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**NOCTURNAL BIRD MIGRATION AND DISRUPTED SLEEP/WAKE CYCLE**

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# NOCTURNAL BIRD MIGRATION AND DISRUPTED SLEEP/WAKE CYCLE

by

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**NOCTURNAL BIRD MIGRATION AND DISRUPTED SLEEP/WAKE CYCLE**

**Kristan Gail Singletary, Ph.D.**

**The University of Texas at Austin, 2009**

**Supervisor: Yvon Delville**

In most birds, changing photoperiods from winter to spring and from summer to fall have two consequences: increased feeding followed by migratory activity. To date, the neural system controlling the activation of migratory activity remains unknown, though behavioral observations point to a possible mechanism. During the migration season, diurnal songbirds show extensive disruption of their sleep/wake cycle, sleeping during the day and flying at night. In mammals, similarly altered cycles of activity result from blocking orexin expression in the hypothalamus. It is possible that decreased orexin expression is associated with migratory activity in songbirds. In addition, changes in ingestive behaviors and fuel availability may also be associated with disruptions in the sleep/wake cycles of migratory birds. The studies in my dissertation will address these issues through three main specific aims. First, I will determine that orexin systems are conserved in vertebrate brains. Second, I will test the association between orexin and migratory activity in songbirds. Third, I will confirm the association between fuel availability, orexin expression and migratory activity in songbirds.

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# Chapter 1: Introduction

In most birds, changing photoperiods from winter to spring and from summer to fall have two consequences: increased feeding followed by migratory activity. To date, the neural system controlling the activation of migratory activity remains unknown, though behavioral observations point to a possible mechanism. During the migration season, diurnal songbirds show extensive disruption of their sleep/wake cycle, sleeping during the day and flying at night. In mammals, similarly altered cycles of activity result from blocking orexin expression in the hypothalamus. It is possible that decreased orexin expression is associated with migratory activity in songbirds. In addition, changes in ingestive behaviors and fuel availability may also be associated with disruptions in the sleep/wake cycles of migratory birds. The studies in my dissertation will address these issues through three main specific aims. First, I will determine that orexin systems are conserved in vertebrate brains. Second, I will test the association between orexin and migratory activity in songbirds. Third, I will confirm the association between fuel availability, orexin expression and migratory activity in songbirds.

## BACKGROUND

### *1.1 Migratory Behavior*

Changing photoperiods control migratory activity in birds. Transfer from short day- to long day photoperiods induces migratory activity in captive songbirds [Rowan 1925; Farner 1950]. This behavior is referred to as migratory restlessness or Zugunruhe

and is characterized by nocturnal activity including flights, hops, and wing whirring (i.e., rapid wing beating while perching or on the side of the cage) [Helms 1963, King and Farner 1963]. Extensive field studies have determined the migratory flyways, flight distances and speeds of migrating birds. When the photoperiod changes, migratory birds follow routes back north to their breeding grounds for the summer, or back to their wintering grounds down south. For example, depending on the species, the birds might use the Atlantic Coast Route or the Great Plains-Rocky Mountain routes. Birds may fly short distances or long distances with short bouts. Some fly long distances in long bouts depending on the route and availability of refueling resources.

Birds use a combination of different environmental cues to orient themselves when migrating. Birds that migrate during the night can't use the sun to navigate, so researchers determined if they used the stars. Nocturnal migrants were placed in a special cage designed to record the birds' movements [Emlen and Emlen 1966]. The cages contained an inkblot on the bottom and blotting paper on the sides shaped in a funnel with a wire top the birds could see through. The cages were positioned under a planetarium sky oriented to mimic the natural sky. Once these birds exhibited Zugunruhe, the birds marked the blotting paper in the direction they were trying to navigate. When given no light cues the birds moved in a random orientation. This showed that these birds do use starlight as one method to navigate.

Diurnal migrants navigate using the sun as well as magnetic fields and landmarks to guide the way. Whether diurnal or nocturnal migrants, photoperiod is the ultimate environmental cue that triggers migration. There is little research however, on the neural

mechanisms that control migration. In order to investigate these mechanisms, it is important to consider the behaviors that are involved in migration. The two main behaviors that occur upon photoperiod change are increased feeding with a change in dietary preference for high protein, high fat food and ultimately increased activity at night (Zugunruhe).

Along with the increase in food intake there is a dramatic increase in body mass. The amount of weight gained and latency to display Zugunruhe varies depending on the species of passerine and length of migration [Ramenofsky *et al.* 2003; Richardson *et al.* 1995].

Zugunruhe can be quantified by video analysis. There are several methods to measure this behavior but video allows us the most thorough investigation as determined in previous studies [Rattenborg *et al*. 2004]. In addition, nighttime video is possible with infrared illumination, which the birds do not see. Daytime activity including ingestive behaviors and nighttime activity including sleep postures will be recorded.

### *1.2 Sleep Behavior*

In diurnal songbirds, Zugunruhe is also associated with an extensive disruption of the sleep/wake cycle. In the Swainson's thrush, photoperiod-induced migratory restlessness includes increased drowsiness, unilateral eye closure, as well as very brief sleep episodes during the day [Fuchs *et al.* 2005]. In the white-crowned sparrow (WCS), migratory restlessness is also associated with increased daytime drowsiness [Rattenborg *et al.* 2004]. During the night, the migrating white-crowned sparrow has shorter periods

of sleep, extended periods of activity and a shorter latency of REM activity at nightfall [Rattenborg *et al.* 2004]. Together, these observations are reminiscent of sleep disorders, such as narcolepsy, and suggest that migratory activity in songbirds is associated with a disruption of the neural systems controlling the sleep/wake cycle.

### *1.3 Neuroendocrine Systems*

Studies have looked at the correlation between migratory behaviors and hormone or neuropeptide expression. When exposed to long days, nocturnal migrants start to show elevated corticosterone before and in the early hours of Zugunruhe [Landys *et al,* 2004]. High levels have been found in migrating willow tits as opposed to territorial willow tits [Silverin *et al*. 1989]. The Migration Modulation Hypothesis [Holberton 1999] explains that increased corticosterone facilitates fattening by increasing glucose production and having a permissive effect on feeding. Also, skeletal muscle is protected from catabolism during migration due to a reduced corticosterone stress response. Though these studies have shown that corticosterone is involved in metabolism, there is no data to date of any effect on Zugunruhe. Blocking the low affinity glucocorticoid (GC) receptor does not affect migratory activity but does decrease feeding [Landys *et al.* 2004]. Additionally, fasting increases corticosterone and daytime activity but not nighttime activity.

A second hormone, melatonin was also studied in relation to migrations. Melatonin is mainly synthesized in the pineal gland in response to input from the retina and SCN. In birds, encephalic photoreceptors are also found in the lateral septal organ

and pineal gland [Silver *et al*. 1988; Kuenzel *et al.* 1997; Gwinner and Brandstatter 2001]. Peak melatonin levels occur during the night in diurnal and nocturnal animals with duration of the peak dependent on the length of the photoperiod. Interestingly, melatonin levels decrease in amplitude by 30% in the night in birds experiencing migratory restlessness [Gwinner and Brandstatter 2001]. Melatonin is correlated with migratory activity, as suppression of Zugunruhe by refueling after fasting is associated with increasing melatonin levels up to short photoperiod (SD) levels [Fusani and Gwinner 2003]. However, there has been no data to show that melatonin affects migratory activity, as a bird will entrain to other zeitgebers such as food availability in the absence of melatonin or melatonin rhythmicity [Gwinner and Brandstatter 2001].

Other hormones have been studied. Testosterone does not differ between migrants and nonmigrant willow tits. Testosterone does not prevent autumn migration, and high levels (no difference) are found in males and females that are migrating or nonmigrating [Silverin *et al*. 1989]. In addition, while growth hormone is higher in migrating juveniles than nonmigrating juveniles, this difference is associated with metabolism, not Zugunruhe [Silverin *et al*. 1989]. Moreover, sensitivity to Neuropeptide Y (NPY) increases in WCS in long photoperiod (LD), resulting in increased food intake with injections of NPY, but the study did not look at migratory activity [Richardson *et al*. 1995]. Finally, T3 and T4 levels have been shown to increase in the premigratory fattening stage, but decrease by migration in the WCS and Rosy pastor [Pathak and Chandola 1984; Wingfield *et al*. 1996]. Unfortunately, once again no direct effect on migratory activity was tested with thyroid hormones.

These studies have shown a correlation between hormones, metabolism and feeding but have not proposed a neural mechanism how these candidates affect migratory restlessness. In these studies, I am introducing not simply another candidate, but a logical neural mechanism underlying the onset of migratory activity in birds.

### *1.4 Orexin*

The neural systems controlling the sleep/wake cycle are much better understood in mammals than in birds. In mammals, the sleep/wake cycle is stabilized by a key neuropeptide, orexin [Saper *et al*. 2005]. Reduced orexin expression in the brain of mice, rats or humans is associated with symptoms of narcolepsy, such as extended drowsiness and occasional periods of sleep during the day as well as enhanced arousal and short onset of REM sleep at night [Saper *et al*. 2005; Peyron *et al*. 2000; Thannickal *et al.* 2000]. There are two forms of orexin, orexin A and orexin B and two orexin receptors in mammals. Orexin receptor 1 has a higher affinity for orexin A and orexin receptor 2 binds both A and B equally [Sakurai *et al*. 1998]. The role of orexin on the sleep-wake cycle appears to be mediated through orexin-2 receptors, as indicated by studies with electrophysiological thalamic recordings, receptor-saporin lesions, deletion mutations and rescue studies [Lin *et al*. 1999; Willie *et al*. 2001, 2003; Bayer *et al.* 2002; Fujiki *et al*. 2003; Gerashchenko *et al.* 2001]. As such, these studies on the role of orexin on the sleep/wake cycle may be relevant to migrating songbirds, though it is unclear whether orexin B modulates activity cycles in birds. However, it is noteworthy that the neural distribution of orexin B innervation is remarkably similar between songbirds and

mammals [Singletary *et al*. 2006]. In addition, mammalian orexin-2 receptors are the most homologous to the only subtype of orexin receptor (cOXR) found in birds [Ohkubo *et al.* 2003].

We propose that migratory activity in songbirds is associated with a loss of orexin innervation within specific areas of the brain implicated in the sleep/wake cycle of birds. This hypothesis will be discussed in Chapter 3. However, it will be necessary to confirm that orexin systems (neuronal distribution, axonal projections) are comparable between vertebrates, namely mammals and birds. This hypothesis will be discussed in Chapter 2.

### *1.5 The Importance of Diet*

In nocturnally migrating birds, photoperiod change is the environmental cue that signals the beginning of physiological and behavioral changes. These changes include diet preference change, hyperphagia and an increase in body weight and fat mass. The disruption of the sleep/wake cycle ensues with the onset of migratory flight. Orexin has been studied in mammals as involved with feeding and the sleep/wake cycle, thus it is proposed that the orexin system of birds regulates nocturnal migration.

Both feeding and activity are behaviors that change during the premigratory and migratory substages. Migratory birds increase feeding and fatten after a change in photoperiod in order to prepare for migration. Most birds reach a weight or fat mass that signals the onset of migration (King and Farner 1963). If birds are not allowed to increase fat mass and body weight, they may not receive the signal to start or maintain migrating. Birds that are already migrating need to refuel during the day and are hyperphagic when

presented with resources. As the photoperiod changes, birds prefer more nutritious food that will ultimately increase body mass. If birds are not given enough nutritious food to replace stores depleted by migrating, the birds may suppress migration until enough stores or a signal permits them to resume activity. These signals differ depending on the species of migrating bird as migratory patterns and physiological adaptations would determine metabolic needs. I predict that as animals transferred from short to long photoperiods start eating and gaining weight, the orexin system is inhibited, thus causing changes in the sleep/wake cycle in these birds. Additionally, disruption of the maintenance of the migratory behavior would result from a reduction in these signals similar to what is seen during a refueling stopover in a short bout migrant. This possibility was tested and discussed in Chapter 5.

# Chapter 2: Comparative distribution of orexin immunoreactivity in the brain of vertebrates


## ABSTRACT

It is hypothesized that orexin is highly conserved across species and has similar distribution. This is important for us to establish before using a mammalian orexin antibody to describe and compare avian functional neuroanatomy, as avian brains differ from those of mammals and other vertebrates. This hypothesis will be tested by immunohistochemistry to orexin A and B in golden hamster, green tree frog, and house finch and compared with previous fish and reptile studies.


## 2.1 INTRODUCTION

The orexins (hypocretins) are neuropeptides in the incretin peptide superfamily [Alvarez and Sutcliffe 2002] that regulate many processes in mammals. Studies have shown orexin to regulate feeding, stress responsivity, reproduction, and nociception [de Lecea *et al*. 1998; Pu *et al*. 1998; Sakurai *et al*. 1998; van den Pol 1999; Dube *et al.* 1999; Ida *et al*. 2000; Kuru *et al*. 2001; Bingham *et al*. 2001; Archer *et al*. 2002; Karteris *et al.* 2005; Muschamp *et al.* 2007]. In recent years, the primary interest in the orexins has grown given their involvement in mammalian sleep-wake behavior. The sleep/wake cycle is stabilized by a key neuropeptide, orexin [Saper *et al.* 2001]. Reduced orexin expression in mammalian brains is associated with severe disturbances to their sleep-wake cycle including symptoms of narcolepsy, such as extended drowsiness and

occasional periods of sleep during the day, as well as enhanced arousal and short onset of REM sleep at night [Chemelli *et al.* 1999; Lin *et al.* 1999; Peyron *et al.* 2000; Thannickal *et al.* 2000; Downs *et al.* 2007].

There are two forms of orexin as described in Chapter one. Orexin A and orexin B are cleaved separately [de Lecea *et al*. 1998; Sakurai *et al.* 1998] from a single precursor prepro-orexin. Orexin A and B amino acid sequences have been characterized in fish, amphibians, birds and mammals and are highly conserved among these vertebrates [Sakurai *et al*. 1998, Shibahara *et al.* 1999; Alvarez and Sutcliffe 2002; Ohkubo *et al.* 2002; Kaslin *et al.* 2004]. Orexin A is identical in human, rat, mouse, and pig. Orexin B in mice and rats differs from human orexin B by only two amino acids [Sakurai *et al*. 1998]. It is been shown that chicken orexin A and B are 85% and 65% homologous to human orexin A and B, respectively [Ohkubo *et al.* 2002]. In *Xenopus laevis* prepro-orexins are 56% homologous to the human sequence [Shibahara *et al.* 1999]. In zebrafish orexin A and B are 32% and 50% homologous to human orexins A and B [Kaslin *et al.* 2004].

If this conservation also extends to neuroanatomy, perhaps the function of the orexin system is also similar. The orexin system has been extensively studied and is involved in many processes in mammals. However, its role in other vertebrates is unclear. In an effort to confirm the conservation of the distribution of orexin we examined and compared orexin immunoreactive (-ir) cell bodies and fiber distributions among various vertebrates. This includes an examination of a reptile (*Anolis carolinensis*), which has previously not been described in detail. Also, orexin distribution in various closely

related passerines is described. And finally, research investigating orexin receptors in non-mammalian vertebrates is scarce but it is important to begin to consider the conservation and distribution of these receptors in the brain. Using this information we can gain a better understanding of the possible functions of the orexin system across evolution. As such, this short commentary will provide a comparative overview of the distribution of orexin cells and fibers across the brains of vertebrates with reference to function.

## 2.2 METHODS AND MATERIALS

### *2.2.1 Animal Care*

Syrian Hamsters (*Microcetus auratus*): Adult male (*n*=8) hamsters were purchased from Harlan Sprague–Dawley (Indianapolis, IN, USA). All animals were housed in a reversed daylight cycle (14L, 10D, lights on at 9:00 a.m.) and received food and water ad libitum.

House Finch (*Carpodacus mexicanus*): Adult males (n=5) were caught in Tempe, Arizona in September (latitude: 33.414N; longitude: 111.908W). They were housed in an outdoor aviary exposed to ambient temperature and natural photoperiod (10L:14D at time of sacrifice), and received food and water *ad libitum*. Finches were all sacrificed on the same day in December. The Arizona State University Institutional Animal Care and Use Committee preapproved all experimental procedures.

House Sparrow (*Passer domesticus*): Adults n=5, males=2, females=3 were caught in Tempe, Arizona in March (latitude: 33.414 N; longitude: 111.908 W) and

received the same treatment as the House Finch but was exposed to an 8L:16D light cycle.

White-crowned Sparrow (*Zonotrichia leucophrys leucophrys*): Adults n=7, males=4, females=3 and Harris Sparrow (*Zonotrichia querula*): Adults n= 13 were caught in Austin, Texas in February. Sparrows were brought to the laboratory and housed in single cages (16"w x 11" d x 21"h) under short day photoperiod (8L:16D). The animals remained in visual and auditory contact with each other and received food and water *ad libitum*. Temperature in the animal room was approximately 65°F.

Green Tree Frog (*Hyla cinerea*): Adult male frogs (n=5), supplied through Charles Sullivan Inc. (Nashville, TN) were housed in a controlled environment (12L:12D, 21°C) for at least two weeks before sacrifice. The frogs were kept in ten-gallon glass tanks with free access to water. Food (crickets) was provided twice a week. All studies were approved by the IACUC of the University of Texas at Austin and the animals were kept in an AALAC-accredited facility.

### *2.2.2 Immunocytochemistry*

All orexin A and B antibodies were obtained from Santa Cruz Biotechnology, Santa Cruz, CA. The goat polyclonal orexin A and B antibodies are raised against an epitope within the last 100 amino acids of the C-terminus of prepro-orexin and affinity purified. The prepro-orexin epitope is of human origin and identical to the corresponding sequence of mouse origin. The secondary donkey anti-goat was bought from Jackson Immunoresearch, West Grove, PA.

Syrian hamsters under anesthesia (Nembutal) were perfused with saline followed by 4% paraformaldehyde in 0.1M phosphate-buffered saline. Brains were extracted, postfixed at 4 °C overnight and immersed in 20% sucrose in phosphate-buffered saline (wt/vol). Brains were then sliced into 40μm thick sections for free floating immunostaining. Sections were stained with Orexin A (1:4000) or B (1:4000) using a protocol adapted from previous studies [Wommack and Delville 2002].

Birds received an intramuscular injection of 0.1 ml sodium pentobarbital (200 mg/kg; Nembutal, Abbott Laboratories, North Chicago, IL). All birds were perfused transcardially with 0.9% saline containing 0.1% sodium nitrite, followed by 4% paraformaldehyde in 0.1 M phosphate buffer. The brains were taken out of the skull and postfixed in 4% paraformaldehyde overnight at 4° C. Afterwards, the brains were embedded in gelatin following a procedure modified from previous descriptions [Saldanha *et al.* 1994; Deviche *et al*. 2000]. First, the brains were placed in 0.1 M phosphate buffer overnight at 4°C, followed by immersion in a 4% gelatin (175 bloom, Sigma Chemical Co., St Louis, MO) solution for 30 minutes. Afterwards, they were embedded in an 8% gelatin mold and allowed to solidify overnight. Later, brains were immersed in 10% and 20% sucrose solutions for 24 hours each, ending with 48 hours in 30% sucrose solution. They were then frozen on dry ice and cut on a cryostat into 40 μm-thick coronal sections. Three parallel sets of sections were collected for each brain and saved at -20°C in a cryoprotectant [Watson *et al*. 1986].

Free-floating sections were processed by immunocytochemistry for orexin A and B following a protocol adapted from previous studies [Singletary *et al*. 2005; 2006].

After washing in 0.1M phosphate buffer saline, sections were treated with 0.5% hydrogen peroxide to inhibit endogenous peroxidase activity. Sections were washed in 0.1M PBS and blocked in a 10% normal donkey serum solution in 0.1M PBS. Sections were successively incubated in orexin A or B antiserum (1:4000) in a 0.1M PBS/ 0.3% Triton X-100 solution to ensure permeabilization. Sections were washed again and incubated in donkey anti-goat immunoglobulin (1:400). Sections were labeled with 0.5 μg/ml diaminobenzidine (Sigma, St. Louis, MO) after incubation in an avidin-biotinylated peroxidase conjugate (1:100, VectaStain ABC kit, Vector Labs, Burlingame, CA).

Sections were then mounted on gelatin coated slides, dried overnight, dehydrated in gradient alcohols, defatted with xylene (Sigma, St. Louis, MO), and coverslipped with Permount (Fisher Scientific, Hampton, NH). Some stained sections were counterstained with thionin to confirm neuroanatomical identification. Brain regions were identified using avian brain atlases and other descriptive studies [Stokes *et al*. 1974; Aste *et al*. 1998; Medina and Reiner 2000; Brandstatter and Abraham 2003; Krutzfeldt and Wild 2004; Kuenzel 2004; Reiner *et al*. 2004; 2005].

Green tree frogs were rapidly decapitated and brains fixed by immersion in 4% paraformaldehyde in 0.1M phosphate buffer (PB) for 24 hours. The brains were then transferred to a 30% sucrose solution in 0.1M PB for 24 hours. Brains were frozen in an embedding medium (M1 embedding matrix, Thermal Shandon, Pittsburgh, PA) on dry ice and then sectioned in the transverse plane on a cryostat (20μm: thaw-mounted or 40 μm: free-floating sections) at -17°C and mounted onto subbed slides. Each brain was divided into 3 sets of serial slices.

Tissue on slides and free-floating sections were washed in 0.1M phosphate buffered saline (PBS), and immersed in 0.1% $NaBH_4$ for 15 minutes in order to clear the tissue of any residual fixative. Slices were next rinsed again in PBS and preincubated in a 10% normal donkey serum, 0.3% Triton-X 100, 3% $H_2O_2$ and PBS solution for one hour. Sections were then incubated 24 hours in orexin A or B antiserum (1:4000), two serial sets from each brain receiving either antiserum. Afterward, sections were rinsed in PBS and then incubated in a donkey anti-goat secondary antibody (1:400) for one hour. Again, sections were rinsed in PBS and then incubated in an avidin-biotinylated conjugate (1:100, VectaStain ABC kit, Vector Labs, Burlingame, CA) for one hour. Sections were rinsed in a 0.1M phosphate buffered (PB) solution and immersed in a DAB solution for 5 min. Subsequently mounted slices were rinsed in PB and then dehydrated in order to coverslip, and free floating gelatin embedded slices were organized in series, mounted on subbed slides, dehydrated, and coverslipped. Some series of orexin A and B slides were counterstained with cresyl violet or thionin. The distribution of immunoreactive neurons and fibers was mapped through a camera lucida attached to the microscope. The quality of the labeling was similar for both orexin A and B. Neural areas were identified using previous descriptions frog brains [Wilczynski *et al.* 1983; Allison *et al.* 1994; Neary *et al.* 1995].

The specificity of all tetrapod immunostaining was tested through omission of the primary antibody and pre-absorption of the antibody to orexin A or B. For this, the orexin B antibody was incubated overnight in the presence of orexin A or B (sc-8070P or sc-8071P, 40 μg/ml, Santa Cruz Biotechnology, Santa Cruz, CA) before application to tissue

sections. Omission of the primary antibodies and peptide pre-absorption eliminated all immunostaining with exception to the receptor antibodies.

## 2.3 RESULTS

### *2.3.1 Distribution of orexin-ir somata*

In the Syrian hamsters orexin A and B-ir cell bodies were found in a discrete population in the lateral hypothalamus and perifornical area extending caudally towards the dorsomedial hypothalamus (Fig. 2D). In the House finch (Fig. 2B), House sparrow, White-crowned sparrow and Harris' sparrow, orexin A and B-ir neurons were located in a single population centered on the paraventricular nucleus of the hypothalamus (PVN) extending out to the stratum cellulare externum or posterior lateral hypothalamus. In the green treefrog orexin A and B-ir perikarya formed a discrete population centered on the suprachiasmatic nucleus (SCN) (Fig. 2A). Orexin-ir cells extended from the caudal SCN rostrally into the caudal edge of the preoptic area (POA), and a few extended dorsally to the magnocellular preoptic nucleus. On the rostral end of their distribution, orexin-ir cells were located along the walls of the third ventricle. Caudally, the cells were gradually located toward the lateral edge of the SCN. Orexin-ir perikarya had few dendrites and appeared either ovoid or piriform.

### *2.3.2 Distribution of orexin-ir processes*

Orexin A and B-ir fibers were found throughout the brain in concordance with previous findings in the Syrian hamster [Mintz *et al.* 2001]. Highest densities were found in the hypothalamic area surrounding the cells and the midline thalamic area including

the paraventricular nucleus of the thalamus (PVT) and habenular region. Moderate densities of fibers were found in the PVN, the tuberomammillary nucleus (TMN) and the preoptic area. The locus coeruleus (LoC) was also heavily innervated as well as the dorsal raphe. Rostrally, fibers were found extending to the septum and ventral cortex.

In all the birds mentioned here, the distribution of orexin A and B-ir fibers were similarly visible across the brain with the highest density seen within the preoptic area (POA), hypothalamus, thalamus and LoC. Previous research in the house finch details the innervation [Singletary *et al.* 2006]. Orexin-ir projections extended rostrally from the paraventricular nucleus of the hypothalamus (PVN) to the POA, laterally towards the medial striatum, nidopallium, and dorsally along the lateral ventricle towards the mesopallium. Ventrolaterally the fibers project to the optic tectum. Caudally, the highest densities of orexin-ir fibers were found along the third ventricle. The periaqueductal grey, substantia nigra pars compacta and the LoC also showed a high density of orexin-ir fibers. The red nucleus (Ru) is highly innervated on the medial edge, decreasing to a low density towards the lateral edge. Heaviest densities were found in the PVN and along the midline dorsally and ventrally.

In the green treefrog, orexin-ir fibers were visible from the telencephalon to the medulla, with the highest concentrations within the preoptic area and hypothalamus. Previous research in the treefrog details the innervation [Singletary *et al.* 2005]. Rostrally, fibers were observed in the septal areas, the medial and lateral pallium and nucleus accumbens. In the diencephalon, dense orexin-ir fibers were seen in the periventricular and dorsal medial portions of the thalamus. Caudally, moderate orexin-ir

innervation was noted in the laminar nucleus of the torus, the deep layers of the optic tectum, the pretectal gray and extending into the medulla. Fewer orexin-ir fibers were seen in the telencephalon and the metencephalon.

