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REGULATION OF SEASONAL CHANGES IN GENE EXPRESSION MEDIATED BY ADIPONECTIN SIGNALING IN A MIGRATORY BIRD SPECIES

A Thesis in

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ABSTRACT

Prior to migration, birds undergo a period hyperphagia, which serves to increase fat stores for energy-intensive flights. During migration, birds switch from a diurnal to a nocturnal activity cycle, which reduces stress associated with migration (e.g. thermal, dehydration, and predation). While it is widely understood that seasonal changes in behaviors are regulated by circadian and circannual changes in the biological clock, the mechanisms linking changes in the biological clock to changes in behavior remains unknown. My aim was to elucidate the effects of adiponectin on changes in clock and metabolic gene expression between the migrating and non-migrating states. Skeletal muscle tissue was harvested from White Throated Sparrows (*Zonotrichiaalbicollis*) and cultured in an organotypic culture system. Following treatment with adiponectin or vehicle at either ZT0 or 12, western blots and quantitative polymerase chain reaction (qPCR) were used to determine changes in adiponectin signaling and gene expression, respectively.

Western blots displayed adiponectin-induced changes in abundance of pERK and AMPK. Several changes in adiponectin-induced gene expression for CLOCK, per3, casein kinase 2, FABPpm, HFABP, PPARα and PPARγ were noted. Changes in gene expression were notably different depending on migratory statusand time of treatment. Our data suggest that adiponectin signaling can induce changes in expression of biological clock and metabolic genes, and that the pathways eliciting these changes may be different between the migrating and non-migrating life stages of birds.

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ABBREVIATIONS

kDa	=	Kilodalton
M	=	Migrating state
NM	=	Non-migrating state
ZT	=	Zeitgeber Time

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CHAPTER 1

INTRODUCTION

Avian migration

Migratory birds undergo a dramatic shift in both metabolism and behavior to prepare for and sustain themselves during the long journey. During the migrating period, birds shift the phasing of their daily activity, from a diurnal to a nocturnal pattern. Nocturnal behavior persists even when caged (manifested as wing-whirring, commonly called nocturnal migratory restlessness or Zugunruhe), and this affords the opportunity to study the migratory phenotype in the lab [5]. Flying by night allows birds to avoid predators, reduce heat and hydration stresses, and allows opportunities for feeding during the daytime [2]. Despite getting little to no sleep, during this time migrating birds do not appear to suffer from the cognitive and physiological deficits normally caused by sleep deprivation [45].

To fuel long distance flight, migrating birds alter their metabolism [55]. During the premigrating period, birds accumulate lipid stores and shift lipid composition [24]. Lipids are the primary fuel for migration, with protein providing most of the remaining energy requirements [20]. Flight muscles upregulate fatty acid transporters at both the level of the membrane (fatty acid translocase (FAT/CD36) and FABPpm) and the mitochondria [heart type fatty acid binding protein (H-FABP)] to enhance utilization of lipids for energy production [39]. Birds also increase the activity of enzymes promoting fatty acid metabolism. In addition to an increase in lipid metabolism, migrating birds show an increase in mass of pectoralis muscle, the primary flight muscle [44]. The causes of

physiological changes accompanying and supporting biannual migration have been much debated, although the current paradigm is that such changes are mediated by the biological clock.

Rhythmic biological systems

Daily (circadian) patterns of gene expression or physiological process are regulated in the bird by an endogenous biological clock [10]. The circadian system is regulated at two levels: the brain and the cell. Light information is transmitted via deep brain photoreceptors to the suprachiasmatic nucleus. Light also regulates production of melatonin from the pineal gland, whose production is also inhibited by the suprachiasmatic nucleus via the superior cervical ganglion. Pinealectomy alone has been shown to disrupt behavioral rhythms, including migration, in some bird species [40]. As information is integrated, the suprachiasmatic nucleus sends hormonal and neural signals to peripheral tissues to synchronize peripheral rhythms throughout the body.

The molecular circadian system is composed of a "positive arm" and a "negative arm", which feedback on and regulate each other (summarized in Figure 1.1). The positive arm is primarily composed of the genes CLOCK andBrain-muscle-arnt-like 1 (BMAL1). CLOCK and Bmal1 dimerize and interact with E-boxes, sequences (5'-CANNTG-3') found in the promoter regions of genes, resulting in increased expression of those genes. Several hundred genes have been identified which contain E-box sequences [61]. Among those genes with E-box-containing promoters are the period (Per) and cryptochrome (Cry) genes, of which there are two members of each in birds

[10]. The protein products of Per and Cry, which constitutes the negative arm of the biological clock, are produced during the day, dimerize, and move into the nucleus at night to inhibit the activity of the CLOCK:BMAL1 complex at the E-boxes. Activity of the Per:Cry dimer is modulated by Casein Kinases, which phosphorylate Per and Cry, targeting them for degradation. Additionally, the CLOCK:BMAL1 dimer increases expression of the Rev-erbα and RORα genes, which repress and induce expression of BMAL1, respectively. It is believed that the molecular clock influences the changes occurring during and around the migrating period [26, 47]. Prior data from the Bartell lab show differences in the expression patterns of key circadian clock and metabolic genesin livers of migrating vs. non-migratingblackcaps[68]. The same study revealed differences in metabolic gene expression and adiponectin receptor expression dependent upon migratory status. Notably, the higher expression of PPARγ (which promotes adipogenesis) during the day and PPARα and GSK3β (which promote fatty acid oxidation) at night agrees with the paradigm of migrating birds feeding and storing energy as adipose tissue during the day, and using fatty acids for energy at night. Evidence for circadian clock gene expression changing with migratory status has also been shown in other avian species [55]. Additionally, the length (tau) of the underlying circadian pattern of activity is longer in birds during migration [5]. Why the expression pattern of circadian clock genes changes during migration and how this regulates migratory behaviors is not well understood.

