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FAT AND MIGRATION: RELATIONSHIP BETWEEN SEASONAL REGULATION OF ADIPOKINES AND BEHAVIOR

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ABSTRACT

Migration events and associated changes in an individual's behavior patterns occur on a seasonal basis, and are regulated by both circadian and circannual clocks. Although the effects of biological clocks on physiology and behavior are readily observable, their mechanisms and pathways are only slowly being detailed. Certain adipokines, signaling proteins secreted by adipose tissue, including adiponectin and visfatin, may be important to timekeeping systems and act as signals of body condition influencing migratory decisions. Although these adipokines have been investigated in mammals, very few studies have utilized avian species, and currently no work has been published regarding Passerines. Our objectives were to examine circulating levels of both adiponectin and visfatin and to explore potential relationships between these adipokines and the seasonal transition between the non-migratory and migratory life history stages. Captive white-throated sparrows were monitored, under constant photoperiod to rule out the influence of changing light:dark ratios, for changes in plasma levels of the two adipokines across a 24 hour period during the migratory and non-migratory stages. Examination of plasma adiponectin levels using Western blotting revealed that migrating individuals display different circadian patterns of plasma adiponectin compared with non-migrating individuals, which suggests a change in a biological clock regulating adipokine levels. Correlative evidence reveals a strong relationship between plasma adiponectin levels and amount of body fat and nighttime activity in migrating individuals, and mixed effects modeling estimates a reduction in high molecular weight adiponectin of approximately half in migrating individuals. Enzyme-linked immunosorbent assays for visfatin concentration revealed a reduction of approximately 20% in plasma visfatin of migrating individuals. Although this seasonal reduction may be regulated by a circannual clock, we did not detect a circadian rhythm in plasma concentration in either non-migrating or

migrating birds. Plasma visfatin did not vary with subcutaneous body fat. Collectively, the results of these studies provide evidence that seasonal or daily differences in signal from adipokines may provide individuals with physiological information regarding fat deposits and might underlie the seasonal behavioral changes necessary for migration.

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ABBREVIATIONS

ADP	=	Adiponectin
HMW	=	High molecular weight
kDa	=	kilodalton
MI	=	Migrating
NM	=	Non-migrating
WTSP	=	White-throated Sparrow (*Zonotrichia albicollis*)
ZT	=	Zeitgeber Time

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"Now, here, you see, it takes all the running you can do, to keep in the same place. If you want to get somewhere else, you must run at least twice as fast as that!"
-Lewis Carroll

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CHAPTER 1

INTRODUCTION

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Although the evolutionary origin of avian migration is debatable, the adaptive significance of many traits associated with migration is evident. Whether species use migration to exploit seasonal resources, or avoid depletion of resources, time has enabled the evolution of genetic programs which regulate the complex migratory machinery [1]. Most habitats are suitable for birds only during part of the year; food, water, and shelter may be limited during the winter months and therefore may be unable to support usual size populations throughout the year [1]. To cope with the stress of increased competition under conditions of limited resources, many bird species have evolved the capacity for migration to more suitable wintering grounds. Birds undergo a dramatic cascade of interconnected physiological changes to cope with the physical demands associated with migration. These sequential changes include the appearance of hyperphagia, increased fat accruition, and the programmatically defined hypertrophy and atrophy of specific muscles and organs.

The migratory phenotype is comprised of many genetically heritable components: the physiological preparation for, timing of the yearly initiation and termination of migratory restlessness, arrival date at destination sites and the direction of migratory flight are all

previously defined heritable traits [1,2]. These traits have been shaped and modified over evolutionary time scales into the vast array of migratory physiologies we see in nature today. Previous breeding studies of blackcaps by Berthold [3] determined that either sedentary or migrant populations can be produced from a mixed, polymorphic population within a span of six generations. In the past few decades, a portion of the central European blackcap population has begun to overwinter in the British Isles, essentially this change in migratory strategy allows them to take advantage of the increases in winter temperatures and food availability instead of enduring the more arduous journey to Africa [4]. Because this divergent population arrives at the breeding grounds earlier than their cohorts arriving from Africa, assortative mating within the population occurs. The resulting temporal barrier and consequent genetic drift between the populations provide the fuel necessary to eventually drive speciation [5]. Speciation has been documented to occur in this manner through temporal segregation of breeding time in the Madeiran storm-petrel [6]. In more recent years, microevolutionary changes in the timing of migration related to climate change have been documented [7,8,9,10,11].

Avian migration is regulated by environmental factors, and physiological adaptations that have evolved over time. Although features associated with migration including onset, duration, and termination are heritable genetic traits, expression of migration integrates time through the help of biological clocks. Individuals' ability to integrate environmental information allows for a high degree of flexibility in the expression of heritable traits associated with migration and gives us hope that current species will adapt to global change in climate.

Seasonal and Daily Cycles

Although migration may, on the surface, appear to be a direct response to changes in temperature, or a paucity of food, the change in day length has been a more reliable cue that

serves to synchronize seasonal events and initiate migratory behaviors [12]. In temperate zones, time of year can be easily determined by documenting the regular changes in photoperiod. These predictable changes shape and modify the endogenous, genetically determined, circannual rhythms of molt, reproduction, and migration [13]. Other environmental factors, such as temperature, food availability [14] or rainfall may serve to fine-tune specific phenological events. The discovery of Zugunruhe, or nocturnal migratory restlessness, in captive migratory birds has instigated a long period of experimental investigation into the factors that control the timing of migratory events. The ability of passerines to accurately time yearly migratory behaviors, in constant conditions while held in captivity without environmental cues is the clearest demonstration known that an internal, circannual clock regulates seasonal behaviors. Subsequently, scientists have accumulated a substantial wealth of information about the regulation of endogenous circannual and circadian clocks from studies utilizing captive migratory birds (reviewed in [12,15,16,17,18]).

Circannual rhythms have been observed in events associated with migration, such as fat deposition, the seasonal timing of nocturnal restlessness, and alterations in the nocturnal profile of blood borne melatonin (reviewed in [12,18]). Unlike circadian rhythms, which are thought to be generated by a set of positive and negative molecular feedback loops, circannual rhythms may be based upon the completion of certain physiological states coinciding with changes in photoperiod [19]. These clocks provide a consistent internal measure of time to improve coordination with seasons and the ability to respond to changes during those seasons [12]. Although much research has been performed on circadian clocks, comparatively little is known about the proximate control of circannual rhythms. Unsurprisingly, mechanisms underlying circannual rhythms are difficult to study because of the length of their inherent period.

During the migratory period, typically diurnal, small songbirds undergo a dramatic behavioral shift, in which they become nocturnally active, thereby allowing them to migrate at night. Nocturnal migration allows birds to reduce thermal and water stress as well as avoid predation [20]. Changes in daily locomotor activity during migration are characterized by: 1) a phase inversion of locomotor activity (i.e. day-active birds switch to nocturnal migration) [13], 2) an increase in alpha (i.e. duration of daytime activity), 3) an increase in tau (i.e. endogenous period of activity) [21,22], and 4) a drastic reduction and reconsolidation of sleep with no decrease in cognitive or physical performance [23]. Although the timing of migration is regulated by a circannual clock, the daily shift in behavior from being diurnal during the non-migratory period to being nocturnal during migration is regulated by a separate circadian oscillator whose expression is limited to the migratory period [22]. One hypothesis is that during the non-migratory period, the normal production of rhythmic melatonin by the pineal gland keeps the daytime and "Zugunruhe" circadian oscillators tightly coupled. Melatonin acts as a signal to synchronize the electrical firing of individual neurons within the master clock of the suprachiasmatic nuclei, the free-running period lengths of individual cells typically have wide variation [24]. Decreased melatonin production during the migratory period reduces synchronization of suprachiasmatic nuclei neurons and causes decoupling of the two oscillators. This decoupling results in a split in the consolidation of locomotor activity, with one component moving into the nighttime; consequently, decoupling allows the oscillator controlling nighttime activity to be expressed when these two clocks are in their metastable antiphase relationship [22].

Fat as Migratory Fuel

Migrating birds must also make physiological adjustments in metabolic capacity to cope with periods of fasting and increased energetic demands. Changes in metabolic physiology begin well prior to migration when birds become hyperphagic and increase fat deposition for use as the primary fuel source during migratory flight [25]. Once critical levels of fat stores have been reached, birds can begin migration. Pre-migratory fattening is achieved not only by increasing food intake and increasing assimilation efficiency, but also by lowering basal metabolic rate (reviewed in [26,27]) on an endogenous, seasonal basis. In avians, the neurotransmitter neuropeptide Y (NPY) has been demonstrated to stimulate food intake in a dose-dependent manner [28]. Thus, NPY might be important during the pre-migratory period of migrant birds when individuals store fat in preparation for migration. The expression of hyperphagy in seasonal migrants might be the result of circannual differences in levels of NPY, or its sensitivity. Although a long-distance migrant has one of the highest capacities for mobilizing lipids, the conservative minimum lipolytic rate to support migratory flight is 2.4 times greater than at rest [29]. These birds must increase their lipolytic rate to match migratory energy expenditure and supply working flight muscles with the fatty acids required to fuel prolonged endurance flights [29]. Fat deposits are a valuable source of energy during migratory flight as they provide more than twice the calories per gram as carbohydrates or protein and approximately double the amount of metabolic water than protein or carbohydrates [30]. Because the energetic cost of flight increases relative to body mass, fuel storage is a problem that affects energy economy. It is no surprise that migrants store fat as fuel because it is the most energy dense per unit weight. Short-distance migrants may rely less on large fat deposits, as stores may be more easily replenished during daily stopovers along the migratory route [25]. Medium-

distance migrants usually reach peak fat deposition, with enough fat to fuel the entire migration, before initiating migratory flight, and use stopovers when too much fuel is spent on flight in inclement weather or avoiding predators [24]. Long-distance migrants may deposit enough fat during the pre-migratory period to fuel long, non-stop flight to critical stopover sites where whole populations of birds may reside for days to rebuild fat stores before completing their journey [25].

Metabolism and Diet Choice

Diet composition and nutrient selection are important factors relevant to determining the quality of fat stores. Recently, interest has been generated in the study of fat composition and how seasonal changes in diet preference [31] can affect composition of lipid stores and the consequent efficiency of utilization during migration (reviewed in [32]). Seasonal frugivory is a widespread characteristic in passerines. Previous work has suggested that birds might base diet selection on digestive efficiency [33], which could affect foraging behavior prior to and during migration when assimilation and digestive efficiency change along with diet preference [34,35]. During the breeding season many birds rely on invertebrates with high protein content as their main food source; in preparation for migration birds display preference for particular fruits, and discriminate between foods according to sugar or lipid content with species specific preference for certain colors [33,36,37]. Additional preference is shown for foods rich in C_{18}fatty acids (reviewed in [27]). Preference for particular foods can be influenced by genetic predisposition, social transmission of feeding habits, and individual previous experience [37]. Because dietary fats provide fatty acids to be stored in adipose tissue, diet choice affects the structure and quality of fat stores by modifying fatty acid composition of lipids; migrating birds have proportionally higher 18:1 fatty acids (the first number in this designation refers to the number of carbon atoms

in a chain, while the number following the colon refers to the number of double bonds) compared with 18:2 fatty acids, whereas the opposite is found in the fat of non-migrating birds (reviewed by [38]). Differences in fatty acid composition of fat affects exercise performance and are species-specific [37]. Some of the fruits preferred during migration have high dietary lipid contents [39] and birds fed a diet high in fat but low in protein attained the highest daily body mass gain [40]. The combination of decreased basal metabolism, increased metabolic efficiency, and preference for foods that promote lipid storage permit frugivorous birds to accumulate enough energy from nutrient-poor food sources to fuel migratory flights.

Adipose Tissue as an Endocrine Organ

Short-to-medium distance migrants forage at daily stopover sites to maintain fuel stores, or to replenish stores that may have been lost due to flight in inclement weather. When fat stores drop below a defined critical level, individuals are unable to complete the next segment of migration. Consequently, birds will delay migratory flight for multiple days at suitable stopover sites. Individual birds must have some physiologic indicator which informs them that their fuel stores are sufficient to continue or delay their migratory flight. Signals produced by adipose tissue, a true endocrine organ, should therefore be most critical in the decision to delay and refuel or continue migration. Adipose tissue synthesizes and secretes a number of bioactive autocrine, paracrine, and endocrine peptides, collectively referred to as adipokines, including leptin [41], adiponectin [42], visfatin [43], and adipsin [44]. Adipose tissue also expresses receptors for traditional endocrine hormones such as insulin, glucagon, growth hormone, and thyroid stimulating hormone as well as receptors for cytokines including leptin, interleukins, and adiponectin [45]. Adipokines may act in a circadian or circannual manner on target cells located in the brain, muscle, and liver to modulate lipid metabolism and feeding behavior. Specific

receptors, such as PPARγ, which are highly expressed in adipose tissue, liver, and bone may also feedback on the brain and liver to regulate energy balance, insulin sensitivity, and lipotoxicity.

In mammals, the primary signal of satiety and the extent of energy stores emanating from adipose tissue is leptin, which circulates in the bloodstream with circadian rhythmicity [46,47,48] and displays seasonal changes in sensitivity [49,50]. Leptin acts on target cells in the brain to affect changes in appetite and feeding behavior, energy expenditure and metabolism, and increase physiological and behavioral satiation responses during food intake [51,52]. Leptin deficiency reversibly causes increased appetite and hyperphagia in mammals and those with leptin deficiency have higher instances of obesity and greater percentages of body fat compared with control subjects [53]. Studies utilizing mammals have revealed a positive correlation between the amount of circulating leptin and the extent of body fat [54,55]. In rodents, leptin stimulates AMP-kinase activity thereby promoting an increase in fatty acid oxidation [56]. Although leptin receptors have been conserved in avian species [57,58], and exogenous injections of leptin induce physiologic responses (reviewed in [59]), the DNA which encodes leptin has been lost from the avian genome [60,61,62]. In the absence of leptin as a signal of satiety, the regulation of appetite and metabolism in birds must arise from other chemical cues subserving leptin's traditional role.

Adiponectin, another adipokine, is found in high concentrations in the blood plasma. Unlike leptin, adiponectin has been demonstrated to have a negative relationship to body fat in mammals and is often referred to as a signal of starvation. Recent evidence suggests a role for adiponectin in energy homeostasis [63]. Adiponectin has the ability to promote weight loss without decreasing food intake through its ability to increase the expression of proteins important for fatty acid transport and utilization, ultimately increasing fatty acid oxidation [64].

