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# The Behavioral, Biochemical and Genetic Effects of Sleep Deprivation in Zebrafish (*Danio Rerio*)

Jade Catherine Boykin

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# THE BEHAVIORAL, BIOCHEMICAL AND GENETIC EFFECTS OF SLEEP

## DEPRIVATION IN ZEBRAFISH (*Danio rerio*)

by

JADE C. BOYKIN

(Under the Direction of Johanne M. Lewis)

### ABSTRACT

Vertebrate sleep is a universal phenomenon and encompasses an array of conserved behavioral and physiological characteristics. Studies of sleep in humans have shown that sleep deprivation leads to adverse effects to human health and additional stress. Teleosts have emerged as a useful model species to study the vertebrate stress response, given its stress axis has high levels of homology with the mammals, including humans. For this study, the behavior of zebrafish (*Danio rerio*) subjected to acute (24 hours) and chronic (20 days) extended light exposure was monitored and whole-body cortisol was analyzed as an indication of activation of the vertebrate stress response. Additionally, transcriptome responses of key genes in the biosynthetic pathway of cortisol (*P450<sub>scc</sub>* and *11 $\beta$ -hyd*) were analyzed with qRT-PCR to investigate the effects of sleep deprivation at the molecular level. We hypothesized that sleep-deprived zebrafish would display increased levels of activity, suggesting lack of “sleep-like” state, which would lead to higher stress, subsequent cortisol production and upregulation of *P450<sub>scc</sub>* and *11 $\beta$ -hyd* expression when compared to control fish. Zebrafish were found to significantly increase activity during extended light exposures, suggesting a disturbance of sleep/wake behavioral cycles. Interestingly, the behavioral changes were not accompanied with a significant increase in whole-body cortisol in response to acute or chronic extended light exposure. At the transcriptome level, *P450<sub>scc</sub>* was significantly upregulated 13.23-fold in the acute sleep deprivation trial, but no

significant changes in expression were observed in the chronic trial. There were no changes in  $11\beta$ -hyd expression levels found in either extended light treatments. In conclusion, acute and chronic extended light exposure resulted in increased activity throughout subjective night hours, but this behavioral change was not accompanied by an increase in whole-body cortisol concentrations, suggesting that lack of sleep-like state does not result in stress in zebrafish. An upregulation of P450<sub>scc</sub> in the acute extended light exposure suggests cholesterol was being cleaved to be readily available for cortisol production if it was later needed, or to possibly become precursors to sex hormones in order to aid in aggressive behavior.

INDEX WORDS: Vertebrate Sleep, Sleep Deprivation, Stress Response, Cortisol, Zebrafish

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DEPRIVATION IN ZEBRAFISH (*Danio rerio*)

by

JADE C. BOYKIN

B.S., Georgia Southern University, 2014

A Thesis Submitted to the Graduate Faculty of Georgia Southern University in Partial  
Fulfillment of the Requirements for the Degree

MASTER OF SCIENCE

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## DEDICATION

*This thesis is dedicated to my mathematician father, Dr. Charles M. Boykin, who instilled  
curiosity and confidence in me at an early age –*

*I would have never started or finished this educational journey without your guidance;*

*To my caring mother, April Boykin,  
for your unconditional nurture, support and love.*

*To my talented brother, Charles L. Boykin,  
for your genuine character and heart.*

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## CHAPTER 1

### INTRODUCTION

Sleep is a universal phenomenon that is shared by all vertebrates and can be categorized by a suite of behavioral, biochemical, and physiological mechanisms. These mechanisms are all controlled by a daily rhythm of a highly conserved endogenous timing system known as the circadian clock. Clinical and experimental observations have suggested the maintenance and synchronization of this clock to be highly advantageous to humans and other vertebrates (Fuchs & Burgdorf, 2008; Walker, 2008). The daily rhythm of this clock is thought to be so advantageous that even short disruptions can directly result in negative impacts to cognitive functions, physical, and physiological health. Short-term sleep deprivation has a significant deleterious effect across most cognitive domains such as attention, memory, processing speed, and reasoning (Lim & Dinges, 2010). Although sleep is a necessary and large component of human life, there still remains much uncertainty regarding the physiological functions of sleep.

#### *Vertebrate Sleep Pathways*

A wide range of vertebrate sleep patterns exists among the vertebrate clade. In humans, sleep is consolidated and occurs at night for typically 5-8 hours, whereas other vertebrates experience fragmented sleep throughout the 24-hour day and only sleep in small bouts of minutes at a time. In the same sense, some vertebrates sleep in an immobile state, while others remain slightly mobile during sleep, as seen in fish species (Zhdanova, 2011). Despite differences in sleep patterns across taxa, the primary restorative functions of sleep are well conserved (Cirelli & Tononi, 2008; Yokogawa et al., 2007; Zhdanova, 2011).

Sleep in higher vertebrates is typically associated with several characteristics: cycles of electroencephalograph (EEG) patterns, specific postures, periods of reversible quiescence (dormancy), and decreased sensitivity to environmental stimuli (Elbaz et al. 2013). Importantly, sleep is multi-faceted in its regulation by both circadian rhythms for timing purposes and molecular homeostatic mechanisms for sleep deprivation prevention purposes. In mammals, the circadian clock is primarily driven by the hypothalamic suprachiasmatic nucleus (SCN), which computes information from other brain regions, such as regulation of sleep period actions of behavioral arousal on circadian rhythms (Tao et al., 2015; Webb et al. 2014). Metabolic activity in the SCN is found to be high in the daytime and low in the nighttime, and at night the metabolic activity of the SCN can be significantly increased by acute light exposure (Robinson & Reddy, 2014). Studies on humans have shown light to be the most significant disruptor of circadian rhythms and that regular periods of light and dark are important for normal sleep physiology (Ackermann et al., 2013). Disruption of this circadian rhythm pattern can decrease or completely diminish sleep in vertebrates, which can be detrimental to the organism's overall health (Lim & Dinges, 2010).

Sleep can be measured neuro-physiologically in higher vertebrates, but can become challenging to observe and define in lower vertebrates and invertebrates. In organisms such as teleost fishes, sleep has been defined using behavioral parameters (Campbell & Tobler, 1984). Sleep in adult zebrafish (*Danio rerio*) has been characterized as prolonged periods of immobility and/or floating (6 seconds up to 10 minute bouts, depending on age and strain) occurring predominantly in night (dark) hours. These periods of immobility have been generally associated with three

different postures: either floating with their head slightly upward or staying in a horizontal position close to the bottom of the tank, occasionally moving their eyes and fins, or even swimming in a very slow manner. Fish in this “sleep-like” state also display an increased arousal threshold to mild stimulation such as electric shock and/or light exposure, suggesting deep sleep is occurring during this time (Zhdanova et al., 2001; Zhdanova, 2011).

### *Sleep Deprivation*

Adequate amounts of sleep are essential to a healthy life (Blagrove & Akehurst, 2001). The recommended amount of sleep for a normal human adult is eight hours in a 24-hour period (Tune, 1968). Sleep provides a fundamental role in brain functioning, immune control, homeostatic regulation, memory, tissue repair, and thermoregulation (Walker, 2008). Studies of sleep in humans have shown the adverse effects of sleep deprivation on human health by metabolic, physical, psychological, and behavioral parameters (Adamantidis & Lecea, 2008; Cirelli, 2008; Curcio et al., 2006; Livingston et al., 2015). These adverse effects can then lead to greater risks of serious diseases and can also affect learning and memory processes because the restorative properties of sleep are not being performed regularly during sleep deprivation (Curcio et al., 2006).

Lack of sleep and the negative health effects associated with it induces stress in individuals. Additionally, it creates a cycle of stress that impairs the individual from being able to achieve efficient sleep the next day. When an individual does not have adequate amounts of sleep in a given day, the body recognizes this lack of restorative sleep as a stressor. The body then initiates the stress response by boosting the level of secreted corticotropin-releasing stress hormone. The

peptide and satiety hormones the brain releases when it recognizes the body in deep sleep (insulin and leptin) are the same hormones it releases to cease the production of corticotropin-releasing stress hormones (Walker, 2008). Therefore, regular sleep is essential for stress hormone regulation in vertebrates.

### *The Vertebrate Stress Response*

The stress response is one of the most well conserved pathways across taxa and also across species within a taxon, and is responsible for the synthesis and release of primary glucocorticoid stress hormones (reviewed by Wendelaar Bonga, 1997). While the stress axis is well conserved, many studies have shown the activity of the stress axis can be greatly varied by numerous environmental, behavioral, and physiological factors. When an organism experiences a stressor, a suite of behavioral and physiological responses is induced in order to compensate for a perceived threat and attempt to maintain homeostatic equilibrium. The teleostean stress response is comprised of primary, secondary, and tertiary responses (Barton & Iwama, 1991; Wendelaar Bonga, 1997). The primary and secondary responses are typically an acute stress response and are thought to be adaptive for short-term maintenance. Tertiary responses occur when stressors chronically persist for hours or days, and the stress response can instead have negative effects on the organism.

The primary response is the first step after a stressor has been experienced and involves the release of catecholamines from chromaffin cells. This release of catecholamines activates the HPI axis, resulting in the increased synthesis and release of glucocorticoids from steroidogenic interrenal cells. Levels of these hormones vary, with catecholamines rising and peaking within



the first few minutes of experiencing a stressor, whereas glucocorticoids levels rise gradually, making them a more suitable hormone to measure than catecholamines in both resting and stress-exposed states (Jeffrey & Gilmour, 2016). Release of catecholamine and glucocorticoid stress hormones lead to the secondary response at the tissue level. Both primary response hormones function to increase physiological changes to aid in the response to a stressor such as increased cardiac output, oxygen uptake, and mobilized energy resources. Finally, tertiary responses (which often are only reached when a stressor has persisted chronically) have effects at the whole animal or population levels and have the potential to inhibit growth and reproduction, cause malfunction of the immune system, and reduce survival, all while reducing the effectiveness of the organism's response to subsequent or additional stress. Glucocorticoids, rather than catecholamines, are ideal for studying the effects of prolonged stressors, as glucocorticoids persist past the immediate time period a stressor just occurred (Jeffrey & Gilmour, 2016).

### *Project Goals*

Sleep deprivation has been shown to disrupt behavioral cycles in vertebrates. It is likely that prevention of sleep and its restorative benefits are stressful to the organisms, resulting in the increase in production of stress hormones such as glucocorticoids. The primary objective of this study was to provide an integrative description of the effects of acute and chronic extended light exposure, using *Danio rerio* (zebrafish) as a model system. Using extended light exposure as a means of preventing entry into sleep, we first assessed changes to diurnal behavior pattern of the fish by analyzing percent of time spent in an active state, cumulative distance traveled and its position in the tank. To assess if alternations to the diurnal sleep/wake cycle were accompanied

by changes at the physiological level, we measured the primary corticosteroid response, whole-body cortisol concentrations. Additionally, control of cortisol production at the transcriptome level was investigated by analyzing mRNA expression of P450 $scc$  and 11 $\beta$ -hyd, both of which are rate-limiting steps in the biosynthetic pathway of cortisol production. Through this integrative investigation of the response of zebrafish to sleep deprivation, this study has the potential to contribute to the understanding of the effects of lack of sleep in vertebrate organisms.

