THE PENNSYLVANIA STATE UNIVERSITY
SCHREYER HONORS COLLEGE

DEPARTMENT OF ANIMAL SCIENCE

CIRCADIAN SYSTEMS AND AGING IN WHITE THROATED SPARROWS

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SPRING 2019

A thesis
submitted in partial fulfillment
of the requirements
for a baccalaureate degree
in Veterinary and Biomedical Science
with honors in Animal Science

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ABSTRACT

The main objective of my work was to determine how the circadian system responds to aging in White-Throated Sparrows. The study used geriatric sparrows, aged at least 10 years in captivity, and monitored locomotor activity continuously. Birds were held under a 12/12 light dark cycle and then experienced a four hour phase shift forward, followed by a shift back to the original light cycle after entrainment for approximately one month in order to measure their ability to synchronize to new light schedules. The resulting data showed a variety of unique circadian manifestations including arrhythmic, nocturnal, with many birds maintaining their original phase of activity despite a shift in the light cycle. The second was to use MRI to compare the brain structure of three geriatric sparrows to three younger sparrows, aged around 2 years. A 7 Tesla machine was used to perform a series of MRI scans and Avizo 9.0 software was used to calculate volumes of the total brain, ventricles, hippocampus, hypothalamus, and the cerebellum. The older birds exhibited smaller hypothalamic volumes. From the actogram data we propose that the previously reported dawn and dusk oscillators are less coupled and their photic input has been differentially affected in the aged birds. The dawn oscillator correctly shifted and entrained to a new light schedule, while the dusk oscillator had reduced photic input and did not shift accordingly. These results are consistent with the hypothesis that the dusk oscillator relies on circadian time memory instead of reentraining to new light cycles.

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ACKNOWLEDGEMENTS

I would like to thank Dr. Paul Bartell and Dr. Ott, for continued support and guidance throughout 4 years of research. Additionally, Dr. Thomas Neuberger for guidance throughout all the MRIs.

Introduction

Circadian Clocks

Circadian rhythms are found throughout the body; they control feeding, sleep-wake, thermoregulation, hormonal, and cell regeneration cycles (Chang & Guarente, 2013). A circadian rhythm is defined as a biological process that displays an endogenous, entrainable, oscillation around 24 hours in length; it must respond to external cues, such as light, reviewed in Bartell and Moore (2013). Synchronizing cues are known as Zeitgebers, or time givers (Bartell & Moore, 2013). When the light dark (LD) cycle is replaced with constant dim light, therefore depriving the animal of zeitgebers, the bird's activity is still 24 hours (Bartell & Moore, 2013). These finding indicate that there is an endogenous timing mechanism that coordinates activity distribution across the day (Bartell & Moore, 2013). The endogenous timing mechanism is referred to as the circadian clock. The clock is ultimately controlled at the cellular level by a network of clock genes that are also regulated by external stimuli (Bartell & Moore, 2013).

Birds serve as some of the best models of circadian rhythms due to their strong behavioral expression of circadian rhythms. They are easily kept in confinement and continue to strongly express circadian rhythms in captivity, making them ideal models to study as reviewed in Bartell and Moore (2013). The most intense of these rhythms expressed is migration. Migration occurs twice as year as the birds move between their summer breeding grounds and their wintering grounds and requires massive changes in behavior and physiology in order for success (Bartell & Moore, 2013). It is known most of migration occurs at night (Bartell & Moore, 2013). Nocturnal

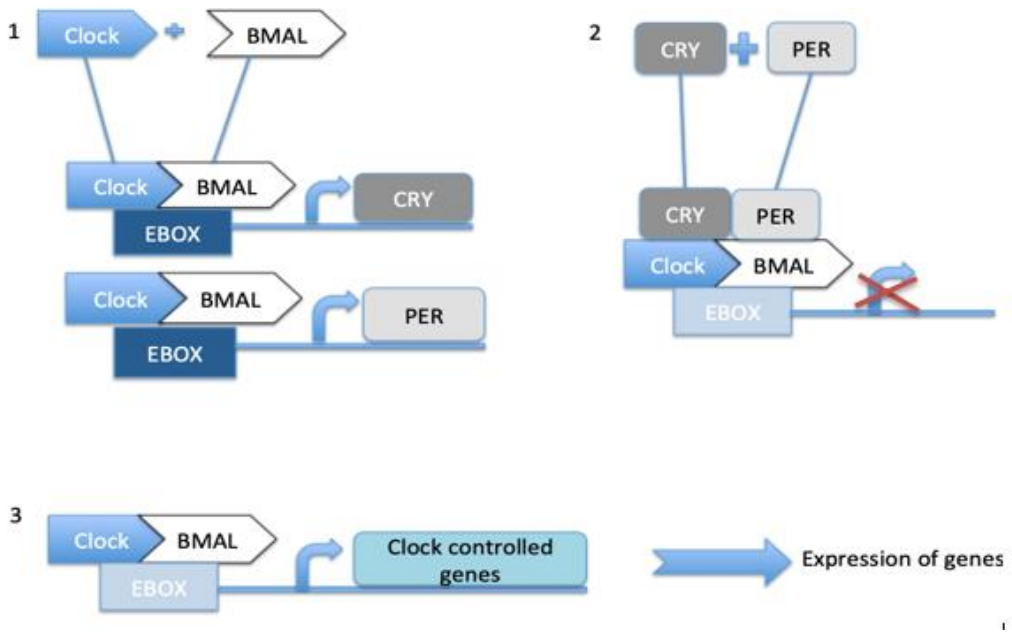
migratory activity can be tracked in laboratories via migratory behavior; this nocturnal migratory behavior is dubbed Zugunruhe (Bartell & Moore, 2013). It means migratory restlessness and is a wing whirring behavior (Bartell & Moore, 2013). During migration, diurnal birds, those that are typically active during the day, become active during both the day and night (Bartell & Moore, 2013). They accomplish this switch from strictly diurnal to both diurnal and nocturnal via the circadian system (Bartell & Moore, 2013). Internal clocks control both the day and nighttime Zugunruhe (Bartell & Moore, 2013). As the bird starts to migrate it lengthens its activity period (alpha) from 24 hours to 27 to 28 hours, eventually increasing activity into the nighttime (Bartell & Moore, 2013).

The circadian clock that controls nocturnal migratory activity is distinct from the daytime clock and their interactions are controlled by seasonal cues (Bartell & Moore, 2013). The seasonal changes in day length determine how the clocks interact and allow for migratory activity to be expressed (Bartell & Moore, 2013). Day length can also be defined as photoperiod (Bartell & Moore, 2013). The light signals must occur at a particular time relative to the circadian clock in order to stimulate migratory activity; this is called photoinducibility (Bartell & Moore, 2013). Laboratories through altering light signals so that light occurs during the bird's perceived night can induce a photoperiodic response (Bartell & Moore, 2013). Perceived night refers to light that falls during what the animal perceives as the dark period. There is another set of oscillators that also track day and night cycles throughout the season. They are two separate but coupled clocks in the brain referred to as morning (M), which tracks dawn, and evening (E) which tracts dusk (Yoshii, Rieger & Helfrich-Förster, 2012). These oscillators have only been partially tracked in the brain of flies (Yoshii, Rieger & Helfrich-Förster, 2012). The different light signals alter the phase angle between the oscillators and allow the animal to adapt its activity to the season's

varying photoperiod (Yoshii, Rieger & Helfrich-Förster, 2012). Phase angle refers to the interval between a phase marker, such as lights on, and a parameter such as start of activity (Yoshii, Rieger & Helfrich-Förster, 2012). The photoreceptors responsible for recording day length and perceiving light are located in the eye, deep brain and pineal gland (Takayoshi et al. 2013). The main components of the circadian system are all located in the brain.

The circadian clock resides within the suprachiasmatic nucleus (SCN) of the hypothalamus, which synchronizes the oscillators to the environment and each other (Herzog & Tosini, 2001). The main clock genes are *circadian locomotor output cycles kaput* (CLOCK), *brain and muscle ARNT-like protein* (BMAL), *cryptochrome* (CRY), and *period* (PER) (Herzog & Tosini, 2001). The clocks function through combined positive and negative feedback loops of clock genes as shown in Figure 1.

Figure 1: Core Circadian Loop Parts 1, 2, and 3 modified from (Bartell, 2010)



Part 1: CLOCK and BMAL heterodimize and activate the E-box in the promoter region of the genes PER and CRY, which causes the transcription of the negative elements, CRY and PER (Herzog & Tosini, 2001). **Part 2**: PER and CRY reenter the nucleus after translation, and repress BMAL and CLOCK activity at the E-box, thus preventing their own transcription (Herzog & Tosini, 2001) CRY and PER naturally degrade in the nucleus and CLOCK and BMAL can once again activate translation (Herzog & Tosini, 2001). The loop then begins again (Herzog & Tosini, 2001). **Part 3**: CLOCK and BMAL can control clock coordinated gene transcriptional activity, which eventually leads to the expression of circadian genes (Herzog & Tosini, 2001).

This entire cycle takes around 24 hours, hence the 24 hour periodicity for circadian rhythm (Herzog & Tosini, 2001). Clock genes are all part of the molecular feedback loop (Herzog & Tosini, 2001). There are many tissues throughout the body that are part of the circadian system and have the ability to generate 24-h periodicities (Herzog & Tosini, 2001).

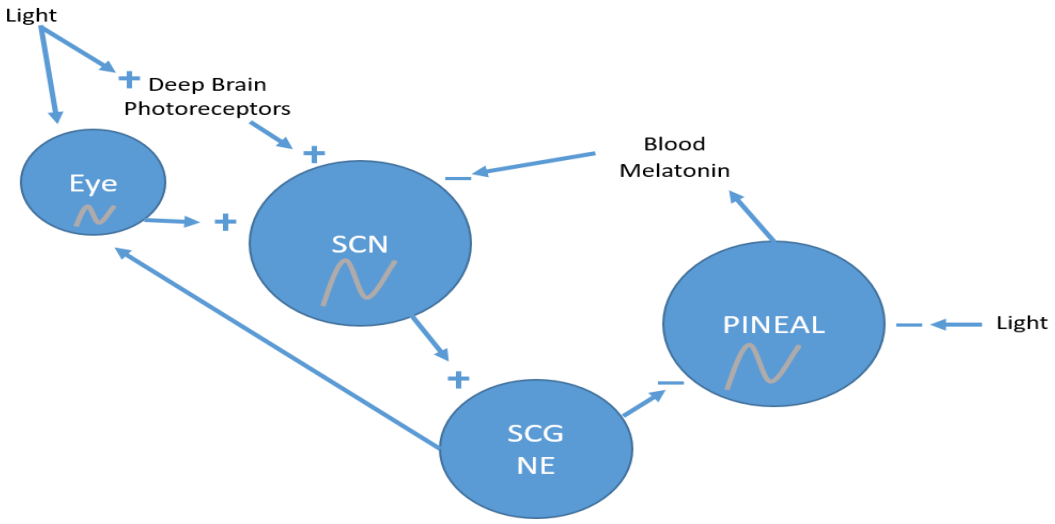
All higher vertebrates express these core molecular feedback loops of the clock; however, there can be some differences at the molecular level. The most notable difference is the protein PER (Dawson, King, Bentley & Ball, 2001). In the avian system there is no PER 1, and birds rely on PER 2/PER3 to perform the functions of PER 1 (Dawson, King, Bentley & Ball, 2001). Mammals however rely heavily on the transcriptional regulation and subsequent negative feedback of PER 1, and PER 3 is of minimal importance (Dawson, King, Bentley & Ball, 2001). Another

divergence is the importance of the SCN. The SCN is the central pacemaker in mammals (Herzog & Tosini, 2001). Removal of the SCN in mammals eliminates daily rhythms such as activity, feeding, etc. and these rhythms can be restored by transplanting the SCN from fetal animals (Ralph, Foster, Davis, & Menaker, 1990). All photic input to the circadian clock comes in through the retinohypothalamic tract after detection by the photoreceptors in the eyes in mammals (Herzog & Tosini, 2001). Birds have a bit more complicated central pacemaker. The SCN is divided into two portions, a visual (vSCN) and medial (mSCN) subgrouping which each have their own photic input (Cantwell & Cassone, 2006). In birds the homolog of the SCN and the pineal gland serve together as the central pacemaker, they coordinate their circadian regulation of events through coordinated outputs described as a neuroendocrine loop (Cassone & Menaker, 1984). These individual components contain coupled circadian oscillators; in each portion multiple inputs from photoreceptors exist and this input leads to the entrainment of the system as a whole (Cassone & Menaker, 1984). One input is the avian eye, another directly by the pineal gland, and finally several sets of photoreceptors are found to be functionally active in or near the hypothalamus within the deep brain (Takayoshi et al. 2013). The photic information is transferred to the SCN through the retinohypothalamic tract or deep brain regions (Takayoshi et al. 2013).

Since the avian system has multiple photic inputs it requires a slightly more complex circadian system. The avian eye is both a photoreceptor and a clock, and can continually modulate the strength of inputs to the central clock based upon its own phasing (Takayoshi et al. 2013). The avian pineal gland serves a circadian pacemaker and coordinates its outputs primarily via the synthesis and release of melatonin into the blood (Takayoshi et al. 2013). The regulation of melatonin biosynthesis is done by circadian regulation of the rate-limiting enzyme in the melatonin biosynthesis pathway, Aralkylamine N-acetyltransferase, AANAT (Tosini, Chaurasia, & Iuvone,

2006). Acutely, light can also degrade AANAT protein, thereby affecting melatonin biosynthesis directly (Tosini, Chaurasia, & Iuvone, 2006). Significant variation exists among birds in the relative roles that the pineal, SCN, and eyes play within the circadian system (Takayoshi et al. 2013). Figure 2 displays the functional aspects of the neuroendocrine loop.

Figure 2: Neuroendocrine Loop, modified from (Cassone & Menaker, 1984) (Zatz, 1991) (Takayoshi et al. 2013)



Light is perceived by lateral eyes, deep brain, and pineal gland (Takayoshi et al. 2013). The light stimulates the SCN (Takayoshi et al. 2013). The pineal produces melatonin at night, which inhibits the SCN (Cassone & Menaker, 1984). Then, during the day, light inhibits melatonin production and allows the SCN to be more active (Cassone & Menaker, 1984). The superior cervical ganglion (SCG) also inhibits melatonin production via norepinephrine production and this process is regulated by synaptic input from the SCN (Zatz, 1991).