Adiponectin levels are reduced in obese mammals, and weight loss and fasting increases adiponectin [65]. Results from studies performed on diabetic and pre-diabetic patients, implicate a role for adiponectin in the development of insulin resistance. Deficiencies in certain adipokines such as adiponectin sensitize tissue to insulin and the administration of adiponectin reverses hyperglycemia and hyperinsulinemia, thereby ameliorating insulin resistance [64,66]. However, differences in the effects of adiponectin are observed between subcutaneous and visceral fat stores, suggesting that regulation may be fat-depot specific. Both Fisher et al. [67] and Lihn et al. [68] demonstrated that adiponectin gene expression is reduced in visceral fat compared with subcutaneous fat in human subjects. However, another group established no difference between the two sites [69]. An inverse relationship has been demonstrated between levels of adiponectin and body fat in both mammals and chicken [70,71,72,73]. Recent studies utilizing migrating birds demonstrate a more complicated relationship. Non-migrating birds display no significant correlation between fat content and plasma ADP, while migrating birds displayed strong positive correlations (Stuber et al. unpublished data). These opposing findings in migrating birds compared with mammals or chicken may suggest a physiological difference between typical obesity and the genetically programmed, temporary obesity experienced seasonally by migratory birds.

Visfatin was originally described in independent laboratories as pre-B cell-enhancing factor 1 homolog, and nicotinamide phosphoribosyltransferase (NAMPT), a rate limiting enzyme in the nicotinamide adenine dinucleotide (NAD^+) biosynthetic pathway which is highly evolutionarily conserved over many taxonomic groups [74,75,76]. Visfatin has no known receptors; however, it may have insulin-mimetic effects, although this is still controversial. Visfatin may play a larger role in metabolic disorders such as diabetes and metabolic syndrome

via its influence on the salvage pathway during NAD biosynthesis [77,78]. By catalyzing a rate limiting component of the synthesis of NAD, NAMPT maintains a reservoir of NAD to function in redox reactions, energy metabolism, and as a substrate for sirtuins, potentially linking metabolism directly to the core circadian molecular clock [77]. Metabolic feedback on the molecular clock may improve the accuracy of timing in daily cycles of energy use [79].

Another adipocyte-derived protein is resistin, whose circulation in plasma is increased in genetic models of obesity, and in diet-induced obesity. Recent studies have demonstrated a role for resistin in the development of insulin resistance in mammals [80]. Injection of resistin into rodents decreased insulin sensitivity, while neutralization of resistin with antibodies improved insulin sensitivity [79]. Decreased serum resistin has been reported in diet induced diabetic and obese mouse models [81] and no difference between genetically obese models and wild-types [82]. As is the case with adiponectin, resistin levels also seem to be regulated in a depot-specific manner, possibly explaining the discrepancies in results. Resistin expression is elevated in visceral adipose, compared with subcutaneous abdominal fat, which follows from obesity related insulin resistance with increased visceral fat deposits [83]. In response to fasting and refeeding, resistin mRNA levels are reduced and elevated, respectively [80,81]. These results suggest that resistin might also have the capacity to function as a sensor of nutritional state.

Along with promoting insulin sensitivity, the nuclear receptor peroxisome proliferator-activated receptor γ (PPARγ) also regulates adipocyte differentiation and metabolism, promoting adipogenesis from mesenchymal stem cells over other cell fates [84,85]. Expression of PPAR follows a diurnal pattern in many metabolically active tissues such as skeletal muscle, adipose tissue, and liver. Located in adipose tissue, PPARγ is naturally activated by an array of substances, including dietary fatty acids [86] and lipoproteins [87] and have the ability to

regulate certain clock genes that modify the molecular circadian clock [88]; thus, PPARγ may act as a lipid sensor. PPARγ is also activated during adipocyte differentiation, converting previously nonadipogenic cells to mature adipocytes [84]. Mutations in PPARγ can lead to lipodystrophy and insulin resistance in humans. By forming a complex with the clock gene Period2, the molecular clock directly regulates the functioning of PPARγ by inhibiting its recruitment to certain promoters. Consequently, knockdown of Per2 leads to increased activation of adipogenic genes [89]. PPARs are also important in sustaining rhythmicity in peripheral clocks, such as those in the liver. PPARα, a clock controlled gene expressing diurnal rhythms in mRNA and protein levels, regulates the transcriptional activity of another clock gene, BMAL1, by binding to a BMAL promoter element. PPARα is required to maintain rhythmicity of bmal1 in the liver and its expression is partly regulated by insulin and glucocorticoids, potentially mediating lipid metabolism in a clock-dependent manner [90].

Peripheral Clocks in Adipose Tissue

Recent interest in how the digestive system communicates with the brain and how signaling molecules affect digestive physiology and behavior has spurred research to elucidate the daily regulation of so-called fat hormones. The effects of fat hormones on energy homeostasis have recently been detailed. Circadian clocks mediate the suite of changes that occur in anticipation of feeding to optimize metabolism, storage, and uptake of nutrients. Clocks also allow for the temporal segregation of metabolic processes that are incompatible with other physiological activities, such as DNA replication and cytokinesis.

Endogenous biological rhythms are generated from the expression and activity of certain “clock genes” which regulate the timing of these rhythms. Simply put, circadian clocks are comprised of a set of genes that encode transcription factors that form interacting positive and

negative feedback loops. Two important genes, Brain-muscle-arnt-like (BMAL), and Circadian Locomotor Output Cycles Kaput (CLOCK) activate the gene expression of Period (Per) and Cryptochrome (Cry) by binding to E-box regulatory sequences in the promoter regions of their DNA [91]. After transcription and the resulting delay during translation, Per and Cry proteins inhibit the transcriptional activity of the BMAL-CLOCK complex, thereby inhibiting their own transcription. Once Per and Cry degrade, the inhibition is removed, allowing CLOCK-BMAL to reactivate transcription [91,92]. Alterations in redox states and gas responsive elements influence the dynamics of the molecular clock and convey information regarding metabolic states [93,94,95]. With the incorporation of additional modifying feedback loops, NAD^+(limited by visfatin) has been implicated in the regulation of the molecular clock through the modification of SIRT1's deacetylase actions on CLOCK [93,96]. The circadian production of visfatin and following diurnal regulation of NAD^+ may therefore provide a mechanism for metabolic factors regulating the molecular clock during migration.

Scientists have found that almost every organ, including liver [97], heart [98], ovary [99] and adipose tissue [100,101], expresses clock genes in a rhythmic fashion, and these peripheral clocks may be driven by a master clock in the brain [102].

It is likely that fat hormones are regulated by a clock in adipose tissue, as other hormones circulate with circadian rhythmicity. Indeed, levels of adiponectin mRNA and receptors display circadian rhythmicity in both visceral and subcutaneous fat explants independent of the master clock of the brain in mammals [103]. Adiponectin also circulates rhythmically in the blood of mammals [104]. Studies utilizing migratory birds, however, have produced results that are dependent upon their migratory status (migrating versus non-migrating) (Stuber et al. unpublished data). As is the case with normal-weight mammals [105], non-migrating birds

display a diurnal variation in plasma adiponectin, with highest concentrations during the day, and the lowest levels at night. Conversely, migrating birds display peak plasma adiponectin levels at night, during the time of migratory activity, and lowest levels during the day, when individuals rest and typically recover from migratory flight, however, like obese mammals, the amplitudes of these rhythms were attenuated (Stuber et al. unpublished data). This shift in the phase and amplitude of circulating adiponectin could be a response to the dramatic behavioral changes migrating birds undertake to reduce the stress associated with migration (typically diurnal songbirds become nocturnally active during the migration period). Visfatin mRNA, like adiponectin, exhibits circadian rhythmicity in the adipose tissue of mammals, peaking during the day, and lowest at night [106]. Visfatin rhythmicity has yet to be examined in migratory songbirds. One of the few studies that have attempted to detail the temporal profiles of resistin demonstrated a circadian rhythm of resistin mRNA expression in adipose tissue, similar to the rhythms in insulin [107]. Resistin mRNA levels increase at night, in anti-phase with serum levels, and decrease during the daytime in nocturnal rats.

Communication between clocks in adipose tissue and the brain is necessary for the expression and control of seasonally adaptive behaviors and physiological modifications important for migration. Information from the environment on photoperiod must be conveyed from brain centers to adipose tissue to mediate pre-migratory fattening on a circannual basis, while information documenting the extent of energy stores must be relayed from adipose stores to the brain in a circadian fashion so that changes in behavior can be elicited by alterations in physiological state. Interestingly, adipose tissue is also innervated by neurons from the sympathetic nervous system [108], thus bi-directional neural communication occurs along with bi-directional humoral communication [109].

Regulation of Feeding Behavior

Energy homeostasis is tightly regulated by complex, highly integrated interactions between the central nervous system and peripheral organs. Physiological feedback systems utilizing adipokines regulate both short and long-term energy balance where signals influence feeding behavior and meal size while other signals are produced in proportion to the extent of fat stores to indicate overall energy reserves and maintain stable adipose fat stores over time. Imbalances in the production or regulation of adipokines may have far reaching consequences on metabolism, inflammation, or cardiovascular function. In mammals, leptin acts on the hypothalamus to provide a satiety signal, suppress food intake, and promote energy expenditure [110]. Without leptin, birds must utilize other signals to regulate weight and energy use. Although the effects of obesity on visfatin levels are debated, its relationship with metabolic syndrome suggests a role in appetite regulation. Cline et al. [111] found that intracerebroventricular injection of recombinant human visfatin significantly increased feeding behavior in a dose-dependent manner without affecting water intake; a higher dose of visfatin prolonged increased feed intake. The fact that an increased feeding response was generated at low doses suggests that visfatin is a fast-acting signal of hunger. The low dose of visfatin (0.025 nmol) used in this study was significantly lower than the dose of NPY (another orexigenic peptide) required to elicit the same response and simultaneously increased locomotor activity, and feeding efficiency [110]. Conversely, in rodents, intracerebroventricular injection of visfatin reduced food intake, increased weight loss, and decreased locomotor activity [112].

The discovery of adiponectin receptors in the paraventricular nucleus of the hypothalamus, which regulates metabolic homeostasis [113], along with their presence in liver and skeletal muscle, suggests a role for ADP in regulating feeding-related behaviors and energy

homeostasis. Studies regarding the effect of adiponectin on food intake have been controversial. Coope et al. [114] demonstrated that intracerebroventricular injection of adiponectin caused a reduction of food intake and body weight in rats. Qi et al. [115] demonstrated that intracerebroventricular injection of adiponectin in mice decreased body weight with no effect on food intake. An additional group demonstrated that peripheral or intracerebroventricular injection of adiponectin increased food intake [116]. Comparison of these studies is confounded by methodology and model organism. Adiponectin may have species, age, sex, dose, or route of administration-specific effects on feeding behavior and energy expenditure. Studies of the direct effects of ADP on feeding behavior have not yet been performed in avian species.

Resistin has been implicated in short-term feeding control. A study by Vazquez et al. [117] established that administration of resistin to rats caused a decrease in food intake and loss of body weight. Again, no studies of the effect of resistin on feeding behavior have been performed in birds.

Because behavioral studies documenting the effects of adipokines have yet to be performed in avian model organisms it is difficult to speculate how birds compensate for the lack of leptin. An attractive hypothesis may be that adiponectin acts in parallel with visfatin as metabolic signals of satiety and hunger. If adiponectin can indeed stimulate fatty acid oxidation without increasing food intake, adiponectin would be a particularly crucial signal for migratory birds. Adiponectin may promote the switch to primarily lipid utilization during the migratory period, at the same time allowing hyperphagia to build or maintain fat stores during migration. Possessing a continuously updated indicator of energy stores during the course of migration would allow birds to determine flight range capabilities and length of stays at stopover sites to rest and rebuild fat stores. When birds fly over lengthy barriers, such as the Gulf of Mexico,

energy reserves are so severely depleted that foraging must commence immediately after landing [118]. Being able to monitor fat stores is critical to the success of an individual's migration as misjudging the extent of energy supply may cause birds to be stranded by exhaustion in poor quality foraging sites and severely delay migration or result in mortality.

Future Directions

Literature describing the fundamental function, action and regulation of fat-derived substances on avian species is lacking. Because hormone-dependent physiologies are often species-dependent, as demonstrated by the conflicting studies on humans, mice, rats, and chicken, fundamental information regarding the biology of fat metabolism needs to be detailed in passerines. These investigations should also focus on sex and age-specific differences in adipokine signaling, as these differences have been documented in mammals [55,119]. Once we have a working knowledge of this highly integrative system and its effects on behavioral ecology can more applied studies can be performed.

Songbird populations have recently been declining and temporal and range shifts in relation to climate change have been established in many migrant species [120]. Conservation efforts have focused mostly on breeding and wintering sites of highly impacted species in response to these changes. However, species at risk may receive greater benefit from more emphasis being placed on conserving high quality stopover sites along the migratory route. Migration is extremely costly for small songbirds, and access to abundant food resources during migration is a requisite for its successful completion. The abundance of migrants decreases as development increases and habitats with greater fruit availability are disturbed [121,122]. Management of areas with high energy fruiting species would help maintain optimal digestive

efficiency and perhaps reduce the accompanying stress of climate change or development on passerine migrants.

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CHAPTER 2

A DESCRIPTIVE ACCOUNT OF SEASONAL DIFFERENCES IN BEHAVIOR PATTERNS OF THE MIGRATORY WHITE-THROATED SPARROW

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ABSTRACT

Avian migration and associated changes in behavior patterns occur on a seasonal basis and are regulated by both circadian and circannual clocks. It is well known that migrating birds must partition behaviors differently, to account for alterations in locomotor activity and sleep cycles. These changes in behavior patterns occur seasonally and are necessary for successful migration. Because of the difficulties related to the study of bird migration in the wild, many studies regarding the physiology of migration have been performed in captivity, where nocturnal migratory restlessness is readily observable. Even so, quantifying the amount and timing of circadian behavior rhythms in migrating and non-migrating individuals has not previously been performed. We used video recordings of a common nocturnal migrant, the White-throated Sparrow (*Zonotrichia albicollis*), to generate a basic ethogram and charts of the timing and frequency of occurrence of each behavior to compare migrant individuals, defined by their stereotypical expression of nocturnal activity, with non-migrating birds.

INTRODUCTION

The discovery of nocturnal migratory restlessness, or zugunruhe, a proxy of migratory flight in the wild, instigated years of work examining avian migration using captive individuals and has produced significant findings regarding the migratory physiology and the endogenous circadian and circannual clocks regulating migration, and the physiological and behavioral adaptations necessary for engaging in migratory activity (see reviews in [1,2,3,4,5]). Detailed analysis of the changes in behaviors associated with the migratory period in captive birds however, has not yet been performed. Adequately describing the behavioral components of the migratory phenotype would provide a starting point for the comparative study of migrants and partial migrants. Behavioral studies beginning at the level of the individual can eventually be incorporated into larger-scale studies of pairwise or other social interactions.