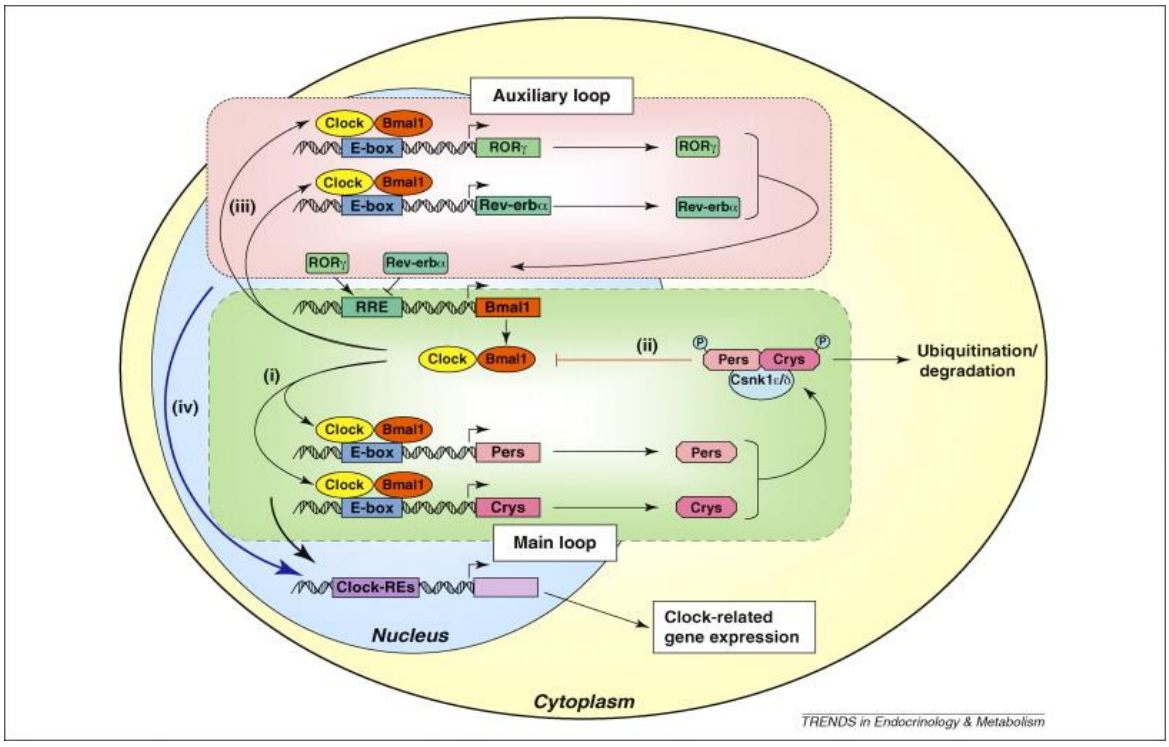

Figure 1.1 Summary of the molecular biological clock. Reproduced from [69].

Circadian systems and metabolism

Avian migration is preceded by a period of hyperphagia, and several physiological changes in the expression of fatty acid transporters facilitate the use of fatty acids as the primary energy source during migration. Disruption of the biological clock is known to impact metabolism. Mice with a mutation of the CLOCK gene, known as Δ19clock mutant mice, show impaired circadian rhythms of serum free fatty acids and glycerol concentrations under 12 hours each of light and darkness (12L:12D) conditions [48]. Under constant darkness, the same mutants have higher serum cholesterol concentrations, and a lack of rhythm of serum free fatty acid and glycerol, which were believed to be caused by disruption in the expression patterns of genes involved in lipolysis, lipogenesis and triglyceride hydrolysis. In addition, Δ19clock

mutants are unable to maintain glycogen stores following a 12-hour fast. Clock mutants not only present abnormal plasma concentrations of metabolites, but also lose circadian rhythms of macronutrient transporters in the small intestine [43]. The impact of circadian clock genes on metabolism does not limit itself to CLOCK. BMAL1 knockout mice have smaller pancreatic islets, leading to impaired insulin production and glucose intolerance, and Rev-erbα can change the amount of glucagon secreted by the pancreas [38, 59]. Overexpression of Cry1 in mice lowers fasting glucose levels, and RORα is known to affect lipid homeostasis by modulating expression of sterol regulatory element-binding protein 1 *(*Srebp-1) and fatty acid synthase (Fas) [23, 65].

Current literature shows that biological clocks and metabolism have a two-way relationship. In the liver, nicotinamide phosphoribosyltransferase (NAMPT) expression is controlled by the biological clock. NAMPT is an enzyme used in the rate limiting step of NAD+ synthesis. NAD+ in turn modulates activity of Sirtuin 1, which in turn modulates acetylation of Bmal1 and Per2 [3, 42]. PGC-1α, a known regulator of metabolism and mitochondrial biosynthesis, increases expression of Bmal1 and Rev-erbα [32]. Although lesions in the suprachiasmatic nucleus in the hypothalamus lead to dysregulation of peripheral clocks, indicating that the central clock is indeed important in synchronizing peripheral clocks, peripheral clocks can also be decoupled from the central clock via diet composition and feeding schedule [50]. Fasting increases the expression of NAMPT and Sirtuin 1, which regulates the circadian clock [22, 62]. A high-fat diet can disrupt the expression of Rev-erbα and CLOCK in peripheral tissues and the hypothalamus [25, 58]. Restricting feeding of mice to the light period, during which mice typically sleep, entrains peripheral clocks but not central clocks, and can

lead to metabolic disruptions, including increased hepatic lipid accumulation, lower plasma glucose concentrations and higher plasma concentration of free fatty acids and triglycerides [63]. Notably, when given a high-fat diet during the dark phase only, mice gain less weight compared with feeding *ad libitum*, giving evidence that central and peripheral clocks need to be working in phase with each other for metabolism to function normally. Given that metabolic changes impact the biological clock, it is reasonable to expect that the lipid accumulation occurring in the pre-migrating period could have an impact on biological clocks, which in turn may impact behavioral changes occurring during migration.

Adipose-derived hormones

Far from being simply a store of energy in the form of lipids, adipose tissue is dynamically producing hormones and cytokines (adipokines or adipocytokines) [70, 71]. Currently, known adipose-derived hormones include adiponectin, leptin, omentin and resistin, among others. Of these, the genes for omentin and resistin may not exist in avian species [72]. In mammals, the primary metabolic effects of adiponectin are to inhibit gluconeogenesis and promote of β-oxidation [12]. Adiponectin, which is typically inversely correlated with adipose tissue mass, is known to regulate satiety via stimulating neurons in the brain which release pro-opiomelanocortin (which suppresses food intake) and inhibiting neurons which release neuropeptide Y and Agouti-related peptide (which promote food intake) [52]. Outside of basal metabolic regulation, literature suggests adiponectin is implicated in the regulation of metabolic syndrome, cardioprotection, osteogenesisand reproduction [16, 17, 21, 49]. Similar to mammals, studies in birds show an inverse relationship between adiponectin and adipose tissue

mass [91]. Adiponectin also is an important regulator of adipose tissue and fatty acid metabolism in birds [92, 93, 94, 95]. Additionally, adiponectin has been shown to have a role bone metabolism, cancer and reproduction in birds [96, 97, 98, 99, 100, 101]. Adiponectin is also seen as a regulator of circannual metabolic and behavioral changes in migrating birds (see "Adiponectin Signaling", below).