## CHAPTER 2

### BEHAVIORAL RESPONSES OF ZEBRAFISH (*Danio rerio*) TO ACUTE AND CHRONIC PERIODS OF EXTENDED LIGHT EXPOSURE

#### INTRODUCTION

Zebrafish display a diurnal nature of sleep, similar to that in humans, but in contrast to the nocturnal rhythms of rats or mice commonly used in sleep research. Like humans, zebrafish display daily cycles related to photoperiod, with the highest locomotor activity in day light hours and lowest locomotor activity in the nighttime hours, whereas rats/mice have highest activity in night hours and lowest activity in day light hours (Hurd et al., 1998; Tovin et al., 2012). In higher vertebrates, with a cerebral cortex, sleep is typically defined using polysomnographic recordings such as electroencephalogram. However, such approaches are not feasible in lower vertebrates. Instead researchers rely upon observed behavioral parameters to identify when organisms enter a sleep-like state, hereafter referred to as sleep (Campbell and Tobler, 1984; Elbaz et al., 2013; Zhdanova, 2011). These key behavioral sleep criteria include: (1) immobility periods that are associated with specific posture(s); (2) quick reversibility to wakefulness; (3) increased arousal threshold to external stimuli; (4) sleep-rebound following sleep deprivation, and (5) preference for nocturnal or diurnal sleep (Zimmerman et al., 2008). These behavioral criteria were used to establish a working singular definition of sleep in adult zebrafish, which collectively defined sleep as a minimum of 6 seconds of immobility (Yokogawa et al., 2007). Because of these recognizable features that are also key indicators of higher vertebrate sleep, zebrafish sleep behavior can be concluded to be similar to other previously studied vertebrates, making it a suitable model to study the physiological mechanisms underpinning sleep.

Similar to mammals, zebrafish sleep is regulated via with circadian mechanisms, involving a complex machinery of an intrinsic circadian clock that includes several core clock genes and proteins that serve as transcription factors. These transcription factors are organized in complex feedback loops to maintain 24-hour oscillations (Reppert & Weaver, 2002). Circadian clock genes regulate sleep/wake and are part of a molecular mechanism that is well-conserved across most fish species and other vertebrate classes (Cahill et al., 1998; Pando & Sassone-Corsi, 2002; Kaneko et al., 2006).

Sleep mechanisms in zebrafish are also regulated by homeostatic processes (Zhdanova, 2011), which can be altered in response to sleep deprivation. After several hours of forced wakefulness during subjective night, adult zebrafish display reduced activity levels and increased sensitivity to arousal stimuli (i.e. light) (Zhdanova, 2011; Yokogawa et al., 2007). Sleep deprived adult zebrafish inefficiently performed cognitive tasks, as compared to control zebrafish that were not rest deprived using continuous light (Yu et al., 2006). They also became hyperactive for some time following periods of sleep deprivation (Zhdanova et al., 2001).

Mature zebrafish sleep patterns and how they are affected by sleep deprivation is still not fully understood. It is possible that maturing zebrafish develop abilities to have microepisodes of sleep, allowing them to move continuously and be unaffected by sleep deprivation. While previous studies analyzed embryo responses to extended light for periods of  $\leq 24$  hours to a maximum of 8 days, the responses of adult zebrafish to long term/chronic ( $> 8$  days) extended light has yet to be investigated. As such, our study will take a novel approach by investigating the behavioral response to extended light exposure treatment (20 days) with mature zebrafish.

This will allow us to further investigate the remarkable ability of zebrafish to seemingly bypass the need for sleep when challenged with constant bright environmental illumination or other external stimuli causing sleep deprivation (Yokogawa et al., 2007).

The objective of the current study was to compare the effects of acute and chronic extended light exposure on the behaviors associated with sleep/wake states in adult zebrafish (*Danio rerio*). To accomplish this, *D. rerio* were monitored using video-tracking software during regular photoperiods and experimental photoperiods (24 hours of continual light exposure). Activity measurements of a regular “natural” photoperiod helped establish the natural sleep behavior cycle over a 24-hour period. Following this, behavioral patterns of zebrafish were tracked during an acute (24 hours) and chronic (20 days) continual light exposure to investigate if the duration of an extended light bout has compounding effects on the zebrafish’s behavior, or if the zebrafish is able to adapt to the extended light. Given the current research conducted on zebrafish and sleep behavior, we expected the highest locomotor activity in light hours and the lowest locomotor activity in dark hours during normal photoperiods. In zebrafish exposed to acute extended light exposure, we expected constant locomotor activity for the full 24-hour observation period. Whereas, during chronic extended light exposure we expected constant activity during the initial stages of the 20-day trial with decreasing active states as the trial persisted and as the fish became acclimated to the experimental conditions.

## METHODOLOGY

### *Animals and Holding Conditions*

All experiments were conducted following the guidelines set by IACUC and all protocols were approved by the Georgia Southern University Animal Care Committee (IACUC # I15015). Zebrafish (*Danio rerio*) derived from the Tuebingen (TU) line were bred and raised at Georgia Southern University. All mature fish were 150-210 days of age (sexually mature), approximately 2-3 cm in length and ranged from 0.3-0.4 g in mass at the start of each experiment. Depending on the experiment, fish were housed either individually (2.8 L) or grouped (6.0 L) in transparent aquaria tanks (Aquateering, Inc. San Diego, CA) with filtered, recirculating water at a target temperature of 28.5°C (absolute range 25.5-30°C). Fish were fed live brine shrimp daily in the late afternoon. Water was constantly recirculated through a system of biological, particle, carbon, and UV sterilization filters (Aquateering, Inc. San Diego, CA) at a rate of approximately 45.4 liters/min. Water quality was checked twice weekly for a target pH of 7.0-7.5 and target ammonia (NH<sub>3</sub>) <0.02 mg/L. Fish were held under a 14-hour light:10-hour dark (14:10 LD) cycle [lights on 0900 h; lights off 2300 h EST]. Lighting in the holding rooms was from fluorescent ceiling lights, with luminance of approximately 275 lux.

### *Experimental Setup and Photoperiod Conditions*

Acute Extended Light Exposure (N=36): The experiment was completed in three runs with 12 zebrafish at a time held in individual tanks (2.8 L). On a given run, zebrafish used in the extended light exposure study were transferred to tanks in a separate room, whereas zebrafish used in the control group were transferred to new tanks within the same room. All zebrafish were allowed two days to acclimate after transfer to their experimental tanks. All experimental tanks

were supplied with the same water conditions as described for the holding tanks. In each of the three runs, the control group (n=6) was held under the same photoperiod as the fish were exposed to prior to the experiment [14-hour light:10-hour dark]. The experimental group (n=6) was switched to an extended light exposure [24-hour light:0-hour dark]. Six fish from each run (three control, three extended light exposure) were video-recorded for behavioral analysis.

Immediately after the 24-hour experimental period all fish were euthanized in lethal doses of MS-222 and flash frozen in liquid nitrogen for analysis of physiological and molecular markers of stress (Chapter 3). All acute extended light exposure trials were run in December 2015.

Chronic Extended Light Exposure (N=36): The chronic experiment was completed in one single run with the six zebrafish used for behavioral analysis housed individually (2.8 L tank) and the remainder of the fish used for cortisol and molecular analyses held at a stocking density of 15 zebrafish per tank (6.0 L tank). For the single chronic run, eighteen zebrafish used in the extended light exposure study were transferred to tanks in a separate room, whereas the eighteen zebrafish used in the control group were transferred to new tanks within the same room. All zebrafish were allowed two days to acclimate after transfer to their experimental tanks. The control group (n=18) was held under the regular holding conditions photoperiod [14-hour light:10-hour dark]. The experimental group (n=18) was switched to an extended light exposure [20 days; 24-hour light:0-hour dark]. Immediately after this experimental period fish were euthanized in lethal doses of MS-222 and flash frozen in liquid nitrogen for analysis of physiological and molecular markers of stress (Chapter 3). The chronic extended light exposure trial was run in February 2016. The original chronic extended light exposure study was planned

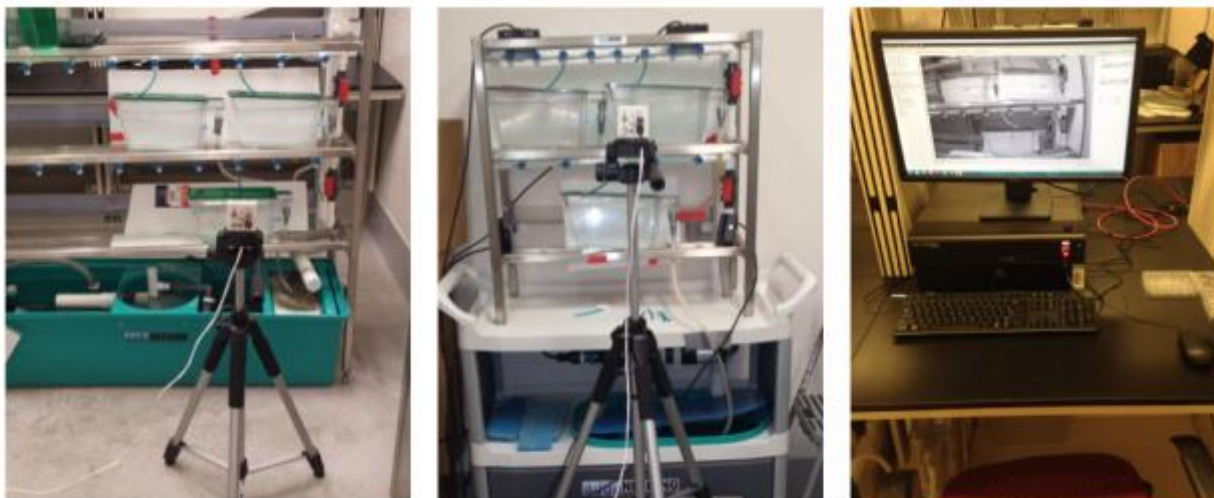
to be 28 days, but was stopped after 20 days as fish began to exhibit signs of serious ill health (as per IACUC requirements).

### *Behavioral Data Acquisition*

Activity of the fish and their position in tank was digitally recorded using EthoVision XT 11.5 (Noldus Information Technology; Netherlands). In brief, fish were distinguished from the background using difference of color (dark fish against a bright, white background), and activity within the tanks were tracked by pixel-level changes frame by frame to estimate behavioral parameters. Tank arenas were individually calibrated using the known tank length and width before analysis of behavior was analyzed. The software tracked fish movement by changes in pixelation frame-by-frame (5 frames/second).

During the experimental runs, the video-tracking program analyzed various behavioral endpoints: duration (%) in awake state, cumulative distance traveled (cm), and duration (%) in bottom of tank. Detailed definitions of each endpoint can be found in Table 2.1. As per the published literature, sleep or sleep-like state was defined as a continuous interval of immobility for  $\geq 6$  seconds (Zhdanova et al. 2001, Yokogawa et al. 2007, Singh et al. 2013). Set-up consisted of Ikegami ICD-49 monochrome cameras and infrared lighting (for dark hours) (Figure 2.1). All trials (acute and chronic) began at 0900 EST and ran for 24 hours. Lighting in holding rooms was regulated by automatic timing systems set for the specific photoperiod.





**Figure 2.1.** Ethovision XT system and tank set-up for experimental (left picture) and control (middle picture) trials. One camera was used to record video for three tanks, which were illuminated in dark using infrared lighting. Video recordings and zebrafish activity was analyzed on EthoVision software (right picture).

**Table 2.1.** Summary of behavioral endpoints and their significance measured in extended light photoperiod trials, based on video-recorded data with EthoVision XT.

