# INVESTIGATION OF CIRCADIAN CLOCK IN PERIPHERAL TISSUES AND IMMUNE-CIRCADIAN INTERACTION IN THE DOMESTIC FOWL, *Gallus domesticus*

A Dissertation

by

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Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

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December 2012

Major Subject: Poultry Science

## ABSTRACT

The circadian system provides living organisms a means to adapt their internal physiology to constantly changing environmental conditions that exists on our rotating planet, Earth. Clocks in peripheral tissues are referred to as peripheral which may participate in tissue-specific functions. The first step to investigating the circadian regulation in the peripheral tissues of avians was to examine for the presence of avian orthologs of core components of the molecular clock using Quantitative real time (qRT-PCR) assays.

We investigated the avian spleen for daily and circadian control of core clock genes and regulation of the inflammatory response by the spleen clock. The core clock genes*, bmal1*, *bmal2, per2*, *per3* and *clock* displayed both daily and circadian rhythms. Proinflammatory cytokines TNFα, IL-1β, IL-6 and IL-18 exhibited daily and circadian rhythmic oscillations. A differential expression of proinflammatory cytokine induction was observed in the spleen undergoing lipopolysaccharide (LPS)-induced acute inflammation. Exogenous melatonin administration during inflammation seems to enhance some and repress a few inflammatory cytokines, implying that melatonin is pleiotropic molecule.

To compare and contrast the role of peripheral clocks in regulating energy balance and reproduction in layer vs. broiler chicken, the visceral adipose tissue (VAT), ovary and hypothalamus were examined for the presence of core clock genes were investigated in these two lines of poultry birds. Quantitative RT-PCR was employed to examine daily

control of core clock genes in these three peripheral tissues over a 24hr period. The layer hens exhibit rhythmic oscillations in the mRNA abundance of the core clock genes in the VAT, ovary and the hypothalamus. The hypothalamus and VAT of the broiler hens exhibit rhythmic mRNA abundance of the core clock genes. However, the clock genes in the ovary of the broiler pullets exhibit marked reduction in their amplitude and rhythms over a 24hr period. The broiler hens are prone to poor energy balance, obesity and reproductive capacity. In summary, these data provide evidence for a functional link between the circadian clock and the ovary by determining clock gene regulation under conditions of disrupted or eliminated reproductive function vs. normal reproductive output.

## DEDICATION

I dedicate my dissertation work to my beloved parents and baby-sister. They have been my biggest support system, helping me through all the ups and downs I have been through.

## ACKNOWLEDGEMENTS

I heartily thank my committee, Dr Ko, Dr Berghman, Dr Caldwell and especially my committee chair Dr Walzem, for their tireless encouragement, support, and guidance. Dr Walzem, I dedicate my sincere thanks and acknowledgements to you for all your timely cooperation and immense amount of patience.

I also thank my previous advisor, Dr Mike Bailey and post-doctoral fellow, Dr Louis Morgan for their guidance and cooperation with regards to my research work. I also thank the faculty, staff, and students of the Poultry Science Department as a whole, to which I have always felt bonded in camaraderie and shared purpose.

Finally, I thank my parents, sister, brother-in-law and all my friends for their unwavering support during my journey as a graduate student.

## NOMENCLATURE

| | |
|---|---|
| 5HT | 5-hydroxy tryptamine, serotonin neurotransmitter |
| ACTH | Adrenocorticotropic hormone |
| AC | Adenylate cyclase |
| AANAT | Arylalkylamine N-acetyltransferase |
| ANS | Autonomic nervous system |
| ANOVA | Analysis of variance |
| AR alpha, beta | Adrenergic receptors alpha, beta |
| B cells | Bursal lymphocytic cells |
| bHLH | Basic-helix-loop-helix |
| BMAL1 | (*Bmal1)* Brain and muscle ARNT-like protein |
| *bmal1* | Brain and muscle ARNT gene or mRNA |
| bZIP | Basic leucine zipper (Transcription factor E4BP4/NFIL3) |
| C-box | Clock box |
| CAMK1 | Calcium/calmodulin-dependent protein kinase - 1 |
| CBS | CCA1-binding site |
| CCA1 | Circadian clock associated 1 |
| CCG/CCGs/ccgs | Clock controlled gene/genes |

| | |
|---|---|
| CCS | Central clocking system |
| cDNA | Complementary Deoxyribonucleic acid |
| cAMP | Cyclic adenosine monophosphate |
| ChiP | Chromatin immunoprecipitation |
| CK (1, 2) | Casein Kinase (1, 2) |
| CLOCK | (Clock/CLK) Circadian Locomotor Output Cycles Kaput protein |
| *clock* | Circadian Locomotor Output Cycles Kaput gene or mRNA |
| clock | Refers to core clock genes and/or circadian clock |
| CLOCK/BMAL1 | (Clock/Bmal1) heterodimer protein/transcription factor |
| *clock/bmal1* | Clock/Bmal1 genes |
| CNS | Central nervous system |
| CRE | Cyclic-AMP response element |
| CREB | CRE binding protein |
| CRY | (*Cry*) Cryptochrome protein |
| *cry* | Cryptochrome gene or mRNA |
| CT | Circadian times |
| DC | Dendritic cells |
| DD | Constant darkness |

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|---|---|
| DM | Dim light |
| DMH | Dorsal medial hypothalamus |
| DMV | Dorsal motor nucleus of the vagus |
| EtBr | Ethylene bromide |
| FEO | Feed-entrainable oscillators |
| FRP | Free-running period |
| FRQ | Frequency protein |
| *frq* | Frequency gene or mRNA |
| Fw | Forward primer |
| GABA | Gamma-amino butyric acid |
| GAPDH | Glyceraldehyde 3-phosphate dehydrogenase |
| GC | Glucocorticoid |
| GnRH | Gonadotropin-releasing hormone |
| GR | Glucocorticoid receptors |
| HPA | Hypothalamic-pituitary-adrenal axis |
| HPG | Hypothalamus-pituitary gland-gonad |
| Hr/hr/hrs | Hour/Hours |
| IML | Intermediolateral cell column |
| LD | Light-dark cycle |
| LH | Luteinizing hormone |
| LL | Constant light |
| LPS | Lipopolysaccharide |

| | |
|---|---|
| LBP | LPS-binding protein |
| MAPK | Mitogen activated protein kinase |
| Mel1a, Mel1b | Melatonin receptors type1a, type1b |
| mRNA | Messenger ribonucleic acid |
| mSCN | Medial suprachiasmatic nucleus |
| NAD/NADP | Nicotinamide adenine dinucleotide/phosphate |
| Nampt | Nicotinamide phosphoribosyltransferase |
| NE | Norepinephrine neurotransmitter |
| NIH-3T3 | Mouse embryonic fibroblast cell line |
| NK | Natural killer cells |
| NR | Nuclear receptors |
| NPAS2 | Neuronal PAS domain-containing protein 2 |
| O-GC | Ovarian granulosa cells |
| O-TC | Ovarian theca cells |
| PARP-1 | Poly (ADP-ribose) polymerase-1 |
| PCOS | Polycystic ovarian syndrome |
| PER | (*Per*) Period protein |
| *per* | Period gene or mRNA |
| pGEMT | Parental vector for TA cloning of PCR products |
| PKA | Protein kinase A (cAMP dependent) |
| PKC | Protein kinase C |

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|---|---|
| PPARγ | Peroxisome proliferator-activated receptor (gamma) |
| PPRE | PPARγ response elements |
| PRC | Phase response curve |
| qRT-PCR | Quantitative real-time polymerase chain reaction |
| *qPer* | Quail Period gene |
| Rev | Reverse primer |
| Rev-erb-alpha | Orphan nuclear receptor |
| RHT | Retino-hypothalamic tract |
| RGCs | Retinal ganglion cells |
| RORE | ROR element |
| RGZ | Rosiglitazone |
| SCN | Suprachiasmatic nucleus |
| SCG | Superior cervical ganglion |
| SIRT1 | Silent mating type information regulation 2 homolog-1 |
| SNS | Sympathetic nervous system |
| T cells | Thymic lymphocytic cells |
| TLR | Toll-like receptors |
| UPC2 | Uncoupling protein2 |
| VAT | Visceral adipose tissue |

| | |
|---|---|
| vSCN | Visual suprachiasmatic nucleus |
| WT | Wild type |
| ZT | Zeitgeber time |

**List of cytokines:**

Interleukin 2-IL2

Interleukin 6- IL6

Interleukin10- IL10

Interleukin 18-IL18

Interleukin 1beta - IL-1β

Tumor necrosis factor alpha-TNF-α

Granulocyte-macrophage colony-stimulating factor-GM-CSF

Interferon-gamma-IFN-γ

The interferon-α/β receptor-IFNRs

Chemotactic cytokine receptors-CCRs

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# 1. INTRODUCTION

## 1.1 General overview

Living organisms on earth have to compete for limited resources within the environment; hence they have evolved adaptations which enable them to occupy distinct niches. The environment of these niches is dynamic in nature, which force the organisms to evolve adaptation strategies in order to thrive. Some of the environmental factors that create specific niches include day/night cycles, fluctuation of temperature, rainfall, and ocean tides etc. Organisms capable of sensing and processing changes in their external environmental factors may evolve endogenous mechanisms which help them anticipate and adapt to these changes. Changes in an external environment may be a daily event (occurring over a period of 24 hours, viz day/night cycles), a monthly event or an annual event (viz fluctuations of temperature, rainfall etc.). Depending on the external periodic event it is sensitive to, a physiological event in an organism may evolve a cycle/rhythm whose period matches the length of the cycle of the external event. Hence, a physiological event may exhibit a period length of about a day (circadian), about a month (circalunar), about a year (circannual), or about a tidal cycle (circatidal). The word circa is of Latin origin meaning "about" or "approximately". Rhythms/cycles of a physiological event in a living organism are referred to as "biological rhythms", and an internal time-keeping system which helps generate and/or maintain these rhythms is referred to as a "biological clock". The study of biological clocks is known as "chronobiology" (chrónos comes from Ancient Greek meaning "time").

Chronobiology attempts at exploring and understanding the components, properties and organization of biological clocks within various species of organisms at molecular and organismal level. Scientists believe that biological clocks help living organisms to proactively organize their physiology and behavior than in a responsive manner to the external time cues (Lowrey and Takahashi, 2004; Reppert and Weaver, 2002; Takahashi et al., 2008). A periodic event known to have remarkable influence on almost all living organisms inhabiting the surface of planet earth is the "day/night cycle". This is because, for billions of years earth has been rotating on its axis with a period of approximately 24hrours (hr)/rotation resulting in a daily rhythm of ~ 12 hr of sunlight and 12 hr of darkness. Therefore, organisms have been exposed to day/night cycles since their origin on the surface of planet earth. The day/night cycles have exerted selective pressure on living organisms resulting in the evolution of some form biological time keeping machinery/mechanisms which help them anticipate, sense, process, utilize and eventually adapt to in these 24 hr daily solar cycles.

Studies on timekeeping mechanisms indicate that they are fundamental and ubiquitous phenomena found in living organisms at almost all levels of phylogeny ranging from single-cell organism to highly complex multicellular organisms. In fact, Jean Jacques d'Ortous deMairan a french astronomer was the first scientist who pointed out the evolutionary relationship between the external solar cycles and internal physiological events in 1729. He observed daily leaf movements in the plant *Mimosa pudica* (a heliotrope) and noticed that these movements continued under constant darkness (Sweeney, 1987; DeCoursey, 2004). This implied that the leaf movement cycles were

endogenous in nature and did not require light cues to remain rhythmic.

Biological rhythms expressing a period of ~ 24 hr are referred to as circadian rhythms (circadian comes from Latin circa, meaning "about" and diem, meaning "day; coined by Franz Halberg). It is necessary to emphasize that circadian rhythms are endogenously generated rhythms that continue to oscillate in absence of any external input/cues (constant condition e.g. constant darkness, constant light) (Chandrashekaran, 1998). The time required for one circadian oscillation to occur in constant condition is known as "free-running period" (FRP). Under normal circumstance circadian rhythms are not free-running as constant conditions are not maintained. Thus, rhythms need to be synchronized to the external environment by a process known as "entrainment".

Endogenously generated biological rhythms are a reflection of adaptations made to take advantage of the physical changes generated by the movements of the earth and moon and their revolutions around the sun. An internal time-keeping system that generates and/or maintains circadian rhythms in an organism is known as a "circadian clock." Circadian clocks have evolved owing to the presence of persistent rhythms in environmental conditions that organisms are exposed to. One such persistent event is the rotation of planet earth on its own axis resulting in daily cycles of light and dark of ~ 24 hr. Circadian clocks are not capable of measuring 24hr cycle with high precision and need to be synchronized periodically to the geophysical time. An external factor that acts as a timing cue for a circadian clock and helps synchronize length and phase of an endogenous rhythm to that of the external factor is referred to as a "zeitgeber" (from German, zeit meaning "time" and geber, meaning "giver"; synchronizer; coined by Aschoff, 1965.

There are several different zeitgebers including light, temperature and food that are capable of resetting a circadian clock. Light is one of the most powerful zeitgebers capable of entraining and synchronizing circadian clock in light-sensitive organisms such that the period and phase of the circadian rhythms are approximately same as those of external light/dark cycles. Some organisms are directly responsive to sunlight such as, single-cell algae, cynobacteria, fungi and plants (Sweeney & Hastings, 1960; Lee et al., 2000; Sommer et al., 1989). In higher order organisms light can reach and influence (or entrain) clock containing cells either directly or indirectly (Plautz et al., 1997). In organisms with highly complex nervous system, light receptive elements have evolved in such a way that cells containing circadian clocks are concentrated in areas of nervous system containing light-transducing cells (Buijs et al., 2003). For instance, in birds the retina and pineal gland possess light receptive cells or photoreceptors (Binkley et al., 1971) that are sufficiently sensitive to respond to amounts of light passing through the skull (Foster et al., 1984; Menaker and Underwood, 1976).

However in mammals, the central nervous system (CNS) receives light signals only after they have been transported from the retina through the retino-hypothalamic tract (RHT).The light is transduced into chemical energy in the form of glutamate secretion (Moore and Lenn 1972; Morin 1994; Ding et al., 1997). An important group of light receptive proteins called cryptochromes (Cry) is an essential part of the mammalian molecular clockwork machinery. Cryptochromes are highly conserved across plants and animals with similar function i.e. light reception (Van der Horst et al., 1999).

Biological clocks are ubiquitously present in living organisms across almost all levels of phylogeny and the molecular make up is almost fundamentally similar. These two findings indicate that biological rhythms have evolved to provide significant advantages over the course of time. Scientists believe that there are two primary adaptive reasons for the evolution of biological clocks. Firstly, they provide temporal organization of physiological functions in an organism which help in optimizing the efficient balance of energy acquisition and consumption. Secondly, temporal organization may synchronize the organism with changes in the external environment.

For instance, diurnal animals synchronize their sleep-wake cycles with the sunrise-sunset cycles such that "early risers" have a higher probability of finding food, mating partners, escape from predators, reduced competition for resources with species sharing their niches etc. Additionally, different biological processes within an organism require temporal organization for normal functioning. A functional internal clock helps in production of physiological and behavioral rhythms which match the external environmental cycles ensuring increased chances of survival (Hurd and Ralph, 1998; Klarsfeld and Rouyer, 1998; Miller et al., 2004; Pittendrigh and Minis, 1972).

Although the anatomical location of a clock varies among species, a circadian system can be generalized as a "three-component model" comprising of, 1) an input signal 2), a circadian pacemaker and 3), an output signal. Input signals are timing cues (zeitgebers) received from the external environment. These are either directly or indirectly conveyed to second component of the model, the pacemaker. The pacemaker then sends signals to rest of the body and synchronizes the overall physiological and

behavioral events. A circadian clock is also defined as a system of components which interact to produce a rhythm with a defined length of period (τ).

Systems such as these are also known as "circadian oscillators", and often referred to as an "oscillator." In higher organisms almost all cells have an oscillator/ clock as a part of their cellular machinery. These cell-autonomous clocks are capable of generating their own rhythms. Thus, complex multicellular organisms host multiple oscillators arranged in a hierarchy. On top of this hierarchy is an oscillator carrying a molecular clock capable of generating a rhythm of ~ 24 hr even in absence of a zeitgeber and is known as a "pacemaker." A pacemaker is sometimes referred to as "master clock/oscillator". Master clocks send neuronal and hormonal signals that act as coupling signals for the rest of the oscillators. Master clocks get this name because their disruption or destruction results in the disruption or loss of biological rhythms making several physiological and behavioral events fall out of rhythm. Organisms with disrupted clock synchrony may have reduced fitness or survival. Cell-autonomous clocks which are under the regulation of master clock are referred to as "peripheral oscillators or peripheral clocks." In the current dissertation, for easy clarification we will use the term "peripheral clocks" to describe peripheral oscillators.

Although peripheral clocks are capable of generating their own rhythms, their phase and period are synchronized at organismal level by the master clock. Hence, there seems to be a hierarchy in the arrangement and regulation of clocks in complex organisms. (Welsh et al., 2004; Yoo et al., 2004; Ko and Takahashi, 2006; Lowrey and Takahashi,

2004; Reppert and Weaver, 2002) with master clocks "helping" the peripheral clocks remain synchronized at the organismal level.

Circadian clocks prepare an organism for tasks that occur over a course of 24hr. For instance, predator animals require that energy-generating organs and muscles are primed in order to successfully hunt prey. Hence, a wide spectrum of physiological parameters including sleep-wake cycle, hormone secretion (e.g. adrenocorticotrophic hormone, cortisol etc.), heart beat and body temperature fluctuate over a period of 24hr. To hunt a prey, a predator requires simultaneous optimization of alertness (via its sense organs and brain) and agility (via muscles and skeleton). Function of each of these peripheral organs is under the regulation of their respective peripheral clock. The master clock senses timing cues from external environment and sends out synchronizing signals to the peripheral clocks in rest of the body. This results in synchronization of all the physiological events in a temporal manner enabling a predator to be awake and alert when the probability of finding its prey is highest.

Output signals from a circadian clock are often referred to as "overt rhythms" or "overt outputs" seen as physiological/behavioral patterns that are indirect markers of an internal clock. Some of these overt outputs are observable and/or measurable. Depending on the species of animal, different types of overt rhythms can be recorded and studied as an indicator of an operating functional circadian clock. Some commonly assayed rhythms in laboratory animals are, wheel-running activity, feeding-activity, drinking-activity, perching -activity in birds capable of flying, oviposition, body temperature fluctuations,

circulating levels of hormones in blood, transcription and translation of clock controlled genes (CCGs/ccgs).

While some assays require manual collection and processing of data, there are a few assays which are collected using standardized automated techniques. For example, wheel-running activity in mammals (viz mice, hamsters, guinea-pigs) is measured by counting number of revolutions of the wheel. The wheels have microswitches which get activated when the animal starts to run on the wheel. These microswitches feed the counts to a computer through a data acquisition board. The data collected is processed using computer software programs such as ClockLab software (Actometrics, Evanston NJ) and printed out as an actogram. An actogram may be single- plotted (Figure 1A) or double plotted (Figure 1B). In a single plotted actogram, each horizontal line represents 24hr period (Figure 1A) while in a double-plotted actogram (Figure 1B) two consecutive 24 hr periods are stacked next to each other. In Figure 1B the first horizontal line represents day 1 and day2. Activity bouts are represented as histogram bars (sum of wheel revolutions per discrete time interval) in a 24hr period (Figure 1A and B). Depending on experimental requirements, animals may be housed in different types of light cycle regimens for various lengths of time (LD, light:dark cycle; DD, constant darkness; LL, constant light; DM: dim light etc.) in order to study the pattern of their physiological and behavioral rhythms.

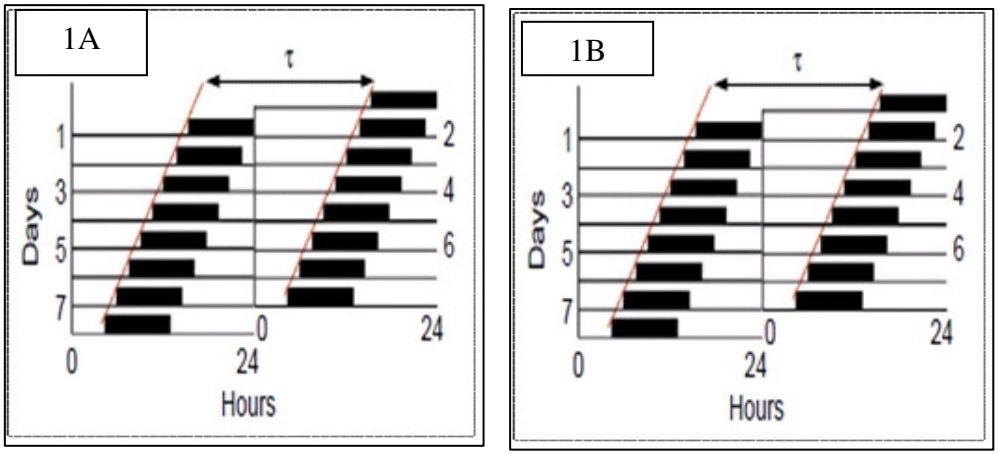


Figure 1. Actogram of wheel-running activity in mice. (A) Actogram of wheel-running activity of mice housed in 7 days of LD followed by 9 days in DD. The bar at the top of the actogram represents the light cycle of the LD portion of the experiment. The lights are on at 5:00 hr and off at 17:00 hr. Bouts of activity are represented as histogram bars (sum of wheel revolutions per discrete interval of time) across a 24hr period of the experiment. Each horizontal line is a single 24hr period of experiment. (B) Graph represents wheel-running activity of mice housed in DD for 15 days (Jud et al., 2005).

The light cycles are represented in form of a bar at the top of an actogram where in, an open bar represents light while a closed bar represents dark period of lighting regimen (Figure 1A). Plotting wheel running activity for consecutive days and stacking them reveals the pattern of locomotor activity of an animal model housed in a specific light cycle over an entire period of experiment. In Figure 1B, red lines are drawn passing through the points of daily onset of wheel-running activity. The space between the red lines of the double plot corresponds to the circadian period length "tau" (τ). To test if light really has the capability of resetting a clock, light pulses applied during early subjective

night or late subjective night should change the phase of a circadian rhythm. The phase shifts due to light pulses is represented in actograms of wheel-running activity of mice housed in 12:12hr LD for 7 days (Figure 2).

## 1.2 Fundamental properties of circadian rhythms

Presence of circadian clocks across almost all phylogeny implies that they did provide an adaptive advantage during the course of evolution although the ancestral origins and entire evolutionary history is still not completely known. Despite their disparate origins, structural organization and molecular composition among phylogenetically diverse organisms (Dunlap 1999, Bell-Pederson et al., 2005) biological clocks share three common fundamental characteristics that define the formal properties of circadian rhythms.

### *1.2.1 Endogenous nature*

The first defining characteristic of circadian rhythms is that they are generated endogenously with a period ($\tau$) of approximately 24 hr under constant conditions (Pittendrigh, 1961). True circadian rhythms are intrinsic in nature, and persist under constant conditions when no external zeitgebers/stimuli are present to influence them. Under such conditions the circadian rhythms exhibit an FRP close to but not exactly 24 hr. The length an FRP depends on the molecular properties running the clock and species of the organism (Bünning, 1977; Pittendrigh, 1981).

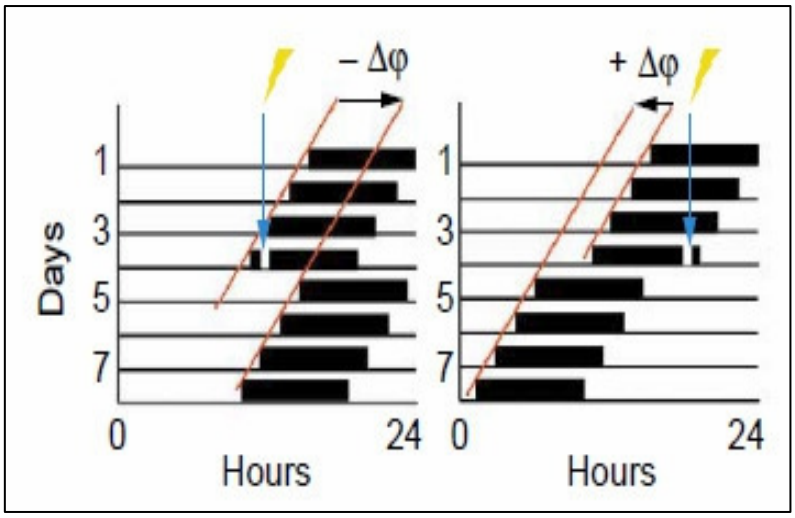


Figure 2: Effect of light pulse on phase of activity in mice. Applying light pulse during the activity phase (night) alters onset of the subsequent locomotor activity cycles. Applying a light pulse applied in the early part of the night (arrow in left panel) delays onset of the subsequent activity cycle resulting in a 'phase delay' characterized by a negative Δφ (phase angle difference). By contrast, a light pulse applied in the late portion of the activity phase (night) induces phase advances characterized by a positive Δφ (arrow in right panel). These alterations in circadian phase implies that light is capable of entraining (resetting) the clock (Jud et al., 2005).

### *1.2.2 Entrainment*

Another fundamental property of circadian rhythms is their ability to be "entrained", or synchronized to external zeitgebers of certain period (T) range. There are several environmental stimuli that can act as zeitgebers including light, temperature, food and social interaction to name a few (Pittendrigh and Minis, 1964; Aschoff et al., 1971; Stephan, 2002). Photic entrainment pathways are the most extensively studied circadian clock inputs as light appears to be the most ubiquitous and effective zeitgeber (Pittendrigh, 1981). Circadian rhythms capable of entraining to external zeitgebers seem to confer adaptive benefits to the organism. These types of circadian rhythms enable necessary levels of plasticity that help the organism to adapt/tune its internal rhythms to the external dynamic environment. Although circadian oscillators can sustain their own rhythms, they cannot operate independently. They need help from other oscillators to remain functional and/or entrain properly. Yet, there are special set of circadian oscillators known as the "pacemakers" that generate and sustain their own rhythms. A pacemaker can synchronize rhythmic outputs from other oscillators and can be entrained to external cues. In chronobiology, circadian clock is sometimes interchangeably used for circadian oscillator by several authors.

Having an entrainable circadian clock helps the organism adjust and to thrive in relation to its niche. However, the mechanisms by which entrainment occurs are not completely understood. There are two different theories that propose two different mechanisms of entrainment for circadian clocks namely, parametric and non-parametric entrainment (Pittendrigh, 1981). In parametric model, the zeitgeber (classic e.g. light)

entrainment occurs continuously such that a constantly changing angular velocity results in a new phase trajectory. This model predicts that a light pulse/stimulus will shift the phase of a circadian rhythm by altering the τ. The non-parametric model, on the other hand, states that it's the timing of the exposure to a light stimulus is key to which shifts the phase of the circadian rhythm without changing its velocity (Pittendrigh, 1981).

Although both mechanisms play a role in entrainment of the circadian clocks, studies show that non-parametric mechanism is sufficient to entrain animals, (Pittendrigh, 1981). There is substantial quantitative data to prove the relationship between the direction of phase-shift and time of light pulse applied to entrain a circadian rhythm. This relationship is generally illustrated in form a graph referred to as the "phase response curve" (PRC) (DeCoursey PJ, 2000). In PRC experiments, animals are maintained under constant conditions where their circadian rhythms "free-run" in absence of external zeitgeber. Animals are exposed to short pulses of light either during early subjective night or late subjective night. These light pulses may reset the phase of the circadian rhythms, referred to as "phase-shift" (ΦΔ).The phase shifts are measured in form a graph known as "phase response curve (PRC). In PRC, the extent of phase shift is plotted as a function of the time at which light pulse was applied under a free-running condition (circadian time or CT). PRC results from several studies show that there is differential effect of light when applied at different phases of a circadian cycle (Daan and Pittendrigh, 1976). A light pulse during subjective day in constant darkness (DD) has little/no effect on length of the circadian period. A light pulse during early subjective night (scotophase) leads to a phase-delay, while applying a light pulse during late subjective night causes a phase-advance. In

addition to phase-advance and phase-delay, the magnitude with which a phase can shift depends on the time at which the light pulse was applied. The PRC graphs measuring the magnitude of phase-shifts can have either of two distinct shapes, Type1 or Type 0 curves. Each animal species exhibit a PRC graph of either Type 1 (small shifts in phase) or Type 0 curves (large shifts in phase) depending on their circadian evolutionary history (Daan and Pittendrigh, 1976).

### *1.2.3 Temperature compensation*

Third fundamental feature of a circadian clock is their ability to maintain a near constant period of oscillations over a relatively broad range of temperatures. The rates of biochemical reactions hence, physiological processes are highly dependent on temperature. It implies that rate of a reaction should either significantly increase or rapidly decrease with a rise or fall in temperature respectively. The relationship of the rate of reaction versus the temperature is referred to as $Q_{10}$. Generally the $Q_{10}$ value of most of the biochemical reaction is around 2-3, which implies that for every 10°C change in temperature the reaction rate changes 2-3 folds respectively. If this is true for circadian rhythms as well, then on a very hot or a very cold day the circadian clock should function very rapidly or very slowly respectively. A circadian clock of this type has not been observed in nature and so seems not to be beneficial to an organism. Interestingly, the average $Q_{10}$ for circadian rhythms is ~ 1.1, which is significantly less than what is observed for most of the other biological processes within physiological temperature ranges (Kalmus, 1940; Pittendrigh, 1954; Pittendrigh, 1961).This property allows the circadian clock to function normally over a

wide range of varying temperatures and is hypothesized to represent a fundamental homeostatic mechanism. This phenomenon has adaptive benefits for the organism but the mechanism of temperature compensation is largely unknown (Pittendrigh et al., 1973).

### 1.3 Adaptive advantages of circadian clock

Time has a cyclic nature on earth and it impinges its effect on all its animate and inanimate objects For instance, organisms living on high latitude and temperate zones are exposed to annual cycles of cold winters and warm summers with varying lengths of photoperiod throughout the year (Pianka, 1973; Pittendrigh, 1993). Similarly, organisms at the sub-tropical and tropical zones face annual cycles in rainfall, atmospheric pressure and wind (Pianka, 1973). The abiotic environment exhibit daily cycles too, such as daily change in the intensity and quality of sunlight, which influences the photosynthetic activity of photo-receptive living organisms. Daily cycles also exist in the electromagnetic spectrum of sunlight, for instance cyclical changes are seen in the levels of ultraviolet, infra-red and gamma radiation to which the living organisms are exposed. Hence, living organisms are continuously exposed to rhythmic selective pressures from the environmental factors, limited resources required for sustenance, competitors, predators, prey, mates; all of which keep changing on a daily and annual basis.

Owing to selective pressures, biological clocks are believed to have evolved for two primary adaptive reasons. Firstly, they provide temporal organization of important biological and physiological functions e.g. cell division, cell repair, energy consuming cellular processes are compartmentalized into specific times of the day when energy

demands are lower for efficient use of energy and balance. Secondly, temporal organization of internal functions helps synchronize the organism to its external environment. For instance, predators are most active when their prey is out foraging, thereby increasing their chance of finding food and preventing wastage of energy by hunting at wrong times of the day. Within an organism, all biological processes are temporally organized to optimize energy usage and proper physiological functioning. Studies show that loss of clock function results in significantly reduced fitness with reduced life span and poor reproductive function (Hurd and Ralph, 1998; Klarsfeld and Rouyer, 1998; Miller et al., 2004; Pittendrigh and Minis, 1972).