Leptin is another adipose-derived hormone which regulates metabolism. In mammals, leptin positively correlates with adipose tissue mass [30], regulates food intake via opposing effects of adiponectin, inhibits neurons which release pro-opiomelanocortin and stimulates neurons which release neuropeptide Y and Agouti-related peptide [6, 15]. Similar to adiponectin, leptin is also implicated in metabolic syndrome, cardioprotection and reproduction [11, 14, 53]. Previously, leptin was believed to not be produced in birds, but recent research has identified functional leptin genes in several bird species [73, 74, 75, 76, 77]. Interestingly, these foundational papers suggest the tissue expression pattern of leptin in birds is variable depending on the species. Similar to mammals, leptin has been shown to mediate changes in appetite and metabolism [78, 79, 80, 81, 82, 83, 84]. Leptin signaling in birds has also been shown to impact immune and reproductive functions [85, 86, 87, 88, 89, 90]. Although leptin injections have been shown to impact feeding behavior in wintering White Throated Sparrows, it has no impact on feeding behavior, fatty acid transporter expression or lipid metabolism enzyme activity in the migrating period [64].

Adiponectin signaling

Adiponectin is a candidate hormone for regulating migratory changes in behavior, metabolism and clock gene expression in birds. Premigratoryhyperphagia results in an increase in fat pad mass and a change in fat pad composition, and a reasonable expectation is that the production of hormones changes [24]. Prior research from the Bartell lab shows a nearly 12 hour shift in the plasma profile of adiponectin as the migrating state of White Throated Sparrows changes, from having a peak during the day and a nadir during the night in non-migrating birds to a nadir during the day and a peak during the night of migrating birds [51]. Additionally, the amplitude of the plasma adiponectin concentration is reduced during the migratory state.

Signaling of adiponectin (summarized in Figure 1.2) occurs through two receptors, adiponectin receptor 1 (AdipoR1) and adiponectin receptor 2 (AdipoR2), which change in expression in the liver dependent on migratory state (Herestofa et. al., in review). Adiponectin can also signal through T-cadherin [12]. Signalingthrough AdipoR1 and AdipoR2 is primarily mediated through adaptor proteins, which interact with the intracellular portion of adiponectin receptors, of which four are known: adaptor protein containing pleckstrin homology domain, phosphotyrosine binding (PTB) domain and leucine zipper motif 1 (APPL1); endoplasmic reticulum protein 46 (ERp46); receptor for activated protein kinase C1 (RACK1); and casein kinase 2 (CK2) [8]. Of these, both RACK1 and CK2 regulate circadian clock genes [37, 46]. Adiponectin regulates lipid and glucose metabolism through a variety of pathways, including the p38-mitogen actived protein kinase (MAPK), protein kinase B (AKT), acetyl-coA carboxylase (ACC), peroxisome proliferator-activated receptor alpha (PPARα) and AMP-activated protein

kinase (AMPK) pathways, as well as modulating translocation of CD36, one of the fatty acid transporters that causes changes in gene expression and enzyme activity between migrating and non-migrating birds [13, 16, 18, 28, 35]. These signaling pathways also modulate biological clock function and expression of biological clock genes. ERK inhibits transcriptional activity of the CLOCK:BMAL1 complex and induces Rev-erbα expression [66,58]. Additionally, ERK induces expression of Per1 [41]. The AMPK pathway phosphorylates Cry1 and induces PPARα expression, thereby promoting PER3 expression [9, 29, 67]. Studies in humans have also found a correlation between expression of casein kinase 1 and circulating adiponectin [60]. Recent literature also supports the role of adiponectin as a regulator of the circadian clock in mammals and suggests a link between metabolic disorders, adiponectin disruption, and clock gene expression [4, 19].

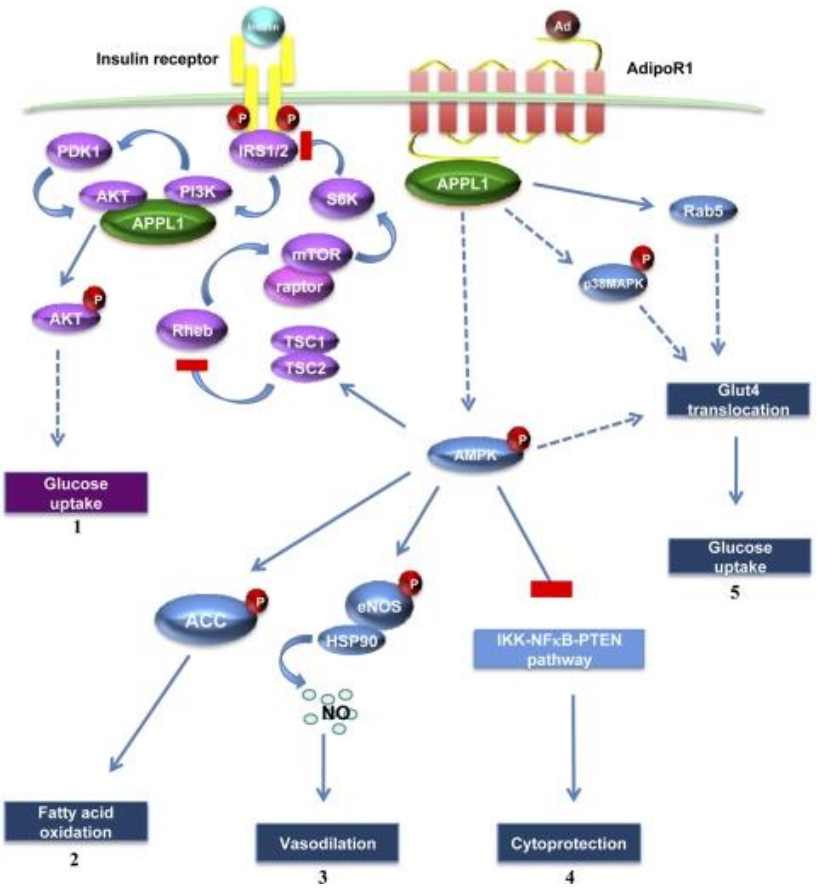

Figure 1.2 Summary of adiponectin signaling pathways. Through APPL1, the ERK (here, MAPK), AMPK and AKT signaling pathways are activated. Reproduced from [13].

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AIMS OF THIS STUDY

The regulatory mechanisms responsible for behavioral and physiological changes birds experience prior to and during migration have yet to be fully determined. This work is intended to elucidate the potential regulatory role of adiponectin in changing clock gene expression and metabolic gene expression in birds and how migratory status affects adiponectin signaling.