Removal of the pineal gland from birds abolishes circadian rhythmicity in constant light conditions and modifies their entrainment under different photoperiods. These effects are due to the interacting mechanisms of the previously described neuroendocrine loop. After a pinealectomy, a population of oscillators that can still affect locomotor output remains, but only for a short period of time (Takahashi & Menaker, 1982). After a pinealectomy birds were placed in total darkness and became arrhythmic in 7-10 days (Takahashi & Menaker, 1982). The persistence of rhythm past the loss of the pineal suggests that there are damped oscillators, which are oscillators that fade with time (Takahashi & Menaker, 1982). The oscillators are coupled

together and in turn their phasing and strength of coupling are regulated by the pineal (Takahashi & Menaker, 1982). Coupled oscillators refers to oscillators that affect each other's expression and in this instance how much they affect each other is regulated by the pineal gland (Takahashi & Menaker, 1982). These dampened oscillators are most likely in the SCN (Takahashi & Menaker, 1982). As proposed by Gwinner in 1978, the pineal contains a self-sustained oscillator that drives a population of weakly coupled, damped oscillators located in the SCN (Takahashi & Menaker, 1982). Both the pineal and the damped oscillator receive photic input for entrainment (Takahashi & Menaker, 1982). Enright (1980) proposed that the pineal is not a circadian oscillator, but rather contributes to the coupling among an ensemble of self-sustained oscillators or 'pacers' (Takahashi & Menaker, 1982).

Entrainment

Entrainment due to light results from the ability of light to affect the expression of circadian rhythms. Light is the primary zeitgeber for birds (Bartell & Moore, 2013). It can cause an advance or delay of the circadian system, as observed by locomotor activity patterns (Zee, Rosenberg & Turek, 1992). For most experiments animals are kept on a constant LD cycle to allow their circadian system to entrain. Time is expressed in zeitgeber time (ZT) when birds are held under a Light:Dark cycle. When the lights first come on it is ZT 0, with succeeding time until the end of the day expressed in hours. The time it takes for the animal to begin locomotor activity each day while entrained, is considered the phase angle of entrainment (Zang et al. 1996). The time of lights on or off can be shifted to allow the researchers to see the circadian response to these changes, in a process called a phase shift. When the animals receive light early in their perceived "day" it

causes a delay shift; light late in their perceived "night" causes an advance shift (Zee, Rosenberg & Turek, 1992). The shift in activity typically occurs over a few days until the animal becomes fully entrained to the new light dark cycle. The time required to entrain to a new phase can be measured and computed, and this measured time is referred to as degree of clock lability (Zee, Rosenberg & Turek, 1992).

The duration of time required for reentrainment depends on the amount of the shift, the direction of the shift, and the phase-angle of entrainment (Wever, 1966). Birds were found to reentrain more rapidly during a period shortening than a phase lengthening, due to their natural periodicity of less than 24 hours (Wever, 1966). Aschoff's rule, as expanded on by Wever, states that under constant light conditions alpha shortens in nocturnal organisms and lengthens in diurnal organisms (Wever, 1966). This difference is because if the dark period is lengthened, then the mean intensity of light is decreased over the entire period and therefore the oscillation lengthens the period (Wever, 1966). Conversely, if the dark-time is shortened, the mean light intensity is increased over the period and the oscillation shortens alpha in nocturnal animals (Wever, 1966). In both examples the circadian period is changed in a direction that naturally accelerates the process of reentrainment (Wever, 1966).

Aging also effects entrainment and rhythm expression in many ways. The amplitude of a rhythm is an estimation of the "strength" of the circadian system and its ability to resist change (Turek, Penev, Zhang, Van Reeth & Zee, 1995). The amplitude of older animals is lower, suggesting the coupling between the activity-rest cycle and zeitgebers are weakened (Turek, Penev, Zhang, Van Reeth & Zee, 1995). This result is found across several species of birds and mammals (Scarbrough, Losee-Olson, Wallen & Turek, 1997). An increase in the magnitude of phase shifts induced by light pulses and a loss of responsiveness to phase shifting of zeitgebers is

considered to be the pronounced result of a weaker circadian system in older animals (Scarbrough, Losee-Olson, Wallen & Turek, 1997).

Aging may also reduce the effect of short days (Scarbrough, Losee-Olson, Wallen & Turek, 1997). There is a marked age related difference between the activity onset of young and older hamsters (Scarbrough, Losee-Olson, Wallen & Turek, 1997). The middle aged and old hamsters became active 1 to 2 hours earlier in short light on periods than the younger hamsters (Scarbrough, Losee-Olson, Wallen & Turek, 1997). Under extended lights on periods, all the age groups achieved the net daily phase shift; they did this by balancing both small phase delays and advances (Scarbrough, Losee-Olson, Wallen & Turek, 1997). The possible reason for this result is that under the extended bright light, age-related advances in locomotor activity are masked. Under the shortened light cycle these advances are more pronounced as the oscillators reduced their coupling strength and the advances lead to an expansion of locomotor activity in the older animals (Scarbrough, Losee-Olson, Wallen & Turek, 1997). Young hamsters were found to show a daily advance during entrainment and the older hamsters will entrain by delays or simply not respond (Scarbrough, Losee-Olson, Wallen & Turek, 1997). Changes in photoperiod alter the strength of coupling of the circadian oscillators and these changes underlie the fragmentation of the activity in the expanded lights on found in the older hamsters (Scarbrough, Losee-Olson, Wallen & Turek, 1997).

Multiple studies have shown animals typically remain able to entrain to changing LD cycles throughout their life (Zee, Rosenberg & Turek, 1992). An age related advance in the onset of alpha was again seen in a stable LD cycle (Zee, Rosenberg & Turek, 1992). After the animals underwent an 8-hour advance or delay shift of the LD cycle, the middle-aged hamsters resynchronized much more rapidly after a phase advance than the younger hamsters (Zee,

Rosenberg & Turek, 1992). These results mean that more light falls in the delay region giving the apparent result of a more rapid reentrainment, following the work of Aschoff and Wever (Zee, Rosenberg & Turek, 1992). The findings are consistent with previous research that found the free running period of locomotor activity was shorted as the hamster's age (Zee, Rosenberg & Turek, 1992). The reduction of the free running period is hypothesized to be due to the effects of aging on the various components of the master clock (Zee, Rosenberg & Turek, 1992).

What is Actually Aging in the Circadian System?

The link between circadian function and health is recognized in many fields of disease, especially cancer, sleep disorders, and diabetes (Chang & Guarente, 2013). Therefore properly functioning circadian expression is an important part of healthy people (Chang & Guarente, 2013). There are several circadian issues seen in older animals. Thermoregulation is an animal's ability to maintain its body temperature despite changing external conditions it is linked to the circadian system (Aujard et al. 2006). Many aged animals exhibit a lack of thermoregulation, including birds and humans (Aujard et al. 2006). Older animals consistently select for warmer environments over varying photoperiods (Aujard et al. 2006). Reduction in duration of sleep bouts, decreases in sleep efficiency, reduced circadian rhythms amplitude, and abnormal phase responsiveness to phase resetting signals is found in many studies (Garau, Aparicio, Rial, Nicolau & Esteban, 2006).

As animals age, they become less sensitive to zeitgebers, however the retinohypothalamic tract (RHT) pathway is usually morphologically unaltered, suggesting the issue lies in the master clock instead of the eyes and light perceiving pathways (Zee, Rosenberg & Turek, 1992). There is a substantial research on what part of the master clock could be responsible for these changes.

SCN activity is restored with implants of fetal SCN into the older animals, making the SCN itself a major point of studies (Turek, Penev, Zhang, Van Reeth & Zee, 1995). Complete or partial SCN lesions produce changes in the circadian rhythms similar to the elderly (Garau, Aparicio, Rial, Nicolau & Esteban, 2006). While many old rats still maintained 24-hour rhythms, some became arrhythmic indicating the circadian system was no longer responsive to light or functioning properly (Stat et al. 1993). There was also a dampening of the mean peak neuronal firing rates in old rats compared to young rats (Stat et al. 1993). This change means the neurons themselves change as the animal ages (Stat et al. 1993). This decreased amplitude is reflected in the actograms, measures of animals' locomotor activity outputs (Stat et al. 1993). The decreased amplitude represents a disruption in the coupling among, or deterioration of, the SCN cells. This decreased oscillation mimics the same oscillation produced from lesions of the SCN and causes the differential disruptions (Stat et al. 1993).

Sirtuin-1 NAD-dependent deacetylase (SIRT1) is part of the core circadian clock machinery (Chang & Guarente, 2013). SIRT1 directly activates the transcription of BMAL1 and leads to an increase of the amplitude of its expression (Chang & Guarente, 2013). SIRT1 also alters the stability of PER2 (Chang & Guarente, 2013). A metabolic target of CLOCK-BMAL is NAMPT, an enzyme required for the synthesis of an SIRT1 cofactor, which is needed for rhythmic SIRT1 activity (Chang & Guarente, 2013). SIRT1 works in conjugation with NAMPT and PPARγ coactivator 1α (PCG-1α) to make a circadian amplifying loop (Chang & Guarente, 2013). SIRT1 was observed to have decreased levels in the SCN of aged mice; this correspondingly leads to a decrease in BMAL as well (Chang & Guarente, 2013). Therefore, the aged mice display a decrease in their intrinsic period and an ability to re-entrain due to alterations in their core molecular clock (Chang & Guarente, 2013).

Vasoactive intestinal polypeptide (VIP) and arginine-vasopressin (AVP) are major peptides of the circadian clock. VIP and AVP peak levels in the SCN of aged lemurs were shifted significantly (Aujard, Cayetanot, Bentivoglio & Perret, 2006). In adult animals VIP peaked during the night and AVP peaked during the second part of the day, while in aged lemurs the AVP peaked in the beginning of the night and VIP peaked early in the day (Aujard, Cayetanot, Bentivoglio & Perret, 2006). These changes were reflected as an increase in daytime activity and in an advanced activity onset (Aujard, Cayetanot, Bentivoglio & Perret, 2006).

Most age-related impairments in the circadian cycles are attributed to reduced melatonin production in both mammals and birds (Garau, Aparicio, Rial, Nicolau & Esteban, 2006). These reduced levels of melatonin are due to alterations to the pineal gland's physiology during aging (Garau, Aparicio, Rial, Nicolau & Esteban, 2006). In older mammals a decrease in the amount of N-actyl transerfase, which catalyzes the rate-limiting step in the melatonin biosynthesis pathway, was observed (Garau, Aparicio, Rial, Nicolau & Esteban, 2006). Melatonin synthesis is dependent on serotonin (5-HT), and therefore increasing tryptophan, which can raise 5-HT levels, can also increase melatonin synthesis (Garau, Aparicio, Rial, Nicolau &, 2006). Ingestion of L-tryptophan and melatonin reduced the nocturnal activity in both old and young ring doves, and greatly increased the efficiency of sleep in the older ring doves (Garau, Aparicio, Rial, Nicolau & Esteban, 2006). However, only the highest doses of melatonin and tryptophan were effective (Garau, Aparicio, Rial, Nicolau & Esteban, 2006). These results likely correspond to the observed higher activity of indoleamine (2,3)-dioxigenase (IDO), which degrades tryptophan in elderly humans, a reduced permeability of the blood brain barrier to tryptophan in aged animals, and the age-related reduction in the number and sensitivity of serotonin receptors (Garau, Aparicio, Rial, Nicolau & Esteban, 2006). The increased amplitude in circadian rhythms after supplementation of l-

tryptophan and melatonin further suggests that in older animals neither compound is produced at sufficient levels for reliable clock expression (Garau, Aparicio, Rial, Nicolau & Esteban, 2006).

An important implication of the direct interaction between the SCN and l-tryptophan was the c-fos expression in ring doves (Garau, Aparicio, Rial, Nicolau & Esteban, 2006). C-fos is an immediate early gene product found in the brain after neuronal firing activity and is a standard marker of neural activation; it is at high levels during spontaneous waking or during sleep deprivation (Garau, Aparicio, Rial, Nicolau & Esteban, 2006). The basal expression of c-fos was reduced by 47% in aged dove's SCN when compared to the young doves (Garau, Aparicio, Rial, Nicolau & Esteban, 2006). C-fos was decreased after administration of melatonin and l-tryptophan during the first hours of the dark period; this is in line with previous findings that melatonin and serotonin inhibit neuronal firing of light-activated cells and demonstrates that the administering of these products directly reduces SCN activity and helps restore normal circadian expression (Garau, Aparicio, Rial, Nicolau & Esteban, 2006). Fos- proteins and cylic-AMP are believed to moderate effects of light on the circadian clock (Zang et al. 1996). Older hamsters were less light sensitive and produced less Fos-protein and phosphorylated less cylic-AMP than their younger counterparts (Zang et al. 1996). The photic threshold for the induction of c-fos is correlated with the threshold of phase shifting effects of light (Zang et al. 1996). The older hamsters were less responsive to phase shifts than younger hamsters, except during the highest light intensity (Zang et al. 1996). These results correspond with previous studies that note older animals being less photosensitive (Zang et al. 1996).

The depletion of brain monoamines, like serotonin, in young animals can induce changes in the responsiveness of the circadian clock that mimic those of aged animals (Turek, Penev, Zhang, Van Reeth & Zee, 1995). Melatonin and serotonin concentrations in young animals were

higher than the older ring doves (Paredes et al. 2006). There was a clear negative correlation between bouts of activity and serum melatonin levels in both young and older animals (Paredes et al. 2006). Plasma melatonin is at high levels during the night and during the inactive phase of behavioral cycles in passerines (Paredes et al. 2006). Serotonin during wakefulness is responsible for initiating a cascade of post-synaptic genomic processes, which in turn leads to homeostatic regulation of slow wave sleep (Paredes et al. 2006). The correlation between activity levels and serotonin during the day was positive, but much stronger in the young animals (Paredes et al. 2006). The results indicate a relationship between the old animal's melatonin and serotonin rhythms and the decline in their respective expression (Paredes et al. 2006). All of these results combined indicate that the SCN is less responsive in aged animals and produces less of the products, which maintain circadian robustness, and this, in turn, leads to a decay of the circadian system as a whole. All of these studies show that many parts of the clock age and there is not just one part that ages and affects circadian expression.

Circadian Clocks and Memory

Time learning (TL) refers to an animal's ability to remember important events that occur in a predictably changing environment (Mulder, Gerkema, & Van Der Zee, 2013). Animals, including birds, employ TL over many activities, but it is best seen in resource localization and predatory avoidance (Mulder, Gerkema, & Van Der Zee, 2013). Bees served as the original TL model. Gould discovered that honeybees store all information involved in flower location and recognition based on time of day (Gould, 1987). Holloway and Wansley discovered that independent of the time of day when training occurs, retention of the training is most optimal in

multiples of 24 hours later (Mulder, Gerkema, & Van Der Zee, 2013). Fear conditioning, conditioned place preference, and conditioned place avoidance show this effect (Mulder, Gerkema, & Van Der Zee, 2013). The fact that animals do not have to remember the time of day implies that the animals automatically remember the time and its periodic retention suggests time memory (Mulder, Gerkema, & Van Der Zee, 2013). TL is believed to be controlled by the circadian system and referred to as cTL (Mulder, Gerkema, & Van Der Zee, 2013).