The ~ 24 hr rotation of earth on its axis exposes its living organisms to exceedingly periodic environmental conditions such as, 24 hr cycles of predictable light and temperature cycles. Hence, life on earth has had to adapt its physiology to these geophysical cycles by evolving circadian clocks (Avivi et al., 2002). Functional clocks allow organisms to keep track of time and the ability to control the timings of their physiological, metabolic and behavioral cycles on a daily basis. Biological clocks are ubiquitously present in almost all living organisms. A set of genes and proteins form the genetic basis of clock, which carryout almost similar functions across different species of living organisms (Woelfle et al., 2004). Owing to their genetic basis and the presence of variation, circadian clocks are heritable in nature and are subject to natural selection (Paranjpe et al., 2005). Although the evolutionary origin of clocks is still unknown, their ubiquitous presence in nearly all living organisms implies that they have been positively selected via natural selection over millions of years (Paranjpe et al., 2005). Hence,

presence of a functional clock must have conferred certain fitness advantages to organisms which may be helping them to out-compete organisms with dysfunctional or no clock. Recent experiments elucidate the adaptive qualities of clock and these studies propose two theories to explain how organisms have gained a fitness advantage from possessing functional clocks, namely, the internal synchronization and the external synchronization value (Sharma, 2003; Paranjpe et al., 2005).

The internal synchronization theory posits that circadian clocks allow organisms to temporally organize their multiple internal metabolic and physiological processes through endogenously generated oscillations (Sheeba et al., 2002; Sharma, 2003). For instance, clocks help in controlling the timings of incompatible internal processes which require different temporal conditions in order to function properly (Sharma, 2003). In cyanobacteria, nitrogen fixation and photosynthesis are incompatible biochemical processes, the former being oxygen sensitive and the latter one generating oxygen. Cyanobacteria is a unicellular organism therefore, it does not have compartments/organelles to physically separate these processes. Thus, these must occur at two different times of the day. This is made possible by generation of endogenous oscillations which ensure that these two phenomenons do not co-occur (Woelfle et al., 2004). Thus, the coordinating force behind the alternating nitrogen fixation and photosynthesis cycles in Cyanobacteria is the internal circadian clock.

Genetic studies in Drosophila melanogaster indicate that traits that are non-adaptive tend to disappear within about 100 generations. The rate of disappearance will be even faster if the trait was deleterious to the species. If intrinsic theory of

synchronization is correct, then possessing a functional clock should prove advantageous to organisms living under constant as well as cyclic environmental conditions. Studies in D. melanogaster supports this assumption, wherein, flies housed in constant conditions for more than 600 generations continued to exhibit strong circadian rhythms in timings of oviposition (egg laying) and eclosion (adults emerging from pupal cases) (Sheeba et al., 2002). Hence, the persistence of circadian rhythmicity implies that there must be some adaptive advantages conferred by the clock to the flies (Sheeba et al., 2002). Persistent circadian rhythms have also been noted in animals living under naturally constant conditions such as the subterranean mole rat which lives in total darkness for its entire life (Avivi et al., 2002).

In 2002, Beaver et al, studied the regulation of reproductive efficiency by the circadian clock in *D. melanogaster*. They compared the reproductive output of wild-type versus the arrhythmic mutant flies (flies with non-functional clock system). A 40% reduction of progeny due to fewer egg-laying and higher unfertilized egg-laying was seen in the mutant flies. The male mutant flies also expressed reduced release of sperms. Thus, the disruption of functional clock in reproductive tissues leads to reduced sperm and egg production (Beaver et al., 2002). This decrease in reproductive output in mutant flies would have dramatically reduced their fitness and have been selected against this trait.

Another theory that explains the adaptive advantage of having a functional clock in terms of better survival rate and reproductive efficiency is the theory of external synchronization. According to this theory, clocks allow organisms to coordinate their

internal physiological, metabolic and behavioral cycles with the external environmental signals (zeitgebers) via a phenomenon termed entrainment (Sharma, 2003). Entrainment allows an organism to tailor the timings of internal processes to appropriate times of the day. Animals capable of these temporal partitions are better in surviving and passing on their circadian clock genes to their offspring (Sharma 2003). Of the several zeitgebers, light-dark cycle is considered as the predominant entrainer of clock because several cellular functions seem to be strongly affected by light (Nikaido & Johnson, 2000). For instance, *Chlamydomonas reinhardtii*, unicellular algae have ultraviolet light (UV-light) sensitive cellular processes that involved in cell division and DNA replication. These processes occur during night time to avoid disruption by UV irradiation. Not surprisingly *C. reinhardtii* is most sensitive to UV light in the evening hours. A functional circadian clock in these algae allows them to successfully predict the onset of night hours and successfully synchronize their internal processes (Nikaido & Johnson, 2000).

Synchronization to the external signals not only provide the adaptive advantage of synchronizing the internal cellular processes to the correct time of the day, but also the capacity to synchronize behavioral patterns to the external zeitgebers. This ability ensures that the organisms with a functional clock are more likely to find food, mates and avoid predators or competition with species living in the same niche (DeCoursey et la., 2000). In 2000, DeCoursey et al., demonstrated how the ability to synchronize behavior to external zeitgebers may have provided a fitness advantage to animals. They studied the effects of rendering a clock dysfunctional by ablating one of the entraining pacemakers, SCN in diurnal eastern chipmunks. The SCN-ablated chipmunks exhibited inability to

synchronize their behaviors to external zeitgebers. They then went ahead and compared the mortality rates of the SCN-ablated chipmunks to the chipmunks with intact SCN in both, laboratory (stress-free) and field conditions. There was no significant difference in laboratory groups, implying that entrainment is not required for survival under stress-free conditions. However, in field conditions, the SCN-ablated chipmunks survived significantly fewer days. About more than 80% of the SCN-ablated chipmunks exhibited increased nighttime restlessness, predisposing them to increased predation (field condition) to the nocturnal predators when compared to their otherwise normally diurnal SCN-intact counterparts(DeCoursey et al., 2000). The inability SCN-ablated chipmunks to coordinate their sleep-wake cycles to the external day-light cycles lead to a dramatic increase in their mortality rates and reduced fitness when compared to the rodents with functional timing systems.

Hence, studies suggest that circadian clocks have adaptive advantages resulting in their positive selection during evolution. These adaptive advantages may fall into either of the two categories: the internal synchronization and the external synchronization value. Both these theories may hold definite merits and could have easily worked together to give animals with functional clock an evolutionary advantage in a highly dynamic environment like that of the planet earth's. These two coordinating forces have helped organisms not only synchronize the internal processes to one another, but also to their external zeitgebers.

### 1.4 The avian circadian clock

As noted earlier, the circadian clock comprises of three basic components 1), a

central pacemaker 2), input pathways capable of modulating pacemaker function and 3), output pathways that relay information from the oscillator to biological processes. The endogenous nature of a circadian clock and generation of circadian rhythms under constant conditions occurs due to the presence of a pacemaker. A pacemaker functions autonomously, and dictates the rhythmic output of a circadian system. At its core, a pacemaker has molecular feedback loops that are under autoregulation and therefore provide "self-sustained" rhythm generation (Dunlap, 1999). The central pacemaker needs signals/cues from the external environment or a zeitgeber in order to be synchronized or entrained. Sensory structures capture and transfer the information from the zeitgeber which are then transduced by the machinery, referred to as the input pathways. After receiving the input signals the central pacemaker becomes entrained and exhibits alteration in circadian phase. This altered/adjusted circadian phase is then relayed as output pathways/signals. The output signals then reach and change the rhythms of downstream biological processes generally seen as an alteration in physiological and/or behavioral rhythms. Experiments in chronobiology are designed to explore one of the three components in circadian clock machinery.

All organisms that harbor a functional circadian clock contain these fundamental clock components. However, the circadian organization at molecular and cellular levels may vary from species to species owing to evolution. In some species of organisms, all the three components of circadian clock may be localized in the same cell (e.g cyanobacteria, fungi). Yet, in highly complex organisms, circadian clocks have distributed themselves among physiologically specialized cells. These specialized cells may be spatially

segregated, but they interact with each other and act as a circadian clock. This kind of arrangement can be seen in several animal species, ranging from Drosophila to human beings.

The generic circadian model can be applied to the avian species as well, but birds exhibit a highly specialized and complex circadian system when compared to mammals. Mammals have a multiple circadian oscillators with the Suprachiasmatic Nucleus (SCN) being the circadian pacemaker also referred to as the master/central clock that sets pace for the functional hierarchy. The SCN is self-autonomous and entrains oscillators present in remaining parts of the body (Yamazaki et al., 2000). The avians on the other hand seem to host not one, but three neural pacemakers and their associated photoreceptors. Photoreceptors are located in the pineal gland, retina, lateral septum and tuberal hypothalamus. The three independent neural pacemakers that utilize these photoreceptors are found in - the hypothalamus (avian SCN), pineal gland and retina (Gwinner et al., 1978). Each pacemaker-photoreceptor set has its own input pathway-pacemaker-output pathway forming an individual clock network. The three individual clocks (the avian SCN, pineal gland and retinal clock) interact with one another and function as a single unit, this functional unit is also referred to as the central clocking system (CCS) (Vinod et al., 2004). The degree of coupling between the three clocks varies greatly among the avian species. Within an individual bird the coupling is highly dependent on the nature of input signals received from an external zeitgeber (Reierth et al., 1999; Brandstätter et al., 2000; Underwood et al., 2001). Thus, the avian circadian system is a complex network of three mutually coupled pacemakers synchronized to the external LD by associated

photoreceptors.

### *1.4.1 Pineal gland*

Unlike the pineal gland in mammals, the avian pineal gland is a photoreceptive pacemaker. The pineal gland secretes melatonin, an indoleamine hormone and thus exercises its control not only on the distant cells and tissues expressing melatonin receptors but also on the CCS (Underwood, 1990; Cassone, 1998). The position of pineal gland in the hierarchy of the CCS varies among the avian species. Removal of pineal gland (pinealectomy) totally abolishes locomotor activity in passerine birds (e.g. house sparrows) (Ebihara and Kawamura, 1981; Fuchs, 1983; Gaston and Menaker, 1968; Gwinner, 1978; McMillan, 1972; Pant and Chandola-Saklani, 1992). However, in columbiforms (e.g. pigeon), pinealectomy does not entirely abolish the locomotor rhythms (Ebihara et al., 1984), and in galliforms (e.g. chicken, quails) there is no effect on the locomotor activity (Simpson and Follett, 1981; McGoogan and Cassone, 1999) under constant conditions.

The pineal gland remains rhythmic in-vitro conditions and can be entrained to external zeitgebers (Menaker et al., 1997; Oishi et al., 2001; Natesan & Cassone, 2002). Each individual pineal cell (pinealocyte) has a clock capable of generating sustained circadian rhythm (Nakahara, et al., 1997).Melatonin is synthesized in a rhythmic fashion in intact animals and cultured cells held under constant conditions and therefore, are a circadian clock output of the pineal gland (Takahashi et al., 1980; Zatz et al., 1988; Murakam et al., 1994).

Pineal gland hosts light-input pathways which includes opsin-based photoreceptive molecules (e.g., pinopsin, Okano et al., 1994; melanopsin, Natesan & Cassone, 2002), photoisomerases and signal transduction mechanisms (Kasahara et al., 2002). The chick pineal expresses *clock, bmal1*, *bmal2, cry1, cry2, per2* and *per3* genes (Larkin et al., 1999; Chong et al., 2000; Okano et al., 2001; Yamamoto et al., 2001; Bailey et al., 2002). The chick pineal does not express *per1*, instead a transcription factor, E4bp4 (basic leucine zipper (bZIP) transcription factor) capable of suppressing transcription of *per2* and is expressed in opposite phase of *per2* in LD and DD.

Melatonin is the most reliable circadian output of the pineal gland. Melatonin levels are lowest during subjective day, and reach a peak at night in LD, and in subjective night in DD and LL. The duration and amplitude of melatonin secretion by pineal gland is controlled by the intensity and length of light period in LD cycle. Hence, melatonin rhythms can provide daily and annual information to birds (Kumar & Follet, 1993; Brandstatter et al., 2000; Kumar, 2002).

The clock in pinealocytes regulates melatonin biosynthesis at transcription and post-translation levels. Pineal transcriptome studies reveal that around 382 genes in LD and 128 genes in DD express rhythmic oscillations with atleast two-fold change (Bailey et al., 2003). The transcripts include genes involved in melatonin biosynthesis, circadian rhythm generations, phototransduction, immune and stress responses. Hence, the pineal possess genes that encode proteins involved in regulation of several important physiological functions which were previously not known.

### *1.4.2 Retina*

The avian retina has circadian clocks that drive local ocular physiological rhythms including rhythmic turnover of photoreceptor outer segments (Pierce et al., 1993), electrophysiological properties (McGoogan and Cassone, 1999; Binkley et al., 1971; Ko et al., 2003), as well as rhythmic biosynthesis of melatonin and dopamine (Adachi et al., 1995; Binkley et al., 1971; Hamm and Menaker 1980, Reppert and Sagar, 1983; Doyle et al, 2002). The rhythms of clock genes and melatonin biosynthesis remain circadian in retinal cell cultures under constant conditions (Toller et al., 2006). Thus, retina too possesses several properties similar to those of the pineal gland both, *in vivo* and *in vitro*. In some avian species (pigeons and quails) both pineal gland and the retina are major contributors of circulating blood plasma-melatonin (Underwood et al., 1984; Oshima et al., 1989). However, in galliforms (chicken) less than 1% of locally produced retinal-melatonin reaches the systemic circulation.

Enucleation (removal of eyeballs/retina) leads to disruption and/or total loss of circadian activity in some avian species (Underwood and Siopes, 1984). In these cases, the disruption/loss of rhythms can be rescued by exogenous melatonin administration an observation that implies that retina controls the circadian rhythms via rhythmic melatonin release (Underwood et al, 2001). In galliforms (chicken) enucleation abolishes locomotor activity (Nyce and Binkley, 1977) although retina retains ~ 99% of the locally synthesized melatonin (Cogburn et al., 1987; Reppert and Sagar, 1983). This implies that in chicken and quail, retina regulates circadian rhythms in distant organs via neural pathways (Underwood et al., 2001). A very critical function of retina is integration and relay of

photoperiodic information to the SCN via retinal ganglion cells (RGCs) and the retinohypothalamic tract (RHT) (Cassone et al., 1988).

The RHT has glutamatergic processes from the RGCs that innervate the avian SCN directly. The RGCs can relay the photic information via RHT and entrain the SCN. Additionally, the RGCs have different kinds of opsin photopigments that help generate local circadian rhythms that in turn can entrain the avian SCN. Hence, retina is acts as a local and systemic pacemaker, and can entrain the SCN via humoral and neurological pathways (Provencio et al., 2000; Bailey and Cassone, 2004). It is relatively difficult to investigate the precise role of avian eyes in circadian system, because blind birds retain light input to the CCS via extra-retinal photoreceptors in the pineal gland and deep-brain tissues. However, several studies indicate that retina hosts autonomic oscillator and play a role in regulation of retinal and physiological rhythms. The eyes of quail (Underwood et al., 1991), chicken, (Reppert & Sagar, 1983) and pigeons (Oshima et al., 1989) synthesize and secrete melatonin in a circadian fashion at subjective night. The retinal cells in quail eye are tightly coupled to each other and work in synchrony to drive circadian rhythms indicating the presence of autonomous clocks (Steele et al., 2003).The retinal photopigments such as melanopsin, rhodopsin and opsins are under circadian regulation (Bailey & Cassone, 2004). Clock genes such as *cry1*, *cry2* and *clock* exhibit rhythmic circadian oscillations (Bailey et al., 2002; Haque et al., 2002). Retina has several well characterized circadian outputs which exhibit rhythmic oscillations such as, rhythmic neuronal activity of retinal cells (McGoogan & Cassone, 1999), arylalkylamine N-acetyltransferase (AANAT) involved in melatonin biosynthesis (Bernard et al., 1997), and

melatonin biosynthesis which is independent of pineal and brain clocks.

### *1.4.3 Suprachiasmatic nucleus*

In addition to retina and pineal gland, the hypothalamus in central nervous system (CNS) of the bird hosts the avian homolog of mammalian SCN. The avian SCN has two sets of functional structures: medial hypothalamic nucleus also known as medial SCN (mSCN) while lateral to the mSCN is the visual SCN (vSCN) (Cantwell and Cassone, 2006). These structures are connected via neuronal projections and have a contiguous cellular distribution. The vSCN exhibits metabolic and electric rhythmicity (Lu and Cassone 1993, Cantwell and Cassone, 2006). The vSCN receives innervations from the RHT and expresses melatonin receptors (Cassone et al., 1995) Administration of exogenous melatonin inhibits the metabolic activity of vSCN (Cassone and Brooks, 1991; Lu and Cassone, 1993; Cantwell and Cassone, 2006). The role of avian SCN is species specific as SCN lesioning (SCN ablation) results vary among species to species. In quails, pigeons and sparrows, SCN lesion abolishes locomotor activity (Ebihara and Kawamura, 1981; Simpson and Follett, 1981; Takahashi and Menaker, 1982; Yoshimura et al., 2001). Unlike mammals where SCN is sufficient to maintain circadian rhythms under constant conditions, birds require all the three neural pacemakers SCN, pineal gland as well retina to do so (Underwood et al, 2001).

### *1.4.4 Extraocular photoreceptors*

In addition to having more than one pacemaker, the avian circadian system also

involve photoreceptors in regions other than the eye, known as the Extraocular photoreceptors. These photoreceptors are a part of the input pathways that transduce photic information to entrain/synchronize an oscillator/pacemaker. These Extraocular photoreceptors are distributed throughout the central nervous system. Hence, birds possess multiple functioning entrainment pathways and each of these pathways is sufficient to entrain them. For example, Underwood et al., (2001) demonstrated that birds can be entrained to light-dark (LD) cycle even after they have been enucleated.

A unique feature of avian pineal gland is the presence of photoreceptors. Pinealocytes are sensitive to light and respond by rhythmic circadian secretion of the hormone melatonin. The hormone melatonin in turn entrains the bird. Natesan (Natesan & Cassone, 2002) suggest that the photopigments, melanopsin and pineal-specific pinopsin may be involved in mediating this photosensitive response. Yet again, in some birds, enucleation and pinealectomy has no effect on photic-entrainment capability, suggesting that CNS hosts photoreceptors in other areas referred to as the deep-brain photoreceptors. Underwood et al, (2001) demonstrated that the ventral hypothalamus hosts rhodopsin-containing photoreceptors which may be responsible for this phenomenon.

### 1.5 The neuroendrocrine loop

The avian circadian system comprises of multiple-oscillators, ocular and extraocular-photoreceptors forming the complex CCS network. These components are spatially distributed throughout the CNS with multiple input-pathways. Although the pacemakers are semi-autonomous and regulate local processes independently, these

oscillators are capable of influencing each other's oscillation by through several feedback loops. Cassone and Menaker (1984) hypothesized what is known as a "neuroendocrine loop" model to explain how the different pacemakers and input-pathways in avians couples and communicate with each other in order to function as a single circadian clock unit. Unity in function requires the CCS to employ neural and humoral signals to remain physiologically connected. Each of the oscillators in the CCS is referred to as a "damped oscillator." Briefly, the pineal gland, and in a few species the retina, secrete melatonin into the blood stream. The melatonin binds to the melatonin receptors on the SCN which results in general inhibition of SCN activity (Cassone et al., 1987), this is the humoral loop. The SCN communicates with the pineal gland via polysynaptic neural pathway, wherein, the SCN synapses with the hypothalamic paraventricular nucleus (PVN). The descending projections from the PVN innervate the intermediolateral cell column (IML) in the thoracic spinal cord. The IML innervates with the superior cervical ganglion (SCG) in finally synapses with the pineal gland (Moore, 2003). An activated SCN causes the release of norepinephrine (NE) at the nerve terminals synapsing pineal gland. Adrenergic receptors on pineal gland bind to NE and inhibit melatonin biosynthesis. During the day, the SCN rhythmically releases NE at the nerve terminals which then inhibits the production of melatonin in the pineal gland. At night the pineal gland rhythmically secretes melatonin which then reduces the SCN activity.

Studies in mammals and birds indicate that melatonin affects circadian clock function and sleep. However, studies by Abraham et al. (2003), Yasuo et al. (2002), and Poirel et al. (2003) indicate that acute melatonin administration has no effect on clock gene

expression in SCN. If rhythmic clock gene expression is required for generation of circadian rhythms, it implies that metabolic and clock gene expressions are two separable properties of circadian clock function. The SCN has a large population of fibrous astrocytes which express melatonin receptors. It is hypothesized that melatonin affects the metabolism of astrocytes via melatonin receptors. The astrocytes with altered metabolism now alter the SCN clock gene expression which in turn affects their metabolic rhythms, since knockdown of clock reduces the amplitude and increases the period length of glucose uptake rhythms in SCN2.2 cell lines but with no alteration in transcriptional rhythmicity. Therefore, in birds SCN is activated by RHT during the subjective day. The activated SCN mediates several downstream processes via neuronal, sympathetic and hormonal outputs. One of the targets of sympathetic activity is the pineal gland. The melatonin biosynthesis by pineal gland is inhibited by both sympathetic NE and by light. Since the SCN is an oscillator, its outputs wanes by dusk, thereby disinhibiting melatonin biosynthesis by the pineal gland. The pineal gland releases melatonin during subjective night which induces its physiological effects by binding to the cells and tissues expressing melatonin receptors. One of the targets of melatonin is the astrocytes which are present within the SCN and rest of the brain. Melatonin decreases glycolytic activity and increases glycogen biosynthesis in the astrocytes. The glycogen biosynthesis during subjective night builds energy stores required for brain activity during the subjective day. This change in metabolic activity may affect the SCN clock gene expression, but the mechanism by which this happens is not completely understood as of yet. Just like the SCN, the pineal gland is an oscillator too whose output wanes as dawn approaches, thereby removing the

disinhibition on SCN activity.

Therefore, according to the neuroendocrine loop model, it is the mutual coupling of the damped SCN, astrocytes and pineal gland oscillators that keeps them robustly oscillating in a rhythmic fashion (Cassone and Menaker, 1984). Apart from the SCN and pineal, the third oscillator, retina adds one more layer of complexity to the neuroendocrine loop. It couples with the SCN and relays photic info via RHT and in some species secretes melatonin that in turn can regulate SCN function.

## 1.6 Molecular basis of biological clocks

Centuries of almost similar selective pressure has led to the evolution of similar endogenous timing mechanism in organisms in nearly every phylum ranging from eubacteria to humans. These endogenous timing mechanisms may have different layers of complexity and composition in different phylum, but they all serve a common purpose namely, to enable the organism predict temporally defined environmental changes and to coordinate complex internal biochemical and physiological processes to respond to them (Pittendrigh 1993; Bell-Pedersen et al., 2005). Remarkably, the cellular and molecular bases of these systems are highly conserved, especially among phylum vertebrata (Panda et al., 2002).

### *1.6.1 A brief history*

French astronomer Jacques de Mairan in 1729 was the first scientist to document the phenomenon of circadian rhythm in a plant, *Mimosa pudica* (Sweeney, 1987;

DeCoursey, 2004). He introduced the idea that plants and animals respond to the environmental cycles owing to their innate ability of internal timing, and it was not just a passive exogenous reaction. Almost after a century, several pivotal studies and findings spurred back interest in the study of biological clocks in the scientific society. Erwin Bünning a pioneering scientist demonstrated that photoperiodic time measurements are controlled by internal mechanisms and hypothesized that circadian rhythms have adaptive value in organisms (Bünning and Moser, 1969). Sweeney and Hastings 1960 demonstrated rhythmic bioluminescence in unicellular algae Gonyaulax under constant condition. Kramer in 1952 coined the term "biological clock" to explain the phenomenon of navigation in migratory birds (Kramer, 1952; DeCoursey, 2004). Colin S. Pittendrigh, investigator of circadian clock in Drosophila and Jürgen Aschoff, investigator of locomotor activity in mice, are the two principal scientists who are considered as the founders of modern biological rhythm research who developed the concepts and key principles of circadian clocks (Daan 2000; Daan et al., 1976). Circadian biology got its much deserved attention when Colin Pittendrigh delivered a lecture titled "Circadian rhythms and the circadian organization of living systems", whereby it recognized as a science which was very much applicable to humans and medical science (DeCoursey, 2004).

The biological clock in any given living organism has a set of "core clock genes". The number of core clock genes, their orthologs, arrangement and functional importance may vary from species to species. Historically, the discovery of the "period gene" in Drosophila was the first stepping stone in understanding the genetic and molecular basis of biological clocks. This remarkable breakthrough was made by Ronald Konopka and

Seymour Benzer while studying a mutant screen in *Drosophila melanogaster* (Konopka and Benzer, 1971). The mutant groups were studied for the persistence of two circadian behaviors, time of pupal eclosion and locomotor activity rhythms. The flies exhibited one of the three phenotypes: lengthened circadian period, shortened circadian period and arrhythmic. The three phenotypes were complemented by a single locus, referred to as the "*Period*" (*per*) gene. Later on, several additional core clock genes were uncovered including *timeless* (*tim*), *cycle* (*cyc*), *doubletime* (*dbt*) and *cryptochrome* (*cry*).

Work by Feldman and Hoyle (Feldman and Hoyle, 1973) in the filamentous fungus *Neurospora crassa* lead to the discovery of "Frequency" (*frq*) gene which is required for persistence of rhythmic conidiation. Martin Ralph and Michael Menaker (Ralph and Menaker, 1988) discovered that a mutation in a single, autosomal locus tau can dramatically shorten the period of their circadian locomotor rhythms. Thus, scientists discovered that even a single gene mutation could disrupt a complex behavioral rhythm. These discoveries forayed a large-scale mutant screening in mice for biological clock mutations (Vitaterna et al., 1994). Consequently, genetic and mutation studies have been applied and dozens of other core clock genes have been identified in normal and mutants of several model organisms such as *Neurospora*, *Arabidopsis*, hamsters, mice, rats and fish (Dunlap, 1999).Genetic studies of core clock genes reveal that although these organisms are greatly separated by genetic makeup and belong to different phyla, the fundamental processes (if not sequences) which drive their circadian rhythms are highly conserved (Dunlap, 1999).

A feature that is common among all the organisms possessing diverse core clock gene systems is the presence of transcription/post-translational feedback loops. These

loops ensure generation of circadian rhythms of high amplitude and a period length of nearly 24 hr. Scientists have developed real-time bioluminescent and fluorescent reporters that help visualize circadian rhythms in tissues and cells (Hastings et al., 2007). These techniques help in understand the coupling of oscillators within a population of cells which are rhythmic. In 2005, Nakajima's lab demonstrated that it is possible to construct a circadian oscillator in the absence of gene transcription (Nakajima et al., 2005).

### *1.6.2 The mammalian molecular clock*

The mammalian circadian oscillator is a network of interlocking transcriptional-translational feedback loops working in driving a rhythmic expression of core clock genes (Reppert and Weaver, 2002). The products of these genes (i.e. clock proteins) are required for the generation and maintenance of circadian rhythms within all clock containing cells in an organism.

The core clock components of mammalian circadian molecular clock can be divided into two sets, the positive and the negative elements. The core clock genes, CLOCK (Circadian Locomotor Output Cycles Kaput, Clock) and BMAL1 (Brain and muscle Arnt-like protein-1, Bmal1) form the positive arm of the circadian clock and they belong to the family of basic-helix loop helix-PAS (bHLH-PAS) containing transcription factors. These two form a heterodimer and bind to the E-box cis regulatory enhancer (CRE) elements and lead to rhythmic transcription and translation of their target clock genes, *period* (*per1, per2, per3*) and *cryptochromes* (*cry1* and *cry2*). *Period* and *cryptochrome* genes form the negative elements of the feedback loop. The period and

cryptochrome proteins form heterocomplexes and translocate back into the nucleus and inhibit their own transcription (Gallego and Virshup, 2007). In addition to the positive and negative feedback loops, a third regulatory feedback loop also participates in the molecular circadian network. The third loop is formed by orphan nuclear receptors REV-ERBα (Rev-erb alpha, an orphan nuclear receptor; Rev-erbα) and RORα (Retinoid-related orphan receptor alpha (ROR alpha; RORα) (Gallego and Virshup, 2007). The proteins of positive elements Clock/Bmal1 heterodimer regulate the expression of these orphan nuclear receptors. In the nucleus, REV-ERBα protein competes with RORα protein to bind with ROR-responsive element (RORE) located in the promoter region of *bmal1* gene. Binding of RORα at the RORE activates *bmal1* transcription, whereas REV-ERBα binding represses it. Hence, the rhythmic expression of *bmal1* is under the positive and negative regulation of RORs and REV-ERBs respectively. This forms the secondary feedback loop referred to as the "stabilizing loop".

Apart from these three transcriptional-translational feedback loops, the circadian clockwork also employs several post-translational modifications for normal functioning. It's believed that post-translational modifications may be responsible for the ~ 24 hrs length of the circadian period (Gallego and Virshup, 2007). Casein kinase members (CK1 ε and CK1 δ) are required for the phosphorylation and degradation of the protein Period. Mutation in CK1 ε results in shorter free-running period in hamsters (Lowrey et al., 2000). Over expression of CK1 ε and CK1 δ causes moderate shortening of circadian period in mammals (Akashi et al., 2002). Inhibition of enzyme glycogen synthase kinase-3(GSK-3) results in shortening of mammalian circadian period (Hirota et al., 2008). Mitogen -

activated protein kinase (MAPK) and cAMP-dependent protein kinase (PKA) pathways induce *per1* induction (Travnickova-Bendova et al., 2002). Resetting of mammalian circadian clock requires protein kinase C (PKC)-mediated phosphorylation of Clock (Shim et al., 2007). Mutation in an F-box containing E3 ligase (Fbx13) causes improper ubiquitination and degradation of CRY proteins. The resulting CRY protein accumulation causes lengthening of circadian period (Busino et al., 2007). Post-translational modification of Bmal1 such as sumoylations, acetylation and phosphorylation are critical for nuclear accumulation of Bmal1 and circadian function (Tamaru et al, 2009).

### *1.6.3 The avian molecular clock*

Avian circadian biology is an emerging field of chronobiology. Several clock genes have been cloned in several species of birds such as, chicken, Japanese quail, pigeon, sparrows to name a few. However, little is known about the mechanisms of molecular clock functions in the birds. Birds do not seem to have the clock gene, *per1*. Negative elements of circadian clock *cry1*, *cry2*, *bmal1* and *bmal2* are found to rhythmic in chicken and sparrow.

When compared to mammals, avians have a complex CCS comprising of mutually coupled, three autonomous and anatomically distinct oscillators with multiple photic-input pathways. The avian pineal gland and retina express rhythmic oscillations in melatonin biosynthesis. The pineal melatonin reaches circulating blood stream, and in some avian species retinal melatonin also reaches the blood circulation. There are significant differences in transcriptional regulation of avian and mammalian biological clocks. In

chicken, core clock genes are differentially regulated within pineal gland and retina (Bailey et al, 2003; Bailey et al., 2004). This implies that molecular clockwork function differently in different pacemakers within the same organism. Thus avian circadian clock networks are functionally complex and require more in depth analysis for greater understanding. At molecular level, genomic and transcriptional analyses reveal a highly conserved network of clock genes. The avian core clock genes are orthologous to the core clock genes identified in insects and mammals (Bailey et al., 2003; Karagnis et al., 2008, 2009). Avian orthologs of core clock genes include *clock*, *bmal1*, *bmal2*, *per2*, *per3*, *cry1*, *cry2* and *cry4* (Chong et al, 2000; Yoshimura et al, 2000; Brandstatter et al, 2000; Yamamoto et al., 2001; Bailey et al., 2001; Fu et al., 2002; Chong et al., 2003; Yasuo et al., 2003; Mouritsen et al., 2004; Helfer et al., 2006).