RESPONSE OF CULTURED MUSCLE TO ADIPONECTIN FROM MIGRATORY BIRDS DEPENDS UPON TIME OF DAY AND MIGRATORY STATUS

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ABSTRACT

Twice each year, many birds undergo a period of hyperphagia to prepare for migration toward either warmer (during Autumn) or cooler latitudes (during Spring). The migratory period is characterized by a utilization of fatty acids as the primary fuel source, and a switch from a diurnal pattern of activity to a near 24-hour period of activity. Causes for these physiological and behavioral changes are currently unknown, but it is believed changes in the biological clock, in part, help regulate migratory activity and physiological processes associated with migration. We investigated whether skeletal muscle in White Throated Sparrows responds differentially to adiponectin depending upon migratory status and time of day in order to determine if acute adiponectin exposure is capable of inducing changes to the molecular biological clock and genes regulating metabolism. Adiponectin-stimulated protein abundance of pERKand AMPK showed moderate changes with treatment. We also observed changes in gene expression in adiponectin-treated samples. PPARγ expression was

increased while PPARα and Per3 expression was reduced in muscle samples from non-migrating birds at ZT12, and with the migrating group treated at ZT0, compared with the non-migrating group treated at ZT0. CLOCK expression was higher; and CK2, FABPpm and H-FABP expression was lower; in muscle from migrating birds treated at ZT12 compared with ZT0. PPARγ and CK2 expression was lower in muscle of migrating birds treated at ZT12 compared to muscle from non-migrating birds treated at ZT12. Our data suggest that adiponectin signaling can induce changes in expression of biological clock and metabolic genes, and that the pathways eliciting these changes may be different between the migrating and non-migrating life stages of birds. Further research utilizing cell culture and *in vivo* studies can help elucidate the impact of adiponectin as a regulator of biological changes occurring during migration.

INTRODUCTION

Many bird species undergo a dramatic series of physiological and behavioral changes twice each year to prepare themselves for the physiologically daunting task of migration. Prior to migration, birds undergo a period of hyperphagia in which they accumulate adipose stores to serve as energy for their flights [1, 11]. At the time of migration, birds transition from a diurnal pattern of activity (active during the day and sleeping at night) to a near 24-hour period of activity. The additional nocturnal activity, known as Zugunruhe or nocturnal migratory restlessness, is characterized by long bouts of wing-whirring at night and feeding during the day while caged. The cause of these changes is currently unknown, but they are believed to be mediated, in part, by changes in output regulated by the biological clock [12, 21, 27].

A potential candidate for modulating the activity of the biological clock and metabolism across the year is adiponectin, an adipose-derived hormone. Prior research from Stuber et. al. (2013) shows a near 12-hour shift in the peak plasma concentration of adiponectin in White Throated Sparrows [25]. In nonmigrating White Throated Sparrows, adiponectin plasma concentration has a peak during the day and a nadir at night, whereas migrating White Throated Sparrows have a peak during the night and a nadir during the day, as well as a smaller amplitude of adiponectin plasma concentration across the day. Several downstream signaling molecules in the adiponectin pathway are known to regulate biological clock genes, in addition to genes regulating metabolism. ERK is known to induce PPARγ and Rev-erbα expression, which in turn inhibits expression of BMAL1 and CLOCK [30, 31, 32]. AKT stimulates GSK3β expression, while ERK inhibits transcriptional activity of the CLOCK:BMAL1 complex [16, 22]. Prior work shows the AMPK pathway phosphorylates Cry1 and induces PPARα expression, thereby promoting PER3 expression [4, 13, 14]. Both RACK1 and Casein Kinase 2, which are adaptor proteins in the adiponectin signaling pathway, also regulate the molecular biological clock [33, 34]. The abundance of adiponectin-signaling pathways which impinge upon the biological clock suggest adiponectin can be a potent regulator of clock gene expression.

We used skeletal muscle slice explant cultures to investigate the potential for short-term adiponectin treatments to alter the expression of clock genes and genes regulating metabolism. It was expected adiponectin treatment would induce expression of CRY1, PER3, CD36, FABPpm, HFABP, GSK3β, PPARα and PPARγ. Expression of CLOCK and BMAL1 was expected to decrease with adiponectin treatment, and

expression of CK1ε and CK2, both regulators of the biological clock, were expected not to change.

MATERIALS AND METHODS

Bacteria-derived Recombinant Chicken Adiponectin

Chicken adiponectin cDNA was previously cloned into a Novagen His-tag pET plasmid vector and transfected into BL21(DE3)*Escherichia coli* competent cells [see 35]. Cells which tested positive for the adiponectin insert were used for producing large quantities of cells in serum-free culture. Protein from these cultures was extracted as a supernatant (see the protocol in the "Western Blot Assay" section) and the histidine-tagged recombinant chicken adiponectin was separated and purified using Ni-NTA column chromatography. The resulting purified solutions were then dialyzed (buffer composition: 25 mM HEPES, 150 mMNaCl, 1 mM $CaCl_2$, pH 7.4) at least three times.

Housing of Animals

White-Throated Sparrows were acquired via mist-nets during Fall 2014 and Spring 2015 in Centre County, PA. Following quarantine, birds were housed in either a large 8' x 8' x 8' cage or in individual cages in order to verify migratory behavior. Birds were allowed access to feed (Mazuri Small Bird Maintenance) and water *ad libitum*. Birds were kept at a 12.5L:11.5D light schedule. For all procedures, timing was measured in Zeitgeber time (ZT), with ZT0 corresponding to lights on.

Determination of the migratory phenotype

Individual birds kept in the smaller cages were monitored at night via infrared cameras. Migrating phenotype was determined by the presence of Zugunruhe, which was defined as the presence of at least three hours of nocturnal wing whirring and hopping for three consecutive days. Recorded video was scored by two independent observers.

Tissue procurement and treatment

Birds were euthanized at ZT6 or ZT18 by cervical dislocation. Pectoralis muscle was excised and placed in ice-cold Modified Tyrode's Solution (MTS, NaCl 136 mM, KCl 5.4 mM, $MgHPO_4$ 1 mM, Glucose 10 mM, $CaCl_2$ 0.9 mM, HEPES 5 mM, pH 7.4). Skeletal muscle was cut parallel with the fibers into 300 µm-thick sections using a vibratome and transferred to six-well plates containing culture medium (DMEM/F-12 (with glutamine and HEPES) (Gibco 11039-021) and 1% penicillin/streptomycin). Tissue slices were cultured at 39°C and 5% CO_2. At ZT12 or ZT0, tissue slices were given recombinant chicken adiponectin (final concentration 25 µg/ml) or vehicle (the buffer solution used for dialysis). Tissue were extracted at either 20 minutes (for gene analysis) or 60 minutes (for protein analysis) after treatment, flash frozen on dry ice and stored at -80°C.