It is unknown how animals actually use the circadian system in memory (Mulder, Gerkema, & Van Der Zee, 2013). However, time of day and its relevance to cognitive function has been around for a while. It is called Zeitgedachtnis, or time memory and in the 1950s Kramer did experiments in starlings and found that birds use an internal time of day mechanism, eventually discovered to be the circadian system, to select the appropriate orientation relative to the position of light (Mulder, Gerkema, & Van Der Zee, 2013). The circadian system is believed to "stamp" memories with time of day as a contextual feature to form associations with other contextual features (Mulder, Gerkema, & Van Der Zee, 2013). These clocks are then consulted to see if the time stamps match the actual time of day (Mulder, Gerkema, & Van Der Zee, 2013). This consulted clock function is what underlies cTL (Mulder, Gerkema, & Van Der Zee, 2013).

Time learning seems to be facilitated by high response costs or high reward, especially when it comes to food, and the stimulation to not make incorrect decisions motivates the need for a time contingency (Mulder, Gerkema, & Van Der Zee, 2013). They found that the circadian gene CRY is necessary for cTL in mice and this finding confirms the circadian nature of cTL (Mulder, Gerkema, & Van Der Zee, 2013). Hippocampus-dependent contextual and spatial learning is unaffected in PER1,2 mutant mice (Mulder, Gerkema, & Van Der Zee, 2013). These results might not be the most relevant for birds due to their reliance on PER 2,3 instead, but nonetheless suggest

that a decrement in learning by having an abolished circadian clock is not at the core of the process, but rather that the clock is responsible for the time of day effects. TL research can be especially useful in aging models. It was found that neither the SCN nor the adrenals are required for circadian time learning in mice, crediting the idea cTL is outside the master clock (Mulder, Papantoniou, Gerkema, & Van Der Zee, 2014). They also found that abrupt shifts in the food-entrainable oscillator's cues affected cTL and that light pulses more severely affected TL performance (Mulder, Papantoniou, Gerkema, & Van Der Zee, 2014). This result means that cTL is more sensitive to timing manipulation with light and food, but that it does not likely reside in the SCN (Mulder, Papantoniou, Gerkema, & Van Der Zee, 2014). Previous zeitgebers also influence future behavior of a circadian oscillator and this "memory" of zeitgebers can span across generations in flies and plants (Boikoglou et al. 2011). More recent work found that the hippocampus may have its own circadian clock (Mulder, Gerkema, & Van Der Zee, 2013). Memory deficiencies occur in clock knockouts and memory formation and consolidation were shown to depend on circadian reactivation in the hippocampal neurons (Mulder, Gerkema, & Van Der Zee, 2013). It was proposed that training functions similarly in the hippocampal neurons as light activation and clock resetting (Mulder, Gerkema, & Van Der Zee, 2013). It is likely that the hippocampal clock is modulated by events and is affected by input from the SCN (Mulder, Gerkema, & Van Der Zee, 2013).

This system might be a key for behavioral changes during aging. For example those who have Alzheimer's are often disoriented in time and place and episodic memory is among the first memories affected (Mulder, Gerkema, & Van Der Zee, 2013). TL may be responsible for these early symptoms (Mulder, Gerkema, & Van Der Zee, 2013). It is hypothesized that cTL will become less reliable with circadian system deterioration. In order to buffer this loss, animals may

switch their dominant strategy to guide behavior and older mice were found to rely on sequence based behavior instead of cTL (Mulder, Gerkema, & Van Der Zee, 2013). This is consistent with previous studies that found aged and sleep deprived animals, both of which weaken the circadian system, rely more on alternative strategies for reversal learning such as procedural memory instead of the hippocampus (Mulder, Gerkema, & Van Der Zee, 2013).

MRI

In any aged animal morphological changes to the brain can be expected. Magnetic resonance imaging (MRI), is a noninvasive way to visualize and measure these morphological changes via volume calculation. Birds differ greatly in brain structure compared to mammals and are rarely imaged using MRI (Behroozi et al. 2018).

Relaxation time constants are a measure of how long it takes for thermal equilibrium of nuclear magnetization in a sample to return after an initial excitation pulse (Bloch Equations, 2018). These times are measured as longitudinal (T1) and transverse (T2) relaxation ties (Behroozi et al. 2018). T1 and T2 matter because they dictate the signal contrast and signal-to-noise ratio (Behroozi et al. 2018). These parameters are required to distinguish between types of tissues and allow the tissue to appear distinct on the images produced from the MRI (Behroozi et al. 2018). Birds have pneumatic bones and these air pockets result in distorted images (Behroozi et al. 2018). Therefore, only rapid acquisition with refocused echoes or RARE scans can be used with an avian skull (Behroozi et al. 2018).

RARE is also called fast spin echo or turbo spin echo (TSE/FSE, 2018). It allows for multiple phase-encoding lines to be acquired during each TR interval and can reduce the time spent

imaging (TSE/FSE, 2018). It also can improve the signal-to-noise ratio and improve spatial resolution (TSE/FSE, 2018). It relies on T1 and T2 relaxation times to produce the signal-to-noise ratio and contrast (Behroozi et al. 2018). In T2, fluid and fats typically appear as bright spots on the images while tissue is dark gray in color and air is black (TSE/FSE, 2018).

It is likely that older avian brains will appear much like elderly person's brains. White matter changes and microbleeds are very common in elderly patients especially those with Alzheimer's disease (Cavalieri, Schmidt & Schmidt, 2012). The white matter becomes more heterogeneous as the patients age and white matter hyperintensities develop (Cavalieri, Schmidt & Schmidt, 2012). They appear in various spots around the brain or halo around spaces. Microbleeds, which appear as bright white spots, are often caused by arteriosclerosis. Also observed is cerebral amyloid angiopathy, which is when amyloid plaques, often the beta variant, form on the walls of blood vessels and lead to greatly increased risks of brain bleeding (Cavalieri, Schmidt & Schmidt, 2012). Both of these issues appear brighter in color on the T2 scans (Cavalieri, Schmidt & Schmidt, 2012). One can also expect expanded ventricles in the brain and less white matter overall, a common pathology of brains with Alzheimer's and elderly patients (Cavalieri, Schmidt & Schmidt, 2012).

Materials and Methods

Actograms

The birds used in this experiment were mixed gender wild caught White-throated sparrows (*Zonotrichia albicollis*). They were captured from The Pennsylvania State University's Arboretum. All animal procedures were reviewed and approved by the animal care and used committee at Pennsylvania State University (protocol #45558-1). White- throated sparrows are diurnal birds. The geriatric set were 8 sparrows that were caught 10 years ago, making them at least 11 years old. The average lifespan of a White-throated sparrow, if surviving past the first year, is around 9 years, with some of the oldest wild caught sparrows living to 14 (Lutterming & Love, 2015). During the experiment one of the sparrows died, Sparrow 7, so only 7 sparrows were part of the final information. The birds were housed in their own room at the Poultry Research Extension Center. They were kept in individual cages within an open face black box with separate food and water dishes. The water dishes were changed daily and cleaned weekly; the food dishes were refilled as needed and cleaned weekly. The birds were numbered 1-8 according to their number in the VitalView software. Only the geriatric sets of sparrows were used in the actogram results.

The birds were kept on a 12:12 LD cycle throughout the experiment. Lights-on was at 0 ZT/8am and lights-off was at 12 ZT/20pm. This light cycle was maintained for a steady state. On Sunday November 19, 2017 the lights were shifted forward for 4 hours producing a lights on at 12pm and lights off at 12 am. This new light cycle was maintained for 37 days. The 37 days was

to allow enough time to entrain and collect blood and was due to the timing of school breaks and scheduling. The lights were then shifted back 4 hours and the original light cycle was restored with lights on at 8am and lights off at 8pm.

The bird's activity was recorded using the VitalView Data Acquisition System from MiniMitter Inc. As per the VitalView Data Acquisition System Instruction Manual, there was one actometer, switch closure device, per cage. When the animal broke an infrared beam of light, it caused the micro switch to close. This data then was transmitted to the QA-4. The QA-4 allows four switch-closure devices to be monitored at once. The number of closures per minute from the QA-4 was accumulated by the DP-24 Data port. DP-24 communicates this information to the PC. VitalView software on the PC displays the information. The activity was logged every minute with each movement equaling one. The software was set up with all eight cages in a group and each animal corresponding to a group member and light beam was selected as the data parameter. To view the data, a data collection monitor was selected. The data collection monitor allowed viewing of the system and gave an ability to see if any cages were not running properly.

To begin data collection, start data collection and then standard were selected for group one. Data were typically collected on a weekly basis and processed later in the same week. To collect data, data collection was stopped and restarted after the data were downloaded to a flash drive. The data were processed using the software program ActiView. The data were loaded into ActiView and automatically converted into an actogram. The proper lights on and off were selected and the base activity level for each cage was chosen to give an appropriate actogram. A periodogram was made using the periodogram tool for birds 5 and 8 to show that 5 was arrhythmic. Bird 7 died during the experiment; therefore bird 7 does not have an actogram. Bird 5 has an

activity base level of 5 instead of 0 due to noise on the actograms all others have an activity threshold of 0.

From the actograms one can calculate phase angle and the speed of the oscillator during reentrainment. To calculate the phase angle of entrainment, the time in minutes between the zeitgeber and alpha was averaged over several days. This was done for birds once stable in their rhythm from before the time shift, during the time shift, and after the time shift for both lights on and lights off. The second piece of data was the speed of the oscillator. The speed of the oscillator is determined by averaging the minutes per day the bird shifts when entraining and then the dividing the average by the number of days it took to entrain. To analyze the periodogram the peak correlation output was recorded. The periodogram shows how robust a bird's circadian cycle is and the length of their period. In birds that are well entrained to a 12:12 LD, the peak correlation will be at 24 hours.

MRI

Since many of the original birds passed away due to old age between the portions of the experiment and due to funding constraints only 6 birds could receive an MRI. The second cohort of sparrows was wild caught and banded in the spring of 2016. Each bird was at least 3 years of age since they were all caught as adults. The sparrows were also captured from The Pennsylvania State's University Arboretum. They were kept in a room with a 12:12 LD, 8 am-8pm cycle. They share a room with visual contact with Northern Wheatears, but no physical contact. They were in a large group cage with plastic trees. They had two separate, group food and water dishes. The water dishes were changed daily and cleaned weekly; the food dishes were refilled as needed and

cleaned at least weekly. Only three of the younger cohort sparrows were chosen for the MRI. Their leg bands differentiated the birds. One was had a red band, the other a silver, and the final was unbanded.

The older sparrows were kept in the same room and therefore same light cycle as the younger cohort for the MRI experiments. They had their own smaller, separate group cage. They were fed the same food as before and as the younger cohort. The numbers given to them for the MRI experiment do not correspond with the numbers recorded for the Actogram portion.

To perform the MRI, birds were anesthetized with Halothane and dexmedetomidine and placed in a specially designed 3D printed holder. Their feathers were trimmed around their ears to secure the ear bars in place, fixing the head in holder. The holder was screwed in to keep the birds steady and their heads were secondarily secured with tape. Once in the holder they still received isoflurane. The holder was placed in the MRI machine and their positioning was confirmed to be correct. The six MRIs processed were the six successful MRIs.

The birds underwent a high field MRI using the Varian 7 tesla horizontal magnet. The MRI has a 31 cm horizontal bore system, with a Varian Direct Drive console and four receiver channels. The MRI sequences chosen were Flash, Press, Field Map, RARE, and finally the DTI itself. The longest sequence was diffusion tensor imaging (DTI) which allows fiber tracking and visualization of the flow of water. The total run time was usually around 3 hours. RARE files were used for image segmentation.

Avizo 9.0 was used to segment and automatically calculate the volumes of the segments. The RARE file needs to open for the segmentation. In WordPad the RARE set of files the was opened to define the dimensions. $PVM_Matrix = (2) gives the first two parameters for dimensions. Then $PVM_SPackArrNSlices= (1) gives the third parameter, number of slices. All

of the MRIs had 32 slices except for the last two which each had 40. In Avizo 9.0, pdata were selected from the same number file where RARE was located. 2dseq2 was selected and the data were opened as raw data. When the raw data parameters window appeared, 16-bit unsigned was selected. The dimensions obtained from the Rare Method file were inserted into the window. The next step was to define voxel size. To determine voxel size, $PVM_SpatResol= (2) was found in the Rare method file and the resulting number was multiplied by 100 and rounded to the nearest whole number. The third parameter was .5 and represents 500 microns, 500 was entered as the last parameter. After all the parameters were correct, segmentation began.

The resulting volumes from segmentation were exported to Excel from Avizo 9.0 material statistics function. All volumes were automatically calculated besides total brain volume, which needed the whole brain plus all other segments added to it. The volumes were averaged within the cohort and then compared across the two groups. A two-sample equal variance t-test was done to determine the p value. The standard p value of .05 was used as the cutoff for significance. The excel formula used was =T.TEST(array1, array2,tails,type).

There were 6 areas of interest for the segmentation. The first was the cerebellum. It was chosen because it is an anatomically distinctive region and can provide an accurate volume comparison between the younger and older cohort. The second area of interest was the hippocampus. The hippocampus is also the site of short to long term memory conversion and has been shown to shrink with aging (O'Shea, Cohen, Porges, Nissim, & Woods, 2016). Therefore it was of great interest to the experiment. It is a distinctive area on the MRI and provides a way to compare the two groups. The third area was the hypothalamus. This area was chosen due to its circadian function and the fact it houses the SCN. Finally the ventricles were chosen since they appeared to be much larger in the older cohort and demonstrate visible physical changes to the

brain's structure as the bird's age. The whole brain was also calculated to provide a final area of comparison.

Results

Actograms

The monitoring system was running for much longer than the experiment. There were a few power failures and machine breakdowns. The time period of October 30, 2017 to January 14, 2018 is the relevant time frame. The actogram copies the information twice so only one column is need for analysis.