Although the entire biochemical details are not well known in birds, studies indicate that the birds too express "positive elements" genes *clock* and *bmal1*. The avian *clock* and *bmal1* genes are transcribed rhythmically in the nucleus and translocated to the cytoplasm. The Clock and Bmal1 proteins dimerize and reenter the nucleus to activate the transcription of negative elements *per2*, *per3*, *cry1*, *cry2* as well as other ccgs that rhythmically affect downstream processes. Genomic analysis of chick pineal and retina express the orthologous clock genes but at different phases. For instance, pineal gland expresses *bmal1*, *bmal2* and *clock* at subjective night, while retina expresses only *bmal1* in a rhythmic fashion. Furthermore, pineal gland expresses the negative elements *per2*, *per3*, *cry1* and *cry2*, but retina expresses only *per3* and *cry1*. The expression profiles of the avian clock orthologous genes are not consistent with the mammalian molecular model.

Although avian orthologs of core clock genes exhibit rhythmic oscillations, not all of these mRNA transcripts are in phase with their mammalian counterparts (Bell-Pedersen et al., 2005; Helfer et al., 2006). For example, the mRNA levels of "negative elements" *per3* and *cry1* are expressed at 180° out of phase to each other, *per3* peaking at night and *cry1* peaking during the day. This implies that oscillator-feedback-loop model is not universally similar across the vertebrate classes. Hence, further studies gene and protein level are required to elucidate the entire molecular mechanism of the circadian clock in birds.

### 1.7 Peripheral clocks

For a long time it was believed that circadian clock existed only within the SCN/master pacemaker. It was the work of Balsalobre et al. in 1998 and Yamazaki et al. in 2000 that successfully demonstrated the presence of circadian genes in Rat-1 fibroblast cells and tissue explants respectively (Balsalobre et al., 1998; Yamazaki et al., 2000). However, explants from peripheral tissues and fibroblast cell cultures progressively fall out of phase and become incoherent in oscillations (Balsalobre et al., 1998; Yamazaki et al., 2000). Studies in SCN-lesioned animals showed reduced and/or abolished rhythms of clock genes in the peripheral tissues. These lead to the belief that the central pacemakers are absolutely indispensable for the peripheral clocks to function (Sakamoto et al., 1998; Akhtar et al., 2002; Terazono et al., 2003). Contradictory to this belief, Nagoshi et al. and Welsh et al. in 2004 (Nagoshi et al., 2004; Welsh et al., 2004) demonstrated that the clocks in the fibroblast cells are cell-autonomous and self-sustained. The lung explants expressed *per2* rhythms for more than 20 days and that SCN lesion did not abolish rhythms in

peripheral tissues. Additionally peripheral clocks are resilient to cell division, acute stress, temperature-fluctuations and general transcription rate (Nagoshi et al., 2004; Dibner et al., 2009; Saini et al., 2011). However, peripheral clocks in mice subjected to SCN-ablation show lack of coordination along with large differences in phase from tissues to tissues (Yoo et al., 2004). Thus, SCN is required for relaying signals that help phase organize the peripheral clocks in SCN-intact animals. Hence, the peripheral clocks are considered as dampened oscillators which require the master clock, SCN to synchronize their rhythms in a robust manner. In addition to the signals from SCN, there are several other signaling pathways that can transiently synchronize cellular oscillators which include serum shock, glucocorticoids and temperature pluses (Balsalobre et al., 2000; Balsalobre et al., 1998; Prolo et al., 2005; Yagita and Okamura, 2000).

### *1.7.1 Central vs. peripheral clocks*

The master pacemakers and peripheral clocks do exhibit rhythmic expression of various clock genes with almost similar molecular mechanisms running their clocks. They both employ transcription-translational feedback loops and post-translational modifications for generation of overt rhythms, yet, there are a few important differences between the central and peripheral clocks. A few of these differences include the redundancy of positive arm of the circadian clock in the SCN which is absent peripheral clocks. The *clock* in the SCN is quite dispensable such that, in absence of *clock*, another positive element, NPAS2 can substitute in functionally. This phenomenon explains the locomotor activity in *Clock*-deficient mice under constant darkness (DeBruyne et al.,

2007). The second difference between the SCN and peripheral clocks is the capacity to communicate phase information between individual cells. The individual cells making up the SCN are capable of communicating with each other through neuronal and paracrine signals. These signals keep all the cells within the SCN coupled (synchronized) and hence, they oscillate robustly in the same phase (Welsh et al., 2010). This coherence in phase is observed in SCN ex-vivo for days to weeks.

Thirdly, members of ROR family (a, b and c) exhibit different patterns of expression across peripheral tissues (Akashi and Takumi, 2005; Guillaumond et al., 2005; Liu et al., 2008; Sato et al., 2004). For instance, RORc is rhythmically expressed in peripheral tissues while in the SCN it is not expressed it at all; RORb is expressed in the SCN and retina but is absent in other peripheral tissues; RORa exhibits robust oscillations in the SCN and is almost dampened in the peripheral tissues. Hamilton et al. reported that mice lacking functional RORa, called "staggerers" (Hamilton et al., 1996) express normal levels of Bmal1 rhythms in peripheral tissues; suggesting that ROR proteins may contribute to rhythmic activation of Bmal1 in a tissue-specific manner (Emery and Reppert, 2004; Sato et al., 2004).

Fourth, deficiency of Clock suppresses the rhythmic amplitude of Rev-erbα in liver, but has little effect in the SCN. Clock-deficient liver has elevated levels and robustly rhythmic Per1 when compared to its wild type mice. However, the levels of Per1 are lowered in dampened in Clock-deficient SCN when compared to the wild type. Further, Clock-Ä19 mutant mice exhibit disruption of *per2* mRNA rhythms in liver and muscle with severely reduced levels in the kidney and heart (Noshiro et al., 2005). Thus, it appears

that the activity of transcription factors promoting circadian gene expression patterns varies from target gene and /or type of tissue (DeBruyne et al., 2006). The peripheral tissues also display vast variations in transcriptional circadian regulation of ccgs as revealed by microarray studies (McCarthy et al., 2007; Miller et al., 2007; Panda et al., 2002; Storch et al., 2002). The tissue-specific differences may imply that the molecular clocks vary in their intrinsic rhythmic properties across the tissues. Additionally, these differences in the peripheral clock properties may also be due to differences in tissue-specific input signals and/or regulatory mechanisms in the clock output pathways. Peripheral clocks are regulated and synchronized by several signaling pathways in addition to the neuronal and humoral signals from the SCN (Kornmann et al., 2007; Stokkan et al., 2001; Zambon et al., 2003).

### *1.7.2 Entrainment and synchronization of peripheral clocks*

In most animals, neuronal pacemakers are primarily entrained by light. In some species, this entrainment may occur via direct exposure of the pacemaker tissue to the photic stimuli, as in the avian species which have photoreceptive pineal gland and retinae. In case of mammals, photic signals are relayed via RHT to the SCN. The peripheral clocks generally do not get entrained by direct light cues. They get entrained by either of these two mechanisms: 1) output signals derived from the SCN; 2) externally derived non-photic stimuli. Immortalized SCN 2.2 cells when co-cultured with NIH 3T3 fibroblasts can drive downstream oscillations in clock gene expression and glucose uptake via humoral signals (Allen et al., 2001). In addition to synchronizing the peripheral clocks indirectly through

sleep-wake cycles and body temperature, the SCN can also entrain the peripheral clocks via direct signaling pathways (viz. humoral and neuronal pathways) (Guo et al., 2005). The SCN regulates plasma-glucocorticoid levels on a daily basis via the hypothalamic-pit adrenal axis (Oster et al., 2006). Glucocorticoids are capable of resetting and phase shifting cycles of peripheral clocks by binding to glucocorticoid receptors (GR) expressed in the tissues. An additional direct synchronization pathway employed by the SCN is the autonomic nervous system (ANS) (Buijs et al., 2009) wherein the SCN resets the peripheral clocks (such as adrenal gland, liver) via light signals. Glucocorticoids may be one indirect method by which the SCN can entrain the peripheral clocks (Balsalobre et al., 2000; Le Minh et al., 2001; Stratmann and Schibler, 2006).

Apart from photic-stimuli, food-intake can also act as a primary external zeitgeber, especially for the oscillators in the liver, stomach and heart underscoring the importance of peripheral circadian clocks in the temporal orchestration of metabolism. These types of clocks are referred to as feed-entrainable oscillators (FEO). In mice and rats, feed restriction can uncouple peripheral rhythms in the liver and heart from those of the SCN without shifting the phase of the SCN itself (Damiola et al., 2000; Hara et al., 2001; Stokkan et al., 2001).Another very interesting mechanism that can entrain circadian clocks in the peripheral tissues is the cellular redox state (NAD/NADP oxidative state) known as the metabolic entrainment pathway(s) (Rutter et al., 2001). McKnight et al. suggest that the NAD(P)H/NAD(P)$^+$ ratio affects the binding of CLOCK/NPAS2-BMAL1 heterodimers to the promoter regions of clock gene sequences (Rutter et al. 2001). Silent mating type information regulation 2 homolog-1(SIRT1), a NAD$^+$-dependent histone

deacetylase may be another candidate for connecting cellular metabolism to circadian gene expression (Asher et al. 2008; Nakahata et al. 2008). Silent mating type information regulation 2 homolog, SIRT1 deacetylates various transcription factors and coactivators (Zschoernig and Mahlknecht 2008) and influences the circadian expression of several clock genes (Asher et al. 2008; Nakahata et al. 2008). Poly (ADP-ribose) polymerase-1 (PARP-1), another $NAD^+$-dependent enzyme, has been implicated in the phase resetting of liver clocks. PARP-1 adds poly (ADP-ribose) residues to Clock protein in a diurnal manner, and whereas PARP-1 and affects the kinetics of phase adaptation to feeding rhythms (Asher et al. 2010; Bellet and Sassone-Corsi et al., 2010) (Figure 3).

Another surprising zeitgeber capable of synchronizing peripheral clocks in mammals is temperature fluctuations. Shallow fluctuations in temperature rhythms imitate fluctuations in body temperature rhythms, and can maintain previously induced rhythms in peripheral clocks and can induce phase shifts without affecting the phase of the SCN (Brown et al., 2002).

### *1.7.3 Physiological importance and roles of peripheral clocks*

After the discovery of clock genes in master oscillators, scientists started looking for clock gene expression in rest of the cells in the body. It quickly became apparent that circadian clocks ticked if not in all, but almost all peripheral cells and tissues. This phenomenon was seen in several vertebrate and non-vertebrate species popularly used for circadian studies (Balsalobre et al., 1998; Yamazaki et al., 2000). As discussed earlier, the central and peripheral clocks have several similar and dissimilar properties in terms of

mechanism of action, molecular components, synchronization, entrainment, input-output signals and physiological significance to name a few. Cultured cells and explants from tissues such as, lung, kidney, spleen, pancreas, heart, stomach, skeletal muscle, thyroid gland and adrenal gland exhibit robust circadian oscillations in clock genes (Yagita et al., 2010). However, not all tissues or cells exhibit similar or persistent cyclic rhythms, such as; the thymus gland and testis in mice are arrhythmic. It is hypothesized that the thymus and testis are largely comprised of rapidly multiplying and differentiating cells which may not exhibit a functional circadian oscillators (Alvarez et al., 2005; Liu et al., 2008).For instance, embryonic stem cells (ES cells) may be considered as the least differentiated cells incapable of generating circadian oscillations. Yagita et al. demonstrated that ES cells become rhythmic once they undergo differentiation implying that there is some sort of cross-talk between the cellular differentiation program and the circadian clock components (Yagita et al., 2010).

A large number of key physiological functions exhibit daily oscillations in the peripheral tissues. There are several examples which support this observation. Endobiotic and xenobiotic detoxification in the liver, kidney and small intestine; lipid and carbohydrate metabolism in the liver, muscle and adipose tissue; renal plasma flow and production of urine in the kidneys; blood pressure and rate of heart beats in the cardiovascular system exhibit recordable daily rhythmic oscillations.

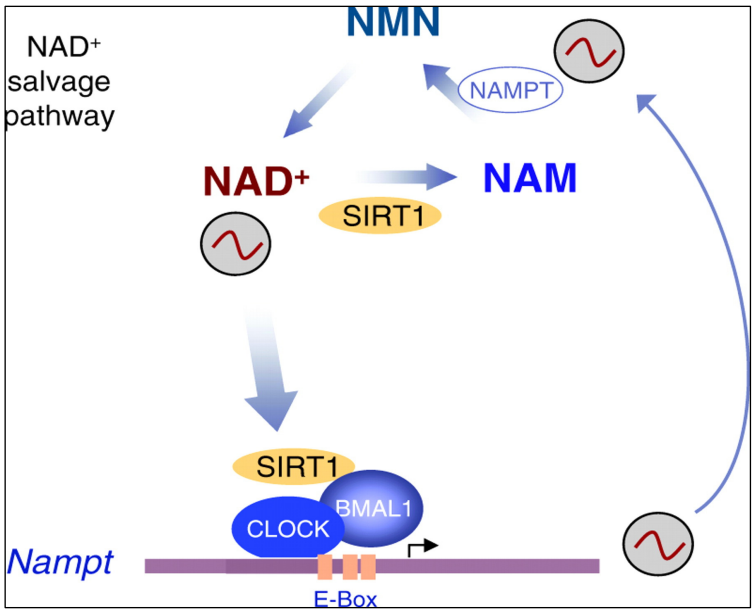


Figure 3. The circadian feedback loop between SIRT1 activity and Nampt transcription The Clock/Bmal1 heterodimer bind to the E-box of Nampt gene. Clock acetylates Bmal1 and local histone tails which promotes transcription of Nampt. The resulting Nampt enzyme increases synthesis of NAD+, which activates the SIRT1. Meanwhile, the Clock/Bmal1 heterodimer induce transcription of *cry1* and *per2* too. The Cry1/Per2 heterodimer, serving as co-repressors of *Clock/Bmal1*, turns off the transcription of *Clock/Bmal1* and Nampt. The activated SIRT1 is subsequently recruited to the transcriptional machinery by Clock. Here SIRT1 deacetylates Bmal1 and Per2. This deacetylation promotes the degradation of both proteins. As such, the promoter region of Nampt is re-primed and the gene is ready for the next round of Clock-dependent induction (Bellet and Sassone-Corsi, 2010).

The old belief of hierarchical organization of the circadian system has been revisited and revised owing to the findings of cell-autonomous peripheral clocks. It was believed that oscillators in the higher centers send out neuronal, humoral and signaling molecules and control the rhythms of peripheral tissues. In absence of the signals from master oscillators, the peripheral tissues are totally incapable of generating any circadian rhythms of their core clock genes and ccgs related to their physiological functions.

Disruption in the master oscillators or their signals was sufficient to throw off the functions and rhythms in the peripheral tissues. Time and again several studies have shown otherwise. Technically, a peripheral oscillator can generate its own rhythm and regulate the physiological functions within its cell/tissue without having to depend on the signals/cues from the master clock. For instance, kidneys, liver and gastrointestinal tract (GIT) can have their rhythms and functions synchronized by the time of food-intake. These tissues host what are known as food entrainable oscillators (FEO) which do not need any other external signal or cues from the master clock to function rhythmically. However, the peripheral clocks in different tissues and cells along with their physiological outputs must be in sync for an organism to survive. This harmony and synchrony is brought about by the master clock. Albrecht and colleagues proposed the "orchestra model of circadian organization" in complex multicellular organisms. According to this model, the master clock sends out neural, humoral and substrate signals to "orchestrate" or synchronize the oscillators present in the peripheral tissues (Dibner et al, 2009). Studies in several tissues and cell-lines indicate that peripheral clocks are not passive players and may have critical roles in regulating the rhythms of local physiological functions. It is

therefore, critical to understand the role of peripheral clocks in each tissue, and their relationship to another in order to comprehend the role of peripheral clocks in circadian physiology.

It is difficult to distinguish the roles of peripheral clocks when master oscillators (such as the SCN) are intact. The SCN continuously synchronizes the phases of peripheral clocks thus influencing downstream physiological outputs. To go around this situation, scientists study circadian transcriptomes of tissue/cell explants and apply tissue/cell type selective manipulation of local clock machinery and look for changes in alterations in physiological outputs.

Transcriptome studies show that several clock genes are expressed in a tissue specific manner, and some clock genes have opposite effects in different tissues indicating that all peripheral clocks are not alike. Additionally, of the 5-10% transcriptome that are rhythmically expressed across the peripheral tissues, there is very little overlap in these genes. This diversity implies that the peripheral clocks are unique to each tissue, hence a particular tissue and cell is able to carry out its own unique functions. Each tissue-specific or cell-specific oscillator exercises its effects on distinct clock-controlled pathways thus diversifying its role in physiological and molecular regulation. The physiological significance in having different peripheral clocks regulating diverse physiological and cellular functions goes back to support the theory of "temporal compartmentalization of biochemically incompatible processes" and organization of successive compatible processes for maximum energy efficiency in a well-orchestrated manner in the course of a normal day. Thus, the peripheral clock tends to become an interpreter, messenger and

organizer helping the cross-talk between the central clock and peripheral physiological processes.

What are the mechanisms that the peripheral clocks use in reception of central clock oscillators and regulation of peripheral physiological functions are under a lot of scrutiny lately. Cell/tissue specific knockout of clock genes and ccgs have revealed very interesting results. For instance, Takahashi and colleagues rescued the expression of Bmal1 expression in *Bmal1*-deficient mice. They showed that activation of *bmal1* is required in the brain to restore the overall circadian behavioral rhythms, but restoring *bmal1* expression in muscles was sufficient to rescue normal wheel-running activity (McDearmon et al., 2006). Lamlia et al. demonstrated that liver-specific *bmal1* leads to dysfunction in glucose and lipid metabolism (Lamlia et al., 2008). Disruption of pancreatic-*bmal1*expression causes defective β-cell function leading to diabetes-like state (Marcheva et al., 2010). In the cardiovascular system, Bmal1 helps in regulation of blood pressure and thrombogenesis (Westgate et al., 2008).Disruption of peripheral oscillator in adrenal gland alters the glucocorticoid (GC) rhythms (Oster et al., 2006; Son et al., 2008). Genome-wide transcriptome profiling studies in peripheral tissues such as heart, liver and adrenal gland indicate that around 5% to 10% of genes are under circadian regulation and expressed rhythmically (Panda et al., 2002; Ueda et al., 2002).Several of these genes are involved in regulation of important physiological functions across different tissues.

### *1.7.4 Lessons from studies on various peripheral tissues and their clocks*

Weitz et al. employed Cre-lox technology and *bmal1* allele with loxP flanking

exon 8 of the gene to inactivate the *bmal1* gene in retina and liver. The resulting phenotype exhibited disturbances similar to those of whole-body *bmal1*-knockout (*bmal1*-KO) mice (Storch et al., 2007). These retina-specific *bmal1*-KO mice exhibited normal circadian gene expression everywhere except in the retina. The retina in these mice showed abnormally low electrical activity especially when exposed to light leading to impaired retinal visual processing. Even in the presence of intact SCN in these KO mice, the animals expressed loss in circadian rhythms of ~ 90% of the otherwise rhythmically oscillating genes. Similar results were seen in SCN ablated (SCNx) retina-specific *bmail1*-KO mice. Although, the role of retinal-*bmal1* in regulating visual processing and electrical activity in retina is still under investigation it is clear that a functional retinal clock is required for processing photic information and not the SCN (Storch et al., 2002; Storch et al., 2007).

Liver is one of the most extensively studied tissues in terms of circadian transcription and ccgs. Around 1000 circadian transcripts are rhythmic in liver of which several genes encode of key enzymes involve in metabolic pathways, energy homeostasis, detoxification and several other biochemical processes. In 2008, Lamia et al. studied the effects of liver-specific *bmal1*- KO in mice on glucose metabolism. These models exhibited hypoglycemia and increased glucose clearance. Kornmann et al. showed that over expression of Rev-erbα in liver prevents the expression of Bmal1 along with the loss of rhythms of several clock-controlled genes in the tissue (Kornmann et al., 2007).

An important organ involved in glucose metabolism is the pancreas and specifically the β-cells. Marcheva et al. examined *bmal1*-deficient pancreatic islets in-vitro to study the

importance of their core clock machinery. These cells were much smaller and had defective insulin secretion. In the in-vivo models, the islet-specificb *bmal1*-deficient mice expressed severe phenotype with markedly reduced glucose tolerance, severely reduce insulin production and a very high blood glucose levels throughout the day. In a normal pancreatic cell with functional circadian clock, Clock/Bmal1 heterodimers bind to the E-box of Nicotinamide phosphoribosyltransferase (Nampt) gene. The clock acetylates Bmal1and promotes the transcription of Nampt. The Nampt enzyme increases NAD synthesis, which induces activation of sirtuin1 (SIRT1). The Clock/Bmal1 heterodimer promote the transcription of *cry1* and *per2* too. The Cry1/Per2 heterodimer bind to the promoter regions of *clock/bmal1* and Nampt and suppress their transcriptions. The Clock recruits the activated SIRT1 which in turn deacetylates Bmal1 and Per2 proteins leading to their degradation. The degradation removes the negative feedback on the Nampt and thus priming to the next round of Clock-dependent transcription (Marcheva et al., 2008). The SIRT1 is an important enzyme which has large number of target molecules, some of these molecules participate in energy homeostasis, metabolism, cancer and aging. In glucose metabolism, the SIRT1 induces insulin production in β-pancreatic cells by inhibiting uncoupling protein2 (UPC2) and increasing exocytosis of insulin-laden globules. In *bmal1*-mutation and deficiency, there is no SIRT1 activation, which leads to reduced insulin production and diabetes-like symptoms.

Another important peripheral tissue known to regulate several metabolic and physiological functions is the adrenal gland. The adrenal gland is part of the hypothalamic pituitary-adrenal axis (HPA axis) and receives humoral and neuronal signals from the

hypothalamus and pituitary and releases glucocorticoids (GCs) accordingly. The adrenal cortex releases the GCs under the influence of SNS and ACTH (adrenocorticotropic hormone) via the HPA axis. Hence, it was initially considered that adrenal gland was a passive organ under the strict regulation of the hypothalamus and pituitary gland with regards to GC secretion on a daily basis. GCs are strong signaling molecules, capable of shifting the clock gene expression in peripheral organs such as liver, kidney and heart (Balsalobre et a., 2000). Surgical removal/ablation of adrenal gland (adrenalectomy) induces loss/dampening of rhythmic expression of several genes in the liver (Oishi et al., 2005). Corticosterone of the adrenal cortex modulates gluconeogenesis and lipid metabolism in the liver, cardiovascular functions and immune functions (Kemppainen & Behrend, 1997). Oster et al. investigated if the adrenal clock helped/ played any role in the modulatory and regulatory capacities of the adrenal gland (Oster et la., 2006). They observed that several genes involved in the biosynthesis of corticosterone are clock controlled (Oster et al., 2006). They used transplantation studies (adrenals from WT or *Per2/Cry1* KO grafted into adrenal ablated mice; adrenal-specific *Per2/Cry1*KO grafted to WT). It was observed that both adrenal and SCN must have functional clocks for normal rhythmic GC synthesis. Adrenal explants suggest that the clocks in adrenal gland gate its sensitivity to ACTH on a rhythmic basis (Oster et al., 2006). They demonstrated that the local clock is tightly linked to the steroidgenic pathway/biosynthesis of glucocorticoid via StAR (steroidogenic acute regulatory proteins). The cyclic expression of StAR (a rate limiting gene of steroid biosynthesis) is under the direct control of Clock/Bmal1 heterodimer. StAR is an adrenal gland-specific ccgs, thus resulting in cyclic rhythms in GC

production (Figure 4).

Nakamura et al. investigated the role of Clock and Bmal1 in the cardiovascular system. They found that Clock/Bmal1 heterodimer transactivates peroxisome proliferator activated receptor-γ (PPARγ). The PPARγ is a member of the superfamily of nuclear receptor ligand-activated transcription factors which participates in glucose and lipid metabolism. It also has vasoprotective action and regulates blood pressure in the cardiovascular system (Nakamura et al., 2008). Wang et al. highlighted the importance of vascular and endothelial PPARγ expression over a period of 24hrs of blood pressure and heart rate. Smooth muscle and endothelial cells lacking PPARγ exhibited reduced rhythms of Bmal1, blood pressure and heart rate. They showed compelling evidence cardiac clock and PPARγ are required for normal cardiac functioning. Namely, PPARγ exhibits robust rhythms which precede Bmal1. Chromatin immunoprecipitation (ChiP) assay revealed that PPARγ interacts with PPRE (PPARγ response elements) sites on the Bmal1 promoter. Lastly, rosiglitazone (RGZ), a PPARγ activator induces Bmal1 expression in mouse endothelial cell line (Wang et al., 2008; Wang et al., 1995). In addition to studies in endothelial cells, Bray et al. and Duran et al. studied the role of circadian clocks in the cardiomyocytes functions (Bray et al., 2008; Durgan et al., 2006).

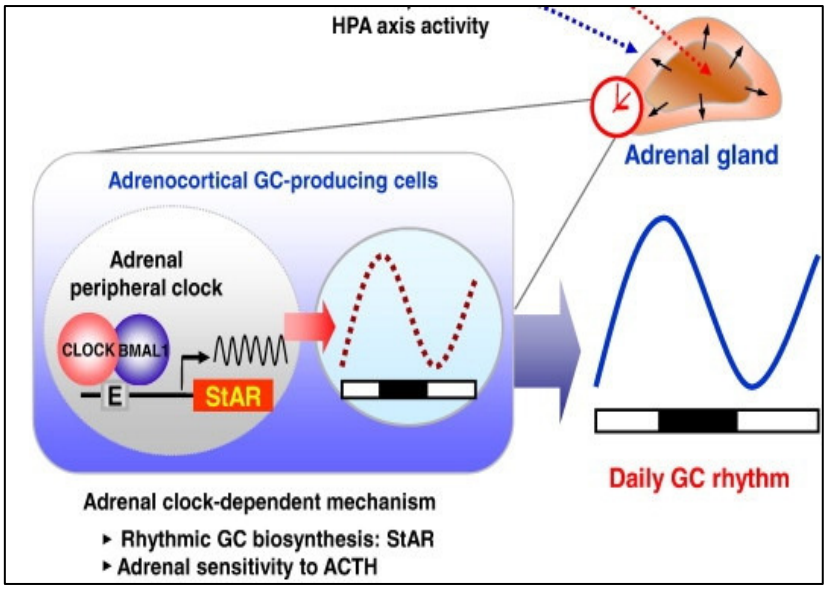


Figure 4. The peripheral adrenal clock). The adrenal local clock is tightly linked with the steroidogenic pathway. StAR is a rate-limiting gene of steroid biosynthesis. The cyclic expression of StAR is directly controlled by the CLOCK:BMAL1 heterodimer. Thus, StAR is an adrenal gland-specific clock-controlled gene; consequently, resulting in daily oscillation in steroidogenesis which contributes to the generation of the robust GC rhythm (Son et al., 2011).

Mice which lack *clock* gene in their cardiomyocytes express lack of rhythmicity in myocardial gene expression, glycogenesis, β-adrenergic signaling, triglycerides metabolism, epinephrine induced contractility, heart-rate, and repression of cardiac output (Bray et al., 2008; Durgan et al., 2006). Hence, disrupting various players in the circadian clock in tissues such as fat, kidney, adipose tissue, ovary should demonstrate the requirement of a responsive and functional circadian peripheral. Hence, disrupting the clock specifically in other tissues, such as fat, kidney, etc., should further demonstrate the requirement of responsive and functional peripheral circadian clocks in other physiological systems.

## 1.8 Specific objectives

### *1.8.1 Objective 1*

To investigate rhythmic immune and clock properties in avian spleen. We will identify and characterize oscillations in spleen and test the hypothesis that the circadian clock rhythmically regulates immune tissue function in chicken. Temporal expression profiles of immune response and core clock genes in immune tissues will be identified and characterized. We will examine the daily regulation of important proinflammatory cytokines namely, TNF-α, IL-1β, IL-6 and IL-18.

### *1.8.2 Objective 2*

Effects of LPS treatment upon immunity, temporal difference in the immune

response and immune tissue functions. We will test the hypothesis that the inflammatory response in spleen is under the regulation of circadian clock. Birds will be immune-challenged at mid-day and mid-night to examine time-of-the day difference in inflammatory response by examining the temporal expression profile of pro-inflammatory cytokines TNF-α, IL-1β, IL-6 and IL-18.

### *1.8.3 Objective 3*

Effects of melatonin on inflammatory response. The pineal gland secretes melatonin which regulates several physiological functions in the peripheral tissues. Melatonin therefore, acts as signaling molecule synchronizing timing information between the central and peripheral clock. We will investigate the effect of exogenous melatonin administration upon the daily dynamics of the inflammatory response in spleen.

### *1.8.4 Objective 4*

Investigation and comparison of circadian clock gene expression and rhythms in the hypothalamus, ovary and adipose tissue of egg-type and meat-type hens. The demonstration of circadian rhythmicity in peripheral tissues and cell types and the ability of excess energy to impact them is an extremely powerful observation for deciphering the timing of reproductive physiology. While much is known regarding the contributions of the hypothalamic-pituitary-gonadal (HPG) axis and hypothalamic oscillator to ovarian function, little is known regarding the necessity of peripheral clock and their coordination of daily ovarian physiology. In the present study we examine the daily control of core

clock genes within central and peripheral clocks in two distinct lines of hens and present a novel model for studying circadian control of ovarian physiology in normal and obesity prone subject.

# 2. CIRCADIAN CLOCK REGULATION OF IMMUNE FUNCTION IN AVIAN SPLEEN*

## 2.1 Introduction

Higher organisms exhibit molecular circadian oscillators in peripheral tissues and cell types similar to those witnessed in the central nervous system. These peripheral clocks regulate the timing of local physiological functions. One such key physiological function under investigation in our laboratory is immune defense. Circadian pacemakers may regulate the immune system via neuroendocrine signaling which includes hormones cortisol and melatonin. However, the role of local clocks in in regulating the immune system and inflammatory response. Studies in animal models show that immune system parameters exhibit time of day-dependent variations. These variations may be coordinated by the peripheral clock at multiple levels. The molecular mechanisms governing communication between the circadian system and the immune system are not completely understood and are under active investigation. With the identification of the core clock genes in immune tissues, and rhythmic oscillations of cytokine genes, it can be hypothesized that the clock may be important for directing daily immunological functions.

Several cell cycle genes such as *Wee1*, *cyclinD1*, and *c-Myc* are regulated by rhythmic core clock genes. Therefore, direct effect on tumorogenesis is possible upon

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disruption of circadian rhythms. In rodents, the clock gene '*period*' has been implicated in tumor suppression and DNA repair. The *per2*-mutant mice lack daily rhythmic expression of an important immune factor Interferon-γ (IFN- γ) in the spleen when compared to wild-type mice. The *bmal1*- deficient mice have impaired B cell development, while *per2*-mutant mice show very poor response to LPS-induced endotoxic shock. These data strongly suggest that circadian clocks are key regulators of the immune functions.