During the migratory period, small songbirds undergo a drastic change in their behavioral repertoire[6]; diurnal songbirds become nocturnally active, to limit migratory flight to the night time. Consequently, a rearrangement of sleep cycles is necessary to account for increased locomotor nocturnal migratory activity [7]. The seasonal timing of behavior is modulated by a circannual clock and maintained, on a daily basis, by alterations in the function of circadian clocks. Although we know that individuals partition behaviors during migration, an accurate account of the changes in the appearance of select behaviors is lacking. The aim of this study is to provide an account of the timing of behavior patterns displayed by a common migrant with emphasis on the differences between the migratory and non-migratory periods as defined by nocturnal or exclusively diurnal activity patterns, respectively.

The White-throated Sparrow (WTSP) (*Zonotrichia albicollis*) is one of North America's most well-studied birds. Abundant in numbers, the species is found throughout Canada, south of

the tree line and the Eastern United States with fewer populations west of the Rocky Mountains. Winter populations extend as far south as northern Mexico and Florida [8]. Breeding and winter ranges overlap in northeast United States and south-eastern Canada. WTSP range is limited primarily by precipitation and minimum January temperature [8]. The species is monotypic, with no subspecies; WTSP are closely related to Juncos and will sometimes hybridize with them as well as White-crowned Sparrows [8]. WTSP prefer edge habitats, brushy fields, overgrown pasture, and second growth forests. During the breeding season these birds feed primarily on insects, supplementing with seeds and fruit; during migration WTSP are frugivores; overwintering, WTSP feed mainly on seeds and fruit. Although migratory routes are not documented in detail, almost all populations are migratory (short-medium distance, nocturnal migrants) [8]. In Pennsylvania, Spring migration occurs from April to June, and Autumn migration from November through December [9]. Adult birds display Prealternate molt on wintering grounds before and sometimes during spring migration. Adults undergo a Prebasic molt of all primaries, secondaries, and retrices on the breeding grounds before beginning autumn migration. There is abundant scientific literature citing the use of WTSP and closely related species in studies of migration using both captive and wild individuals (e.g. [5,7,10,11]). We observed individual migrating and non-migrating birds, where birds are nocturnally active, and diurnal, respectively, for continuous 10 day segments. We describe the behaviors observed in individual birds and discuss the significance of the amount and timing of various behaviors between the two migratory conditions.

MATERIALS AND METHODS

WTSP were mist-netted in Centre County, PA during the spring of 2008 (see Appendix A for use of individual birds) (USFWS #MB170276-0; PAGC #COL00194). Sparrows were housed individually in wire-grated bird cages (9" x 12" x 15.5", Top Wing®) in the Poultry Education and Research Center (The Pennsylvania State University, University Park, PA). After capture, birds were maintained in a 12hr:12hr L:D photoperiod and had access to food and water *ad libitum*. Birds were monitored with infrared motion sensors (Quorum International; VitalView, Sunriver, OR) mounted above each cage and video recordings were taken continuously with cameras equipped with infrared lights (Q-See®) beginning autumn 2009. Individuals were visually isolated from other conspecifics but within auditory contact.

Video recordings were made during Fall 2009- Fall 2010 to capture the middle portion of both the migratory and non-migratory periods of different individuals (3 individuals were observed during both the migratory and non-migratory periods; 2 individuals were observed in only one of the migratory conditions). We defined the migratory period as the times of year where birds are nocturnally active; birds are exclusively diurnal during the non-migratory period. The behaviors of birds were observed for a continuous 10 day period during the non-migratory (n=4) and migratory (n=4) periods. Start dates of each 10 day segment were randomly assigned, never occurring at the transition from one migratory condition to the other. Behaviors were scored as either occurring (score=1) or not occurring (score=0) every 15 minutes for 10 days; infrared cameras enabled us to observe nighttime behavior without affecting expression.

Behavioral Analyses

Polar plots displaying the occurrence of each behavior at different times of the day were constructed by averaging occurrence scores over 10 day sampling periods of individuals using the analysis program SigmaPlot® for Windows version 11.0 [12]. We then plotted the scores as percentages of individuals displaying the behavior at each time of day. We also calculated the average number of 15 minute intervals per day spent displaying each behavior, and averaged values over each 10 day sampling period per individual and separated scores by group. Scores from the 10 day sampling period of each WTSP were used to calculate the mean ± SE for select behaviors of each individual (see Appendix B).

RESULTS

Ten behaviors were defined in WTSP (Table 1.) and behaviors were assigned to one of five functional categories: maintenance, locomotor activity, vigilance, recovery, and communication.

Table 1. The following behaviors were observed in captive White-throated Sparrows during either the migrating or non-migrating periods. Behaviors with expression limited to the migratory period are indicated with an *.

Functional category	Behavior	Description
Maintenance	Feeding	From inside food dish or perched on rim, head is bent towards food to peck and eat pellets.
	Preening	Straightening and cleaning feathers with beak.
	Bathing	Splashing water from drinking dish to wet feathers using either the beak or wings.
Locomotor activity	Flying	Flapping movement from perches, floor, or food dishes to outer walls of cage and back.
	Hopping	Movement without flapping between perches and dishes, or across perches.
	Wing-whirring*	Quick beating of wings while lightly perched; body is sometimes stretched to full length.
Vigilance	Looking around	Head rotates back and forth, surveying the surroundings while perched.
Recovery	Resting	Little body movement, eyes droop; sometimes the head hangs or slowly bobs.
	Sleeping	Perched, eyes closed, head tucked under wing.
Communication	Singing	Head tilted upwards with beak open during song.

Because our cameras did not support audio recording, singing was not used in analysis, as it was difficult to distinguish singing in all individuals. The only behavior not observed in non-migrating individuals was wing-whirring (WW), which was to be expected as it is a stereotypical behavior that defines the migratory period along with increases in other nocturnal locomotor

behaviors. In addition to behaviors described in the ethogram, all individuals exhibited a similar response immediately following the lights being turned off: each day, individuals would hop from one end of the perch to the other in decreasing distance, frequently stretching their bodies upward, until they settled on one end to sleep (eyes closed, head tucked under wing) or rest (eyes droop, head hangs or slowly bobs). Total number of 15 minute intervals of activity expression plotted based on lights on or off are given as bar charts (see Appendix B).

Polar Plots

Maintenance

Amount and timing of maintenance behaviors (feeding and preening) were similar during the migratory and non-migratory periods. Non-migrating birds appear to spend more intervals preening during the day than migrating birds (Fig. 2.1). Feeding behaviors were virtually never displayed at night (over the 10 day sample period, non-migrating birds displayed feeding behavior either once or twice, and feeding behavior occurred in migrating birds between zero and six times).

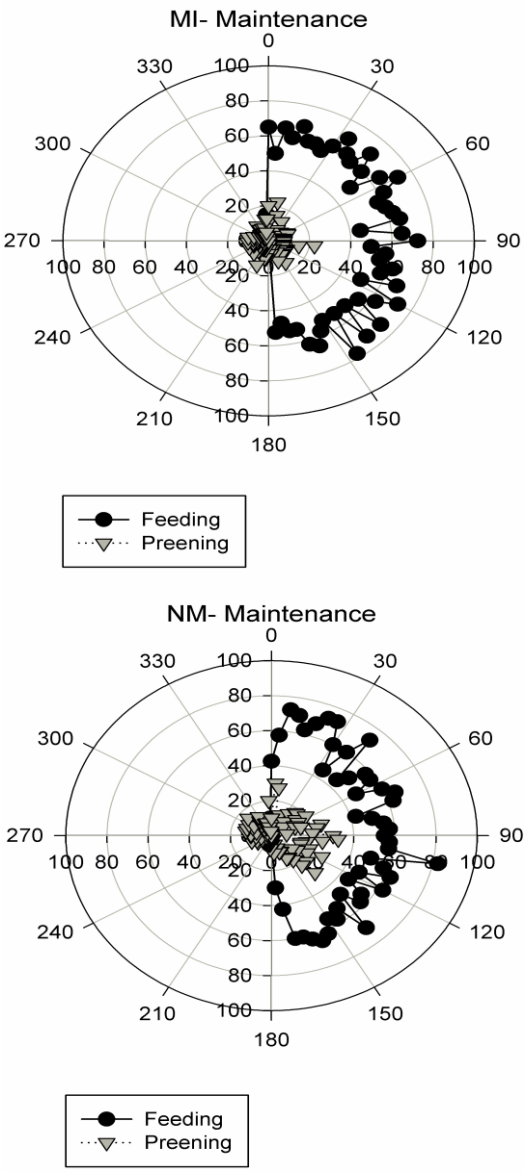

Figure 2.1. Polar plot of average individual and group maintenance behaviors of migrating (MI) (n=4) and non-migrating (NM) (n=4) White-throated Sparrows. 360 degrees corresponds to a 24 hour day where 1-180 degrees is the time when lights are on, and 181-360 degrees is the time when lights are off. Points close to the center of the plot indicate lower levels of behavior expression; points further from the center indicate higher levels of behavior expression.

Locomotor activity

During the daytime, hopping was similar between the groups; flying, however, occurred more frequently in migrating birds during the day (Fig. 2.2). At night, migrating birds displayed an even greater amount of hopping, flying, and wing-whirring (Fig. 2.2). Migrating birds generally

began displaying nocturnal migratory restlessness approximately 1.5 hours after lights-off, with the greatest expression during the second half of the night and frequently continued until lights-on.

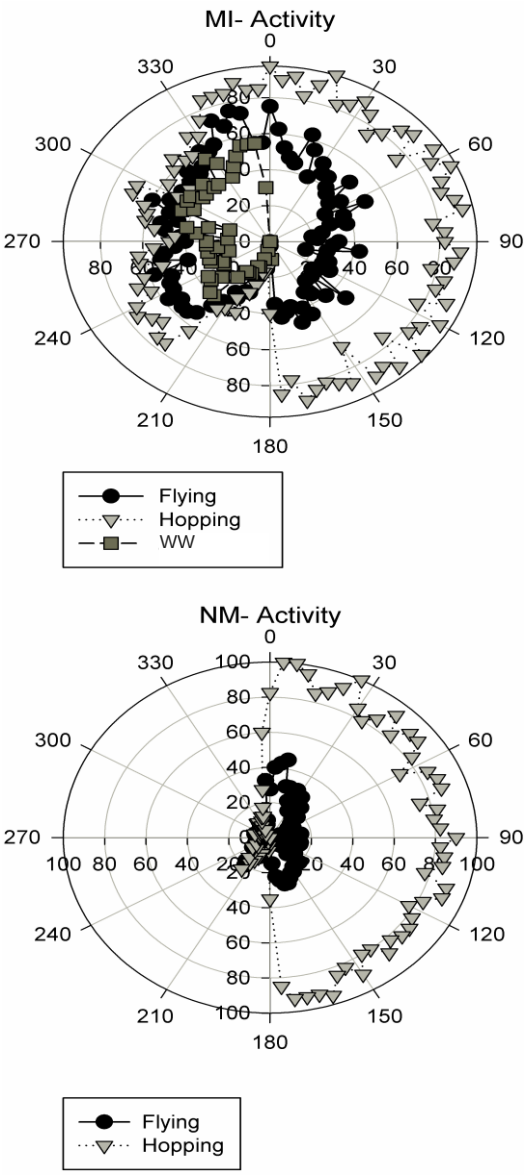

Figure 2.2. Polar plot of average individual and group locomotor behaviors of migrating individuals (MI) (n=4) (hopping, flying, and wing-whirring) and non-migrating White-throated Sparrows (hopping and flying). 360 degrees corresponds to a 24 hour day. Points close to the center of the plot indicate lower levels of behavior expression; points further from the center indicate higher levels of behavior expression. WW= wing-whirring

Vigilance

Although daytime vigilance was similar between groups, non-migrating birds appeared more vigilant earlier after the lights were turned on, than migrating individuals, while migrating birds were more vigilant immediately preceding lights-off (see Fig. 2.3 circles). Migrating birds also displayed more nocturnal vigilance behavior than non-migrating birds.

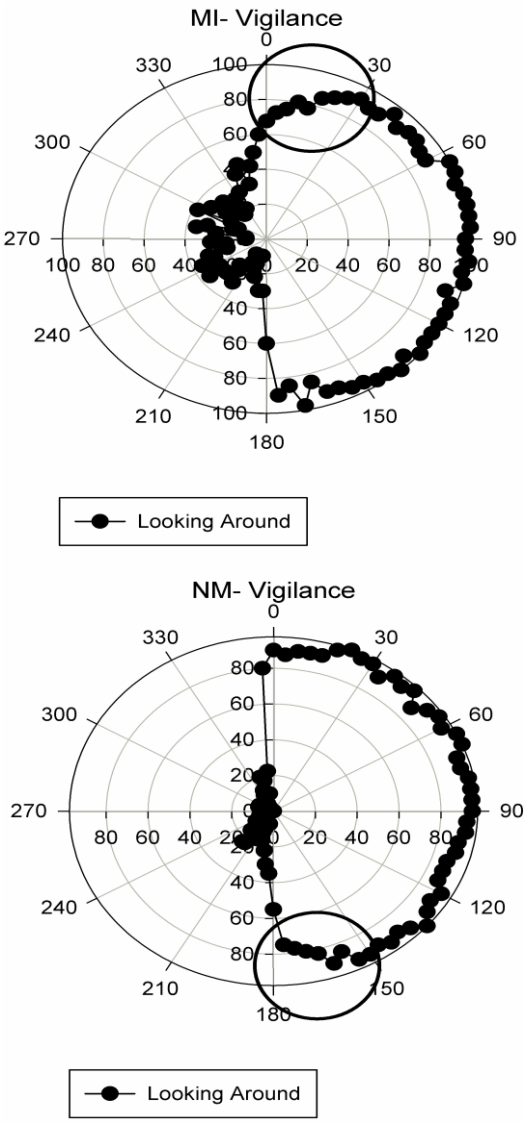

Figure 2.3. Polar plot of average individual and group vigilance behavior of migrating (MI) (n=4) and non-migrating (NM) (n=4) White-throated Sparrows. 360 degrees corresponds to a 24 hour day. Points close to the center of the plot indicate lower levels of behavior expression; points further from the center indicate higher levels of behavior expression. Circles: see Discussion.

Recovery

The distribution of sleep over the course of the night appeared different between the two groups of birds. Migrating birds went to sleep almost immediately after the lights were turned off, while non-migrating birds spent considerable time resting before displaying true sleep and continued to sleep until just before lights-on (Fig. 2.4).