Quantitative polymerase chain reaction (qPCR)

RNA was extracted via homogenization of 0.1 g. oftissue in 1 ml of Ribozol, followed by a 5 min. room temperature incubation.Samples were then centrifuged at 5,000xg at 4°C for five minutes and the supernatant extracted. Next, 200 µl of

chloroform was added to the supernatant, shaken vigorously for 15 seconds, followed by a three minute room temperature incubation. The solution was centrifuged at 4°C for 15 minutes and the upper aqueous phase extracted. An equal quantity of 70% ethanol in nuclease-free water was added to the solution and the sample was further processed using a GeneJet RNA purification kit (Thermo K0731). A ThermoFisherNanodrop machine was used to measure RNA concentration.

Prior to cDNa synthesis, 1 µg of RNA per sample was treated with 1 µl of ThermoFisherDNAse I (EN0521) and 1 µl of ThermoFisherDNAse I reaction buffer (B43) (10 µl total volume) in a thermocycler for 30 minutes at 37°C, followed by adding 1µl of EDTA (50 mM) and incubating for 10 minutes at 65°C to stop the reaction.The same tube was then used to synthesizecDNA using the Superscript IV First-Strand Synthesis System (Invitrogen 18091050), according to the manufacturer'sprotocol. Both DNAse treatment and cDNA synthesis were performed using a Biorad MJ Mini Thermal Cycler (PTC-1148).

Primers for qPCR were designed with Primer Express and previously tested for specificity and linearity. Briefly, primer sequences were searched via BLAST to determine specificity of the sequences to the target genes. qPCR using three amounts of cDNA (1, 10 and 100 µg) was used to determine linearity, and analysis of melting curves were further used to determine specificity. Expression of genes of interest were quantified by qPCR using 5 µl of SYBR Green Super Mix, 2.4 µl of Nuclease-free water, 0.3 µl of both forward and reverse primers (300 nMfinal concentration for each) and 20 ng of cDNA (2 µl of 10ng/µl stock). Reactions were done in triplicate in a 96 well plate using a 7500 Fast Thermocycler. An initial denaturation of 20 seconds at 95°C was

followed by 40 cycles including 3 seconds at 95°C, 10 seconds at 10°Cand 30 seconds at 63°C. Relative expression was quantified using the $2^{-\Delta\Delta Ct}$ method, with β-actin as the housekeeping gene. Targets included the clock genes Cry1, Per3, Clock, BMAL1, Casein kinase 1ε and Casein kinase 2; the metabolism-regulating genes PPARα, PPARγ and GSK-3β; and the fatty acid transporter genes H-FABP, FABPpm and FAT/CD36. See Supplementary Table 2.1 for primer sequences.

Western Blot Assay

Protein was extracted using 1 ml. of RIPA lysis buffer (Santa Cruz 24948) mixed with 20 µl of Protease Inhibitor Cocktail(Sigma P8340) and 10 µl each of Phosphatase Inhibitor Cocktails 2 (Sigma P5726) and 3 (Sigma P0044). Protein was quantified via a BioRad protein assay according to manufacturer specifications and absorbance was read at 595 nm using a FLUOstar Omega plate reader.

Protein samples were mixed with 5 µl of 4x LDS Sample Buffer (Invitrogen NP0007), 2 µl of 10x Reducing Agent (Invitrogen NP0004) and MilliQ water (20 µl total volume) and denatured in a boiling water bath for 10 minutes. An Invitrogen 4-12% Bis-Tris gel (Invitrogen NW04120Box) was loaded into a XCellSureLockMiniCell (Invitrogen), with the inner chamber filled with 200 µl of 1x MOPS SDS running buffer (Invitrogen NP0001, diluted in MilliQ water) with 500 µl of antioxidant (Invitrogen NP 0005) and the outer chamber filled with 600 µl of 1x MOPS SDS running buffer (Invitrogen NP0001, diluted in MilliQ water). The denatured protein solution was added to the gel wells and the apparatus was set to 200 volts and ran until the dye in the solution reached the foot of the gel. Inside of an XCell II Blot Module, protein transfer

was performed (for 1 hour at 30 volts and 250 mAmp.) on PVDF or nitrocellulose membranes, depending on the protein to be detected. Membranes were washed three times in MilliQ water, blocked for two hours at room-temperature on a shaker with 5% Non-fat Dairy Milk (NFDM) in TRIS-buffered saline with 0.01% Tween-20 (TBST). Membranes were then incubated with primary antibody overnight at 4°C. Antibodies were diluted in 5% BSA in TBST, (see Supplementary Table 2.2 for Catalog numbers and dilution factors). The next day, membranes were washed in TBST (twice quickly, once on a shaker for 15 minutes and three times for 5 minutes) and incubated with HRP-conjugated secondary antibody (anti-rabbit IgG, Pierce 31462), diluted in 5% NFDM in TBST (see Table 2.2 for dilution factors), for 1 hour at room temperature and subsequent washing (same as prior to using the secondary antibody). Membranes were incubated with Pierce ECL2 chemiluminescent reagent and scanned for fluorescence on a Molecular Dynamics Storm scanning system. α-tubulin was used for normalization (both antibodies diluted in 1x casein in TBST).

Western blots were used to assess changes in the relative abundance of total and phosphorylated forms of, MAPK, AKT and ERK, as well as the relative abundance of APPL1. See Supplementary Table 2.2 for data on antibodies.

Data analysis

For gene expression analysis, comparing before and after treatment, data was analyzed for significance using Welch's T-test. Outliers in gene expression data were excluded due to unusually high Ct values, disparity in consensus of replicate wells or identified via a Grubb's test for outliers. Significance was set at $P<0.05$.

RESULTS

Gene expression data from skeletal muscle slice cultures are presented in Figures 2.1 and 2.2 and show the $2^{-\Delta\Delta Ct}$ fold change of that gene after adiponectin treatment compared to vehicle-treated tissue from the same bird. PPARγ expression was increased while PPARα and Per3 expression was reduced in muscle samples from non-migrating birds at ZT12, and also with the migrating group treated at ZT0, compared with the non-migrating group treated at ZT0. CLOCK expression was higher; and CK2, FABPpm and H-FABP expression was lower; in muscle from migrating birds treated at ZT12 compared with ZT0. PPARγ and CK2 expression was lower in muscle of migrating birds treated at ZT12 compared to muscle from non-migrating birds treated at ZT12.