<b>Endpoint (units)</b>	<b>Definition</b>	<b>Interpretation</b>
Cumulative distance traveled (cm)	Cumulative distance the zebrafish traveled in 4-hour time bins within the tank	Reflects general motor functions in batches of time; trends in behavior can be observed when batching time points
Duration of time spent in state of movement (%)	“Not Moving” state defined as continuous immobility for 6+ seconds at 1.5 cm/s; “Moving” state defined as movement at 1.51+ cm/s	Indicates restful state or wakeful state in zebrafish, which can be defined as states of mobility
Duration (%) of time spent in bottom of tank	Frequency of zebrafish entering bottom half of tank	Reflects preference for bottom zone of tank in zebrafish during changes to external environment

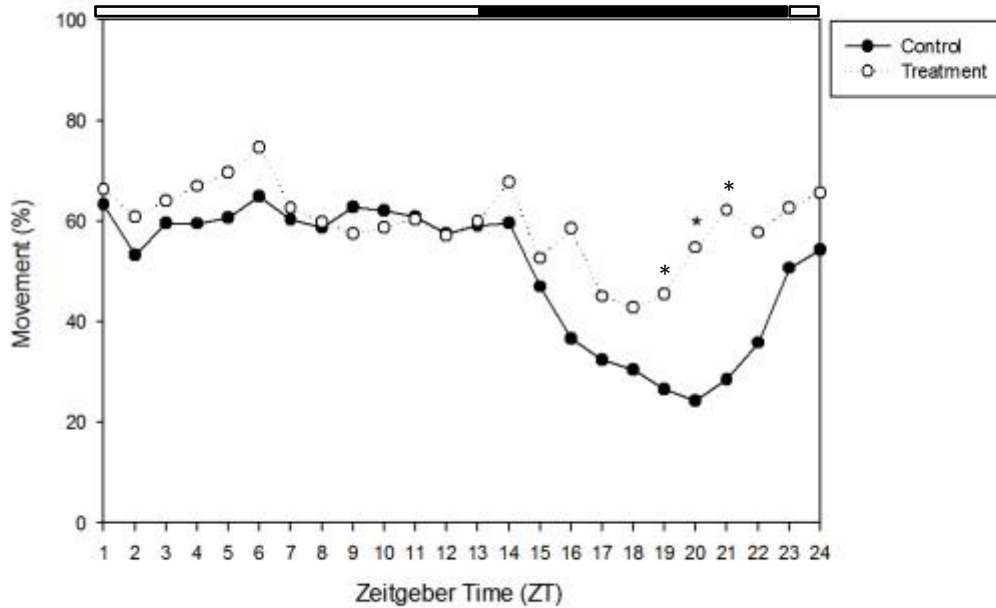
### *Data Presentation and Statistical Analysis*

For clarity sake all behavioral data is presented on the scale of Zeitgeber Time (ZT), which is the experimental time starting at the onset of each 24-hour recording trial. Statistical significance between control and experimental groups for behavioral endpoints were determined via an Independent Samples *t*-test ( $p \leq 0.05$  deemed significant). For duration of time spent in moving state and in bottom zone of tank, differences between control and experimental groups were calculated every hour for the 24 hours of the trial. To determine statistical differences between treatments for the distance moved, data were binned in four-hour groups and an Independent Samples *t*-test was run for each four-hour bin. All analyses were conducted using JMP 12 (SAS Institute, Inc. Cary, NC).

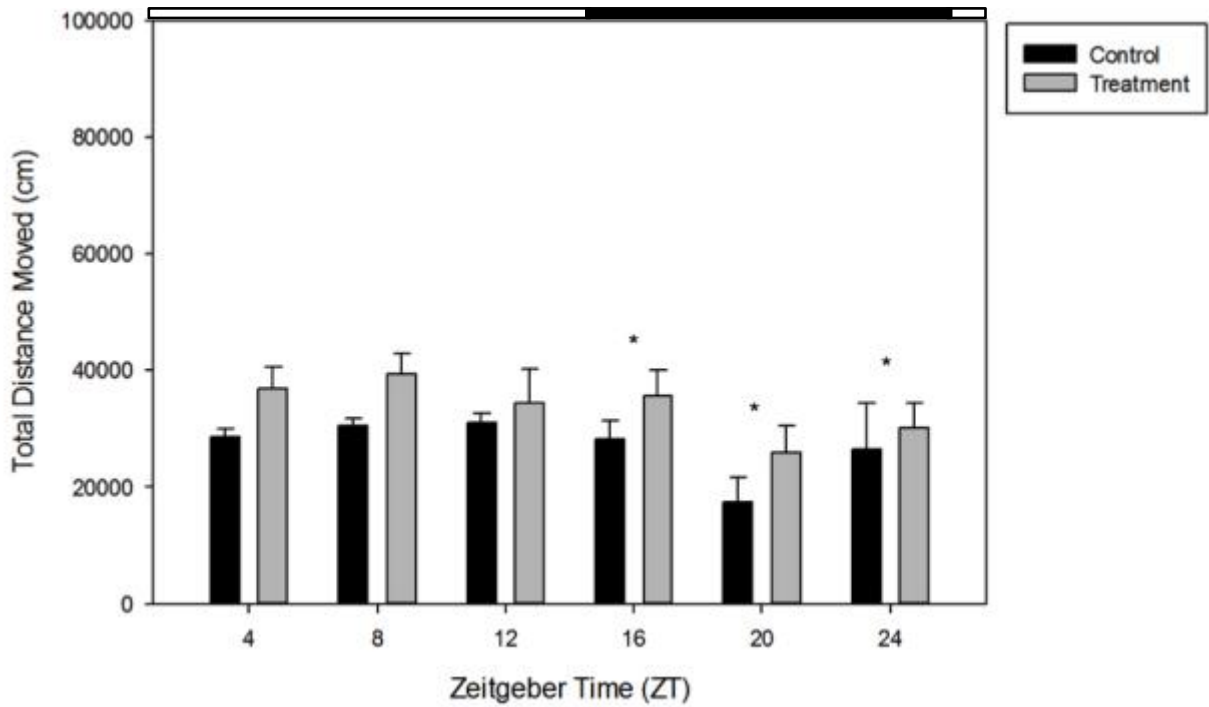
## RESULTS

*Natural (Control) Photoperiod:* During daylight hours (ZT0-ZT13) the zebrafish exposed to a natural photoperiod (control) in both acute and chronic experiments spent approximately 60-70% of their time in an active state (Figures 2.2 & 2.5). At the onset of night (lights off; ZT14-ZT23), the zebrafish exhibited an immediate decrease in their activity, spending 30-40% of their time in an active state. Once light returned (ZT23-ZT24), zebrafish showed a slight increase in time spent in the active state (Figure 2.2 & 2.5). The cumulative distance moved (cm) also decreased during night, with zebrafish swimming from 3500-4500 cm every four hours during daylight hours and approximately 1000-2000 cm every four hours during the night hours (Figures 2.3 & 2.6). Control zebrafish spent approximately 60% of their time in the bottom half of the tank during daylight hours and spent approximately 40% of their time in the bottom half of the tank during night hours (Figures 2.4 & 2.7).

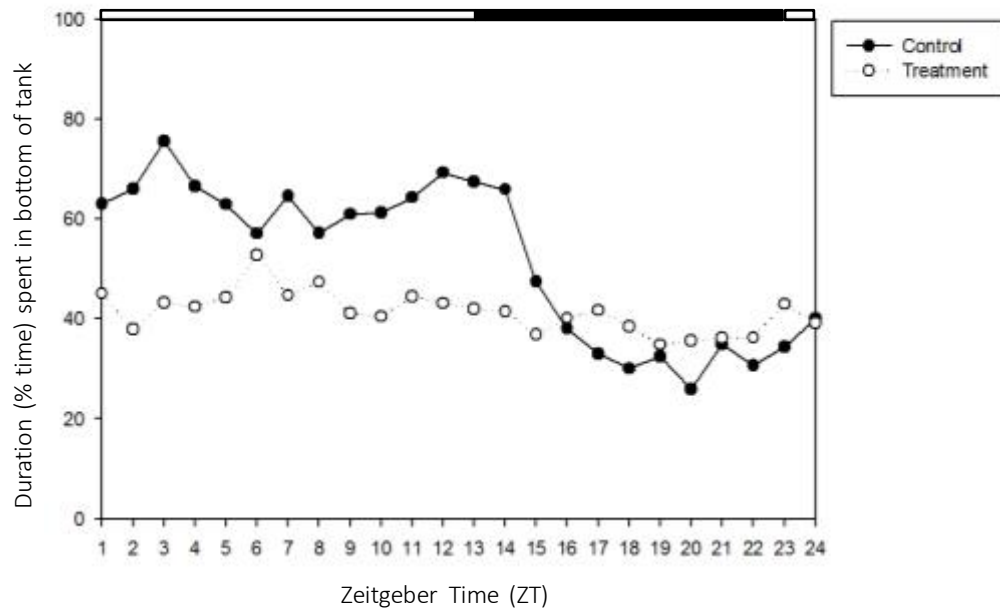
*Acute Extended Light Exposure:* Zebrafish in the acute extended light exposure group behaved similarly to zebrafish held under a natural photoperiod during daylight hours (ZT0-ZT13), however they showed marked differences in their behavior during subjective night hours (ZT14-ZT23). These zebrafish experienced a significant increase (~15%) in time spent in active state during ZT19-21 ( $p < 0.05$ ; Figure 2.2) and significantly increased the cumulative distance moved to 3000-3500 cm every four hours during ZT12-ZT24 ( $p < 0.05$ ; Figure 2.3). Acute extended light exposure zebrafish spent approximately 40% of their time in the bottom half of the tank during subjective daylight and night hours (Figure 2.4).



**Figure 2.2.** Behavioral observations of state of activity with acute extended light exposure [24 hr-light:0-hr dark]. The percentage of time spent moving (awake) over a 1-hour period (cumulative duration %) was recorded over the 24-hour cycle. Zeitgeber Time (ZT) refers to the time from the start of the experiment. Data are presented as means of each time point (n=9) with significant difference between treatments, tested every hour, represented by an asterisk ( $p \leq 0.05$ ).

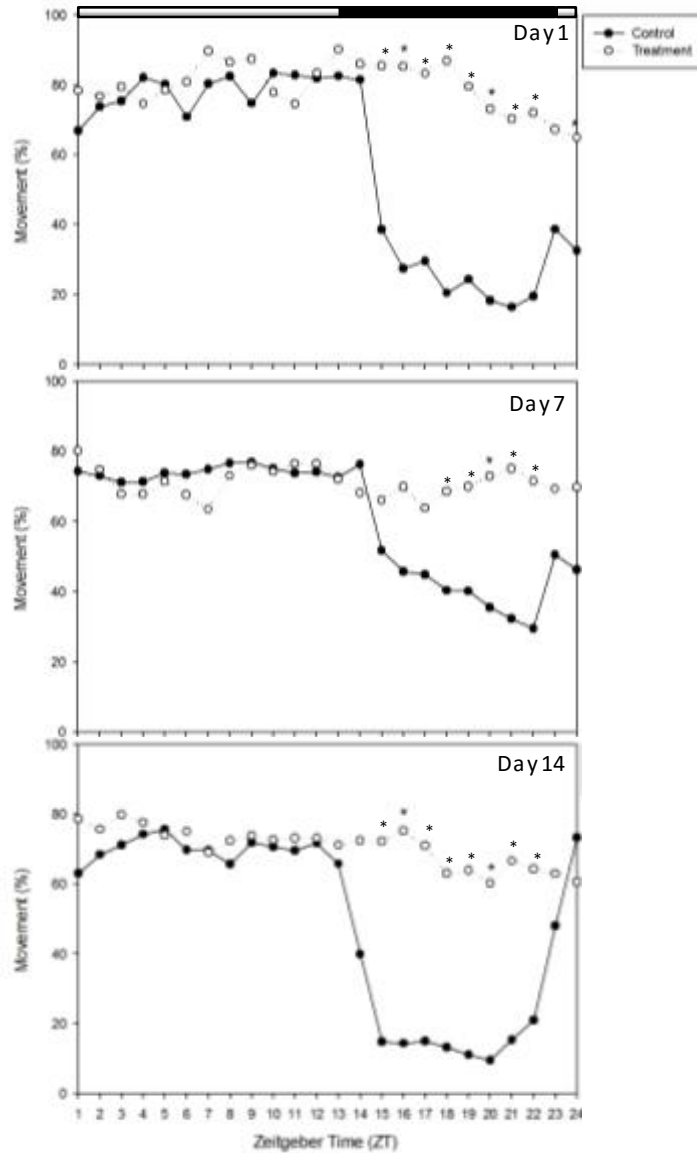


**Figure 2.3.** Behavioral observations of distance moved with acute extended light exposure [24-hr light:0-hr dark]. The cumulative distance moved (cm) over a 4-hour period was recorded over the 24-hour cycle. Data are presented as means of each time point  $\pm$  SE (n=9) with significant difference between treatments, tested every 4 hours, represented by an asterisk ( $p \leq 0.05$ ).