## 2.4. CONCLUSION

### *2.4.1 Functional implications of orexin-ir neuronal distribution*

Our studies on the distribution of orexin-ir neurons in the Syrian hamster, passerines, and the green tree frog support previous findings for the conservation of the orexin system across vertebrates. However orexin-ir distribution varies slightly across vertebrates, suggesting the orexin system may support different functions based on diverging physiological needs. For example, in rodent brains orexin neurons are centered on the lateral hypothalamus (LHy) including the perifornical area, which is involved in feeding behavior and the sleep/wake cycle [Peyron *et al.* 1998; Cutler *et al*. 1999; Nambu *et al*. 1999; McGranaghan *et al.* 2001; Mintz *et al*. 2001]. However, in sheep, only a few are seen in the lateral hypothalamus [Iqbal *et al.* 2001] and the orexin neurons in this LHy subpopulation project to the preoptic area apparently to regulate gonadotropin releasing hormone (GnRH). The majority of orexin cells are concentrated on the dorsomedial hypothalamus (DMH), which regulates feeding, the sleep/wake cycle and the baroreceptor reflex [McDowall *et al.* 2006]. It is possible that the orexin system may have different primary functions in different animals. It is also likely that due to differences in brain morphology several brain areas in one animal may accomplish the same functions as one area in another animal. Studies show that administration of orexin

B does induce feeding in sheep [Sartin *et al*. 2001] and in the pig [Dyer *et al.* 1999] as in rodents. In the pig, orexin neurons are seen in the perifornical areas, DMH and posterior hypothalamus with a few seen in the anterior hypothalamus (AH) and POA suggesting a division of roles in feeding, arousal, reproduction or fluid homeostasis [Su *et al.* 2008]. Is orexin an overarching gatekeeper to homeostatic maintenance? Does the orexin system divide into several differing functional subpopulations as the evolution of more complex behavior emerges? If so, the mammalian species would likely have the most diverse and complex functions regulated by the orexin system. Indeed, the mammalian orexin sequence is thought to have evolved more rapidly than other vertebrates [Alvarez and Sutcliffe *et al.* 2002].

We also have to consider that neighboring areas and connectivity may dictate how orexin neurons function. Depending on the area of the brain, different neurotransmitters or neuropeptides are present and co-localize with orexin neurons, influencing separate or supplemental function. In rats, 94% of orexin neurons are co-localized with dynorphin in the LHy, which suppresses GABAergic input to the tuberomammillary neurons. These histaminergic neurons are known to increase arousal via orexin input [Bayer *et al.* 2002; Huang *et al.* 2001]; therefore, dynorphin facilitates the effect of the orexin excitatory stimulus [Chou *et al.* 2001; Eriksson *et al.* 2004]. Separate glutamate and orexin immunoreactive vesicles are present in the orexin projections to the tuberomammillary nucleus, which may increase the excitatory input depending on concurrent release [Torrealba *et al.* 2003]. Orexin cells are also co-localized with Narp, a neuronal pentraxin known to regulate AMPA glutamate receptor clustering [Reti *et al.* 2002]. At least 50%

of orexin cells are considered to be glutamatergic based on VGLUT2 immunostaining in the rat. The authors of that study also consider that the function of subsets of orexin neurons may be dependent on the neurochemical phenotypes [Rosin *et al.* 2003]. Other examples indicate that the orexin system may be important in reproduction in some mammalian species. Six percent of orexin cells are co-localized with GnRH neurons in the pig hypothalamus [Su *et al.* 2008]. Many studies have also shown orexin to modulate reproduction in rodents [Pu *et al.* 1998; Kohsaka *et al.* 2001; Campbell *et al.* 2003] as in other mammals. Considering the co-localization and connectivity of orexin neurons though, it is likely orexin plays a larger role in the sleep/wake cycle or energy metabolism. Therefore, although orexin is highly conserved across these mammals it is not homogeneous and may suggest separate or additional functions.

Also, environmental pressures unique to a species may facilitate evolution of systems in order to adapt. The threat of predation or the timing of food availability can alter the sleep durations and cycles of animals. Sleep stages and durations are different in a predator vs. prey, where the predator can afford to enter deeper stages of sleep but the prey needs to remain vigilant [Lima *et al.* 2005]. Though the proteins regulating these behaviors may be somewhat conserved the sensitivity to receptors or protein expression may vary depending on circadian or circannual states or sensory information [Richardson *et al.* 1995; Taheri *et al.* 2000; Archer *et al.* 2002; Chen and Randeva 2004]. Receptor selectivity may also change over the course of evolution, which may alter the potency and therefore function of the orexins [Shibahara *et al*. 1999].

Terrestrial vs. aquatic vertebrates have different environments so would likely

have differing behavioral homeostatic fluid balance mechanisms [Feder and Burggren 1992] or neuromotor patterns during feeding [van der Leeuw *et al.* 2001]. Understanding the specialization of function of the orexin system or the addition of functions can also be used to tease out phylogenetic lineages.

In most species, orexin-ir cells are periventricular and found in neurosecretory areas. The PVN is a neurosecretory area in reptiles and mammals with connections to the median eminence and pituitary [Kawata and Sano 1982; Smeets *et al*. 1990; Propper *et al*. 1992; Luo *et al*. 1995]. The rostral PVN in birds also contains neurosecretory cells [Panzica *et al*. 1982; Kiss *et al*. 1987; Panzica *et al.* 1999; Absil *et al*. 2002]. In fish and amphibians, neurosecretory areas are found in the POA and SCN [Goossens *et al*. 1977; Conway and Gainer 1987; Smeets and Gonzalez 2001; Duarte *et al*. 2001]. The fact that areas containing orexin-ir cells in non-mammalian vertebrates have a neurosecretory function suggest that the neuropeptide, although found in different brain regions, play a similar role. Orexin-ir cells in many mammals are found outside of the neurosecretory PVN in the LHy. It is interesting to note that both areas contain neurons that regulate feeding behaviors. There are second-order target neurons in the PVN that inhibit feeding [Olson *et al*. 1991; Kow *et al.* 1991], but in the LHy the second-order neurons, which include orexin, induce feeding [Sakurai *et al*. 1998; Dube *et al*. 1999]. It is likely that in other vertebrates both inhibition and activation of feeding are primarily regulated by areas that differ in specialization in comparison to mammals. Indeed, though the avian brain is organized somewhat differently than mammals, studies have shown the neuronal

populations to be comparable to mammals [Karten 1997; Medina and Reiner 2000; Jarvis *et al.* 2005].

In birds, orexin-ir neurons are located in one paraventricular population that expands to the stratum cellulare externum or posterior lateral hypothalamus [Rattenborg *et al.* 2001; Ohkubo *et al.* 2002; Singletary *et al.* 2006]. Orexin-ir neurons were located in similar areas regardless of phylogeny or domestication in birds. Given the location of cells, studies were done to determine if orexin was involved in the regulation of feeding as in mammals. However, while food deprivation up-regulates prepro-orexin in rats [Cai *et al.* 1999; Karteris *et al.* 2005], fasting does not enhance orexin expression in Japanese quail [Phillips-Singh *et al*. 2003]. Furthermore, while injections of orexin A increase feeding in rats [Dube *et al*. 1999], and infusions of orexin A increase feeding in goldfish [Volkoff *et al*. 1999], no orexigenic effect has been seen in chicken or pigeons [Furuse *et al.* 1999; Simao de Silva *et al.* 2008]. It is also interesting to note that unlike mammals, a receptor that preferentially binds orexin A in chickens has not yet been identified [Ohkubo *et al*. 2003]. Though only one receptor has been found in birds, it is the most similar to orexin receptor 2 which binds A or B with the same affinity. Several studies in mammals suggest a role for orexin A as orexigenic and orexin B as a modulator of the sleep/wake cycle [Sakurai *et al.* 1998; Fujiki *et al.* 2003], but these roles could be dependent on the receptors that have evolved in different vertebrates [Chen and Randeva, 2004]. In addition, orexin A injections in pigeons increase arousal and orexin antagonist injections increase sleep behavior in sparrows [Simao de Silva *et al.* 2008; unpublished observations] similar to mammals. It is possible that as the orexin system evolved in

birds, the regulation of the sleep/wake cycle was conserved but feeding modulation was lost.

In green anole lizards, orexin-ir cells have been found more caudally as a single population located along the third ventricle within the periventricular nucleus [Farrell *et al.* 2003]. In turtles, orexin-ir neurons are predominantly found in the median eminence (ME) extending to the periventricular hypothalamic nucleus [Eiland *et al.* 2001]. This closely resembles the population seen in birds, but in birds this neuronal population extends laterally. Studies determining structure or function have not been completed in reptiles but from cell and fiber location it is likely that orexin regulates homeostasis.

In amphibians, the distribution of orexin-ir neurons reported varies between urodeles and anurans as well as between suborder of frogs. In the green tree frog, orexin-ir cells consist of a single population localized along the third ventricle and mostly within the SCN. In *Xenopus laevis*, orexin-ir neurons are centered in the ventral hypothalamus. In *Rana ridibunda* the neurons are predominantly found in the suprachiasmatic nucleus. It is interesting to see in a urodele, the axolotl, the cells are located in the posterior preoptic nucleus, extending to the SCN and rostral ventral hypothalamus [Suzuki *et al.* 2008]. Rostrally, smaller orexin-ir neurons are located close to the third ventricle and seemed to be in contact with the cerebral spinal fluid, which is similar to what is reported in turtles [Eiland *et al.* 2001]. Caudally, larger orexin-ir neurons were located more laterally away from the ventricle. Location and differences in size of these subsets of neurons indicates partitioning of function.

In goldfish, while there is a consensus that a group of orexin-ir cell bodies is located along the third ventricle, some studies reported an additional group in a more lateral brain region [Huesa *et al.* 2005; Nakamachi *et al.* 2006]. In medaka, orexin-ir cell bodies have a similar distribution to the goldfish [Amiya *et al.* 2007]. There is inconsistency among the findings in zebrafish as well. In one zebrafish study, orexin cells are distributed into two separate populations, one along the third ventricle within the preoptic area and SCN, and the other within the anterior hypothalamus [Kaslin *et al.* 2004]. Recently however, researchers have shown only one population in the rostral medial hypothalamus using a species-specific antibody [Faraco *et al.* 2006]. Discrepancies between species may be due to phylogenetic differences, although within species this could reflect different techniques or species-specific antibodies available. Studies have shown that injections of orexins increase feeding in goldfish [Volkoff *et al.* 1999; Nakamachi *et al.* 2006] and locomotor activity [Nakamachi *et al.* 2006]. In fasted goldfish the expression of orexin mRNA is increased as well as the number of orexin-ir cells [Nakamachi *et al.* 2006]. The orexin system has been more thoroughly studied in zebrafish. Similar to birds, there is only one orexin receptor found in zebrafish that is most homologous to the mammalian orexin receptor 2 [Prober *et al.* 2006]. The orexin system has also been implicated in the regulation of the sleep/wake cycle in zebrafish. In orexin receptor mutant zebrafish, a decrease in sleep is found with sleep fragmentation. Additionally when orexin A was injected decreased locomotion and increased sleep was observed [Yokogawa *et al.* 2007]. In contrast, overexpression of orexin in zebrafish larva

decreases sleep [Prober *et al.* 2006]. This is conflicting but may be explained by the age of the zebrafish and the development of the orexin system.

### *2.4.2 Functional implications of orexin-ir fiber distribution*

*Mammals*

In addition to orexin cell body location, orexin-ir fiber distribution can help further elucidate the physiological roles of orexin. Generally, in mammals, there is dense orexin-ir innervation of the hypothalamus, midline thalamic areas, and locus coeruleus, whereas, the hippocampus, cortex and cerebellum are sparsely innervated. However in some cases, orexin-ir fiber distribution differs slightly depending on species. For example, dissimilar to rats, orexin projections are absent in the hamster SCN though they are seen in the surrounding areas [Mintz *et al.* 2001; this study]. This result is interesting given the overwhelming evidence for the role of orexin in the sleep/wake cycle and the circadian function of the SCN.

Research initially suggested a main role for orexin in feeding but studies now show that orexin's role on feeding is dependent on the time of day and state of the animal [Estabrooke *et al*. 2001; Cai *et al.* 2002; Espana *et al.* 2002]. In addition, orexin is thought to increase arousal via the TMN in anticipation of food, distinct from other modes of arousal [Torrealba *et al*. 2003, but see Mieda et al. 2004]. Overall, the high degree of conservation in orexin-ir distribution and the physiological studies referenced earlier suggest that orexins are involved in general physiological and/or homeostatic mechanisms.

*Birds*

The apparently conserved neural distribution of orexins suggests that these peptides play similar roles among birds including galliformes, columbiformes and passeriformes. The distribution of immunoreactive neurons and fibers in this study was consistent with previous descriptions in birds using various methods of mRNA analysis and immunohistochemistry with different orexin antibodies [Rattenborg *et al.* 2001; Ohkubo *et al*. 2002, 2003; Phillips-Singh *et al*. 2003; Singletary *et al*. 2006].

High densities of orexin-ir fibers were observed across the hypothalamus, thalamus, and preoptic area similar to mammals. In addition, the auditory system is highly innervated, with the highest densities seen in the nucleus intercollicularis (ICo) and torus semicircularis (located just rostral to the ICo). In lizards and frogs, the torus semicircularis is moderately innervated [Farrell *et al.* 2003; Galas *et al.* 2001; Singletary *et al.* 2005]. The SCN and LHN are also highly innervated, areas thought to have circadian and visual functions in birds similar to the mammalian SCN [Brandstatter and Abraham 2003]. This is also consistent in lizards and frogs [Farrell *et al.* 2003; Galas *et al.* 2001; Singletary *et al.* 2005]. In addition, areas known to be involved in vigilance and movement in mammals, such as the LoC, the periaqueductal grey (PAG), Ru and substantia nigra pars compacta (SNc) are highly innervated in the passerine brain by orexin-ir fibers. Taken collectively, the results suggest that orexin is important for sensory and motor processing and circadian function in birds and other vertebrates.

There are a few discrepancies in orexin-ir fiber distribution between birds and mammals. For example, in passerines the Ru is innervated where fibers are scarce in

mammals [Peyron *et al.* 1998]. Also, virtually no fibers were seen in the pedunculopontine tegmentum (PPT) in passerines, but this area in mammals is highly innervated [Peyron *et al.* 1998; Cutler *et al.* 1999; Nambu *et al.* 1999; McGranaghan *et al*. 2001; Mintz *et al*. 2001]. However, as a moderate density of orexin-ir fibers surrounds the periphery of the PPT in house finches, one cannot rule out the possibility of synaptic contact just outside the area. The PPT regulates atonia during REM sleep in mammals [Takakusaki *et al.* 2004; Takakusaki *et al.* 2005]. Interestingly, the nuchal (neck) muscles in birds, but not mammals, elicit electromyographic activity during REM sleep [Rattenborg *et al.* 2004]. An association between the absence of orexin-ir fibers in the PPT and the absence of atonia may not be limited to nuchal muscles, but also exist in other muscle groups.

*Reptiles*

In reptiles, the orexin-ir fibers are most prevalent in the medial and periventricular areas of the hypothalamus [Eiland *et al.* 2001; Farrell *et al.* 2003]. To date there are no physiological studies of the effects of orexin on reptiles and amphibians. Therefore, we can only speculate the function of orexin based solely on orexin-ir cell location and fiber distribution. In turtles orexin-ir neurons located in the ME send processes that protrude and likely secrete orexin directly into the CSF where it may be involved in endocrine function [Eiland *et al*. 2001]. In addition, in the anolis, orexin-ir fiber distribution also extended into thalamic areas, mainly the midline thalamus and habenula, which are thought to relay sensory information to the cortex. These areas are also important in the maintenance of biological rhythms in mammals and have similar connections. The optic

tectum and torus semicircularis in the lizard were also innervated similar to amphibians [Galas *et al.* 2001; Singletary *et al.* 2005; Suzuki *et al.* 2008]. Orexin-ir fibers were not found in the cerebellum similar to other vertebrates. Despite the fact that there is a greater quantity and lateral extent of orexin-ir fibers in birds compared to lizards there was overall a similar distribution near the midline. This may mean a conservation of function between birds and lizards but that in birds, the larger innervation pattern would indicate additional functions of orexin.

*Amphibians*

In anurans, most of the orexin-ir innervation was located in the diencephalon and mesencephalon whereas the hippocampus, cerebellum and cortex are sparsely innervated. The distribution of orexin-ir innervation in the brain of *Hyl*a is consistent with other vertebrates. In *Hyla,* as in mammals, orexin-ir fibers innervate the hypothalamus, preoptic area, thalamus, septum, and parts of the striatum, midbrain and hindbrain [Nambu *et al.* 1999; Mintz *et al.* 2001]. This innervation was also similar to the *Rana* and *Xenopus* but was more expansive [Shibahara *et al.* 1999; Galas *et al.* 2001]. However, few orexin-ir fibers were found in the *Rana* septum in contrast to the green tree frog and *Xenopus.* Also, orexin-ir projections were absent in the anterior and central thalamic nuclei of the *Xenopus*, which were moderate to dense in the *Rana* and *Hyla.* These latter frogs are considered terrestrial frogs and belong to the same superfamily of Neobatrachia, but the *Xenopus* is considered aquatic and belongs to the Mesobatrachia superfamily. In the urodele axolotl, the orexin-ir is found predominantly in the hypothalamus and mesencephalon but not the thalamus. Also, innervation of the SCN was scarce in contrast

to what has been reported in the anurans. However, there was a higher density of orexin-ir innervation found in the mesencephalon than in anurans. Even with these differences, the overall similarity of orexin-ir innervation across vertebrate suggests that the orexin system is involved in general physiological regulation.

This degree of conservation of the orexin system is interesting considering the differing specializations of these various vertebrates. For example mammals and birds thermoregulate internally; mammals, birds and reptiles are amniotic; mammals, birds, reptiles and amphibians are tetrapods. The earliest evidence of the emergence of the orexin system is seen in fish [Alvarez and Sutcliffe 2002].

*Fish*

In goldfish orexin-ir fibers are predominantly found in the ventral telencephalon, diencephalon and dorsal white zone of the optic tectum. Few fibers are found in the vicinity of the locus coeruleus and dorsal raphe [Huesa *et al.* 2005; Nakamachi *et al.* 2006], which indicates that orexin may be involved in feeding but not the control of vigilance, arousal or serotonergic modulation. However, orexin may be involved in only hunger-related arousal in goldfish, as these areas are not activated during mammalian food anticipatory behavior [Torrealba *et al.* 2003].

This is in contrast to the innervation seen in one zebrafish study [Kaslin *et al.* 2004] and in mammalian studies [Date *et al.* 1999; Brown *et al*. 2001]. In the zebrafish, orexin-ir innervation was found in the aminergic systems including the locus coeruleus, dorsal raphe, histaminergic neurons and cholinergic systems [Kaslin *et al.* 2004] but more recent studies show that that may be the result of non species-specific antibodies

[Yokogawa *et al.* 2007]. Furthermore, the sole orexin receptor found in the zebrafish is not expressed in the locus coeruleus, serotonergic or histaminergic neurons. This lack of orexin-ir innervation and absence of orexin receptor in these wake promoting areas are crucial in explaining the surprising role of orexin on the sleep/wake cycle in zebrafish.

*Further Considerations*

The orexin peptides have highly conserved sequences across vertebrates. However, the receptors for these peptides are not as conserved or have not been found yet. Interestingly, *Xenopus* orexin B has a higher affinity *in vitro* for human receptor 1 and 2 than human orexin B but *Xenopus* orexin receptors have not been characterized yet. Only one receptor has been found in birds and in zebrafish, both of which are most homologous to the mammalian orexin 2 receptor. The functions of the orexin system are also dependent on the receptor type and distribution, the co-localization of orexin cells, and the areas to which the cells project.

Additionally, behavioral state may modulate the system according to the species' unique adaptations or gender. Peripherally there are sex differences in the orexin system as well [Ohkubo *et al.* 2003; Silveyra *et al.* 2007]. Cerebral spinal fluid levels of orexin change diurnally, and the activity of orexin neurons change throughout the day [Estabrooke *et al.* 2001; Martinez *et al.* 2002]. Orexin and orexin receptor expression changes in response to food deprivation [Nakamachi *et al.* 2006; Chen and Randeva *et al.* 2004] or photoperiod in sheep [Archer *et al.* 2002].

Overall, it is important to consider that differences in the orexin system within and between species could arise due to these factors. Many of the studies done thus far do

not include information on the time of sacrifice or time of injection of drugs made to alter the orexin system. Many of the studies include animals in different photoperiods and different reproductive stages. Antibodies and techniques vary and can become problematic if comparisons are not all done with species specific antibodies and sensitive imaging techniques, though the obvious limitation is availability. Despite the fact that many caveats exist, any information that can be garnered proves beneficial for furthering the basic understanding for the role of orexin in human and all vertebrate physiology.

### *2.4.3 Summary*

Because there are general similarities in cell location it is logical to assume some similar function of orexin across vertebrates. In the increasing complexity of the homeostatic mechanisms within and across each taxon, or introduction to unique environmental pressures, it is likely that the orexin system became more complex. The orexin system can be better explained by understanding the function of the areas that the orexin cells, fibers and receptors are found. In this study, we have contributed a detailed description of the orexin cell and fiber distribution across vertebrates. Though much work needs to be completed to determine the receptors in non-mammalian vertebrates, we can begin to narrow down function by comparing distributions of cells and fibers, orexin sequences, and the few physiological studies available. Given the information today the orexin system seems to regulate general homeostatic function across vertebrates, with specialization of function depending on the species.

**Fig. 2.1**
Photomicrographs showing comparative distribution of orexin cells across vertebrates

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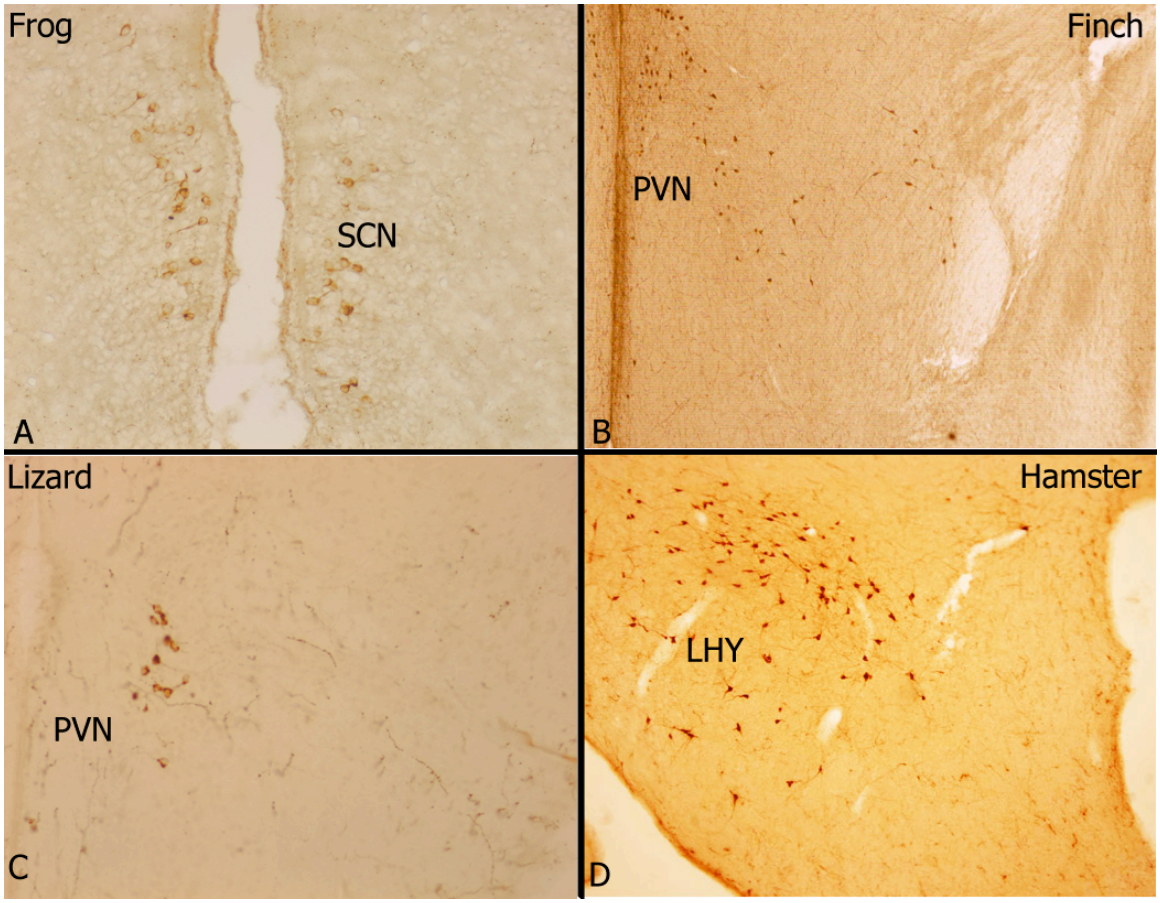


# Orexin-ir fiber distribution:

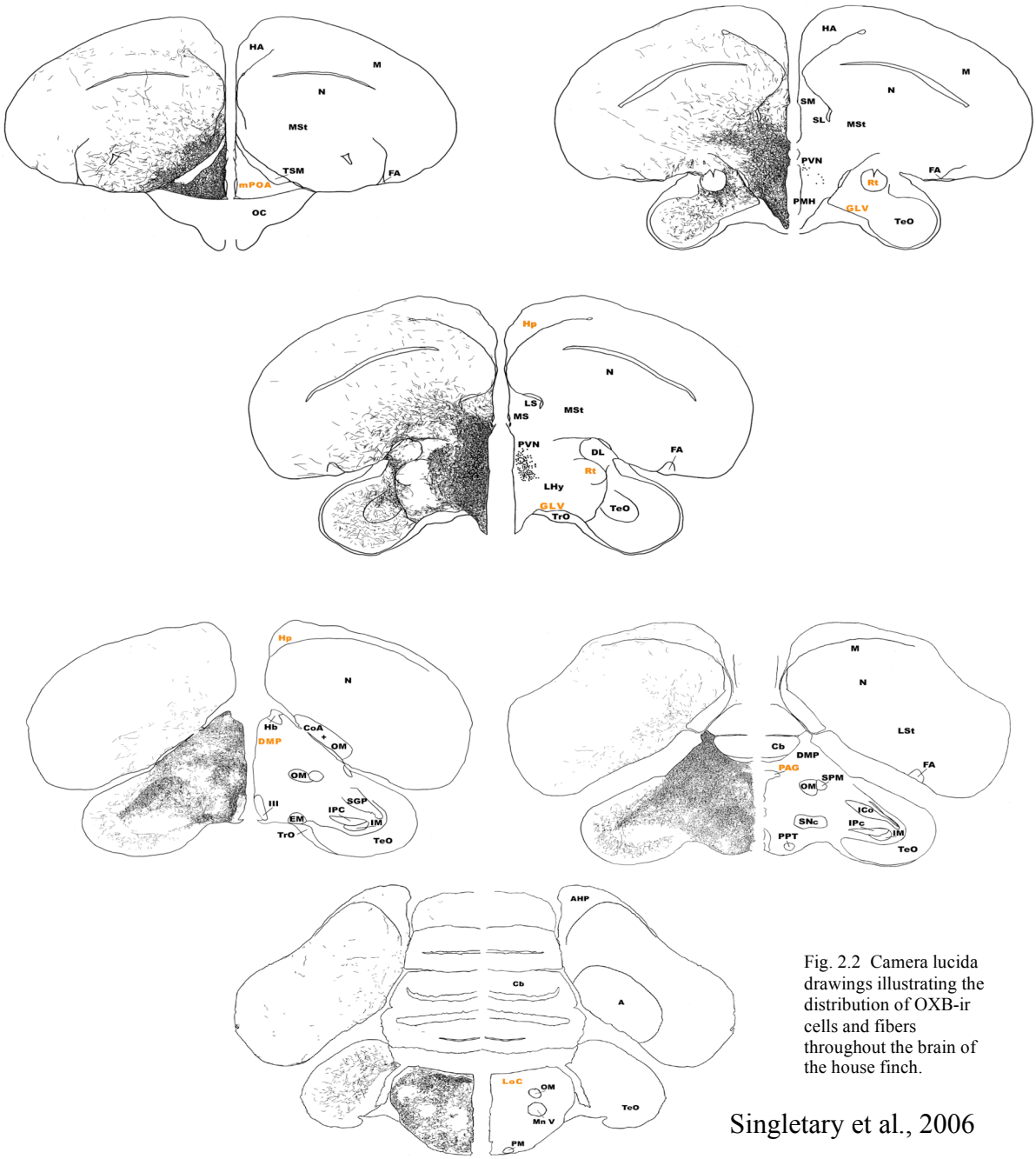



Fig. 2.2 Camera lucida drawings illustrating the distribution of OXB-ir cells and fibers throughout the brain of the house finch.