Figure 3: Actogram of Sparrow 1

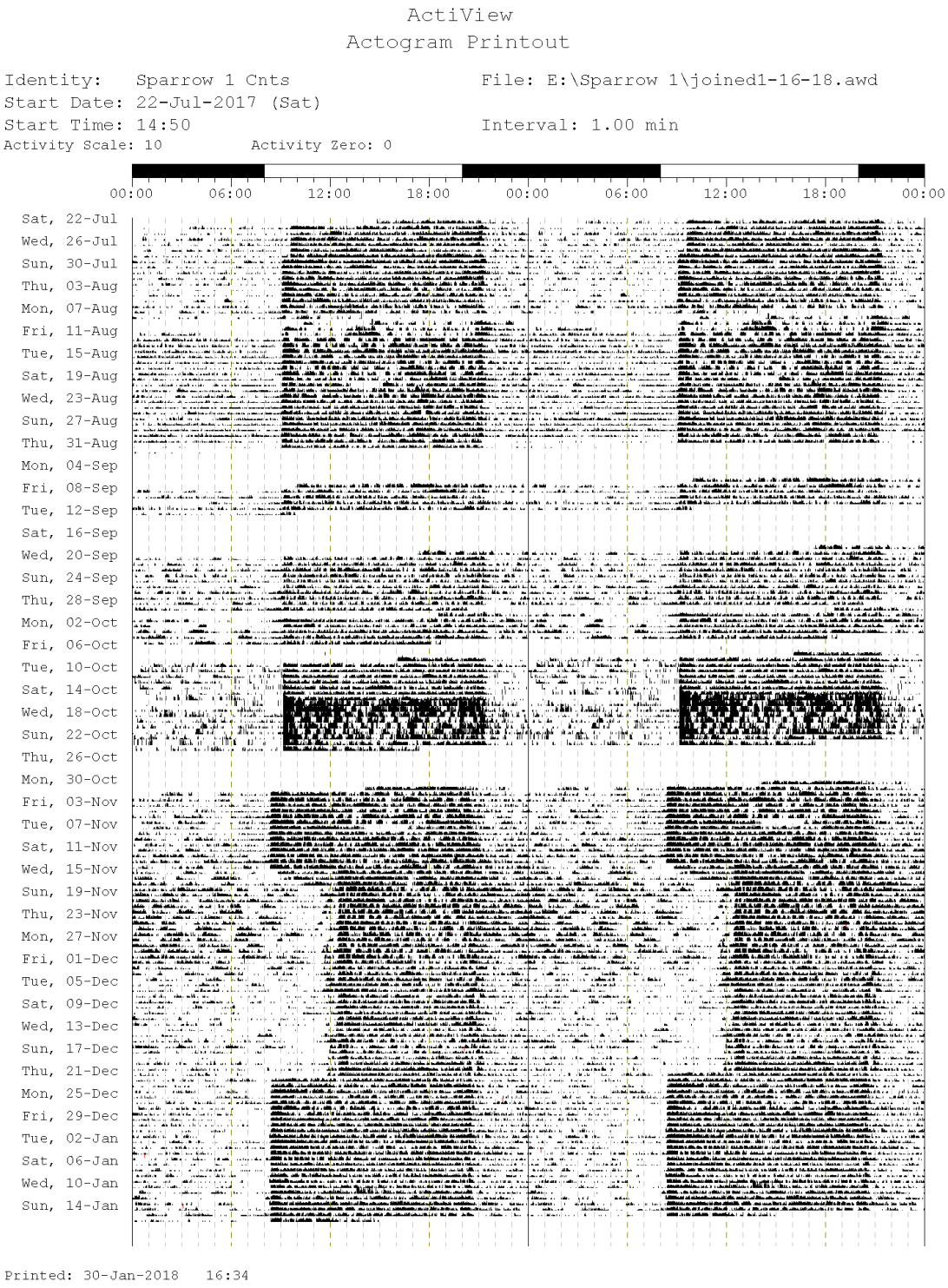


Table 1: Circadian Data, Sparrow 1

Sparrow 1	Lights On (Minutes)	Lights Off (Minutes)
Phase Angle of Entainment, Before	24	-56
Phase Angle of Entrainment, During	30	156
Phase Angle of Entrainment, After	30	-54
Speed of Oscillator, Before	48	37
Speed of Oscillator After	100	0

Bird 1 showed an interesting entrainment. The light cycle shift was a full four hours. The bird however maintained its entrainment to the previous light cycle for the dusk as seen in Figure 3. It did a phase shift forward but only in the AM. The PM phase shift never occurred despite the lights being on until midnight. It still stopped most activity at 8pm instead of 12am when the lights actually went out. It did undergo a time period during the shift where it tried to entrain on the forward shift but settled on a shortened alpha of 8 hours instead of 12. When the lights switched back to the original LD cycle it immediately reentrained and did not show any adjustment or entrainment to the previous lights off cue and expanded its alpha back to 12 hours. Bird 1 Circadian Data were taken from the actograms. The -156 represents a compression of alpha due to dusk not synchronizing.

Figure 4: Actogram of Sparrow 2

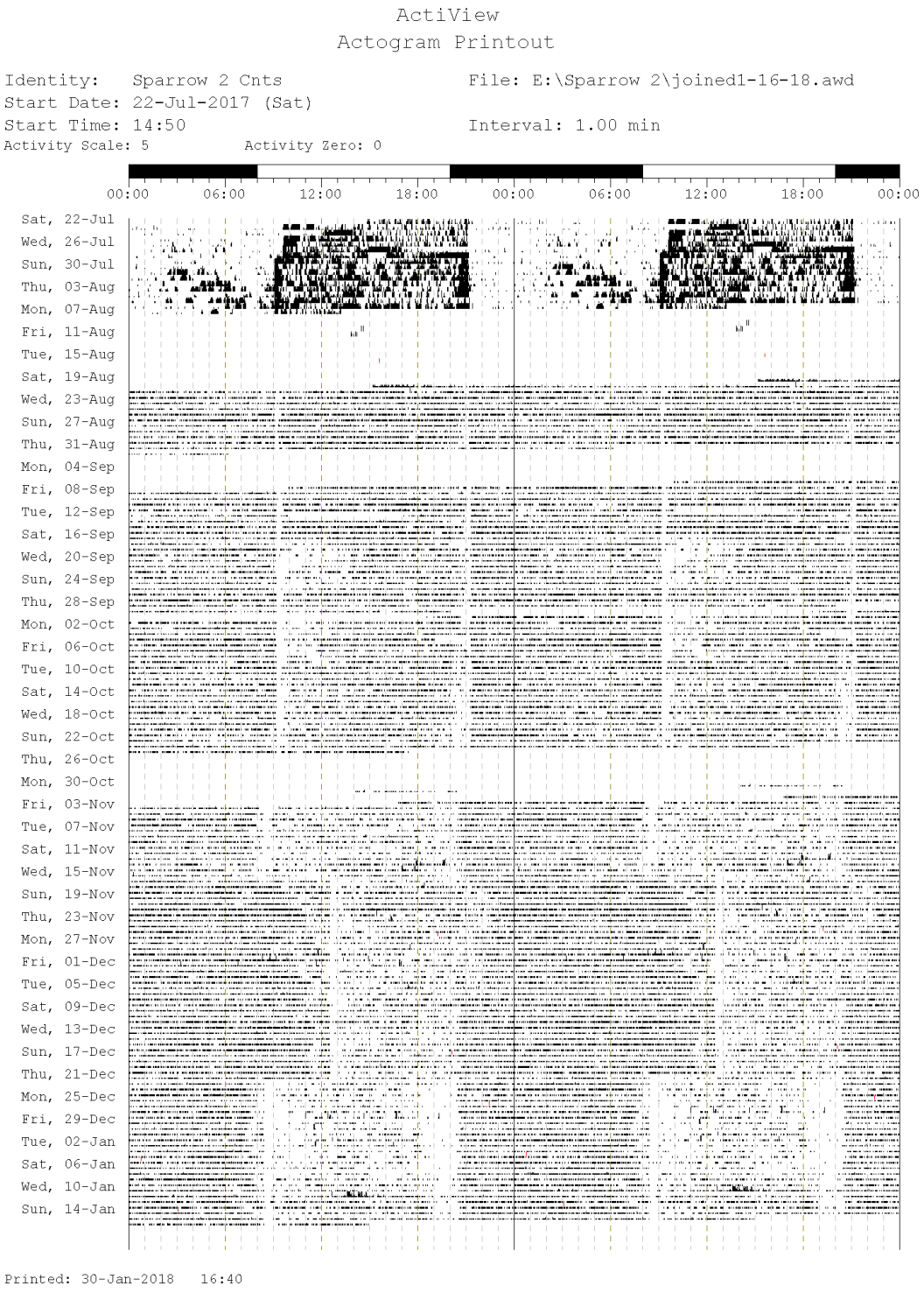


Table 2: Circadian Data, Sparrow 2

Sparrow 2- Nocturnal	Lights On (Minutes)	Lights Off (Minutes)
Phase Angle of Entainment, Before	0	32
Phase Angle of Entrainment, During	30	-210
Phase Angle of Entrainment, After	20	-210
Speed of Oscillator Before	120	24.3
Speed of Oscillator After	0	240

Bird 2 was the most interesting of the birds. It was fully nocturnal and had an opposite rhythm of the all the other diurnal birds. White- throated sparrows are supposed to be diurnal. It showed for a short time the full four-hour shift forward and then like bird one reentrained on the previous light cycle but in reverse. It fully reentrained to an expanded alpha with 12am being the cue for activity and following 12pm being the cue for inactivity. The full four-hour shift forward though for a time shows that the lights were working correctly for the experiment, despite all the other birds shortening their free running period. It, like Bird 1, almost immediately reentrained during the phase shift back four hours as reflected in the speed of the oscillator.

Figure 5: Actogram of Sparrow 3

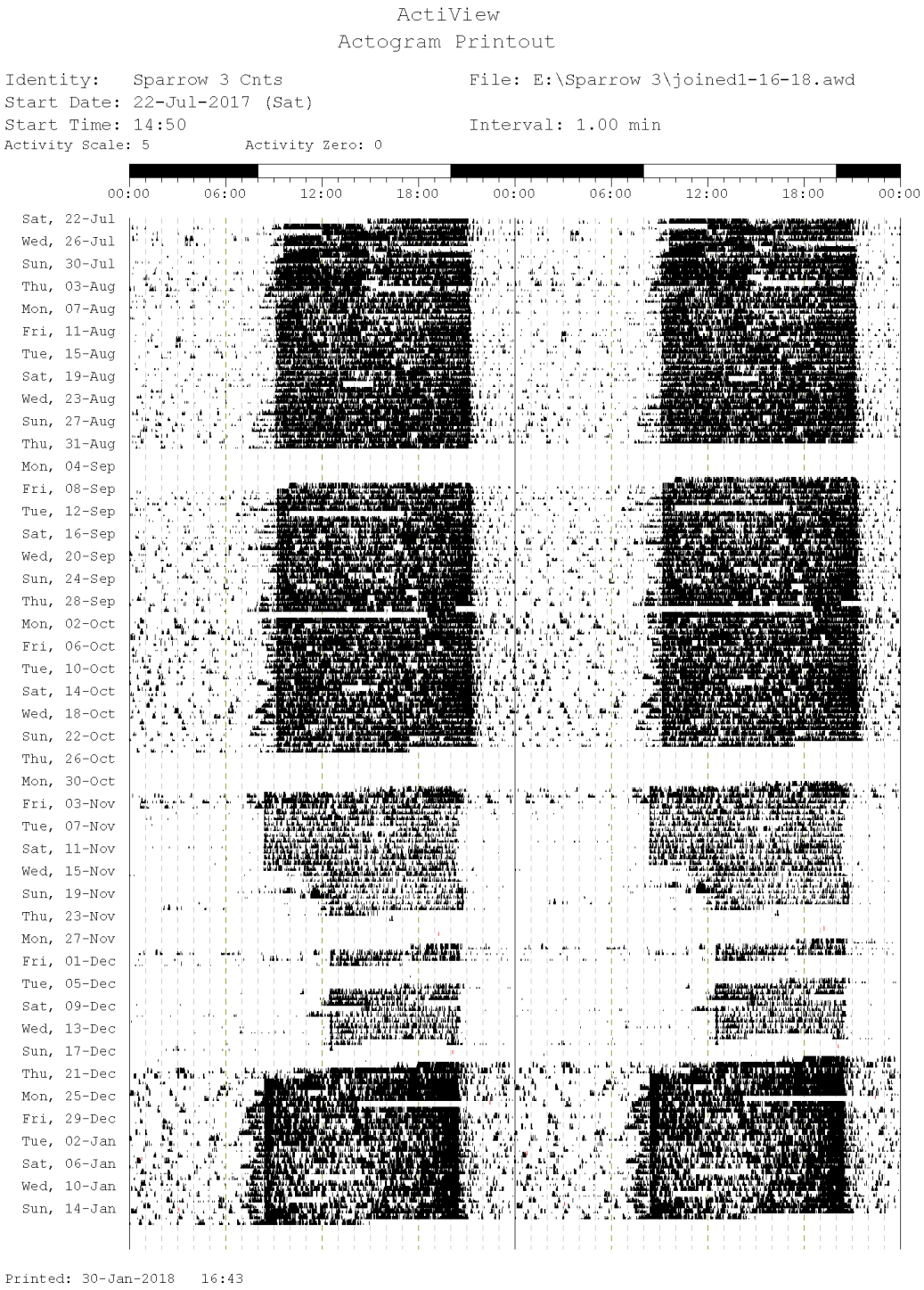


Table 3: Circadian Data, Sparrow 3

Sparrow 3	Lights On (Minutes)	Lights Off (Minutes)
Phase Angle of Entainment, Before	20	26
Phase Angle of Entrainment, During	25	-205
Phase Angle of Entrainment, After	20	37
Speed of Oscillator Before	61	5
Speed of Oscillator After	125	0

Bird 3, upon the four-hour shift forward, took some time to adjust to the light on. It never adjusted to the new time of lights off however. It just shortened alpha to around 8 hours similar to bird 1. However it extended its activity by around 20 minutes. This shift was not enough to be active until ZT13. It almost immediately reentrained to lights on during the shift back, but kept extra activity post lights off when compared to the original.