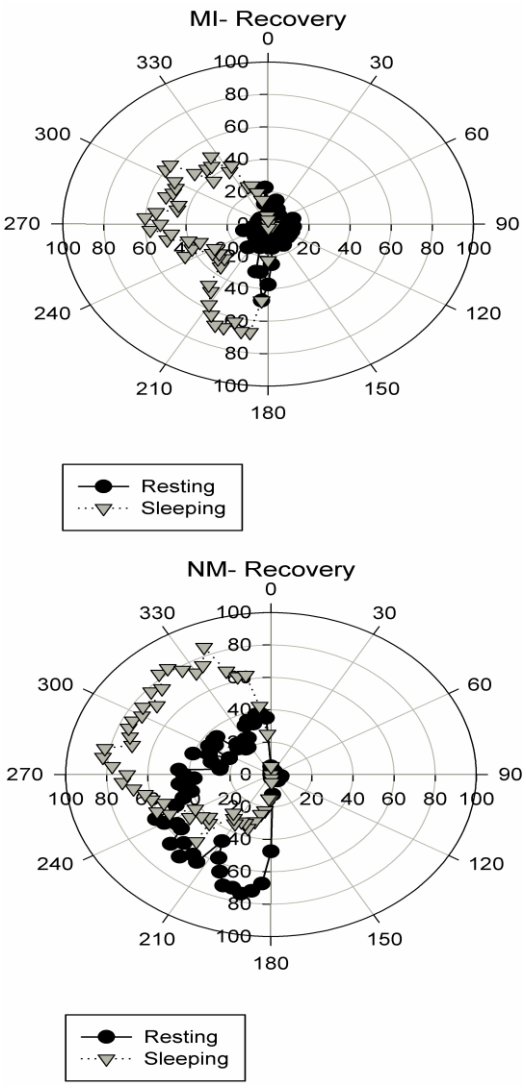

Figure 2.4. Polar plot of average individual and group recovery behaviors of migrating (MI) (n=4) and non-migrating (NM) (n=4) White-throated Sparrows. 360 degrees corresponds to a 24 hour day. Points close to the center of the plot indicate lower levels of behavior expression; points further from the center indicate higher levels of behavior expression.

Sleep replaced resting, shortly after lights-off, in non-migrating birds, while the greatest amount of sleeping behavior was partitioned to the second half of the lights-off period. Sleep occurred in migrating birds immediately after lights-off and towards the middle of the lights-off period. Migrating birds did not rest much during the night but would sleep in-between bouts of locomotor activity. Migrating birds also displayed increased amounts of resting behavior throughout the day compared with non-migrating birds.

DISCUSSION

In this study we developed an ethogram of the basic behavioral repertoire of captive White-throated Sparrows. Our ethogram represents the basic behaviors of individual birds and can be used as a framework to conduct analysis of pairwise interactions, population-scale behavioral studies, or comparisons between species. Although many of the basic behaviors presented here are not new observations, a formal account of the temporal organization of behavior patterns on a daily basis has not previously been compiled.

As is the case with any organism, the effects of captivity must be considered when interpreting results from behavioral observations. The lack of many environmental and social factors may change the complexity, frequency, or occurrence of behavior patterns. Notwithstanding, captivity offers the opportunity to record the most fundamental behaviors, in great detail over long, continuous periods of time. This is especially important as studying behaviors of birds during actual migration is currently not possible in the wild. Because these birds are not exposed to seasonal environmental cues, their circannual rhythms might become uncoupled from what is observed in wild conspecifics. Furthermore, the birds used in this study were in captivity for approximately 1.5 years prior to behavioral data collection, potentially further uncoupling yearly rhythms. However, 3 of the 4 migrating birds used in this study displayed Autumn migration at the appropriate time of year compared with Pennsylvania populations; the 4th migrating bird was displaying migration approximately 2 months later than the average wild, Pennsylvania conspecific (see Appendix A). Intra-individual differences in circannual rhythm will become increasingly apparent the longer individuals are kept away from seasonal environmental cues.

We used this ethological study to investigate differences in behavioral patterns between birds that were migrating and those that were not. We recorded ten discrete behaviors, one of which, wing-whirring, is expressed exclusively during the migratory period and is a behavior that we used to define migrating individuals. Visual evaluation of polar plots allowed us to observe differences in behavior partitioning and pattern between the two migratory conditions.

Patterns of maintenance behaviors were visually similar in migrating and non-migrating individuals. Because WTSP deposit most of their migratory fat stores during pre-migratory hyperphagia, it is not unusual that migrating individuals do not differ in their feeding patterns compared with non-migrating birds [13]. Increases in feeding and assimilation efficiency, along with decreased basal metabolic rate might allow migrating birds to maintain high levels of body fat given similar feeding behavior as non-migrating birds. Data on feeding should be considered with the caveat that most free-living migratory birds exhibit changes in food preference between the migratory and non-migratory states [14,15]. Allowing our captive birds access to high-quality food *ad lib*, year round could mask any changes in feeding-related behaviors between the migratory conditions. Individuals are able to maintain high levels of subcutaneous fat during the migrating period (unpublished data) by foraging during daytime hours between relatively short migratory flights suggesting that increased intake in food per feeding bout or alterations in metabolic output may occur [16].

Preening patterns were similar between migratory conditions; non-migrating birds displayed slightly greater preening activity during the daytime, perhaps to maintain newly molted feathers. Previous work in swallows has demonstrated that time spent preening increases during the early Spring, and decreases during the breeding season [17]. These differences could be responses to differences in exposure to parasites, which increase in the spring and re-

prioritization of time budgets during incubation and rearing of nestlings during the breeding season. Maxson and Oring found that during the breeding season, male spotted sandpipers preen more than females [18]. Maxson and Oring [18] propose that because females have higher energy requirements during breeding than males, and consequently will spend more time actively foraging, males have more time available for other behaviors such as preening. Three of the non-migrating birds used in this study were observed before they began autumn migration. In isolation, these birds are unable to breed, although they do display seasonal cycles of growth and regression of reproductive organs (unpublished data), thus, the increase in preening behavior we observed might be in response to a complete molt of body and flight feathers occurring after the reproductive period but prior to autumn migration. Although in some agonistic situations between individuals, preening is often a displacement behavior [19,20], individuals observed in this study often displayed autochthonous preening after bathing.

Interestingly, one migrating individual arrested nocturnal migratory activity for one night during the 10 day review period, which could be equivalent to a stopover in the wild. It is interesting to note that the day prior to stopover, we observed lower than average feeding behavior during the day, and lower than average migratory restlessness preceding the stopover. Fat stores are a bird's primary source of fuel during migratory flight and even a short period of poor foraging is able to induce a change in migratory behavior [4,21,22]. The extent of subcutaneous fat deposits determines the length of stopover in free-living migrants [23,24,25]. In this individual, one day of arrested migration followed one day of reduced feeding. After one night of stopover, feeding behavior was back to observed usual levels.

As expected, nighttime locomotor activity appeared to be greater in migrating birds and migratory restlessness included increased hopping, flying, and wing-whirring. Although hopping

behavior was similar in migrating and non-migrating birds, it is unclear why migrating individuals displayed increased daytime flying.

Migrating and non-migrating birds display interesting differences in vigilance behavior at the transitions between light and dark (Fig. 2.3 circles). Migrating birds are comparatively more vigilant than non-migrating birds near the transition from day to night, just before they are about to commence nocturnal migration. Unlike non-migrating birds at this time who are settling down inconspicuously to sleep, migrating birds are going to be active throughout the night. Nocturnal migration may be conspicuous to nocturnal predators therefore migrants might need to be more vigilant at this time and during the night. During migration, birds are continuously presented with novel situations: novel foraging sites, novel predators, novel climate, etc. We might expect that during this life history stage, migrants display different amounts of exploratory behaviors, vigilance, or neophobia [26]. Similarly, Mettke-Hofmann discovered seasonal differences in novelty response during two life history stages, courting and breeding of Red-rumped Parrots [27]. Differences in vigilance behavior observed in our WTSP might be due to endogenous circannual changes in neophobia. Captive migrating individuals were not exposed to novel surroundings or situations during the migratory period but appeared to display different pattern and amount of vigilance behavior than captive non-migrating birds.

Along with such a drastic increase in overall locomotor activity, migrating birds must partition sleep and rest differently to account for a reduction in time available for rest. The importance of sleep for repair, memory consolidation, and homeostasis is well documented, although not well understood [28,29,30]. Sleep deprivation comes with many negative side-effects, including reduced cognitive and physical performance, the worst of which is mortality. These negative effects however, are not generally observed in migrating birds. WTSP, like other

nocturnal migrants, seem to have developed behavioral and physiological adaptations to compensate for reduced nocturnal sleep. One method of behavioral compensation is to display sleep-like behavior throughout the daytime [31]. Because normal bouts of avian nocturnal sleep can be short in duration, lasting only a couple minutes in some cases [32], even short bouts of rest during the daytime should partially compensate for loss of nighttime sleep. Presumably, daytime rest does not significantly increase risk of predation as individuals remain vigilant between bouts of rest. Birds may also exhibit daytime unihemispheric sleep [7,33,34]; however, we were unable to detect this behavior while limited to a one-camera perspective. Birds may further compensate for reduced sleep during the migrating period by decreasing the amount of time spent in early stages of sleep and spend greater proportions of time in REM or deeper stages of sleep. Indeed, we observed more nighttime resting behavior in non-migrating individuals compared with those migrating as opposed to "true" sleep. These compensatory behaviors may enable birds to overcome the negative effects of sleep deprivation and maintain high performance levels, greater even than during the non-migrating period [7].

ACKNOWLEDGEMENTS

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CHAPTER 3

DIFFERENTIAL REGULATION OF ADIPOKINES MAY INFLUENCE MIGRATORY BEHAVIOR IN THE WHITE-THROATED SPARROW (*ZONOTRICHIA ALBICOLLIS*)

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ABSTRACT

White-throated sparrows increase fat deposits during pre-migratory periods and rely on these fat stores to fuel their bodies during migration. Previous work has concluded that the size of these temporary subcutaneous fat deposits influences the onset of migration and the length of delays at individual stopover sites during the course of migration where birds rest and rebuild fat stores. Lean birds have prolonged stays at stopover sites whereas fat birds generally leave after a single day of rest. Here, we present evidence that a change in signal from adipokines might act as an indicator of body condition, thereby influencing an individual's decision to commence migratory flight, or to delay and refuel. We quantified plasma adiponectin and visfatin levels across the day in birds held captive under constant photoperiod. The circadian profiles of plasma adiponectin in non-migrating birds are approximately inverse of profiles from migrating birds. Adiponectin levels are positively correlated to body fat, and body fat is positively correlated to amount of

nocturnal migratory restlessness. Visfatin levels appeared constant on a circadian basis and did not correlate with fat deposits however, a reduction in concentration occurs during the migratory period. The data suggest a significant change in the biological control of adipokine expression between the two migratory conditions and we propose a role for adiponectin, visfatin and adipose clocks in the regulation of migratory behaviors.

INTRODUCTION

Small songbirds have evolved many strategies to cope with the costly rigors of long-distance migration. During the pre-migratory period, physiological and endocrine systems change in preparation for migration; birds become hyperphagic and alter their metabolism resulting in increased fat deposition [1,2]. Fat provides more than twice the calories per gram and approximately double the amount of metabolic water as protein or carbohydrates [3].

Avian migrants rely heavily on circannual and circadian clocks to regulate migration and the processes of these clocks are shaped by changes in photoperiod. Biological clocks and photoperiodic timers dictate the initiation, duration, termination, and organization of the migratory period (see reviews in [4,5,6,7,8]). Hyperphagia and pre-migratory fattening are important aspects of the physiological preparation for long-distance flight and these preparations are all under the control of biological clocks [1,9,10].

Pre-migratory hyperphagia and fattening may lead to changes in metabolic signals circulating in the blood of individual birds. Consequently, changes in adipokine signaling could be utilized to initiate migration once fat stores reach a critical level. The migratory programs of many small songbirds consist of segments of nocturnal flights and subsequent daytime stops to rest and refuel. Although many small passerines commence migratory flight after peak fat deposition has been reached [1], unforeseen circumstances during migratory flight, such as adverse wind and weather, may prompt increased fuel use. Flights during successive nights under poor conditions may lower fuel reserves below critical thresholds, requiring birds to delay longer at stopover sites to replenish fat stores [11].

The circannual rhythm of migration is shaped by changes in photoperiod, indicating an influence of environmental cues in regulating this program. The degree to which photoperiodic

cues directly regulate migratory behaviors changes among taxa and vary even within the same genus [12]. The appearance of "nocturnal migratory restlessness", commonly called Zugunruhe, in otherwise diurnal animals is under the direct control of an endogenous circadian clock [13,14]. In *Zonotrichia albicollis*, the white-throated sparrow (WTSP), it has been demonstrated that circadian clocks and photoperiodic timers regulate the seasonal appearance of Zugunruhe [12,15,16]. Clocks are generated by molecular rhythms in the expression and activity of so called "clock genes" which regulate behavioral rhythms through positive and negative autoregulatory feedback loops [17]. Molecular clocks are influenced by metabolism, in particular through alterations in redox states and gas responsive elements [18,19,20]. The molecular oscillations of endogenous clocks convey rhythmic information to peripheral systems and modulate system-specific rhythmic behaviors [21]. Rhythmic expression of canonical clock genes has been observed in peripheral organ systems, including adipose tissue [22,23]. Because migratory behaviors arise from the interface of circadian and circannual clocks, inter-clock communication must occur. Clocks located in the periphery may play a role in imparting feedback information to alter, synchronize or re-set clocks in the brain based upon specific internal conditions.

Historically, adipose tissue was thought to function exclusively as a storage depot for energy. More recently, the role of adipose tissue as an endocrine organ secreting adipokines has been detailed [24,25,26]. Adiponectin (ADP) is a hormone secreted by adipocytes and regulates energy homeostasis [27]. Consequently, adiponectin may play a role in the switch to lipid metabolism as the main source of energy fueling migratory flight. ADP is a self-aggregating protein that circulates in the plasma as trimer, hexamer and (primarily) as a high molecular weight isoform (HMW) [28]. The function of specific isoforms remains unclear, as studies have produced conflicting results [27,29]. It is generally accepted, however, that HMW isoforms of

ADP are considered to be most physiologically protective for their anti-atherogenic and anti-inflammatory properties [30,31]. Both normal-weight human subjects and rodent models display circadian rhythmicity of plasma ADP levels and ADP gene expression and plasma ADP concentrations increase after weight loss [10,32,33]. Calvani et al. [30] also observed that the daily expression of plasma ADP in obese subjects had smaller amplitudes compared with normal weight subjects. Although ADP is secreted primarily by adipose tissue, ADP mRNA is also expressed in various other tissues including the liver, skeletal muscle, kidney, pituitary gland, and diencephalon, suggesting autocrine or paracrine roles for this hormone [34]. Because of its role in stimulating fatty-acid oxidation [27], ADP could potentially act as a signal of body condition during the migratory period, a time when birds rely on fat as their main energy source. Adiponectin could signal the brain to modulate energy expenditures during food intake and fasting. We hypothesize that ADP levels are regulated by a biological clock, similar to other autocrine factors such as prolactin [35], serotonin [36], and melatonin [37,38]. Furthermore, a change in the circadian rhythm of ADP signaling, whether in amplitude or phase, etc., between non-migratory and migratory conditions could influence migratory activity by communicating changes in adiposity to the clock in the brain which regulates Zugunruhe.