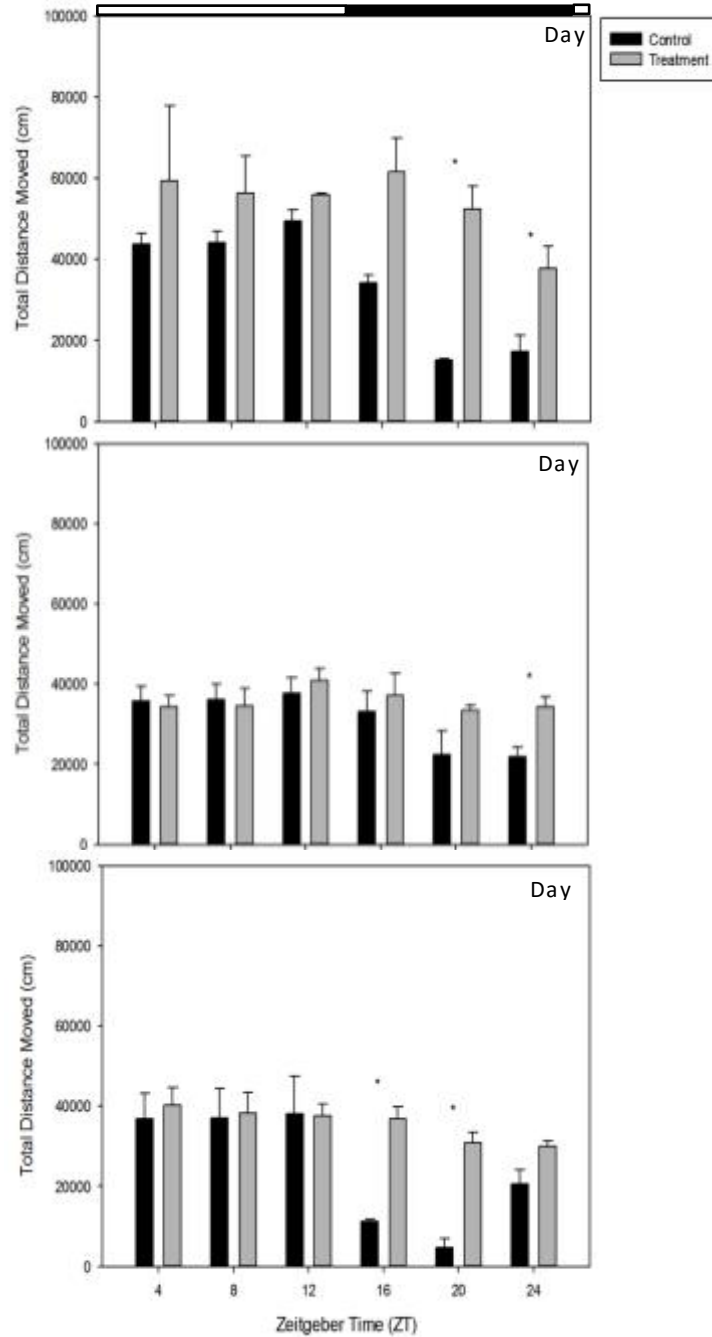
**Figure 2.4.** Behavioral observations of cumulative duration (% time) in the bottom half of the tank with acute extended light exposure [24-hr light:0-hr dark]. The cumulative duration over a 1-hour period was recorded over the 24-hour cycle. Data are presented as means of each time point (n=9) with significant difference between treatments, tested every hour, represented by an asterisk ( $p \leq 0.05$ ).

*Chronic Extended Light Exposure:* Zebrafish in the chronic extended light exposure group behaved similarly to zebrafish held under a natural photoperiod and acute extended light exposure during daylight hours (ZT0-ZT13), however they showed marked differences in their behavior during subjective night hours (ZT14-ZT23). On Day 1, zebrafish experienced a significant increase (~45%) in time spent in active state during ZT15-ZT22 ( $p < 0.05$ ; Figure 2.5) and significantly increased the cumulative distance moved by ~2500-4000 cm every four hours during ZT16-24 ( $p < 0.05$ ; Figure 2.6). During Day 7, zebrafish experienced a significant increase (~35%) in time spent in active state during ZT18-ZT22 ( $p < 0.05$ ; Figure 2.5) and significantly increased the cumulative distance moved by ~1000 cm every four hours during ZT20-ZT24 ( $p < 0.05$ ; Figure 2.6). During Day 14, zebrafish experienced a significant increase (~55%) in time spent in active state during ZT15-ZT22 ( $p < 0.05$ ; Figure 2.5) and significantly increased the cumulative distance moved by ~2500 cm every four hours during ZT12-ZT20 ( $p < 0.05$ ; Figure 2.6). Days 1, 7, and 14 chronic extended light exposure zebrafish spent approximately 40-50% of their time in the bottom half of the tank during subjective daylight and night hours (Figure 2.7).

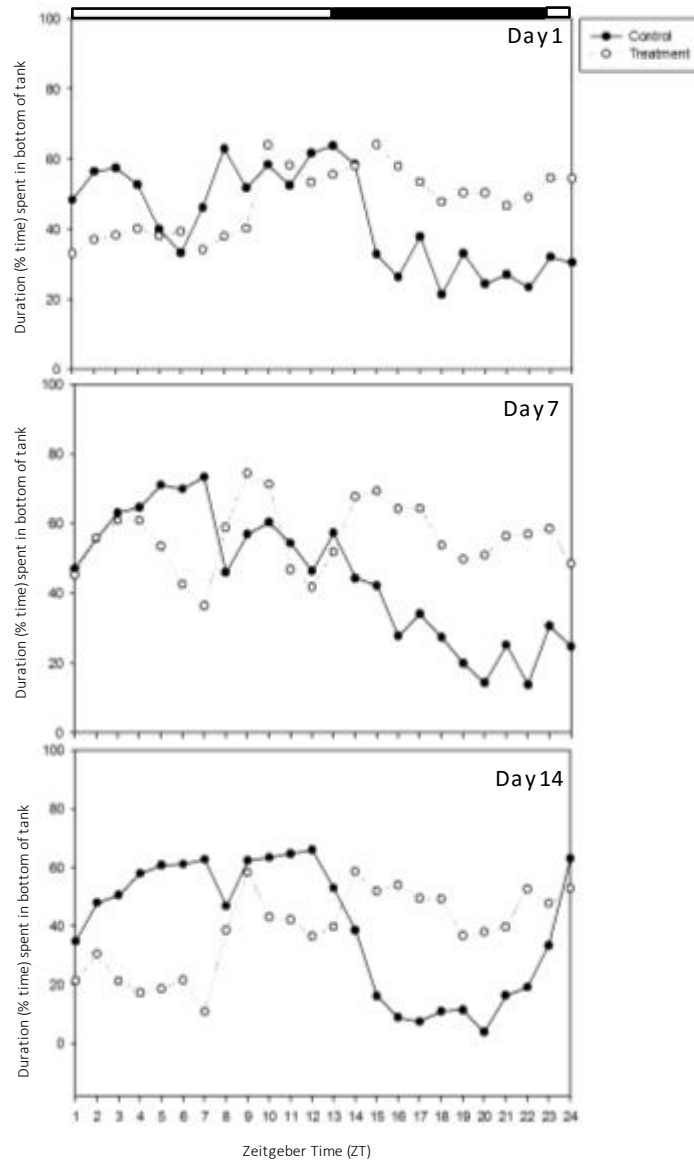


**Figure 2.5.** Behavioral observations of state of activity with chronic extended light exposure [20 days; 24-hr light:0-hr dark]. The percentage of time spent moving (awake) over a 1-hour period (cumulative duration %) was recorded over a 24-hour cycle. Data are presented as means of each time point (n=3) with significant difference between treatments, tested every hour, represented by an asterisk ( $p \leq 0.05$ ).





**Figure 2.6.** Behavioral observations of distance moved with chronic extended light exposure [20 days; 24 hr light:0 hr dark]. The cumulative distance moved (cm) over a 4-hour period was recorded over the 24-hour cycle. Data are presented as means of each time point  $\pm$  SE (n=3) with significant difference between treatments, tested every 4 hours, represented by an asterisk ( $p \leq 0.05$ ).



**Figure 2.7.** Behavioral observations of cumulative duration (% time) in the bottom half of the tank [20 days; 24 hr light:0 hr dark]. The cumulative duration (%) of time spent in the bottom of the tank over a 1-hour period was recorded over the 24-hour cycle. Data are presented as means of each time point (n=3) with significant difference between treatments, tested every hour, represented by an asterisk ( $p \leq 0.05$ ).

## DISCUSSION

Larval and mature zebrafish exhibit daily sleep/wake patterns tightly linked with the presence or absence of light (Chiu & Prober, 2013) and these patterns are consistent with other diurnal species such as mice (Ohta et al., 2005). The mature zebrafish measured in our study showed increased activity in day hours and decreased activity in night hours when exposed to a natural photoperiod [14-hour light:10-hour dark] (Figures 2.2, 2.5). This observation was additionally supported with zebrafish showing decreased cumulative distance moved during night hours (Figures 2.3, 2.6). Zebrafish sleep/wake cycles and their timings are thought to be controlled by a circadian clock (Shang & Zhdanova, 2007), which is highly sensitive to light exposure (Egan et al., 2009). This evidence provides further support that sleep and arousal states, along with their associated behaviors, are indeed conserved across the animal kingdom, from invertebrates such as *C. elegans* (Saigusa et al., 2002) and *Drosophila* (Hardin, 2005), to members of the various vertebrate classes (Allada & Siegel, 2008; Cirelli & Tononi, 2008).

The importance of light in regulating the circadian rhythm of sleep/wake states was further demonstrated in our study with acute continuous light exposure [24-hour light:0-hour dark]. Zebrafish experiencing acute extended light exposure significantly increased their active state by 15% during subjective night, when compared to control fish exposed to a natural photoperiod (Figure 2.2). In addition, zebrafish in the acute extended light exposure treatment significantly increased cumulative distance moved by approximately 3-fold during subjective night hours (Figure 2.3). This suggested that even for an acute amount of time, extended light exposure disrupted typical sleep/wake behavior observed in mature zebrafish experiencing a natural photoperiod. This observation was consistent with previous studies that found extended light

exposure increased movement and decreased sleep-like state in larval and mature zebrafish (Yokogawa et al., 2007; Zhdanova, 2011).