Figure 6: Actogram of Sparrow 4

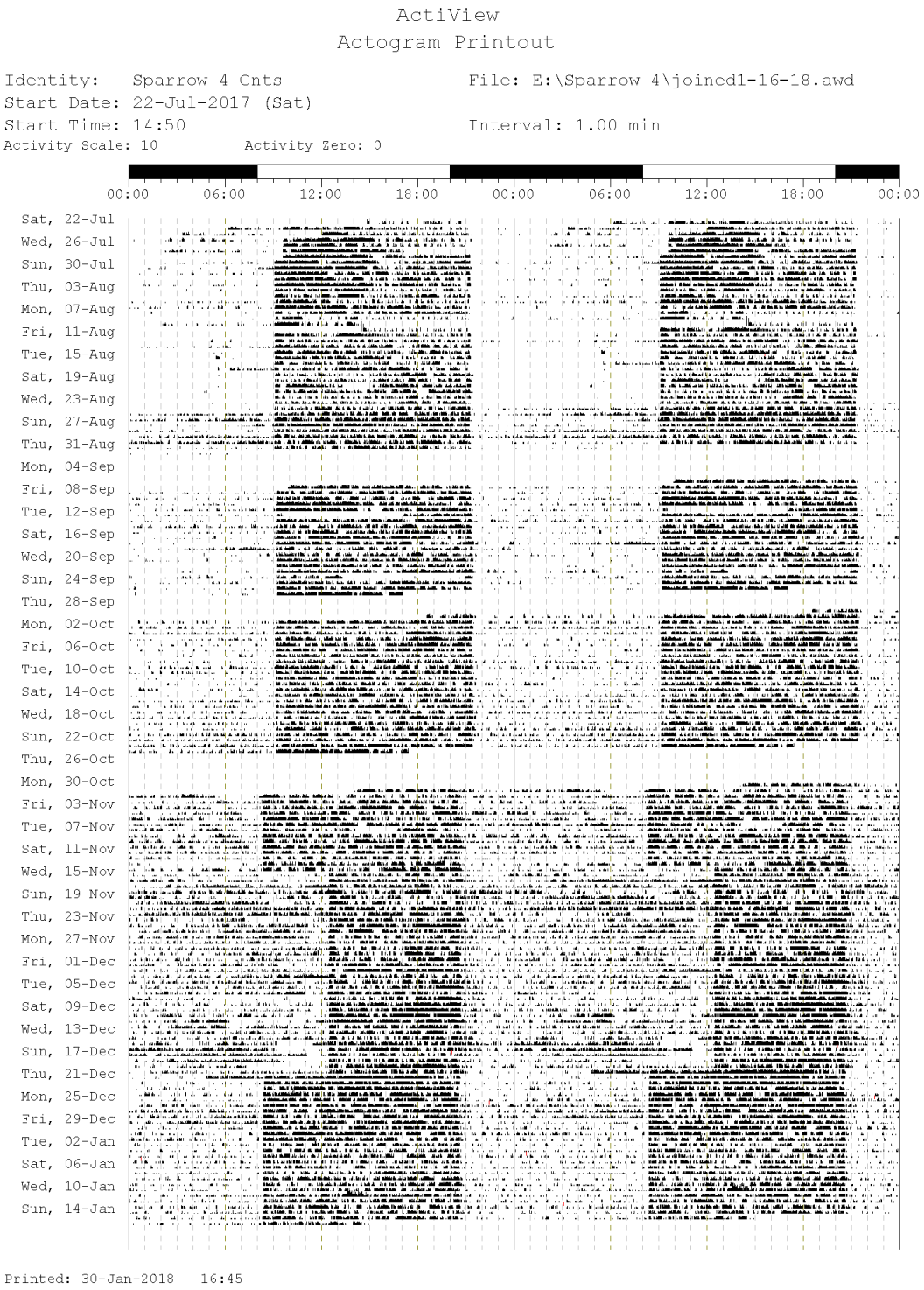


Table 4: Circadian Data Sparrow 4

Sparrow 4	Lights On (Minutes)	Lights Off (Minutes)
Phase Angle of Entainment, Before	16	27
Phase Angle of Entrainment, During	23	-194
Phase Angle of Entrainment, After	20	18
Speed of Oscillator Before	240	5
Speed of Oscillator After	240	8

Bird 4 was very similar to bird one. It did a phase shift forward but only in the AM. The PM phase shift never occurred. It stopped most activity at 8pm instead of 12am when the lights actually went out. It almost immediately entrained on the forward shift and settled on a shortened alpha of 8 hours instead of 12. When the lights switched back to the original LD cycle it immediately reentrained and simply lengthened its period back to 12 hours.

Figure 7: Actogram of Sparrow 5

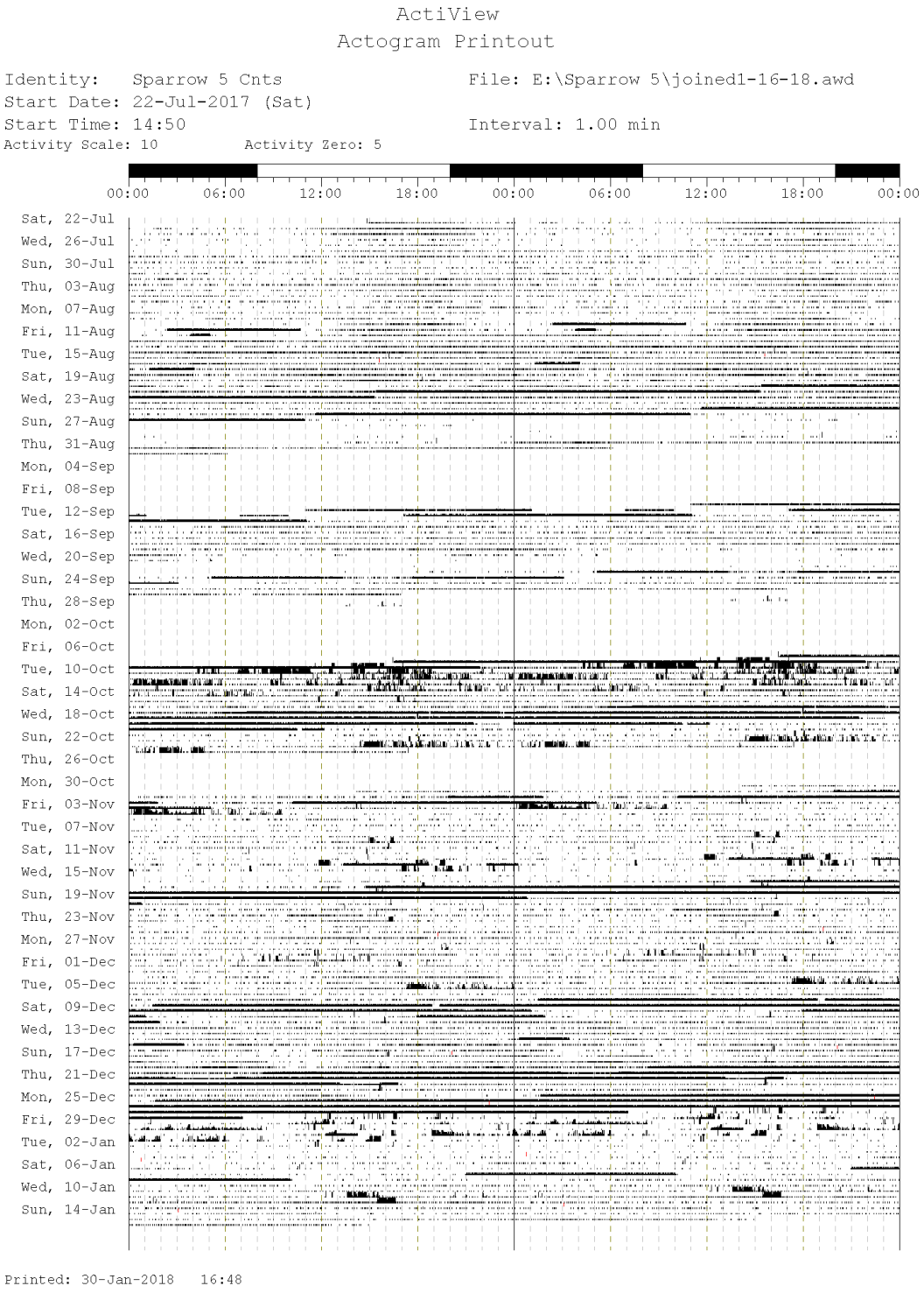
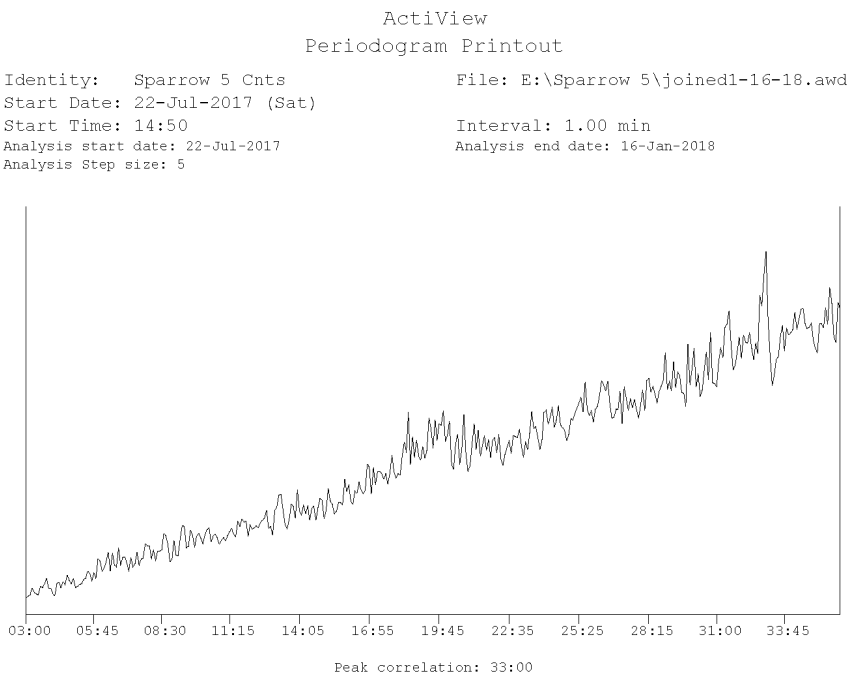


Figure 8: Periodogram of Sparrow 5



Bird 5 was arrhythmic despite being exposed to the same light cycle as all the other birds in the same room. It never entrained and just kept constant small movements. This lack of rhythm is reflected in Figure 8. The periodogram shows how robust a bird's circadian cycle is and the length of their period. In birds that are well entrained to a 12:12 LD, the peak correlation will be in 24 hour. Bird five lacks a peak showing that is in fact not entrained to any light cues or running a 24 hour internal period.

Figure 9: Actogram of Sparrow 6

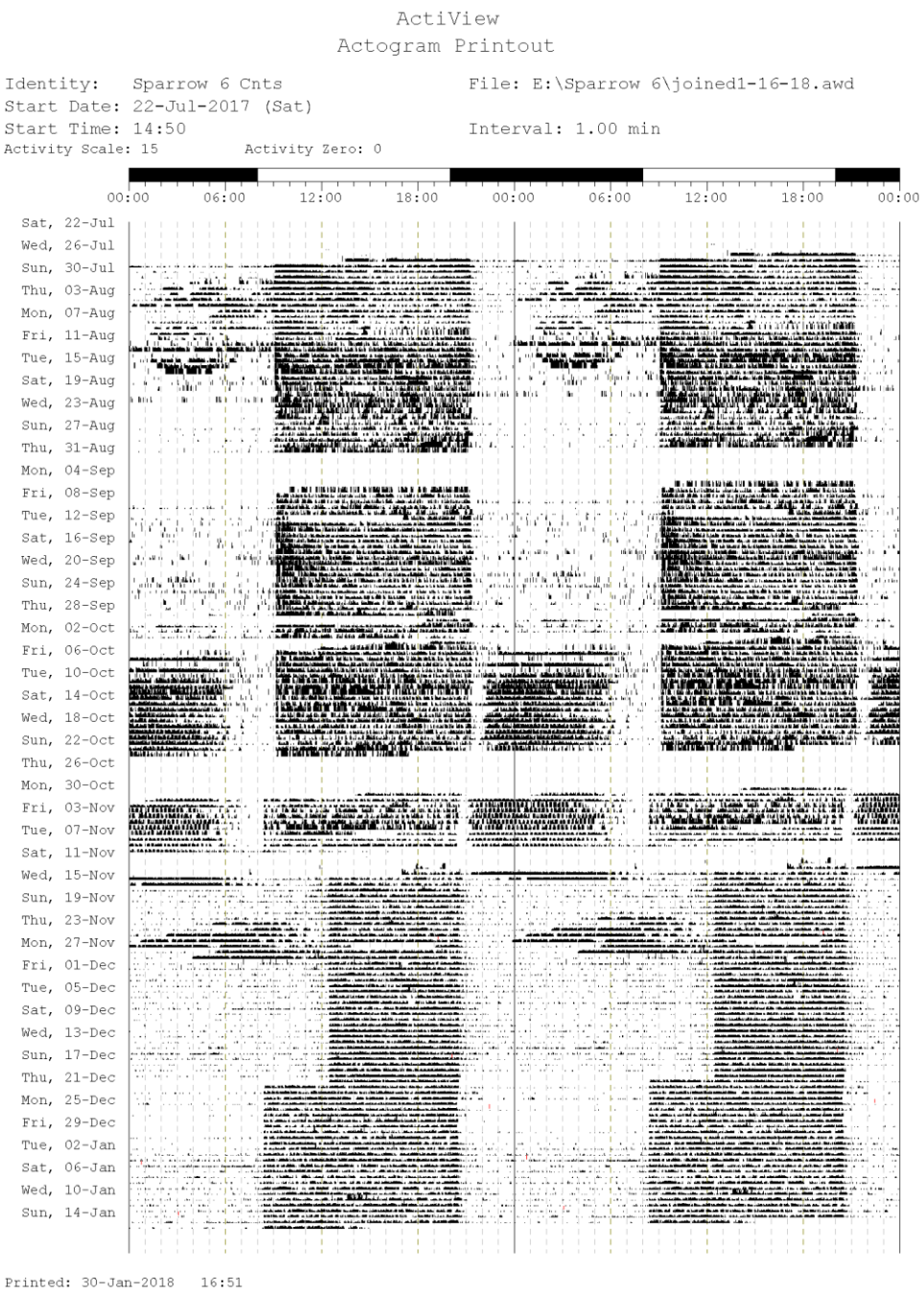


Table 5: Circadian Data, Sparrow 6

Sparrow 6	Lights On (Minutes)	Lights Off (Minutes)
Phase Angle of Entainment, Before	20	35
Phase Angle of Entrainment, During	20	-200
Phase Angle of Entrainment, After	15	23
Speed of Oscillator Before	NA	NA
Speed of Oscillator After	240	5

Bird 6 was very similar to bird 3. After the four-hour phase shift forward, it took some time to adjust to the light on. It never entrained to lights off however. It just shortened alpha to around 8 hours similar to bird 1. However it extended its activity by around 10 minutes. This shift was not enough to be active until ZT13. It almost immediately reentrained to lights on during the shift back, but did not keep as much activity post lights off when compared to the original. What was unique was that it did show some migratory patterns before the light shift and underwent a period of activity during the night similar to the actograms of migrating birds. It stopped showing any migratory activity after it fully entrained to the new light cycle.