Another potentially important adipokine, visfatin, has been implicated in directly linking metabolism to the molecular clockwork. As a rate-limiting enzyme in the NAD+ biosynthetic pathway, visfatin maintains a reservoir of NAD to function in redox reactions, metabolism, and to act as a substrate for sirtuins. In order to function, SIRT1 requires NAD, whose availability fluctuates with metabolic state. In mammals, SIRT1 binds directly to the CLOCK/BMAL dimer complex and is necessary for transcription of core clock genes in the generation of circadian rhythms. Thus, visfatin plays an indirect role in providing metabolic information to biological

clocks by regulating the production of NAD. Changes occur in the biological clockwork of nocturnal migrants during migration [14]. A change in visfatin signals between the non-migratory and migratory periods would provide different information about metabolic state and potentially influence migratory behavior. Although controversial, visfatin, which currently has no known receptors, has also been labeled an insulin mimic.

We used Western Blotting and enzyme-linked immunosorbant assay techniques to quantify the levels of two adipokines in the blood plasma of a common migratory bird, the white-throated sparrow, *Zonotrichia albicollis*, and compared plasma levels of these adipokines at different times of the day and during the migratory and non-migratory periods. Because white-throated sparrows migrate at night, migratory behavior could be quantified by measuring the amount of nocturnal activity present in individuals while caged. Nocturnal migratory restlessness in white-throated sparrows is under the direct control of a circadian clock [15,16]. Unlike migratory behaviors in other *Zonotrichia* species, nocturnal migratory restlessness in WTSP is regulated by biological clocks, photoperiod, and the changes in daily expression of locomotor activity [2,12,15,16,39,40]. Subcutaneous fat scores, a reliable measure of adiposity in birds [41,42], were taken to determine the levels of migratory fattening. Our results provide new evidence regarding the roles of adipose signaling in birds and that adipose signaling may be used to regulate circannual and circadian migratory behaviors.

MATERIALS AND METHODS

Housing of animals

White-throated sparrows were captured in Centre County Pennsylvania during spring 2008 using mist nets (see Appendix A) (USFWS #MB170276-0; PAGC #COL00194). Wild-caught sparrows were housed at the Poultry Education and Research Center (The Pennsylvania State University, University Park, PA) in a large, environmentally controlled indoor aviary. Birds were maintained under a 12hr light, 12hr dark photoperiod and were provided with food and water *ad libitum*. Birds (n=10 ADP study; n=17 visfatin study) were transferred to individual wire grated bird cages (9" x 12" x 15.5", Top Wing®) in auditory contact with other birds and were allowed to acclimate for at least 2 weeks before experimentation began. All procedures were performed in accordance with standards approved by the Pennsylvania State University Institutional Animal Care and Use Committee (IACUC #27105, and #29985).

Locomotor and behavioral activity

Infrared motion sensors (Quorum International) were mounted above each cage to record locomotor activity. Locomotor data were collected in 5 minute bins using the software program, VitalView (Mini Mitter, Respironics) and evaluated for daily activity with the software package, ActiView (Mini Mitter, Respironics). Migratory status was determined using the locomotor activity record by noting exclusively diurnal patterns of activity (non-migrating), and periods of excessive nighttime activity (migrating), as has been previously reported for WTSP [15,16].

Nocturnal migratory restlessness is apparent in caged individuals as increased nocturnal activity, including flightless wing-whirring which corresponds to migratory flight in free-living birds. We used infrared video cameras (Q-See®) to continuously record the behaviors of

individual birds. Video recordings were used to confirm when individual birds were exhibiting nocturnal migratory restlessness [14].

Fat scoring

We utilized the subcutaneous fat score classification system developed by A. Kaiser [41], previously tested and validated by Soxhlet extraction in similar species of either the same order (Passiformes) or family (Emberizidae) [42]. This classification system has been shown to produce highly repeatable results by ranking fat scores from 0 (no fat) to 8 (extremely fat) by subcutaneous fat load in the furcular depression, breast muscles, and abdomen. Birds were evaluated bi-weekly, between the times of 3 and 4 ZT (ZT=zeitgeber time, time since lights on) to control for any daily changes in fat deposits. This method is widely practiced in field studies of small songbirds [61,62,63,64].

Western blot analysis of plasma ADP

Blood samples (~50µl) were collected from migrating (n=7) and non-migrating (n=5) birds from the brachial vein with heparinized capillary tubes during autumn 2009 and spring 2010. Samples were collected at four different times of day, in 6hr intervals beginning with ZT0 (time of lights on) during one continuous migratory or non-migratory period. We allowed three days between each blood drawing for recovery. Samples were centrifuged at 2500rpm for 20 minutes at 4°C and the plasma collected and stored until use at -80°C.

Using the Novex mini gel system and NativePAGE gels (Invitrogen®) we performed gel electrophoresis according to the manufacturer's instructions. Serial dilutions of plasma were performed to optimize the amount of protein loaded per well. A standard curve was generated from the signal intensity measures of seven different plasma dilutions to determine the linear range (r^2=0.97) of the standard curve. The 1:20 plasma dilution was chosen for further use from

within the linear range of the standard curve. Plasma samples were prepared by adding 5ul of 1:20 diluted plasma sample to 4X Sample Buffer, G-250 Sample Additive, and deionized water. 10 µl of sample mixture was loaded into NativePAGE 4-16% Bis-Tris gel with NativeMark unstained (Invitrogen®), and Novex Sharp pre-stained (Invitrogen®) protein standards to provide a molecular mass reference, and a 1:100 dilution of broiler chicken plasma was included as a positive control.

After separation, proteins were electrotransferred to a polyvinylidenedifluoride membrane. To check the uniformity of transfer, the gel was stained using Coomassie Blue. Membranes were incubated in SuperBlock Blocking Buffer (Pierce) for 1hr at room temperature, incubated in a custom generated primary antibody against a keyhole limpet hemocyanin conjugate in the N-terminal domain of chicken adiponectin (Rabbit anti-cADN; 1:2500) [43,65] overnight at 4°C, and incubated in secondary antibody (Pierce anti-rabbit IgG-HRP 1:10,000) at room temperature for 1hr. Enhanced chemiluminescence detection was performed on transparency film with ECL Plus Detection Reagent (Amersham) and imaged on the STORM 860 optical scanner using blue wavelength fluorescence. To determine the specific immunoreactivity of anti-adiponectin antibody, primary antibody was preadsorbed with chicken adiponectin protein [43].

Enzyme-linked immunosorbant assay (ELISA) analysis of plasma visfatin

Blood samples (~200 µl) were collected from the brachial vein using heparinized capillary tubes from migrating (n=8) and non-migrating (n=9) individuals. Samples were collected at four different times of day (ZT 0, 6, 12, 18) to assess circadian rhythmicity. We allowed at least 2 days between sampling for the birds to recover. Blood samples were centrifuged at 2500rpm for 20 minutes at 4°C and the plasma was stored until use at -80°C.

Undiluted plasma visfatin concentrations were determined by ELISA (Phoenix Pharmaceuticals, Burlingame, CA, Visfatin C-Terminal (Human) Kit), following the manufacturer's instructions. The use of this kit for detecting avian plasma visfatin was previously validated in chicken [58]. Samples and controls were assayed in duplicate using two plates (intra-assay variation: plate 1= 11.6%; plate 2= 5.1%). Samples from both migrating and non-migrating birds were interspersed on both plates as a safeguard against nondemonic intrusion. Samples were read at 450nm using the FLUOstar Omega microplate reader; standard curves were generated using 4-parameter logistics based on blank corrected averages of duplicates. Sample volumes of one time point in two individuals were inadequate and not used for analysis.

Data analysis

The ECL signal intensity of ADP bands from Western blots was quantified (Image Quant 5.1) in arbitrary intensity units. Protein amount was determined by Bradford assay and signal intensity was normalized to individual protein concentrations. A calibration curve was constructed for each Bradford protein assay using five dilutions of bovine serum albumin in triplicate, including water blanks, using Bio-Rad Protein Assay Dye Reagent Concentrate. Sparrow plasma samples were diluted 1:100 to fall within the linear range of BSA standard curves and assayed in duplicate. Two birds exhibiting migratory behaviors ceased to exhibit zugunruhe during the blood collection period and were removed from analysis. Total plasma adiponectin levels were quantified and additional analyses were performed to quantify individual multimeric bands. Assumptions of homoscedacity and normality were assessed by evaluating normal probability plots. Non-normally distributed samples were $\log_{10}$ transformed to accommodate this assumption; bands that did not meet the normality assumption after

transformation were analyzed using nonparametric statistics (Kendall tau rank correlation). Average total ADP (sum intensity of all isoforms), individual isoforms of ADP, and visfatin concentrations were analyzed using linear harmonic regression in cosinor analysis using the program CircWave [66] to determine the presence of a circadian rhythm.

To accommodate dependency among levels of hormone sampled over a short time series, we employed a mixed modeling approach to examine the importance of migratory status (migrating vs. non-migrating), time of day (ZT 0, 6, 12, 18), sex (visfatin data only) and fat score in predicting hormone levels. To identify the most important variables in predicting levels of plasma HMW ADP, we compared five linear mixed-effects models using second order Akaike information criteria (AICc) to correct for small sample sizes, fitted in the software program "R" 2.10.1 [67] which is based on log-likelihood and the number of model parameters to quantify the evidence of support for each proposed model. The AICc value provides a measure of distance between competing models and allows for model comparison. Akaike weights are similar to probabilities and describe the weight of evidence in support for each model in minimizing the distance from the "true model" and allows for comparison of evidence for each model in a group of models. This weight can be interpreted as the relative probability of the model given the data [68,69]. A model with an Akaike weight approaching 1 is unambiguously supported by the observed data [70]. We began by considering a Full model ("Full"), including all three explanatory variables. We dropped the least important variable (time) and considered a model with migratory status and fat ("Status+Fat"), and a model with an interaction between group and fat ("Interaction"). Lastly, we considered models with migratory status ("Status") and fat ("Fat") individually. To account for the effects of autocorrelation in repeated sampling over time, we used an AR1 error structure and included "individual" as a random effect. The model with the

smallest relative AICc value indicates the best model of those proposed. Residuals were examined to assess homogeneity and verify normality. Examination of residuals versus fitted values ensured that the model fit our data well.

Six mixed-effects models were evaluated to predict plasma visfatin in white-throated sparrows. Migratory status, fat score, and sex, were included as fixed effects while individual and time of day were included as random effects. We used time as a random effect because plasma visfatin did not display circadian rhythmicity. We considered a Full model ("Full"), including all three fixed effects, an interaction model between migratory status and fat ("Interaction"), an additive model of migratory status and fat ("Status+Fat"), an additive model of sex and fat ("Sex+Fat"), as well as models with migratory status ("Status") and fat ("Fat") alone. Residual plots were used to evaluate fit of the models.

We calculated correlations between circulating plasma ADP isoform levels and fat score, and the ratio of daytime to nighttime activity and fat score with "R". We calculated this activity ratio rather than raw nighttime activity values to correct for individuals with varying baseline activity levels. Data for correlations of ADP and fat score were grouped by migratory disposition (exhibiting migratory restlessness or not) and ADP levels were evaluated at ZT 6 (the time closest to when fat scores were collected). Correlations between fat score and activity ratio were performed based on values averaged by individual. Because the fat classification system used in this study has a relatively large category size, and the distribution of ordinally measured fat scores is normal, ordinal fat score values were treated as continuous [71,72].

RESULTS

Migratory restlessness in captive birds

Birds were held under constant photoperiod (12L:12D); as such, observed measurements between seasons and across the day were not due to differences in photoperiod. We know that the endogenous circannual program continued to run in these individuals based upon the presence of seasonal changes in molt, migratory restlessness, and reproductive behaviors (unpublished data). Repeated changes in nocturnal activity observed in captivity approximated autumn and spring migratory periods of free-living conspecifics and indicate an endogenous control of migratory rhythms (unpublished data). Migratory restlessness in captive birds is manifested as increased nocturnal activity, including flightless wing-whirring, and corresponds to actual migratory flight in the wild. In *Zonotrichia albicollis*, migratory behavior is expressed as the presence of nocturnal locomotor activity concomitant with little change in daytime activity [15,16]. This is different than the locomotor profiles reported for other *Zonotrichia* species in captivity (i.e. a complete switch to exclusively nocturnal activity) [2,12]. Changes in nocturnal activity were quantified using infrared motion sensors and video cameras were used to validate migratory disposition (Fig. 3.1).

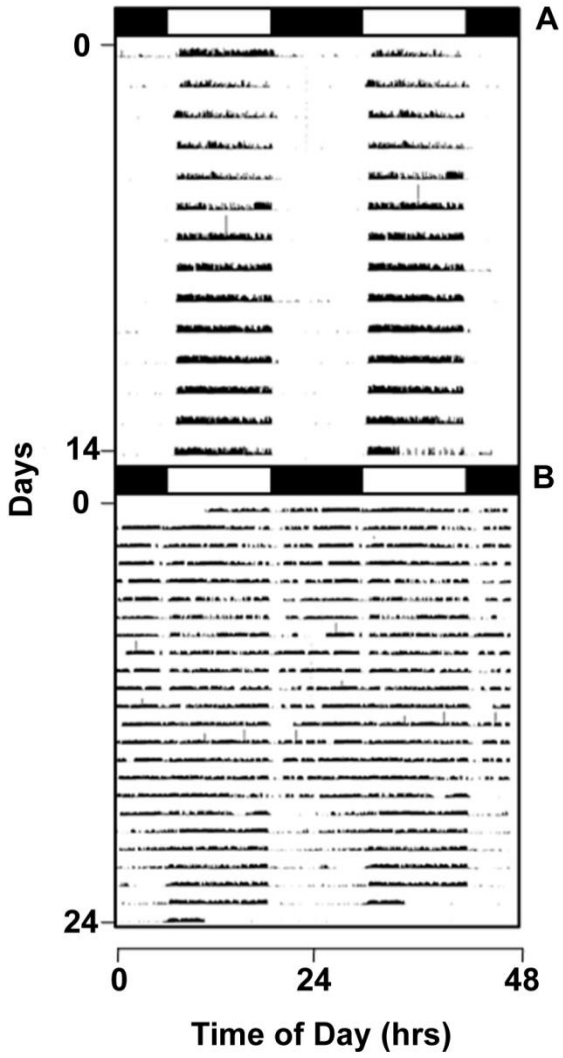

Figure 3.1. Locomotor activity of non-migrating and migrating white-throated sparrows.