The molecular mechanisms governing communication between the circadian system and the immune system are not completely understood and are largely under investigation. In order to understand the progression of several inflammatory disorders and disease conditions, it is critical to understand how the disruption of circadian timing mechanisms leads to dysfunctional immune response.

Therefore, the aim of this research proposal was to investigate daily and circadian regulation of immunological function by the circadian clock in our animal model, the domestic chicken (*Gallus gallus*). We chose an important peripheral tissue critically involved in t avian immunological functions, the spleen. The avian spleen was examined for expression patterns of core clock genes and temporal regulation of the inflammatory responses over a 24-hour period. Hence, avian spleen was systematically investigated to test whether, i) its core clock genes exhibit daily and/or circadian rhythms, and ii) pro-inflammatory cytokines genes exhibit daily and/or circadian rhythms. The study provided a novel functional evidence for the presence of core circadian clock genes in the avian immune tissue, and its possible role in temporal regulation of immune tissue physiology.

## 2.2 Background and significance

The rotation of earth on its axis and revolution around the sun exposes its living organisms to the light/dark cycle. Synchronization of the internal environment to the external environment's 24-hour periodicity has resulted in evolution of the molecular circadian clocks of living organisms on planet Earth. As a result, living organisms exhibit circadian rhythms in their physiology and behavior. The word "circadian" stands for circa diem in Latin, which means "about a day." Living organisms have complex neural and molecular mechanisms which enable them to anticipate and adapt to the dynamic daily environmental changes. Living organisms have endogenous timing system known as the circadian clock. The circadian clocks are capable of regulating behavioral and physiological functions on a daily basis by complex signaling mechanisms.

These circadian rhythms exhibit a period of approximately 24 hours which persist even in the absence of external timing cues. Higher organisms have specialized cells in certain areas of brain, eye or optic lobes which are capable of generating circadian rhythms. These cells make up the master pacemaker/central clock. The master pacemaker functions as a regulator of several physiological, metabolic and behavioral processes (Ralph et al., 1990). Studies indicate that peripheral organs and cells such as heart, liver, kidney, skin and some cultured cells carry their own circadian clock machinery (Yamazaki et al, 2000; Keller et al, 2009). The local clock machinery is referred to as the peripheral oscillator/clock. The phases of peripheral clocks might be synchronized by the master pacemaker, perhaps by neuroendocrinal signals. Although peripheral clocks may be under

the regulation of central/master pacemaker, reports indicate that these peripheral clocks may be involved in the regulation of local physiological processes (Schibler, 2006; Lamia et al., 2008 & Yamazaki et al., 2000). In addition to light, feeding and ambient temperature can also act as powerful zeitgebers (German for time givers) for the peripheral clocks (Damiola et al. 2000; Brown et al. 2002).

The central and peripheral clocks share a fundamental mechanism of rhythm generation consisting of interlocking transcriptional/translational feedback loops involving clock genes. Molecular studies have identified several core clock genes (Glossop and Hardin, 2002; Reppert and Weaver, 2002 & Hastings et al., 2007). These core clock genes have been grouped into "positive" and "negative" elements. In mammals, the positive elements Clock and Bmal1 undergo transcription and translation. The Clock and Bmal1 protein dimerize to form Clock/Bmal1 dimer binds to the E-box motifs of negative element genes such as *per1, per2*, *cry1* and *cry2*.The negative elements proteins form oligomers and get translocated to the nucleus where they inhibit the binding of Clock/Bmal1transcription factors to the E-box, thereby inhibiting their own transcription. Several avian orthologs of these mammalian clock genes have been identified (Abraham et al., 2002; Bailey et al., 2002, Yasuo et al., 2003).

In mammals, there are several auxillary loops that act on the primary feedback loops. One such interlocking loop involves the nuclear receptors (NRs), REV-ERB and ROR (Preitner et al., 2002; Emery & Reppert 2004). Nuclear receptors render the clock responsive to several circulating hormones (e.g. cortisol, estrogen, and melatonin), nutrient signals (e.g fatty acid derivatives, retinoids) and redox status of the cell (NAD: NAD+

ratio). These feedback loops and auxillary cycles provide an approximate period of 24-hours, and drive the rhythmic expression of several clock-controlled and clock-modulated genes, which in turn mediate circadian rhythms in behavior and physiology in the organism.

As mentioned earlier, the presence and rhythmic expression of clock genes in peripheral tissues implies that the molecular clocks reside in the central nervous system as well as in peripheral tissues. These local clocks may be involved in the regulation of biological processes in the local tissues (Earnest and Cassone, 2005; Hastings et al., 2008). One such physiological function that may be under the regulation of circadian clock is the Immune system. Studies in mammals (mice, hamsters, guniea-pigs etc) indicate that disruption of circadian clock gene expression in peripheral tissue may induce cancer (Fu and Lee 2003), obesity (Shimba et al. 2005) and cardiovascular disorders (Young 2006), all of which have links to immune function.

Halberg et al. demonstrated about 50 years ago that mice have diurnal variation in susceptibility to endotoxic shock (Halberg et al., 1960). In addition to susceptibility to infection (Cutolo et al., 2005), course of a disease (e.g rheumatoid arthritis, asthma), clinical diagnostic parameters and drug-therapy too show time-of-day dependence (Sutherland et al., 2003), indicating the importance of the circadian system in regulating immunological responses (Smolensky et al., 1999). Several functions and parameters in the immune system exhibit time-of-day dependent variations, e.g., lymphocytic proliferation (Esquifino et al., 1996), natural killer (NK) cell activity, (Arjona and Sarkar, 2005), humoral immune response (Fernandes et al., 1976), cytokine levels (Young et al.,

1995) and serum cortisol levels (Krieger 1975).

Studies show that the circadian system and immune system are capable of crosstalk with each other. Immune markers such as IL-2, IL-10, GM-CSF, CCR2, IL-6, IL-1β, TNF-α, MCP-1/JE, IFN-γ and IFNRs are under circadian regulation (Young et al., 1995; Lundkvist et al., 1998; Talkane et al., 2002; Hayashi et al., 2007). The *per2* mutant mice lack rhythm in IFN-γ expression (Arjona & Sarkar, 2006) and are unable to produce IL-10 and IFN-γ upon endotoxic shock induced by LPS (Liu et al., 2006). The *bmal1*-KO show very early aging, chronic inflammation, corneal inflammation and reduced number of circulating lymphocytes (Kondratov et al., 2006). Mice deficient in *cry1* and *cry2* genes exhibit exacerbated cytokine production and joint swelling upon arthritic induction (Hashiramoto et al., 2010).

Several functions and parameters in the immune system have been determined to be dependent on time-of-the day for instance lymphocytic proliferation (Esquifino et al., 1996) , as well as proliferation of natural killer (NK) cell activity (Arjona & Sarkar, 2005), humoral immune response (Fernandes et a., 1976), rhythms in absolute and relative numbers of circulating white blood cells and their subsets (Kawate et al,, 1981), cytokine levels (Young et al., 1995), and serum cortisol (Krieger, 1975). Additionally, the variation based on time of the day in susceptibility to infection (Shackelford & Feigin, 1973), course of diseases in rheumatoid arthritis (Cutolo et al., 2005) or asthma (Sutherland et al., 2003), clinical diagnostics parameters as well patterns in response to therapy based on time of the day etc. uncover the integral role the circadian system plays in immunological responses (Hause et al., 1999). Kornmann et al., suggest that both cell-autonomous and systemic

pathways may participate in relaying timing information (Kornmann et al., 2007) it is largely unknown how the circadian system and the immune system communicate. It is speculated that clock-controlled factors such as cortisol and melatonin or innervations by the autonomic nervous system may regulate gene expression and protein activity (Ralph et al., 1990), but the possibility that the local clocks in immune cells may directly control cellular immune functions cannot be ruled out either. Hence, the aim of this part of the study was to gain a deeper understanding of the mechanisms regulating circadian immunological rhythms on a systemic level. To this end, we investigated the spleen tissue to test (i) for the presence of an autonomous circadian clock, (ii) whether such a clock regulates circadian immune functions. The aim of this study was to decipher the role of circadian clock in the dynamics of pathophysiology of immune response.

## 2.3 Materials and methods

Melatonin and lipopolysaccharides (LPS from Escherichia coli 0111:B4) were obtained from Sigma-Aldrich.

### *2.3.1 Animals*

Day-old male chicks, *Gallus gallus*, Hy-line Brown, were obtained from Hyline International (Bryan, TX). For daily and circadian studies over 7different timepoints, the chicks were housed photoperiod of LD 12:12hr for 3 weeks. Food and water was made available *ad libitum*. A total of n = 63 for daily study and total of n = 63 chicks were used for the circadian experiment. Seven timepoints tested in 12:12 hr LD photoperiod were

ZT0, ZT3, ZT6, ZT12, ZT15, ZT18, and ZT21 (ZT: zeitgeber time, lights on at ZT0; lights off at ZT12). For the circadian study, chicks were held in constant darkness (dark-dark phase, DD) for 3 days prior to sacrificing and collecting tissue samples under dim red light. The circadian timepoints used for sampling were CT0, CT3, CT6, CT12, CT15, CT18, and CT21 (CT: circadian time). All the birds were sacrificed by CO2 asphyxiation and tissues collected at each timepoint were immediately placed on solid CO2 and stored at -80°C until use. Three pools of tissue were prepared at each time point, each of which was composed of three spleens (n = 9 per time point). Animal use and care protocols were in accordance with NIH guidelines.

### *2.3.2 Quantitative real-time polymerase chain reaction*

From each tissue pool Total RNA (4 µg/sample) was extracted using TRIzol protocol (Invitrogen), according to manufactures instructions. To remove contaminating genomic DNA, the total RNA was subjected to DNase treatment using TURBO DNA-free (Ambion). Ribonucleic acid quantification was assessed using an Eppendorf Biophotometer (Eppendorf). Using 1 µg of DNase-treated total RNA as starting material, synthesis of cDNA was performed following the High Capacity cDNA Reverse Transcription Kit protocol (Applied Biosystems). The qRTPCR determinations were made using a LightCycler 480 (Roche). Each reaction of 20µl volume contained 0.5 µM primers, SYBR Green mastermix (Roche), and cDNA, according to the manufacturer's instructions. Each incubation step consisted of an initial denaturation step at 95°C for 10 minutes, followed by 40 cycles of a 95°C denaturation for 15 s, 30 s annealing at 63°C,

then extension at 72°C for 30 s. The list of primers used for the daily and circadian expression studies using qRTPCR are described in Table 1. All the primer pairs generated a single product of the predicted size as indicated by agarose gel electrophoresis. Their specificity was demonstrated by melting curve analysis (Tm) during every qRTPCR run. Typically ~25 cycles were necessary to detect amplification of the product. All qRTPCR assays were linear ($r^2 > 0.99$) from $10^1$ to $10^7$ copies. Internal standards were used to determine transcript numbers. They were prepared by cloning target PCR products into pGEMT Easy vectors (Promega).

Clones were verified by performing direct sequence analysis. The plasmid DNA was digested, followed by agarose gel electrophoresis (2.0%, w/v) for visual verification of correct product sizes and staining with ethidium bromide (EtBr, 0.5 µg/ml). To generate standard curves, a set of 100-fold serial dilutions of each internal standard ($10^1$-$10^7$ copies/2 µl) was prepared. The transcript numbers were determined by using a 2 µl sample of a 10-fold dilution of cDNA prepared as mentioned above. The values were then normalized to the number of Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) copies (Table 1 and Table 2).

Table 1. List of primers used for qRT-PCR for core clock gene expression study

| Target | Identity | Sequence | Accession # | Size |
|---|---|---|---|---|
| Bmal1 | Fwd | ggaattccaggaggaacaaga | AF205219 | 60 |
| | Rv | ttcttcagcaatcatccttcc | | |
| Bmal2* | Fwd | gaagtccggtataaaccttcgtt | AF246958 | 65 |
| | Rv | gcagccctaaggattaactgtct | | |
| Clock | Fwd | acacgcatgatagaggcaaa | AF246959 | 62 |
| | Rv | tgttcttgaattttccgcaact | | |
| Cry1* | Fwd | cggacctgtacaaaaaggtaaaa | NM_204245 | 61 |
| | Rv | agctggccatagagggagag | | |
| Cry2 | Fwd | tctggcgggagtttttctac | NM_204244 | 60 |
| | Rv | cctccatgcgatcaaacttc | | |
| Per2 | Fwd | cgaggtcaggggggttctact | NM_204262 | 61 |
| | Rv | gatatcagcttgctgctcagg | | |
| Per3* | Fwd | tttaggctctcactcctgtgaa | AY046567 | 60 |
| | Rv | ttgctgtttttcccactgtct | | |
| GAPDH* | Fwd | ggagtccactggtgtcttcac | NM_204305 | 64 |
| | Rv | cttagcaccacccttcagatg | | |

Fwd = forward primer; Rv = reverse primer; Size = expected amplicon size. Primer pairs that also spanned an intron are indicated by an *.

Table 2. List of primers for qRT-PCR for proinflammatory cytokine gene expression study

| Target | Identity | Sequence | Accession # | Size |
|---|---|---|---|---|
| IL-1β | Fwd | ggtggccatgaccaaact | NM_204524 | 61 |
| | Rv | caggtcgctgtcagcaaag | | |
| IL-2 | Fwd | gagtgcacccagcaaactct | NM_204153 | 66 |
| | Rv | ttcagtttctttcttcagagtaacca | | |
| IL-6* | Fwd | caggacgagatgtgcaagaa | NM_204628 | 64 |
| | Rv | tgttccggacgagcatct | | |
| IL-12b | Fwd | ccaccgaagtgaaggagttc | NM_213571 | 63 |
| | Rv | cgtgggtcttagcagacagg | | |
| IL-18* | Fwd | agagcatgggaaaatggttg | NM_204608 | 60 |
| | Rv | ccaggaatgtctttgggaac | | |
| TNFα | Fwd | acaaaatttgcaggctgtttc | AY765397 | 60 |
| | Rv | ctgaaataaacaggcacaaaagag | | |
| GAPDH* | Fwd | ggagtccactggtgtcttcac | NM_204305 | 64 |
| | Rv | cttagcaccacccttcagatg | | |

Fwd = forward primer; Rv = reverse primer; Size = expected amplicon size. Primer pairs that also spanned an intron are indicated by an *.

## 2.4 Results

### *2.4.1 Daily rhythms of clock genes in avian spleen*

The avian pineal gland expresses circadian clock and immune genes on a daily and circadian basis. This prompted us to investigate if peripheral immune tissue, spleen harbors and rhythmically expresses core clock genes on daily and circadian basis (Bailey et al., 2003; Bailey et al., 2004). From Figure 5 it is evident that the core clock genes in spleen exhibit 24 hr oscillations in mRNA abundance in a robust manner. Investigation of putative negative elements, the *cry* and *per* genes, reveals daily oscillations with 2-5 fold amplitudes with higher abundances occurring during the late night for *cry1* (pANOVA < .001; pcosinor = .009), *cry2* (pANOVA = .003; pcosinor < .001), *per2* (pANOVA < .001; pcosinor = .005), and *per3* (pANOVA < .001; pcosinor = .008). Analysis of putative positive elements, clock and the bmals, too reveals a daily pattern of rhythmicity in the spleen. The *clock* mRNA attained maximal abundance during the early night (pANOVA < .001; pcosinor < .001) while *bmal1* is highest during the late night to early day period (pANOVA = .007; pcosinor = .01). The *bmal2* exhibited peak expression at night time (pANOVA = .01; pcosinor = .03), however it was not as robust as the other core clock genes nor in excess of a 2-fold rhythm. Birds do not seem to exhibit *period1* (*per1*) gene; hence it was not examined in our study (Table 1).

### *2.4.2 Circadian rhythms in clock genes in avian spleen*

The core clock genes exhibit display robust oscillations under free running

conditions (DD). The *cry1* (pANOVA < .001; pcosinor = .001), *cry2* (pANOVA = .006; pcosinor = .005), *per2* (pANOVA < .001; pcosinor < .003), and *per3* (pANOVA =.03; pcosinor = .006), *bmal1* (pANOVA < .001; pcosinor = .003), *bmal2* (pANOVA < .001; pcosinor = .003), and *clock* (pANOVA < .001; pcosinor = .002) exhibited maximum amplitude at late subjective night and early day (Figure 5). We demonstrate for the first time that the avian spleen expresses circadian clock genes on a daily and circadian manner. To our knowledge this is the first demonstration of daily and circadian clock gene regulation in an avian immune tissue (Figure 5 and Figure 6).

**2.5 Discussion**

In recent years, the field of chronobiology and chronotherapy has garnered a lot of scientific and medical attention. This attention stems from the studies that indicate that circadian clocks are tightly coupled with several aspects of immune function, which includes regulation of cytokine production, proliferation and trafficking of leukocytes and apoptosis (Gudewill et al., 1992; Jones et al., 1992; Lemmer et al., 1992; Sother et al., 1995; Oishi et al., 2006; Smolensky et al., 1999; Smolensky & Portaluppi1999). However, molecular pathways and structures, or timing signals bridging the communication between circadian clocks and immune system are not yet known in their entirety. Studies are on to complete the puzzle of relationship between circadian clock dysfunction and the resulting aberrations in physiological homeostasis.In the current study we provide important insight to circadian control of inflammatory mechanism in the avian model. The knowledge should help in opening avenues towards deciphering circadian control of immune tissues

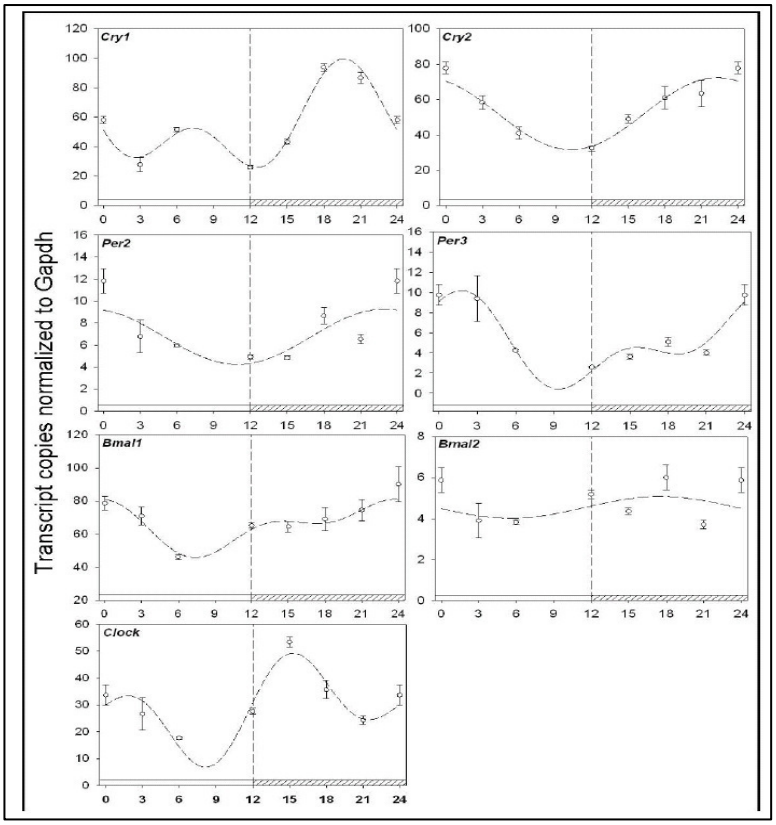


Figure 5. Quantitative RT-PCR analysis of core clock genes in spleen under 12:12LD. Plotted open circles represent the mean ± SEM in each study group. Dashed line represents fitted plot of cosinor analysis utilizing linear harmonic regression. Values are represented as the number of transcript copies/1000 Gapdh transcripts. Abscissa labels indicate ZT timepoints for 3 hrs under LD 12:12hr photoperiod; ZT0 = lights on; ZT12 = lights off. Open bar on the bottom of the x-axis indicates light period, while crosshatched indicates darkness. The *cry* and *per* genes harbor daily oscillations ~2-5 fold in amplitude with higher abundances occurring during the late night for *cry1* (pANOVA < .001; pcosinor = .009), *cry2* (pANOVA = .003; pcosinor < .001), *per2* (pANOVA < .001; pcosinor = .005), and *per3* (pANOVA < .001; pcosinor = .008). The *clock* and the *bmals*, also express a daily pattern of rhythmicity in the spleen. The *clock* mRNA exhibited peak during early night (pANOVA < .001; pcosinor < .001) while *bmal1* peaked during late night to early day period (pANOVA = .007; pcosinor = .01). The *bmal2* peaks at nighttime (pANOVA = .01; pcosinor = .03), however its not as robust as other clock genes nor in excess of a 2-fold rhythm.

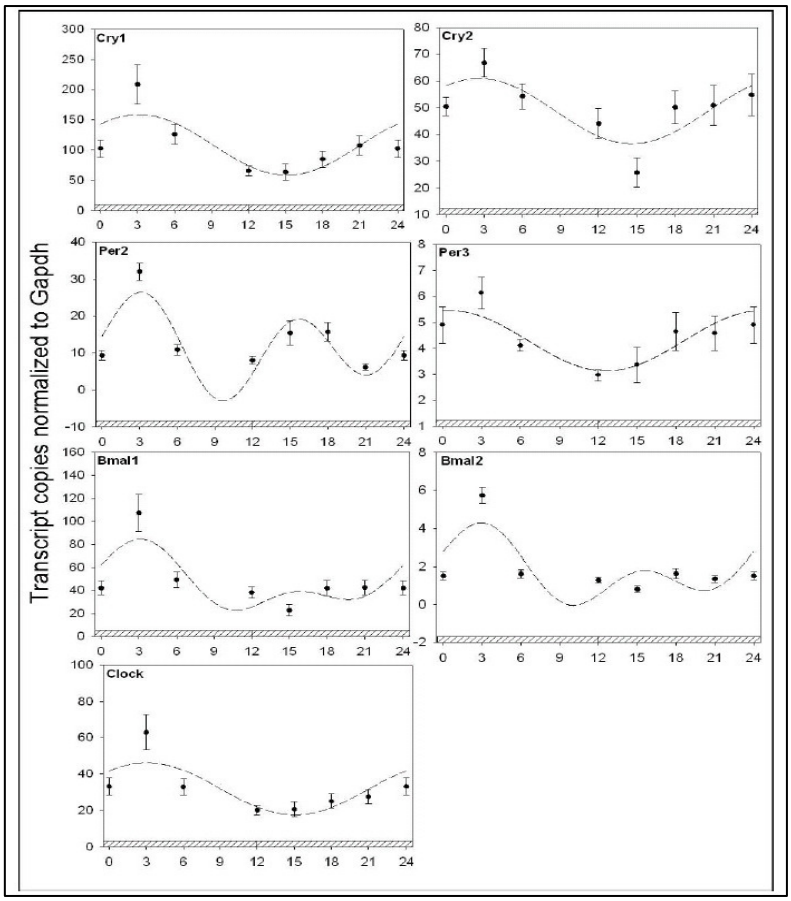


Figure 6. Quantitative RT-PCR analysis of core clock genes in spleen under DD. Plotted dark circles represent the mean ± SEM in each experimental group. The dashed line represents the fitted plot of cosinor analysis utilizing linear harmonic regression. Values are represented as the number of transcript copies/1000 Gapdh transcripts. Abscissa labels indicate CT timepoint for 3 hrs under DD conditions; crosshatched indicates darkness. The *clock* genes exhibit 24 hr rhythmicity with peak amplitudes in the late subjective night and early day for *cry1* (pANOVA < .001; pcosinor = .001), *cry2* (pANOVA = .006; pcosinor = .005), *per2* (pANOVA < .001; pcosinor < .003), and *per3* (pANOVA = .03; pcosinor = .006), *bmal1* (pANOVA < .001; pcosinor = .003), *bmal2* (pANOVA < .001; pcosinor = .003), and *clock* (pANOVA < .001; pcosinor = .002).

and their immunological rhythms.

### *2.5.1 Daily and circadian regulation of clock genes in the spleen*

The avian and pineal gland exhibit rhythmic oscillations of core clock genes on a daily and circadian basis (Bailey et al., 2003; Bailey et al., 2004; Bailey et al., 2008). This and several other studies have prompted the notion that molecular clocks reside in peripheral tissues and cells outside the master clock (Earnest & Cassone, 2005; Hastings et al., 2007; Reppert & Weaver, 2002). Molecular clocks in the peripheral tissues may be required for regulating local peripheral physiology (Lamia et al., 2008). In this study we demonstrated that the avian spleen harbor core clock genes. These core clock genes exhibit daily and circadian oscillations. We found interesting observations firstly; there is no strict anti-phase of positive and negative elements. This kind of pattern has been demonstrated in Drosophila and mammalian models as well. The avian spleen Bmals are expressed coincidentally with the Cry genes. The mRNA expression pattern of these core clock genes is similar, but not exactly identical, to those demonstrated in the avian pineal gland (Bailey et al., 2003). The *bmal* and *cry* genes in the avian pineal gland are expressed rhythmically peaking at the same time of the day. This data was confirmed by northern blots. Although we do not have protein data in the spleen study, however comparing mRNA temporal expression profiles of core clock genes in avian spleen to those of avian pineal gland, mammals and flies strongly suggest that other rhythmic mechanisms are in place which regulate molecular rhythms among these model systems.

Spleen is a highly dynamic tissue composed of several sub-population of cells (B

cells, T cells, macrophages, dendritic cells, natural killer cells). Each sub-population of cell serves different functions. Each cell type may possess its own cell-autonomous molecular clock. The overt spleen inflammatory function may be a result of independent timing signals originating from the sub-population of different cell types. Hence, additional studies are required to study the molecular clocks and their functions in different sub-population of the spleen tissue.

### *2.5.2 Daily and circadian regulation of proinflammatory cytokine in the spleen*

Proinflammatory cytokines IL-1b, IL-6, and TNF are key regulators of early inflammatory response during infection or inflammation (Schluger & Rom, 1997). Understanding the regulation of cytokines under different photoperiodic conditions in avian spleen and the role of melatonin in modulating their expression patterns under normal and inflammatory states is integral to deciphering the dynamics of the inflammatory response (Semaeva et al., 2010). In the current study we demonstrate that several proinflammatory cytokine genes exhibit daily and circadian oscillations in avian spleen (Figure 7 and 8; Table 3 and 4). The results suggest that circadian clock in avian spleen may regulate the inflammatory response on a temporal basis.

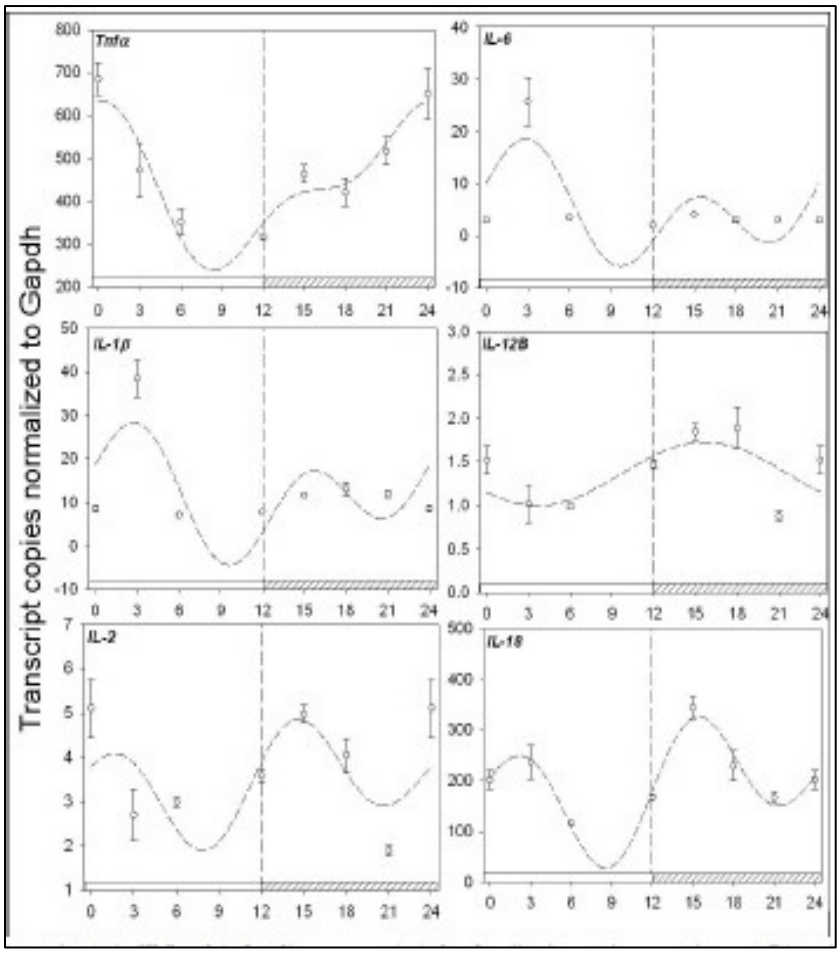


Figure 7. Quantitative RT-PCR analysis of cytokine genes in spleen under 12:12LD. Plotted open circles represent the mean ± SEM in each experimental group. The dashed line represents the fitted plot of cosinor analysis utilizing linear harmonic regression. Values are represented as the number of transcript copies/1000 GAPDH transcripts. Abscissa labels indicate ZT time values every 3 hrs under LD 12:12 conditions; ZT0 = lights on; ZT12 = lights off. Open bar at the bottom of x-axis indicates the light period, while crosshatched indicates darkness. TNFa mRNA show peak levels at dark-light transition (pANOVA < .001; pcosinor .003), IL-1b (pANOVA < .001; pcosinor < .001), and IL-6 (pANOVA < .001; pcosinor < .001) mRNA displayed peaks at ~ZT3. IL-18 (pANOVA < .001; pcosinor = .003) achieved peak levels before midnight (ZT15). IL-2 and IL-12b mRNAs did not exhibit a >2-fold rhythm in LD, although their mRNA levels fluctuated during 24hr period.