Previous studies investigated the effects of continual light exposure for up to eight days and found complete lack of circadian rhythm patterns (Yokogawa et al., 2007). However, because zebrafish are thought to be well-adaptive to environmental disruptions (Zhdanova, 2011), eight days may be insufficient for understanding long-term effects of continuous light exposure to zebrafish behavior and physiology. Our study aimed to address the hypothesis that mature zebrafish may be able to override the cue for continual activity/wake-state during long-term continuous light exposure and re-establish a circadian rhythm pattern. This was tested by exposing mature zebrafish to a chronic light exposure trial of 20 days [20 days; 24-hour light:0-hour dark]. To our knowledge, this is the longest period of extended light exposure zebrafish have been challenged with. Zebrafish experiencing chronic extended light exposure had similar activity during Days 1, 7 and 14. Chronic extended light exposure zebrafish significantly increased active state by approximately 45% (Day 1), 35% (Day 7) and 55% (Day 14) during the majority of subjective night hours, when compared to control fish (Figure 2.5). In addition, zebrafish in chronic extended light exposure significantly increased cumulative distance moved by approximately 2500-4000 cm (Day 1), 1000 cm (Day 7) and 2500 cm (Day 14) during the majority of subjective night hours (Figure 2.6). Our behavioral observations of zebrafish exposed to chronic extended light exposure followed similar trends to our observations of zebrafish exposed to acute extended light exposure. These findings suggested that mature zebrafish are affected by continuous light, but are still not able to fully regain regular circadian

rhythm behavioral patterns even after 14 days of continuous light exposure, as all days of the chronic light exposure trial had activity that was statistically similar.

The duration of the chronic extended light exposure (20 days) make this study unique in that it allowed us to observe the behaviors associated with long-term continuous light previously not observed in other studies. Previous studies have shown that when exposed to continuous light, mature zebrafish do not experience regular characteristic cycles of sleep/wake states. After being held under these abnormal conditions eight days, zebrafish still lacked a clear circadian pattern (Yokogawa et al, 2007). Our study reconfirmed these previous findings with consistent loss of circadian behavioral patterns in chronic extended light conditions for 14 days. These observations suggested that zebrafish sleep patterns respond rather dramatically to continuous light exposure. The ability to survive during intense periods of disrupted circadian rhythm, unlike many mammalian vertebrates, suggests that there could be adaptive underlying physiological and transcriptome-level strategies in play during such challenging conditions (e.g. similar to that of regional distribution of sleep found in aquatic mammals and birds) (Zhdanova, 2011). In regional distribution of sleep, the brain allows some areas to control behavior while others sleep, allowing for constant activity and some level of alertness. This type of unihemispheric behavior would allow the fish to be adaptive in continually fluctuating environments, such as under water, which could be advantageous to survival.

Additionally, qualitative observations of the zebrafish's overall condition showed declining health of zebrafish with time spent in the chronic extended light exposure. After seven days of extended light exposure, some zebrafish began displaying redness of gills, which increased

dramatically as the trial progressed. These same zebrafish also began losing vibrancy of color, becoming paler. When the first mortality occurred on day 20, the study was suspended (as per IACUC requirements). Although qualitative, these observations of the negative effects of chronic extended light exposure on physical condition suggested that the fish were entering a state of overall poor health. It was possible that these declining physical conditions of the fish exposed to chronic extended light exposure were a result of tertiary levels of the stress response. The tertiary level of the stress response is manifested after continuous activation of the stress axis and will often result in whole-body alterations such as loss of muscle mass and color variation (Barton & Iwama, 1991).

The zebrafish sleep/wake cycle is associated with rhythms in behavior activity, postural changes, and environmental changes, which contribute to day/night rhythms of neuroendocrine hormones. Disruption of this cycle could be considered stressful to zebrafish. In addition, the internal circadian timing system, independent of sleep and wakefulness, has a notable affect on many hormones and production of them (which also can be disrupted and contributing to the mentioned day/night rhythms of hormone levels). Under naturally entrained conditions, the behavioral cycle and circadian system are synchronized and levels of hormones are appropriately controlled, but disrupting this behavioral cycle and circadian system with external environmental conditions (such as continuous light) could have profound adverse effects on physiological processes (i.e. endocrine functions) which may result in overall health problems for the zebrafish (Morris et al., 2012).

## CONCLUSION

Results from this study reconfirmed that extended light exposure prevents zebrafish from entering periods of restful states, shown by increased activity and distance swam during subjective night. These results provide further support that the presence or absence of light is critical in regulating sleep/wake cycles in zebrafish. It was shown that zebrafish lack the ability to adapt to this change in their environmental light as fish maintained elevated activity and increased swimming throughout the 20-day exposure. These results provide new information on the ability of the mature zebrafish to survive even with diminished regular circadian rhythm behavioral patterns that allow for rest during subjective night.

Our study observed declining health of zebrafish exposed to 20 days of chronic extended light exposure, which suggested a tertiary level of the stress response. This observation led us to question the effects of extended light and lack of sleep at the physiological level. With obvious declining health in continuous light, it was possible that continuous light was stressful to the mature zebrafish. This led the study to investigate the effects of acute and chronic extended light exposure on zebrafish stress level at a physiological and molecular level, which were research areas lacking concise clarification.

## CHAPTER 3

### EFFECTS OF SLEEP DEPRIVATION ON CORTISOL PRODUCTION AND CORTISOL SYNTHESIS PATHWAY GENE EXPRESSION IN ZEBRAFISH (*Danio rerio*)

#### INTRODUCTION

Sleep deprivation causes an internal disruption that is known to cause behavioral and subsequent physiological changes in an organism. Studies of sleep in humans have shown the adverse effects of sleep deprivation to human health by metabolic, physical, psychological, and behavioral parameters (Adamantidis & Lecea, 2008; Cirelli & Tononi, 2008; Curcio et al., 2006; Livingston et al., 2015). In short, lack of sleep in many vertebrates induces negative consequences such as decreased cognitive abilities and motor functions, resulting in neuroendocrine stress responses (Kahn-Greene et al., 2007). Zebrafish display diurnal patterns of sleep, similar to humans, and are strong models to study the physiological effects of sleep deprivation (Zhdanova, 2011). Indeed, we have found that zebrafish sleep patterns can be manipulated in laboratory settings using continuous light exposure on both an acute and chronic basis (Ch. 2). Extended light exposure (e.g. lack of dark cycle of photoperiod) resulted in zebrafish displaying significantly increased active state and cumulative distance moved during subjective night. The present study aimed to investigate if the increased activity during subjective night hours in response to extended light exposure in mature zebrafish was accompanied by signs of stress at the physiological level.

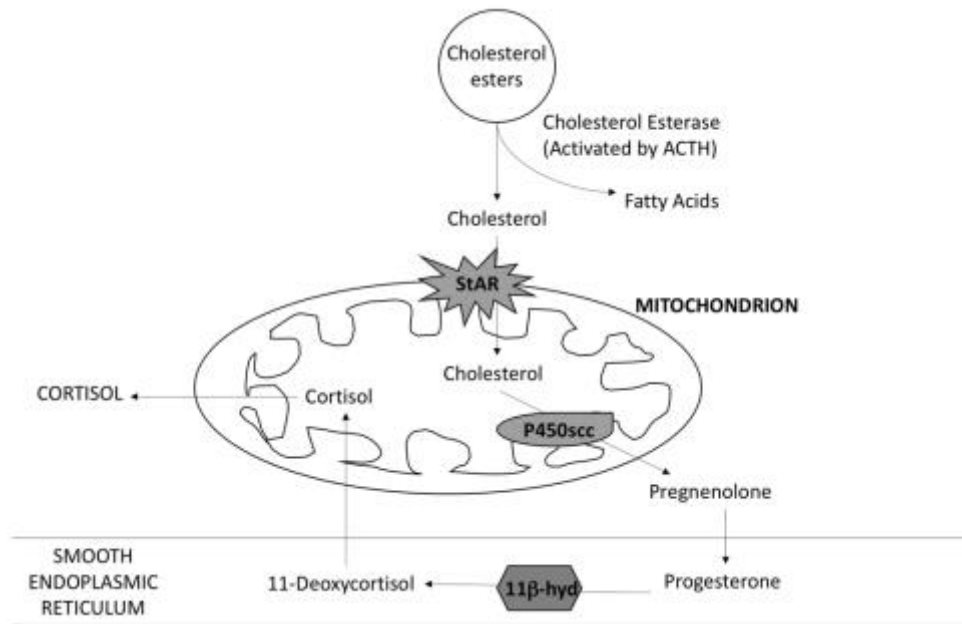
In vertebrates, exposure to a stress or stressor results in a triggering of the vertebrate stress axis. This involves a suite of behavioral and physiological response that compensate for a perceived



threat and attempt to maintain homeostatic equilibrium, making it important to the overall well-being of the organism. One of the major end points of the stress response is the production of glucocorticoids (e.g. cortisol or corticosterone) and therefore, the appearance of elevated glucocorticoids in the blood stream is often used as an indicator of the stress response being initiated. While elevation of glucocorticoids has substantial benefits to vertebrates on the short-term (such as gluconeogenesis, fat breakdown, arousal, decrease in inflammation), chronic elevation can negatively affect growth rate, stress responsiveness, and anxiety-related behaviors (Jeffrey & Gilmour, 2016; Roumestan et al., 2004).

In teleostean fish, cortisol is the principal corticosteroid and is a key contributor to many physiological processes including growth, immunoregulation, energy maintenance, and reproduction (Mommsen et al., 1999). When experiencing a stressor (such as predator attack, chemical or extended light exposure, etc.), protein kinase A (PKA) becomes activated and facilitates the conversion of cholesterol ester to free cholesterol (Figure 3.1) (Jefcoate et al., 1992). During HPI axis activation the corticotropin-releasing factor (CRF), produced in the hypothalamic preoptic area (POA), stimulates the pituitary gland corticotropic cells to secrete adrenocorticotropin hormone (ACTH) (Figure 3.1). ACTH ultimately regulates cortisol synthesis and secretion (Tsalafouta et al., 2014). Circulating ACTH binds to melanocortin-2 receptor (MC2R) of the interrenal cells of the kidneys, which lead to moving cholesterol to the inner mitochondrial membrane as the first and rate-limiting step of cortisol synthesis, which is an action facilitated by the StAR enzyme (Figure 3.1) (Jeffrey & Gilmour, 2016). Transcription of StAR is known to increase during acute stress response (Clark et al., 1994; Stocco, 2001). The cholesterol side-chain cleavage enzyme (P450<sub>scc</sub>) cleaves cholesterol to pregnenolone (Figure

3.1) (Aluru & Vijayan, 2006; Hagen et al., 2006; Mommsen et al., 1999). Both the movement of cholesterol from the outer to the inner mitochondrial membrane by StAR (Stocco, 2001) and the cleavage of cholesterol by P450<sub>scc</sub> are considered rate-limiting in cortisol synthesis (Bernier et al., 2008; Mommsen et al., 1999), with StAR shuttling hydrophobic cholesterol across the aqueous membrane being the primary rate-limiting step (reviewed by Stocco, 2001). Finally, cortisol synthesis occurs through a multi-step enzyme catalyzed reaction chain, ending with the conversion of 11-deoxycortisol to cortisol catalyzed by the enzyme 11-Beta hydroxysteroid dehydrogenase (11 $\beta$ -hyd) (Aluru & Vijayan, 2006). Cortisol then enters its target cells throughout the body by passive diffusion where its action is mediated by the ligand-activated transcription factors glucocorticoid receptor (GR) and the mineralcorticoid receptor (MR) (Prunet et al., 2006). In chronic stress situations, activation of the stress response and cortisol production via steroidogenic genes occurs. However, their exact mechanisms and activation timing during varying durations of stress remain unclear (Sewer & Waterman, 2002).



**Figure 3.1.** The zebrafish cortisol synthesis pathway involving the shuttling of precursors between mitochondria and the endoplasmic reticulum. ACTH: adrenocorticotropic hormone; StAR: steroidogenic acute regulatory protein; P450<sub>scc</sub>: cholesterol side-chain cleavage enzyme; 11 $\beta$ -hyd: steroid 11-beta-hydroxylase.