Double-plotted actograms displaying locomotor activity recorded from representative non-migrating (**A**) and migrating (**B**) white-throated sparrows under a constant 12hr light and 12hr dark photoperiod. White and black bars above each actogram represent hours of lights-on and lights-off, respectively; thickness of lines represents levels of activity. WTSP are diurnal during the non-migratory period; Zugunruhe was directly observed in migrating individuals using infrared videography during hours of lights-off.

Detection of plasma adiponectin isoforms

To detect multiple isoforms of ADP in white-throated sparrow plasma, we performed Western blot analysis under native conditions (Fig. 3.2A). Strong immunoreactive bands were detected in sparrow plasma at approximately 720kDa (which we refer to as HMW), regardless of migratory status and sex.

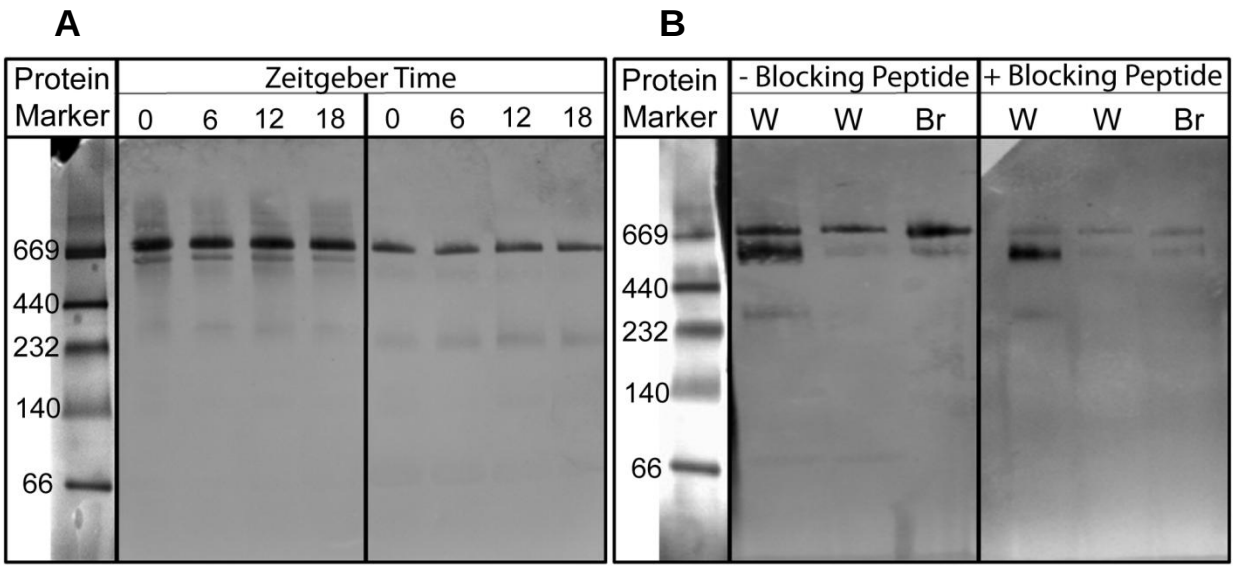

Figure 3.2. Multiple isoforms of white-throated sparrow plasma adiponectin.

Western blot analysis of plasma ADP under native conditions. **A**, representative data from two white-throated sparrows where plasma samples were collected at four times of day (ZT 0, 6, 12, 18). **B**, white-throated sparrow (W) and broiler chicken (Br) plasma separated under native conditions. Primary anti-chicken antibody was either not preadsorbed (- blocking peptide), or preadsorbed with adiponectin peptide (+ blocking peptide). A high molecular weight protein marker was included to determine molecular weight (kDa) of adiponectin isoforms.

This finding is consistent with the molecular weight and intensity of HMW bands formed in chicken [43]. Weaker bands of approximately 330kDa were also detected regardless of migratory status and sex. Bands of same molecular weights were previously validated as adiponectin in chicken plasma [43]. All females displayed bands of approximately 540kDa. Additionally, other trace bands at approximately 67, 136, 450, 670, and 750 kDa were occasionally observed. Multiple isoforms of ADP were observed in the white-throated sparrow, some of which had not been observed in the domestic chicken; although, multimeric bands of similar size were observed in Japanese quail (*Coturnix japonica*) (data not shown). Preadsorption of anti-chicken adiponectin antibody to chicken adiponectin peptide reduced immunostaining of 720 and 540 kDa bands, and eliminated immunostaining of other bands (Fig. 2B) except for bands of 330 kDa which were removed from analysis. All parametric statistics were performed on normalized, log

transformed total (sum intensity of all individual isoforms), log transformed HMW ADP, and log transformed visfatin which met the assumptions of normality. Non-parametric statistics were used to analyze normalized molecular weight bands of 67, 136, 450, 540, 670, and 750 kDa ADP, as their expression levels were not normally distributed.

Rhythmic expression of adiponectin

Using cosinor analysis, we determined that non-migrating birds exhibited rhythmic mean total (average cosinor fit: $p = 0.06$, mesor ± SE: 0.54 ± 0.06) (Fig. 3A) and HMW ($p < 0.05$, mesor ± SE: 0.45 ± 0.06, respectively) (Fig. 3C) levels of ADP in circulating plasma (Fig. 3), suggesting that regulation of ADP concentration in plasma is accomplished using a biological clock. Greatest levels of total and HMW ADP occurred at Zeitgeber time (ZT: time since lights on) 3.5, and ZT 3.6, respectively, and lowest levels occurred at ZT 15.5, and ZT 15.6, respectively, according to the best fit cosinor function. The average level of plasma HMW ADP approximately doubles from the migrating condition to the non-migrating condition. ADP intensity approximately doubled between peak and trough levels throughout the day. No statistically significant circadian rhythm of average total and HMW ADP levels in migrating individuals (mesor ± SE: 0.45 ± 0.03, 0.25 ± 0.04, respectively) was detected, however, a sinusoidal trend was evident such that the peak levels of ADP in migrating birds appeared to be damped and phase-inverted compared with non-migrating birds, even though photoperiod was the same in both groups (Figs. 3.3B and 3.3D).

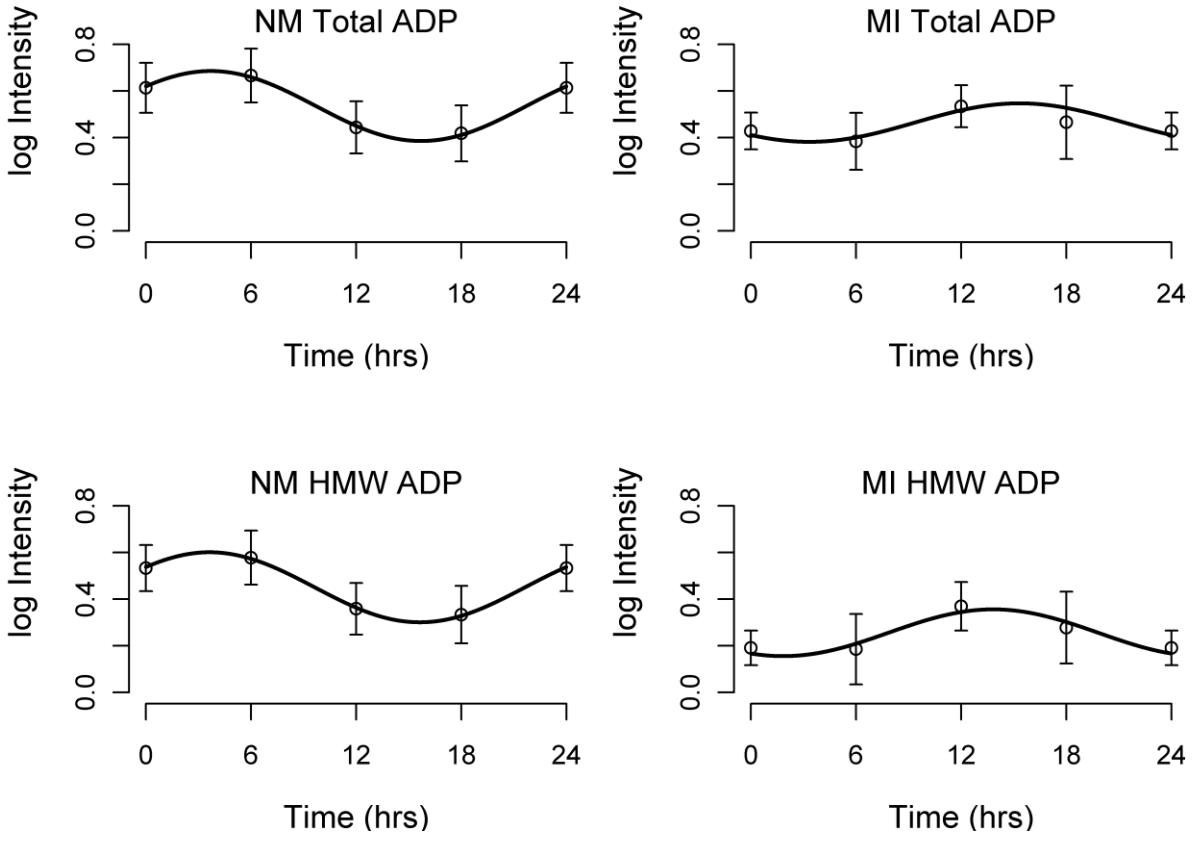

Figure 3.3. Circadian secretion of plasma adiponectin.

Cosinor analysis of (A) total plasma ADP in non-migrating birds, (B) total plasma ADP in migrating birds, (C) HMW plasma ADP in non-migrating birds, and (D) HMW plasma ADP in migrating birds. The solid wave shows the best-fit curve determined by linear harmonic regression. Individual data points are plotted as mean ± SE of actual measurements. ZT0 has been double-plotted as ZT24 for visualization.

Plasma ADP levels of migrating birds were lowest during the day and greatest at night. Non-migrating birds also exhibited rhythmic expression of 136 kDa ADP (p= 0.03, mesor ± SE: 0.03 ± 0.004) however, peak levels were estimated to be ZT 16.3, and the lowest levels at ZT 4.35, which is phase-inverted when compared with total and HMW ADP in non-migrating birds. Plots of residuals versus predicted values demonstrated no violations of original assumptions.

Mixed-effects modeling of HMW adiponectin expression

Of the five mixed models considered, the best model contains only one fixed parameter: Migratory Status (Table 1). The evidence in support of this model was moderate (w= 0.55), with lower support for the Fat and Status+Fat models (w= 0.24, 0.20, respectively), and very little, or no support for the Interaction and Full models (w= 0.01, 0.00, respectively). Despite the appearance of a small absolute effect size on a log scale ($\beta_1 \pm$ SE: -0.20 ± 0.13), we consider migratory status to be an important factor in predicting HMW ADP levels, as a reduction of approximately half the relative amounts of HMW ADP in plasma of migrating birds is considerable.

Table 3.1.Model selection results to predict plasma HMW adiponectin levels in the white-throated sparrow.

Model[a]	$\mathbf{K_i}$	$\mathbf{AICc_i}$	$\mathbf{\Delta_i}$	$\mathbf{w_i}$
MigratoryStatus	5	9.61	0	0.55
Fat	5	11.21	1.60	0.24
MigratoryStatus+Fat	6	11.59	1.97	0.20
Interaction	7	17.38	7.77	0.01
Full	7	22.82	13.21	0.00

[a]*N* = 40; K is the number of parameters included in each model; AICc is the second order Akaike's information criteria; Δ_i is the difference between the AICc values of each model and the model with the lowest AICc; w_i is the weight of evidence for each model.

Our data demonstrate that a change in migratory disposition may underlie the complexity of the relationship between this fat hormone and body condition which may ultimately affect migratory behavior.

Correlative analysis of plasma adiponectin and fat score

A strong positive correlation between ZT 6 HMW ADP and fat score was detected in migrating birds (HMW: r= 0.93; $p< 0.05$). Strong (τ= -0.84) negative correlations were detected

in 540kDa ADP in migrating birds and in 136kDa ADP of non-migrating birds (p= 0.05). We detected a negative correlation between fat score and the ratio of daytime to nighttime activity in migrating birds (τ = -0.79, p= 0.11) however, sample sizes were too small for statistical significance.

On average, non-migrating birds had a fat score 1 fat class lower than migrating birds (t-test: p< 0.05; 95% confidence interval: -1.58, -0.44). We did not detect a sexual dimorphism in the average levels of plasma ADP, as has been observed in mammals [29], however, our sample of females was small (n=3).

Plasma visfatin

Plasma visfatin levels ranged from 0.34ng/mL to 70.55ng/mL (mean= 12.01; med.= 9.37ng/mL) (not log-transformed). We do not consider visfatin rhythmic although, when values were averaged by time, migrating individuals as a group trended towards rhythmicity (p< 0.10).

Results from mixed-effects modeling of plasma visfatin are displayed in Table 2. Of the models considered, the best model contains only one fixed effect: MigratoryStatus. Evidence for this model was strong (w= 0.82), with less support for the Status+Fat model (w= 0.12) and little or no support for the remaining models. The Status model estimates a reduction of 0.62ng/mL (SE= 0.18) (log scale) between the migrating and non-migrating groups, approximately a 20% reduction.

Table3. 2. Model selection results to predict plasma visfatin concentrations in the white-throated sparrow.

Model[a]	**K_i**	**$AICc_i$**	**Δ_i**	**w_i**
MigratoryStatus	5	131.31	0	0.82
MigratoryStatus+Fat	5	135.05	3.74	0.12
Full	6	137.78	6.47	0.03
Interaction	6	138.09	6.78	0.03
Fat	7	140.65	9.34	0.01
Sex+Fat	7	142.87	11.56	0.00

[a]*N* = 66; K is the number of parameters included in each model; AICc is the second order Akaike's information criteria; Δ_i is the difference between the AICc values of each model and the model with the lowest AICc; w_i is the weight of evidence for each model.