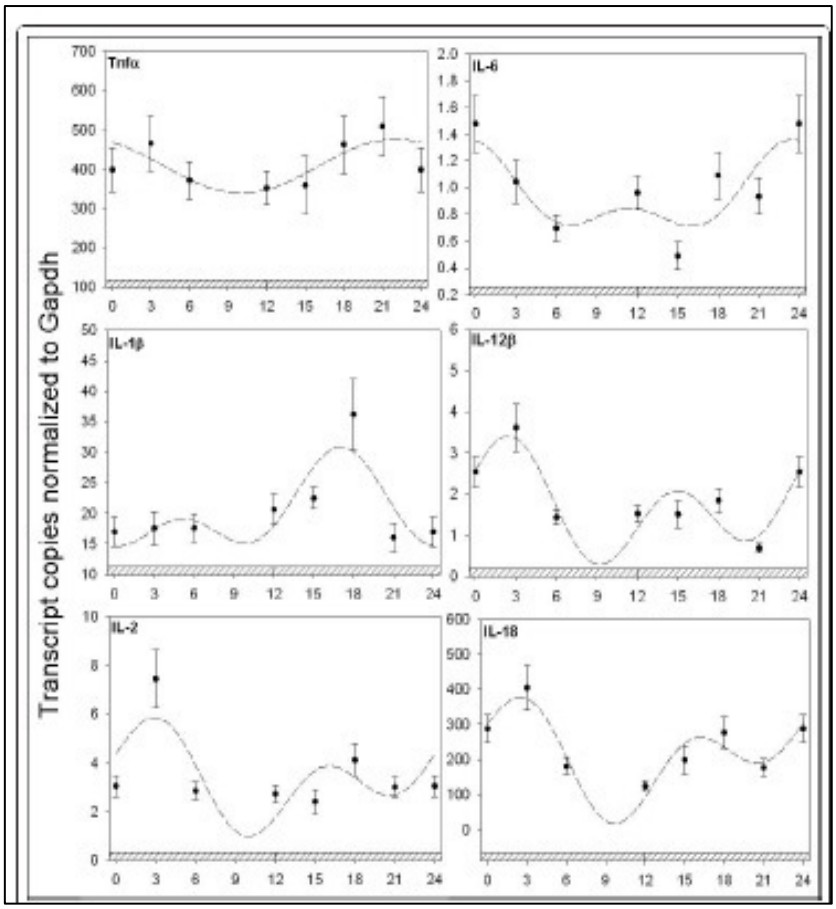


Figure 8. Quantitative RT-PCR analysis of cytokine genes in spleen under DD. Plotted dark circles represent the mean ± SEM in each study group. The dashed line represents the fitted plot of cosinor analysis utilizing linear harmonic regression. Values are represented as the number of transcript copies/1000 GAPDH transcripts. Abscissa labels indicate CT timepoints for every 3 hrs under DD conditions; crosshatched indicates darkness. TNFa became statistically arrhythmic at a >2-fold change. IL-1b (pANOVA = .004; pcosinor = .003) and IL-6 (pANOVA < .001; pcosinor = .003) mRNAs maintained robust circadian rhythms with peak levels at subjective midnight and subjective day respectively. IL-2 (pANOVA < .001; pcosinor = .003), IL-l2b (pANOVA < .001; pcosinor = .002) and IL-18 (pANOVA < .003; pcosinor = .006) exhibit their peak mRNAs at early subjective day.

**Table 3.** Quantitative RT-PCR values of core clock and cytokine genes in spleen under 12:12LD

| Gene | LD max. | LD min. | Δ |
|---|---|---|---|
| **Bmal1** | 75 | 44 | **1.7** |
| **Bmal2** | 7.6 | 3.3 | **2.3** |
| **Cry1** | 99 | 26 | **3.8** |
| **Cry2** | 88 | 27 | **2.9** |
| **Clock** | 41 | 18 | **2.3** |
| **Per2** | 14 | 3.1 | **4.6** |
| **Per3** | 10 | 2.3 | **4.3** |
| **IL-1β** | 40 | 3.6 | **11** |
| **IL-2** | 4.3 | 2.2 | **2** |
| **IL-6** | 28 | 2.1 | **14** |
| **IL-12b** | 2.2 | 0.9 | **2.4** |
| **IL-18** | 355 | 141 | **2.5** |
| **TNF** | 689 | 240 | **2.9** |

Table 4. Quantitative RT-PCR values of core clock and cytokine genes in spleen under DD

| Gene | DD max. | DD min. | Δ |
|---|---|---|---|
| **Bmal1** | 85 | 25 | **3.4** |
| **Bmal2** | 6.7 | 0.8 | **8.3** |
| **Cry1** | 203 | 6 | **3.2** |
| **Cry2** | 70 | 26 | **2.7** |
| **Clock** | 62 | 20 | **3.1** |
| **Per2** | 36 | 6.6 | **5.6** |
| **Per3** | 5.8 | 2.6 | **2.3** |
| **IL-1β** | 36 | 14 | **2.7** |
| **IL-2** | 5.1 | 1.9 | **2.7** |
| **IL-6** | 26 | 2.1 | **13** |
| **IL-12b** | 1.9 | 0.9 | **2.2** |
| **IL-18** | 404 | 116 | **3.4** |
| **TNF** | 651 | 315 | **2.1** |

# 3. EFFECT OF SYSTEMIC ADMINISTRATION OF LIPOPOLYSACCHARIDE ON TEMPORAL EXPRESSION PROFILE OF CLOCK GENES IN AVIAN SPLEEN*

## 3.1 Introduction

The presence of cellular clocks in tissues and cells of the immune system suggests that the peripheral clocks fulfill a local regulatory function from the studies on peripheral clocks of other tissues (Lamia et al., 2009). The proinflammatory cytokine genes (Figure 7 and Figure 8) showed daily and circadian rhythmic oscillations in spleen. Presence of such definitive temporal rhythms in cytokine gene expression implies that there might be systemic and/or autonomous circadian clock regulation.

Halberg and colleagues found that rate of mortality by LPS-induced endotoxic shock in mice was dependent on the time of the day (Halberg et al., 1960; Liu et al., 2006). We hypothesized that the immune tissue-specific mechanisms such as, response to induced acute systemic inflammation may be under temporal regulation in birds too. During inflammation immune tissues release several signaling molecules in the form of pro inflammatory cytokines. The LPS interacts via the LPS-binding protein (LBP)-LPS complex (TLRs) on the spleen tissue to induce downstream signaling pathways leading to transcription of several pro-inflammatory cytokines. Therefore, we

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investigated the effect of systemic administration of LPS on spleen to test whether the immune/inflammatory response to bacterial endotoxin is time-of-the day dependent. If there was a temporal regulation of inflammatory response in the spleen, what possible signaling molecule might be modulating this inflammatory response?

### 3.2 Background and significance

Administration of lipopolysaccharide (LPS or endotoxin) systemically or intravenously (i.v.) is a powerful method of challenging the immune system. This acute inflammation results in increase in the concentrations of different cytokines. The effects of LPS vary with the dose and mode of administration. LPS administration generally leads to fever, acute-phase responses and septic shock (Rivest et al., 2000). Marpegan et al in 2009 replicated the classic chronotoxicity by Halberg (Halberg et al., 1960). They compared and contrasted the mortality rates, types of pro-inflammatory cytokine induced, and effect on locomotor activity in two groups of mice injected with LPS at two different times of the day (zt11 and zt19). They compared these results to two more groups of mice housed in constant darkness (DD), to determine whether similar results persisted in the absence of light. As expected, the mice in LD expressed differential susceptibility to LPS-induced endotoxic shock depending on time of the day. There was no temporal difference in mortality rates in mice housed in DD. There was only transient effect on locomotor activity with no modification in circadian period and phase. In LD mice, LPS injection at ZT11 induced levels of IL-1b, IL-6 when compared to ZT19. Mice with high LPS-mortality at zt11 had high levels of IL-1b and IL-6 when compared to zt19. No such

information is available for the chicken, although it is reasonable to think that such processes occur in this species. To gain direct evidence in this regard we hypothesized that the spleen, an important peripheral immune tissue, possesses an endogenous circadian clock and that LPS administered at different phases of the clock would vary in inflammatory effects.

## 3.3 Materials and methods

Effect of clock phase on LPS toxicity was tested by comparing the effects of intravenous LPS administration at midday (zt6) to those at midnight (zt18).

### *3.3.1 Animals*

Day-old male chicks, Hy-line Brown (n = 144) were housed under 12:12 hr LD photoperiod for 5 weeks until their weight reached ~0.5 kg. Of the 144 birds, 72 birds were used to evaluate acute systemic inflammatory response at midday, defined as ZT6. For this, an intravenous (IV) injection of 1.5 mg/kg LPS in 100 ul of saline was administered to 36 birds (test group), while 100 ul/bird of saline vehicle control was administered to an additional 36 birds (control group). Spleen tissues were collected 5 minutes post-injection, and then for every 1 hour for next 3 hrs (n = 9 per sampling, n = 36 per condition) in test and control groups. A 3 hr time frame has proven to be an appropriate time course to analyze cytokine induction as a result of LPS stimulation (Miller, 1997).

The remaining 72 birds were similarly injected with LPS (n = 36) (1.5 mg/kg

in100 ul saline, test group) or saline (n = 36, control group) as midnight, defined as ZT18. Spleens were harvested 5 minutes following the injection and then every hour for 3 hrs (n = 9 per sampling, n = 36 per condition) in the test and control groups at the zt18 timepoint.

### *3.3.2 Quantitative real-time polymerase chain reaction*

From each tissue pool Total RNA (4 µg/sample) was extracted using TRIzol protocol (Invitrogen), according to manufactures instructions. To remove contaminating genomic DNA, the total RNA was subjected to DNase treatment using TURBO DNA-free (Ambion). Ribonucleic acid quantification was assessed using an Eppendorf Biophotometer (Eppendorf). Using 1 µg of DNase-treated total RNA as starting material, synthesis of cDNA was performed following the High Capacity cDNA Reverse Transcription Kit protocol (Applied Biosystems).

The qRTPCR determinations were made using a LightCycler 480 (Roche). Each reaction of 20µl volume contained 0.5 µM primers, SYBR Green mastermix (Roche), and cDNA, according to the manufacturer's instructions. Each incubation step consisted of an initial denaturation step at 95°C for 10 minutes, followed by 40 cycles of a 95°C denaturation for 15 s, 30 s annealing at 63°C, then extension at 72°C for 30 s. The list of primers used for the daily and circadian expression studies using qRTPCR are described in Table 1. All the primer pairs generated a single product of the predicted size as indicated by agarose gel electrophoresis. Their specificity was demonstrated by melting curve analysis (Tm) during every qRTPCR run. Typically ~25 cycles were necessary to detect amplification of the product. All qRTPCR assays were linear ($r^2 > 0.99$) from $10^1$ to $10^7$

copies. Internal standards were used to determine transcript numbers. They were prepared by cloning target PCR products into pGEMT Easy vectors (Promega). Clones were verified by performing direct sequence analysis.

The plasmid DNA was digested, followed by agarose gel electrophoresis (2.0%, w/v) for visual verification of correct product sizes and staining with ethidium bromide (EtBr, 0.5 µg/ml). To generate standard curves, a set of 100-fold serial dilutions of each internal standard ($10^1$-$10^7$ copies/2 µl) was prepared. The transcript numbers were determined by using a 2 µl sample of a 10-fold dilution of cDNA prepared as mentioned above. The values were then normalized to the number of Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) copies.

### *3.3.3 Statistical analysis*

Analysis of variance (ANOVA) (Sigmaplot) was used for analyzing times series data involving the 7 timepoints. The cosinor analysis was done using linear harmonic regression (CircWave software) (Oster et al., 2006). Student-Newman-Keuls method was used to estimate significant differences among means. Average changes in cytokine levels in the LPS and melatonin experiments were subjected to two-way ANOVA (Sigmaplot) and multiple comparisons vs. control group (Holm-Sidak method) for each time point. Statistical significance is based on $P < 0.05$.

## 3.4 Results

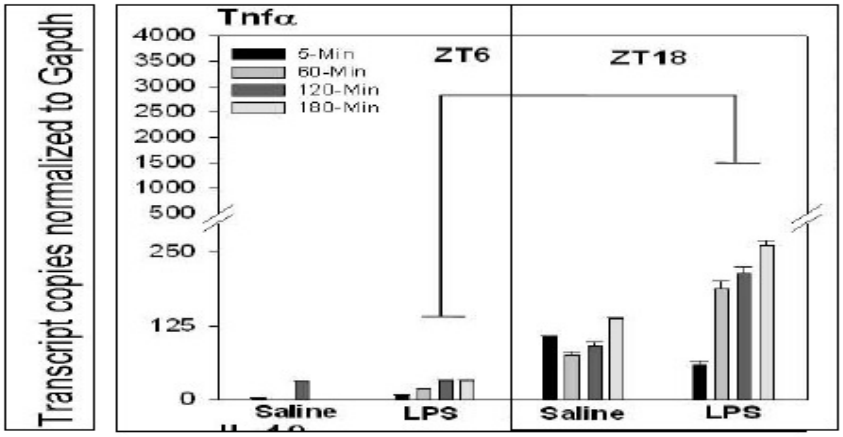
### *3.4.1 Daily regulation of the inflammatory response in avian spleen*

Our experiments demonstrate that several inflammatory cytokines oscillate in a daily and circadian manner in spleen (Figure 6 and 7). Therefore, we hypothesized that the circadian clock may regulate the inflammatory response in the avian spleen. Time-of-day-dependency of inflammatory response in spleen was studied performed by challenging the birds with LPS at midday and midnight followed by assaying proinflammatory cytokine mRNA expression profiles. The results indicated that one set of cytokines exhibits greater overall induction during the night vs. the day, implying daily regulation of inflammatory response (Figure 9 a-f). For instance, TNFα exhibits a ~8-fold greater overall induction at midnight (ZT18) versus midday (ZT6) following immune challenge (p, ANOVA < 0.001) (Figure 9 a). However, an LPS injection at ZT6 (pANOVA = 0.5) lead to a mere 2.5-fold average induction of TNFα levels in the three hrs when compared to ZT18 (p, ANOVA < 00.001) vs. saline control. A closer examination reveals that the magnitude of TNFα induction at midnight is a reflection of daily TNFαregulation, as the TNFά levels of saline group at ZT18 is higher than those at ZT6 saline group (Figure 9 a). The IL-18 cytokine induction exhibits patterns similar to those of TNFά cytokine. Around 7-fold greater overall induction at midnight (ZT18) vs. midday (ZT6) following immune challenge (p, ANOVA < 00.001) (Figure 9 b, Table 5) was seen. However, only a 3-fold average induction of IL-18 levels was observed when LPS was administered both ZT6 (pANOVA < 0.001) and ZT18 (pANOVA < 0.001) vs. saline control (Figure 9b, Table 5). IL-1b

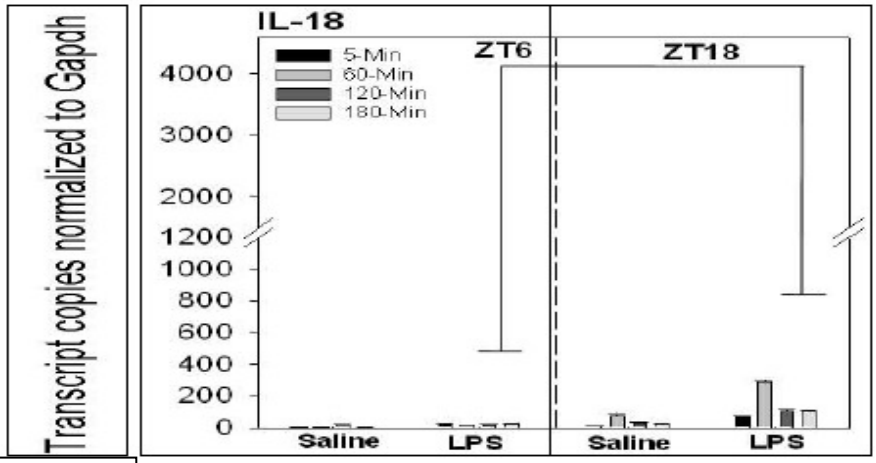
(Figure 9 c) and IL-6 (Figure 9 d) harbor exhibited a very different daily inflammatory response profiles. When compared with TNFα and IL-18, the IL-1b and IL-6 did not express variation in induction at midnight vs. midday following LPS stimulation. However, the magnitude of IL-1b and IL-6 induction after LPS injection is higher at midnight than midday.

Quantitatively, a 15-fold (p, ANOVA < 0.001) vs. 6-fold (p, ANOVA < 0.001) for IL-1b, and 331-fold (p, ANOVA < 0.001) vs. 14-fold (p, ANOVA < 0.001) for IL-6 were observed (table 5). The mechanism to explain this phenomenon may be circadian clock regulation of inflammatory response. Cytokines IL-1b and IL-6 exhibit reduced levels at night when compared to daytime (Figure 9 c and d). The cytokines IL-2 (Figure 9 e) and IL-12b mRNAs (Figure 9 f) did not have statistically different inductions following LPS injection at either ZT6 or ZT18. It implies that these cytokines are produced downstream of the acute inflammation pathway (Klein et al., 1997).

Summarily, cytokines IL-1b and IL-6 cytokines released in response to an inflammatory response are in an anti-phase relation to the cytokines IL-18 and TNFα. This temporal difference in cytokine inductions in avian spleen may be due to circadian clock control of inflammatory response.

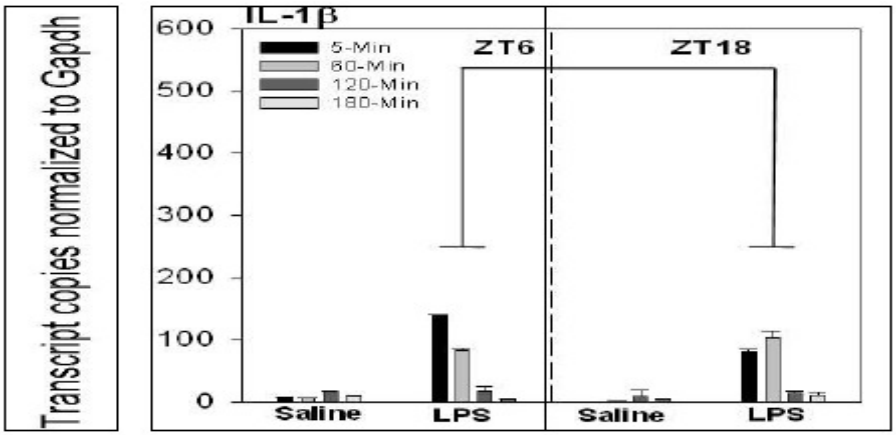



**9a**




**9b**

Figure 9. (a-f) Effects of acute LPS administration upon cytokine induction in the spleen at midday vs. midnight. Plotted values represent the mean ± SEM in each experimental group. Values are represented as the number of transcript copies/1000 GAPDH transcripts following the respective treatments, lipopolysaccharides (LSP), or saline (Sal), ZT6 = midday; ZT18 = midnight.
*Statistical significance is based on $P < 0.05$.

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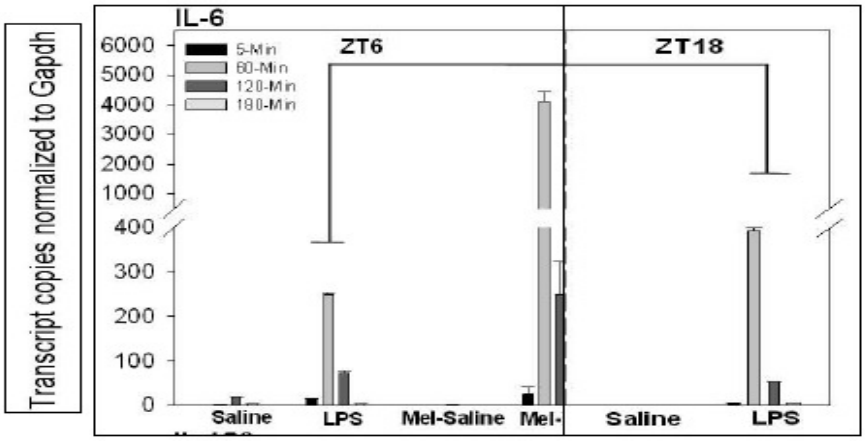
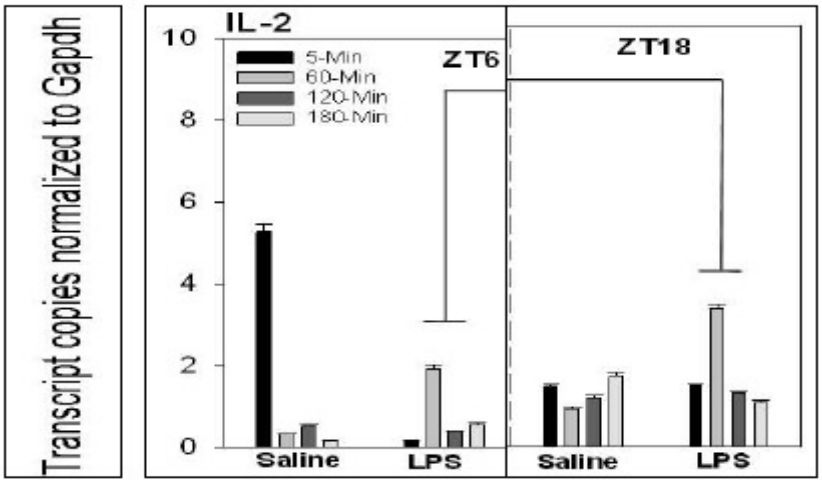
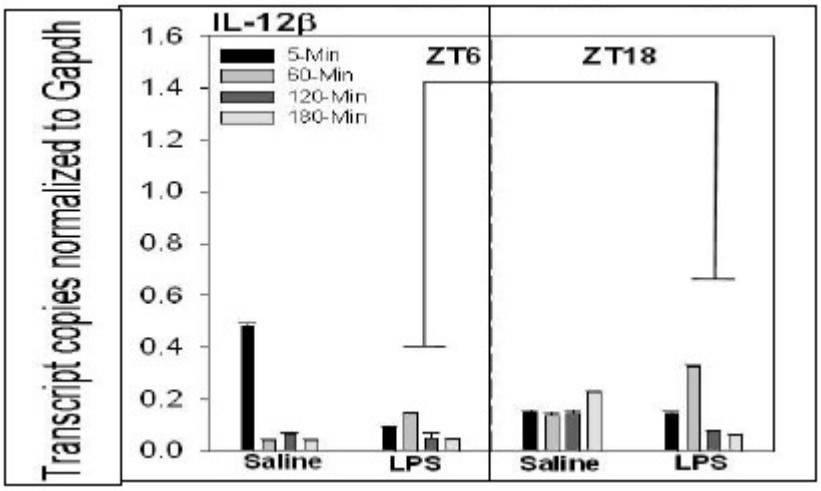



**9d**

Figure 9 Continued.




**9e**




**9f**

Figure 9 Continued.

Table 5. Quantitative RT-PCR values of cytokine genes in spleen under LPS induced inflammation at midday vs. midnight

| **Il-1β** | Avg. | Avg. | Δ |
|---|---|---|---|
| ZT6 LPS vs. Saline | 60 | 10 | 5.6 |
| ZT18 LPS vs. Saline | 52 | 3.5 | 15 |
| ZT18 Saline vs. ZT6 Saline | 3.5 | 10 | 0.3 |
| ZT18 LPS vs. ZT6 LPS | 52 | 60 | 0.9 |
| **IL-6** | Avg. | Avg. | Δ |
| ZT6 LPS vs. Saline | 85 | 6.2 | 14 |
| ZT18 LPS vs. Saline | 115 | 0.4 | 331 |
| ZT18 Saline vs. ZT6 Saline | 0.4 | 6.2 | 0.1 |
| ZT18 LPS vs. ZT6 LPS | 115 | 84 | 1.4 |

| **TNFα** | Avg. | Avg. | Δ |
|---|---|---|---|
| ZT6 LPS vs. Saline | 23 | 9 | 2.6 |
| ZT18 LPS vs. Saline | 180 | 102 | 1.8 |
| ZT18 Saline vs. ZT6 Saline | 102 | 9 | 11 |
| ZT18 LPS vs. ZT6 LPS | 180 | 23 | 7.9 |
| **IL-18** | Avg. | Avg. | Δ |
| ZT6 LPS vs. Saline | 162 | 26 | 6.1 |
| ZT18 LPS vs. Saline | 379 | 83 | 4.6 |
| ZT18 Saline vs. ZT6 Saline | 83 | 26 | 3.2 |
| ZT18 LPS vs. ZT6 LPS | 379 | 161 | 2.3 |

Table 5. Continued.

| IL-12b | Avg. | Avg. | Δ |
|---|---|---|---|
| ZT6 LPS vs. Saline | 0.2 | 0.2 | 1 |
| ZT18 LPS vs. Saline | 0.2 | 0.2 | 1 |
| ZT18 Saline vs. ZT6 Saline | 0.2 | 0.2 | 1 |
| ZT18 LPS vs. ZT6 LPS | 0.2 | 0.1 | 2 |

| IL-2 | Avg. | Avg. | Δ |
|---|---|---|---|
| ZT6 LPS vs. Saline | 0.8 | 1.6 | 0.5 |
| ZT18 LPS vs. Saline | 1.7 | 1.3 | 1.4 |
| ZT18 Saline vs. ZT6 Saline | 1.3 | 1.6 | 0.8 |
| ZT18 LPS vs. ZT6 LPS | 1.8 | 0.8 | 2.3 |

### 3.5 Discussion

In this study, inflammatory responses by LPS-induced acute systemic inflammation were compared and contrasted. A set of 6 different proinflammatory cytokines were investigated for their mRNA expression profiles when immune challenged at two different times of the day (midday vs. midnight). Of the 6 proinflammatory cytokines investigated, TNFα and IL-18 exhibited significant greater overall induction at midnight (ZT18) versus midday (ZT6) following immune challenge due to elevated levels for each during the night (Figure 9 a and b).

Molecular studies to link the direct interaction between circadian clock immune system have helped to reveal a few key players. These key players, in turn, help us understand the phenomenon of temporal gating of the immune system on a daily (LD) and circadian (DD) basis. For instance, REV-ERBα mediates clock control of multiple cellular metabolic pathways, including hepatic lipid metabolism and regulation of sterol regulatory element-binding protein in mice (Martelot et al., 2009). It seems that the circadian homeostasis, metabolism, and immune responses share common pathways in human beings (Bechtold et al., 2010). Findings by Gibbs et al. reveal that REV-ERBα is capable of regulating innate immunity in the lungs (Gibbs et al., 2009). Suppressing the *bmal1* gene in macrophages in mice removed the temporal gating of endotoxic-induced cytokine response in both cell-culture and in vitro studies in mice. Similar results were seen, when Rev-Errbα expression was suppressed in vivo and in vitro. Circadian gating of endotoxin response was lost in rev-erbα$^{-/-}$ mice and in cultured macrophages from these animals, despite maintenance of circadian rhythms within these cells. They also

used human macrophages to study the effects of knocking down rev-erbα. The results show that it was sufficient to modulate the production and release of proinflammatory cytokine, IL-6. Hence, macrophage clockwork provides temporal gating of systemic responses to endotoxin, and identifies REV-ERBα as the key link between the clock and immune function. Thus, REV-ERBα may represent a unique therapeutic target in inflammatory disease (Martelot et al., 2009). Thus these types of studies will help in understanding chronotoxicity and chronotherapeautic measures.

We speculate that the daily control of cytokine gene expression may be under master clock and/or peripheral clock regulation. The systemic timing mechanism to explain nocturnal abundance of TNFά and IL-18 cytokines may be attributed to several signaling molecules released by circadian clock, such as glucocorticoids, norepinephrine and melatonin to name a few. For our next study we selected the hormone, melatonin to study its immunomodulatory effect on inflammatory response in the avian spleen. We hypothesized that that melatonin is immunomodulatory molecule and, day time administration of melatonin prior to LPS-induced systemic inflammation results in mRNA expression profiles of proinflammatory cytokines very different to those seen in Figures 5a-f. Upon priming the birds with melatonin 1-hour prior to LPS injection during midday, the mRNA expression profiles of the proinflammatory cytokines should ideally look like that of expression profiles of test group birds zt18-LPS injection (Figure 9 a-f). The test birds at zt18 have circulating nocturnal melatonin hence, mimicking the zt6 birds injected with exogenous melatonin.

# 4. ROLE OF MELATONIN ON TEMPORAL REGULATION OF INFLAMMATION IN THE AVIAN SPLEEN*

## 4.1 Introduction

In dissertation sections 2 and 3, we demonstrated that the avian spleen exhibits rhythmic oscillations of several proinflammatory cytokine genes on a daily and circadian basis (Figures 6, 7). We suggest that the circadian clock regulate an inflammatory response by regulating cytokine genes on a daily basis. Our results show that the cytokines TNFά and IL-18 mRNA levels peak at night in normal, healthy birds. This explains the phenomenon a very high induction of TNFα and IL-18 at midnight (ZT18 LPS challenge) when compared to midday (ZT6 LPS challenge) (Figure 5). It is still unclear if the regulation of inflammatory response in under master clock or peripheral clock (in the spleen).

Melatonin is one of the most important signaling molecules released by the master clock which is rhythmically secreted at night by the pineal gland. Melatonin impinges its temporal effects via melatonin receptors in target cells. Hence, the nocturnal abundance of TNFa and IL-18 cytokines may be due to the hormone melatonin binding to melatonin receptors in the splenocytes. Nocturnal peak of melatonin is observed in all vertebrate species, regardless of their diurnal or nocturnal behavior.

The magnitude and duration of nocturnal increase in melatonin secretion is

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dependent on the length of the dark-phase.

Hence, melatonin acts as an entrainment cue for multiple biological functions (Cassone, 1998). Melatonin's capability in regulating antioxidant defense and immune system has been documented in a few studies (Reiter & Maestroni, 1999). The immunomodulatory role of melatonin has been examined by several scientists. The results however have been conflicting with no particular consensus regarding immune-modulation function of melatonin. Studies in rats indicate pro-inflammatory role of melatonin, while pinealectomy produces an opposite effect, resulting in a reduction of immune parameters (Maestroni et al., 1986; McNulty et al., 1990). Opposite results were observed in mice studies. Inhibition of melatonin synthesis using propanolol (adrenergic beta1-receptors) and of p chlorophenylalanine administration (a tryptophan inhibitor) in mice results in significant reduction of the primary antibody response to sheep red blood cells (Maestroni et al., 1986). Melatonin studies in some bird species failed to exhibit an alteration in immune modulation activity (Skwarlo-Sonta, 1999). The role of melatonin in immune-regulation cannot be ruled in avian spleen. Hence, we hypothesized that melatonin may play a role in regulating the levels of pro-inflammatory cytokine genes on a daily basis via melatonin receptors on splenocytes.

**4.2 Background and significance**

Molecular mechanisms governing communication between the circadian system and the immune system are still largely under investigation (Kornmann et al., 2007). These cross talk "conversations" may involve important signaling molecules such as

glucocorticoids, melatonin, and cytokines. The inflammatory responses may be gated at a local level by the peripheral clock within a tissue or a cell. For example, mice peritoneal macrophages exhibit rhythmic expression of clock genes, autonomous gene oscillations, and exhibit regulation of inflammatory response to lipopolysaccharide (LPS) (Hayashi et al., 2007; Keller et al., 2009; Gibbs et al., 2009). Keller et al., demonstrated that mouse macrophages cell cultures exhibit rhythmic expression cytokines as well as genes that are involved in LPS response pathways, suggesting a direct influence of the circadian clock on immune responses (Keller et al., 2009).

In the avian circadian system, like in the other higher vertebrates, overt circadian rhythms are regulated by a set of neural and neuroendocrinal structures. Work of Gaston and Menaker proved that the pineal gland is necessary for maintenance of self-sustained locomotor and body temperature rhythms in house sparrows, Passer domesticus (Gaston and Menaker, 1968). The avian pineal gland has circadian oscillators as well as photoreceptors which are necessary for the generation of circadian rhythms of melatonin secretion in vitro and entrainment of these rhythms to the external light/dark photoperiods (Takahashi et al., 1980).