Given the robust behavioral response of zebrafish to extended light exposure (Ch.2), we predicted that the loss of typical sleep/wake patterns due to extended light exposure was triggering activation of the stress response. Extended light exposure was found to significantly increase duration (%) of time spent in active state and subsequently decrease duration (%) of time spent in inactive state in zebrafish. Additionally, the cumulative distance moved (cm) increased in mature zebrafish during subjective night, when sleep-like state and inactivity was typically experienced. Disruption of established circadian rhythm patterns is known to have little effects on the stress response for acute durations, such as 3-6 hours (Sigurgeirsson et al., 2013), but the effects from chronic durations remained uncertain. The objective of our study was to observe the effects of acute and chronic extended light exposure on cortisol production and transcriptome level expression of cortisol synthesis genes. We hypothesized that increased whole-body cortisol concentrations and the rate-limiting steps of the cortisol synthesis pathway gene expression (*P450<sub>scc</sub>* and *11 $\beta$ -hyd*) would be increased in both acute and chronic extended light exposure treatments.

## METHODOLOGY

### *Animals & Experimental Light Exposures*

Zebrafish (*Danio rerio*) derived from the Tuebingen (TU) line were bred and raised at Georgia Southern University. All fish were 150-210 days of age and sexually mature, approximately 2-3 cm in length and ranged from 0.3-0.4 g in mass at the start of each experiment. The zebrafish used for the cortisol and gene expression analysis were exposed to the acute and chronic extended light exposure trials at the same time as the zebrafish used for the behavioral analysis.

As such all holding conditions and experimental exposures were as described for the zebrafish in Chapter 2.

In brief, mature zebrafish were exposed to either acute or chronic extended light exposures. For the acute extended light exposure, an individually housed control group [14-hour light:10-hour dark] (n=18) and an individually housed acute extended light exposure group [24-hour light:0-hour dark] (n=18) were compared to measure the effects on the stress response from 24 hours of continuous light. All fish (control and treatment, n=9 each) were sacrificed following behavioral experiments (Ch.2) for whole-body cortisol analysis. The additional 18 fish (control and treatment, n=9 each) exposed to the same acute exposure treatment were sacrificed for gene expression analysis.

For the chronic extended light exposure, a group housed control group [14-hour light:10-hour dark] (n=18) and a group housed chronic extended light exposure group [20 days; 24-hour light:0-hour dark] (n=18) were compared to measure the effects on the stress response from 20 days of continuous light. For both the control and chronic treatment groups, the three fish used for behavioral observations were the same fish later used for whole-body cortisol concentrations (note: the six fish in both control (n=3) and treatment (n=3) groups used for behavioral observations were housed individually for videotaping purposes). For both groups, an additional six fish were used for whole-body cortisol concentrations and an additional nine fish were used for gene expression analysis.

All zebrafish were euthanized with lethal doses of tricaine mesylate: MS-222, buffered 1:2 with  $\text{NaHCO}_3$  to make a final concentration of 150 mg/L in a process lasting 2-3 minutes. After euthanasia, zebrafish were weighed, sexed, transferred to cryo-vials, flash frozen in liquid nitrogen and stored at  $-80^\circ\text{C}$  until analysis.

#### *Analysis of Whole-Body Cortisol Extraction*

Whole frozen zebrafish were powdered over liquid nitrogen with a mortar and pestle followed by further homogenization with a PowerGen 125 electric homogenizer (Fisher Scientific, Waltham, MA, USA) on ice in 500  $\mu\text{L}$  of ice cold 1X PBS (800mL; 8g NaCl, 0.2g KCl, 1.44g  $\text{Na}_2\text{HPO}_4$ , 0.24g  $\text{KH}_2\text{PO}_4$ ; pH 7.4). After homogenization, the rotor blade was rinsed with an additional 500  $\mu\text{L}$  of 1X PBS into the 1.5 mL Eppendorf tube containing the homogenate. Cortisol was extracted from the homogenate based on a protocol adapted from Jeffrey & Gilmour (2016). To summarize, 2 mL of ethyl acetate was added to each sample homogenate and vortexed thoroughly for 1 minute. Samples were centrifuged at 3,500 RPM at  $4^\circ\text{C}$  for 5 minutes. This extraction with ethyl acetate was repeated three times. After the final extraction, 1600  $\mu\text{L}$  of supernatant was transferred to a 10 mL glass tube (Kimble) and evaporated under a forced nitrogen gas stream. Extracts were reconstituted with 1 mL of 1X extraction buffer [from Neogen cortisol enzyme-linked immunosorbant assay (ELISA) kit, Lexington, KY, USA] and incubated overnight at  $4^\circ\text{C}$  until analysis by ELISA (Neogen) the following day. Cortisol levels were determined in duplicate on extracted samples using an enzyme-linked immunosorbant assay (ELISA) kit (Neogen, Lexington, KY, USA), and a SpectraMax Plus 384 spectrophotometer (Molecular Devices, Sunnyvale, CA, USA) at a wavelength of 650 nm at room temperature ( $22^\circ\text{C}$ ). Concentrations of samples were determined by comparing the binding

efficiency to that of a set of serially diluted cortisol standard. In addition to the experimental samples, four mature zebrafish were used to validate the efficiency of the cortisol extraction protocol by spiking non-experimental homogenates with 2  $\mu$ l of 10 ng/mL of C-106 Cerilliant cortisol stock solution (Sigma Aldrich). The extraction protocol was performed identically for spiked samples as for non-spiked samples and the efficiency was determined based on the ability to effectively recover the known amount of cortisol stock solution using the protocol. Our average extraction efficiency was 94.3%, which was within the accepted range in previous studies that examined whole-body cortisol levels in zebrafish (Ramsay et al., 2006).

#### *Preparation of RNA and genomic DNA from whole body*

To extract total RNA from the zebrafish, whole-bodies were powdered with liquid nitrogen and then homogenized in a 1.5 mL Eppendorf tube in TRIzol reagent (Invitrogen) with sterile, disposable pestles and a hand-held homogenizer (50-90 mg tissue:1 ml TRIzol). Resulting homogenates were used for total RNA extraction following the TRIzol manufacturer's protocol with modifications. Homogenates were centrifuged at 12,000 g for 10 minutes at 4°C and supernatant was transferred to a new 1.5 mL Eppendorf tube and left to incubate at room temperature (RT) for 5 minutes. Samples were extracted two times by adding 200  $\mu$ l of chloroform (per 1 mL Trizol) to each sample and hand inverting for 5 minutes. Samples were centrifuged at 12,000 g for 15 minutes at 4°C. Resulting supernatant was transferred to a new 1.5 mL Eppendorf tube and an additional 200  $\mu$ l of chloroform was added to each sample with extraction following. 400  $\mu$ l of 100% isopropanol was added to each aqueous phase sample and incubated for 10 minutes at RT. Samples were centrifuged at 12,000 g for 10 minutes at 4°C. Resulting supernatant was removed from the tube, leaving only the RNA pellet, which was

washed with 1 mL of 75% ethanol to improve purity. Pellet and wash was centrifuged at 7,500 *g* for 5 minutes at 4°C and pellet was air dried for 5-10 minutes. Once dried, pellet was re-suspended in 50 µl RNase-free H<sub>2</sub>O and incubated in a heat block for 10 minutes at 57-59°C. RNA concentration was measured US A260/280 and A260/230 NanoDrop UV spectrophotometer (NanoDrop Technologies). All total RNA samples were stored at -80°C until analysis.

*cDNA Synthesis, Sequencing of Target Gene Amplicons, and Quantitative Reverse Transcription-Polymerase Chain Reaction Analysis*

cDNA was synthesized from 2 µg of total RNA in a 20 µl reaction using random primers [200 ng (Invitrogen)] and RevertAid H<sup>-</sup> MuL V Reverse transcriptase [200 U/µl (Fisher Scientific)] with the manufacturer's 5X reaction buffer and RNase OUT [40 unit/µl (Invitrogen)] at 42°C for 60 minutes. Negative control reverse transcriptase (NRT) and no-template control (NTC) reactions were performed on a subset of RNA samples from each treatment. Complementary DNA (cDNA) was diluted in nuclease-free H<sub>2</sub>O to a final volume of 50 µl. cDNA concentration was measured US A260/280 and A260/230 NanoDrop UV spectrophotometer (NanoDrop Technologies). Samples were stored at -20°C until QRT-PCR analysis.

PCR primers for genes of interest (GOI) were adapted from previous studies (Table 3.1). Using the primers for GOI and housekeeping gene (18S), we performed RT-PCR amplifications using a Mastercycler thermal-cycler (Eppendorf) in a 25 µl reaction that contained 0.1 µl Platinum®Taq (Life Technologies, Grand Island, NY), 0.5 µl of cDNA (1,000 ng of input RNA) and 10 µM of both forward and reverse primers. Duplicate reactions were completed on the thermal-cycler for



3 minutes at 94°C, followed by 38 cycles of 30 seconds at 94°C, 30 seconds at 58°C, and 30 seconds at 72°C, and a final extension of 15 minutes at 72°C. Negative controls were included during each run and contained all components of the reaction except the cDNA template, which was replaced with DEPC-treated nuclease-free water (Life Technologies, Grand Island, NY). PCR products were separated electrophoretically on 1.0% agarose gels stained with ethidium bromide alongside a 1 Kbp ladder (Fisher Scientific) to verify amplification of a single amplicon free of primer dimers. Bands of appropriate molecular size were used to verify that primers were targeting the correct genes.

GOI transcript expression levels were quantified by QRT-PCR on the QuantStudio 6 (Life Sciences Solutions) using SYBR Select Master Mix (Life Technologies). Pooled samples were taken from fish in both treatments (acute and chronic light exposure) and both control photoperiods, which each fish contributing equally to the pool. Each primer set was subjected to quality testing, which included dissociation curve to confirm there was only a single peak and amplification efficiency calculation [ $E = 10^{(-1/\text{slope})}$ ; Pfaffl 2001] based on 5-point fivefold dilution standard curves. The amplification efficiencies were: P450scc (99.5%), 11 $\beta$ -hyd (96.4%) and 18S (98.0%). These efficiencies represent the average of that obtained from acute, chronic and control photoperiod pools. Quantification cycle ( $C_q$ ) values obtained from the mastercycler were used to generate a standard curve and to calculate  $R^2$  values, amplification efficiency, and efficiency present. Primers were checked for primer dimers via a melt curve analysis. The primer sequences and respective amplicon sizes are reported in Table 3.1. Furthermore, threshold cycle ( $C_t$ ) values of NRT and NTC were at least four cycles greater than  $C_t$  values from the most dilute standard.

**Table 3.1.** Primers used in quantitative real-time RT-PCR in zebrafish (*Danio rerio*).

<b>Gene</b>	<b>Primer (5' – 3')</b>	<b>Amplicon size</b>	<b>Accession number</b>	<b>Reference</b>
18S	F – ggc ggc gtt att ccc atg acc R – ggt ggt gcc ctt ccg tca att c	702 bp	FJ915075	Jeffrey & Gilmour (2016)
P450 <sub>scc</sub>	F – agg gcc atc acc cca ata g R – cca ggc ctt ccc ttc ttt tag	82 bp	NM152953	Alderman & Bernier (2009)
11 $\beta$ -hyd	F – gct cat gca cat tct gag ga R – tgt gct gaa ggt gat tct cg	102 bp	AY279108	Ings & Van Der Kraak (2006)

Amplification was carried out in duplicate in a 12.5  $\mu$ l reaction containing 2  $\mu$ l template (2,000 ng of input RNA), 0.25  $\mu$ l – 0.5  $\mu$ l primer (2.5  $\mu$ M), 6.25  $\mu$ l SYBR Green Master Mix (Fisher Scientific), and a volume of RNase/DNase-free H<sub>2</sub>O to bring total volume to 12.5  $\mu$ l. The real-time analysis program consisted of 1 cycle of 50°C for 2 minutes, 1 cycle of 95°C for 10 minutes and 40 cycles of (95°C for 15 seconds and 60°C for 1 minute), followed by dissociation analysis (+1% increases every 30 seconds from 60°C to 95°C). On each plate, for every sample, the target gene and endogenous control were tested in duplicate. The fluorescence threshold cycle (C<sub>T</sub>) was determined automatically using the QuantStudio 6 Software.