Singletary et al., 2006

## Chapter 3: Photoperiod Induced Changes in Orexin Innervation in the Brain of a Migratory Songbird, the White-Crowned Sparrow


### ABSTRACT

During migration, diurnal songbirds show extensive disruption of their sleep/wake cycle, sleeping during the day and flying at night. In mammals, similarly altered cycles of activity result from blocking orexin expression in the hypothalamus. We hypothesized that nocturnal migratory activity in songbirds is associated with reduced orexin innervation within key areas of the brain. Changes in orexin B immunoreactivity were quantified in the brains of migratory (white-crowned sparrows, *Zonotrichia leucophrys*) and non-migratory (house sparrows, *Passer domesticus*) birds before and after a change in photoperiod to induce migratory activity. In the white-crowned sparrow, there was no change in orexin B innervation within areas constituting the ascending arousal system, such as the locus coeruleus, in association with migratory activity. Orexin B innervation in the midline thalamic area (specifically the dorsomedial posterior thalamus, DMP) also did not change in birds exposed to long days and performing migratory activity. This area is thought to be homologous to the paraventricular thalamus in mammals, which regulates the sleep/wake cycle. A decrease was not observed in the DMP of sedentary house sparrows after transfer from short to long days. In addition, orexin innervation in the DMP of white-crowned sparrows did not change after migratory activity ceased. This midline thalamic nucleus integrates afferents from the ascending arousal system. It was hypothesized that changes in orexin innervation in this area could play a critical role in the activation of migratory activity and the regulation of the sleep/wake cycle in

songbirds. Data from these studies do not support a role for orexin in the regulation of migration. The role of the orexin system on the sleep/wake cycle of migratory birds remains in question.

## 3.1 INTRODUCTION

Changing photoperiods control migratory activity in birds. Transfer from short day- to long day-photoperiods induces migratory activity in captive songbirds [Rowan 1925; Farner 1950]. In diurnal songbirds, migratory activity is also associated with a disruption of the sleep/wake cycle [Rattenborg *et al.* 2004; Fuchs *et al.* 2006]. These observations are reminiscent of sleep disorders, such as narcolepsy, and suggest that migratory activity in songbirds is associated with a disruption of the neural systems controlling the sleep/wake cycle.

The neural systems that regulate the sleep/wake cycle are better understood in mammals than in birds. As discussed in Chapters one and two, in mammals, the sleep/wake cycle is stabilized by a key neuropeptide, orexin [Saper *et al.* 2001]. We know in mammals that there are primarily two groups of neurons that control sleep or wake. The wake promoting neurons of the LoC, the tuberomammillary nucleus (TMN) and raphe activate the awake state and inhibit the sleep promoting neurons of the ventrolateral preoptic area (VLPO). A third group of neurons, the orexin neurons, excite and maintain the wake "on" state. Remove this maintenance, and inhibition of the sleep state is removed thus there is an imbalance or a flip flop. This flip flop is the model we will focus on these studies. Visually, this resembles a seesaw or teeter board. On one end

of this seesaw are the wake promoting neurons. On the other end are the GABA and galanin neurons of the VLPO which are inhibited during the awake state. When these sleep "on" neurons promote sleep, they also inhibit the awake neurons. The orexin neurons are also inhibited by the VLPO neurons thus inhibiting the awake neurons directly and indirectly, promoting sleep. Without these orexin neurons the switch between the two states is unstable.

We showed in Chapter 2 that the distribution of the orexin neurons and fibers are comparable between vertebrates, and other studies have shown the structure to be similar. The potential for function to also be conserved was high and we began our experiments. Though other hormones and peptides have been associated with migratory activity, there have been no neural mechanisms found that cause this behavior to occur. Orexin has been shown to regulate feeding and the sleep/wake cycle in mammals and these behaviors change during the migratory stage in white-crowned sparrows. Assuming that the disruption in the sleep/wake cycle of these migrating birds is a narcoleptic-like state, the orexin system was a good candidate to begin investigating in association with Zugunruhe. To test predictions that migratory activity in songbirds is associated with changes in the orexin system, we compared the density of orexin B-immunoreactive (–ir) fibers in brains of white-crowned sparrows under migratory or non-migratory conditions. Various brain areas were important to analyze based on functions necessary for migration. Vision, although hindered by the lack of sun, would be necessary to navigate by the night sky. Brain areas involved in vision such as the nucleus rotundus and ventrolateral geniculate nucleus could function differently during migration. The

hippocampus would be used to remember landmarks and navigation routes. The preoptic area is important in mammalian sleep and possibly in birds. The locus coeruleus and periaqueductal grey (PAG) areas are known to be involved in locomotion and movement which are intense during the migratory stage. The PAG is also thought to be involved in mammalian paradoxical sleep [Sastre *et al.* 1996]. The mSCN in birds is thought to be a circadian oscillator which would direct activity during changing photoperiods. Thalamic areas such as the dorsointermedial posterior and ventromedial thalamic areas receive input from many brain areas and are well-situated to relay to many other areas. One area we will focus on is the dorsomedial posterior thalamus (DMP). The medial region of the avian DMP is thought to be functionally homologous to the mammalian paraventricular thalamic nucleus (PV) [Csillag and Montagnese 2005]. The PV integrates inputs from SCN and SCN relay areas with autonomic structures such as the NTS, and relays in the PB and PAG [Watts *et al*. 1987; Watts and Swanson 1987; Moga *et al*. 1995; Krout and Loewy 2000a; 2000b; Kawano *et al*. 2001; Van der Werf *et al.* 2002; Saper *et al.* 2005]. These connections suggest that the PV regulates sympathetic and parasympathetic function through outputs to hypothalamic and brainstem areas. In addition, the PV projects to limbic forebrain and hypothalamic areas, suggesting a role in the control of attention and motivation [Peng *et al*. 2004; Kelley *et al.* 2005].

The PV may also play a role in the control of sleep/wake cycles [Sewards and Sewards 2003; Ishibashi *et al*. 2005]. It receives input from the monoaminergic neurons, indicating a role in the regulation of wakefulness [Kirouac *et al.* 2005] and is active during the subjective day in diurnal animals [Novak and Nunez, 1998; Novak *et al.*

2000]. Furthermore, the PV receives a high density of projections from orexin cells and contains numerous orexin-2 receptors [Peyron *et al.* 1998; Novak *et al.* 2000; Marcus *et al.* 2001; Kirouac *et al*. 2005]. In addition to its role in the sleep/wake cycle, the orexin system is proposed to be involved in the modulation of autonomic function as fibers are found in the NTS, the Arc, and the dMnX [Date *et al*. 1999]. The PV in mammals has been suggested to regulate arousal depending on energy needs and circadian input [Timofeeva and Richard 2001; Kelley *et al.* 2005]. Thus, the DMP may be an area that serves as important integrator of energy homeostasis and the avian sleep/wake cycle.

Similar connections have been seen in the avian DMP, suggesting functional similarities. The DMP receives input from autonomic and monoaminergic ascending arousal systems and also projects to the forebrain and limbic structures [Güntürkün and Karten 1991; Veenman *et al.* 1997; Montagnese *et al*. 2003; Reiner *et al.* 2005; Csillag and Montagnese 2005]. Reciprocal connections exist between the substantia nigra pars compacta and the DMP; the ventral pallidum and the DMP; the ventral tegmental area and DMP; and the septal region and DMP. The DMP also receives a high density of orexin innervation [Singletary *et al.* 2006].

In addition, the mSCN is indirectly connected to the DMP via the POA and PVN, further supporting its role in relation to diurnal cycles. The avian mSCN is thought to regulate avian circadian rhythmicity and has efferents and afferents similar to the mammalian SCN, though the avian mSCN shows more connections [Cantwell and Cassone 2006]. Both the mammalian and avian SCN receive orexin fiber innervation [Date *et al*. 1999; Singletary *et al*. 2006]. The role of orexin is ideal here as two

processes that change in migrating birds are the sleep/wake cycle and control of metabolism. Taken altogether, the DMP is strategically located to regulate changes in the sleep/wake cycle as well as autonomic function.

In addition, we looked for photoperiod-induced changes in the brain of the house sparrow, as we predicted that any changes observed in the white-crowned sparrow (WCS) would not be found in a sedentary species.

## 3.2 MATERIALS AND METHODS

### *3.2.1 Animals and treatment*

Adult and juvenile white-crowned sparrows (*Zonotrichia leucophrys leucophrys;* n=49, males=22, females=27) were caught using seed-baited potter traps (Country Cages, Penngrove, CA) in Austin, Texas (latitude: 30.17 N; longitude: 97.44 W) in February. Sex was determined at sacrifice as these birds are monomorphic. Juveniles with brown and cream crown stripes were distinguished from adults with black and white stripes. This species adapts well to captivity and exhibits migratory restlessness when exposed to a long photoperiod simulating spring [Farner 1950; Ramenofsky *et al.* 2003]. Sparrows were brought to the laboratory and housed in single cages (16"w x 11" d x 21"h) under short day photoperiod (8L:16D). Birds were in visual and auditory contact with each other and received Home Grown™ chow (Country Acres Feed Company, Brentwood, MO), white millet (Tomlinson's, Austin, TX) and water *ad libitum*. Temperature in the animal room was approximately 65°F. All sparrows were allowed to acclimate for at least a month under short days before data collection. Experimental procedures were

approved by the University of Texas Institutional Animal Care and Use Committee and the animals were housed in an AALAC-accredited facility.

House sparrows (*Passer domesticus*) were used as a non-migratory control and do not show Zugunruhe in response to photostimulation [Eyster 1954]. Adult house sparrows (n=10, males=4, females=6) were caught in Tempe, Arizona in March (latitude: 33.414 N; longitude: 111.908 W). They were housed individually (cage size: 16"w x 11" d x 21"h) in visual and auditory contact under a short day photoperiod (8L:16D). The room temperature was set at 65°F and sparrows received mealworms twice a week, white millet, Mazuri Finch Food (PMI, Richmond, Indiana), and water *ad libitum*. All sparrows acclimated for at least a month under short days (SD). All experimental procedures with this species were approved by the Arizona State University Institutional Animal Care and Use Committee.

### *3.2.2 Experimental Design*

In the first study, after the period of acclimation, a group of white-crowned sparrows (SD, n=7) was sacrificed while subjected to short days. Another group (LD, n=7) was transferred from short to long day photoperiods (from 8L:16D to 16L:8D) to induce migratory activity. Behavioral activity was recorded during the night (see below for details) and birds were sacrificed after confirming their behavioral state over 5-7 consecutive days: Either pre-migratory condition (under short day photoperiods) or migratory condition (after a few weeks of exposure to long day photoperiods). This study was replicated (SD, n=6) (LD, n=7).

In the second study, we looked at the association of orexin innervation with maintenance and termination of migratory behavior. Over time, birds exposed to a long photoperiod exhibit different levels of activity representing different time points in their migratory behavior. Birds show an initial peak in activity and decrease over time apparently associated with arrival at the breeding grounds [Helms 1963; Kuenzel and Helms 1974; Smith and Norment 2005]. Consequently, we included birds sacrificed during the peak of migratory activity (Peak Migration; n=5), towards the end of the migratory cycle (Late Migration; n=6), and after migratory activity had ceased (Post Migration; n=6). These stages parallel the vernal migratory life history sub-stages mentioned in earlier studies [Jacobs and Wingfield 2000; Ramenofsky *et al.* 2003] and are defined below.

In white-crowned sparrows, a 20-50% increased body weight and resulting fat accumulation typically precedes migratory activity after transfer to long days [King and Farner 1963; Wingfield *et al*. 1996; Ramenofsky *et al*. 2003]. As an added measure, body weights and fat scores were also recorded in this second study. Subcutaneous fat deposits can be easily seen and measured by blowing on the feathers to expose the furcular region. The scale used ranged from 0-5 where 0 indicated no fat in the furculum, 1 some fat, 2 the furculum was lined with fat, 3 the fat filled the furculum to slightly concave, 4 the furculum is flush with fat and 5 is bulging over [Wingfield and Farner 1978].

In the third study, non-migratory house sparrows were housed under short days for a period of habituation. The house sparrows were then separated into two groups.

One group was kept under short days (8L:16D, SD, n=5) and the other group (LD, n=5) was transferred to a long day photoperiod (16L:8D). All groups contained similar proportions of males and females. All animals were sacrificed one month later. Behavioral activity was not recorded in these animals, as house sparrows do not perform Zugunruhe after transfer from short to long day photoperiods [Eyster 1954].

### *3.2.3 Behavior*

White-crowned sparrows were videotaped with Sony digital video cameras for one hour at night under infrared illumination (Super Nightshot and Nightshot Plus with additional infrared lights if necessary depending on the camera) when exposed to short and long days to confirm their migratory status. Videotaping began at least two hours after lights off. Time points for video recording were chosen to fit within the nightly window of Zugunruhe based on earlier reports [King and Farner 1963; Ramenofsky *et al.* 2003]. In the first study, Zugunruhe or migratory behavior was analyzed as percentage of time active during the one-hour observation periods. This activity included wing whirrs, flights, and hops. In the second study each of these behaviors were analyzed separately as frequencies, as the birds would start flying and hopping at night a few days before they exhibited wing whirring.

In the second study, migration substages were defined based on the number of wing whirrs per hour. The Pre Migration substage was characterized by birds exhibiting no nocturnal activity. Peak Migration was characterized by intense nightly activity. Animals were consistently awake for more than 90% of one-hour test periods and performed over 100 wing whirrs per hour over four consecutive nights. During the Late

Migration substage, birds were consistently awake at least 10% of the one-hour test periods and performed less than 100 wing whirrs per hour averaged over four nights. The Post Migration substage was characterized by sparrows that had ceased nocturnal migratory activity (no wing whirrs) and were active or alert for less than 10% of the one-hour nightly test periods.

Active wakefulness was defined as sparrows exhibiting behaviors ranging from flying, hopping, foraging, and pacing. Alert wakefulness was defined as birds moving their head in an erect posture or immobile, but with both eyes open. These behaviors have been previously described [Amlaner and Ball 1994; Fuchs *et al.* 2006]. Birds perched immobile and in a sleep posture with neck tucked in and ruffled feathers or head tucked in backwards were considered as sleeping. This data was not analyzed.

### *3.2.4 Immunocytochemical Labeling*

Birds received an intramuscular injection of 0.1 ml sodium pentobarbital (200 mg/kg; Nembutal, Abbott Laboratories, North Chicago, IL) after a one-hour period of observation to confirm their migratory state (white-crowned sparrow) or were anesthetized by inhalation of Metofane (house sparrow). All birds were perfused transcardially with 0.9% saline containing 0.1% sodium nitrite, followed by 4% paraformaldehyde in 0.1 M phosphate buffer. The brains were taken out of the skull and postfixed in 4% paraformaldehyde overnight at 4° C. Afterwards, the brains were embedded in gelatin following a procedure modified from previous descriptions [Saldanha *et al*. 1994; Deviche *et al*. 2000]. First, the brains were placed in 0.1 M phosphate buffer overnight at 4°C, followed by immersion in a 4% gelatin (175 bloom,

Sigma Chemical Co., St Louis, MO) solution for 30 minutes. Afterwards, they were embedded in an 8% gelatin mold and allowed to solidify overnight. Later, brains were immersed in 10% and 20% sucrose solutions for 24 hours each, ending with 48 hours in 30% sucrose solution. They were then frozen on dry ice and cut on a cryostat into 40 μm-thick coronal sections. Three parallel sets of sections were collected for each brain and saved at -20°C in a cryoprotectant [Watson *et al.* 1986].

Free-floating sections were processed by immunocytochemistry for orexin B following a protocol adapted from previous studies [Singletary *et al.* 2005; 2006]. After washing in 0.1M phosphate buffer saline, sections were treated with 0.5% hydrogen peroxide to inhibit endogenous peroxidase activity. Sections were washed in 0.1M PBS and blocked in a 10% normal donkey serum solution in 0.1M PBS. Sections were successively incubated in orexin B antiserum (1:4000, sc-8071 Lot #A100, Santa Cruz Biotechnology, Santa Cruz, CA) in a 0.1M PBS/ 0.3% Triton X-100 solution to ensure permeabilization. The goat polyclonal orexin B antibody is raised against an epitope within the last 100 amino acids of the C-terminus of prepro-orexin and affinity purified. The prepro-orexin epitope is of human origin and identical to the corresponding sequence of mouse origin. The antibody can be used in avian tissue, as chicken orexin B is 65% homologous to human orexin B [Ohkubo *et al*. 2002]. This antibody has been previously validated for immunohistochemical use in amphibians and birds in our laboratory [Singletary *et al*. 2005; 2006]. Sections were washed again and incubated in donkey anti-goat immunoglobulin, 1:400, Lot # 70722 Jackson Immunoresearch, West Grove, PA). Sections were labeled with 0.5 μg/ml diaminobenzidine (Sigma, St. Louis, MO) after

incubation in an avidin-biotinylated peroxidase conjugate (1:100, VectaStain ABC kit, Vector Labs, Burlingame, CA).

Sections were then mounted on gelatin coated slides, dried overnight, dehydrated in gradient alcohols, defatted with xylene (Sigma, St. Louis, MO), and coverslipped with Permount (Fisher Scientific, Hampton, NH). Some stained sections were counterstained with thionin to confirm neuroanatomical identification.

The specificity of the immunostaining was tested through omission of the primary antibody and pre-absorption of the antibody to orexin B. For this, the orexin B antibody was incubated overnight in the presence of orexin B (sc-8071P, 40 μg/ml, Santa Cruz Biotechnology, Santa Cruz, CA) before application to tissue sections. Omission of the primary antibody and peptide pre-absorption eliminated all immunostaining. The distribution of immunoreactive neurons and fibers in this study was consistent with previous descriptions in birds using various methods of mRNA analysis and immunohistochemistry with different orexin antibodies [Ohkubo *et al*. 2002, 2003; Phillips-Singh *et al*. 2003; Singletary *et al*. 2006].

### *3.2.5 Quantification of Innervation*

The density of immunoreactive fibers was quantified using a light microscope (Nikon Eclipse E600) and NIH Image 1.62 through gray level thresholding, as previously explained [Shipley *et al*. 1989; Delville *et al.* 1998]. Background was minimized using a histogram to determine standard peak values and camera illumination was kept constant. We quantified orexin-ir fiber density in each subject by sampling from six to eight consecutive sections of each selected brain region. The results were expressed as the area

($mm^2$) covered by orexin-ir fibers within the sample surface (a 100 $\mu m^2$ square). These measures were averaged for each animal and the averages were compared between groups. All measurements were made without knowledge of the experimental treatment.

Brain regions were identified using avian brain atlases and other descriptive studies [Stokes *et al.* 1974; Aste *et al.* 1998; Medina and Reiner 2000; Brandstatter and Abraham 2003; Krutzfeldt and Wild 2004; Kuenzel 2004; Reiner *et al*. 2004; Singletary *et al.* 2006]. Landmarks used for this study are based on previous experience with orexin immunolabeling in birds and on Nissl stained sections used to confirm and identify areas labeled by orexin [Singletary *et al*. 2006]. Brain regions were chosen based on observation of innervation or sleep/wake function. These areas included the locus coeruleus (LoC, just dorsolateral to the fasciculus longitudinalis medialis), the periaqueductal grey (PAG, just ventrolateral to the cerebral aqueduct), the medial suprachiasmatic nucleus (mSCN, located ventral to the preoptic area at the base of the third ventricle), the medial preoptic area (POM, just lateral to the preoptic paraventricular magnocellular nucleus), and the hippocampus (Hp, located between the lateral ventricle and midline at the level of the anterior commissure). Other areas include non-specific and specific thalamic nuclei such as the dorsomedial posterior thalamus (DMP, located below the habenula and next to the wall of the third ventricle), the dorsointermedial posterior thalamus (DIP, located ventrolateral to the DMP and next to the dorsolateral anterior thalamus, pars medialis), the ventromedial thalamic area (VMA, located ventromedial to the interstitial nucleus of Cajal and just above the medial reticular formation) (Fig. 3.5). Specific thalamic nuclei included the ventral lateral geniculate

nucleus (Glv, located just above the optic chiasm ventromedial to the lateral anterior thalamus) and the nucleus rotundus (Rt, located in between the lateral forebrain bundle and the tectothalamic tract at the level of the anterior thalamus).

### *3.2.6 Data Analysis*

In the first study, parametric data (percentage of time awake and orexin B-ir fiber density) were analyzed using independent Student's *t* tests (two-tailed) for each measure and each brain region. In the second study, fiber density and body weights were analyzed with independent one-way ANOVAs followed by Fisher PLSD post-hoc tests. In addition, non-parametric data such as daytime and nighttime frequency of behaviors were analyzed by Mann-Whitney tests (two-tailed). Fat scores were analyzed using Kruskall-Wallis tests and followed by Mann-Whitney. Some of these data were repeated, but the animals were sacrificed across time and all comparisons were, therefore, analyzed with non-repeated statistics. In the third study, orexin B-ir fiber density in the DMP was analyzed using Student's t-test (two-tailed).

## 3.3 RESULTS

### *3.3.1 Behavior*

As previously shown [King and Farner 1963; Ramenofsky *et al*. 2003], photoperiod influenced the nocturnal activity of white-crowned sparrows. Under short photoperiods, birds showed no nocturnal migratory activity, did not perform any flights, hops or wing whirrs, and they exhibited sleep postures. In contrast, birds exposed to long photoperiods exhibited nocturnal restlessness (flights, hops or wing whirrs). In the first

study, birds displayed these behaviors approximately 70% of the time while they were videotaped before sacrifice. These animals also paced and moved their heads, but these activities were not quantified in this study. The behavioral difference between groups was statistically significant [$t(12)=6.56$, $P<0.001$ total activity] (Fig. 3.1).

In the second study, the latency to exhibit migratory behavior varied individually but the migratory substages were clearly observable. After transfer to long photoperiods, birds would start to pace, hop, and fly at night. Many flights started with bill up behavior as described in previous studies [Ramenofsky *et al*. 2003]. As the migratory substage advanced, wing whirrs were also observed. On average birds exhibited peak nocturnal activity within two to three weeks of transfer from short to long days. During the peak migration substage, animals were awake the entire time observed and wing whirring lasted the entire observation period for most individuals. Two weeks later most birds were only awake 64% of the time observed and exhibited 40 wing whirrs on average. The frequency of wing whirrs and percentage of time awake gradually decreased until there was none seen and birds would sleep during the entire observation period (Fig.3.2). Most birds ceased migratory behavior within two to three months.

Variations in nighttime activity were associated with changes in daytime activity. The intensity of the daily activity exhibited by premigratory birds changes after they start to migrate at night. During daytime, birds in the Peak migratory substage exhibited decreased frequency of flights and hops compared with birds that did not show nocturnal restlessness ($Z=2.575$, $P<0.05$; $Z=2.714$, $P<0.01$; respectively; Fig.3.3).

Migratory substages were also associated with changes in body weights and fat scores. Body weight increased 11% between the Pre and Peak migratory condition but was not significant across substages. The average fat scores increased 37% between the Pre Migration and Peak Migration $F(3, 43)=3.807$, $P<0.05$; (Fig.3.4). During Late and Post Migration fat scores decreased to levels similar to those recorded in premigratory birds under short photoperiods.

### *3.3.2 Orexin-IR*

The distribution of orexin-ir cells and fibers in white-crowned and house sparrows is similar to that in the house finch [Singletary *et al*. 2006]. Orexin B-ir neurons were located mainly along the walls of the third ventricle. They extended rostrally from the paraventricular nucleus (PVN) into the POA. At the level of the PVN, the distribution of orexin B-ir cells extended laterally into the lateral edge of the lateral hypothalamus (LHy) with a few perikarya seen ventrally towards the ventromedial hypothalamus (VMH) and some just dorsal to the LHy (Fig. 3.5). Orexin B-ir fibers were widespread throughout the brain. Innervation density was highest in the diencephalon and mesencephalon along the ventricles and midline (Fig.3. 5). A moderate to high density of orexin B-ir fibers was seen in the metencephalon and a low to moderate density of fibers was seen in the telencephalon.

In the first study, orexin B-ir innervation density was quantified in various brain areas. We did not detect significant changes in innervation in these areas (Fig 3.6). We also quantified other thalamic areas that contained dense innervation (Fig 3.6). The

dorsomedial posterior thalamus (DMP) along the midline was well innervated. We found a 50% decrease in the DMP of the LD group [$t(12)=2.50$, $P<0.05$] in one study(Fig.3.6, 3.7) but no significant difference when pooled with the data from the replication study. In comparison, there was no significant difference between groups in other midline and thalamic areas (Fig. 3.6).