DISCUSSION

Our results demonstrate programmatic variations in the expression of the adipokines adiponectin and visfatin between the migratory and non-migratory conditions in white-throated sparrows. Adiponectin secretion is regulated by a circadian clock, likely in adipose tissue, during non-migrating periods, and expression either becomes uncoupled from a circadian clock, or the rhythm of ADP secretion is damped and phase-inverted when birds are migrating. Although we do not know what aspect of the biological clockwork causes such regulatory changes in ADP, one may speculate that ADP output is a manifestation of changed clockwork in the adipose tissue of migrants. Although we did not detect a circadian rhythm of plasma visfatin concentration, we did observe a reduction in plasma visfatin during migration. Because visfatin is able to communicate metabolic information to the molecular clock, this change in visfatin expression might modify the output of the biological clock.

During the course of the day, rates of lipogenesis and lipolysis vary to accommodate changes in energy demands [44]. Recent evidence demonstrates the existence of molecular circadian clocks located within adipose tissue [45,46]. Clocks in adipose tissue can be influenced by feeding regimens or central pacemakers through multiple modalities [47] and may precipitate changes in sensitivity to factors promoting either storage or mobilization of triglycerides in anticipation of feeding, fasting, or exercise [45]. Alteration or uncoupling of the clock in adipose tissue from clocks in the brain could arise from changes in the hormonal signals released by adipose cells, thereby permitting expression of seasonal behaviors such as hyperphagia or migration, and restricting glucose metabolism while allowing lipid metabolism to increase. Our data suggest that communication between clocks in adipose tissue and the clock in the brain

regulating Zugunruhe may influence the changes in locomotor profiles observed during migration via changes in either ADP or visfatin expression.

Associations between sleep-wake cycles and weight regulation have been well documented, although the mechanisms linking the two processes are not well-understood. If clocks in adipose tissue are able to communicate with clocks in the brain, particularly the separate circadian clock controlling Zugunruhe [14], its disruption might account for the dramatically altered sleep-wake cycles associated with nocturnal migration. We suggest a role for clocks in adipose tissue regulating behavior, but further experimentation is needed to elucidate the mechanisms regulating peripheral oscillators in fat and the precise mechanisms regulating how they can influence behavior.

The physiological situation regarding adipokines in non-migrating birds may be analogous to the lean condition in birds that interrupt their migratory journey at stopover sites during the migratory period. Birds of poor body condition often delay for multiple days at stopover sites and display behaviors more characteristic of non-migrating birds; at stopover sites, birds will forage during the day, and suppress the expression of nocturnal migratory restlessness until fat stores have been rebuilt [48]. During this time at a stopover site, the clock regulating migratory restlessness may again be coupled to the master clock, causing suppression of migratory activity. Due to these similarities, we could extend our conclusions regarding adipokines in non-migrating birds to birds delaying their journey at stopover sites for multiple days, although further investigations are warranted. Because the duration of an individual's delay at a stopover site is largely determined by the amount of subcutaneous fat stores [11] which are strongly correlated with ADP, adiponectin may serve as an indicator of body condition for these birds, thereby delaying migratory flight. Visfatin, however, did not correlate with fat score in

either non-migrating or migrating individuals. Mixed model estimates predict a decrease in visfatin in migrating birds which display increased locomotor activity. In mammals, visfatin causes decreases in locomotor activity, decreases in food intake, and weight loss [49]. Therefore, it would be beneficial to migrating individuals to have reduced visfatin, thereby permitting the greater activity levels, increased food intake, and weight gain associated with the migratory period.

Although still controversial, many scientists are of the opinion that birds do not produce leptin, a fat hormone with the primary role in mammals of signaling energy sufficiency and adiposity, even though avian leptin receptors exist [50,51,52]. Adiponectin may subserve the role of leptin in birds, as it has been hypothesized to act on the brain and with peripheral tissues to mediate ingestive behaviors and energy homeostasis. Consequently, by controlling physiological changes associated with hyperphagia and increased fat deposition, biological clocks may modulate signals of body condition that could inform an individual whether it has enough fuel stored to commence or continue migration.

The shape of the circadian ADP profile approximates the daily profile of locomotor activity. In migrating birds of appropriate body condition, a low ADP profile during the day coincides with little daytime activity, and a higher ADP profile during the night coincides with the time of migratory flight. Conversely, the profile of ADP in diurnal, non-migrating birds is highest during the day, and lowest during the night. Compared to non-migrating birds, the phase shift in levels of ADP in migrating birds might be particularly adaptive for regulating lipid metabolism. Because adiponectin stimulates lipid metabolism without suppressing food intake [53,54], it would be beneficial for migrating birds to increase ADP production at night. This hypothesis is supported in Fig. 3B, where it is demonstrated that birds signal to increase lipid

metabolism during times of migratory flight. When migrating birds rest and refuel during the daytime, ADP levels are decreased, thereby allowing fat synthesis and preservation to occur until migratory flight continues [55]. In non-migrating birds, ADP levels are greatest during the day potentially allowing lipid metabolism to coincide with times of increased activity. Additionally, ADP levels are reduced at night when birds are sleeping, thereby preserving fat stores. One might artificially alter daily light:dark cycles to examine whether the ADP rhythm would follow changes in the phase of activity/sleep.

Correlations between HMW ADP and fat score, a proxy of body condition, in migrating birds are particularly intriguing because studies of mammals and chicken have demonstrated that levels of ADP decrease during obesity [43,56,57]. Our findings here demonstrate a positive correlation between fat and HMW ADP, the predominant ADP isoform in avian species. However, our scoring results were based solely on subcutaneous fat. The conflicting correlative findings regarding obese mammals and temporarily obese migrating birds may suggest that there are physiological differences between the genetically programmed, temporary subcutaneous fat stores maintained by birds to deal with the enormous energetic costs of migration and typical obesity in humans. In chicken, food deprivation leads to a significant decrease in ADP gene expression in adipose tissue [34], however, fasting has no effect on plasma ADP concentrations. Our results regarding non-migrating passerines are in opposition to these findings in chicken and demonstrate that plasma ADP is low at night when birds are not actively foraging (fasting), which could be a response to conserve fat stores until plasma ADP is high during the day, and food is available. Our results regarding migrating birds are similar, however, to those observed by Gomez-Abellan et al. [33], where the amplitude of ADP rhythms are attenuated in obese human subjects. Temporarily obese migrating white-throated sparrows display an average

rhythm with a smaller amplitude compared with non-migrating individuals. Our results also agree with the findings of Calvani et al. [32], that plasma ADP concentrations increase after weight loss in human subjects. The circadian profile of non-migrating sparrows also matches the diurnal profile of normal-weight human subjects [32]. ADP is positively related to fat score which also displays a negative correlation with the ratio of daytime to nighttime activity, a relationship recently confirmed by Fusani et al. [48]. Migrating birds with low fat stores display greater daytime activity, perhaps foraging to replace lost fat, and migrating birds with greater fat stores spend more time night-active, displaying migratory restlessness.

Very few studies have examined avian visfatin. Work performed in chicken has demonstrated that visfatin is expressed to a greater degree in skeletal muscle compared with visceral fat [58]. Thus, as our findings show, we might expect a small effect of fat deposition on plasma visfatin concentrations. Visfatin from skeletal muscle may act in a paracrine fashion to regulate glucose utilization locally. During migration, a birds' primary source of energy comes from the oxidation of adipose fatty acids (see [59]). Glucose is relatively unimportant as a fuel source in the long-distance endurance flights of migrating birds. In humans, visfatin has the ability to increase glucose uptake, playing a role in glucose homeostasis [60]. A seasonal decrease in plasma visfatin coinciding with the migratory period could reduce glucose utilization and allow for a greater reliance on and utilization of fat stores and fatty acids to fuel migration.

Our results provide evidence that a biological clock modulates the physiological transitions from non-migratory to nocturnal migratory behaviors. Adipokine signals may play a significant role in the initiation, continuation, and termination of nocturnal migration in small songbirds by indicating the amount of available fuel from fat and the overall body condition. Future experimentation utilizing restricted feeding schedules could be performed to force a

"stopover" in captive birds during the migratory period. Analysis of plasma samples collected during forced stopovers could determine whether the change in the ADP profile observed in migrating versus non-migrating birds is applicable to stopover situations as well. Experiments to localize ADP receptors in the brain would also provide valuable information as to the specific location of neural substrates regulating the transition of behaviors in migratory birds. As this is the first study of the adipokines adiponectin and visfatin in migrant birds, further investigation of these and other adipokines in migrant species that store and use fat differently than the white-throated sparrow, such as shorter or long-distance migrants that cross large geographical barriers with non-stop flight, would be relevant to a more complete understanding of the role of adipokines in metabolism and migration.

It is unclear from this study whether a change in adipokine signal drives a change in migratory disposition, influencing initiation or termination of migration or stopover delays, or a change in migratory status drives change in adipokine signals. However, because of adiponectin's close relationship with body fat, as well as migratory disposition, it is possible that ADP drives change in migratory disposition and changes in other adipokines such as visfatin make up the long list of adaptations necessary for successful migration.

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CHAPTER 4

CONCLUSIONS

It is important to conduct fundamental research on the regulation of lipid metabolism in migratory birds to clarify seasonal differences in regulation, and understand the underlying mechanisms and their consequences. Performing this basic research would be useful in predicting what hardships migratory birds might face when exposed to environmental change. On a seasonal basis, biological clocks regulate the transition from non-migratory to migratory periods and consequent modifications to the physiological underpinnings allowing for successful migration. Thus, biological clocks may regulate the altered expression of lipid metabolism observed in birds while migrating via their control over the production of adipokines. The objectives of the studies presented here were to increase our knowledge of the behavioral adaptations accommodating migration in white-throated sparrows, and to evaluate the roles of certain metabolic signals in the migratory and non-migratory stages of these birds. The studies presented here provide a framework for our understanding of the role of adipokines in migration as studies of adiponectin and visfatin have never been performed in migratory animals. This research provides a necessary starting point for the development of more complex evaluations of the activity of adipokines in birds utilizing different migratory strategies.

The first study presented here details the behavioral repertoire of the white-throated sparrow and compares the daily expression of behaviors of individuals during the migrating and non-migrating period. We define migration in captive birds removed from seasonal environmental cues as those individuals displaying nocturnal migratory restlessness; non-migrating birds display diurnal activity patterns. Although this is the most stereotypical definition of avian migration in captivity, traits do not evolve in isolation. The migratory

syndrome of many small songbirds involves coordinated evolution of a suite of behavioral, physiological, and morphological traits. Similar to the appearance of migratory restlessness in captive birds, there also seem to be seasonal changes in other behaviors adaptive to migration. These include changes in both timing and occurrence of behaviors such as locomotor activity, recovery behaviors, and maintenance behaviors (Stuber et al., Chapter 2). To avoid stressors including high temperature, increased predation, and poor wind conditions, many small songbirds migrate at night. It follows that with the addition of a large nighttime activity component, there is less time available for sleep. Migrating birds reorganize sleep patterns and budget the most recovery time to sleep, which may be more physiologically protective than resting behavior. Migrating birds also display more resting behavior during the daytime, which may help to pay the sleep debt incurred by drastically reduced nighttime sleeping. Birds also seem to display seasonal changes in vigilance behavior which might be evidence of trade-offs between traits. Differences in partitioning of certain behaviors might minimize trade-offs between migration and other daily activities. Observed individual variation suggests plasticity in behavioral traits that might allow for adaptation to current environmental change.

In addition to seasonal changes in behavior, migrating birds undergo drastic physiological change to cope with the rigors of migratory flight. One fundamental physiological change is the switch to fat metabolism as the main source of fuel for migration. Although a variety of physiological factors are known to influence adipokines, migratory status is the best predictor of plasma adiponectin (along with fat), and plasma visfatin (Stuber et al. Chapter 3). Large changes in the profiles of plasma adipokines may be necessary to accommodate drastic changes in metabolism and energy source that occur during migration. It seems as though more than one adipokine is important to the success of migration therefore we might see changes in many

adipokines during the transitions between the migratory and non-migratory periods. A change in only one adipokine might increase the probability that an individual would express migratory behavior, however, changes in multiple signals seem to be necessary for migration. A causal relationship between adipokines and behaviors would need to be established to solidify these notions. Adiponectin had a very strong positive relationship with subcutaneous body fat, a critical migration energy store. Future studies using short-distance migrants who rely less on pre-stored fat, or long-distance migrants crossing geological barriers that prevent stopovers for refueling might generate different results than what we see from these studies of medium-distance migrants. Having knowledge of the physiological regulation of lipid metabolism and energy utilization in migration could shed light on different conservation strategies, perhaps to maintain critical sites that provide high quality forage essential for building and maintaining fat stores.

Plasma adiponectin but not visfatin varied over time, cycling with a period of 24hours and displaying a seasonal trend towards phase inversion coinciding with changes in migratory status (Stuber et al. Chapter 3). This circadian rhythmicity suggests regulation by a biological clock, perhaps in adipose tissue itself, communicating with clocks in the brain. Adiponectin levels in blood plasma seem to parallel locomotor activity where adiponectin peaks during times of day when birds are most active. Adiponectin is able to stimulate lipid metabolism, the main source of fuel during migration, thus it makes sense that adiponectin would be highest at times of day when birds require the most energy. Because of the strong relationship between adiponectin and subcutaneous body fat in migrating birds, these individuals may use levels of circulating adiponectin as an indicator of the extent of current fat stores to gauge whether or not they have enough energy to continue migrating.

Although plasma visfatin did not vary with time, mixed effects modeling estimates a seasonal reduction in visfatin where migrating birds have approximately 20% less visfatin compared with non-migrating birds. This suggests that a circannual clock may regulate this change in visfatin based on migratory status. Because visfatin plays a role in glucose metabolism, we might expect that a reduction in its concentration during migration would be adaptive. Exclusively during migration, white-throated sparrows utilize fat stores as their primary energy source, rather than glucose or other fuel types. A reduction in visfatin may allow for a greater utilization of fatty acids for energy and decrease reliance on glucose metabolism. Unlike the adipokine adiponectin, we did not observe a statistically significant relationship between body fat and visfatin. This may be due to the fact that in chicken, and perhaps all birds, visfatin is expressed to a greater degree in skeletal muscle.

This work documented both behavioral and physiological changes that white-throated sparrows have evolved to accomplish seasonal migration. Relatively little is known about adiponectin and visfatin in avian species, and no studies regarding these adipokines have previously been performed using migratory birds. Our results provide evidence that biological clocks acting on both circadian and circannual levels may enable migratory birds to undergo the vast physiological and behavioral changes observed between the non-migratory and migratory life history stages by orchestrating temporally integrated biological systems.