Western blot data (see Figure 2.3) show similar signals from total AKT (60 kDa) and little to no signal from phosphorylated AKT in all groups (Figure 2.3a). Bands from phosphorylated ERK was higher in adiponectin-treated samples compared to vehicle-treated samples (Figure 2.3b and c). Bands for phosphorylated AMPK (Figure 2.3d and e) were also absent, but bands for total AMPK were almost always greater in adiponectin-treated samples compared to vehicle-treated samples. Western blot bands for APPL1 were extremely weak (Figure 2.3f).

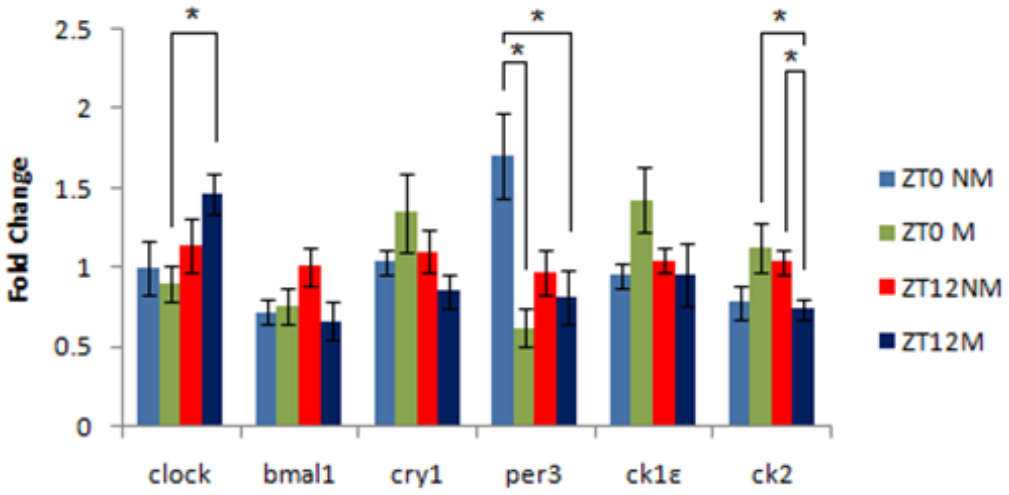

Figure 2.1. Fold changesin clock gene expression of adiponectin-treated skeletal muscle slice cultures with respect to vehicle treated cultures. Expression levels were compared from tissue from the same bird. Welch's T-test was used for analysis. ZT0 and ZT12 refer to treatment time, "M" indicates the tissue was from a bird in the migrating life stage and "NM" indicates the tissue was from a bird in the non-migrating life stage. Significance at $p<0.05$ is denoted by *. Error bars represent standard error of the mean. N = 6 – 9 animals per group.

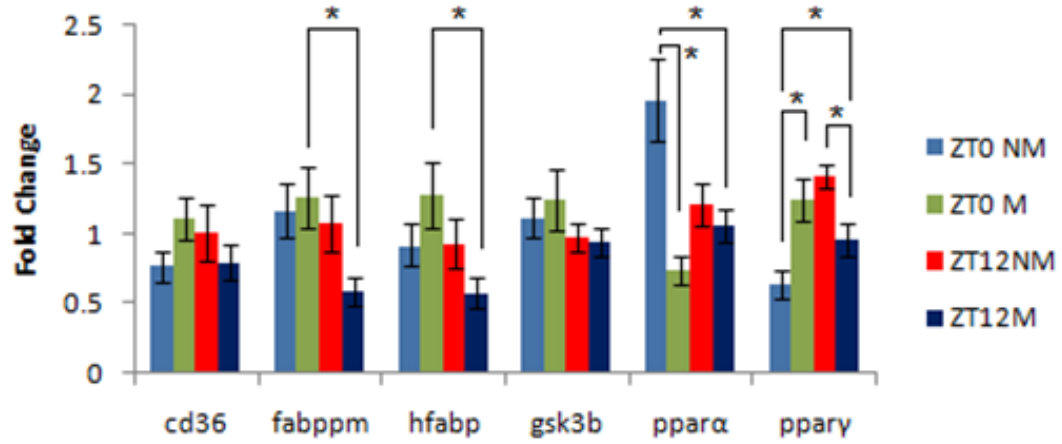

Figure 2.2. Fold changesin metabolic gene expression of adiponectin-treated skeletal muscle slice cultures with respect to vehicle treated cultures. Expression levels were compared from tissue from the same bird. Welch's T-test was used for analysis. ZT0 and ZT12 refer to treatment time, "M" indicates the tissue was from a bird in the migrating life stage and "NM" indicates the tissue was from a bird in the non-migrating life stage. Significance at $p<0.05$ is denoted by *. Error bars represent standard error of the mean. N = 6 – 9 animals per group.