GOI (gene of interest) messenger RNA (mRNA) expression levels were normalized to *18S*, and on each plate, for every sample, the GOI and normalizer gene were run in duplicate. *18S* was chosen as the reference gene as QRT-PCR results showed that *18S* mRNA levels did not change with acute or chronic sleep deprivation (data not shown). Relative transcript abundance was calculated for each sample according to the equation  $R = E_{\text{target}}^{\Delta C_t \text{ target (control - sample)}} / E_{\text{ref}}^{\Delta C_t \text{ ref (control - sample)}}$  (Pfaffl, 2001) with an average of sample “AC4” being used as control value (i.e. calibrator). This relative transcript abundance was then used to identify fold changes between treatment and control groups. When there were differences between the control and experimental groups, relative fold change is reported.

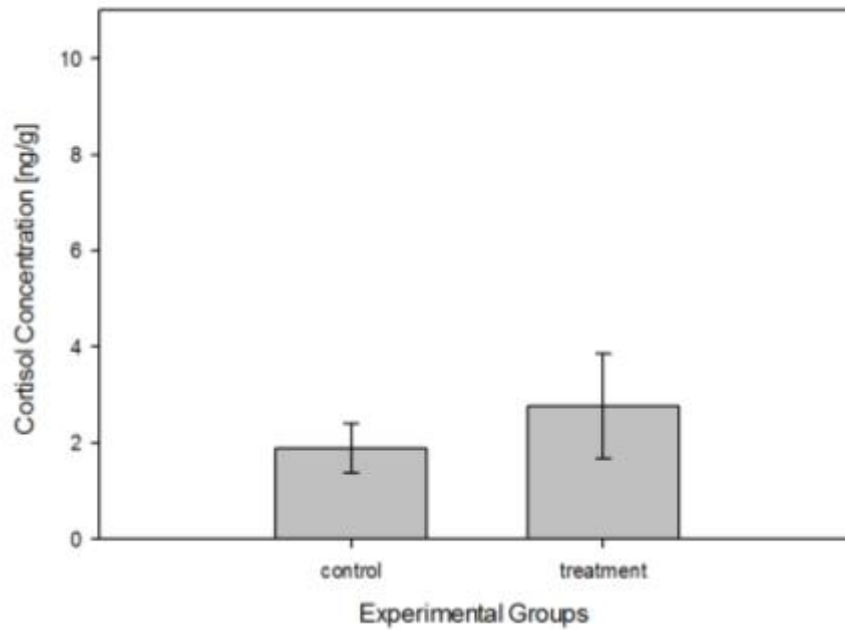
### *Statistical Analysis*

Statistical significance between control and experimental groups for whole-body cortisol concentrations and QRT-PCR gene expression data was determined using JMP 12 (SAS

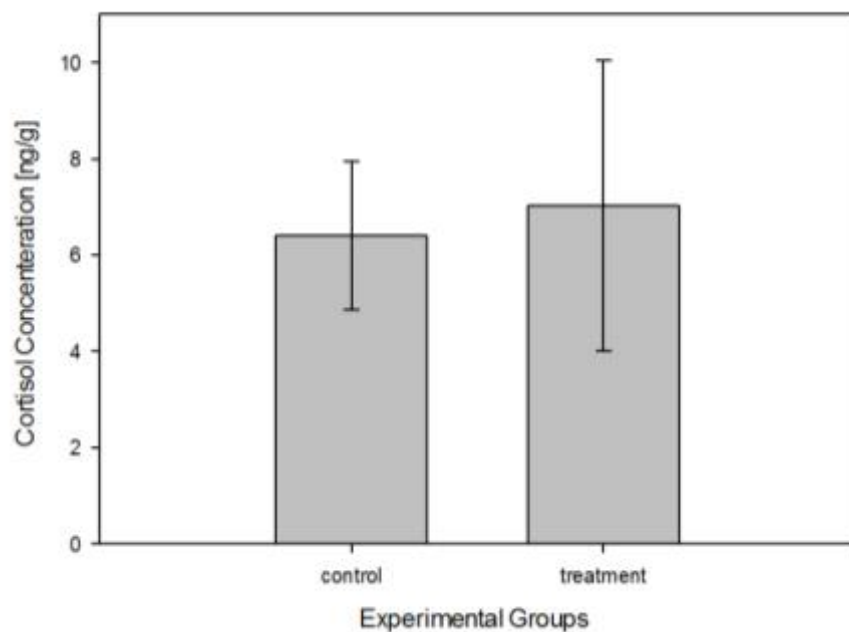
Institute, Inc. Cary, NC). Data was analyzed via an Independent Samples *t*-test to determine if a statistical difference existed between the experimental and control groups for whole-body cortisol concentrations and relative expression levels of GOI. All data were reported as mean concentrations/relative expression  $\pm$  standard error (SE). Differences among groups were considered significant when  $p \leq 0.05$ .

## RESULTS

Extended light exposure did not have a significant effect on whole-body cortisol levels in zebrafish in either the acute or chronic treatment groups, when compared to their control groups (Fig 3.2,  $p = 0.1168$  acute exposure; Fig. 3.3,  $p = 0.8587$  chronic exposure). Additionally, there was no significant difference found in whole-body cortisol when comparing fish used in the acute and chronic trials (control and experimental groups together) (Figs 3.2, 3.3;  $p = 0.5830$ ).

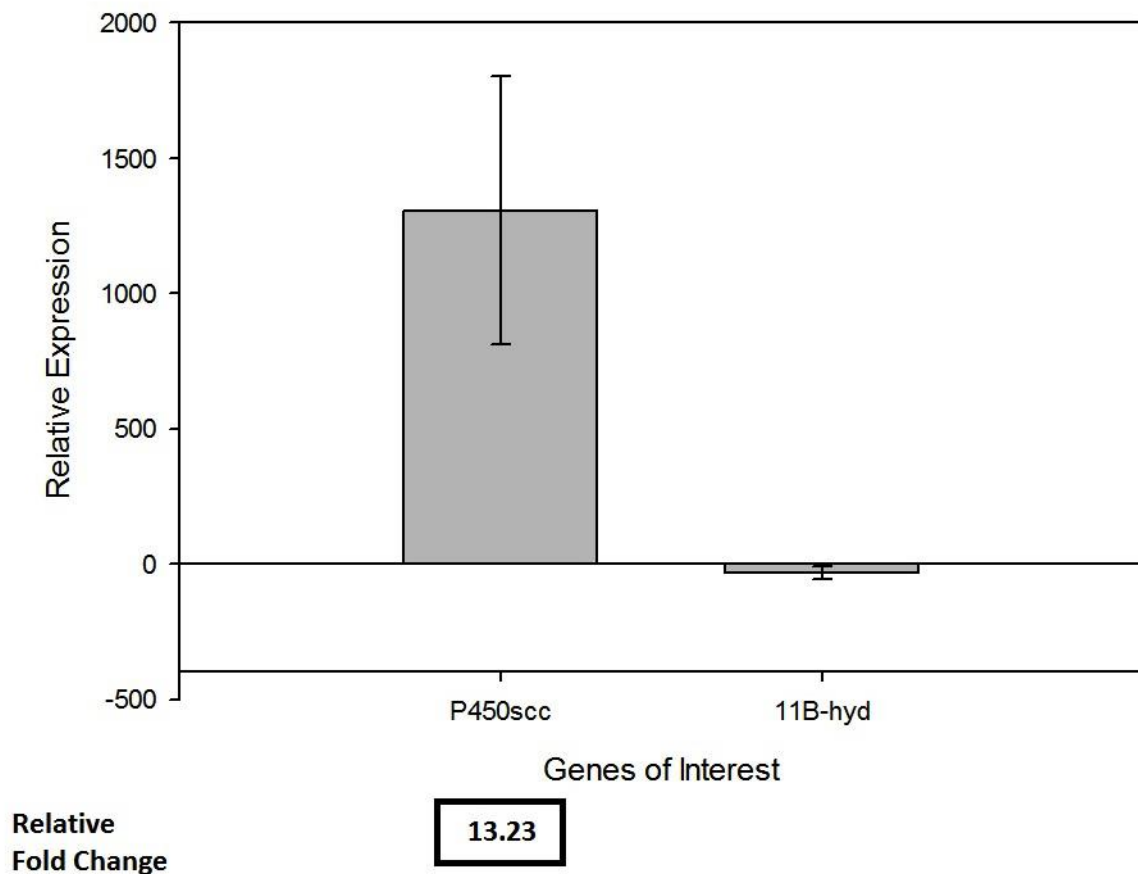


**Figure 3.2.** Average whole-body cortisol concentrations (ng/g) in zebrafish (*Danio rerio*) held under control [14-hour light:10-hour dark] or acute extended light exposure [24-hour light:0-hour dark]. There was no significant effect of acute extended light exposure on whole-body cortisol concentrations of zebrafish. Data presented as mean  $\pm$  SE (n=9).

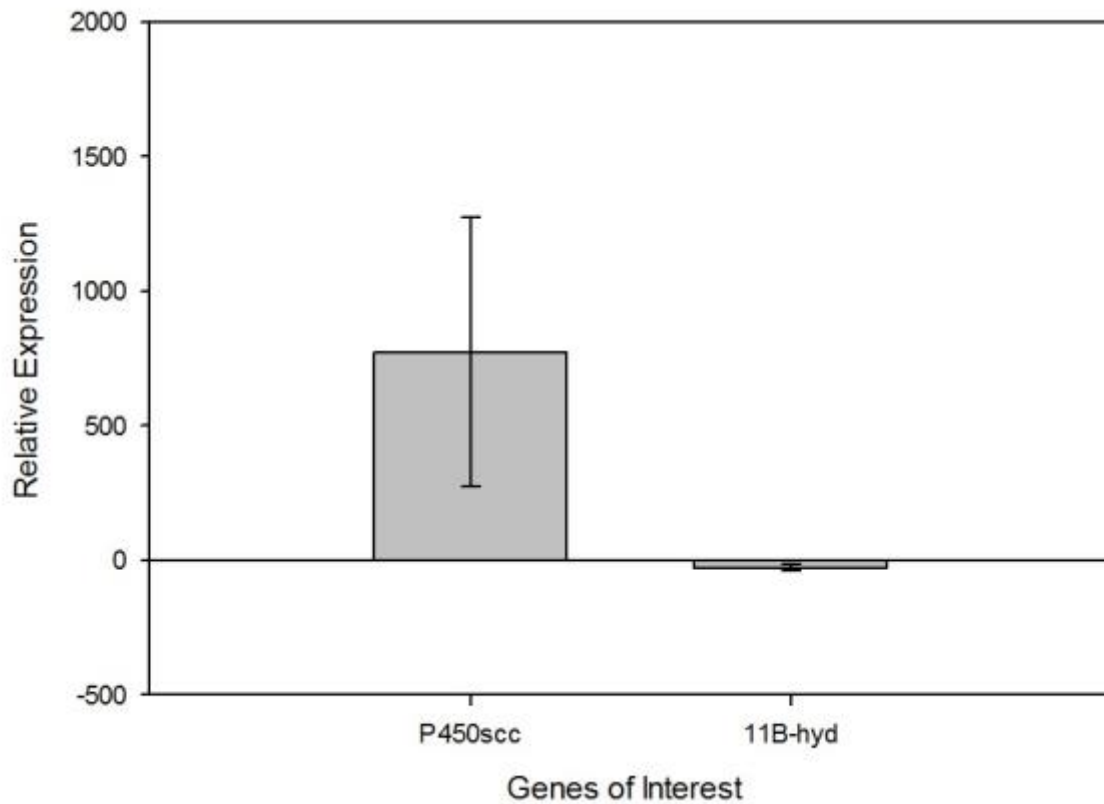


**Figure 3.3.** Average whole-body cortisol concentrations (ng/g) in zebrafish (*Danio rerio*) held under control [14-hour light:10-hour dark] or Day 20 chronic extended light exposure [20 days; 24-hour light:0-hour dark]. There was no significant effect of chronic extended light exposure on whole-body cortisol concentrations of zebrafish. Data presented as mean  $\pm$  SE (n=9).

