration by 20mg/L. Thus, it is possible that the gradual exposure to the substance attenuated any anxiety behaviors. However, to draw a better

conclusion, it would be necessary to evaluate the effects of prolonged caffeine administration on anxiety behavior under normal sleep and waking rhythms.

Fish in the DD condition entered an equivalent number of sleep bouts relative to the LD group during the day phase (Table 3). Prior to the onset of the dark phase on disruption day, the lights were turned on to the DD group to allow for the washout of sleep from the night prior. It is possible that the washout and the initial light change (dark light) signaled the beginning of a new day. And may have altered the resulting sleep and activity profile. Further research is needed to evaluate the sleep and wake profile of zebrafish in a sustained dark period that occurred as an extension of the typical night phase, i.e. to keep begin evaluating behavior at 10 pm (the typical onset of the dark phase), and maintain the zebrafish under dark conditions for a 24-hour period. Relative to the LD condition, the DD fish exhibited similar anxiety profiles in both the novel tank and the open field task, suggesting that maintaining zebrafish under extended dark conditions is not an inherently stressful event.

The zebrafish model provides a unique combination of neural simplicity and behavioral complexity that allows for the systematic study of memory (Arthur & Levin, 2001), learning (Collier et al., 2014), and anxiety (Richendrfer, Pelkowski, Colwill, & Creton, 2012; Stewart et al., 2012), as well as the effects of psychoactive compounds on such tasks (Khan et al., 2017). In the present study, we have demonstrated the ability of ambient lighting to disrupt the onset of sleep and have provided the first evidence of the ability of a sustained caffeine exposure to reduce the drive for sleep. We did not evaluate

any sleep rebound effects following sleep disruption day, though this data would provide meaningful insight to the role of ambient lighting and caffeine on sleep activity.

APPENDIX A – IAUC Approval Letter

THE UNIVERSITY OF
SOUTHERN MISSISSIPPI.

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE
118 College Drive #5116 | Hattiesburg, MS 39406-0001
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INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE NOTICE OF COMMITTEE ACTION

The proposal noted below was reviewed and approved by The University of Southern Mississippi Institutional Animal Care and Use Committee (IACUC) in accordance with regulations by the United States Department of Agriculture and the Public Health Service Office of Laboratory Animal Welfare. The project expiration date is noted below. If for some reason the project is not completed by the end of the approval period, your protocol must be reactivated (a new protocol must be submitted and approved) before further work involving the use of animals can be done.

Any significant changes should be brought to the attention of the committee at the earliest possible time. If you should have any questions, please contact me.

PROTOCOL NUMBER:	**14111301**
PROJECT TITLE:	**Assessing Sleep Behavior in Zebrafish**
PROPOSED PROJECT DATES:	**11/2014-9/2016**
PROJECT TYPE:	**New**
PRINCIPAL INVESTIGATOR(S):	**David Echevarria**
DEPARTMENT:	**Psychology**
FUNDING AGENCY/SPONSOR:	**N/A**
IACUC COMMITTEE ACTION:	**Full Committee Approval**
PROTOCOL EXPIRATON DATE:	**September 30, 2017**

Frank Moore, Ph.D.
IACUC Chair

November 15, 2014
Date

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