Due to initial changes seen in the DMP I determined whether orexin B innervation density increases in the DMP as a function of the migratory substage. We analyzed orexin B density in the DMP of birds sacrificed during peak migratory activity, late migratory activity, and post migration. Orexin B-ir innervation of the DMP did not change significantly from one substage to another [$F(2,15)=1.627$, $P>0.1$] (Fig. 3.8).

In the third study, we quantified orexin B-ir fibers in the DMP of the non-migratory house sparrow. Orexin-B immunoreactivity throughout the brain was similar to that in the white-crowned sparrow, with cells primarily located in the paraventricular nucleus. The innervation density did not differ between SD and LD groups of house sparrows (Fig. 3.9).

**3.4 DISCUSSION**

In the white-crowned sparrow, migratory behavior is characterized by a disruption in the sleep-wake cycle. Migratory passerines show enhanced nocturnal activity (migratory restlessness) and a reduction in sleep time with short onset REM activity compared to non migratory birds [Rattenborg *et al.* 2004, Fuchs *et al.* 2006]. During the day, extended periods of drowsiness [Rattenborg *et al*. 2004] and short bouts of sleep

[Fuchs *et al*. 2006] were also reported. The migrating birds in our study showed a disruption in their sleep-wake cycles as they were active at night and exhibited less daytime locomotor activity when in migratory than non migratory condition. The behavior exhibited by birds during migration is similar to the narcoleptic-like symptoms demonstrated by mammals with low to no orexin function. As orexin stabilizes the sleep/wake cycle in mammals [Saper *et al.* 2001], we hypothesized that nocturnal migratory activity would be associated with reduced orexin innervation in particular brain regions. We found orexin fibers widespread and similarly distributed throughout the brain of the white-crowned sparrow, as is the case in other songbirds [Singletary *et al.* 2006]. The density of orexin innervation in most areas of the brain analyzed was similar in migrating and non-migrating birds. Indeed, no change in DMP orexin B innervation was found in response to transfer from SD to LD. Thus, changes in orexin B-ir in the DMP are not associated with behaviors exhibited by migratory sparrows as photoperiods change from SD to LD.

In addition, these fibers did not increase in the DMP with the termination of the behavior. It is possible that certain species have decreases in orexin expression in order to adapt to a change in season that requires different mechanisms for survival, such as an increase in vigilance or activity. Sparrows breeding at northern latitudes require longer periods of time awake [Newman *et al.* 2007] and the sparrows may maintain vigilance at night. Establishing and defending territory, mating, and tending to young when exposed to a long photoperiod may require constant random spurts of activity. It has been suggested that short naps or periods of drowsiness would be adaptive to reduce sleep

drive and cope with a disrupted sleep wake cycle [Rattenborg *et al*. 2004; Fuchs *et al*. 2006]. A continuation of the disrupted sleep/wake cycle would, therefore, be beneficial in this breeding stage as well. However, the sleep/wake cycle was no longer disrupted in the birds that we examined after termination of migratory behavior. They did not experience the natural external stimuli requiring increased vigilance or activity. They also were still capable of breeding. It is likely that the birds may need a permissive signal from final stages of the breeding condition in order to reset orexin levels, such as a peak in reproductive hormones or proximate factors such as their youth fledging [Muschamp *et al*. 2007]. At the very least, these data indicate that changes in orexin innervation of the DMP are not associated with the onset of nocturnal migratory activity or with the continuation or end of migratory activity.

Finally, we predicted that any changes in orexin B innervation in the white-crowned sparrow brain may result from a change in day length to which birds were exposed, independent of migratory activity. In sheep, photoperiod regulates orexin gene expression in the hypothalamus [Archer *et al*. 2002]. However, we note that house sparrows that were subjected to a change in photoperiod similar to that experienced by white-crowned sparrows did not show a difference in orexin B innervation in the DMP. These data suggest that orexin expression is not regulated by photoperiod in migrating or nonmigrating birds. Other brain areas will have to be measured in the future.

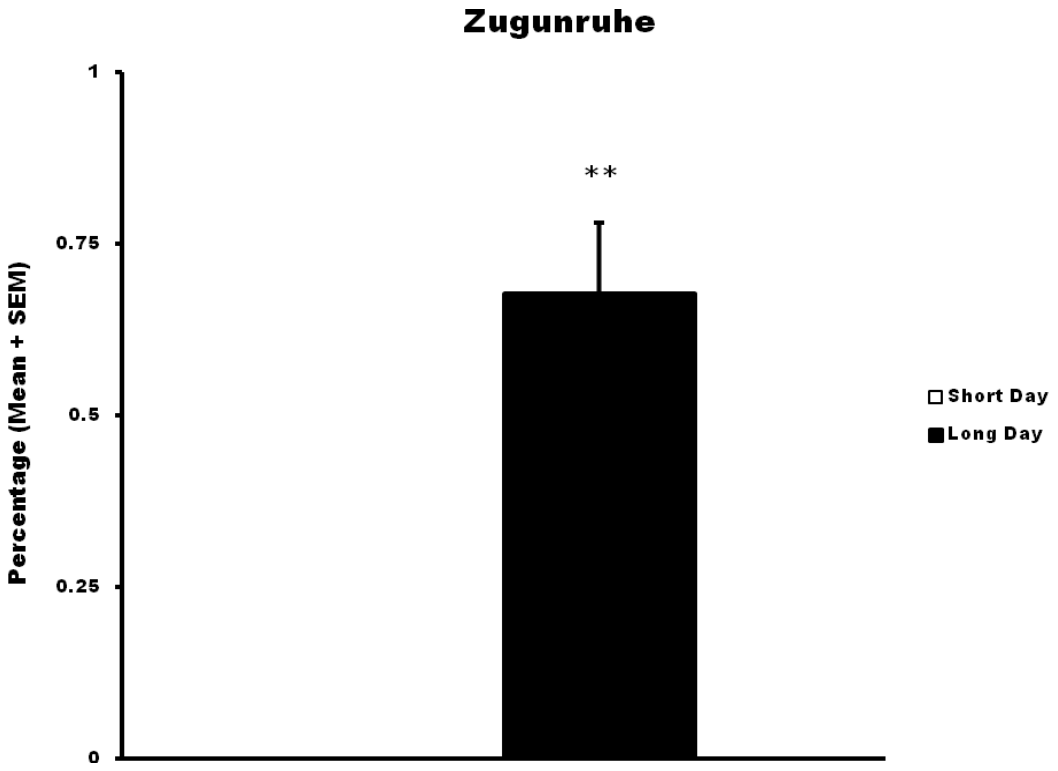
In summary, a decrease in orexin B innervation in the DMP is not associated with onset of migration. Our data has not provided a hypothesis for the mechanism underlying

the onset of migratory activity. However, the study has not ruled out the role of orexin on the avian sleep/wake cycle and will be addressed in the next chapter.

## Figure 3.1

Percentage (Mean $\pm$ SEM) of one hour samples of time that migratory restlessness (Zugunruhe) was exhibited in short or long photoperiods. Birds in short day did not display Zugunruhe.

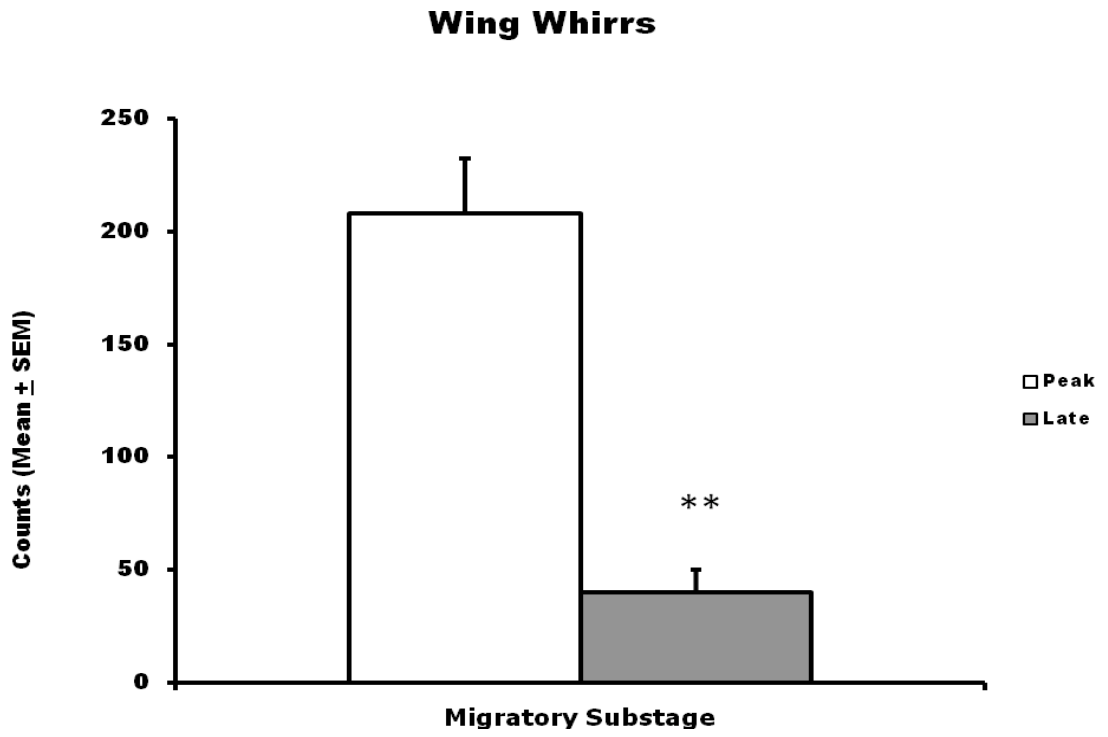
** indicates mean difference is significant at 0.001



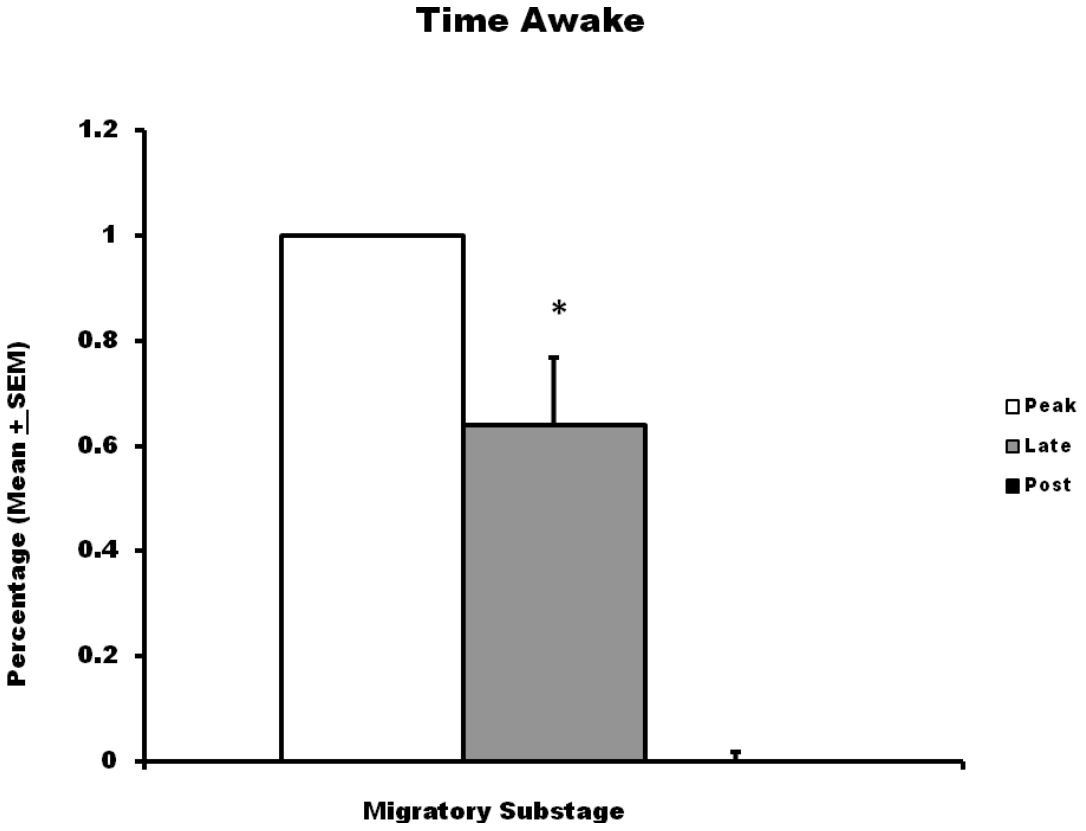
## Figure 3.2

Criteria for inclusion in substages. (A) Number of wing whirrs (Mean ± SEM) observed in one hour samples of time during Peak and Late migratory substages. No wing whirrs were observed in Pre or Post migratory substages. (B) Percentage of one-hour samples of time (Mean ± SEM) representing active or alert wakefulness as observed during the night across migratory substages. ** indicates mean difference is significant at 0.001; * indicates mean difference is significant at 0.05.

**A**



**B**

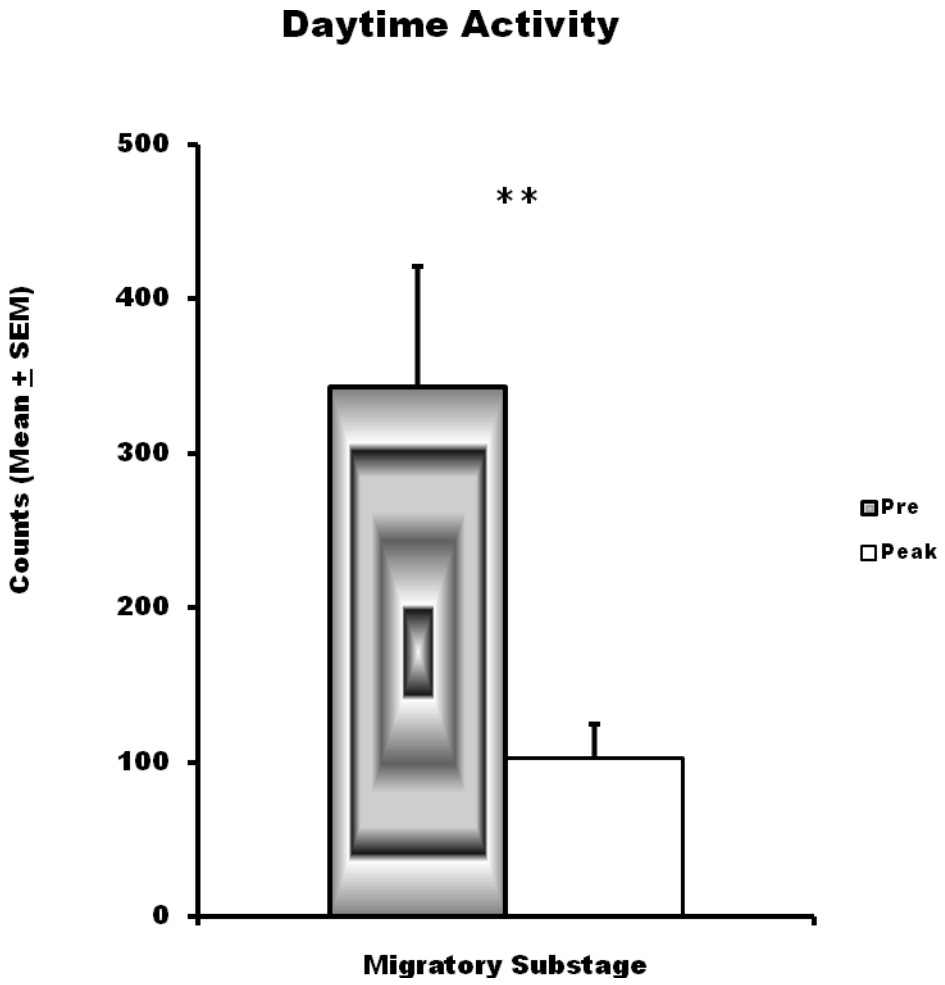

Time Awake
1.2
1
0.8
0.6
0.4
0.2
0
Percentage (Mean ± SEM)
*
Peak
Late
Post
Migratory Substage

## Figure 3.3

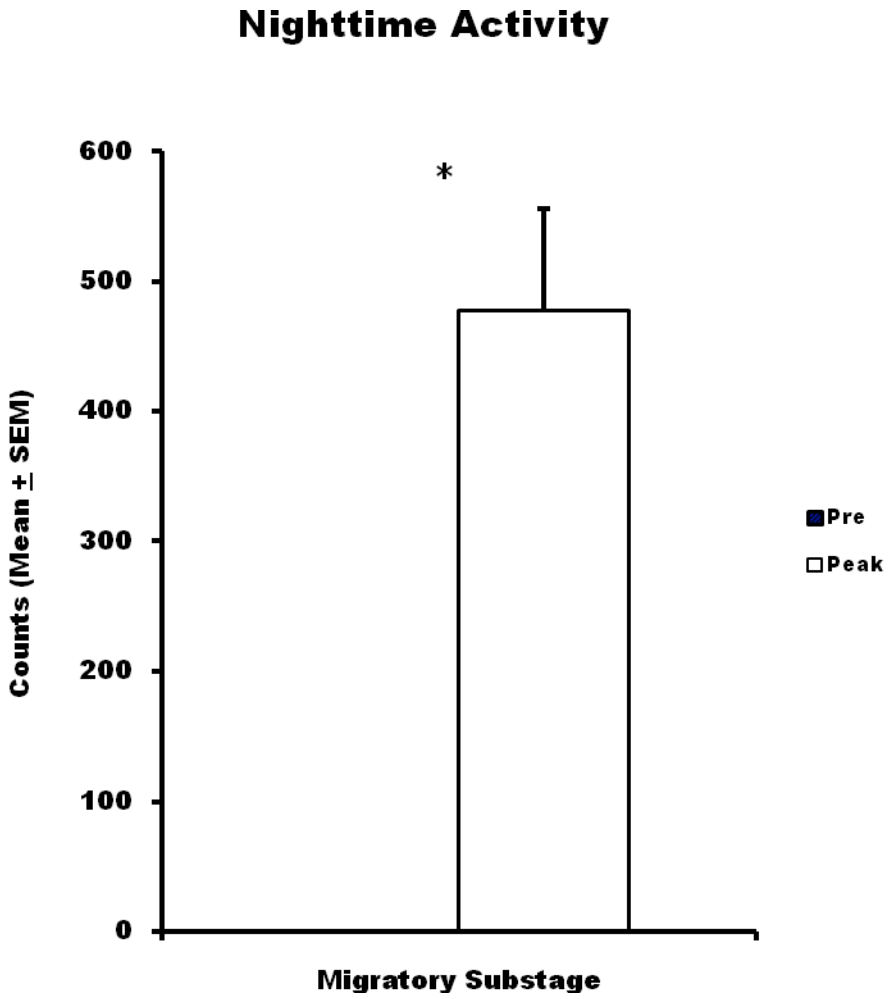
Activity measured before (Pre) and during the Peak migratory condition.

(A) Daytime activity counts (Mean ± SEM) measured as flights and hops. (B) Nighttime activity (Mean ± SEM) measured as flights and hops. ** indicates mean difference is significant at 0.001; * indicates mean difference is significant at 0.05.

**A**



**B**

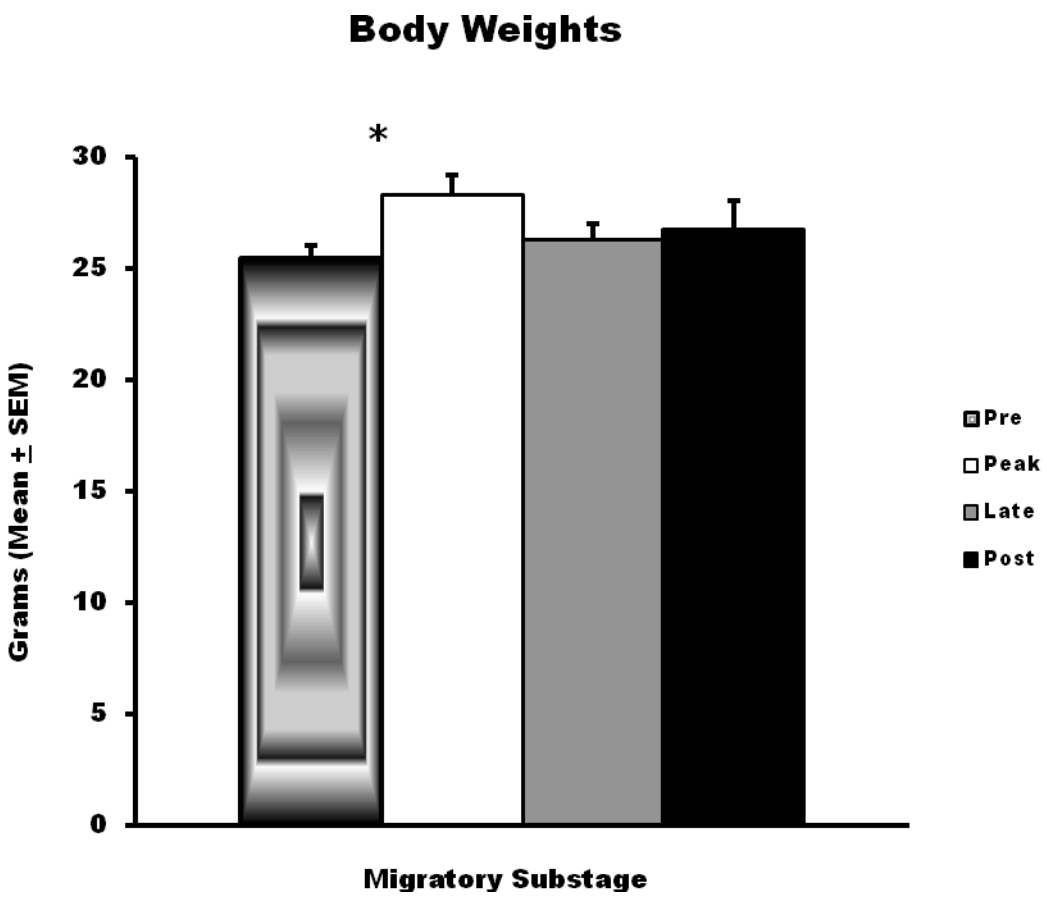

Nighttime Activity
600
500
400
300
200
100
0
*
Counts (Mean ± SEM)
Migratory Substage
Pre
Peak

## Figure 3.4

Physiological measures across migratory substages.

(A) Body weights and (B) fat scores (Mean ± SEM). * indicates mean difference is significant at 0.05.

**A**



**B**

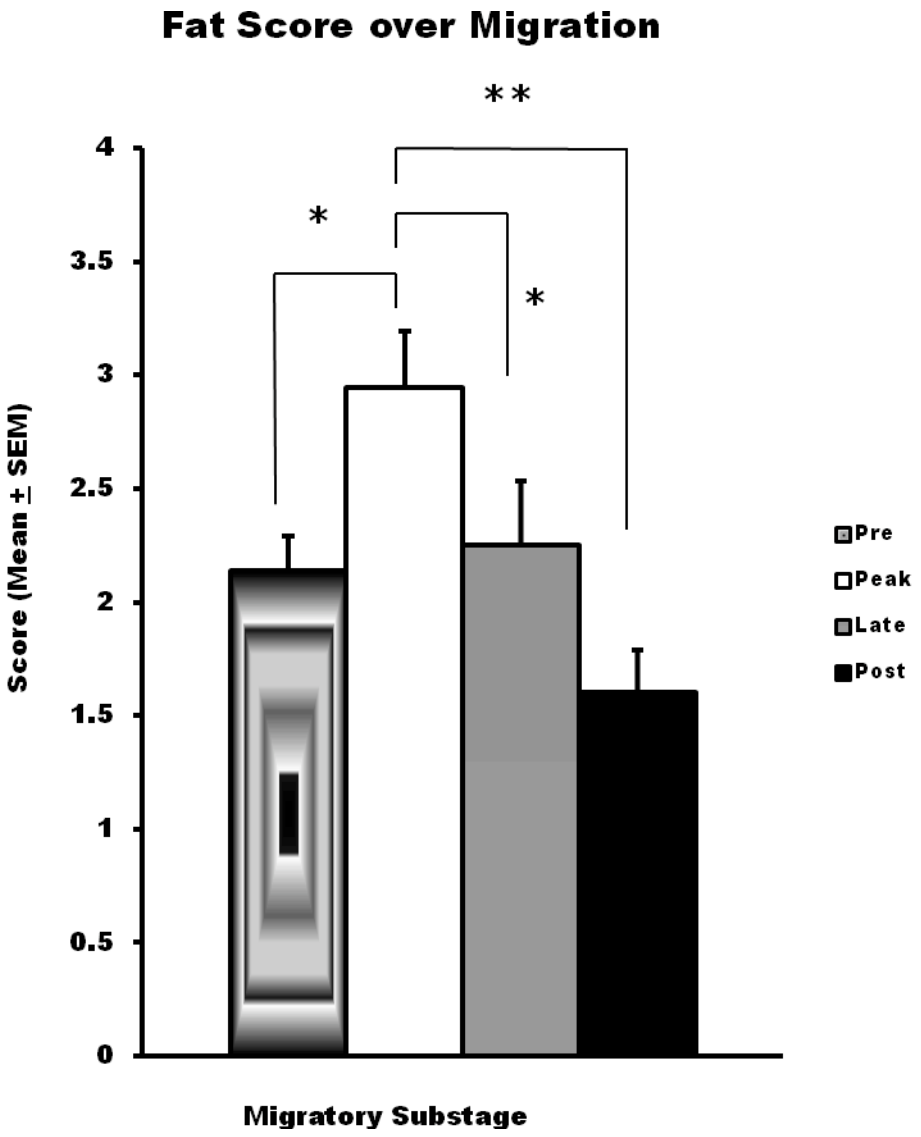

Fat Score over Migration
**
*
*
4
3.5
3
2.5
2
1.5
1
0.5
0
Score (Mean ± SEM)
Pre
Peak
Late
Post
Migratory Substage

**Figure 3.5**
Camera lucida drawings of the distribution of orexin B cells and fibers in the hypothalamus (**A)** and thalamus (**B)** of the white-crowned sparrow. Cells are shown on the right side with the nomenclature. Abbreviations: CoA, anterior commissure; DIP, dorsointermedial posterior thalamus; DMP, dorsomedial posterior thalamus; Hb, habenula; Hp, hippocampus; LHy, lateral hypothalamus; N, nidopallium; NIII, nervus oculomotorius; OM, tractus occipitomesencephalicus; PPT, pedunculopontine tegmental nucleus; PVN, paraventricular hypothalamus; TeO, optic tectum; Tu, tuberis; VMA, ventromedial thalamic area; VMH, ventromedial hypothalamus.