Acute extended light exposure resulted in a significant upregulation of P450<sub>scc</sub> by 13.23-fold (Figure 3.4;  $p = 0.0478$ ) when compared to control fish, but no significant changes of 11 $\beta$ -hyd (Figure 3.4;  $p = 0.1823$ ). There were no significant changes in gene expression at Day 20 of the chronic exposure of P450<sub>scc</sub> (Figure 3.5;  $p = 0.9969$ ) or 11 $\beta$ -hyd (Figure 3.5;  $p = 0.2739$ ).



**Figure 3.4.** Relative expression levels of the stress hormone pathway genes P450<sub>scc</sub> and 11 $\beta$ -hyd in acute extended light exposure zebrafish. Expression of P450<sub>scc</sub> was significantly upregulated 13.23-fold when compared to the control group ( $p = 0.0478$ ). Data presented as mean  $\pm$  SE (n=9),  $p \leq 0.05$  significant.



**Figure 3.5.** Relative expression levels of the stress hormone pathway genes P450scc and 11 $\beta$ -hyd in Day 20 chronic extended light exposure zebrafish. There was no significant effect of chronic extended light exposure on expression levels of either P450scc or 11 $\beta$ -hyd. Data presented as mean  $\pm$  SE (n=9),  $p \leq 0.05$  significant.



## DISCUSSION

Zebrafish are sensitive to changes in environmental light from that of their normal photoperiod, with extended light exposure preventing entrance into a sleep-like state. Previous studies analyzed the effects of extended light exposure for a maximum of eight days. Our study aimed to analyze the effects of chronic extended light exposure for 20 days. We confirmed that extended light exposure disrupts behavioral circadian cycles on both a long-term and short-term basis (Ch. 2). Zebrafish exhibited increased durations of time in active state during subjective night throughout the chronic extended light exposure trial, suggesting they did not acclimate to the extended light exposure even by Day 14. Given the homeostatic mechanisms of regular sleep patterns that maintain a consistent cycle, we hypothesized that these behavioral changes and absence of circadian rhythm patterns were stressful to the zebrafish. Little had been done to investigate effects of sleep deprivation at the physiological level, particularly long-term effects. The objective of our study was to investigate if extended light exposure, in particular chronic periods of extended periods of extended light, activated the stress axis and subsequently, increased whole-body cortisol concentrations and transcriptome-level gene expression of the rate-limiting steps of cortisol production: P450*scc* and 11 $\beta$ -hyd.

Although acute extended light exposure did not cause a significant increase in whole-body cortisol concentrations (Figure 3.2), qRT-PCR analysis suggested that underlying changes were occurring in the biosynthetic pathway. Upregulation of downstream genes, such as 11 $\beta$ -hyd, was not observed (Fig. 3.5), however, P450*scc* is the initial rate-limiting steps of cortisol synthesis and was significantly upregulated 13.23-fold (Fig. 3.4). P450*scc* cleaves cholesterol into pregnenolone and 11 $\beta$ -hyd is the final rate-limiting step of cortisol synthesis that converts 11-

deoxycortisol to cortisol. These results suggest that 24-hour continuous light may have triggered the synthesis of cortisol as part of a stress response at a molecular level, but that it was not manifested at the physiological level. It is possible that cortisol may have been measurable at a later time point in the acute treatment study (36 hours later, 48 hours later, etc.) (Wendelaar Bonga, 1997), or that *P450<sub>scc</sub>* was upregulated as an anticipatory effect of a stress response but the effect at the protein level was never observed (Wendelaar Bonga, 1997; Hagen et al., 2006).

Chronic extended light exposure resulted in increased activity and distance moved, even after several weeks of exposure (Ch. 2). These results were interpreted as evidence for continuous circadian rhythm disruption and sleep deprivation. Sleep deprivation for 20 days, as zebrafish were exposed to in this study, was thought to be stressful to the zebrafish. In fact, human sleep studies have found that it is difficult to sustain sleep deprivation for even a few days and impossible to sustain for over a week (Mignot, 2008). Indeed, our originally planned 28-day study had to be cut short on day 20 of the chronic trial due to declining overall health of the zebrafish. These qualitative observations indicated that extended light exposure was leading to tertiary level stress response, resulting in whole-body alterations. Tertiary level stress response is typically manifested with increased physiological response, such as elevated cortisol concentrations. This, combined with the fact that our acute extended light exposure trial showed early signs of the stress response being recruited by the upregulation of *P450<sub>scc</sub>* suggested that chronic extended light exposure would result in induced elevated cortisol concentrations and expression levels of cortisol synthesis pathway genes *P450<sub>scc</sub>* and *11 $\beta$ -hyd*. However, chronic extended light exposure resulted in no changes to whole-body cortisol concentration, when compared to control light exposure (Figure 3.3). Additionally, no change in relative expression

levels of P450<sub>scc</sub> or 11 $\beta$ -hyd (Figure 3.5) were observed in chronically sleep-deprived fish. These transcriptome-level results suggest that even after 20 days of being exposed to continuous light, the zebrafish stress axis was not being activated.

An alternate explanation for the upregulation of P450<sub>scc</sub> after 24 hours, but not after 20 days, could be that the cholesterol that was moved through the mitochondrial membrane by StAR enzyme and then later cleaved into pregnenolone by P450<sub>scc</sub> was being used for the production of an alternate end point other than cortisol. Rather than being used to synthesize cortisol by 11 $\beta$ -hyd, the pregnenolone and/or progesterone that was produced from P450<sub>scc</sub> could have instead been utilized in other parts of the zebrafish endocrine system to aid in aggression behaviors. In addition to being precursors to the stress hormone cortisol, pregnenolone and progesterone are also the precursors to primary sex hormones estradiol and testosterone (Harvey et al., 2005). Estradiol and testosterone are responsible for several reproductive functions and associated behaviors – with one notable side effect being aggression (Hill & Robert, 2003). Several studies have found correlations between these primary sex hormones and aggression (Tollman & King, 1956; Persky et al., 1971; Van de Poll et al., 1988; Trainor et al., 2008). Due to these studies linking sex hormone level changes and aggression in mammals and zebrafish, the up-regulation of P450<sub>scc</sub> in this study could be indicative of the use of cholesterol for the production of primary sex hormones in order to aid in aggressive behaviors in zebrafish and not cortisol as originally proposed. Further studies could pursue this hypothesis by measuring aggressive behaviors and levels of associated estradiol and testosterone of zebrafish exposed to continuous light.

Our study did not show strong indications of extended light exposure causing stress in mature zebrafish, which could be due to the high flexibility of the zebrafish response to environmental disruptions (i.e. continuous light). Zebrafish are known to be highly active and social organisms, making it possible they were already equipped to be able to easily adapt to continuous light because in a natural setting, they are already responsive to sudden environmental disturbances (social interactions, predator avoidance, etc). Also, it is possible that chronic extended light exposure, though disruptive to behavioral patterns, is not considered stressful to zebrafish. Further studies analyzing the ability of zebrafish to maintain physiological homeostasis would be needed to fully understand the underlying mechanisms helping the zebrafish cope with the robust behavioral changes that occur in response to chronic continuous light. Transcriptome-level gene expression of key regulators of the zebrafish circadian rhythm, such as *Clock1a* and *Bmall1a*, may help fill gaps in the knowledge of how zebrafish are able to forego periods of prolonged rest during chronic extended light.

## CONCLUSION

Extended light exposure, regardless of the duration (acute or chronic), does not result in the production of excess cortisol at the whole-body level. This can be interpreted as extended light does not cause stress to the zebrafish. However, our study did see significant upregulation of *P450<sub>scc</sub>*, a rate-limiting step in the biosynthetic pathway of cortisol, in response to acute extended light exposure. If not being used for cortisol production, it is possible that the product from *P450<sub>scc</sub>* (pregnenolone and/or progesterone) was being used for alternate purposes such as the production of sex hormones to aid in various reproductive and/or behavioral response, such as aggression. It can also be interpreted that zebrafish are able to cope with extended light

exposure due to changes in the key regulators of the circadian rhythm. Further research needs to be done to fully understand the underlying processes and mechanisms that are allowing zebrafish to adapt to extended light exposure on a physiological and transcriptome-level.

## CHAPTER 4

## SUMMARY AND FUTURE DIRECTIONS

The purpose of this study was to analyze the effects of extended light exposure to zebrafish (*Danio rerio*) on a behavioral, physiological, and transcriptome level. The goal being to use information gained from studying the effects of sleep deprivation on a simpler vertebrate model, the zebrafish, to provide clarification for some of the gaps in knowledge of the effects of lack of rest in humans. It was determined that zebrafish did show significantly increased active state and cumulative distance moved during subjective night hours in 24 hours of extended light exposure. Even more interesting, those significant behavioral changes continued to be observed after two weeks of chronic extended light exposure, suggesting that the zebrafish were not behaviorally adapting to the extended light in order to regain normal rest periods. These behavioral observations led us to investigate if the stress response was also being activated from extended light exposure.

While whole-body cortisol was not affected in response to extended light exposure in mature zebrafish, underlying mechanisms may allow the fish to cope this potential source of stress. However, this could not be confirmed without measuring the cortisol synthesis pathway, since changes could have started to occur at the molecular level but did not have time to manifest at the protein level. Indeed, our study did observe a significant up-regulation of the rate-limiting step in cortisol synthesis, P450<sub>scc</sub>, in acute extended light exposure zebrafish. The final rate-limiting step that ultimately produces cortisol, 11 $\beta$ -hyd, did not show any expression changes. It

is possible that the product of *P450<sub>scc</sub>* could have been for alternate purposes in sex hormone production and aggression, but that prediction remains uncertain in the current study.

Extended light exposures did result in robust changes to sleep/wake behavior in zebrafish, but did not result in significant changes to cortisol production due to complete stress axis activation. This conclusion suggests that there are other underlying mechanisms that allow the zebrafish to adapt to extended light exposure. It is possible that underlying changes to the key regulators of the zebrafish circadian rhythm (*Clock1a* and *Bmall1a*) were occurring during extended light exposures that allowed the zebrafish to forego the need for typical rest durations (Cahill, 2002). In mature zebrafish, the rest phase of the circadian rhythm is heavily influenced by melatonin production, which has release timing that is stimulated by *CLOCK/BMAL* interactions (Adamantidis & de Lecea, 2008). In zebrafish Z3 retinal cells, light directly inhibits the *CLOCK/BMAL*-dependent transcription, therefore functioning in the light entrainment of the circadian clock in mature zebrafish (Tamai et al., 2007; Hirayama et al., 2007). Therefore, zebrafish circadian systems contain both circadian oscillators and photoreceptive mechanisms that enable entrainment by light:dark (LD) cycles. This suggests that continuous light could have such profound effects on the zebrafish circadian clock activation and melatonin production that they are not able to undergo periods of rest, and this lack of rest does not activate the stress axis. Ongoing research is examining the regulatory mechanisms of the circadian rhythm during adaptation to extended light exposure and lack of rest. It is possible that the key regulator genes of the zebrafish circadian rhythm, *Bmall1a* and *Clock1a*, have decreased expression in order to decrease or cease melatonin production and therefore allow the zebrafish to forego the need for regular rest periods.

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