Figure 10: Actogram of Sparrow 8

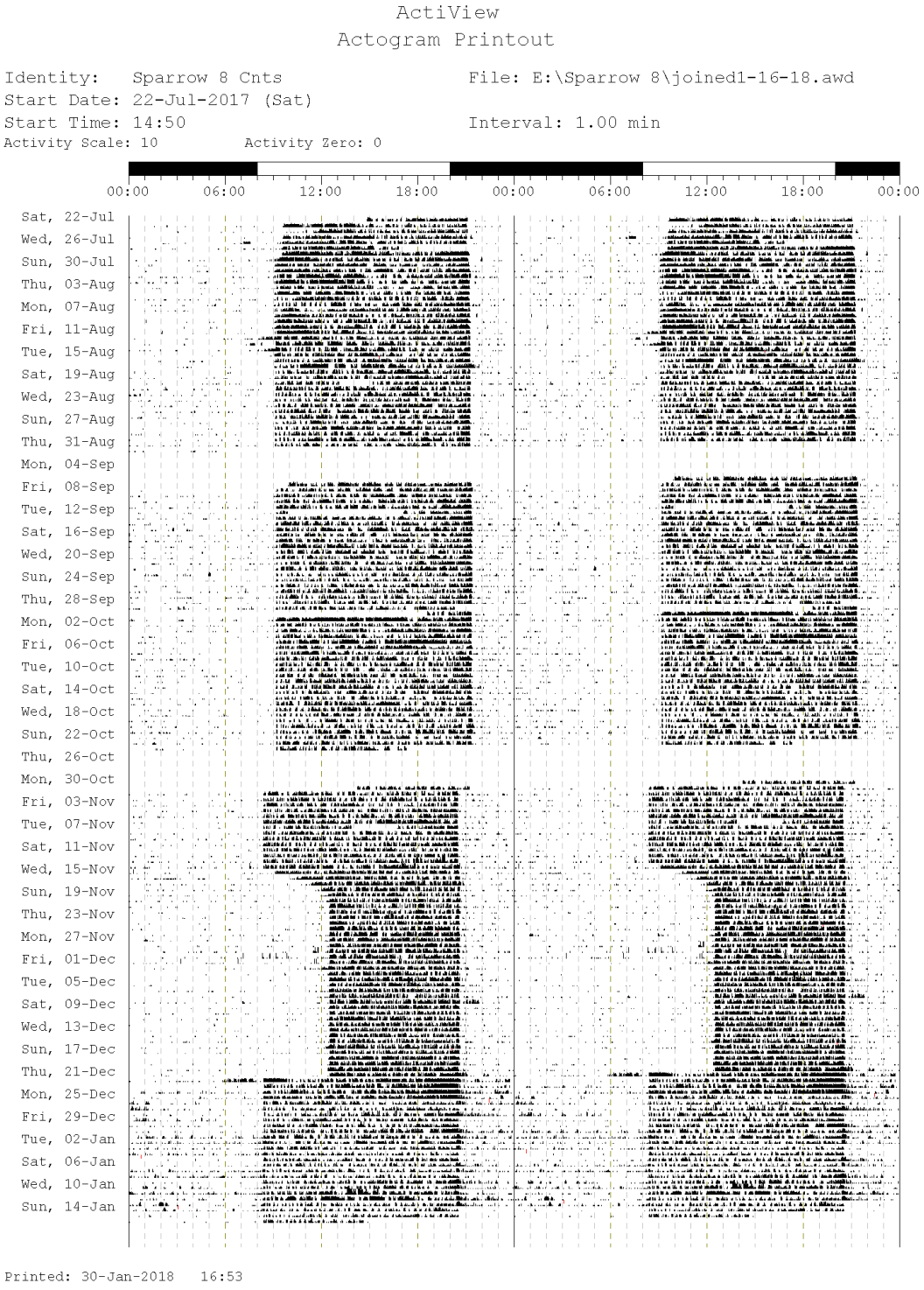
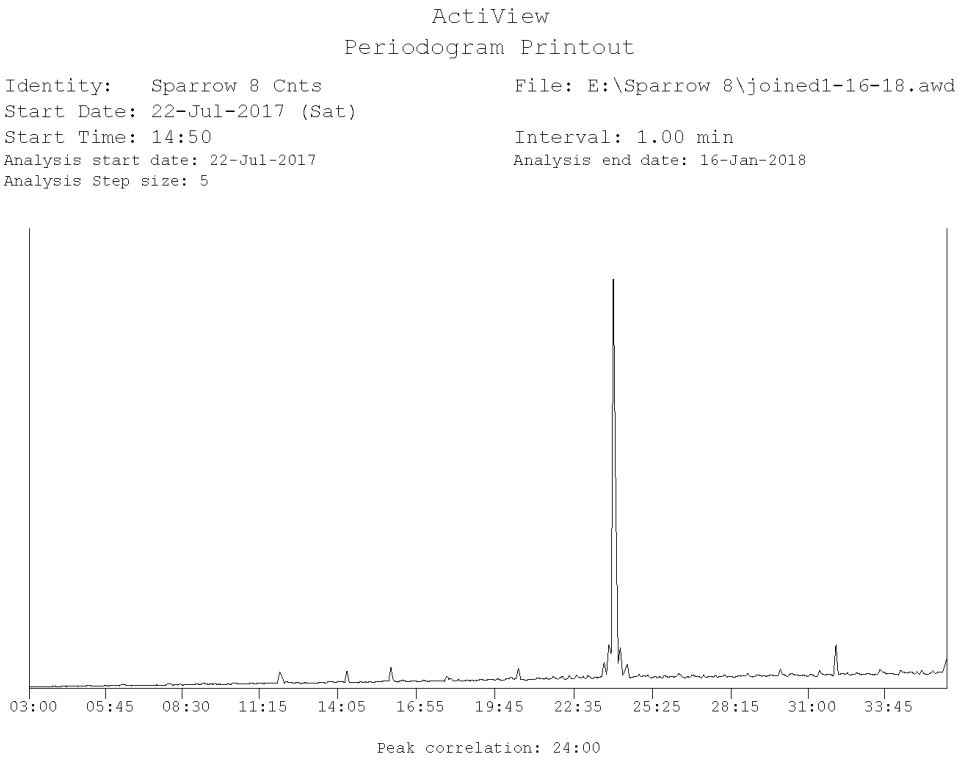


Table 6: Circadian Data, Sparrow 8

Sparrow 8	Lights On (Minutes)	Lights Off (Minutes)
phase angle, before	20	30
phase angle, during	29	-205
phase angle, after	21	20
speed of oscillator, before	60	2
speed of oscillator, after	305	3

Figure 11: Periodogram of Sparrow 8



Bird 8 showed a very similar entrainment to Bird 1. Upon the four-hour cue forward, this bird took some time to adjust to the light on. It never entrained to lights off however. It just shortened alpha to 8 hours. It never showed any activity past ZT 12, despite the shifted dark cues. It immediately reentrained on the shift back. The periodogram showed how well the bird was entrained and its free running period. In birds that are well entrained the peak correlation will be in 24 hour, which is exactly where this bird's peak is.

MRI

Each segment for volume is color-coded. The cerebellum is orange. The hippocampus is light blue. The ventricles are green and the hypothalamus is purple. The whole brain is pink, but is not shown anywhere but in Figure 14 due to clarity issues for all the other MRI images. All MRI images, unless specified, were taken from the young bird, red band's segmentation.

Figure 12: Complete Brain Segmentation

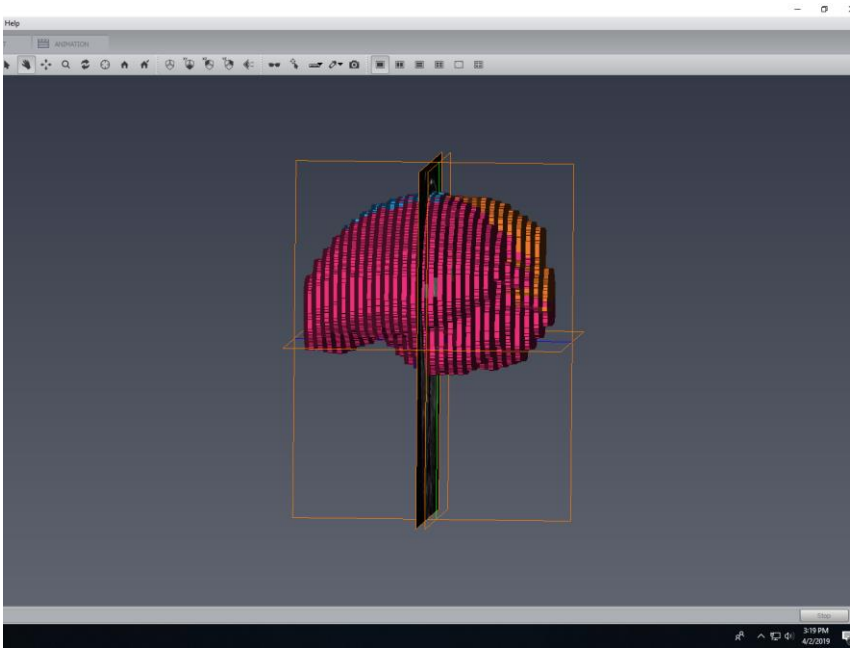


Figure 12 demonstrates the overall shape of the avian brain and the various how the various sections fit together.

Figure 13: Segmentation, Anterior View

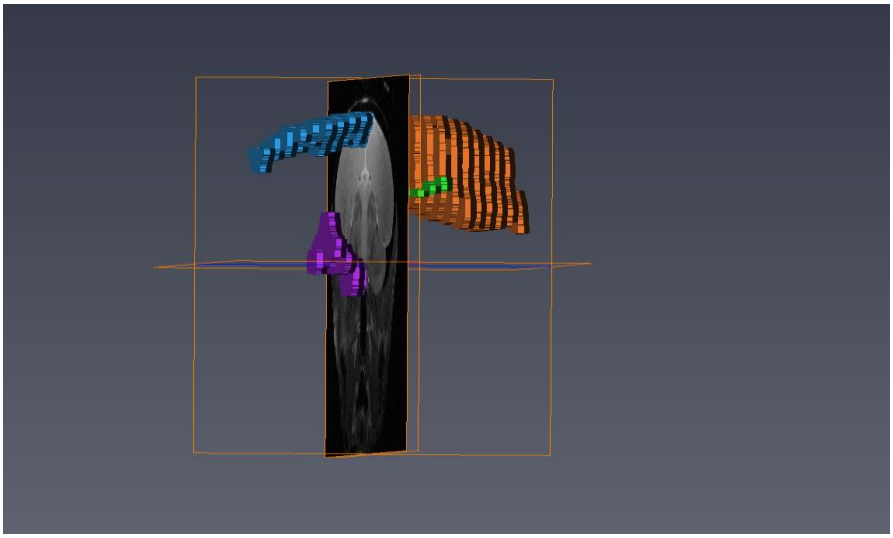


Figure 13 is the hippocampus and hypothalamus and cerebellum from an anterior focused view of brain.

Figure 14: Segmentation Posterior View

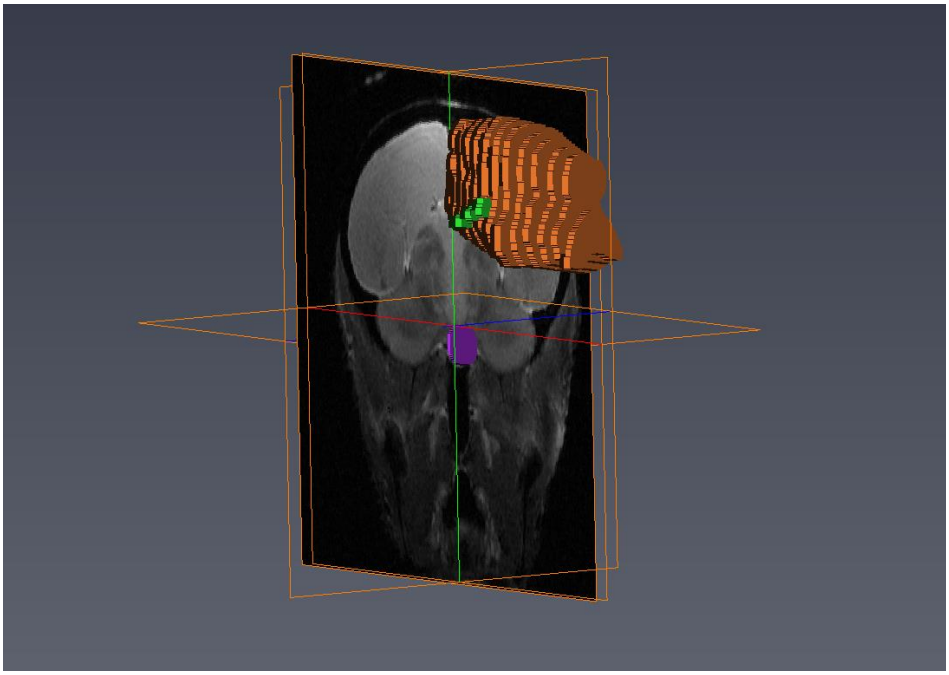


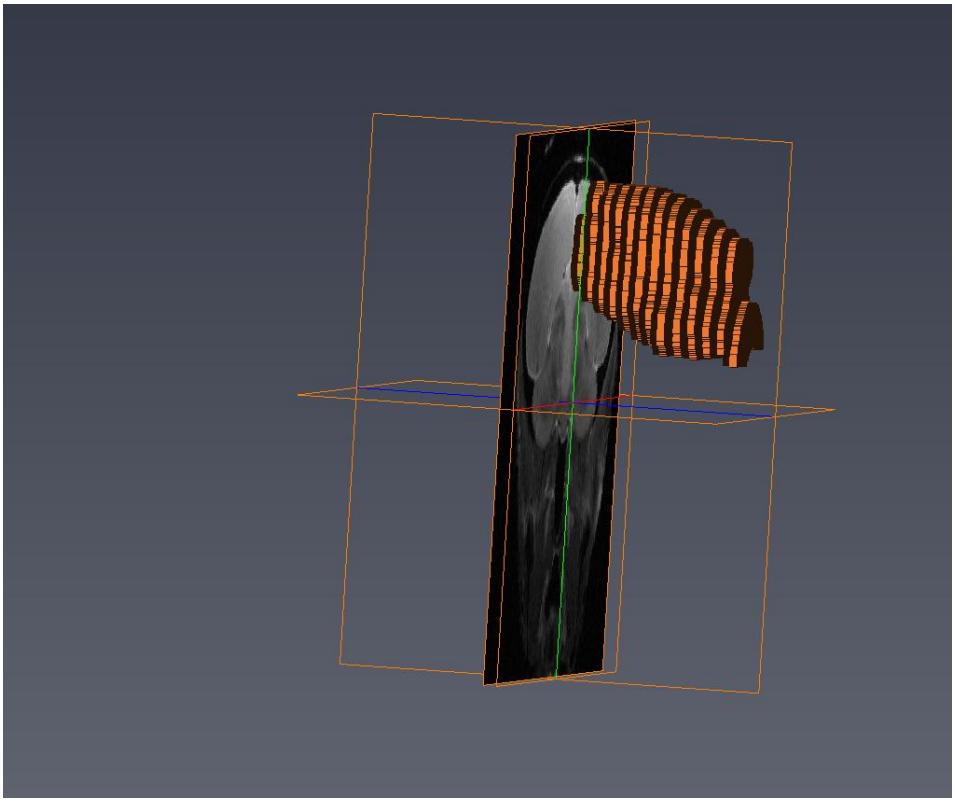
Figure 14 gives a view of the whole brain from a posterior view to demonstrate the position of the ventricles and cerebellum and hypothalamus.