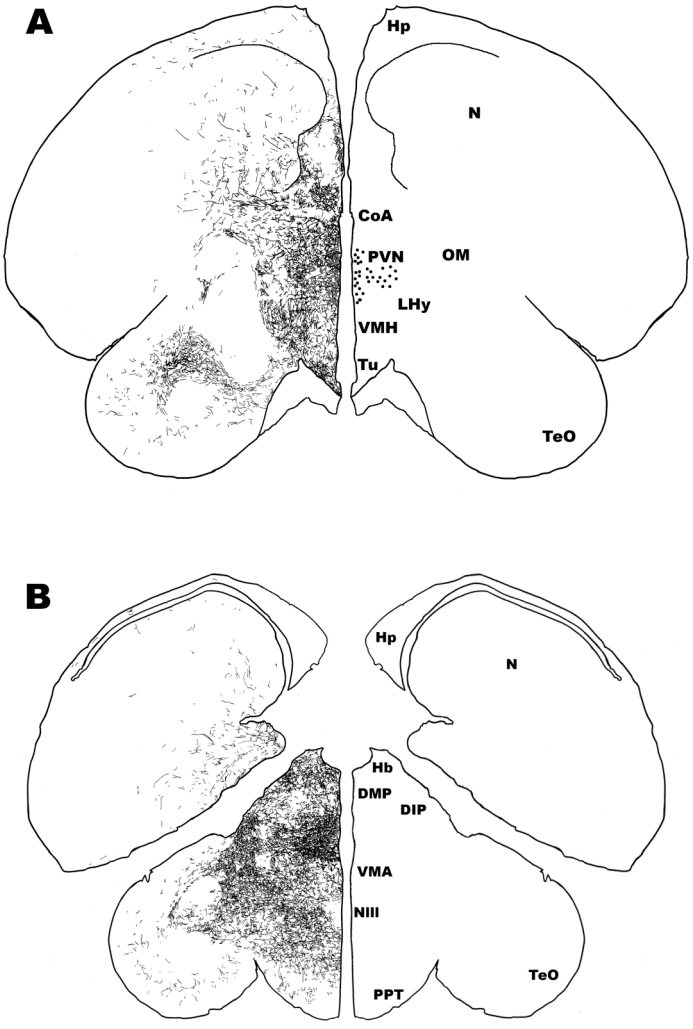
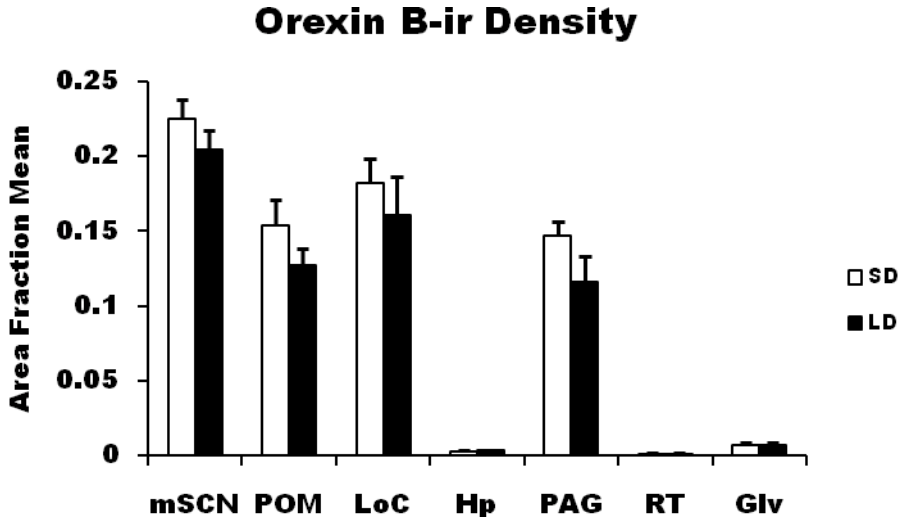
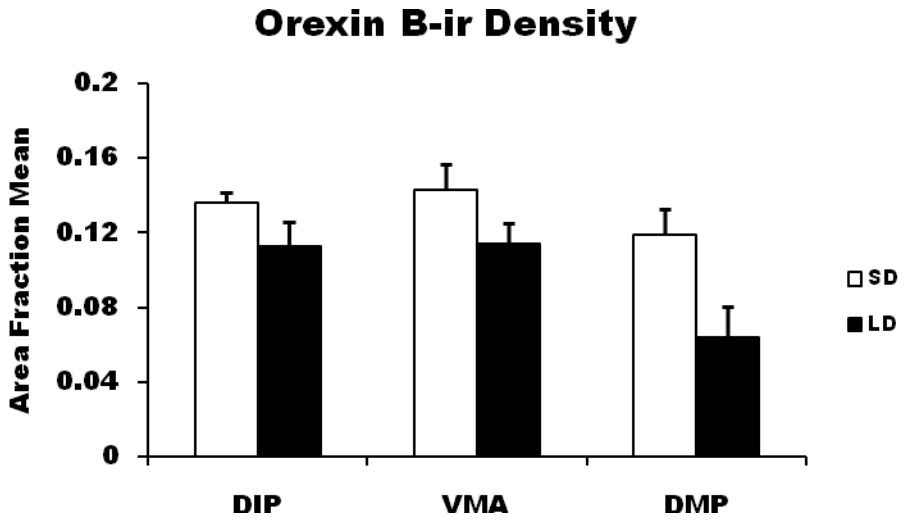


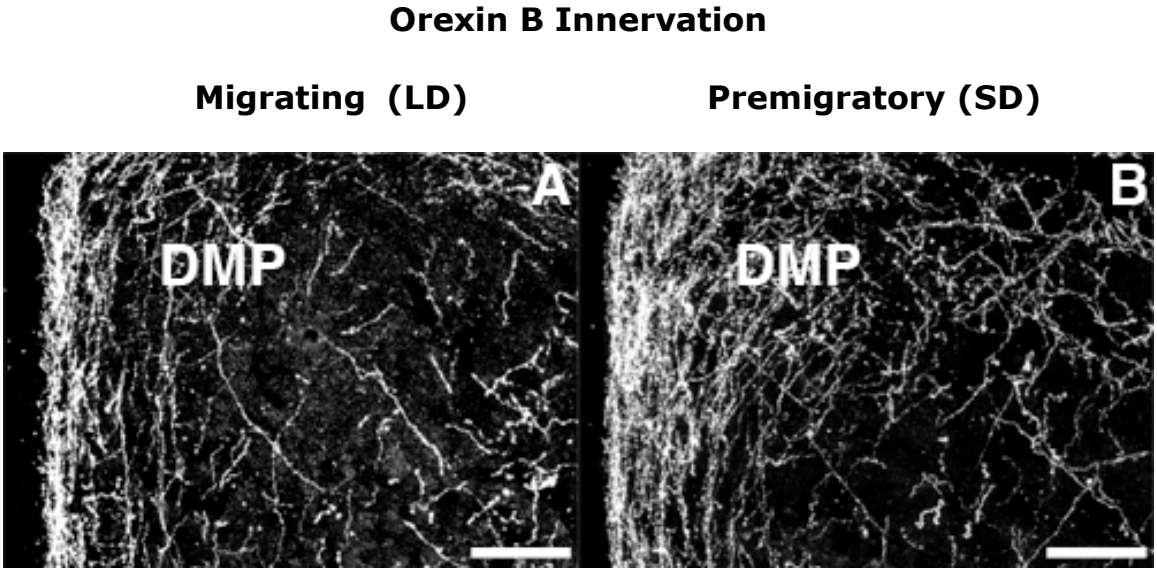
**Figure 3.6**
Comparison of orexin B innervation (Mean Area $\mu m^2 \pm$ SEM) in selected areas of the white-crowned sparrow brain between long day and short day groups (A) and (B). Abbreviations: DIP, dorsointermedial posterior thalamus; DMP, dorsomedial posterior thalamus; Glv, ventral lateral geniculate nucleus; Hp, hippocampus; LoC, locus coeruleus; mSCN, medial suprachiasmatic nucleus; PAG, periaqueductal grey; POM, medial preoptic area; Rt, nucleus rotundus; VMA, ventromedial thalamic area.
* indicates mean difference is significant at 0.05



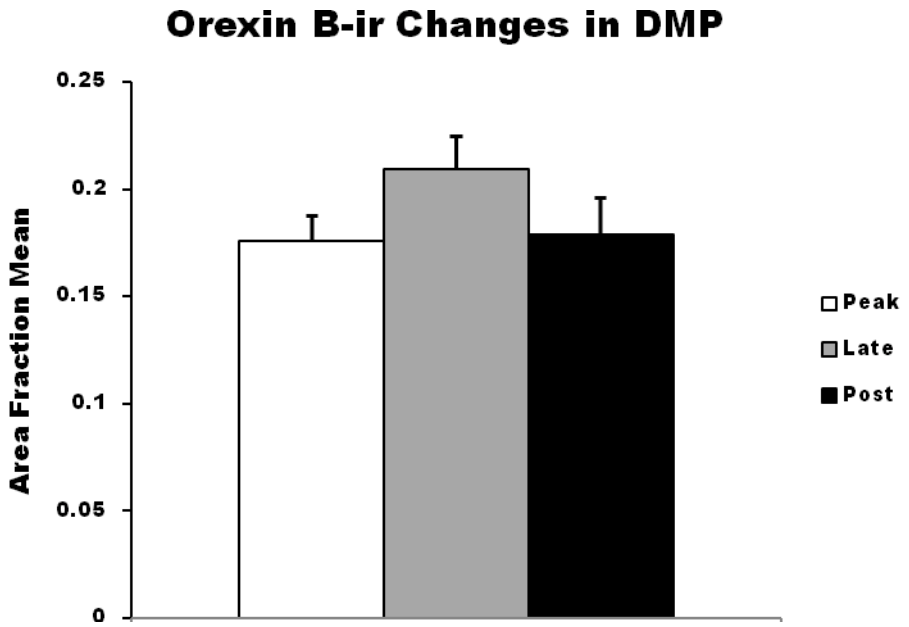
## Figure 3.7

Representative darkfield photomicrographs depicting orexin B-ir in the dorsomedial posterior thalamus (DMP) during long day (**A**) and short day (**B**) photoperiods. Scale bars: 100μm at 20X.



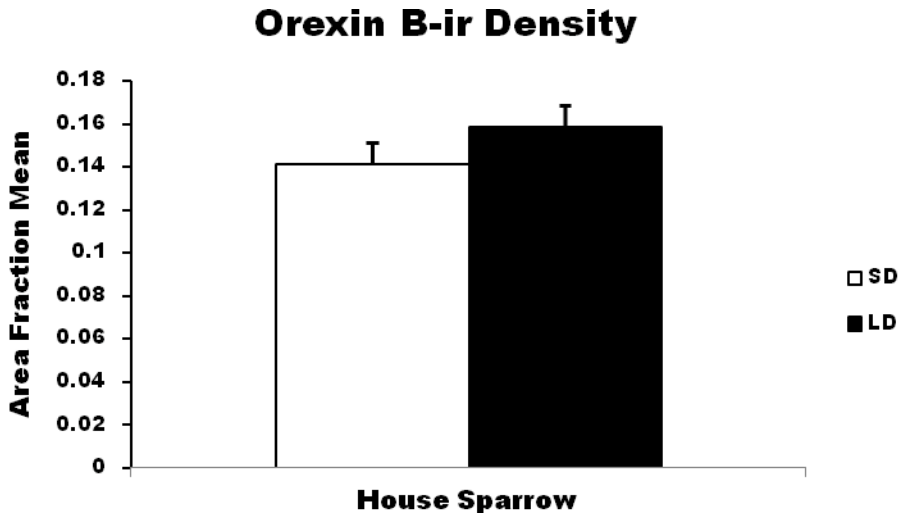
## Figure 3.8

Comparison of orexin B innervation (mean area percentage ± SEM covered with immunostained fibers) in the dorsomedial posterior thalamus (DMP) across migratory substages.



## Figure 3.9

Comparison of orexin B innervation (Mean Area Fraction $\mu m^2 \pm$ SEM) in the dorsomedial posterior thalamus of the house sparrow during long and short day photoperiods.



## Chapter 4: A Dual Orexin Receptor Antagonist Modulates the Sleep/Wake Cycle in White-crowned Sparrows


### ABSTRACT

The orexin/hypocretin system modulates the sleep/wake cycle in mammals but little is known of the function of orexin in birds. Recently in pigeons, orexin A injections had arousing effects and decreased sleep postures. We hypothesized that a dual orexin receptor antagonist would increase sleep postures and decrease daytime activity in sparrows. Additionally, we predicted a disturbance of nighttime sleep resulting in the expression of nocturnal migratory behavior. White-crowned sparrows kept in a short day photoperiod (8L:16D) were injected intramuscularly with a low (6mg/kg) or a high (30mg/kg) dose of Almorexant. Sleep postures and daytime activity were monitored by video and behavior was analyzed. We compared behaviors immediately after injection and throughout the day and night. Additionally, sparrows switched to a long day photoperiod were given oral administration and analyzed.

There was a significant increase in sleep postures and decrease in activity exhibited by the sparrows in the high dose group in the first thirty minutes after injection ($P<0.05$). Total activity and sleep postures did not significantly change during the day or night. These results indicate that the orexin system is not involved in the initiation of migratory activity. This study does indicate the orexin system may modulate the sleep/wake cycle similarly in mammals and birds.

## 4.1 INTRODUCTION

Few studies have examined the function of the orexin system [Dube et al. 1999] in birds and are focused mainly on orexigenic effects based on what has been found in mammals. Though orexin A is thought to be orexigenic in mammals, no avian studies have been able to conclude this role for orexins [Ohkubo et al. 2002; Furuse et al. 1999]. However synthetic mammalian orexin A injections did have arousing effects and decreased sleep postures in pigeons [Simao da Silva et al. 2008]. As such it is likely that the orexin system does modulate wakefulness in birds. Manipulating the orexin system in mammals can alter wakefulness. Humans receiving a dual competitive orexin receptor antagonist (Almorexant, Actelion, France) displayed more sleepiness and less wakefulness within the first hour after administration. In vitro Almorexant blocks intracellular calcium increases in cells overexpressing orexin receptors when administered with orexin. This dual antagonist is a non-peptide that crosses the blood brain barrier so peripheral injections can be done or can be used orally without invasive stereotaxic equipment [Brisbare-Roch *et al.* 2007]. This dual antagonist would be a good tool to test our predictions in birds. We hypothesized that a dual orexin receptor antagonist would increase sleep postures and decrease daytime activity in white-crowned sparrows.

As we have shown in Chapter three, transfer from short day- to long day-photoperiods induces migratory activity in captive songbirds and a disruption of the sleep/wake cycle [Rattenborg *et al*. 2004; Fuchs *et al*. 2006]. The sleep disruption of a migrating passerine is reminiscent of narcoleptic-like behavior due to a lack of orexin

expression, so it is hypothesized that administration of a dual orexin receptor antagonist will initiate the beginning of Zugunruhe outside of the influence of photoperiod.

## 4.2 MATERIALS AND METHODS

### *4.2.1 Animals and treatment*

Adult white-crowned sparrows (*Zonotrichia leucophrys leucophrys;* n=33 were caught using seed-baited potter traps (Country Cages, Penngrove, CA) in Austin, Texas (latitude: 30.17 N; longitude: 97.44 W) in February. This species adapts well to captivity and exhibits migratory restlessness when exposed to a long photoperiod simulating spring [Farner, 1950; Ramenofsky *et al.* 2003]. Sparrows were brought to the laboratory and housed individually (cage size: 16"w x 11" d x 21"h) in visual and auditory contact under a short day photoperiod (8L:16D). Birds received Home Grown™ chow (Country Acres Feed Company, Brentwood, MO), white millet (Tomlinson's, Austin, TX) and water *ad libitum*. Temperature in the animal room was approximately 65°F. All sparrows were allowed to acclimate for three months under short days before data collection. Experimental procedures were approved by the University of Texas Institutional Animal Care and Use Committee and the animals were housed in an AALAC-accredited facility.

### *4.2.2 Experimental Design*

In the first study, after the period of acclimation, four groups of white-crowned sparrows (n=8 per dose group) were injected intramuscularly for five consecutive days while subjected to short day photoperiod. Groups were balanced by weight. During

short photoperiod, 4 groups received either no dose, a low dose 0.15mg/25g, high dose 0.75mg/25g, or pH matched saline vehicle in the morning 2 hours after lights on. Activity, sleep postures, and ingestive behaviors were analyzed and recorded immediately after administration and during the rest of the day and night.

In the second study, 2 groups were given a month washout, reassigned to new groups (n=8 per dose group) and then transferred from short to long day photoperiods (from 8L:16D to 16L:8D) to induce migratory activity. Groups received an oral administration of a high dose 0.75mg/25g or saline vehicle in the morning 2 hours after lights on and also recorded as in the first study.

Body weights, fat scores and food consumption were recorded in both studies. Subcutaneous fat deposits can be easily seen and measured by blowing on the feathers to expose the furcular region. The scale used ranged from 0-5 where 0 indicated no fat in the furculum, 1 some fat, 2 the furculum was lined with fat, 3 the fat filled the furculum to slightly concave, 4 the furculum is flush with fat and 5 is bulging over [Wingfield and Farner, 1978].

### *4.2.3 Behavior*

White-crowned sparrows were videotaped with Sony digital video cameras during the day and at night under infrared illumination (Super Nightshot and Nightshot Plus with additional infrared lights if necessary depending on the camera). Four camera signals were routed via RCA cable to a surveillance digital video recorder with screen splitter. Analog signal was converted to a digital signal as an MPEG2 and burned to DVD-R Video was manually analyzed by researchers blind to groups using an XBOX 360 which

allowed for panning and zooming in on the birds and controlling brightness. A 48" plasma TV (Samsung) also helped improve behavior analysis. Behavior was analyzed as duration of time active or sleeping for twenty sequential minutes after administration and an average of five minute samples of duration over the rest of the day and night. The twenty minute behavioral analysis started ten minutes after the researcher left the animal room. The active behavior was defined as any high or low activity such as flights, hops, pacing, preening, and head movements. The sleep behavior was characterized by puffy round/circular body shape, ruffled feathers, head facing forward or head facing backwards under wing. Legs are not visible and the eyes are closed. Birds perched immobile before exhibiting a sleep posture or in a sleep posture but with eyes open are considered as drowsy.

Ingestive behaviors include drinking and feeding. Feeding was recorded as time spent at feeder. These behaviors are separated based on previous studies showing leptin analogs affecting food intake and ingestive behaviors (Dridi et al. 2000). Drinking was recorded as counts of dip and billups (swallowing) combined.

#### *4.2.4 Data Analysis*

The Stopwatch software (CBN, Atlanta) was used to quantify behaviors. In examining the effect of antagonists compared with vehicle or non-handled, parametric data were analyzed using one-way ANOVAs followed by LSD post-hoc tests for each behavioral and physiological measure. $P<0.05$ was accepted as significant and $P\leq 0.1$ was considered a trend.

## 4.3 RESULTS

### *4.3.1 Short Day Behavior*

No Zugunruhe was observed in any bird in the SD. In the sparrows exposed to short day photoperiod (SD) there was a significant increase in duration of sleep postures compared to vehicle within the first thirty minutes after high dose injection (P=0.018) but not low dose (P=0.543) (Fig.4.1B). There was also a significant decrease in duration of awake behaviors immediately after high dose injection (P=0.044) but not low dose (P=0.141) (Fig.4.1A). There was a significant decrease in drowsiness in the low dose group (P=0.015) (Fig.4.2).

Total awake behaviors and sleep postures did not significantly change over the next 24hrs. during the day or night for the animals receiving high doses of orexin receptor antagonists. There was a trend of decrease, however in wakefulness during the day (p=0.068) and a trend of increase in sleep postures during the day (p=0.054).

In the low dose group there was a significant decrease in drowsiness during the day (p=0.0006) (Fig.4.2). At night there was a significant increase in awake behavior (p=0.024) (Fig.4.3) and decrease in sleep postures (p=0.018) in the low dose. The awake behavior seen here consisted of head movements, preening and an occasional hop.

### *4.3.2 Long Day Behavior*

Behavior was analyzed after one day of exposure to long day photoperiod (LD) and after five days of exposure to LD with the antagonists being orally administered every day. After the first day of LD exposure and within the first thirty minutes of administration a significant decrease in awake behaviors (p=0.01) and increase in sleep

postures was observed (p=0.01) (Fig. 4.4A; 4.4B). During the day the wakefulness remained low compared to vehicle (p=0.001) and sleep postures remained high (p=0.002) (Fig. 4.5A, 4.5B). During the night there were no differences in behavior as all birds slept.

In the sparrows exposed for five days to LD there was no significant increase in sleep postures or awake behavior compared to vehicle within the first thirty minutes after oral administration. However total wakefulness during the day decreased significantly (p=0.006) and sleep postures significantly increased (p=0.007) (Fig. 4.6A, 4.6B). During the night there were no significant differences in behaviors. Only one bird exhibited Zugunruhe but was in the non-handled group.

During the SD there was no significant difference in time spent at the feeder or counts of drinking between groups within the first thirty minutes after injection. After the first day of LD exposure there was no significant differences in drinking or duration at the feeder after administration, although a trend of decrease is seen in the high dose compared to vehicle (p=0.07). After five days of LD exposure and within the first thirty minutes of oral administration there was no significant difference compared to vehicle. In comparison to non-handled however, experimental groups were significantly decreased (p=0.004) (Fig.4.7).

### *4.3.3 Physiology*

During the SD or LD there was no significant change in the weight of food consumed between groups (data not shown). There was no significant percentage change

in body weight from baseline to after the first injection day or baseline to 5 days of injection compared to vehicle in the SD groups. During the LD on the first day after oral administration, body weight significantly decreased in high dose group compared to vehicle ($p=0.004$). After four days of administration in LD this significant decrease in body weights was still observed in the experimental group ($p=0.004$) (Fig.4.8).

## 4.4 DISCUSSION

The results from this study do not support a role for the orexin system in initiating migratory activity in white-crowned sparrows. However, this study indicates the orexin system may modulate the sleep/wake cycle similarly in mammals and birds. Additionally, these data support previous findings in pigeons suggesting the orexin system is involved in modulating arousal [Simao da Silva et al. 2008]. Using a mammalian dual orexin receptor antagonist, we were also unable to find a role for the orexin system in regulating ingestive behaviors. Although only one orexin receptor has been found in birds, using an antagonist that blocks both mammalian orexin receptors can help to elucidate the role of the orexin system in the sleep/wake cycle of birds.

During the SD, intramuscular injections in the morning increased sleep postures and decreased awake behaviors within the first thirty minutes in the high dose group. This effect was not maintained during the day and returned to baseline within three hours. These results are similar to what is seen in mammals given this dose of Almorexant, but the effect is more transient in our birds [Brisbare-Roch et al. 2007]. This is likely due to the higher metabolism of passerine migratory birds [Jetz et al. 2008] and injections allow

quick entry into the bloodstream. Drowsiness significantly decreased in the low dose group but no other behaviors significantly changed. When compared to non-handled however there was no significant difference in drowsiness behavior. Additionally the low dose group was significantly more awake and exhibited less sleep postures than vehicle at night.

During the LD, after the first day of LD exposure, oral administration increased sleep postures and decreased awake behaviors within the first thirty minutes. Interestingly this effect was maintained for eight hours and closely resembled the results seen in mammals. This could be explained by an oral administration as opposed to an IM injection, the possibility that the orexin system changes in response to an increased photoperiod or a combination of the two. Oral administration is the method used by the Actelion researchers, so this is a likely explanation. In addition, the orexin system is known to change seasonally in mammals [Archer et al. 2002] and other neuropeptide systems in birds differentially respond to changes in photoperiods [Richardson et al. 1995].

After the fourth day of LD exposure and the fourth sequential day of oral administration, there was no significant increase in sleep postures and no decrease in awake behaviors within the first thirty minutes. However throughout the rest of the day there was a significant decrease in wakefulness and increase in sleep behavior. This lack of initial effect may be due to morning sleep inertia experienced by all groups as the birds' hormonal and body temperature circadian rhythms may not be synchronized to the photoperiod yet. The significant increase in sleep and decrease in wakefulness in the

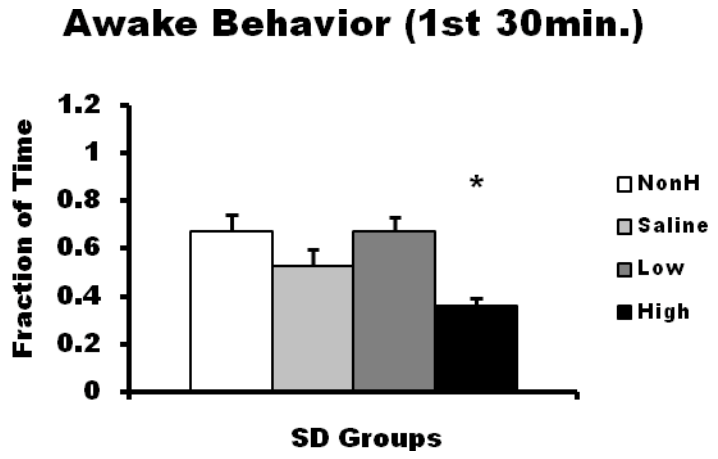
experimental group was seen the very next hour and maintained for about eight hours later. No significant differences were seen in nighttime behaviors. This is consistent with the findings in mammals whether administered at the beginning or end of wake period [Brisbare-Roch et al. 2007].

We hypothesized that blocking the orexin system would disrupt the white-crowned sparrow's sleep during SD and lead to Zugunruhe. This did not happen so we can conclude that blocking the orexin system with a mammalian dual orexin receptor antagonist does not initiate migratory activity. It is likely the antagonist does block the avian orexin receptor as we saw a decrease in wakefulness during the active period. We could not rule out the possibility that the blocking of the orexin system itself may not be sufficient to initiate migratory behavior but first requires activation by a change in photoperiod. We switched the photoperiod to LD and administration on the very next day yielded no Zugunruhe, nor four days of administration afterwards. Once again wakefulness was suppressed during the wake period. It is possible that afternoon administrations before the sleep period might induce Zugunruhe but Almorexant given to rats and dogs before their sleep period resulted in no behavioral change [Brisbare-Roch et al. 2007]. Also, in mammals orexin levels in the CSF are at minimal levels at the end of the sleep period and maximum at the end of the wake active period. Blocking the receptors would be most effective in the beginning of the wake period. It is likely the avian orexin CSF levels have a similar diurnal rhythm, but continuous administration using orexin receptor antagonist implants would circumvent this unknown.