In birds, the pineal gland secretes melatonin which peaks at night and is an important regulator of physiological events (Klein 1997; Klein 2007). One such event suggested being under circadian clock regulation and melatonin hormone signaling is the immune system (Carillo-Vico et al., 2005). Several studies have shown the immunomodulatory effect of melatonin in chronic inflammatory conditions and tumors. Lissoni demonstrated that simultaneous administration of melatonin with IL-2 amplified

IL-2's lymphocytosis and anti-tumor efficiency in several types of tumors (Lissoni 2000; Lissoni et al., 2003).Melatonin also seems to modulate biological activity and toxicity of the anti-tumor cytokine TNF-α (Lissoni, 2000). Patients with chronic inflammation such as rheumatoid arthritis and asthma express rhythmic symptoms (Cutolo & Maestroni, 2005; Sutherland, 2003). The increase in rhythmic symptoms may be due to disturbance in the clocks regulation of the immune response resulting in an increase of proinflammatory cytokine production. The increase in circulating cytokine levels may be due to increased melatonin levels resulting in inflammation. Hence, melatonin could be the functional link between the central/master circadian clock and immune tissue function. The molecular mechanisms by which melatonin regulates the immune system are largely unknown and are being actively investigated by various scientists.

The current research proposal thus aimed at examining mechanisms of regulation between the circadian clock and immune system in the domestic chicken, Gallus gallus. It should help in elucidating the role of clock and melatonin in regulation of immune system. The proposal explored the hypothesis that the circadian clock, melatonin and immune system are tightly coupled in function such that the clock is capable of modulating immune tissue physiology and the immune response on a temporal basis.

**4.3 Materials and methods**

Effect of melatonin on LPS-mediated acute inflammatory responses at midday (ZT6)

### *4.3.1 Animals*

Day old male Hy-line Brown birds (n = 72) were housed in a 12:12hr LD photoperiod for approximately 5 weeks until their weight reached ~0.5 kg. The birds were injected with an intramuscular injection (IM) of 100 µL saline (n = 36, control group) or 100 µL saline containing sufficient melatonin to provide 100 µg melatonin/kg bodyweight (n = 36, study group) at ZT5. The melatonin dose gives rise to plasma concentrations that mimic physiological nighttime concentrations in the chicken (Cassone, 1986). One hour post-melatonin (study group) or post-saline (control group) injection, an intravenous (IV) injection of LPS (1.5 mg/kg, 100 ul) was delivered to half the animals (study group) and saline vehicle to other half (control group). Spleen tissues were harvested 5 minutes following the second injection and then every hour for 3 hrs (n = 9 per sampling, n = 36 per condition) from the study and control groups. A 3 hr time frame is considered sufficient for LPS -induced cytokine production (Miller et al., 1997).

### *4.3.2 Quantitative real-time polymerase chain reaction*

From each spleen tissue pool Total RNA (4 µg/sample) was extracted using TRIzol protocol (Invitrogen), according to manufactures instructions. To remove contaminating genomic DNA, the total RNA was subjected to DNase treatment using TURBO DNA-free (Ambion). Ribonucleic acid quantification was assessed using an Eppendorf Biophotometer (Eppendorf). Using 1 µg of DNase-treated total RNA as starting material, synthesis of cDNA was performed following the High Capacity cDNA Reverse Transcription Kit protocol (Applied Biosystems). The qRTPCR determinations were made

using a LightCycler 480 (Roche). Each reaction of 20µl volume contained 0.5 µM primers, SYBR Green mastermix (Roche), and cDNA, according to the manufacturer's instructions. Each incubation step consisted of an initial denaturation step at 95°C for 10 minutes, followed by 40 cycles of a 95°C denaturation for 15 s, 30 s annealing at 63°C, then extension at 72°C for 30 s. The list of primers used for the daily and circadian expression studies using qRTPCR are described in Table 1 and Table 2. All the primer pairs generated a single product of the predicted size as indicated by agarose gel electrophoresis. Their specificity was demonstrated by melting curve analysis (Tm) during every qRTPCR run. Typically ~25 cycles were necessary to detect amplification of the product. All qRTPCR assays were linear ($r^2 > 0.99$) from $10^1$ to $10^7$ copies.

Internal standards were used to determine transcript numbers. They were prepared by cloning target PCR products into pGEMT Easy vectors (Promega). Clones were verified by performing direct sequence analysis. The plasmid DNA was digested, followed by agarose gel electrophoresis (2.0%, w/v) for visual verification of correct product sizes and staining with ethidium bromide (EtBr, 0.5 µg/ml). To generate standard curves, a set of 100-fold serial dilutions of each internal standard ($10^1$-$10^7$ copies/2 µl) was prepared. The transcript numbers were determined by using a 2 µl sample of a 10-fold dilution of cDNA prepared as mentioned above. The values were then normalized to the number of Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) copies.

### *4.3.3 Statistical analysis*

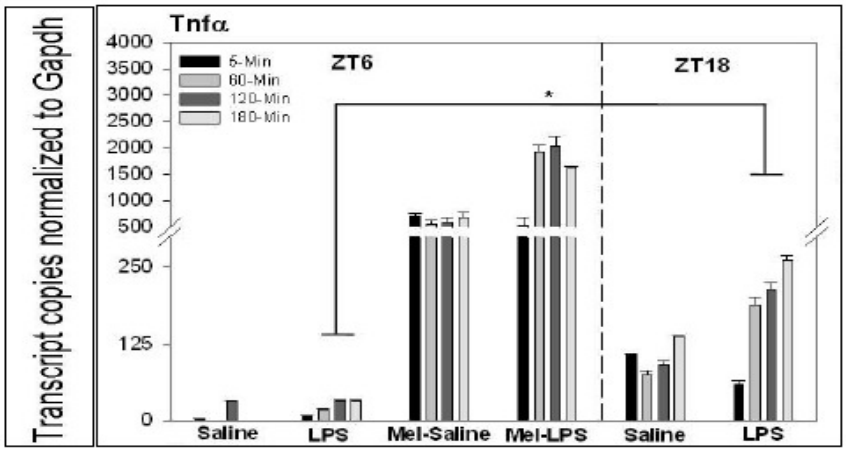
Analysis of variance (ANOVA) (Sigmaplot) was used for analyzing times series

data involving the 7 timepoints. The cosinor analysis was done using linear harmonic regression (CircWave software) (Oster et al., 2006). Student-Newman-Keuls method was used to estimate significant differences among means. Average changes in cytokine levels in the control and melatonin-LPS experiments were subjected to two-way ANOVA (Sigmaplot) and multiple comparisons vs. control group (Holm-Sidak method) for each time point. Statistical significance is based on $P < 0.05$.

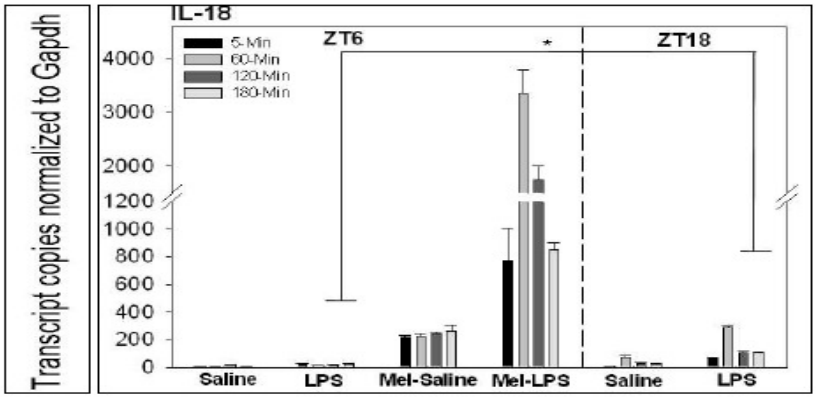
## 4.4 Results

### *4.4.1 Melatonin control of the inflammatory response*

The melatonin hormone is released at night time by the pineal gland and is a transducer of timing information the peripheral tissues and cells which have melatonin receptors. Thus, melatonin seems have to an impact on a wide range of physiological functions throughout the body (Klein et al., 1992; Pevet, 2003). Hence, melatonin represents as an attractive model for studying the role of central clock in synchronization the peripheral clocks and their functions. Hence, in this study we investigated the effects of exogenous melatonin administration upon the daily dynamics of inflammation in the avian spleen. Melatonin administered at midday mimicking nighttime physiological levels resulted in a 70-fold increase (p, ANOVA < 0.001) and 34-fold increase (p, ANOVA < 0.001) in case of TNFα and IL-18, respectively vs. control (Figure 10 a and 10 b; Table 6). Administration of melatonin prior to LPS injection leads to a 170-fold increase (p, ANOVA < 0.001) in TNFα mRNA levels. This enhanced induction effect appears to be a reflection of the

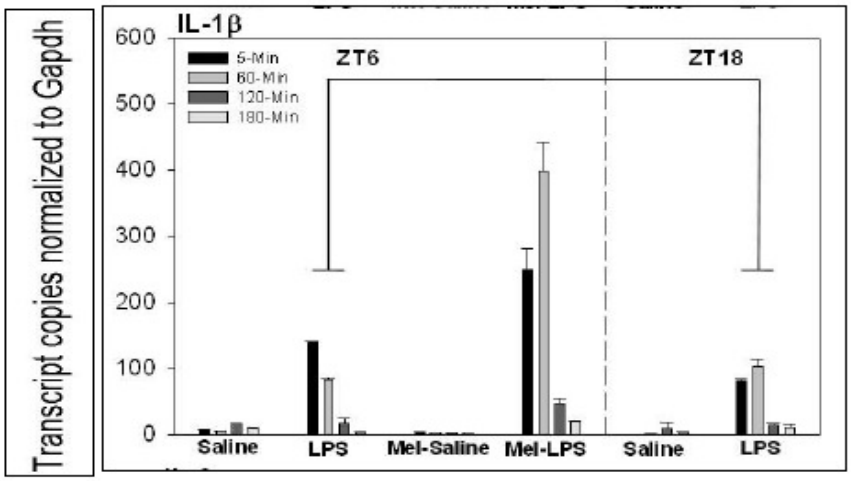



**10a**

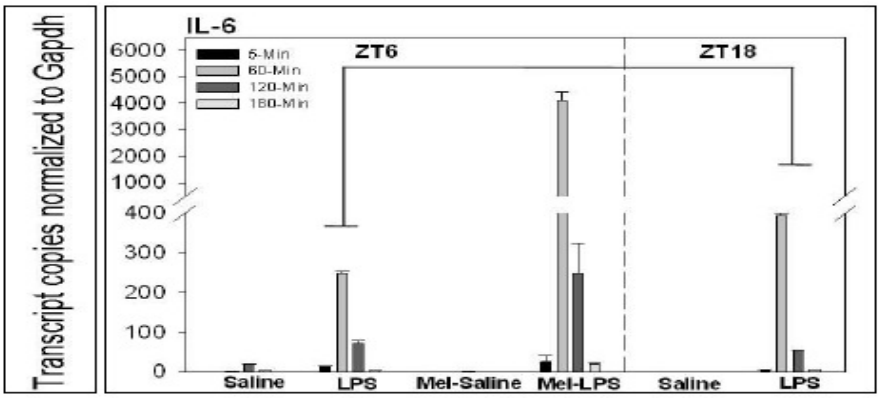



**10b**

Figure 10. (a-f) Effects of acute melatonin and LPS administration upon cytokine induction in the spleen at midday vs. midnight. Plotted values represent the mean ± SEM in each experimental group. Values are represented as the number of transcript copies/1000 GAPDH transcripts following the respective treatments, lipopolysaccharides (LSP), melatonin (Mel), or saline (Sal), ZT6 = midday; ZT18 = midnight. Melatonin was administered one hour prior to challenge with LPS or saline (ZT5). *Statistical significance is based on $P < 0.05$.




**10c**




**10d**

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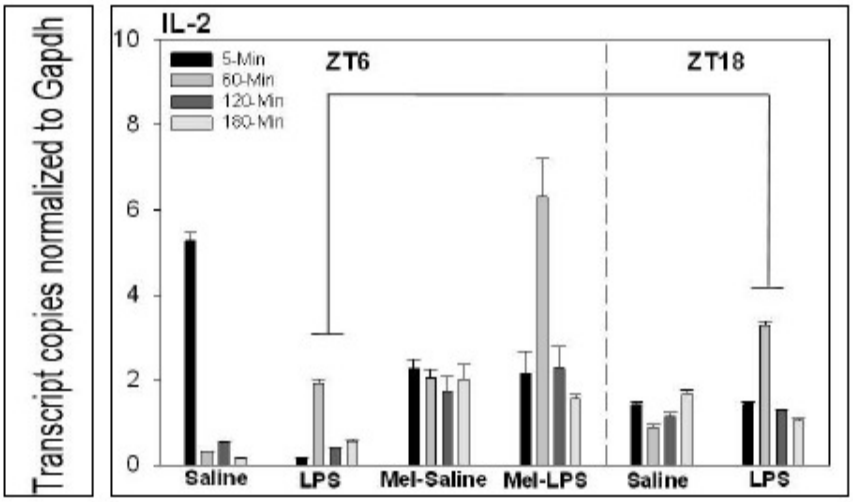
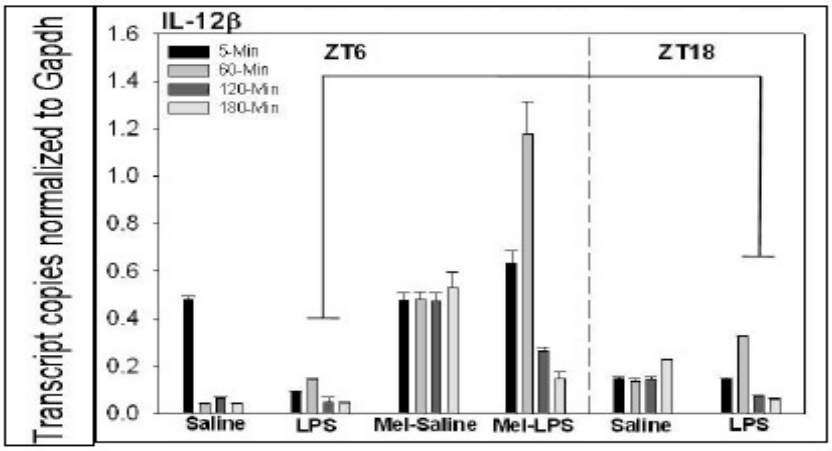



**10e**




**10f**

Figure 10 Continued.

combined actions of both molecules. For instance, TNFα increases by 2.5-fold following LPS stimulation alone at midday, while melatonin injection elicits a 70-fold increase. Combining the melatonin and LPS should theoretically induce a 175-fold induction in TNFά. This value is very close to what we observed in our results, a 170-fold increase. Similarly, IL-18 exhibited an average 3-fold increase following LPS injection at ZT6 (p, ANOVA < 0.001), 34-fold increase upon melatonin injection at ZT6 (p, ANOVA < 0.001,) and about 217-fold (p, ANOVA < 0.001) after melatonin and LPS injection at ZT6. Hence, we observed a synergistic effect of melatonin on LPS-induced inflammatory induction of TNFά and IL-18 cytokine genes mRNA levels. The cytokines IL-1b and IL-6 behave in an opposite fashion to that of TNFα and IL-18. The IL-1b and IL-6 mRNA levels decreased in levels following melatonin administration at midday (Figure 10 c and 10 d, Table 6). Melatonin injection prior to LPS led to a massive increase in IL-1b and IL-6 mRNA levels vs. melatonin ZT6 study group. Quantitatively, IL-6 increased by 14 fold (p, ANOVA < 0.001) upon LPS injection at ZT6, but decreased by 11-fold (p, ANOVA < 0.001) following melatonin administration, and increased 176-fold (p, ANOVA < 0.001) upon melatonin+LPS injection, resulting in absolute levels 12-fold higher than LPS treatment alone. Likewise, IL-1b is increased 6-fold (p, ANOVA < 0.001) upon LPS treatment at midday, decreased by 3-fold upon melatonin injection at ZT6, and increased 18-fold (p, ANOVA < 0.001) by both melatonin and LPS treatment at ZT6 (Table 6). The cytokines IL-2 and IL-12b expressed only slight increase in their mRNA levels upon melatonin and melatonin + LPS administration, indicating that although expression of these cytokines is weakly stimulated by melatonin, it does not act in conjunction with LPS

or enhance its effects, at least within the 3 hr time frame of the data sampling. These data reveal that melatonin decreases the expression of IL-1b and IL-6 mRNA at midday but also functions in a synergistic fashion with LPS in the induction of these cytokines during inflammation.

**4.5 Discussion**

This study provides evidence that melatonin is capable of regulating inflammatory response. However, melatonin is not capable of modulating all the pro-inflammatory cytokines selected for the study. Hence, it implies that there may be additional signaling mechanisms that may be regulating the the inflammatory response in the spleen. For instance, IL-1b (Figure 9 c) and IL-6 (Figure 9 d) mRNA levels peak at ZT3, while TNFα (Figure 10a) and IL-18 (Figure 9 b) mRNA peak at subjective night. Additionally, neither IL-1b (Figure 9 e) nor IL-6 mRNA levels are altered upon exogenous administration of melatonin at ZT6 (Figure 9 f).

The results suggest that additional circadian mechanisms of cytokine regulation may be present in the avian spleen. For instance, there may more than one hormone or chemical mediator (NE), which may be regulating some cytokines during the day resulting in diurnal phase differences in immune responses. Thus melatonin may not be the sole mediator of differential immune response in the avian spleen, as hypothesized in Figure 11.

Table 6. Quantitative RT-PCR values of cytokine genes in spleen under LPS induced inflammation at midnight vs. Melatonin and LPS at midday

| **TNFα** | Avg. | Avg. | Δ |
|---|---|---|---|
| ZT6 LPS vs. Saline | 23 | 9 | 2.6 |
| ZT18 LPS vs. Saline | 180 | 102 | 1.8 |
| ZT6 Melatonin atonin vs. ZT6 Saline | 630 | 9 | 70 |
| ZT6 Melatonin + LPS vs. ZT6 Saline | 1528 | 9 | 169 |
| ZT6 Melatonin + LPS vs. ZT6 Melatonin | 1528 | 630 | 2.4 |
| ZT18 Saline vs. ZT6 Saline | 102 | 9 | 11 |
| ZT18 LPS vs. ZT6 LPS | 180 | 23 | 7.9 |

| **IL-18** | Avg. | Avg. | Δ |
|---|---|---|---|
| ZT6 LPS vs. Saline | 162 | 26 | 6.1 |
| ZT18 LPS vs. Saline | 379 | 83 | 4.6 |
| ZT6 Melatonin atonin vs. ZT6 Saline | 236 | 26 | 9 |
| ZT6 Melatonin + LPS vs. ZT6 Saline | 1678 | 26 | 63 |
| ZT6 Melatonin + LPS vs. ZT6 Melatonin | 1678 | 236 | 7.1 |
| ZT18 Saline vs. ZT6 Saline | 83 | 26 | 3.2 |
| ZT18 LPS vs. ZT6 LPS | 379 | 161 | 2.3 |

| **Il-1β** | Avg. | Avg. | Δ |
|---|---|---|---|
| ZT6 LPS vs. Saline | 60 | 10 | 5.6 |
| ZT18 LPS vs. Saline | 52 | 3.5 | 15 |
| ZT6 Melatonin atonin vs. ZT6 Saline | 3.1 | 10 | 0.3 |
| ZT6 Melatonin + LPS vs. ZT6 Saline | 179 | 10 | 16.6 |
| ZT6 Melatonin + LPS vs. ZT6 Melatonin | 179 | 3.1 | 57 |
| ZT18 Saline vs. ZT6 Saline | 3.5 | 10 | 0.3 |
| ZT18 LPS vs. ZT6 LPS | 52 | 60 | 0.9 |

Table 6 Continued.

| IL-6 | Avg. | Avg. | Δ |
|---|---|---|---|
| ZT6 LPS vs. Saline | 85 | 6.2 | 14 |
| ZT18 LPS vs. Saline | 115 | 0.4 | 331 |
| ZT6 Melatonin atonin vs. ZT6 Saline | 0.6 | 6.2 | 0.1 |
| ZT6 Melatonin + LPS vs. ZT6 Saline | 1103 | 6.2 | 176 |
| ZT6 Melatonin + LPS vs. ZT6 Melatonin | 1103 | 0.5 | 1904 |
| ZT18 Saline vs. ZT6 Saline | 0.4 | 6.2 | 0.1 |
| ZT18 LPS vs. ZT6 LPS | 115 | 84 | 1.4 |

| IL-2 | Avg. | Avg. | Δ |
|---|---|---|---|
| ZT6 LPS vs. Saline | 0.8 | 1.6 | 0.5 |
| ZT18 LPS vs. Saline | 1.7 | 1.3 | 1.4 |
| ZT6 Melatonin atonin vs. ZT6 Saline | 2 | 1.6 | 1.3 |
| ZT6 Melatonin + LPS vs. ZT6 Saline | 3.1 | 1.6 | 2 |
| ZT6 Melatonin + LPS vs. ZT6 Melatonin | 3.1 | 2 | 1.5 |
| ZT18 Saline vs. ZT6 Saline | 1.3 | 1.6 | 0.8 |
| ZT18 LPS vs. ZT6 LPS | 1.8 | 0.8 | 2.3 |

| IL-12b | Avg. | Avg. | Δ |
|---|---|---|---|
| ZT6 LPS vs. Saline | 0.2 | 0.2 | 1 |
| ZT18 LPS vs. Saline | 0.2 | 0.2 | 1 |
| ZT6 Melatonin atonin vs. ZT6 Saline | 0.5 | 0.2 | 2.5 |
| ZT6 Melatonin + LPS vs. ZT6 Saline | 0.6 | 0.2 | 3 |
| ZT6 Melatonin + LPS vs. ZT6 Melatonin | 0.6 | 0.5 | 1.1 |
| ZT18 Saline vs. ZT6 Saline | 0.2 | 0.2 | 1 |
| ZT18 LPS vs. ZT6 LPS | 0.2 | 0.1 | 2 |

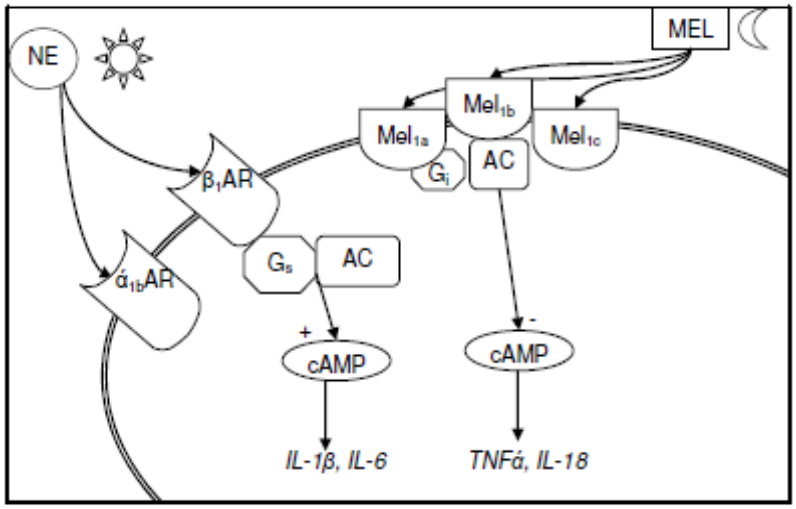



Figure 11. Model showing circadian clock regulation of cytokine rhythms in spleen. The IL-1b and IL-6 cytokines may be upregulated during daytime by norepinephrine (NE) via adrenergic receptors. The TNFά and IL-18 are upregulated during the night time by melatonin via melatonin receptors.

Moore and Siopes (Moore & Siopes, 2000) reported the stimulatory effects of melatonin on cellular and humoral immune responses in quail were opiod-dependent. Additional studies have also shown that melatonin interacts with GCs and testosterone hormone resulting in diurnal variations in immune function (Maestroni et al., 1986; Singh & Haldar, 2007). In the current study we demonstrated melatonin's ability to elicit diurnal changes in inflammatory response avian spleen. We also hypothesize that further study is needed in this area to identify the mechanism(s) and any additional mediators regulated by the circadian clock that are involved in modulating the immune response.

# 5. PERIPHERAL CLOCKS IN AVIAN OVARY AND ADIPOSE TISSUE

## 5.1 Background

Most physiological and behavioral functions of organisms exhibit a daily rhythm of ~24 hrs in length. These rhythmic processes are governed by environmental cues (e.g., daily light intensity and temperature), an endogenous circadian timing system termed the circadian clock, and the interactions between the circadian clock and environmental signals (Bell-Pedersen et al., 2005). In higher organisms, the circadian timekeeping system is comprised of a complex circuitry including a master pacemaker that is located in cells of specific structures of the organism, including regions of the brain (optic lobe) in insects; the eyes in certain invertebrates and vertebrates; and the pineal gland of some non-mammalian vertebrates (Gaston & Menaker, 1968; Sokolove, 1975). In mammals, the master clock resides in the suprachiasmatic nucleus (SCN), which is located at the base of the brain, just above the optic chiasm in the anterior hypothalamus. For quite some time SCN was viewed as the sole master circadian structure responsible for generating ~24 hr rhythms of physiology and behavior.

However, we know now that several peripheral tissues and cells contain molecular clocks similar to those in the brain, termed as peripheral clocks (Reppert & Weaver, 2002). For example, researchers have discovered that circadian rhythms persist in isolated lungs, livers, and other tissues in vitro, in the absence of SCN control (Yamazaki et al., 2000). These studies have led to a progressive shift in the traditional perception of circadian hierarchy, which is now regarded as an integrated timing system involving

peripheral oscillators in tissues and cells throughout the body whose activities are not wholly dependent upon SCN stimulation for coordination.

The fundamental basis for circadian rhythms of physiology lies with the molecular clock consisting of transcriptional/translational feedback loops. In mammals, the molecular clock consists of interlocking feedback loops which regulate circadian gene expression. Transcription factors Bmal1/Bmal2 and Clock/Npas2 for heterodimers and bind to the E-box regulatory elements at promoter regions of *period* (*per1, per2, per3*) and *cryptochrome* genes (*cry1, cry2*), thereby activating their expression. Once translated, PER and CRY repressor proteins form cytoplasmic complexes. This complex translocates to the nucleus and suppresses Bmal/Clock-mediated transcription (Ko & Takahasi, 2006). This interaction provides the framework that enables the intracellular circadian timing system, creating a robust 24 hr timekeeper that drives oscillations in output gene expression.

Although the period of an oscillator is relatively constant with a mean of 24hrs, the phase of the clock can vary greatly between cells and tissues. The core clock gene products are basic helix-loop-helix transcription factors which are capable of binding to E-box promoter sequences of ccgs and regulating their rhythmic expression. These ccgs include several genes engaged in cell signaling pathways, cellular metabolism and cell cycle regulators. It is implied that rhythmic regulation of ccgs by the clock may be critical for normal physiological functioning in a cell/tissue-specific manner (Cassone et al., 1984; Mitchell et al., 2005). (Silver, 1986; Sharp, 1984; Nakao et al, 2007). Hens (Gallus domesticus) and quails (*Coturnix japonica*) in constant light (LL) show persistence of ovipositioning cycles with a ($\tau$) of approximately 27hr. The rhythm of ovulation-

oviposition in birds depends on several factors including, time of LH secretion (Underwood et al, 1997), positive feedback action of progesterone on neuroendocrine system and the rhythmic sensitivity of hypothalamus to the circulating progesterone which in turn depends on the timing of LH secretion (Lesauter et al., 2003). Studies in domestic hens (Lesauter et al., 2003) and Japanese quail (Yoo et al., 2004; Yoo et al., 2005) indicate that the oviposition-ovulation cycles are regulated by a multi-oscillatory system wherein the central clock drives the circadian rhythms of body temperature, and a peripheral clock in the ovary regulates the time of oviposition.

**5.2. Circadian clocks in ovary and adipose tissue**

In a very interesting study in Japanese quail (*Coturnix japonicum*) housed under constant light (LL) and constant darkness (DD), Underwood et al. (Underwood et al., 1997) suggest the presence of a circadian clock in ovary which may regulate the rhythms of core body temperature (CBT). They found that the CBT rhythm exhibit daily and circadian oscillations. However, under LL the CBT starts free-running free with at period > 24hr and gets synchronized to the rhythms of oviposition. The birds under DD cease to ovulate and their CBT rhythms free-ran with a period close to 22hrs. Ovariectomy abolished the CBT rhythms when birds were kept in LL. When birds were not given any photic input, the CBT rhythms split into two periods, a period of < 24 hr and a period of > 24hr. These rhythms are similar to the rhythms of oviposition. This suggests that rhythms of CBT are under the influence of multiple oscillators, one of which may be located in the ovary which is responsible for the period > 24hr (Underwood et al., 1997). It is speculated

that the ovarian clock regulates rhythm of steroid hormone (tau (> 24 hr) which in turn regulates the longer CBT rhythms.

The ovary of Japanese quail and domestic hens host core circadian clocks (Underwood et al., 1997; Yoshimura et al., 2000). Several core clock genes such as *per2*, *per3*, *clock* and *bmal1* have been identified in several peripheral tissues in quail (Yoshimura et al., 2000). Strong diurnal rhythms were observed of quail *per2* (*qPer2*) and *qPer3* mRNA expression in granulosa cells (O-GC) and theca cells (O-TC) of fully mature F1 follicles (Underwood et al., 1997). Rhythms of *qPer2* and a tendency toward cycling in *qPer3*, *clock* and *bmal1* were also detected in F1 follicles from quail housed in constant light. The F1 follicles exhibit robust rhythms in cholesterol transporter steroidogenic acute regulatory protein (StAR) and steroid biosynthetic enzyme 3β-hydroxysteroid dehydrogenase (3-βhsd) (Nakao et al., 2007). The promoter region of StAR in transiently transfected primary cultures of chicken granulosa cells revealed that Clock/Bmal1 transcription factor is capable of activating the transcription of StAR gene (Nakao et al., 2007). Hence, the quail and domestic hen ovary may host molecular clock (perhaps specifically in their O-GC and O-TC) which play a role in temporal regulation of steroidogenesis. Ball suggests (Ball, 2007) a novel hypothesis that the clocks in avian ovary participate in ovulation rhythms by driving rhythmic positive steroid hormone feedback on the hypothalamus. An important hormone regulating the ovulation in birds and rodents is the LH. The LH secretion from the pituitary is under temporal regulation by the master clock (SCN) (Sharp et al., 1984; Silver et al., 1986). Although regulation of LH surge by SCN exists in rodents (e.g. rats, mice and hamsters), a similar phenomenon is

unclear in women. However, female subjects exhibit rhythmic secretion of LH during menstrual cycle. These rhythms correlate strongly with the rhythm of serum cortisol and the L:D photoperiod cycle (Kerdelhue et al., 2002; Garcia et al., 1981; Seibel, 1986). Unraveling these relationships could prove crucial to understanding the role of circadian clock function in diseases that negatively impact fertility.

Autonomous rhythmicity in reproductive structures has only recently been explored. Although the majority of evidence from mammals suggests that some male reproductive structures (e.g. testis) do not contain cell-autonomous clocks (Yoo et al., 2005; Yoo et al., 2006) circadian clocks are present in accessory structures such as the extratesticular ducts (Wilsbacher et al., 2002). Core clock gene expression is also evident in the cells of the ovary, uterus and oviduct (Dolatshad et al., 2009).