Table 7: Total Brain Volume

Birds	Total Brain Volume	P Value for Old vs Young
Young, No Band	8.90E+11	0.193606773
Young Silver	9.10E+11	
Young Red	8.88E+11	
Average Volume Young	8.96E+11	
Old 2	8.37E+11	
Old 5	8.75E+11	
Old 7	8.92E+11	
Average Volume Old	8.68E+11	

Table 7 represents the total volume of the avian brains. It is all the segments and the whole brain segment added together and then averaged and compared across the age groups to give the resulting p-value of 0.194. This value is not significant.

Figure 15: Cerebellum Segmentation



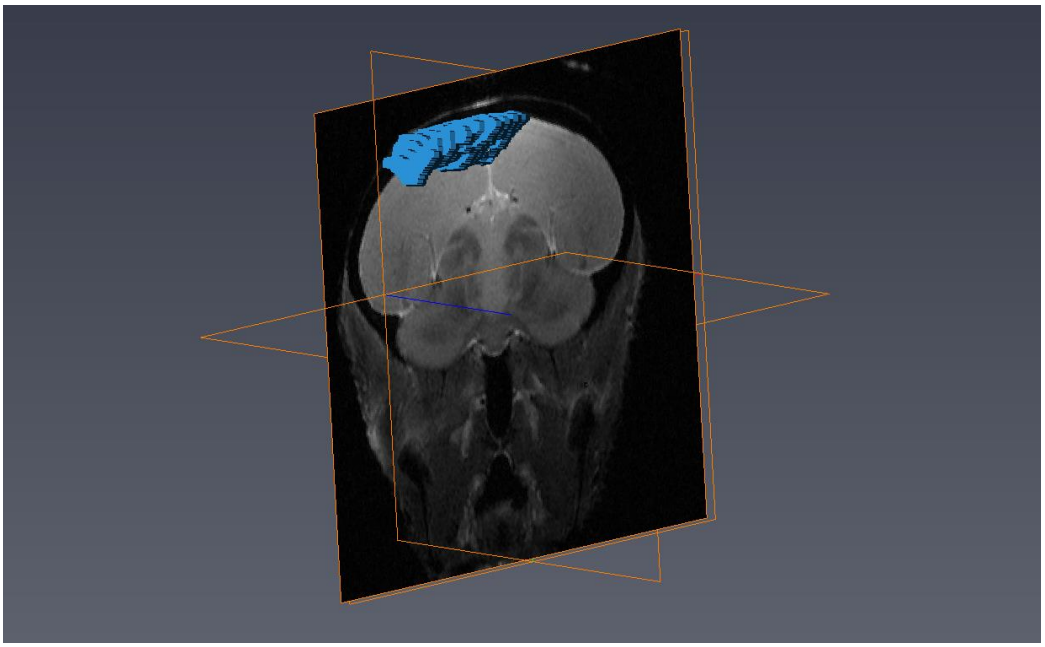
The cerebellum is pictured in orange. It sits posterior to the cerebrum and superior to the thalamus and midbrain.

Table 8: Cerebellum Volume

Birds	Cerebellar Volume	P Value for Old vs Young
Young, No Band	7.92E+10	0.460830594
Young Silver	6.46E+10	
Young Red	8.71E+10	
Average Volume Young	7.69E+10	
Old 2	8.23E+10	
Old 5	9.96E+10	
Old 7	7.36E+10	
Average Volume Old	8.52E+10	

Table 8 represents the volume of the avian cerebellum. It is all the cerebellum segmentation for each age group averaged. The resulting means are compared across the age groups to give the resulting p-value of 0.461. This value is not significant.

Figure 16: Hippocampus Segmentation



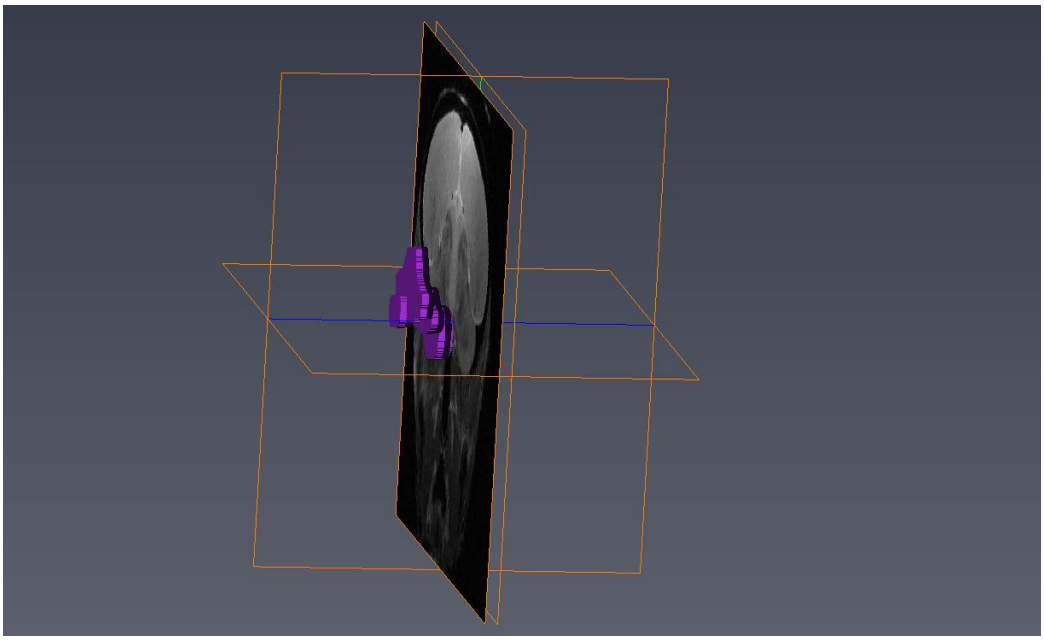
The hippocampus here is shown in blue. In avian species the hippocampus is a v shaped structure.

Table 9: Hippocampus Volume

Birds	Hippocampal Volume	P Value for Old vs Young
Young, No Band	2.75E+10	0.403530427
Young Silver	2.09E+10	
Young Red	1.79E+10	
Average Volume Young	2.21E+10	
Old 2	1.59E+10	
Old 5	1.87E+10	
Old 7	2.23E+10	
Average Volume Old	1.90E+10	

Table 9 represents the volume of the avian hippocampus. It is the hypothalamus segmentation for each age group averaged. The resulting means are compared across the age groups to give the resulting p-value of 0.404. This value is not significant.

Figure 17: Hypothalamus Segmentation



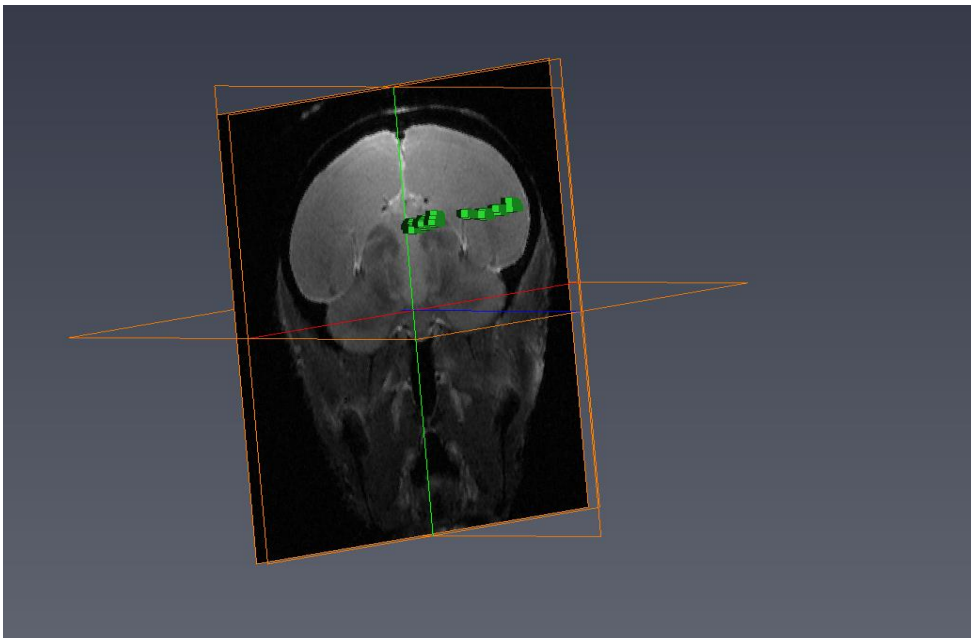
The hypothalamus is pictured in purple. It was the most morphological diverse of the brain structures due to the changes in appearance due to the angle of the sparrow's head in the MRI.

Table 10: Hypothalamus Volume

Birds	Hypothalamic Volume	P Value for Old vs Young
Young, No Band	9.11E+09	0.050936211
Young Silver	1.03E+10	
Young Red	7.80E+09	
Average Volume Young	9.08E+09	
Old 2	4.22E+09	
Old 5	6.21E+09	
Old 7	7.26E+09	
Average Volume Old	5.90E+09	

Table 10 represents the volume of the avian hypothalamus. It is the hypothalamus segmentation for each age group averaged. The resulting means are compared across the age groups to give the resulting p-value of 0.051. This value is significant.

Figure 18: Ventricle Segmentation



The ventricles are shown in green. Ventricles serve to channel cerebrospinal fluid around the brain there are 4 sets total. However, the ventricles were not consistently visible and for this reason only the fourth ventricle was used as a comparison point.

Table 11: Ventricle Volume

Birds	Ventricular Volume	P Value for Old vs Young
Young, No Band	9.58E+08	0.614929466
Young Silver	1.45E+09	
Young Red	3.89E+08	
Average Volume Young	9.33E+08	
Old 2	3.46E+09	
Old 5	3.60E+08	
Old 7	6.68E+08	
Average Volume Old	1.49E+09	

Table 11 represents the volume of the avian hippocampus. It is the ventricle segmentation for each age group averaged. The resulting means are compared across the age groups to give the resulting p-value of 0.614. This value is not significant.

Figure 19: Younger Ventricle Segmentation

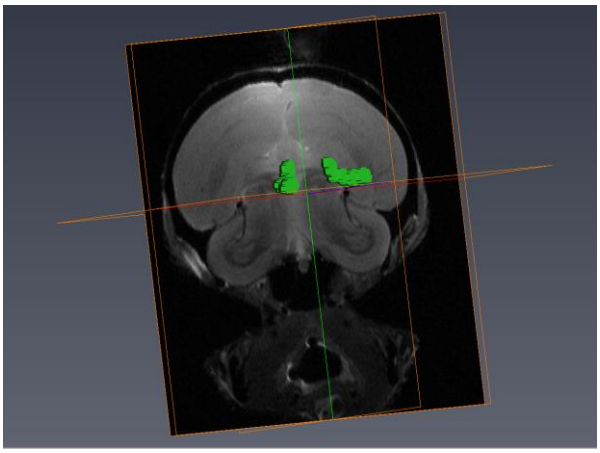
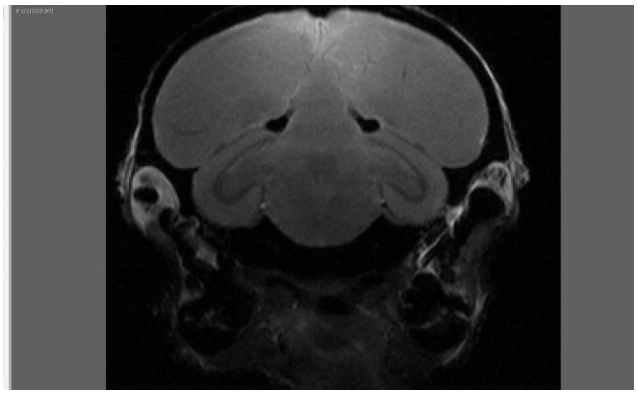


Figure 20: Younger Ventricle



Figures 19 and 20 represent the ventricles of young bird, silver banded. Figure 19 is the segmentation and 20 is a still from an MRI slice. They are provided to give visual contrast of the ventricles between young and old birds.

Figure 21: Older Ventricle Segmentation

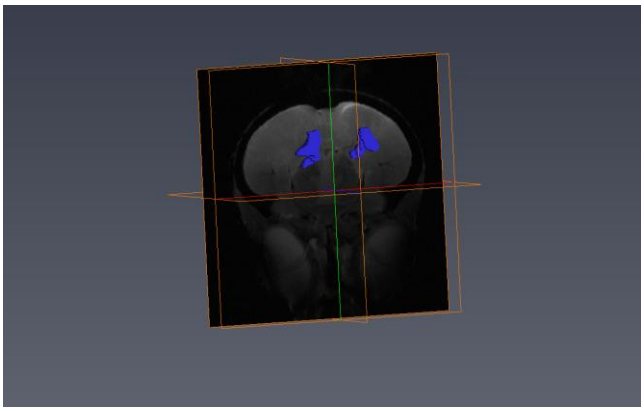
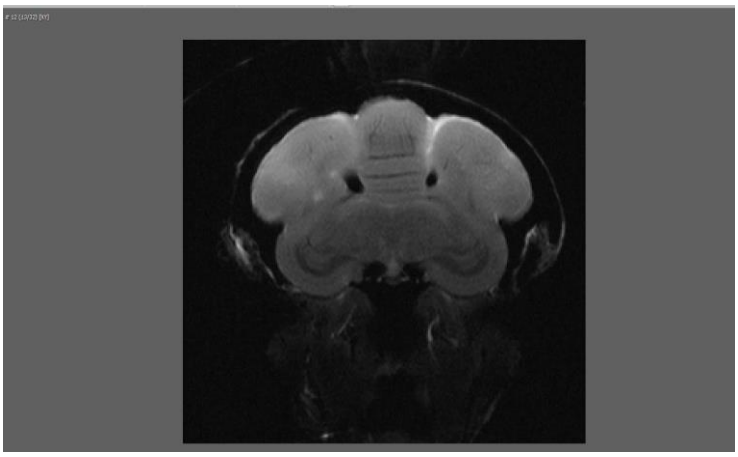


Figure 22: Older Ventricle



Figures 21 and 22 represent the ventricles of old bird 2. Figure 21 is the segmentation and 22 is a still from an MRI slice. They are provided to give visual contrast of the ventricles between young and old birds. Figure 22 shows how the ventricles appear more expansive to the naked eye than Figure 20.