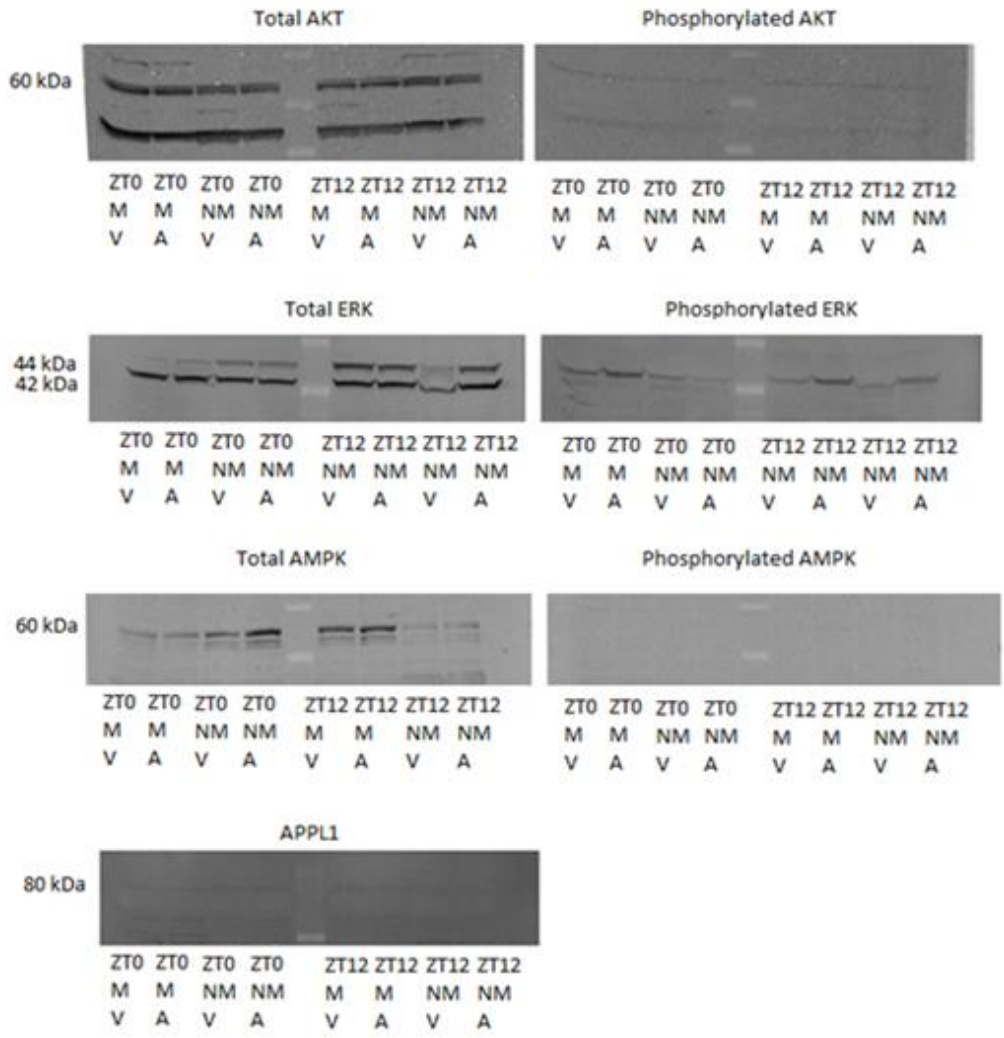

Figure 2.3: Representative protein analysis data. ZT0 and ZT12 refer to treatment time. "M" indicates the tissue was from a bird in the migrating life stage and "NM" indicates the tissue was from a bird in the non-migrating life stage. "V" indicates tissue was treated with vehicle and "A" indicates tissue was treated with adiponectin.

DISCUSSION

We found that the abundance of phosporylated ERK increased notably after adiponectin treatment. As stated previously, the ERK pathway promotes expression of PPARγ, and PPARγ expression also notably increased in all groups compared to the ZT0 nonmigrating group after adiponectin treatment. This suggests that ERK phosphorylation may play an important part of adiponectin signaling and regulation of

metabolism in migrating birds. Additionally, western blots show an increase in the abundance of total AMPK when skeletal muscle is treated with adiponectin. The AMPK pathway, as stated previously, induces expression of PPARα, and PPARα promotes expression of Per3. Figures 2.1 and 2.2 show an interesting similarity between the changes of expression of PPARα and Per3, one explanation for which is that the changes in PPARα expression are helping regulate the changes in Per3 expression.

For the CK2, FABPpm and H-FABP genes, the fold change in gene expression for the migrating group is greater when treated at ZT0 rather than ZT12. Data from Stuber, et. al. (2013) show that the plasma adiponectin concentration in the migrating White Throated Sparrow is higher at ZT12 than ZT0. A higher plasma concentration of adiponectin at this time point indicates a greater amount of signaling through the adiponectin pathways would be occurring at ZT12 than at ZT0, which may cause the tissue to be more sensitive to an addition of adiponectin at ZT0 if receptors are already saturated by endogenous adiponectin. Alternatively, receptors could be downregulated at ZT12, thereby limiting activation. Changes in abundance of adiponectin receptors across the day has been shown. One notable exception to this pattern is CLOCK, which had significantly higher expression in the migrating group at ZT12 compared with ZT0, after treatment. As stated previously, the ERK pathway is capable of stimulating the expression of Rev-erbα, and Rev-erbα has been demonstrated to inhibit CLOCK expression, a possible explanation of why the pattern of expression for CLOCK after treatment bifurcates from that of other genes.

In Figures 2.1 and 2.2, several additional genes had trends towards changes in gene expression. It is possible that additional gene pathways may have been activated

if the time duration was longer between stimulation and harvesting of cultures. In any case, our findings strongly show that adiponectin can increase or decrease expression of genes associated with both the canonical biological clock as well as metabolic activities associated with migration. Additionally, our data show that the effects of adiponectin on muscle are variable depending upon time of day and migratory status of the bird.

In the present study, western blot analysis showed activation of ERK following treatment with adiponectin, as evidenced by increased phosphorylation, along with increases in AMPK abundance. A lack of observed phosphorylated proteins for the other pathways could be explained in several ways. The duration of exposure to adiponectin may have been only long enough to stimulate the ERK and AMPK pathways, while having little to no effect on other adiponectin signaling pathways. Additionally, a greater concentration of adiponectin may need to be used to activate these pathways.

Prior literature indicates slice culture systems are tenable for certain applications. Liver slice culture studies have shown a maintained viability of slices in culture over extended periods, and some studies suggest using liver slice cultures in biomedical research [5, 19, 24, 28, 29). Several studies using slice cultures of ventricular muscle have also shown sustained viability and electrophysiological properties over the course of several days, even with slice thicknesses up to 300 µm [2, 8, 10, 15, 18]. In addition to liver and ventricular muscle tissue, slice culture has been successfully used for several other tissues, including gastrointestinal tissue, spinal tissue, epicardium, prostate and squamous cell carcinoma tissue [3, 6, 17, 20, 26]. One slice culture

experiment utilizing tissue from the suprachiasmatic nucleus found a continuing circadian pattern of expression of CLOCK for several days, though the amplitude did decrease over time [23]. In contrast to liver and ventricular muscle, very few slice culture studies have been performed with skeletal muscle [7, 9]. My research shows that a skeletal muscle slice culture system is viable for treatment based studies. Slice culture systems have an advantage in linking *in vitro* to *in vivo* studies due to the tissue being more similar in composition to the *in vivo* state (i.e. having an *in vivo*-like architecture, with supporting cell types), though the presence of multiple cell types may show different results compared with cell cultures.

Our results further suggest that adiponectin is at least one factor contributing to the regulation of genes involved during the transition from migrating and non-migrating life stages. Changes in gene expression were made all the more intriguing by their presence despite an amount of signaling pathway activation below what was expected. Further experiments utilizing cell culture systems, such as hepatocyte culture and *in vivo* studies, can likely provide additional information regarding metabolic and circadian signaling in birds and should be pursued in the future.