Disruption of circadian clock function seems to affect the fertility in several species (Boden et al., 2010; Kennaway et al, 2005; Miller et al., 2004). Middle aged *per1* and *per2*$^{-/-}$ mice exhibit reduced fecundity and fertility (Pilorz and Steinchner, 2008) while, clock mutant (*clock*$^{\Delta 19}$) mice have lengthy estrous cycles (Miller et al., 2004) and *bmal1*$^{-/-}$ mice luteal cells (LCs) display reduced progesterone production, reduced expression of StAR and prolonged estrous cycle. Scientists suggest that disrupted ovarian clock may be one of the etiologies of one such reproductive dysfunction affecting ~ 6-10% women known as the PCOS (polycystic ovarian syndrome). PCOS ovarian thecal cells (O-TCs) have inherent defect in mitogen activated protein kinase (MAPK) signaling, which might contribute to excess CYP17 gene expression and androgen biosynthesis (McAllister JM, 2006). PCOS involves a vicious cycle wherein hyperandrogenism favors abdominal

adiposity, which further stimulates androgen secretion by the ovaries. It is hypothesized that abnormal clock gene expression in the ovary could affect the expression of steroid hormone biosynthetic enzymes, leading to excess androgen secretion and PCOS (Yildizet al., 2008).

### 5.3 Clocks in ovary and adipose tissue in poultry

The third largest agricultural sector in the United States is occupied by the poultry industry with an annual production of more than 30 billion pounds of meat and 6 billion dozens of eggs from chickens alone (USDA 2005). However, a major factor contributing to limiting the efficiency of meat production is the poor egg production. Genetic selection programs that underlie the gains in early rapid broiler growth have outpaced our knowledge of reproductive biology in currently utilized genetic backgrounds. Comparative studies have been done with regards to fertility and energy-balance between egg-laying and meat-type hens (broiler breeder hens). The broiler breeder hens have a productive egg-laying cycle of about 40 weeks when compared to the 60 week cycle of egg-laying (layer breeder hens) (Cobb-Vantress 2008; International 2009). During these productive egg-laying cycles, the Broiler breeders lay ~173 eggs during the first 40 weeks of production compared to ~234 egg laid by egg-type hens during this same period. Hence, broiler breeder hens have reduced rate and persistency of egg production for a given production cycle. The Broiler breeder hens lay ~180 eggs to the ~ 352 eggs laid by egg-type hens; an approximately 50% reduction in the egg production.

Although these large discrepancies in feed efficiency and egg-production

capacities are well known in the two lines of chicken, the molecular basis are still largely unknown. To maintain optimal egg-production capacity, the broiler breeder hens need strict diet and light-dark cycle managements. Strict restricted-diet to maintain uniform weight among the broiler breeder hens for a productive cycle seems to be the most effective method. Studies show that broiler breeder hens on an ad-libitum diet showed excessive body weight (obesity), doubling of feed intake, reduced egg-production and physiological symptoms similar to human metabolic syndrome (Chen SE, McMurtry JP, Walzem RL.( 2006) Overfeeding-induced ovarian dysfunction in broiler breeder hens is associated with lipotoxicity. Poult Sci.85 (1):70-81. Similar changes are produced in egg-type hens which were force fed by intubating 120% of ad libitum intake daily for periods of up to three weeks (Walzem et al. 1994). The former study provided the first insight into a physiological mechanism that might underlie a large number of observations by others documenting altered hormonal patterns, ovarian dysfunction and impaired egg production in conjunction with untoward weight gain in female Broiler breeders (Yu, Robinson et al. 1992a; Yu, Robinson et al. 1992b; Yu, Robinson et al. 1992c). Hence, the relationship between over-feeding and reproduction and the role of circadian clocks in regulating these physiological events should be able to gap the bridges in understanding this phenomenon.

## 5.4 Investigation of clock genes in layer vs. broiler hens

### *5.4.1 Introduction*

A functional ovary is critical for successful reproduction in broiler breeder hens.

This is the point where the internal balance between growth and reproduction will interface with external management methods to achieve the greatest number of settable eggs. It is postulated that there are two interacting systems that regulate the timing of ovulation (Etches and Schoch, 1984). The first system regulates follicular maturation. The second system regulates the surge of LH and is under the control of circadian clock. It is generally accepted that the ovulation in hen has circadian component. However the extent to which the circadian clock regulates ovulation is still a subject of debate as the identity and description of the circadian oscillators regulating reproduction in poultry remains unknown. The innate genetic disparity in egg production of broiler breeders versus leghorn-type hens represents a unique opportunity to dissect out circadian timing mechanisms controlling ovulation in order to mitigate poor reproductive function in poultry and thus serve as a focal point of our investigations.

Understanding the relationships between circadian clock and reproductive function will help understand the reproductive system under normal and pathological states. In the current study we examined temporal expression profiles of core clock genes of two distinct lines of poultry which differ in their reproductive function and energy storage capacity capabilities (fat). Comparing and contrasting these data will garner us a better understanding of reproductive function regulation by the circadian clocks. The aim of the present research was to understand the daily regulation of reproductive function by the circadian clock through determining the temporal expression patterns of core clock genes in central clock and peripheral tissues of two distinct lines of poultry. If the broiler breeder hens exhibit disrupted circadian clocks within the hypothalamus, adipose tissue and ovary,

it may explain the poor reproductive functioning in this obesity prone poultry line when compared to the layer hen. These data may help establish the circadian link between ovary and ovarian physiology.

### *5.4.2 Materials and methods*

#### *5.4.2.1 Animals*

Day old female broiler chicks, Gallus gallus, Ross x Ross (n=54) were obtained from Brazos Sanderson Farms Hatchery (Bryan, TX). Day old female layer chicks, Bovans (n=54) were obtained from Feather Crest Farms (Bryan, TX). Animals were held in LL at the Texas A&M University poultry research farm till the age of 39 days. On the $40^{th}$ day animals were placed in a LD 12:12 photoperiod for 1 week with food and water continuously available (total n=54 for each experiment involving 6 time points. The 6 time points tested in LD were ZT2, ZT6, ZT10, ZT14, ZT18 and ZT22 (ZT: Zeitgeber time, lights on at ZT0; Lights off at ZT12). All animals were sacrificed by $CO_2$ asphyxiation and tissues (ovary, adipose tissue and hypothalamus) were harvested. The tissues were immediately placed on solid $CO_2$ and stored at -80ºC until use. Three pools of tissue were prepared at each time point, each of which was composed of three tissue samples (n=9 per time point). Animal use and care protocols were in accordance with NIH guidelines.

#### *5.4.2.2 Quantitative real-time polymerase chain reaction*

From each tissue pool, Total RNA (4 µg/sample) was extracted using TRIzol

protocol (Invitrogen), according to manufactures instructions. To remove contaminating genomic DNA, the total RNA was subjected to DNase treatment using TURBO DNA-free (Ambion). Ribonucleic acid quantification was assessed using an Eppendorf Biophotometer (Eppendorf). Using 1 µg of DNase-treated total RNA as starting material, synthesis of cDNA was performed following the High Capacity cDNA Reverse Transcription Kit protocol (Applied Biosystems). The qRTPCR determinations were made using a LightCycler 480 (Roche). Each reaction of 20µl volume contained 0.5 µM primers, SYBR Green mastermix (Roche), and cDNA, according to the manufacturer's instructions. Each incubation step consisted of an initial denaturation step at 95°C for 10 minutes, followed by 40 cycles of a 95°C denaturation for 15 s, 30 s annealing at 63°C, then extension at 72°C for 30 s. The list of primers used for the daily and circadian expression studies using qRTPCR are described in Table 1. All the primer pairs generated a single product of the predicted size as indicated by agarose gel electrophoresis. Their specificity was demonstrated by melting curve analysis (Tm) during every qRTPCR run. Typically ~25 cycles were necessary to detect amplification of the product. All qRTPCR assays were linear ($r^2 > 0.99$) from $10^1$ to $10^7$ copies. Internal standards were used to determine transcript numbers. They were prepared by cloning target PCR products into pGEMT Easy vectors (Promega). Clones were verified by performing direct sequence analysis. The plasmid DNA was digested, followed by agarose gel electrophoresis (2.0%, w/v) for visual verification of correct product sizes and staining with ethidium bromide (EtBr, 0.5 µg/ml). To generate standard curves, a set of 100-fold serial dilutions of each internal standard ($10^1$-$10^7$ copies/2 µl) was prepared. The transcript numbers were

determined by using a 2 µl sample of a 10-fold dilution of cDNA prepared as mentioned above. The values were then normalized to the number of Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) copies.

*5.4.2.3 Statistical analysis*

Analysis of variance (ANOVA) (Sigmaplot) was used for analyzing times series data involving the 7 timepoints. The cosinor analysis was done using linear harmonic regression (CircWave software) (Oster et al., 2006). Student-Newman-Keuls method was used to estimate significant differences among means. Average changes in core clock gene levels were subjected to two-way ANOVA (Sigmaplot) and multiple comparisons vs. control group (Holm-Sidak method) for each time point. Statistical significance is based on $P < 0.05$.

### *5.4.3 Results*

*5.4.3.1 Daily rhythms in the hypothalamus*

The interacting transcriptional-translational feedback loops generate circadian rhythms in several peripheral tissues (Bell Pederson et al., 2005). Examination of the putative negative elements, in layer hen hypothalamus , reveals daily oscillations ~2-3 fold amplitudes with high abundances occurring during the early day for *cry1* (p, ANOVA = 0.003; $p_{cosinor} = 0.002$), and at the dark to light transition for *per2* (p, ANOVA = 0.05; $p_{cosinor} = 0.01$), and *per3* (p, ANOVA < 0.001; $p_{cosinor} < 0.001$) (Figure 12). *Cry2* (p,

ANOVA = 0.4), did not display a statistically significant difference over a 24 hr period in the hypothalamus. Analysis of the putative positive elements reveals a daily pattern of rhythmicity. The *clock* attained maximal abundance during midday (p, ANOVA = 0..004; p, $_{cosinor}$ = 0.01) while *bmal1* (p, ANOVA = 0.02; p, $_{cosinor}$ = 0.02) and *bmal2* (p, ANOVA = 0..03; p, $_{cosinor}$ = 0.03) attained maximal abundance at the light to dark transition (Figure 12).

In the hypothalamus of broiler hens, all clock genes examined displayed mRNA rhythms with 2-7 fold amplitudes over the 24 hr period with peaks occurring at midday for *cry1* ($p_{ANOVA} < .001$; $p_{cosinor} < .001$), *cry2* ($p_{ANOVA} = .09$; $p_{cosinor} = .002$), *per2* ($p_{ANOVA} < .001$; $p_{cosinor} < .001$), *per3* ($p_{ANOVA} = < .001$; $p_{cosinor} < .001$), *clock* ($p_{ANOVA} = .004$; $p_{cosinor} = .001$), *bmal1* ($p_{ANOVA} = .04$; $p_{cosinor} = .007$) and *bmal2* ($p_{ANOVA} = < .001$; $p_{cosinor} < .001$) (Figure 13). The *period1* (*per1*) gene has not been identified in birds and is thus not examined in these studies.

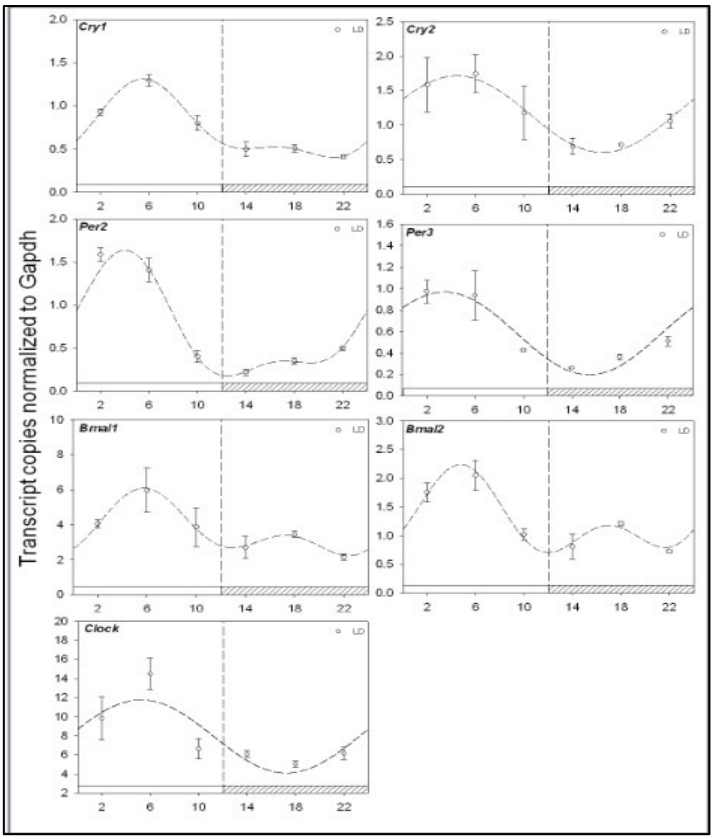



Figure 12. Quantitative RT-PCR analysis of core clock genes in Layer hypothalamus. Plotted open circles represent the mean ± SEM in each experimental group. The dashed line represents the fitted plot of cosinor analysis utilizing linear harmonic regression. Values are represented as the number of transcript copies/1000 Gapdh transcripts. Abscissa labels indicate ZT time values every 4 hrs under LD 12:12 conditions; ZT0 = lights on; ZT12 = lights off. Open bar on the bottom of the x-axis indicates the light period, while crosshatched indicates darkness. The cry and per genes harbor daily oscillations ~2-3 fold amplitudes with high abundances occurring during the early day for *cry1* (pANOVA =.003; pcosinor = .002), and at the dark to light transition for *per2* (pANOVA = .05; pcosinor =.01), and *per3* (pANOVA =<.001; pcosinor <.001). The *cry2* (pANOVA = .4), did not display a statistically significant difference over a 24 hr period. The *clock* mRNA attained maximal abundance during midday (pANOVA =.004; pcosinor = .01) while *bmal1* (pANOVA = .02; pcosinor = .02) and *bmal2* (pANOVA = .03; pcosinor = .03) attained maximal abundance at the light to dark transition.

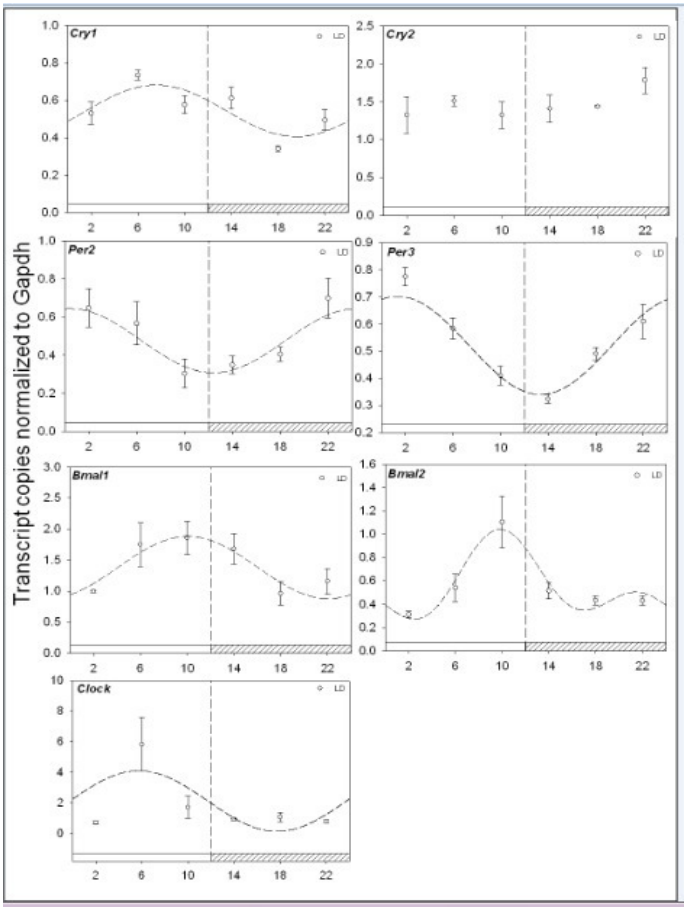



Figure 13. Quantitative RT-PCR analysis of core clock genes in Broiler hypothalamus. Plotted open circles represent the mean ± SEM in each experimental group. The dashed line represents the fitted plot of cosinor analysis utilizing linear harmonic regression. Values are represented as the number of transcript copies/1000 Gapdh transcripts. Abscissa labels indicate ZT time values every 4 hrs under LD 12:12 conditions; ZT0 = lights on; ZT12 = lights off. Open bar on the bottom of the x-axis indicates the light period, while crosshatched indicates darkness. All *clock* genes displayed mRNA rhythms with 2-7 fold amplitudes over the 24 hr period with peaks occurring at midday for *cry1* ($p_{ANOVA} < .001$; $p_{cosinor} < .001$), *cry2* ($p_{ANOVA} = .09$; $p_{cosinor} = .002$), *per2* ($p_{ANOVA} < .001$; $p_{cosinor} < .001$), *per3* ($p_{ANOVA} = < .001$; $p_{cosinor} < .001$), *clock* ($p_{ANOVA} = .004$; $p_{cosinor} = .001$), *bmal1* ($p_{ANOVA} = .04$; $p_{cosinor} = .007$) and *bmal2* ($p_{ANOVA} = < .001$; $p_{cosinor} < .001$).

*5.4.3.2 Daily rhythms in visceral adipose tissue*

Integral functions of energy homeostasis, including the sleep-wake cycle, thermogenesis, and feeding are subject to circadian regulation (Gimble et al., 2011). However, it remains to be elucidated whether these genes are expressed in avian adipose tissue. Existence and daily oscillations of clock genes in layer VAT are evident, as *clock* genes exhibit 24 hr rhythms in mRNA abundance. Examination of the putative negative elements reveals daily oscillations with ~3-10 fold amplitudes with peak abundances occurring during the day for *cry1* ($p_{ANOVA} < .001$; $p_{cosinor} < .001$), and *cry2* ($p_{ANOVA} = .01$; $p_{cosinor} = .007$), and at the dawn transition for *per2* ($p_{ANOVA} < .001$; $p_{cosinor} < .001$), and *per3* ($p_{ANOVA} = < .001$; $p_{cosinor} < .001$) (Figure 14). Analysis of *clock* and the *bmals*, reveals a daily pattern of rhythmicity in VAT. The *clock* attained maximal abundance during the late night ($p_{ANOVA} = .003$; $p_{cosinor} = .005$) while *bmal1* is highest during the late day period ($p_{ANOVA} = .02$; $p_{cosinor} = .04$). The *bmal2* exhibited increased expression during the early day and late nighttime, however there is not a statistically significant difference over a 24 hr period ($p_{ANOVA} = .2$). However, for broiler hens daily core clock gene oscillations in VAT are markedly different (Figure 15). Cry1, the *per* genes and *bmal1*, possess daily oscillations with 2-6 fold amplitudes with higher abundances occurring during the day for *cry1* ($p_{ANOVA} < .001$; $p_{cosinor} = < .001$), *per2* ($p_{ANOVA} < .001$; $p_{cosinor} = < .001$), *per3* ($p_{ANOVA} = .003$; $p_{cosinor} = .001$) and *bmal1* ($p_{ANOVA} = .009$; $p_{cosinor} = .007$). The *cry2* ($p_{ANOVA} = 0.1$), *bmal2* ($p_{ANOVA} = 0.1$), and *clock* ($p_{ANOVA} = 0.09$) mRNA values fluctuated, however none show a statistically significant difference over a 24 hr period (Figure 15).

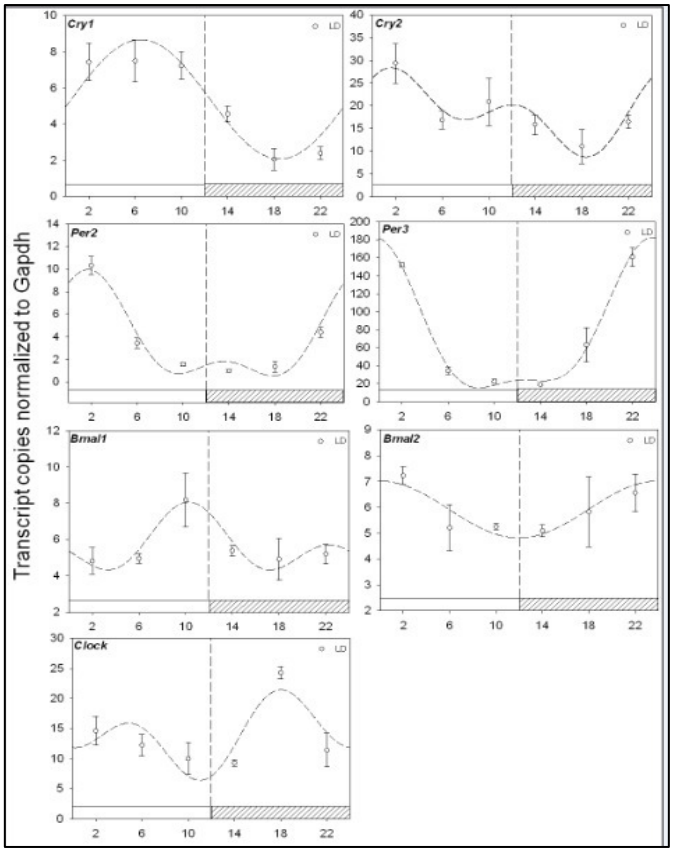



Figure 14. Quantitative RT-PCR analysis of core clock genes in Layer VAT. Plotted open circles represent the mean ± SEM in each experimental group. The dashed line represents the fitted plot of cosinor analysis utilizing linear harmonic regression. Values are represented as the number of transcript copies/1000 Gapdh transcripts. Abscissa labels indicate ZT time values every 4 hrs under LD 12:12 conditions; ZT0 = lights on; ZT12 = lights off. Open bar on the bottom of the x-axis indicates the light period, while crosshatched indicates darkness. Negative elements reveals daily oscillations with ~3-10 fold amplitudes with peak abundances occurring during the day for *cry1* ($p_{ANOVA} < .001$; $p_{cosinor} < .001$), and *cry2* ($p_{ANOVA} = .01$; $p_{cosinor} = .007$), and at the dawn transition for *per2* ($p_{ANOVA} < .001$; $p_{cosinor} < .001$), and *per3* ($p_{ANOVA} = < .001$; $p_{cosinor} < .001$). The *clock* mRNA attained maximal abundance during the late night ($p_{ANOVA} = .003$; $p_{cosinor} = .005$) while *bmal1* is highest during the late day period ($p_{ANOVA} = .02$; $p_{cosinor} = .04$). The *bmal2* exhibited an increased expression during the early day and late nighttime, however there is not a statistically significant difference over a 24 hr period ($p_{ANOVA} = .2$).

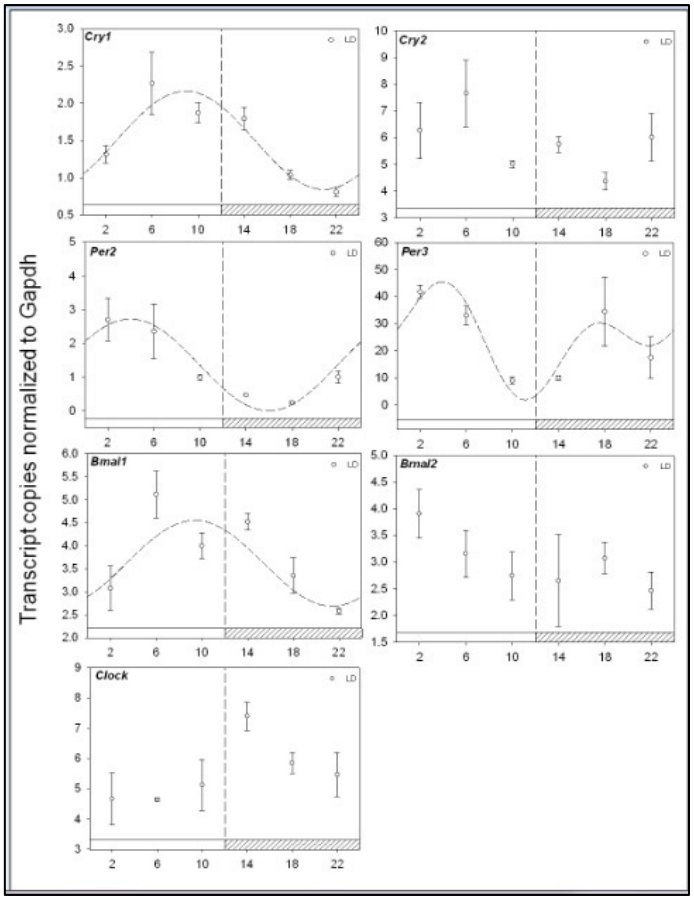



Figure 15. Quantitative RT-PCR analysis of core clock genes in Broiler VAT. Plotted open circles represent the mean ± SEM in each experimental group. The dashed line represents the fitted plot of cosinor analysis utilizing linear harmonic regression. Values are represented as the number of transcript copies/1000 Gapdh transcripts. Abscissa labels indicate ZT time values every 4 hrs under LD 12:12 conditions; ZT0 = lights on; ZT12 = lights off. Open bar on the bottom of the x-axis indicates the light period, while crosshatched indicates darkness. The *cry1*, the *per* genes and *bmal1*, possess daily oscillations with 2-6 fold amplitudes with higher abundances occurring during the day for *cry1* ($p_{ANOVA} < .001$; $p_{cosinor} = < .001$), *per2* ($p_{ANOVA} < .001$; $p_{cosinor} = < .001$), *per3* ($p_{ANOVA} = .003$; $p_{cosinor} = .001$) and *bmal1* ($p_{ANOVA} = .009$; $p_{cosinor} = .007$). The *cry2* ($p_{ANOVA} = 0.1$), *bmal2* ($p_{ANOVA} = 0.1$), and *clock* ($p_{ANOVA} = 0.09$) mRNA values fluctuated, however none show a statistically significant difference over a 24 hr period with a >2-fold rhythm.

*5.4.3.3 Daily rhythms in the ovary*

It is well known that several aspects of reproductive function including ovulation in birds and mammals exhibit circadian characteristics. However, there is very limited evidence for a functional clock in the hen ovary to date (Yamazaki & Takahashi, 2005). For this reason we examined core clock genes profiles in the ovary of hens.). Examination of the *cry* and *per* genes, reveals daily oscillations with amplitude changes of 3-10 fold. Of these, higher abundances occur during the early day for *cry1* ($p_{ANOVA} < .001$; $p_{cosinor} <$ .001), *per2* ($p_{ANOVA} < .001$; $p_{cosinor} <.001$), and *per3* ($p_{ANOVA} =<.001$; $p_{cosinor} <.001$). The *cry2* also had elevated mRNA abundance during the early day however it was not statistically significant ($p_{ANOVA} = .1$) (Figure 16). Analysis of gene expression of the positive elements of the clock, *clock* and the *bmal* genes, revealed that only *bmal2* displayed a significant daily pattern of rhythmicity in the layer ovary ($p_{ANOVA} =.006$; $p_{cosinor}$ =.007) (Figure 16).

Investigation of the broiler ovary revealed a vastly different situation than what occurs in the layer ovary. None of the core clock genes examined displayed a statistically significant difference over a 24 hr period, *cry1* ($p_{ANOVA} = 0.8$), *cry2* ($p_{ANOVA} = 0.1$), *per2* ($p_{ANOVA} = 0.06$), *per3* ($p_{ANOVA} = 0.06$), *bmal1* ($p_{ANOVA} =0.1$), *bmal2* ($p_{ANOVA} = 0.1$), and *clock* ($p_{ANOVA} = 0.9$) (Figure 17).

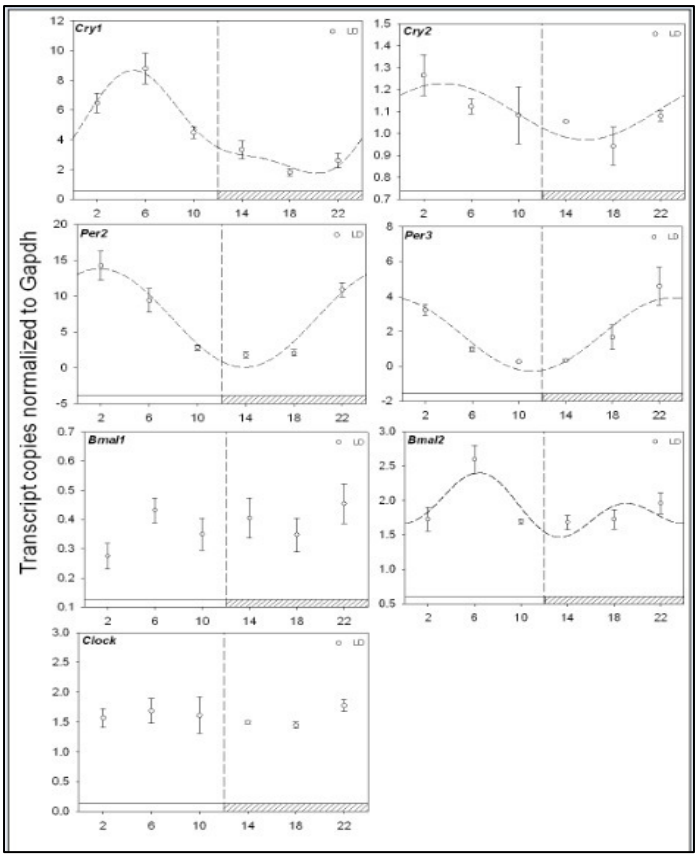



Figure 16. Quantitative RT-PCR analysis of core clock genes in Layer ovary. Plotted open circles represent the mean ± SEM in each experimental group. The dashed line represents the fitted plot of cosinor analysis utilizing linear harmonic regression. Values are represented as the number of transcript copies/1000 Gapdh transcripts. Abscissa labels indicate ZT time values every 4 hrs under LD 12:12 conditions; ZT0 = lights on; ZT12 = lights off. Open bar on the bottom of the x-axis indicates the light period, while crosshatched indicates darkness. The *cry* and *per* genes, reveal daily oscillations with 3-10 fold amplitudes with higher abundances occurring during the early day for *cry1* ($p_{ANOVA} < .001$; $p_{cosinor} < .001$), *per2* ($p_{ANOVA} < .001$; $p_{cosinor} < .001$), and *per3* ($p_{ANOVA} = < .001$; $p_{cosinor} < .001$). The *cry2* also had elevated mRNA abundance during the early day however it was not statistically significant ($p_{ANOVA} = .1$). Analysis of the putative positive elements, *clock* and the *bmals*, revealed that only *bmal2* displayed a significant daily pattern of rhythmicity in the layer ovary ($p_{ANOVA} = .006$; $p_{cosinor} = .007$).

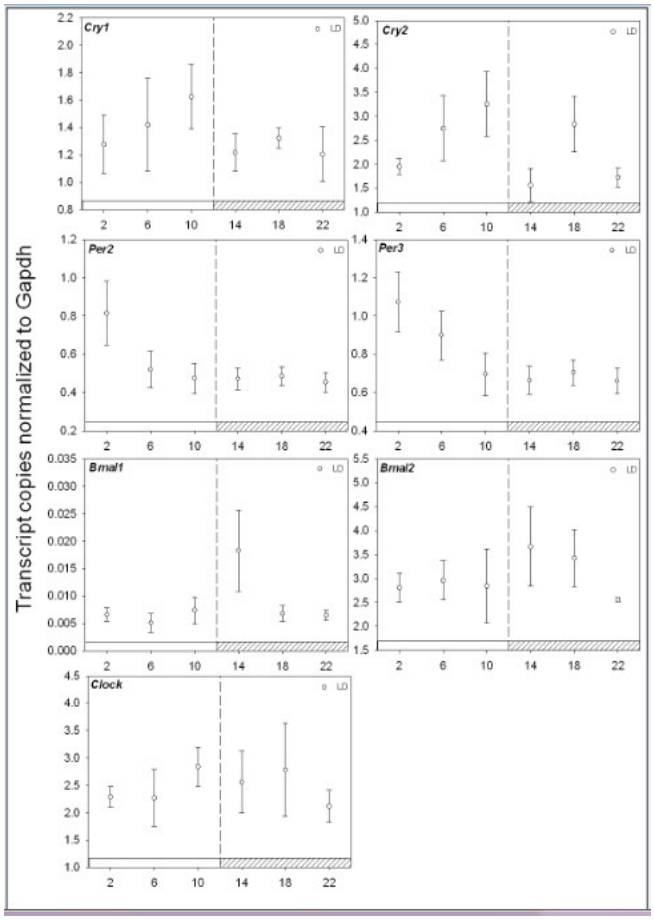



Figure 17. Quantitative RT-PCR analysis of core clock genes in Broiler ovary. Plotted open circles represent the mean ± SEM in each experimental group. The dashed line represents the fitted plot of cosinor analysis utilizing linear harmonic regression. Values are represented as the number of transcript copies/1000 Gapdh transcripts. Abscissa labels indicate ZT time values every 4 hrs under LD 12:12 conditions; ZT0 = lights on; ZT12 = lights off. Open bar on the bottom of the x-axis indicates the light period, while crosshatched indicates darkness.