Figure 23: Older Bird Hyperintensities

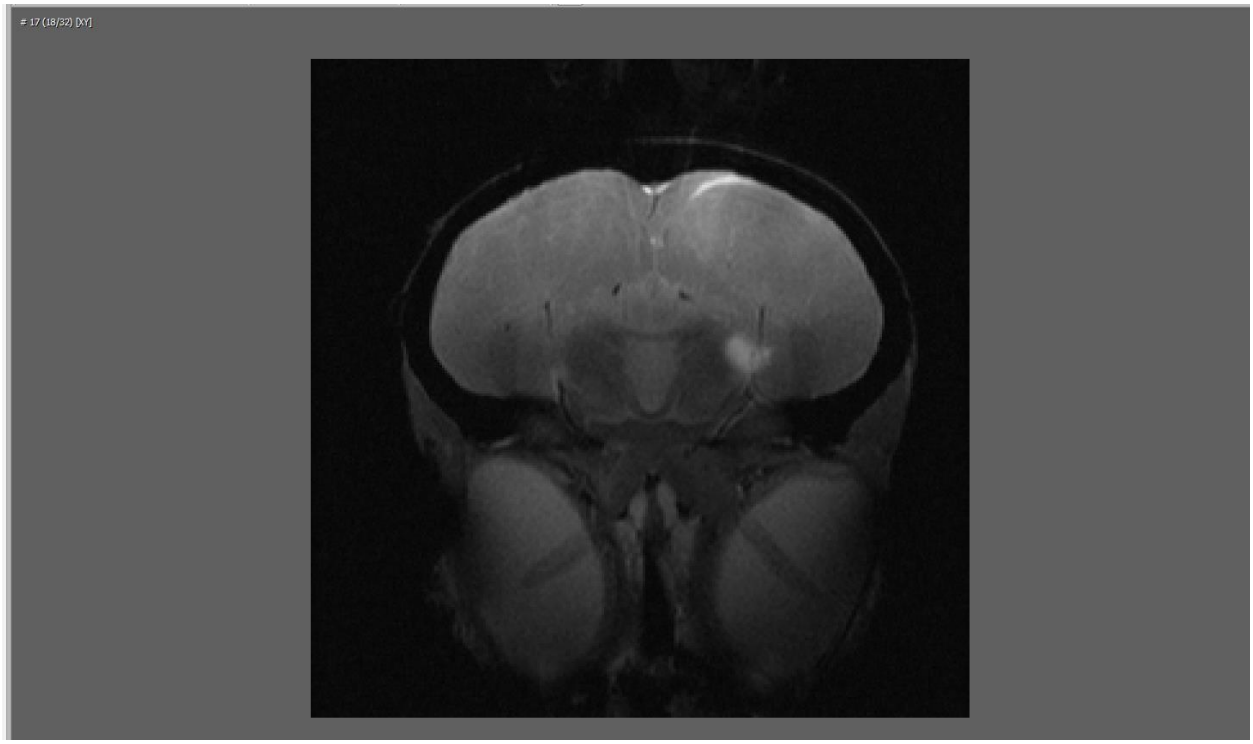


Figure 25 demonstrates the hyperintensities seen only in the older brains. The first hyperintensity is inferior to the hippocampus. The second is superior to the eyes and to the anatomical left of the hypothalamus.

Discussion

This set of experiments encountered several issues along the way. The original intent of the project was to examine the circadian regulation of locomotor activity and also examine melatonin levels in the blood at different ZT times. The melatonin levels would be compared to previous samples collected from the same set of sparrows when they were first caught. A -80 freezer broke before the samples could be processed and a decision was made to not collect blood samples from the birds again. This decision was due to a fear that the birds would not survive the bleedings again due to their age and poor response to bleedings overall. MRI was chosen to replace this original data set. The MRI process was not smooth and took much longer than expected, as bird MRI has not been performed frequently and, as mentioned in the MRI section, various physical features of the bird anatomy need to be overcome in order to get a usable signal. Also due to death of many of the geriatric birds, only three birds from each age group were compared. The older birds died due to old age or were euthanized before the MRI for humane reasons.

The MRI was more of a trial and error process than we would have liked; therefore the six MRI segmented were the most usable of the MRIs. The first MRIs were unusable due to birds escaping the holder. The change in slice numbers was due to the fact that as we did the MRI we found we got much higher resolution on those images. The one bird (bird_nothing) was placed incorrectly in the machine and appeared crooked in the MRI segmentation. There were white spots visible in all of the older birds. This is likely do to the deposition of a magnetically sensitive material in the brain, similar to plaque buildup or after cerebral strokes.

The MRI segmentation is novel in avian species. MRI imaging of birds has been done infrequently, so part of the impetus for this section was to gather information on which MRI sequences that could be used. The modifications to increase the resolution of the RARE scans improved the signal to noise ratio. Something that we could not explain was the fact the ventricles were dark when in the T2 scan, as they are appear bright in typical scans of mammalian brains. However there are many differences between avian and mammalian tissues and these differences are reflected at times in the MR images. For example, we did try varying the T2 relaxation times, to determine the stiffness of the brain tissue, however the slope that was derived was much different than would be expected. One interesting result was the lack of difference in size (see table 11) between the older and younger birds ventricles. The older birds ventricles appeared larger on viewing in the MRI, however, the difference is volume was not statistically significant. This result is probably due the fact that the ventricular volumes were averaged and only one of the birds, Old 5, had visibly much larger ventricles. We also did not add in the other ventricles besides the fourth because of inconsistency in visibility across the birds. The inability to see the ventricles however implies that the bird was alive during the scan since they tend to become diffuse after death. This may contribute the their lack of difference in volume as well since the other ventricles were more consistently visible in the older birds. We did not really expect any major differences in the volumes of other brain areas. It makes sense the total brain volumes were overall the same because the older birds were still overall functional and significant volume changes would be not allow this. The hippocampus appeared morphologically similar across the cohorts, as did the cerebellum. The difference in hypothalamic volume is exciting because that is the area of the brain containing the SCN and therefore the area of most interest in light of the circadian portion of the study. If the old birds actually have a significant difference in their volumes then that shows that the

hypothalamus decreased with aging and would be the major explanation of their actograms since it produced abnormalities similar to SCN lesions. This finding does come with some caveats, as there are only 32 slices for most brains, and the angle of orientation can greatly affect the visibility of the hypothalamus. The most obvious way to improve this set of experiments is to have more birds in both groups and to have a consistent angle of the head to allow more consistencies between the slices for the various birds.

The actogram data were obviously very interesting. The birds showed a wide variety of circadian rhythm. Five was likely arrhythmic due to damage to the SCN's neurons or damage to the SCN's tissue that naturally occurs with aging. Arrhythmicity has been previously reported in aged animals (Stat et al. 1993). The fact that bird 2 the nocturnal bird expanded its alpha shows that the lights were in fact working in the room. All the other birds maintained the previous lights off, which did not line up with the zeitgeber of the four hours later lights off. The compression of alpha is interesting because it is not compression of the free running period, as seen from Figure 11. While previous studies of mammals found a compression of the free running period altogether (Zee, Rosenberg & Turek, 1992) and (Chang & Guarente, 2013). Most bird's phase angle of entrainment did not really vary between the lights on and off. The fact that the lights on cue still entrained the birds implies that the circadian system still had some ability to respond to zeitgebers. What we propose is happening is a modified version of the Takahashi 1982 paper with the pinealectomized birds. The oscillators we are proposing are shown below in Figure 24.

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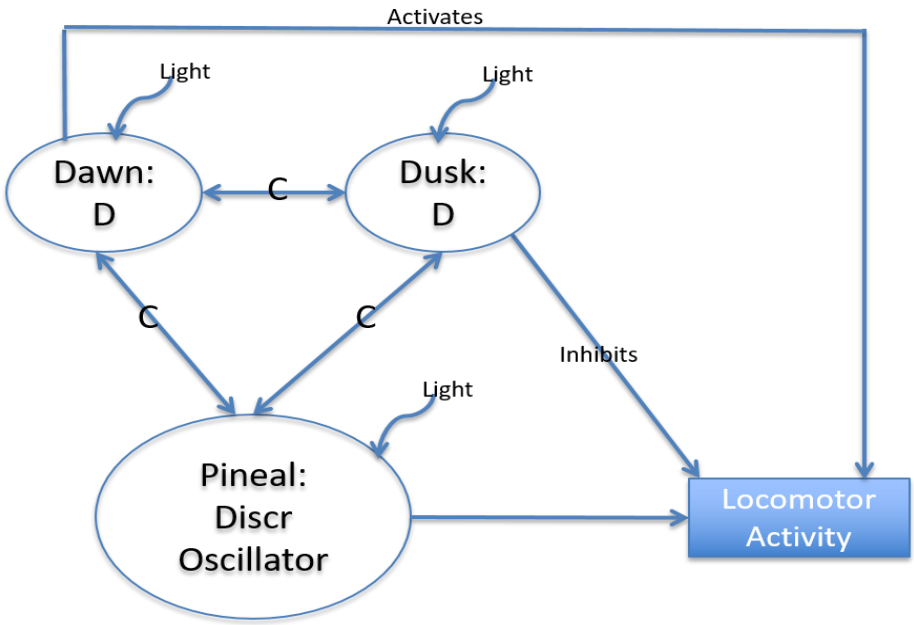
Figure 24: Proposed Oscillators



There are two separate populations of coupled, dampened oscillators: one for the morning lights on, Dawn, and one for the evening lights off, Dusk. Dawn and Dusk function through separate pathways with their own inputs. Dawn is activating of locomotor activity and Dusk is inhibitory. They are most likely located in the SCN. The pineal gland regulates them the according to the neuroendocrine loop of the SCN and is its own discrete oscillator. C signifies that the oscillators are coupled and D refers to Dawn and Dusk being damped.

We hypothesized that the dusk oscillator likely got uncoupled and is no longer responsive to circadian cues about synchrony with the dawn oscillator, in addition to having its own inputs about the timing of the new "lights off" to be abolished or weakened. That would explain the lack of response to circadian cues but only during the lights off. Dusk in our experiment, in addition to becoming uncoupled, seems to "remember" the previous light cycle instead of correctly responding the changing light cues. After effects are when the circadian system displays a memory of the previous zeitgebers before entraining to the new zeitgebers. The birds displayed something similar to an after-effect, but the fact it is so extended implies that there may be more going on. We are proposing that cTL is what is responsible for the bird's behavior and is what is controlling dusk. The bird remembers that the lights off at 12ZT are important and retains the behavior. Since the hippocampus appears well preserved it is likely that cTL could remain intact and take the place

of regular circadian function when the circadian system goes. As found in Mulder, Gerkema, & Van Der Zee (2013), animals can switch their learning systems, such as the elderly mouse switch to ordinal learning. Similar to this switch, the aged birds could have switched from the normal circadian expression to cTL, as the ability for normal circadian expression was lost due to age related pathologies. This hypothesis is strengthened by the fact that there is possibly a circadian oscillator located in the hippocampus (Mulder, Gerkema, & Van Der Zee, 2013), it could take over with cTL in response to the decay of the hypothalamus and the main circadian oscillators within it. The hippocampus showed no differences between the younger and older birds and is therefore likely less affected by aging.

With the decreased volume of the hypothalamus there is likely decay of the SCN as well, including the area of the dusk oscillator. In mammals there are defined topographical regions dedicated to dawn and dusk entrainment (Yoshii, Rieger & Helfrich-Förster, 2012), perhaps this is the situation in birds as well. Indeed, birds do possess the vSCN and mSCN subgrouping of the SCN, which receive information from different photosensitive structures, with the vSCN receiving input from the external eye's photoreceptor and the mSCN receiving input from the deep brain photoreceptors (Cantwell & Cassone, 2006). It is possible that this differential input could be driving the differences that were observed. The SCN, as previous studies have shown (Stat et al. 1993) and (Garau, Aparicio, Rial, Nicolau & Esteban, 2006), has neuronal and spatial decay, so the lower volumes could be a result of both. The fact that most of the birds show this rhythm is also very unique. It might imply that this happens in many geriatric birds and that as oscillators are lost due to age decay the brain has other mechanisms to maintain circadian behavior that it employs. The ability to generate cTL is believed to be a likely candidate since it is located in another part of the brain and, in birds, is another circadian mechanism.

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EDUCATION

The Pennsylvania State University — **University Park, PA**
Schreyer Honors College — Graduation May 2019
Bachelor of Science in Veterinary and Biomedical Science
Minor in Spanish

Puebla Study Abroad Program — **Universidad De Ibero, MX**
Grades Received: A
Summer 2017

- Immersed in Mexican culture and language for 6 weeks with a host family
- Attended courses in Mexican history, culture, and arts all taught in Spanish

Large Animal Veterinary Practices in the Tropics and Wildlife Health Ecology, and Conservation — **CELA Belize, Belize**
Grades Received: A
Summer 2016

- Administered vaccines to cattle, sheep, goats, pigs, and domestic pets
- Performed castrations and obtained blood from pigs, horses, and sheep
- Attended lab courses, lectures, and field research with respective-field experts
- Assisted in the Belize Zoo in their daily activities with all animals and medical care of jaguars, tapirs, birds, and ocelots

VETERINARY EXPERIENCE

Dr. Paul Bartell's Lab, Senior Undergraduate Researcher, University Park, PA — **Spring 2016 – Present**

- Run a circadian aging study in >10 year old white throated sparrows
- Obtain and process blood samples
- Compile and analyze actographs from a self-built monitoring system
- Monitor and maintain health of 50 birds and train new researchers
- Build a meal-worm colony for food supply

The National Aviary, Hospital Intern, Pittsburgh, PA — **Summer 2018**

- Patient care for teaching hospital residents involving a wide range of species from passerines to red-legged seriemas.
- Observing and assisting veterinary staff with medical procedures including sterile wound care, leg deformity care, microscopy, necropsies, and preventative medical care such as nebulizations.
- Independent study and medical rounds with veterinary staff about cases, behavior, anatomy and protocols including pharmacy, treatments/procedures, lab and surgery

The Bridge Spay and Neuter Clinic, Veterinary Assistant, Trevose, PA — **Summer 2015- Present**

- Assist veterinarians in spay and neuter surgeries
- Monitor anesthesia
- Administer vaccines and obtain blood samples

Buckingham Animal Hospital, Volunteer, Buckingham, PA — **2015- Present**

- Shadowed Dr. Kuhn in daily activities in a small animal clinic

RELEVANT SKILLS

Advanced Spanish, Blood Drawing, Slide Preparation and Microscopy for feces, blood, saliva, and SNAP tests

EXTRACURRICULARS

Pre-Vet Club, Membership Chair, University Park, PA — **Fall 2016-Present**

- Membership Chair, Manage the list-serv for the club, coordinate effective club-wide communication, and record keep for points and events
- Stayover Committee Co-Chair, Prepare merchandise and assist in coordinating the stayover event
- Homecoming Chair, Manage and Coordinate the homecoming committee and parade display
- One Health Committee Member, Work to unite human, animal, and environmental health and form a one health branch at the University Park campus

The GLOBE, Vice President, University Park, PA — **Fall 2015 – Fall 2017**

- Manage event planning for community of 70 students
- Coordinate with external groups to plan globally-minded events and facilitate international discussion

Bucks Clucks 4H Poultry Club, Vice President, Bucks County, PA — **2008-2016**

- Kept detailed records of club activities and coordinated community outreach
- Taught mixed age groups about poultry as part of extension programs