Additionally, in SD our evidence does not indicate the orexin system as a modulator of ingestive behaviors. In LD, white-crowned sparrows become hyperphagic and gain weight. However, after 4 days of exposure to LD and four sequential days of oral administration the sparrows were significantly less in weight in comparison to vehicle. Time spent at the feeder, food consumption and drinking counts did not differ between groups. The duration of time spent foraging on the ground was not assessed, which would account for more energy expenditure and would not be fruitful for the birds as the cage floor was wire and would not hold seed accessible. In seasonal animals energy expenditure and metabolism changes depending on the photoperiod. Studies show increased resistance or increased sensitivity to metabolic hormones seasonally depending on the species [Morgan and Mercer 2001]. The metabolic hormone leptin is secreted by fat cells, is an indicator of energy balance and regulates metabolism depending on fat deposition. In Siberian hamsters leptin signaling is overridden in a short photoperiod. In sheep, leptin is transported across the blood-brain barrier differentially depending on the photoperiod [Adam *et al*. 2006]. The orexin system may also be regulated differentially depending on photoperiod. Another example, during LD research has shown that WCS are more sensitive to exogenous NPY resulting in an increase in feeding [Richardson *et al.* 1995]. Combined with previous research it is likely that the orexin system is not involved in feeding during SD in birds, but may be involved in energy storage during the LD in migratory white-crown sparrows. We tested the interaction between energy storage, migration, and orexin in Chapter Five.

**Figure 4.1**
**Comparison of fraction of time awake (A) (recorded as daytime flights and hops) and sleep postures (B) (recorded as sleep postures) in the first thirty minutes after morning injection of Almorexant (6or30mg/kg) or vehicle. *=P<0.05**

**A**




**B**

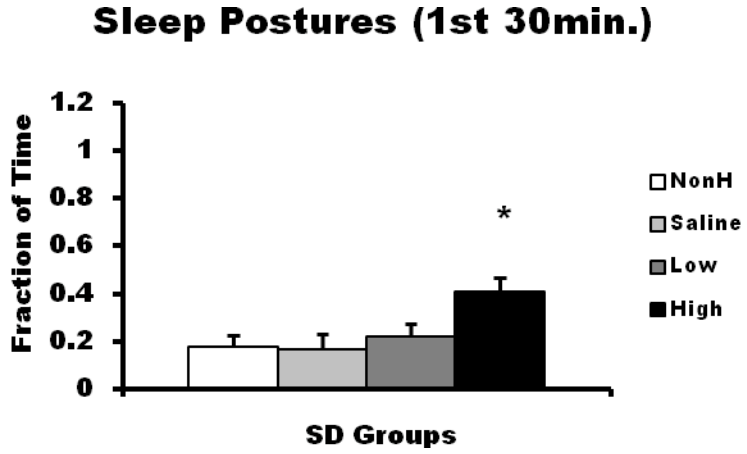
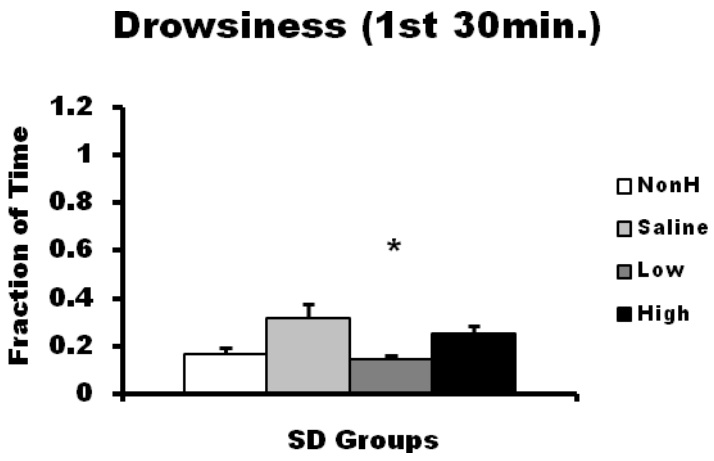


**Figure 4.2**

**Comparison of fraction of time spent in a drowsy state in the first thirty minutes (A) or sampled across the day (B) after morning injection of Almorexant (6or30mg/kg) or vehicle. *=P<0.05**

**A**




**B**

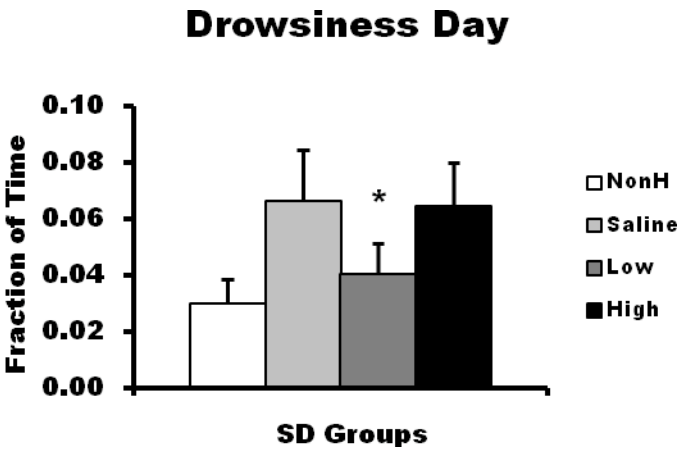


**Figure 4.3**

**Comparison of nighttime awake behaviors (analyzed for 5min. every other hour and averaged) after morning injection of Almorexant (6 or 30mg/kg) or vehicle.**

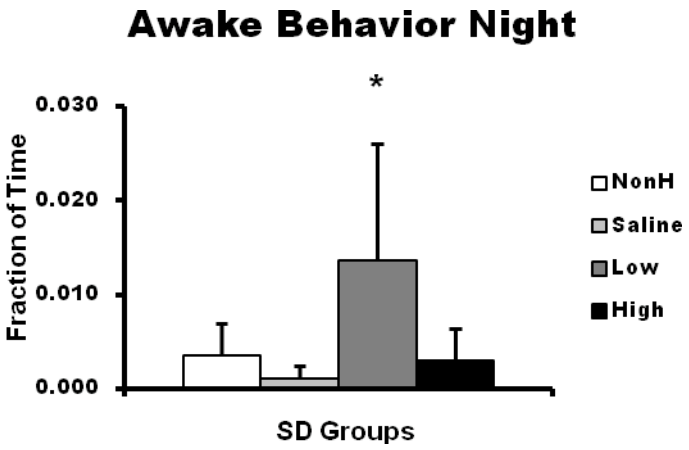
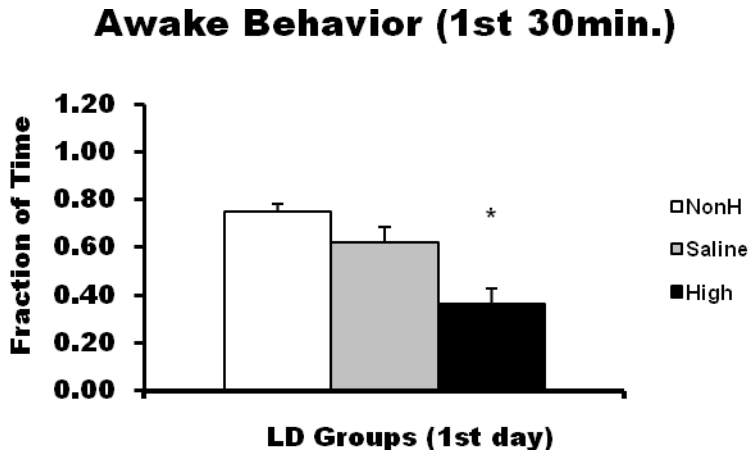


**Figure 4.4**
**Comparison of the fraction of time awake (A) (recorded as daytime flights and hops) and sleep postures (B) (recorded as sleep postures) in the first thirty minutes after morning administration of Almorexant (30mg/kg) or vehicle. *=P<0.05**

**A**




**B**

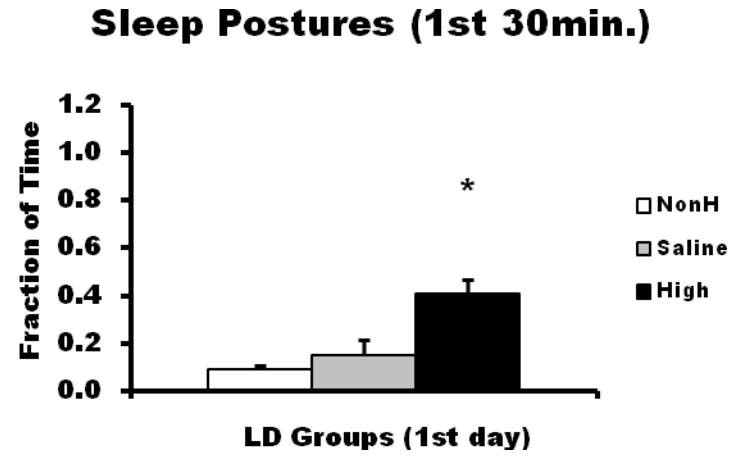
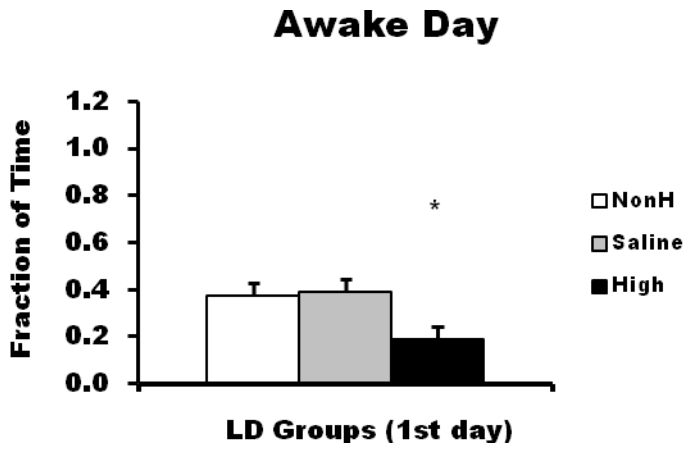


**Figure 4.5**
**Comparison of fraction of time exhibiting daytime awake behaviors (A) and sleep postures (B) (analyzed for 5min. every other hour and averaged) after morning administration of Almorexant (30mg/kg) or vehicle, and after one day of LD exposure.**

**A**




**B**

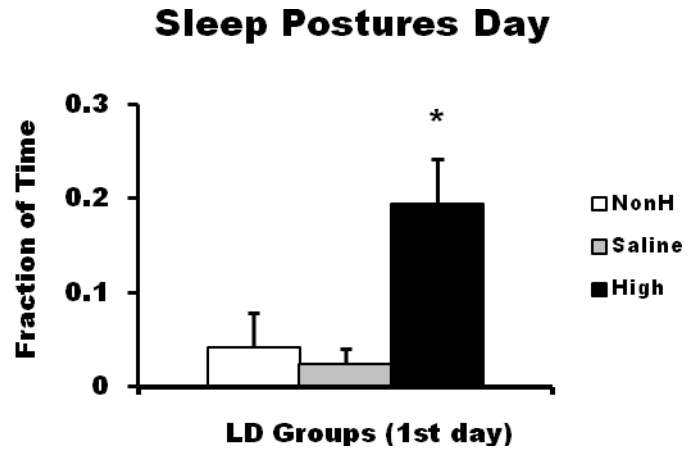


**Figure 4.6**
**Comparison of fraction of time exhibiting daytime awake behaviors (A) and sleep postures (B) (analyzed for 5min. every other hour and averaged) after morning administration of Almorexant (30mg/kg) or vehicle, and after four days of LD exposure.**
**A**

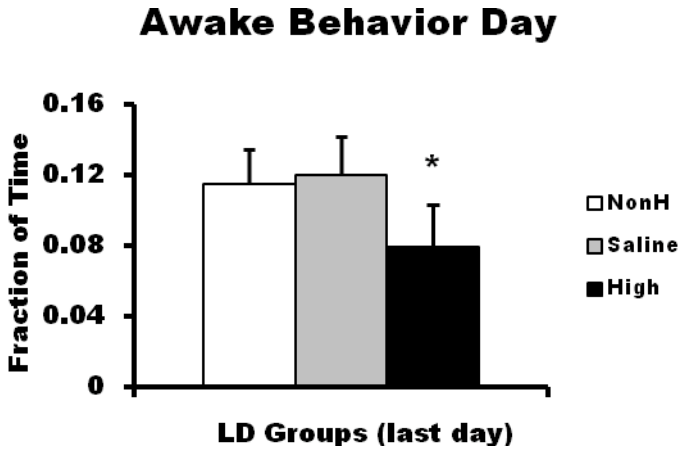



**Figure 4.6B**

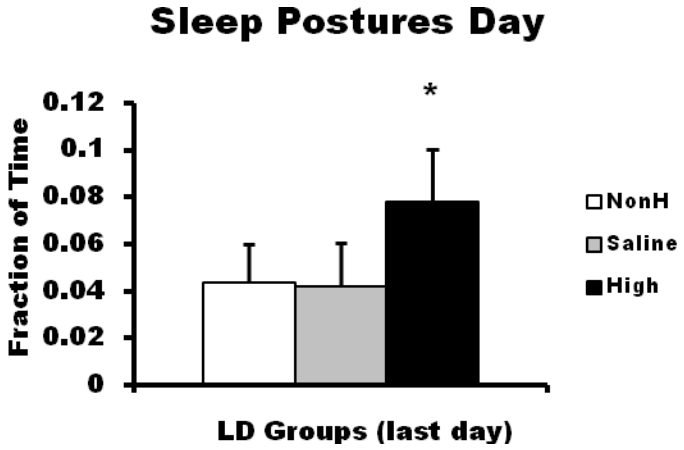


**Figure 4.7**
**Comparison of the fraction of time spent at the feeder.**

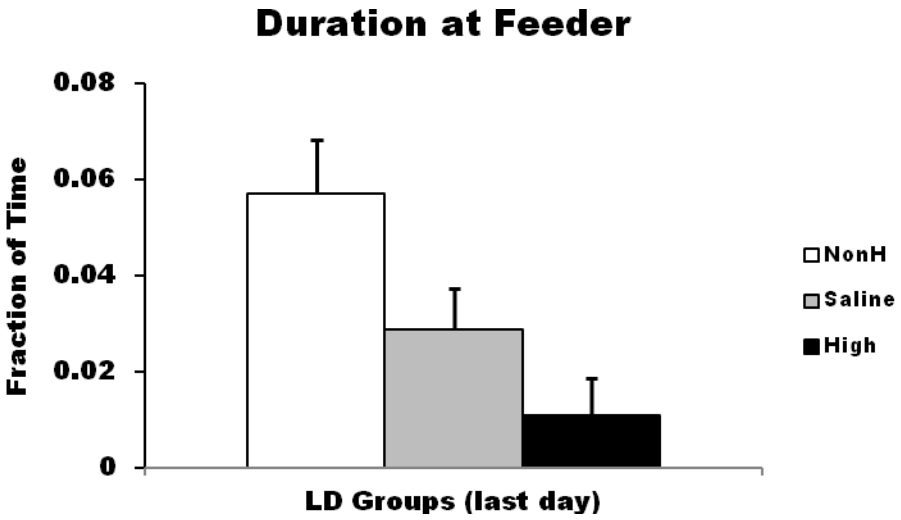
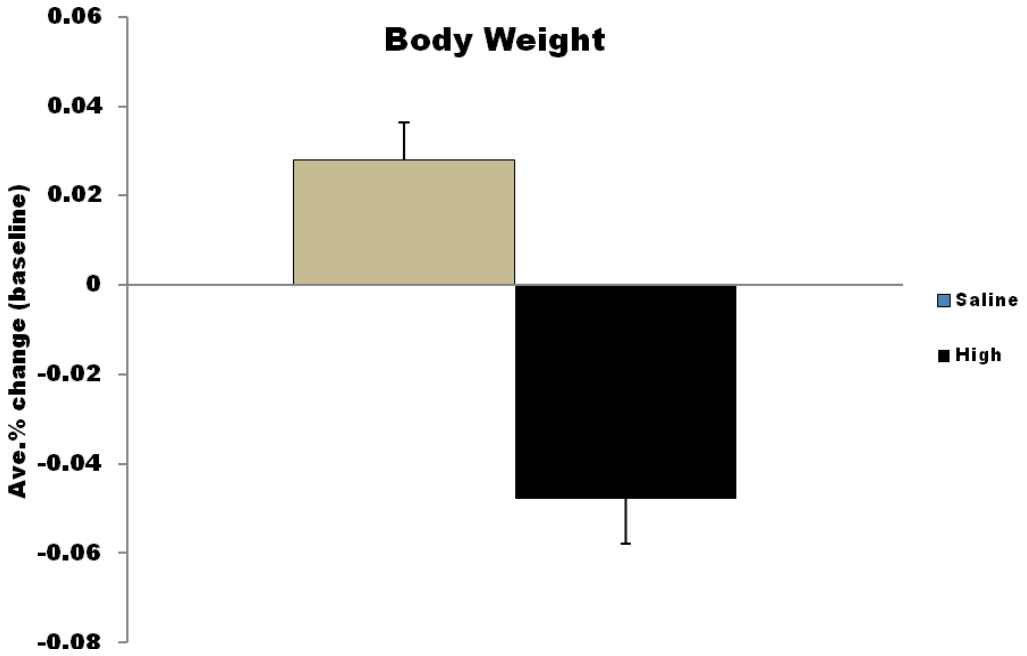



**Figure 4.8**
**Comparison of percentage of change from baseline body weight.**



## Chapter 5: Fuel Availability and Altered Sleep/Wake Cycles in Migrating Sparrows


### ABSTRACT

During the migration season, typically diurnal songbirds show extensive disruption of their sleep/wake cycles, sleeping during the day and flying at night. Along the way, sparrows make stopovers to refuel and rest, assuming a temporary diurnal activity cycle. Fuel availability is a critical factor in the initiation and maintenance of migration in certain species, and may modulate neuroendocrine systems associated with the sleep/wake cycle such as leptin or orexin. This was tested in Harris's sparrows (*Zonotrichia querula)* by changing food quality during their spring migration. We predicted a decrease in food quality (protein and fat content) would inhibit fat deposition, dampen migratory activity, trigger an extended stopover and temporarily restore a diurnal sleep/wake cycle. In mammals, leptin is secreted from adipocytes and inhibits orexin and neuropeptide Y. As a reduction in orexin expression is associated with disruptions in the sleep/wake cycle and metabolism in mammals, it is a likely candidate to examine in the regulation of avian nocturnal migration. We also predicted that inhibition of migratory activity by reduced fuel availability would be associated with increased orexin innervation in specific parts of the brain.

Within a few weeks, exposure to a low protein/fat diet resulted in decreased fat mass and inhibition of migratory activity as compared to controls given enriched diets. Interestingly, animals that stopped migrating reverted to a diurnal cycle of activity, while

migrating controls maintained altered sleep/wake cycles. Additionally we examined the nocturnal activity of another sparrow species, the white-crowned sparrow (*Zonotrichia leucophrys leucophrys)* under the same conditions. These data support the relationship between migratory activity, fuel availability and altered sleep/wake cycles in songbirds.

## 5.1 INTRODUCTION

Nocturnally migrating birds experience disrupted sleep/wake cycles in spring. Daytime activity decreases and nighttime activity and arousals increase compared to winter. They exhibit this migratory restlessness (Zugunruhe) when exposed to a longer photoperiod. Photoperiod change is the primary environmental cue that signals the beginning of physiological and behavioral changes. These changes include diet preference change, hyperphagia and an increase in body weight and fat mass. The disruption of the sleep/wake cycle ensues with the onset of migratory flight. The Harris' sparrow is a long distance migrant and increases in weight by at least 30% before vernal migration. They winter in the southern and midwestern United States and during breeding season these sparrows can be found in Canada to the edge of the tundra. After initiation of the migratory condition, sparrows make stopovers to refuel and rest along the way, temporarily assuming a diurnal sleep/wake cycle until ready to migrate again. This suggests that the Harris' sparrow sleep/wake cycle is variable and closely associated to metabolic hormones or orexigenic neuropeptides.

Preliminary work in our lab for a separate study showed that Harris' sparrows given a winter typical millet diet before and after transfer to a simulated spring

photoperiod did not gain weight or increase in fat deposition. They did not enter Zugunruhe but the white-crowned sparrows (WCS) in the same room on the same diet began exhibiting Zugunruhe regardless of minimal weight gain. When higher fat, high protein chow was added to the diet, the Harris' sparrow began gaining weight and fat. Within a week of chow supplement the Harris' sparrows started to exhibit Zugunruhe (unpublished observations). These sparrows exhibited a high dependence on fuel quality and energy stores for the initiation of Zugunruhe. We considered that these sparrows would be a good model to test the metabolic requirements required for migratory restlessness.

The importance of body composition is seen in other migratory bird research. A study looking at three different species of *Zonotrichia* showed that all Harris' deposited fat before they exhibited Zugurnruhe in contrast to only 80% of golden-crowned sparrows and 25% of the WCS in the study. Additionally, WCS given a restricted access diet before and after photoperiod change exhibited a decrease in Zugunruhe intensity but not abolition, in comparison to ad libitum controls [King and Farner 1963]. In the white-throated sparrow, diet quality is important for quick recovery of body mass at a stopover site [Pierce and McWilliams 2004]. Associations of body composition and initiation or resumption of migratory activity vary depending on the species and migratory state [Biebach 1985; Fusani *et al.* 2008]

Orexin and leptin have both been studied in mammals as involved with feeding and the sleep/wake cycle. Orexin expression is down regulated by leptin, a 16kDa, 146 amino acid circulating hormone secreted from adipose tissue [Lopez *et al.* 2000]. Leptin

is involved in the sleep/wake cycle as direct and indirect inputs to sleep/wake and circadian areas of mammals [Laposky *et al.* 2005; Ahima *et al.* 2000]. Additionally, leptin levels are disrupted in narcoleptic individuals. In mammals, leptin increases energy expenditure [Levin *et al.* 1996; Houseknecht and Portocarrero 1998] and stimulates the sympathetic nervous system [Reidy and Weber 2000]. Orexin and leptin are also known to be reciprocally inhibiting thus we propose that the orexin and leptin systems of birds regulate nocturnal migration. Unfortunately there is no conclusive evidence for the accurate cloning of an avian leptin [Sharp *et al.* 2008]. Initially we can examine the role of the orexin system in migration as the ligand structures have been determined [Ohkubo *et al.* 2002].

We predicted a decrease in food quality (protein and fat content) would inhibit fat deposition, dampen migratory activity, trigger an extended stopover and temporarily restore a diurnal sleep/wake cycle. A reduction in fat deposition would decrease leptin levels thereby disinhibiting orexin and stabilizing the sleep/wake cycle. In Chapter four, though we were unable to affect Zugunruhe by blocking the orexin system in sparrows, we were able to acutely modulate the sleep/wake cycle. We also predicted that the inhibition of migratory activity by reduced fuel availability will be associated with increased orexin B innervation. In addition we compared another migratory sparrow species as previous studies have shown a difference in the fat deposition of the WCS *gambelli* subspecies and Harris' sparrow.

## 5.2 MATERIALS AND METHODS

### *5.2.1 Animals and treatment*

Adult Harris' sparrows *Zonotrichia querula* n=13 and adult white-crowned sparrows (*Zonotrichia leucophrys leucophrys* n=13 will be caught using seed-baited potter traps (Country Cages, Penngrove, CA) in Austin, Texas in February (latitude: 30.17N; longitude: 97.44W). Both of these species are capable of adapting to captivity and exhibit migratory restlessness when exposed to a long photoperiod simulating spring [Farner, 1950; Ramenofsky *et al.* 2003] Sparrows were brought to the laboratory and housed individually (cage size: 16"w x 11" d x 21"h) in visual and auditory contact under a short day photoperiod (8L:16D. Birds received a 1:1 blend of Home Grown$^{TM}$ chow (18% protein; 2% fat; Country Acres Feed Company, Brentwood, MO) and parakeet mix consisting of white millet and oat groats (12% protein; 4.5% fat; Tomlinson's, Austin, TX) and water *ad libitum*. Upon division of groups, one group will only receive the millet (8% protein; 2% fat; Tomlinson's, Austin, TX) as food. All animals received grit in their feed. Temperature in the animal room was approximately 65°F. All sparrows were allowed to acclimate for one month under short days. Experimental procedures were approved by the University of Texas Institutional Animal Care and Use Committee and the animals were housed in an AALAC-accredited facility.

### *5.2.2 Experimental Design*

In the first study examining Harris' sparrows, after the period of acclimation in SD, the photoperiod was switched to a long day (LD) photoperiod of (16L:8D) in order to elicit migratory restlessness. Activity and sleep postures were recorded and analyzed.

Once birds exhibited weight gain and Zugunruhe, the sparrows were balanced by activity and weight and assigned to groups. Birds were divided into an enriched diet (spring diet) group n=7 or a lower protein, lower fat diet (winter millet diet) group n=6 for three weeks. We compared activity levels between groups as well as day vs. night. Behavioral differences were established between groups and brains collected for orexin immunohistochemistry.

In the second study examining WCS the methods were the same except that only nighttime behavior was compared between groups and brains were not collected. The control group (n=7) received the same enriched diet for three weeks and the experimental group (n=6) received millet for three weeks.

Body weights and fat scores were recorded in both studies. Subcutaneous fat deposits can be easily seen and measured by blowing on the feathers to expose the furcular region. The scale used ranged from 0-5 where 0 indicated no fat in the furculum, 1 some fat, 2 the furculum was lined with fat, 3 the fat filled the furculum to slightly concave, 4 the furculum is flush with fat and 5 is bulging over [Wingfield and Farner, 1978]. Blood samples were taken every two weeks but were not analyzed for this study.