APPENDIX A. USE OF INDIVIDUAL BIRDS

ID	Capture Date	Photoperiod (L:D)	Move-in to Isolation Cage	Move-out of Isolation Cage	Use	Sex	Migratory Status	Duration of Experiment
W2	Spring 2008	12:12	Oct. '09	Nov. '10	Exp 1 (Behavior)	F	MI	12/28/09 - 01/06/10
W2	Spring 2008	12:12	Oct. '09	Nov. '10	Exp 1 (Behavior)	F	NM	12/03/09 - 12/12/09
W4	Spring 2008	12:12	Oct. '09	Nov. '10	Exp 1 (Behavior)	M	MI	11/09/09 - 11/18/09
W4	Spring 2008	12:12	Oct. '09	Nov. '10	Exp 1 (Behavior)	M	NM	12/26/09 - 01/04/10
W6	Spring 2008	12:12	Oct. '09	--	Exp 1 (Behavior)	M	NM	11/21/10 - 11/30/10
W7	Spring 2008	12:12	Jan. '10	--	Exp 1 (Behavior)	F	MI	02/02/10 - 02/11/10
W7	Spring 2008	12:12	Jan. '10	--	Exp 1 (Behavior)	F	NM	12/10/10 - 12/19/10
W9	Spring 2008	12:12	Nov. '10	--	Exp 1 (Behavior)	M	MI	11/21/10 - 11/30/10
W1	Spring 2008	12:12	Oct. '09	--	Exp 2 (ADP)	M	NM	Nov. 2009 - Mar. 2010
W2	Spring 2008	12:12	Oct. '09	Nov. '10	Exp 2 (ADP)	F	NM	Nov. 2009 - Mar. 2010
W3	Spring 2008	12:12	Oct. '09	--	Exp 2 (ADP)	F	MI	Nov. 2009 - Mar. 2010
W4	Spring 2008	12:12	Oct. '09	Nov. '10	Exp 2 (ADP)	M	MI	Nov. 2009 - Mar. 2010
W4	Spring 2008	12:12	Oct. '09	Nov. '10	Exp 2 (ADP)	M	NM	Nov. 2009 - Mar. 2010
W5	Spring 2008	12:12	Oct. '09	Nov. '10	Exp 2 (ADP)	F	NM	Nov. 2009 - Mar. 2010
W6	Spring 2008	12:12	Oct. '09	--	Exp 2 (ADP)	M	NM	Nov. 2009 - Mar. 2010
W6	Spring 2008	12:12	Oct. '09	--	Exp 2 (ADP)	M	MI	Nov. 2009 - Mar. 2010
W7	Spring 2008	12:12	Oct. '09	--	Exp 2 (ADP)	F	MI	Nov. 2009 - Mar. 2010
W8	Spring 2008	12:12	Oct. '09	Nov. '10	Exp 2 (ADP)	M	MI	Nov. 2009 - Mar. 2010

ID	Capture Date	Photoperiod (L:D)	Move-in to Isolation Cage	Move-out of Isolation Cage	Use	Sex	Migratory Status	Duration of Experiment
W1	Spring 2008	12:12	Oct. '09	--	Exp 3 (Visfatin)	M	NM	Nov. 2010 - Apr. 2011
W1	Spring 2008	12:12	Oct. '09	--	Exp 3 (Visfatin)	M	MI	Nov. 2010 - Apr. 2011
W2	Spring 2008	12:12	Oct. '09	Nov. '10	Exp 3 (Visfatin)	F	NM	Nov. 2010 - Apr. 2011
W2	Spring 2008	12:12	Oct. '09	Nov. '10	Exp 3 (Visfatin)	F	MI	Nov. 2010 - Apr. 2011
W3	Spring 2008	12:12	Oct. '09	--	Exp 3 (Visfatin)	F	NM	Nov. 2010 - Apr. 2011
W4	Spring 2008	12:12	Oct. '09	Nov. '10	Exp 3 (Visfatin)	M	NM	Nov. 2010 - Apr. 2011
W6	Spring 2008	12:12	Oct. '09	--	Exp 3 (Visfatin)	M	NM	Nov. 2010 - Apr. 2011
W6	Spring 2008	12:12	Oct. '09	--	Exp 3 (Visfatin)	M	MI	Nov. 2010 - Apr. 2011
W7	Spring 2008	12:12	Oct. '09	--	Exp 3 (Visfatin)	F	MI	Nov. 2010 - Apr. 2011
W9	Spring 2008	12:12	Nov. '10	--	Exp 3 (Visfatin)	M	MI	Nov. 2010 - Apr. 2011
W10	Spring 2008	12:12	Nov. '10	--	Exp 3 (Visfatin)	F	MI	Nov. 2010 - Apr. 2011
W11	Spring 2008	12:12	Nov. '10	--	Exp 3 (Visfatin)	F	MI	Nov. 2010 - Apr. 2011
W12	Spring 2008	12:12	Nov. '10	--	Exp 3 (Visfatin)	F	MI	Nov. 2010 - Apr. 2011
W13	Spring 2008	12:12	Mar. '11	--	Exp 3 (Visfatin)	M	NM	Nov. 2010 - Apr. 2011
W14	Spring 2008	12:12	Mar. '11	--	Exp 3 (Visfatin)	M	NM	Nov. 2010 - Apr. 2011
W15	Spring 2008	12:12	Mar. '11	--	Exp 3 (Visfatin)	M	NM	Nov. 2010 - Apr. 2011
W16	Spring 2008	12:12	Mar. '11	--	Exp 3 (Visfatin)	F	NM	Nov. 2010 - Apr. 2011

APPENDIX B. INDIVIDUAL BEHAVIORAL EXPRESSION

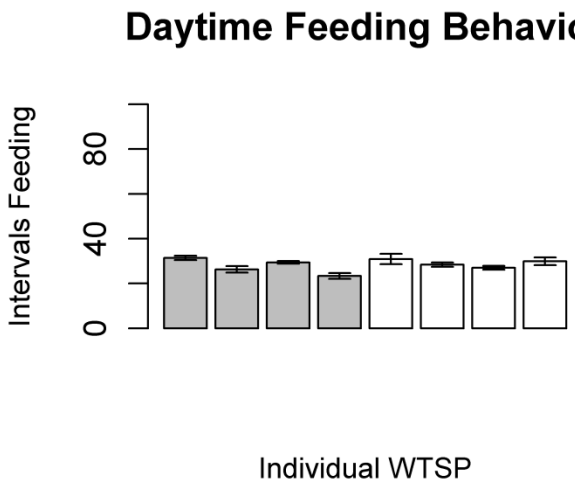

Figure A2.5. Daytime feeding activity (15 minute intervals where feeding behavior is expressed) of non-migrating individuals (n=4; shaded bars) and migrating individuals (n=4; white bars); WTSP in captivity. Individuals are plotted as behavior expression averages over 10-day recording periods ± SE.

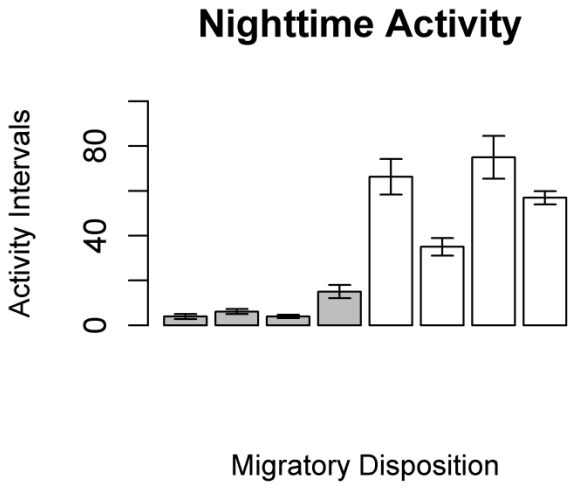

Figure A2.6. Nighttime activity (15 minute intervals where activity behavior is expressed, including hopping, flying, and wing-whirring) of non-migrating individuals (n=4; shaded bars) and migrating individuals (n=4; white bars); WTSP in captivity. Individuals are plotted as behavior expression averages over 10-day recording periods ± SE.

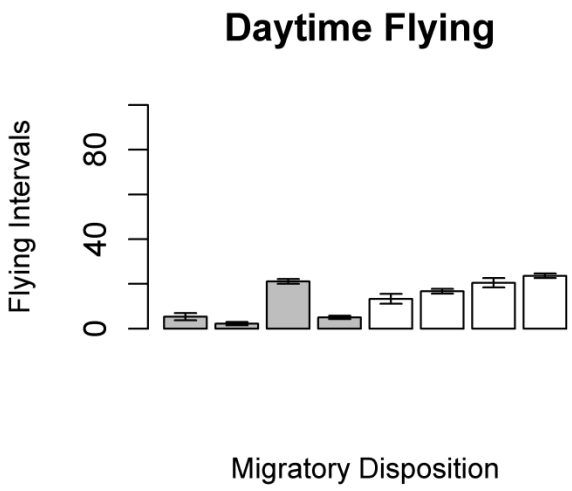

Figure A2.7. Daytime flying activity (15 minute intervals where flying behavior is expressed) of non-migrating individuals (n=4; shaded bars) and migrating individuals (n=4; white bars) during the daytime; WTSP in captivity. Individuals are plotted as behavior expression averages over 10-day recording periods ± SE.

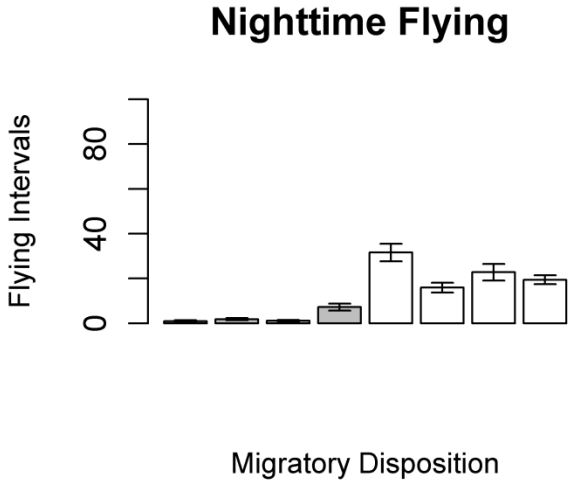

Figure A2.8. Nighttime flying activity (15 minute intervals where flying behavior is expressed) of non-migrating individuals (n=4; shaded bars) and migrating individuals (n=4; white bars) during the night; WTSP in captivity. Individuals are plotted as behavior expression averages over 10-day recording periods ± SE.

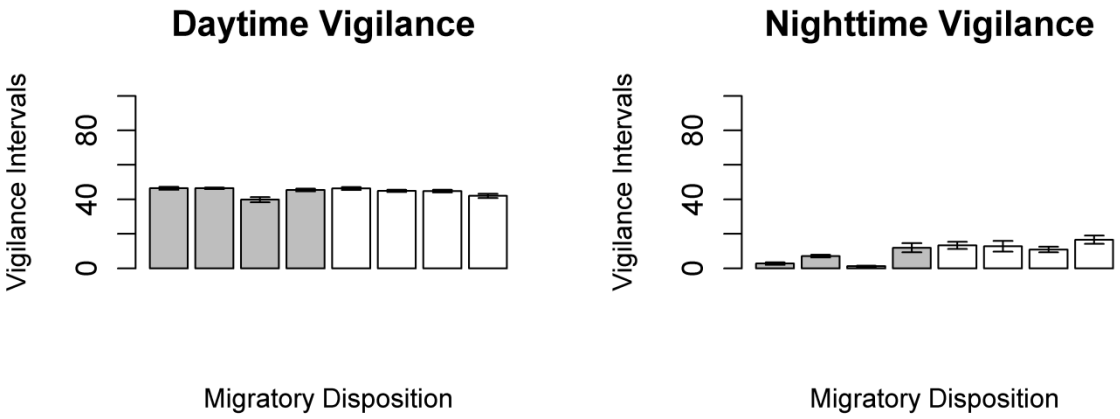

Figure A2.9. Vigilance behavior (number of 15 minute intervals where vigilance behavior is expressed) of non-migrating individuals (n=4; shaded bars) and migrating individuals (n=4; white bars) during the day and night; WTSP in captivity. Individuals are plotted as behavior expression averages over 10-day recording periods ± SE.

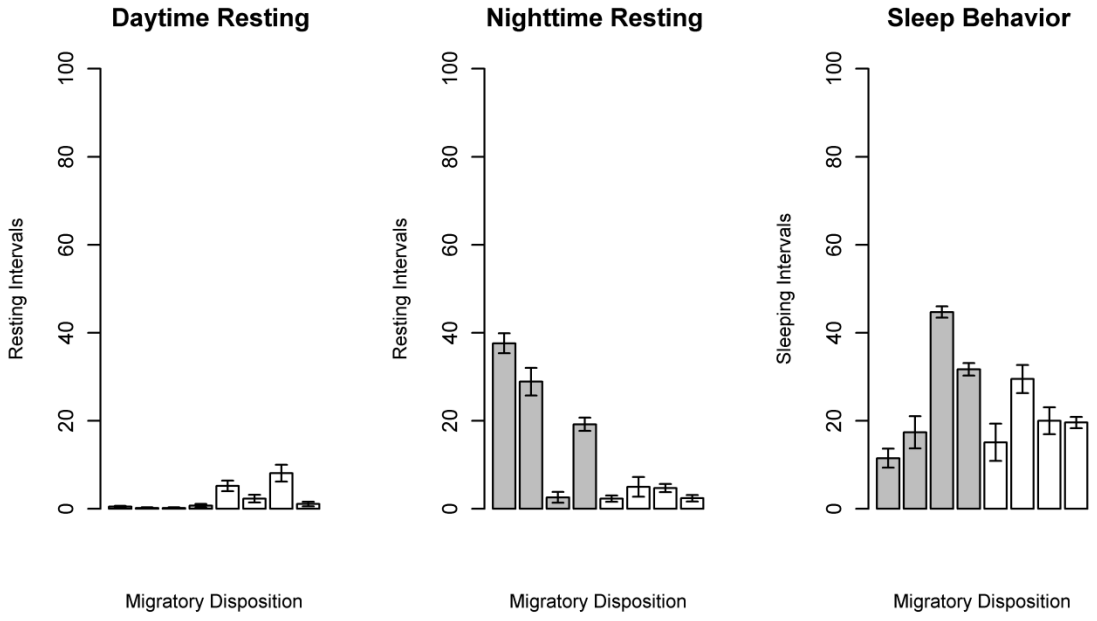

Figure A2.10. Recovery behaviors (number of 15 minute intervals where resting or sleeping behavior is expressed) of non-migrating individuals (n=4; shaded bars) and migrating individuals (n=4; white bars); WTSP in captivity. Individuals are plotted as behavior expression averages over 10-day recording periods ± SE.