### *5.4.4 Discussion*

The role of hypothalamus-pituitary gland-gonad (HPG) axis in maintaining reproductive efficacy is well established. One additional candidate peripheral tissue capable of modulating ovarian function is the adipose tissue owing to its endocrine capacity. It is proposed that normal ovarian function and fertility requires a synchronization of neuroendocrine signals from the HPG axis as well as endocrine signals from the adipose tissue (Gimble et al., 2002). If peripheral clocks are capable of regulating physiological functions within the tissues, then we hypothesize that desynchronization of clock in HPG and/or adipose tissue with that of the ovarian clock should lead to disrupted/reduced efficacy in ovarian function such as ovulation.

In the poultry industry, egg-type chickens have been genetically selected for their prolific ovulation rates and smaller body size. In the meat-type chicken however, rapid growth-rate and heavy bodies are the basis for their genetic selection. But the broiler breeder hens also exhibit detrimental phenotypic features such as, poor ovarian follicular development with disorganized hierarchies among ovarian follicles (Sellix et al., 2010). This makes broiler breeder hens relatively poor egg-layers. Studies indicate that this decreased efficacy may be due to any of these 3 reasons, 1) effects of adipose related hormones, 2) oocyte signaling, and 3) oocyte-granulosa interactions (Gimble et al., 2002). Hence, ovulation may be affected by feed intake and body weight in broiler breeder hens. In the current study we examined temporal expression profile of core clock genes on a daily basis in visceral adipose tissue (VAT), hypothalamus and ovary of 44 day old female egg-type (layer) and meat-type (broiler) pullets. A tissue or cell is considered to have a

functional clock if it expresses positive element *bmal1* in an anti-phase relationship to the negative elements *per1*, *per2*, *cry1*and *cry2* (Bell-Pedersen et al., 2005). We have demonstrated that there is significant cycling of core clock genes in the hypothalamus of layer and broiler pullets, although the expression patterns differed between the two poultry lines (Figure 12and 13). Even though the mRNA expression profiles in layer hypothalamus differed among the six clock genes tested, no obvious anti-phase relationship between the negative element *cry1* and positive elements *bmal1* and *clock* were observed (Figure 12). A similar pattern was reported by Tischkau et al., 2011 in the hypothalamus of 26 week old broiler-hens. The negative elements *per2* and *per3* were in anti-phase with the positive elements *bmal1* and *clock*. We report that in broiler hypothalamus, although all the six clock genes express circadian oscillations, the positive elements (*bmal1* and *clock*) and negative elements (*cry1*, *cry2*, *per2* and *per3*) do not show obvious anti-phase. All six core clock genes peaked during day time and fell during night time (Figure 12).

The clock genes in VAT exhibit daily oscillations in both layer and broiler pullets although the temporal expression profiles are different in these two poultry lines (Figure 14 and 15). The mRNA profile of positive elements *bmal1* and *clock* genes are in anti-phase with the negative elements *cry1*, *cry2*, *per2* and *per3*in layers. However, in broilers *cry1*, *per2*, *per3* and *bmal1* were in same phase with daily abundance occurring during the day, while the mRNA levels of *cry2* and *clock* were statistically insignificant (Figure 15). This comparative study indicates that the layer VAT has a functional clock as opposed to the broiler VAT.

Birds and mammals exhibit circadian characteristics in several aspects of their reproductive function including ovulation. We demonstrated that the layer pullets exhibit daily oscillations in the mRNA levels of core clock genes *cry1*, *cry2*, *per2* and *per3* (Figure 16). The mRNA levels of positive elements *bmal1* and *clock* were statistically insignificant. Studies from F1 follicles in 26 week old chicken ovary exhibit significant cycling of *bmal1*, *cry1* and *per2*. The *cry1* and *per2* were in-phase with each other while bmal1 cycled out-of-phase indicating presence of functional molecular clock. The *cry1* and *per2* peaked at ZT12 while *bmal1* levels were high at ZT0 (Tischkau et al., 2011). In quails, the Per2 peaked during early day and showed a trough during late night while the bmal1 showed no significant oscillation (Nakao et al., 2007). This observation is almost similar to what we saw in the layer-pullet ovary transcription profile for per2 and *bmal1* levels (Figure 16). Yoshikawa & Menaker, 2010, Nakao et al., 2007 and Tischkau et al., 2011 demonstrated that LH is a powerful zeitgeber capable of resetting the ovarian circadian clock in chicken with significant effect on *bmal1* and per2 genes. One possible reason for the lack of significant levels of *clock* and *bmal1* oscillations may be that the birds are sexually immature with ovaries which do not have mature follicles and are unresponsive to LH. In contrast to the layer-pullet ovary, none of the core clock genes exhibited any statistically significant levels in the broiler-pullet ovary. A next interesting study would be to examine the temporal expression profiles of core clock genes in the ovaries of sexually mature broiler hens with a history of poor fertility/reproduction over a 24 hour cycle. Arrhythmic or lack of core clock gene oscillations may indicate loss and may imply that the absence of functional ovarian clock leads to loss of regular ovarian

function in broiler breeder hens. Selix and Menaker (2010) suggest that circadian oscillators exist in the HPG-axis and synchronization between hypothalamus, GnRH (Gonadotropin releasing hormone) neurons, LH surge and ovarian cells are necessary for maintaining normal physiology in the ovary. Disruption of coordination or synchronization within and between these circadian clocks may have significant negative effects on fertility.

Recent studies in mammals and mice demonstrated that adipose tissue is capable of releasing hormones which participate in metabolic as well as reproductive functions. A set of adipose-hormones (adipokines) namely, leptin, adinopectin and resistin may impinge their endocrine effects by binding to their receptors that are expressed in higher brain centers like hypothalamus as well as on ovaries (Mitchell et al., 2005) and thereby regulate ovarian functions. Chicken granulosa and theca cells express AdipoR1 and AdipoR2 receptors which bind to a hormone adiponectin, a hormone released by adipose tissue namely (Chabrolle et al., 2007). Chabrolle et al., 2007 also reported activation of AMPK in response to adiponectin in chicken cultured granulosa cells. Tosca et al., 2006 reported that activation of AMPK reduced progesterone secretion. Adipose tissue function exhibits circadian oscillations in adipokines secretion on daily basis (Gimbel et al., 2011). Disruption of circadian clock in adipose tissue may lead to disruption (of phase and/or levels) of adipokines secretion thus upsetting normal ovarian function.

Excess energy intake leads to excess adipose tissue and obesity-related complications in metabolism and reproductive complications. The adipose tissue secretes several biologically active molecules such as leptin, resistin and adiponectin (Matsuzawa

et al. 2006). Some of these molecules express daily rhythms in their secretion (Gavrila et al. 2003). It is speculated that insulin resistance and metabolic syndrome in obese human beings may be due to altered rhythms in secretion of adipocytokines (Matsuzawa et al., 2006; Stefan et al. 2002).

Study in obese mice showed severe attenuation of adipokine secretion by adipose tissue. The authors suggest that the adipose clock may regulate rhythmic secretion adipocytokines in healthy mice. In obese mice, there is a loss of functional adipose clock resulting in altered secretion of adipokines (Chen et al., 2006; Ando et al., 2005). All of the above indicate the importance of a functional molecular clock in adipose tissue metabolism.

In our current study we compared temporal expression profile of core clock gene mRNA levels between layer-pullet and broiler-pullet in the hypothalamus, ovary and VAT. In broiler-pullet hypothalamus and VAT, several core clock genes oscillations were out-of-phase and a few were at statistically insignificant levels (Figure 13and 15). There were no rhythmic oscillations of any core clock gene mRNA in the broiler-pullet ovary, indicating an absence of a functional clock. These data suggest that immature broiler hens may possess either poorly functional or disrupted circadian clock in their hypothalamus, VAT and ovary with significant physiological implications. Disrupted circadian clock in hypothalamus may lead to improper energy-homeostasis capability thereby disrupting feed-intake and energy balance, a genetic predisposition already seen in broiler birds.

A poor clock in the adipose tissue may lead to disrupted circadian oscillation and secretion of adipokines. These adipokines in turn may impinge their disruptive effects on

ovarian function. Lack of circadian rhythms in core clock genes in the ovaries of broiler pullet also means that physiological functions within the ovary (e.g LH receptive window, ovulation, and steroidogenesis) may be either disrupted or desynchronized leading to poor reproductive capacity.

We thus propose a paradigm in which circadian clocks in the hypothalamus, VAT and ovary may contribute to ovarian function. We hypothesize that synchronization within and between these clocks are required to maintain rheostasis in reproductive events. Disruption of this synchrony might affect the reproductive capacity/fertility of the organism. We suggest that in the broiler breeder hens the circadian clock of the hypothalamus, VAT and ovary are out of sync/out-of-phase leading to an improper timing of the neuronal and endocrinal signaling between the tissues. This lack of coordination may explain the lack of regulation of energy homeostasis, propensity to obesity in absence of feed restriction practice and poor reproductive capacity in broiler hens.

### *5.4.5 Conclusions*

Ultimately, understanding the role of circadian clock and its timing cues in modulating immune function will have significant impacts on the fields on chronobiology, physiology, immunology, and endocrinology. Elucidating the pathways of circadian-immune interaction will provide new insight into the role of the circadian clock and further our understanding of an organism's health.

# 6. CONCLUSIONS AND FUTURE DIRECTIONS

## 6.1 The circadian inflammatory response in the avian spleen

From the results presented in this dissertation (section 2, 3 and 4) and evidence from published literature concur and show that the innate immune response is under circadian control. The results indicate that the avian spleen has a functional circadian clock with its core clock genes showing rhythmic oscillations in a daily (LD) and circadian (DD) manner. Avian spleen is a dynamic tissue with a large turnover of splenocytes, lymphocytes and macrophages. We examined the avian spleen for circadian clock in normal healthy chicks, and in birds undergoing acute systemic inflammation. Results from these studies have been discussed in sections 2 and 3. Interesting observations include daily rhythmic oscillations of mRNA of cytokine genes in the avian. These cytokine genes are proinflammatory in nature. These proinflammatory cytokines have been implicated in several chronic inflammatory diseases, disrupted sleep-wake cycles, obesity and reproductive disorders in several mammalian species including human beings. Several chronic disease conditions in circulating proinflammatory cytokines have been related to disrupted circadian clocks. These studies hypothesize that a functional circadian clock tightly regulates the rhythms of cytokine genes with very critical physiological implications. Long investigated, newer molecular techniques have identified several intriguing pathways of circadian-immune crosstalk that are means for circadian-immune regulation. Although the entire molecular mechanism has not been elucidated yet, there are several pieces of this puzzle that have been discovered as of now which are helping in

understanding these pathways.

Circadian clock in multicellular organism comprises of master oscillators and peripheral clocks. The aim of the current dissertation was to investigate peripheral tissues for the presence of a functional clock and their potential roles in regulating local physiological functions. Isolated immune cells have been shown to oscillate in terms of their raw numbers in the circulation (Arjona and Sarkar 2005; Haus and Smolensky 1999). Tissues from the immune system have been shown to oscillate both as tissue explants (using the PER2::luc mouse) and as mRNA (*mPer2* and *mRev-erbα*) extracted from mice in constant darkness over the 48h (Keller et al. 2009). Furthermore, mediators involved in inflammatory response exhibit oscillations independently and in response to LPS challenge in both human beings and murine models (Keller et al. 2009; Petrovsky et al. 1998).

In vivo studies, like the ones conducted as part of this dissertation cannot rule out the regulation of the central/master clock in the higher centers of the brain and inflammatory response in the avian spleen. Some authors have gone around this issue by isolating various cells from immune-tissues and exposing them to signaling molecules from the master clock (for instance norepinephrine, glucocorticoids, and melatonin etc) in order to compare to expression (gene/proteins) of the important cytokines in explants and cell culture settings that are free of these regulating factors.

In human beings, glucocorticoids (GC) are released in a circadian manner and have a negative correlation with production of IFNγ, TNFα and IL-12. Peak serum GC levels are associated with suppressed cytokine production (Petrovsky et al., 1998). Studies in

human volunteers show that IL-6 and IL-1β expression do not correlate with GC release, but IFN-γ, IL-8 and TNFα do, so it seems that circulating GC concentrations do, affect circulating cytokine concentrations. An association between GC concentration and TNFα production has been shown in serum (Parant et al. 1991), where pre-treatment of adrenalectomised mice with GC did not express TNFα expression, in response to LPS. However, there is no difference in TNFα or IL-6 circadian gene expression between the spleens of intact and adrenalectomised mice treated with LPS over the circadian day (Keller et al. 2009). This suggests that although in vivo cytokine production can be influenced by circulating GC, which is under direct control of circadian clock, yet there are some cytokines, such as TNFα and IL-6, that remain rhythmic even their absence. Furthermore, Keller et al. (2009) showed through an array study that 8% of the macrophage transcriptome is under circadian control with multiple levels of the inflammatory response.

Several genes involved in healing of wounds, stress response and phagocytosis exhibit rhythmic circadian expression. Also genes, whose protein products act in complexes such as cFOS and cJUN (AP-1) and CD-180 and MD-1 (TLR4 inhibitory molecules) are in-phase with each other, suggesting the role of circadian clock regulation. Taken together, although circulating factors such as GC can, and do, affect cytokine production. Thus, the central clock participates in regulating the immune system in several species of lab animals, yet the role of peripheral clocks cannot be ruled out. Inflammatory cells cytokines such as TNFα and IL-6may be under direct regulation of the peripheral clock, rather than the central clock.

The immune-regulation by circadian clock seems to have physiological significance. One hypothesis may be that at different times of the day an inflammatory response may be more harmful (i.e. lethal) than at other times. Doses of TNFα that are enough to kill mice at certain times of the day, can be less harmful during other times (late activity phase/night time) (Hrushesky et al., 1994; Hrushesky et al., 1997). In the Keller study, (Keller et al., 2009), the peak serum TNFα and IL-6 expression occurred during the mid to late subjective day (prior to the onset of activity), as did numbers of macrophages and monocytes, a time that corresponds with a high degree of TNFα induced mortality in mice (Hrushesky et al. 1994). It is interesting to speculate as to what could be the evolutionary advantages of such temporal profile. Perhaps it better prepared an individual against an anticipated bacterial infection at certain times of the day (for instance while foraging of food, hunting, pregnancy etc). Additionally, it would be a waste of energy to maintain a high level of circulating cytokines/chemokine at times of rest and while in relative safety from pathogen exposure. High levels of cytokines just (TNFα and IL-6 ) prior to the onset of activity may be in preparation for moving out of the safety of the nest, a time when the animal could be disorientated and susceptible to attack. In addition to such possibilities there could be one more reason for this tight circadian control on immune system, namely, prevention of hyper-immune response leading to the death of the host. Furthermore, circadian control of the inflammatory response would be beneficial to allow it to keep the phase of the immune-system and related organs in sync with the rest of the organ system in the body.

The immune system can directly influence the core clock as well as the clock

causing the oscillation of cytokine expression, and the relationship is therefore bi-directional. LPS can suppress E-box mediated transcription (Per2, Cry1 and Rev-erbα) via TNFα (Cavadini etal. 2007) which is most likely another 'pro-survival' mechanism as the loss of Per2 has been shown to increase survival after LPS administration (Liu et al. 2006). However, this action is opposite to the effects previously described above where TNFα was less likely to kill when administered during the late subjective night, when PER2 protein is at its highest.

Far more research needs to be carried out before the bi-directional relationship between the inflammatory response, the clock and ultimate survival is clarified. From the LPS-induced inflammatory response studies described in section 3, we hypothesized that a circadian clock output/signaling molecule, melatonin may be one of the candidate molecules capable of regulating the expression of proinflammatory cytokines in the avian spleen. To test this hypothesis, we studied the effects of priming the birds (with melatonin) prior of LPS administration at midday (section 4). Previous studies show intriguing results about immunomodulatory behavior of the melatonin.

Coming back to the study in the current dissertation, there are several experiments that can be added to add the missing pieces to the puzzle of peripheral clock-local immune system regulation." The interesting studies that can be pursued in the future to add to this study include, opposite effects of melatonin are observed on cAMP and IP3 production in the same cell depending on the activation state of cells and the involvement of specific subtypes of melatonin receptors. Avian splenocytes express different types of melatonin receptors. Melatonin binds to the Mel1c receptors decreases intracellular cAMP and

increases IP3 in unstimulated chick splenocytes (Markowska et al. 2004), whereas in LPS/mitogen-stimulated splenocytes it increases cAMP levels and decreases IP3 acting through MT2 receptors (Markowska et al. 2002).

## 6.2 Future directions

A list of interesting studies that can be done in the future that could add more interesting information to the current dissertation include but are not restricted to the following:

### *6.2.1 Experiment 1 to test the effect of neuroendocrinal signaling molecule, norepinephrine on inflammatory response*

Bidirectional communication exists between central nervous system and immune system. A signaling molecule which participates in this cross-talk is the catecholamine, Norepinephrine (NE). During an inflammatory response, immune cells secrete cytokines which activate the brain. This results in stimulation of sympathetic nervous system (SNS) with the release of NE. The NE modulates the level of immune activity and function by binding to the adrenergic receptors present on immune tissues and immune cells. We will investigate the effect of NE upon the daily dynamics of the inflammatory response.

We hypothesize that NE acts as an immunomodulatory molecule and regulates the diurnal levels of pro-inflammatory cytokines. We will test this hypothesis by injecting NE (Sigma-Aldrich) at midday (ZT6) and at midnight (ZT18). Day old birds (n=48) will be placed in 12:12LD photoperiod for approximately 5 weeks (~0.5 kg body weight). The

treatment groups (ZT6, n=12; ZT18, n=12) will get a single IV dose of NE 1 hour before IV injection of LPS (Escherichia coli O111:B4, Sigma-Aldrich; dose: 1.5mg/kg body weight). The control groups (ZT6, n=12; ZT18, n=12) will get a single IV dose of NE 1 hour before IV injection of 100µl of normal saline (0.9% NaCl). Spleen, thymus, bursa of Fabricius will be collected from 3 birds at 0hr, 1hr, 2hr, 3hr post-injection from the treatment and control groups. The tissues will be processed for examining the transcription profiles of immune function genes (TNF-α, IL-1β, IL-6 and IL-18) and clock genes using qRT-PCR.

### *6.2.2 Experiment 2 to determine the effect of pinealectomy upon immune tissue function*

Studies indicate that melatonin has immunomodulatory property and act as pro-inflammatory as well anti-inflammatory molecule. We will investigate effect of the absence of circulating melatonin on the rhythmic transcription profiles of immune function genes (TNF-α, IL-1β, IL-6 and IL-18) in both health and during inflammation.

#### *6.2.2.1 Experiments 2a effect of pinealectomy upon rhythmic immune tissue function in LD cycle*

In domestic chicken, pineal gland is the source of circulating melatonin hormone. We will examine the role of immunomodulatory role melatonin by surgically excising the pineal gland (pinealectomy). Day old birds (n=126) will be placed in 12:12LD cycle for 2 weeks. After 2 weeks, the experiment group (n=63) will be pinealectomized, while the control group (n=63) will undergo sham surgery. The experiment and control group will

get 10 days of recovery period (12:12 LD photoperiod; food and water ad libitum). After 10 days of recovery, pinealectomized and sham birds will be sacrificed (n=9, at ZT0, ZT3, ZT6, ZT12, ZT15, ZT18 and ZT21) by $CO_2$ asphyxiation. Spleen, thymus and bursa of Fabricius will be collected for determining temporal mRNA of immune function genes (TNF-α, IL-1β, IL-6 and IL-18) using qRT PCR.

*6.2.2.2 Experiment 2b effect of pinealectomy upon rhythmic immune tissue function under constant darkness DD*

We will test if immune function genes are under the regulation of melatonin under constant conditions. Day old birds (n=126) will be placed in 12:12LD cycle for 2 weeks. After 2 weeks, the experiment group (n=63) will be pinealectomized, while the control group (n=63) will undergo sham surgery. The experiment and control group will get 10 days of recovery period (12:12 LD photoperiod; food and water ad libitum). After 10 days of recovery, pinealectomized and sham birds will be sacrificed (n=9, at CT0, CT3, CT6, CT12, CT15, CT18 and CT21) by $CO_2$ asphyxiation. Spleen, thymus and bursa of Fabricius will be collected for determining temporal expression profiles of mRNA levels of immune function genes (TNF-α, IL-1β, IL-6 and IL-18) using qRT-PCR.

*6.2.2.3 Experiment 2c study the effect of pinealectomy on inflammatory response*

We will test if the loss of circulating melatonin alters the temporal expression profile of pro inflammatory cytokine genes in birds undergoing inflammatory response. Day

old birds (n=48) will be housed in 12:12LD photoperiod for 2 weeks. At 3 weeks of age, pinealectomy (n=24) and sham (n=24) surgeries will be performed and the birds will be placed in 12:12 LD till they are 5 weeks old. The pinealectomized-treatment group (n=12) and sham-treatment group (n=12) will be injected with a single IV dose of LPS (LPS, Escherichia coli 011:b4; Sigma-Aldrich; Dose: 1.5mg/kg body weight) at mid-day (ZT6, n=12) and mid-night (ZT18, n=12). Spleen, thymus, bursa of Fabricius will be collected from 3 birds at 0hr, 1hr, 2hr, 3hr post LPS injections at ZT6 and ZT18 time points respectively. The pinealectomized-control group (n=12) and sham-control group (n=12) will be administered with 100µl of Normal saline (0.9% NaCl) IV. Spleen, thymus, bursa of Fabricius will be collected from 3 birds at 0hr, 1hr, 2hr, 3hr post normal saline injection at ZT6 and ZT18 time points respectively. We will examine the immune tissues for transcription profiles of pro-inflammatory cytokine genes (TNF-α, IL-1β, IL-6 and IL-18) using in qRT-PCR.

**6.3 Expected results**

Experiment1: If the peripheral clock regulates the immune function genes in time of day dependent manner, then immune challenging the birds at different times of the day should result in exhibition of differential immune response. LPS challenge should attenuate rhythms of immune tissue function and immune response depending upon the time of day.

NE is a catecholamine neurotransmitter capable of regulating immune tissue functions during inflammation. If NE has a pro-inflammatory property during day time,

we should see an increased expression of pro-inflammatory cytokine genes in birds primed with NE followed by an immune challenge with LPS. Conversely, if NE is has anti-inflammatory property, then immune-challenging the birds primed with NE should result in an attenuation of immune response.

Experiment 2a-2c: If melatonin is regulating the rhythms of immune tissue oscillations, then pinealectomy should lead to loss or disruption of these rhythms. Pinealeactomized birds should show loss or disruption in the rhythms of immune function genes in a daily and circadian manner. If melatonin is capable of modulating an inflammatory response, then pinealectomized birds undergoing endotoxic shock should show statistically significant difference in transcription profiles of pro-inflammatory cytokines depending on time of the day when compared to pineal gland-intact birds.

**6.4 To summarize the results from sections 2, 3 and 4**

- In the spleen, core clock genes, as well as genes for proinflammatory cytokines including Tnfα and IL-1β exhibit rhythmic oscillations of mRNA abundance and are under control of the clock.
- Acute melatonin administration at midday induces expression of some, but not all proinflammatory cytokines in the spleen.
- LPS-induced systemic inflammation initiated at midday versus midnight results in a differential immune response of proinflammatory cytokine induction indicating regulation by the clock.
- Exogenous melatonin administration at midday prior to LPS stimulation conveys

pleiotropic effects; enhancing and repressing cytokine induction indicating melatonin functions as both a pro and anti-inflammatory molecule in the spleen. Hence the data suggests that the rhythmic properties of the spleen including the differential immune response to inflammation are mediated by the circadian clock and the hormone melatonin.

The Hypothalamus-Adipose-Ovary clocks in egg-type vs. meat-type breeder hens: a comparative study: Around 5-10% of women suffer from a condition called polycystic ovarian syndrome (PCOS). The primary symptoms being, anovulation/oligoovulation and polycystic ovarian morphology. Although the symptoms are well known, the mechanism of the disease onset are still largely under investigation (Xita and Tsatsoulis, 2006; Ehrmann, 2005). PCOS is often present with another medical condition known as metabolic syndrome. The metabolic syndrome is characterized by obesity, high cardiovascular disease risk, hyperinsulinemia, dyslipidemia and diabetes-like condition (Ehrmann, 2005). It is hypothesized that excessive ovarian androgen secretion may be an underlying cause of PCOS (Balen et al., 2009; Homburg, 2009; Padmanabhan et al, 2006). The excess androgen might bind to the androgen receptors present on the SCN and alter the SCN-regulated circadian rhythms such as body temperature, hormone secretion, metabolic cycles (Butler et al., 2009; Karatsoreos et al., 2007). One more school of thought about etiology of PCOS is presence of abnormal clock gene in the ovary. The abnormal clock gene expression directly impairs the biosynthesis of steroid hormones leading to excess androgen synthesis and PCOS. Scientists studying PCOS in murine models have focused on the role of the HPG axis, including role of the clocks present in the cells forming the HPG axis. It is hypothesized that impairment of clocks in any one part of the HPG axis or

their downstream regulatory pathways may throw-off the delicate balance regulating the reproductive function. Bohler et al., (Bohler et al., 2010) proposed the model which relates adipose tissue with ovarian physiological functions as summarized in Figure 18.

The meat-type broiler breeder hens are known to have poor reproductive capacity and are incapable of energy balance when fed ad libitum. The breed's propensity to obesity and poor reproduction is similar to the females with PCOS associated with excess body fat. Hence, we set out to investigate the adipose tissue (visceral adipose tissue, VAT), hypothalamus and ovary in female broiler breeder chicken for the presence of rhythmic core clock gene expression. These results were compared to the results in egg-type breeder chicken which are known for efficient energy metabolism and reproductive function.

We compared temporal expression profile of core clock gene mRNA levels between layer-pullet and broiler-pullet in the hypothalamus, ovary and VAT. In broiler pullet hypothalamus and VAT, several core clock genes oscillations were out-of-phase and a few were at statistically insignificant levels (Section 5). There were no rhythmic oscillations of any core clock gene mRNA in the broiler-pullet ovary, indicating an absence of a functional clock. These data suggest that immature broiler hens may possess either poorly functional or disrupted circadian clock in their hypothalamus, VAT and ovary with significant physiological implications.

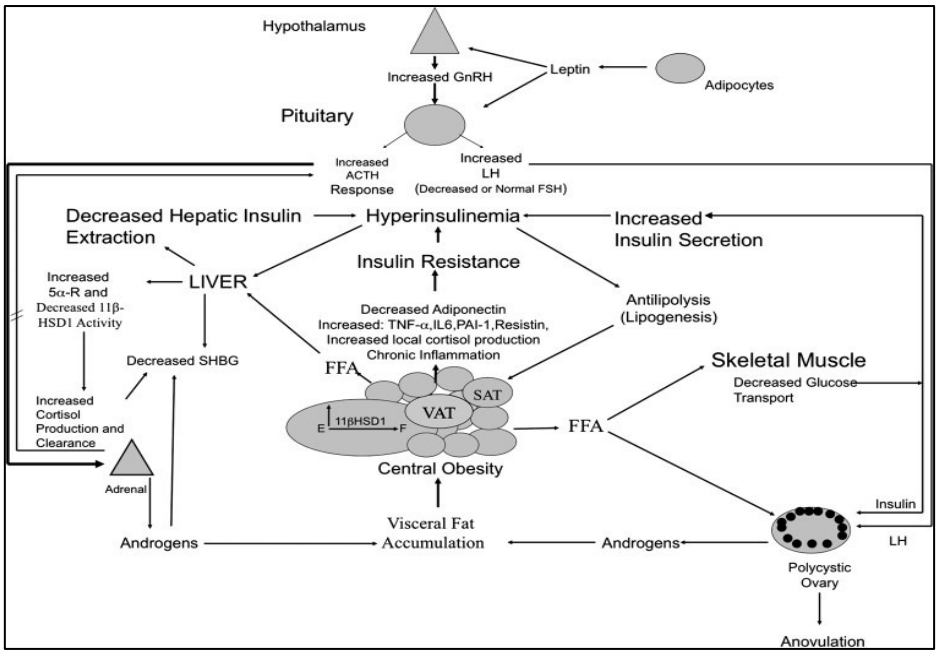



Figure 18. Role of VAT and SAT in PCOS pathophysiology). The above model hypothesizes the possible role of VAT and SAT in the pathophysiology of polycystic ovary syndrome (PCOS). Increase in VAT may result in elevated serum androgen and local cortisol. Adipokine and FFA in liver and skeletal muscle may contribute to insulin resistance. Insulin acts as a co-gonadotropin, amplifying the effect of LH to induce androgen production from the ovary leading to anovulation. Leptin may play a role in the increase in LH secretion although other factors and obesity dampen this effect. VAT = visceral adipose tissue; SAT = subcutaneous adipose tissue; FFA = free fatty acid; HSD = hydroxysteroid dehydrogenase; E = cortisone; F = cortisol; R = reductase; SHBG = sex hormone binding globulin; ACTH = adrenocorticotropic hormone; LH = luteinizing hormone; GnRH = gonadotropin releasing hormone; TNF = tumor necrosis factor; IL = interleukin; PAI = plasminogen activator inhibitor (Bohler et al., 2010).

Disrupted circadian clock in hypothalamus may lead to improper energy-homeostasis capability thereby disrupting feed-intake and energy balance, a genetic predisposition already seen in broiler birds. A poor circadian clock in the adipose tissue may lead to disrupted circadian oscillation and secretion of adipokines. Disordered adipokine secretion may, in turn, provoke additional disruptive effects on ovarian function. Lack of circadian rhythms in core clock genes in the ovaries of broiler-pullet also means that physiological functions within the ovary (e.g LH receptive window, ovulation, and steroidogenesis) may be either disrupted or desynchronized leading to poor reproductive capacity. From the results, we hypothesized that synchronization within and between these clocks are required to maintain rheostasis in reproductive events. Disruption of this synchrony might affect the reproductive capacity/fertility of the organism. We suggest that in the broiler breeder hens the circadian clock of the hypothalamus, VAT and ovary are out of sync/out-of-phase leading to an improper timing of the neuronal and endocrinal signaling between the tissues. This lack of coordination may explain the lack of regulation of energy homeostasis, propensity to obesity in absence of feed restriction practice and poor reproductive capacity in broiler hens.

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