### *5.2.3 Behavior*

White-crowned sparrows were videotaped with Sony digital video cameras during the day and at night under infrared illumination (Super Nightshot and Nightshot Plus with additional infrared lights if necessary depending on the camera). Four camera signals were routed via RCA cable to a surveillance digital video recorder (Samsung) with screen splitter. Analog signal was converted to a digital signal as an MPEG2 and burned to

DVD-R using an LG One-Touch burner. Video was manually analyzed by researchers blind to groups using an XBOX 360 which allowed for panning and zooming in on the birds and controlling brightness. A 48" plasma TV (Samsung) also helped improve behavior analysis. Behavior was analyzed as activity or sleep. This activity included duration of wing whirrs, number of flights, and hops. The sleep behavior (duration) was characterized by puffy round/circular body shape, ruffled feathers, head facing forward or head facing backwards under wing. Legs are not visible and the eyes are closed. Birds perched immobile before exhibiting a sleep posture or in a sleep posture but with eyes open are considered as drowsy and this behavior was not quantified. Any behavioral analysis started ten minutes after the researcher left the animal room for twenty sequential minutes every other hour over the rest of the day and night.

### *5.2.4 Immunocytochemical Labeling*

Birds received an intramuscular injection of 0.1 ml sodium pentobarbital (200 mg/kg; Nembutal, Abbott Laboratories, North Chicago, IL). All birds were perfused transcardially with 0.9% saline containing 0.1% sodium nitrite, followed by 4% paraformaldehyde in 0.1 M phosphate buffer. The brains were taken out of the skull and postfixed in 4% paraformaldehyde overnight at 4° C. Afterwards, the brains were embedded in gelatin following a procedure modified from previous descriptions [Saldanha *et al.* 1994; Deviche *et al*. 2000]. First, the brains were placed in 0.1 M phosphate buffer overnight at 4°C, followed by immersion in a 4% gelatin (175 bloom, Sigma Chemical Co., St Louis, MO) solution for 30 minutes. Afterwards, they were embedded in an 8% gelatin mold and allowed to solidify overnight. Later, brains were

immersed in 10% and 20% sucrose solutions for 24 hours each, ending with 48 hours in 30% sucrose solution. They were then frozen on dry ice and cut on a cryostat into 40 μm-thick coronal sections. Three parallel sets of sections were collected for each brain and saved at -20°C in a cryoprotectant [Watson *et al*. 1986].

Free-floating sections were processed by immunocytochemistry for orexin B following a protocol adapted from previous studies [Singletary *et al*. 2005; 2006]. After washing in 0.1M phosphate buffer saline, sections were treated with 0.5% hydrogen peroxide to inhibit endogenous peroxidase activity. Sections were washed in 0.1M PBS and blocked in a 10% normal donkey serum solution in 0.1M PBS. Sections were successively incubated in orexin A or B antiserum (1:4000) in a 0.1M PBS/ 0.3% Triton X-100 solution to ensure permeabilization. Sections were washed again and incubated in donkey anti-goat immunoglobulin (1:400). Sections were labeled with 0.5 μg/ml diaminobenzidine (Sigma, St. Louis, MO) after incubation in an avidin-biotinylated peroxidase conjugate (1:100, VectaStain ABC kit, Vector Labs, Burlingame, CA). Sections were then mounted on gelatin coated slides, dried overnight, dehydrated in gradient alcohols, defatted with xylene (Sigma, St. Louis, MO), and coverslipped with Permount (Fisher Scientific, Hampton, NH). Some stained sections were counterstained with thionin to confirm neuroanatomical identification.

### *5.2.5 Quantification of Innervation*

The density of immunoreactive fibers was quantified using a light microscope (Nikon Eclipse E600) and NIH Image 1.62 through gray level thresholding, as previously explained [Shipley *et al*. 1989; Delville *et al.* 1998]. Background was minimized using a

histogram to determine standard peak values and camera illumination was kept constant. We quantified orexin-ir fiber density in each subject by sampling from six to eight consecutive sections of each selected brain region. The results were expressed as the area ($mm^2$) covered by orexin-ir fibers within the sample surface (a 100 $\mu m^2$ square). These measures were averaged for each animal and the averages were compared between groups. All measurements were made without knowledge of the experimental treatment.

Brain regions were identified using avian brain atlases and other descriptive studies [Stokes *et al*. 1974; Aste *et al*. 1998; Medina and Reiner 2000; Brandstatter and Abraham 2003; Krutzfeldt and Wild 2004; Kuenzel 2004; Reiner *et al*. 2004; 2005]. Landmarks used for this study are based on previous experience with orexin immunolabeling in birds and on Nissl stained sections used to confirm and identify areas labeled by orexin [Singletary *et al*. 2006]. Brain regions were chosen based on observation of innervation or sleep/wake function. These areas included the locus coeruleus (LoC, just dorsolateral to the fasciculus longitudinalis medialis), the dorsomedial posterior thalamus (DMP, located below the habenula and next to the wall of the third ventricle) and the tuberoinfundibular area (TIA, at the level of the anterior and/or posterior median eminence) [Gompf and Aston-Jones 2008; Meddle and Follett 1997; Steinman *et al.* 2008].

### *5.2.6 Data Analysis*

The Stopwatch software (CBN, Atlanta) was used to quantify behaviors. In examining the effect of decreasing dietary fat and protein compared with an enriched diet, parametric data were analyzed using independent Student's *t* tests (two-tailed) for

each behavioral and physiological measure. $P<0.05$ was accepted as significant and $P \leq 0.1$ was considered a trend.

## 5.3 RESULTS

### *5.3.1 Harris' Sparrow*

In the Harris' sparrow, birds receiving a winter millet diet showed a 67% decrease in nocturnal activity measured as wing whirr duration ($p=0.04$) and a 258% increase in daytime activity ($p=0.03$) after three weeks when compared to enriched diet controls (Fig 5.1). In addition, there was a 204% increase in sleep postures and immobility observed during the night ($p=0.04$) and a 70% decrease during the day ($p=0.03$) in the millet diet group (Fig 5.2).

After three weeks of a millet only diet, the winter diet group had a significant reduction in weight ($p=0.001$) and fat deposition ($p=0.0002$) when compared to the enriched diet group (Fig 5.3). There were no differences in orexin B innervation in any of the brain areas (DMP $p=0.96$; LoC $p=0.37$; TIA $p=0.22$: anterior $p=0.28$; posterior $p=0.22$) analyzed between groups (Fig 5.4).

### *5.3.2 White-crowned Sparrow*

In the WCS study, the winter millet group also decreased nocturnal activity by 76% ($p=.0007$) and increased nighttime sleep by 119% ($p=0.00002$) when compared to enriched diet controls (Fig 5.5). After three weeks of a millet only diet, the winter diet group had a significant reduction in weight ($p=0.001$) (Fig 5.6), however a decrease in fat deposition was just below significant; $p=0.058$).

## DISCUSSION

We hypothesized that reducing the fat and protein content in the diet of Harris' and white-crowned sparrows in migratory condition would reduce Zugunruhe. This expectation is based upon stopover studies showing that fuel availability and quality in some migrants is closely associated with fat deposition and Zugunruhe [Biebach 1995; Yong and Moore 1997; Ramenofsky *et al.* 1999; Pierce and McWilliams 2004; Fusani *et al.* 2009] Additionally, this nocturnal migratory activity in birds is accompanied by a disruption in their sleep/wake cycle [Rattenborg 2004; Fuchs *et al.* 2006].

Indeed, we found a significant reduction of Zugunruhe after three weeks in the Harris' sparrows and WCS given a millet diet. This coincided with an increase in nighttime sleep as well a decrease in body weight. In the millet diet group, Harris' sparrows exhibited less daytime sleep and a reduction in fat deposition as well. These sleep/wake patterns are similar to a premigratory sleep/wake cycle. Studies in mammals have shown that high fat diets disrupt sleep/wake cycles and circadian rhythms of metabolic signals, including leptin and melatonin [Kohsaka *et al.* 2007; for review see Knutson and VanCauter 2008]. It is likely that in our studies, energy expenditure exceeds the nutrients available, thereby decreasing weight, fat and associated metabolic signals that initiate resumption of migratory activity. An extended "stopover" results, similar to what is seen when lean birds stop to refuel. The physiological condition of a bird and availability of food are major factors that determine the length of stopover [Biebach 1985; Fusani *et al.* 2009]. At stopover migratory birds need both protein and fat to rebuild reserves [Pierce and McWilliams 2004] as weight loss may be lean mass

(digestive organs, muscles) as well as fat mass. Physiological changes in the daily rhythm of lipogenic and lipolytic enzymes as well as corticosterone during migration increases the efficiency of fat deposition and energy utilization [Ramenofsky *et al.* 1999]. The flexibility in the sleep/wake cycle of a migratory bird allows a bird to stay at a stopover site to refuel and rest, or take off the very next night if the location is not suitable, dependent on metabolic signals.

The disrupted sleep/wake cycle in many mammals results in physical and cognitive deficiencies but studies have shown the WCS ability to compensate with short bouts of drowsiness [Rattenborg *et al.* 2004] and Swainson's thrushes take short naps [Fuchs *et al.* 2006]. Indeed our Harris' sparrows increased rest during the day when exhibiting Zugunruhe. Although daytime video was not available for this WCS diet study, previously we have shown an increase in daytime rest in WCS exhibiting Zugunruhe. In humans, the resulting sleep disruption from increases in fat or caloric intake seems maladaptive, however in birds this system is advantageous to surviving the extreme requirements of migration.

We also hypothesized that a likely candidate system that regulates the sleep/wake cycle and feeding in mammals, the orexin system, is associated with the changes seen here in migratory activity. Orexin regulates the sleep/wake cycle and feeding in mammals. The role of orexin is ideal here as two processes that change in migrating birds are the sleep/wake cycle and control of metabolism. We looked at orexin B innervations in the locus coeruleus, the tuberomammillary nucleus and the dorsomedial posterior thalamus. Based on mammalian studies, these areas may also be involved in the

avian sleep/wake cycle. The locus coeruleus and tuberomammillary nucleus are part of the ascending arousal system in mammals. Decreases in excitatory orexin innervation to the noradrenergic locus coeruleus (LoC) is thought to be a contributing factor of poor sleep quality in the elderly [Downs *et al.* 2007]. Orexin B-ir axon density was determined in the LoC of young, adult and aged macaques. OXB-ir density was significantly lower in the aged macaques than that observed in the young or adult animals. Additionally, orexin neurons have been shown to innervate and diurnally activate the LoC [Gompf and Aston-Jones 2008].

The tuberomammillary nucleus in mammals consists of neurons expressing histamine. These histaminergic neurons are known to increase arousal via orexin input [Bayer *et al.* 2002; Huang *et al.* 2001]. The absence of histamine results in sleep/wake changes including involuntary sleep and lack of vigilance [Parmentier *et al.* 2002]. Histamine is known to be regulated by leptin [Yoshimatsu et al 1999] a hormone secreted from fat cells which also inhibits orexin and neuropeptide Y (NPY) in mammals. NPY is found in the tuberoinfundibular (arcuate) nucleus and excites orexin neurons, stimulating food intake. Moreover, orexins innervate NPY neurons to increase feeding [Muroya *et al.* 2004]. Sensitivity to NPY increases in WCS in long photoperiod (LD), resulting in increased food intake with injections of NPY [Richardson *et al*. 1995]. The avian tuberal hypothalamus is not as well organized as in the mammal, so the tuberoinfundibular area (TIA) was sampled anteriorly and posteriorly to encompass these nuclei and possibly the ventral medial hypothalamus.

Thalamic areas such as the dorsomedial posterior thalamus (DMP) receive input from many brain areas and are well-situated to relay to many other areas. The medial region of the avian DMP is thought to be functionally homologous to the mammalian paraventricular thalamic nucleus (PV) [Csillag and Montagnese 2005]. The PV integrates inputs from SCN and SCN relay areas with autonomic structures such as the nucleus tractus solitaries and relays in the parabrachial nucleus and periaqueductal gray [Watts *et al*. 1987; Watts and Swanson 1987; Moga *et al*. 1995; Krout and Loewy 2000a; 2000b; Kawano *et al*. 2001; Van der Werf *et al.* 2002; Saper *et al.* 2005]. The PV may also play a role in the control of sleep/wake cycles [Sewards and Sewards 2003; Ishibashi *et al*. 2005]. It receives input from the monoaminergic neurons, indicating a role in the regulation of wakefulness [Kirouac *et al.* 2005] and is active during the subjective day in diurnal animals [Novak and Nunez, 1998; Novak *et al.* 2000]. Furthermore, the PV receives a high density of projections from orexin cells and contains numerous orexin-2 receptors [Peyron *et al.* 1998; Novak *et al.* 2000; Marcus *et al.* 2001; Kirouac *et al*. 2005]. In addition to its role in the sleep/wake cycle, the orexin system is proposed to be involved in the modulation of autonomic function as fibers are found in the NTS, the Arc, and the dMnX [Date *et al*. 1999]. The PV in mammals has been suggested to regulate arousal depending on energy needs and circadian input [Timofeeva and Richard 2001; Kelley *et al.* 2005]. Thus, the DMP may be an area that serves as an important integrator of energy homeostasis and the avian sleep/wake cycle.

Despite the fact that we saw a decrease in Zugunruhe and a return to a "normal" sleep/wake cycle when we administered a low protein, lower fat diet, we do not have any

evidence to support that these changes in sleep/wake cycle are associated with orexin B expression in the DMP, LoC or TIA. This is consistent with our previous findings in WCS that showed no change in OXB innervation density between migrating sparrows in LD and non-migrating sparrows in SD. Taken together, orexin B does not directly regulate the metabolic or migratory activities via these brain areas.

It is possible that if inhibition of the orexin system destabilizes the sleep/wake cycle, this allows quick adaptation to environmental challenges. These may include entering a stopover site with inadequate resources, too much competition for food, or high risk of predation. Resting may not be the best option and the bird decides to keep flying or remain vigilant. Once a bird has entered the migratory condition, a destabilization would have to continue for the span of migration. It is also likely that the demands of lengthy photoperiods, establishing territories, reproduction and parental care would require a destabilized sleep/wake cycle until reaching wintering grounds again. A comparison to birds receiving an enriched or millet diet, before migratory activity commences, could attempt to answer this question.

In addition, orexin A injections in pigeons increase arousal and orexin antagonist injections increase sleep behavior in sparrows [Simao de Silva *et al.* 2008; previous observations] similar to mammals. Though orexin B injections did not have an effect in the pigeons, it is unknown if the compound used was degraded too soon. Mammalian orexin B is a linear peptide and is degraded quicker than orexin A which has two interchain disulfide bonds [Sakurai *et al.* 1998]. Even if this is not the case and orexin B has been found to be ineffective in birds, it would be interesting to consider orexin A as a

candidate before completely ruling out the role of the orexin system in regulating migratory behavior.

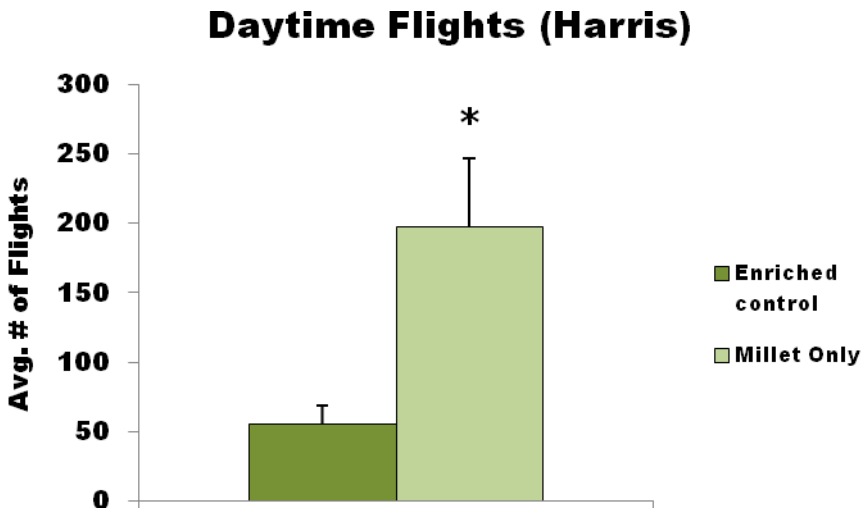
***Summary***

Taken together, these data confirm the importance of fuel quality and availability for migratory activity in this species. Decreasing nutritional content triggers a temporary inhibition of migratory activity, a stopover, which is associated with a return to a diurnal sleep/wake cycle. The process of migrating from winter to summer grounds in birds can be associated with repeated disruptions in their sleep/wake cycles. In these species fuel availability and body composition affect the sleep/wake cycle. Migratory species could be viewed as interesting flexible models for studying factors capable of causing sleep/wake cycle disruptions.

**Figure 5.1**

Comparison of Daytime (A) and Nighttime (B) activity recorded as nocturnal wing whirr duration (migratory activity) and daytime flights between sparrows after 3 weeks of exposure to either an Enriched or low protein low fat diet (Millet).

**A**




**B**

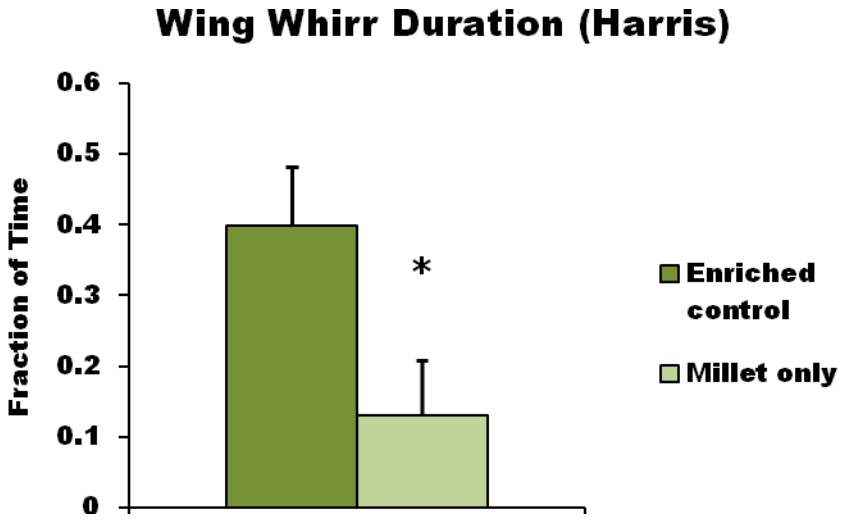
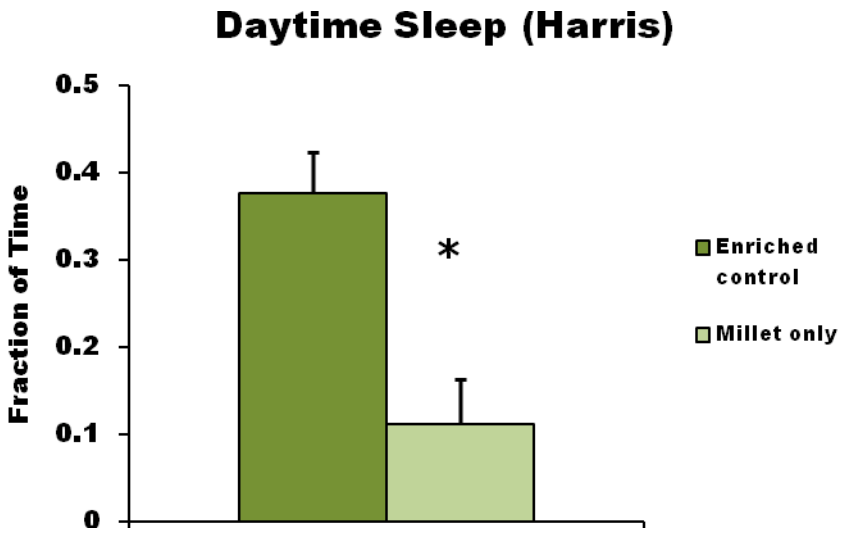


**Figure 5.2**
Comparison of Daytime (A) and Nighttime (B) sleep (analyzed for 20min. every other hour and averaged) between sparrows after 3 weeks of exposure to either an Enriched or low protein low fat diet (Millet).

**A**




**B**

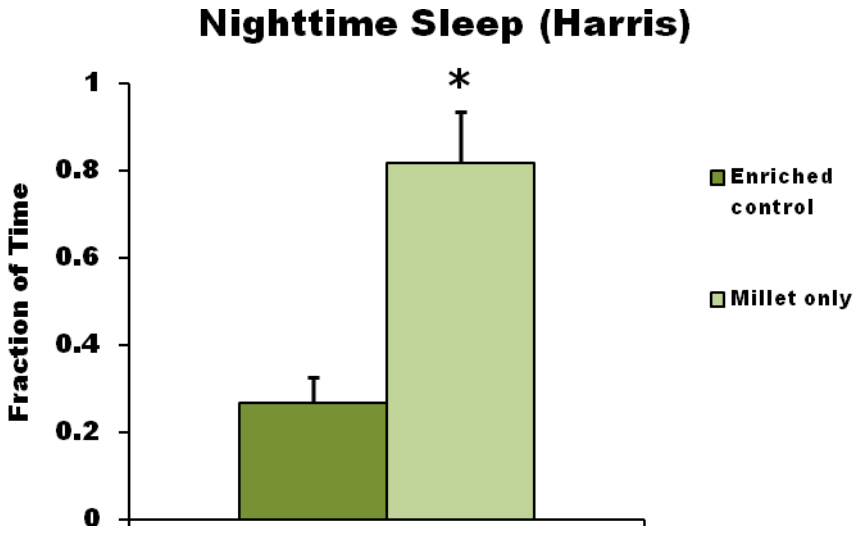
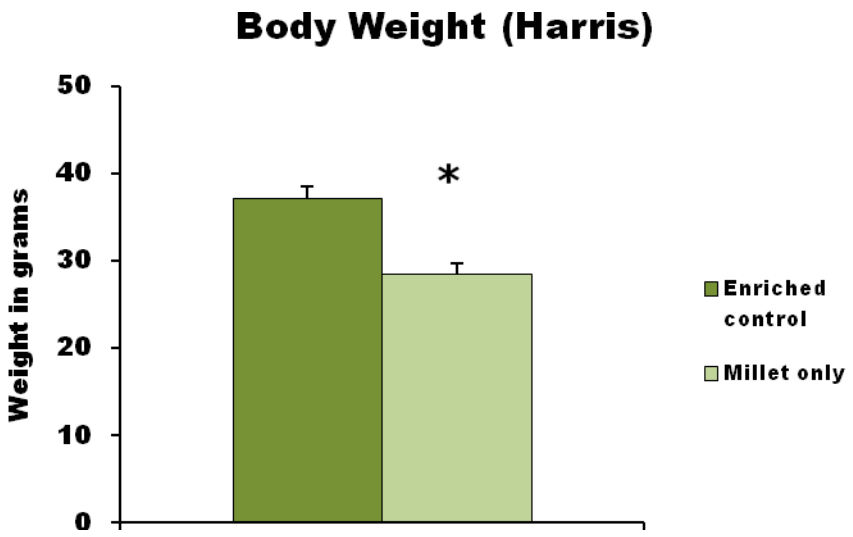


**Figure 5.3**

Comparison of physiological measures between sparrows after 3 weeks of exposure to either an Enriched or low protein low fat diet (Millet). Fat scores were taken from the furculum on a scale of 0-5.

**A**




**B**

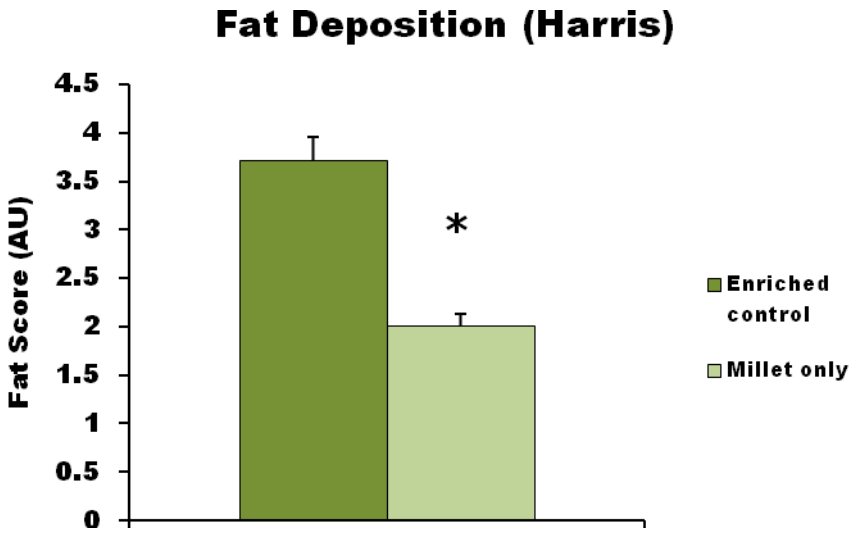
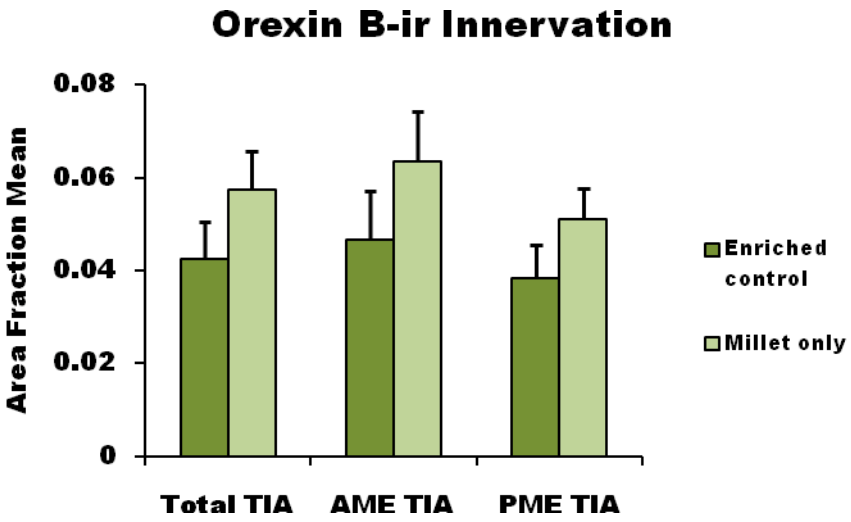
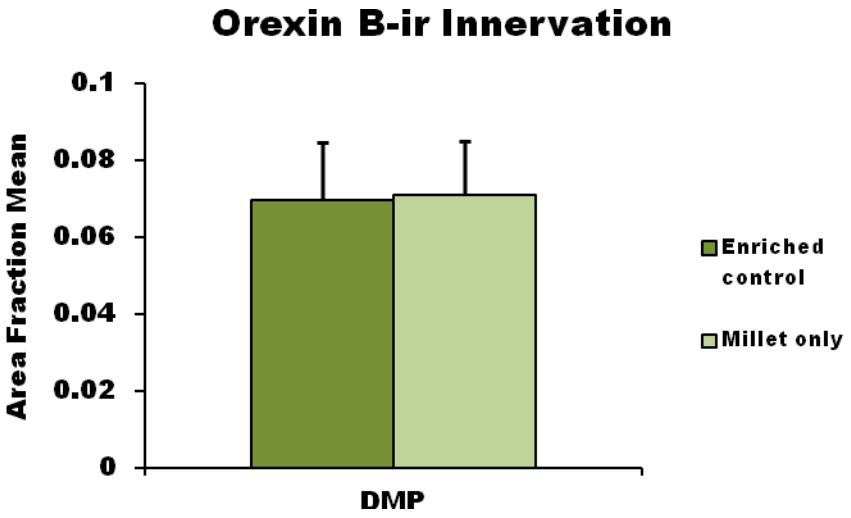


**Figure 5.4**
Comparison of Orexin B fiber innervation in the (B) dorsomedial posterior thalamus, tuberoinfundibular area (A) and locus coeruleus (C) between sparrows after 3 weeks of exposure to either an Enriched or low protein low fat diet (Millet).