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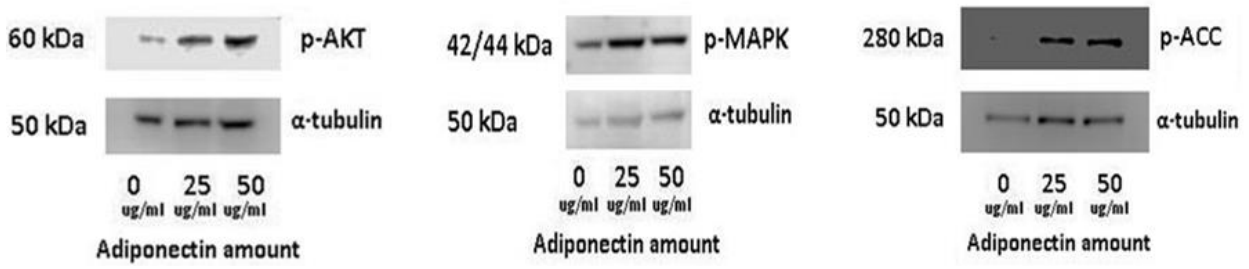
SUPPLEMENTARY DATA

	Forward	Reverse
β-actin	ACCACAGCCGAGAGGGAAAT	TCCTTGATGTCACGCACGAT
BMAL1	ATGGCTGTCCAGCACATGAA	CCATCTGCTGCCCTGAGAAT
Casein kinase 1ε	CAGCTCTTCCGCAACCTCTT	GCTCCCGATCCATACCTCA
Casein kinase 2	AGCTGGATCCACGTTTCAAT	TCACCAGGTGTTGGTTCTCA
Clock	ATGCATCAGGGTTCAGGACA	AATTCCAAACCATCCGCAAG
Cry1	TCTACACAGCGGCGACTAACA	GGAAAACCTGTCCTGCCTTCT
FAT/CD36	CATACTGGGAAGGCCACTGT	CTGTATCCGTGCAGAAGCAA
FABPpm	GTGGAAGGAGTTGGCAGCTA	CTCTCCATACAGCCCCATGT
GSK-3β	GCAGGCTGTGTATTGGCTGA	TGTAGGCGTTCCCAGAACCT
H-FABP	AAGACCCAGAGCACCTTCAA	AACAGCGATGTCTCCTTCC
PPARα	TGGAGATCGTCCTGGTCTTG	TTTGGGAAGAGGAAGGTGTCA
PPARγ	TGTGAAGCCCATCGAAGACA	ATCTGCCTCAGGTCCGTCAT
Per3	GCACAAGCCCTCTGAATGAA	CTGGCTGCAGAAGCAGTTTG

Supplementary Table 2.1: Primer sequences utilized in qPCR analysis.

	Primary Antibody Catalog #, Dilution	Secondary Antibody Catalog #, Dilution
α-tubulin	Sigma T6199, 1:1000	Pierce 31432, 1:1000
ACC	Cell Signaling 3662, 1:2000	Pierce 31462, 1:10000
Phosphorylated ACC	Cell Signaling 3661, 1:500	Pierce 31462, 1:2000
AKT	Cell Signaling 9272, 1:1000	Pierce 31462, 1:5000
Phosphorylated AKT	Cell Signaling 9271, 1:500	Pierce 31462, 1:5000
AMPK	Cell Signaling 2532s, 1:500	Pierce 31462, 1:2500
Phosphorylated AMPK	Cell Signaling 2531s, 1:500	Pierce 31462, 1:2500
APPL1	Cell Signaling 3276, 1:1000	Pierce 31462, 1:5000
ERK	Cell Signaling 9102s, 1:1000	Pierce 31462, 1:5000
Phosphorylated ERK	Cell Signaling 9101s, 1:500	Pierce 31462, 1:5000

Supplementary Table 2.2: Catalog numbers and concentrations of antibodies used in protein analysis.



Supplementary Figure S2.1: Western blots showing the relative abundance of the phosphorylated forms of AKT, MAPK (ERK) and ACC after exposure to 0, 25 or 50 µg/ml of adiponectin. Protein was extracted from White Throated Sparrow hepatocyte cell culture after 20 minutes of treatment.

CHAPTER 3

CONCLUSIONS

My study is the first to show changes in the expression of clock genes and metabolic genesby treatment with adiponectin in a migratory avian model. Although not all known adiponectin-signaling pathways appeared to be activated; the changes we observed suggest that both ERK and AMPK signaling pathways have a significant impact on the regulation of expression of clock and metabolic genes, and therefore the further study of adiponectin as a regulator of migration is warranted. As mentioned in Chapter 1, adiponectin has drawn interest as a potential target for developing treatments for metabolic disorders, and disruption of the clock gene rhythm has been shown to impact metabolism. The potential for a migratory avian model to discern the finer details tying clock gene expression and metabolic disorders together could prove to be an invaluable resource.

A time course of adiponectin treatments, such as in hourly sampling of avian tissue and cell culture following adiponectin treatment, as well as long term studies on adiponectin treatment, in which tissue or cell culturesare treated for multiple days, could help elucidate both seasonally-regulated changes in gene expression due to adiponectin and the adiponectin to entrain circadian rhythms of clock and metabolic genes. Further, as the ERK and AMPK pathways were most prominently affected by adiponectin treatment in these experiments, the use of ERK or AMPK inhibitors in conjunction with adiponectin administration may help determine the roles of specific signaling pathways in regulating individual genes and gene networks.

In vivo experiments should also be pursued to determine how adiponectin impacts behavioral changes and physiological changes associated with specific seasonal behaviors. These studies should include the impact of other biological factors (hormones, cytokines, physical activity, etc.) in modulating how adiponectin modulates gene expression and behavior. The impact of adiponectin on changing the amount of fatty acids being released from or stored into adipose tissue adds a further degree of complexity in determining the differential effects of *ex vivo* versus *in vivo* adiponectin exposure. Effects of adiponectin may be different when injected into cerebrospinal fluid versus those found by modulating levels in blood. Adiponectin is in cerebrospinal fluid, and adiponectin signaling affects neurons which modify appetite; however, it is currently unknown if adiponectin enters the cerebrospinal fluid from plasma, or if adiponectin is produced within the central nervous system.

My work clearly shows the ability of adiponectin to regulate expression of both clock and metabolic genes in skeletal muscle of a migratory bird. The changes in expression of genes were impacted both by the timing of adiponectin treatment and the migratory state of the bird from which the tissue was taken, suggesting a role for seasonality in adiponectin signaling. This provides impetus to further study the interplay of adiponectin signaling, expression of clock genes, and migratory and metabolic states.