**A**




**B**



c

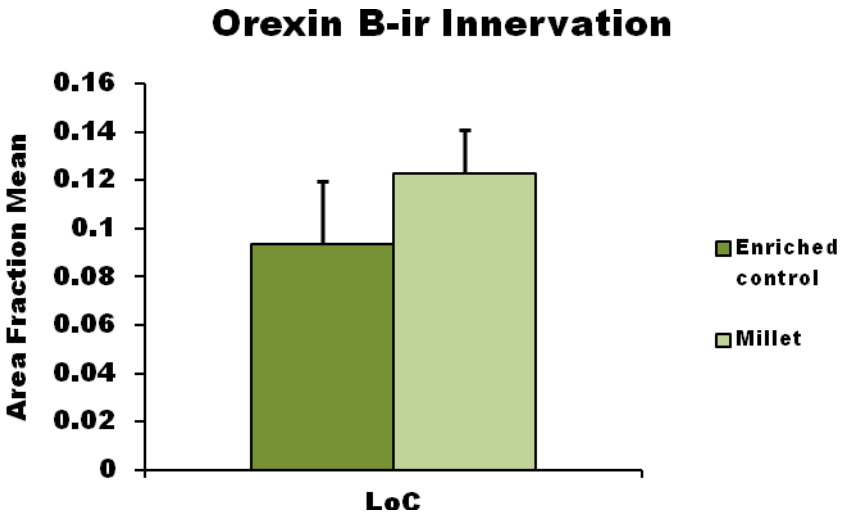
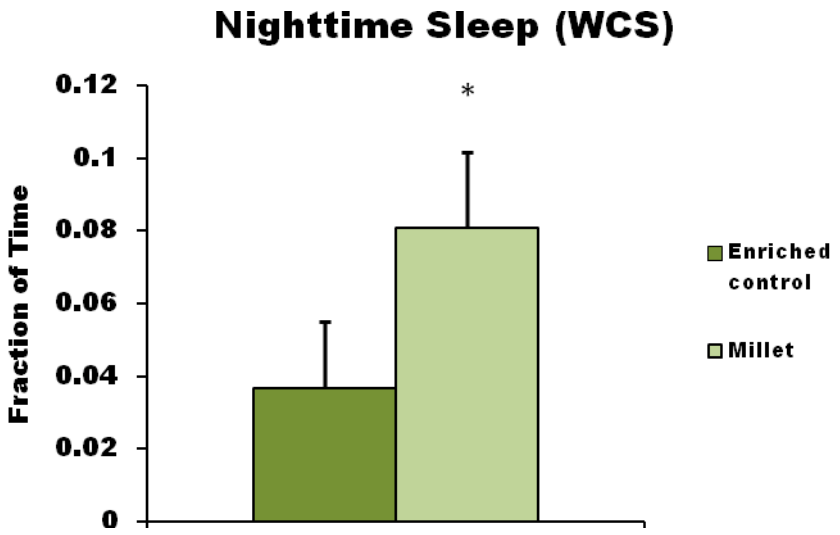

Orexin B-ir Innervation
Area Fraction Mean
0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
LoC
Enriched control
Millet

**Figure 5.5**

Comparison of Nighttime sleep (A) and activity (B) (analyzed for 20min. every other hour and averaged) between sparrows after 3 weeks of exposure to either an Enriched or low protein low fat diet (Millet).

**A**




**B**

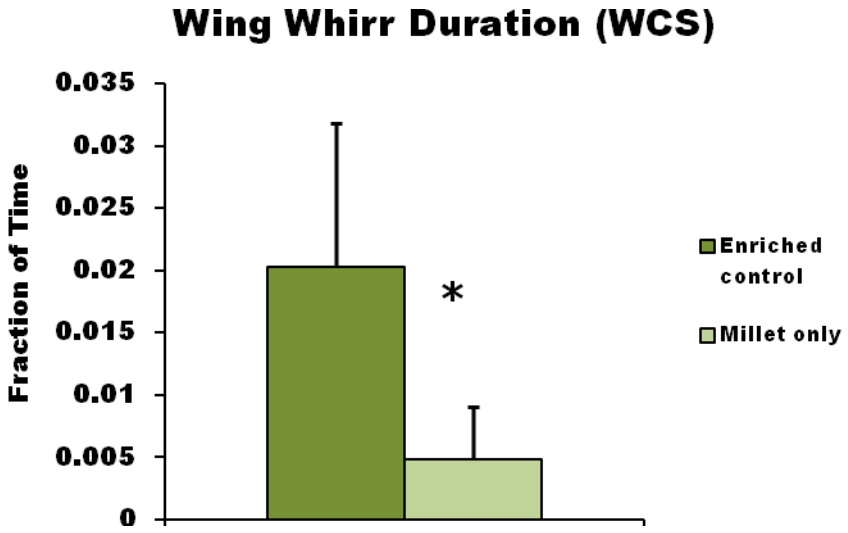
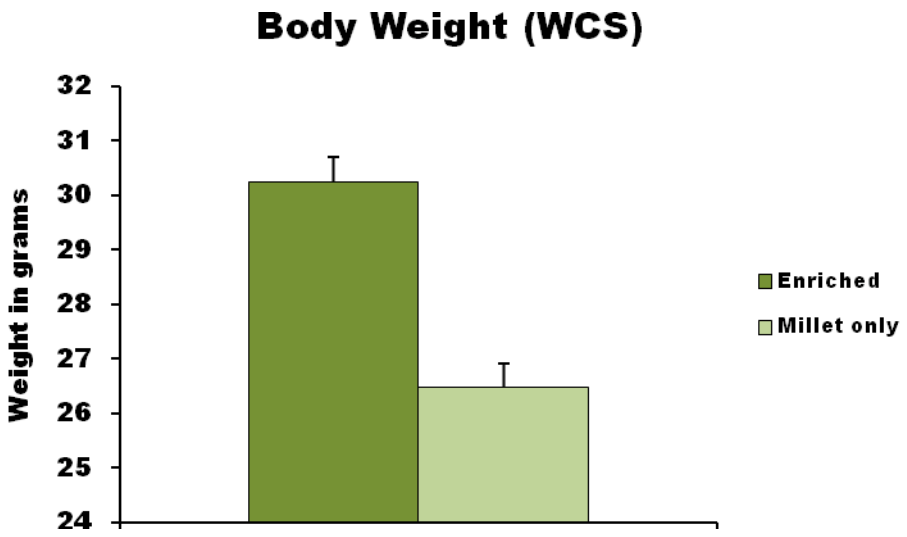


**Figure 5.6**
Comparison of physiological measures between sparrows after 3 weeks of exposure to either an Enriched or low protein low fat diet (Millet).



## Chapter 6: Conclusion

The main goal of this research was to examine the role of the orexin neuropeptide system in the regulation of avian nocturnal migration using several migratory and sedentary songbird species. Additionally these studies sought to further elucidate the interaction of fuel availability, the sleep-wake cycle and migratory activity. In summary, my data suggest a role for the orexin system in the regulation of the avian sleep/wake cycle, but regulation of disruptions in sleep similar to narcolepsy are not supported by any of my data. Therefore I have to accept the null hypothesis that the orexin system is not involved in the initiation or maintenance of migration. It is evident that neural systems associated with satiety affect migratory behavior. Not all migrants fly at night. The orexin system may facilitate the capacity to enter migratory condition but another system likely triggers Zugunruhe. It is evident that not just one mechanism controls migratory activity but a network of sleep and metabolic regulators. My data also suggests that orexin may regulate energy utilization and storage during the LD, and this study is the first to show this function of the avian orexin system. Additionally, the ease with which the sleep/wake cycle can be manipulated in these birds by either photoperiod or diet makes migratory sparrows a novel and convenient animal model to study. A discussion of the findings from each of the studies in chapters two through five is as follows:

In chapter 2, we asked if the orexin system is highly conserved across vertebrates with a similar distribution of cells and projections. Many neuromodulating systems are

well understood in mammals and there are abundant antibodies, antagonists, agonists, etc. to choose from when designing experiments. This is not always the case in non-mammalian animals. The orexin system is not well understood in birds, and it was important for us to ask this question before using mammalian orexin antibodies to describe and compare avian functional neuroanatomy. We used immunohistochemical techniques to determine the location and distribution of orexin A and B in golden hamster, green tree frog, and house finch and compared with previous fish and reptile studies. We found that the location of orexin neurons and processes are highly conserved across species but with minor differences. Similarities in cell location suggest some similar function of orexin across vertebrates. In the increasing complexity of the homeostatic mechanisms within and across each taxon, or introduction to unique environmental pressures, it is also likely that the orexin system became more complex. The orexin system can be better explained by understanding the function of the areas that the orexin cells, fibers and receptors are found. In this study, we have contributed a detailed description of the orexin cell and fiber distribution across vertebrates. Though much work needs to be completed to determine the receptors in non-mammalian vertebrates, we can begin to narrow down function by comparing distributions of cells and fibers, orexin sequences, and the few physiological studies available. Given the information today the orexin system seems to regulate general homeostatic function across vertebrates, with specialization of function depending on the species.

In chapter 3, we wanted to know if a reduction in orexin expression would occur in birds that exhibit migratory restlessness. We quantified orexin B immunoreactivity

within key areas of the brain and videotaped nocturnal activity in migratory white-crowned sparrows, *Zonotrichia leucophrys leucophrys.* Preliminary results showed a significant 50% reduction in orexin B innervation of the DMP in migrating birds, but not in any other area that we looked at. After trying to replicate the data, we saw no difference in any of the brain areas and decided to pool the data. In fact, the innervation densities were almost identical in the DMP. This is difficult to explain. I was unable to determine the age of these birds, which could affect our results. In aging rhesus macaques, one study found an association between a disrupted sleep/wake cycle and a significant reduction in orexin B innervations of the LoC [Downs *et al.* 2007]. However, we also looked at the LoC in both studies, and consistent in both there was nothing significant. Orexin B purportedly is less stable than orexin A and if the brain was not fixed well or took too long to freeze, there would be protein degradation and cellular damage especially in the innermost midline areas, such as the DMP. However, cell counts (unpublished) from the PVN, also midline, did not significantly differ between groups or studies. Sacrifice days and times were similar. Brain slices from both studies were quantified and analyzed twice for consistency. Nevertheless, we cannot support our hypothesis with these findings.

We also considered that if we were to see a change in orexin B innervation, would this be associated with the migratory activity or with a change in photoperiod? We used a sedentary species, the House sparrow (*Passer domesticus)* and subjected them to the same conditions as the WCS. We saw no changes in orexin B innervation in the DMP, as

the densities were almost identical. Thus, photoperiod does not affect the expression of orexin B in the DMP of the house sparrows or white-crowned sparrows.

Next in chapter 3 we looked at orexin B expression over time and looked at changes throughout pre, peak and late stages of migration as well as post migration. We videotaped to determine what migratory stage the bird was exhibiting and sacrificed animals at every stage. Immunohistochemistry and quantification revealed no changes in orexin B innervation of the DMP over the course of migration. All stages including "premigratory" were in LD, and we've established no effect from photoperiod change in the sedentary sparrows. So I would conclude that expression of orexin B in the DMP does not differ due to changes in migratory activity or photoperiod. If the orexin B expression had already reached a low level before our observations, then it may be likely that it would remain dampened, thus allowing the balance between sleep/wake stages to remain plastic. This would accommodate the energetic demands of establishing territories, mating, reproduction, and parental care whilst in a very long photoperiod in their natural environment. But in captivity our birds did eventually return to a diurnal sleep/wake cycle and the photoperiod remained at only 16hrs as opposed to the 20-22 hr photoperiod of the breeding grounds. Speculation aside, data from these studies also do not support a role for orexin in the regulation of migration. We tested orexin B as a possible candidate to examine in this study based on mammalian studies. However recently research has shown that pigeons injected with orexin A, not orexin B, caused increases in arousal [Simao da Silva 2008]. It would be interesting to see if there were any differences in the expression of orexin A versus orexin B during migration. The role of the orexin system

on the sleep/wake cycle of migratory birds remained in question, thus in chapter four we inhibited the orexin system.

In chapter 4 we predicted that migratory activity could be initiated in SD by blocking orexin receptors in migratory birds. Alternatively, if blocking orexin is not sufficient to initiate Zugunruhe without a photoperiod change, it may be necessary to block orexin after photostimulation. In order to answer these questions we injected a double orexin antagonist (mid morning) in birds subjected to SD for five days and later a different set of birds were orally administered the antagonist a day after the photoperiod had been switched to LD and for 4 sequential days. Video was analyzed within the first thirty minutes, sampled over the rest of the day, and sampled over the night.

Blocking orexin receptors in premigratory SD or LD birds did not initiate Zugunruhe at any dose, although a low dose of Almorexant in SD increased wake behaviors during the night. It is possible that a smaller dose of Almorexant can destabilize the sleep/wake cycle. As discussed in Chapter three, orexin is thought to regulate the sleep/wake cycle in mammals similar to a seesaw, which is called the flip-flop model [Saper *et al.* 2003]. During the awake state orexin neurons excite the wake neurons and maintain the wake state. During the sleep state the sleep promoting neurons inhibit orexin and the wake neurons. Without orexin the switch between states is unstable. It may be that these WCS need only a slight reduction in orexin to upset the balance and that the sleep drive is more potent or sleep promoting neurons are more "heavy" on their end of the seesaw. Though we would assume that peripheral administration would affect orexin receptors uniformly throughout the brain, it would be

interesting to see the quantity and location of orexin receptors that were blocked in low vs. high doses. Also, we administered Almorexant for five days in SD and saw no difference in nocturnal activity or sleep in the high dose. If there was any sleep debt before the injections producing a high sleep drive, the five days of increased sleep should have reduced the drive in the high dose group. Interestingly we did not see an increase in wake activity "recovery" suggesting that there may a homeostatic need for more sleep and five days of naps was not enough to reduce the sleep drive. However, this explanation does not take into consideration the strength of the circadian input in WCS.

We did show that blocking orexin receptors in WCS does induce daytime sleep in both SD and LD, but effects are not lasting or consistent. Passerines are known to have a higher metabolism than mammals with the same weight, and experience rapid alterations of digestive and metabolic systems dependent on environmental cues, including photoperiod. There was no effect of Almorexant on food consumption or time spent at the feeder or water cup in SD or LD. This indicates that the orexin system is not directly involved in feeding in birds. However, in LD there was a significant decrease in body weight in the high dose when an increase in body weight from baseline was seen in controls. Once WCS are exposed to a LD they normally gain weight. It is likely that the orexin system responds differentially during different photoperiods. It is possible that as the orexin system evolved in birds, feeding modulation was lost or altered but the regulation of the sleep/wake cycle was conserved. Keep in mind that it is difficult to interpret any results of a mammalian dual receptor antagonist when only one avian orexin receptor has been found.

In chapter 5 we wanted to examine the relationship of food availability and Zugunruhe. Harris' sparrows in migratory condition were given a diet low in protein and fat. After losing fat and weight, Harris' sparrows reverted to a "normal" sleep/wake cycle and stopped exhibiting Zugunruhe. It is interesting to note an inverted effect in mice fed a high fat diet, whereby circadian rhythms of metabolic systems are disrupted including orexin and NPY [Kohsaka *et al.* 2007]. Also, disruptions in human sleep can alter leptin or ghrelin signaling such that when food is available overeating occurs [Knutsen and Van Cauter 2008o].

Leptin is a hormone secreted from adipose tissue and known to reciprocally inhibit orexin neurons, regulating metabolism in mammals. Leptin levels are disrupted in narcoleptic individuals. Leptin also reduces food intake by inhibiting neuropeptide Y (NPY). Leptin is believed to signal the hypothalamus when fat stores are high in migrating dunlins [Kochan *et al.*, 2006]. Migratory birds increase feeding and fatten after a change in photoperiod in order to prepare for migration. Thus it is assumed birds about to migrate will have higher levels of leptin than birds in a non-migratory state and that leptin may be the signal needed to initiate migration.

It has been suggested that leptin modulates migration [Kochan *et al.* 2006; Fusani *et al.* 2009] and indeed would be a good candidate to examine but the controversy concerning the existence of an avian leptin has not been settled [Sharp 2008]. Several studies have shown behavioral effects of mammalian leptin injections and show leptin changes using mammalian ELISA kits. We did inject "chicken" leptin in WCS in short day to initiate migratory behavior and we injected at the switch to long day photoperiod

to reduce latency to Zugunruhe. Our results showed no change in SD and no difference in latency in LD (unpublished). We also injected human leptin antagonist but the behavior has not been fully analyzed. Further work needs to be done to initially establish that there is an avian leptin system before we can make any conclusions or invest further!

It is necessary to look at other migrating sparrows to see if the results seen earlier will be species specific. The Harris' sparrow is a close relative to the white-crowned sparrow; however they are larger and have a narrow migratory flyway (less refueling stations), possibly requiring different homeostatic processes to regulate metabolism and migration. We found that the WCS also reduced Zugunruhe and increased sleep at night when given a low protein low fat diet. Body weights were significantly decreased in both species associated with the decrease in Zugunruhe. The WCS millet group had a decrease in fat deposition however it was not significant. This may reflect a difference in how metabolic signals affect behavior depending on the adaptations of the species [Dawson *et al.* 1993]. Harris' sparrows have a higher resting nighttime metabolic rate than WCS and do not adapt as well to cold temperatures [Norment and Shackleton 1993o] and it is likely that they store more fat before flying north. Male WCS on the other hand will show Zugunruhe when temperatures are dropped which indicates that temperature plays less of a role in the vernal migration. Differences in species' regulation of migration leads one to think that there are two separate mechanisms that control fat deposition and migratory restlessness but they may be triggered by a common signal [King and Farner 1963]. Though retaining a general function of modulating

energy homeostasis and the sleep/wake cycle, the orexin system may have evolved to lose or gain different functions wholly or in part to allow nocturnal migration.

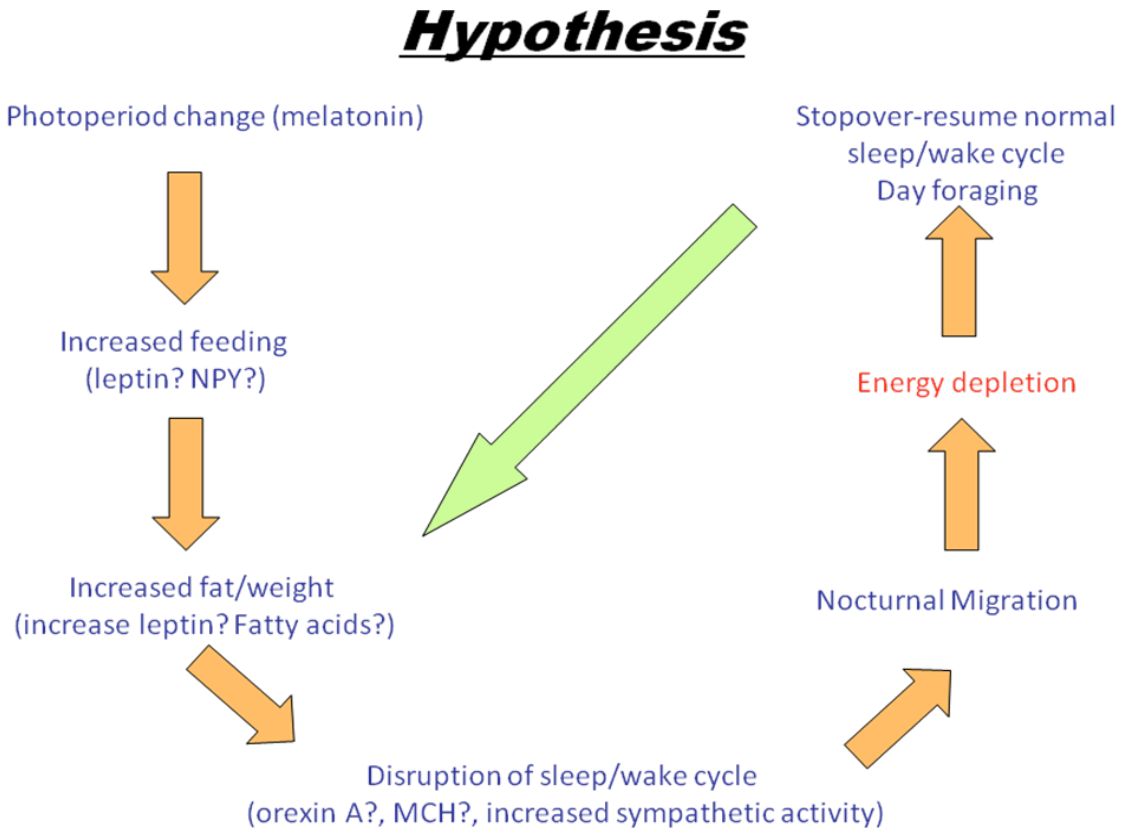
Originally we predicted that the photoperiod change increased feeding and body mass in a long distance, short bout nocturnal migrant. This would lead to a decrease in orexin expression which would result in an imbalance of the sleep/wake cycle. This disruption allows migration to happen but the resulting energy expenditure requires a nocturnal migrant to stopover and refuel. The increase in body mass signals migration to resume this cycle until reaching the breeding grounds. We were not able to show any association of the orexin system modulating Zugunruhe but we were able to show that the orexin system does modulate sleep in white-crowned sparrows.

Based on my data, I believe that it is necessary to restructure my original hypotheses of how orexin works to modulate the sleep/wake cycle in a nocturnal migrant. We know that seasonal changes in melatonin levels are differentially expressed in migrating vs. premigratory blackcaps [Fusani and Gwinner 2003]. This change in melatonin is known to affect feeding in mammals, but other orexigenic hormones are also known to increase feeding in response to a change in photoperiod such as NPY. The resulting increase in body weight and fat deposition produces signals that indicate the energy state of the bird (such as leptin) and these signals affect the sleep/wake cycle. Disruption may be due to a reduction in orexin expression or another neuropeptide such as melanin concentration hormone, which is thought to maintain the sleep state, modulate feeding, and is co-distributed with orexin neurons in mammals. In mammals there is a strong association between satiety hormones, orexigenic and anorexigenic neuropeptides

in relation to the sleep/wake cycle [Morgan and Mercer 2001]. Previous research showing the dynamic metabolism of migrating sparrows suggests that the modulation of migration is complex and involves more than one necessary system, none being sufficient by itself. We have not shown that the orexin system is directly involved in the initiation of migration but we have not ruled out an indirect modulation of either migration or a disrupted sleep/wake cycle.

We further predict that this disruption in the sleep/wake cycle enables the sparrow to migrate. This migration results in a further reduction in melatonin which is thought to dampen the circadian rhythm allowing the sleep/wake cycle to remain plastic [Fusani and Gwinner 2003]. This sleep/wake disruption results in sleep deprivation (which yields increased sympathetic activity in mammals). Leptin is also known to regulate sympathetic activity in mammals and may contribute to the disruption in the sleep/wake cycle. Upon depletion of energy stores, the migrating sparrow is required to stopover and refuel. We have shown the resumption of a normal sleep/wake cycle when sparrows experience a simulated stopover. Melatonin increases during stopover in blackcaps and likely contributes to this resumption. The subsequent increase in body mass and fat deposition continues the cycle until the sparrow reaches the breeding grounds. Fig 6 illustrates these new hypotheses. While our studies here won't validate our original hypotheses, it points us to a new set of hypotheses.

Fig. 6 A schematic of new hypotheses of neural mechanisms of nocturnal migration



## Appendix: Understanding Captive Bird Care

All wild birds brought into the lab were quarantined and given 10μl 1% Ivermectin (Durvet, Blue Springs, MO) topically on nape initially (for mites, fleas, parasites). After a week of acclimation the birds were given a dust bath in 5% Carbaryl (Hi-Yield, Voluntary Purchasing Groups, Bonham, TX). We placed 2 grams in a box and let the bird fly around in the closed box for less than a minute. We applied once, or as needed (for mites, fleas, worms). Care was taken not to get the dust in the feed or water.

Health was assessed every day and liners examined for dropping quality [Tully et al 2000; Books: Avian Medicine, Diseases and Treatment of Caged Birds, Avian Physiology]. In the case of a puffy or lethargic bird, Terramycin (Oxytetracycline HCl, Pfizer, Eston, PA) was administered in water bowls: 3g/L diluted glucose/fructose vitamin water (¾ water and ¼ vitamin water). Birds usually do not drink the antibiotic mix without sweetener and will dehydrate. To address the incidence of individual fickleness, plain water was only offered two hours a day as the birds will not drink the antibiotic water when both are available. Care was given not to administer sucrose, as sparrows cannot metabolize it (hummingbirds can) but they will drink it over nonsweetened water. Treatment should last 7-10 days (for coccidiosis, bacterial infections, injury). If the bird was fickle or if I suspected the bird was not ingesting enough antibiotic, I used antibiotic chow (Chick Starter, PMI Richmond, Indiana) mixed with seed.

If a bird was found residing only on the floor of the cage and responded slowly or was otherwise exhibiting abnormal behavior, it may be too late and was given antibiotic drops by hand. A 1-3ml syringe (1ml of antibiotic mix, 6g/L) was used to administer, drop by drop. The cage was separated from others and the cage wrapped with a heating pad set to low. Antibiotic water and food was placed on the floor for easier access. Shields were hung around the bird cage in order to alleviate stress. The bird was watched closely.

If a bird seemed to be eating a lot but the cage lining showed few droppings, it was possible it had parasites. An anti-parasitic Piperazine-17 (Durvet, Blue Springs, MO) was administered orally in their drinking water sweetened with glucose/fructose. If parasites were seen on or through the flesh (the skin is translucent) the bird received the oral dose from a syringe.

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