

THE PENNSYLVANIA STATE UNIVERSITY

SCHREYER HONORS COLLEGE

DEPARTMENT OF POULTRY SCIENCE

CONTROL OF SEROTONERGIC SIGNALING BY THE DUODENUM

ELIZABETH EBERT-ZAVOS

Summer 2011

A thesis
submitted in partial fulfillment
of the requirements
for a baccalaureate degree
in Animal Sciences
with honors in Animal Sciences

Reviewed and approved* by the following:

Dr. Paul Bartell
Assistant Professor of Avian Biology
Thesis Supervisor

Dr. Troy Ott
Associate Professor of Reproductive Physiology
Honors Adviser

- Signatures are on file in the Schreyer Honors College.

ABSTRACT

The main objective of my thesis was to determine how the circadian clock in the duodenum regulates serotonin synthesis and release in order to regulate circadian rhythms in digestive and skeletal systems. I hypothesized that a circadian clock exists in the duodenum and that this clock controls the biosynthesis and release of serotonin on a daily basis. In this series of experiments, duodenum and plasma from chickens were examined at different times of the day for the purpose of determining if: 1) the duodenum contains a functional molecular circadian clock; 2) the molecular circadian clock regulates genes that control serotonin biosynthesis; 3) the amount of serotonin in the duodenum varies across the day; and 4) serotonin profiles in blood are rhythmic. The circadian regulation of plasma serotonin by the duodenum may represent an additional biochemical signal in the blood encoding time and could be used by target tissues to indicate the status of nutrient absorption, in particular calcium. Serotonin has shown to cause bone degradation in mice and humans by affecting calcium regulation. Because serotonin affects duodenal absorption, the circadian molecular clock could control calcium bioavailability. If this clock can be regulated, it would be possible to modify other clocks to enable laying hens to produce eggs with thicker, stronger, eggshells and also reduce skeletal problems, such as osteoporosis.

TABLE OF CONTENTS

LIST OF FIGURES.....	v
LIST OF TABLES	vi
ACKNOWLEDGEMENTS.....	vii
INTRODUCTION	1
Circadian rhythms	1
Serotonin	7
Melatonin.....	9
Bone remodeling.....	12
Medullary bone	14
Eggshell formation	15
Objectives.....	18
MATERIALS AND METHODS.....	19
Statistics	25
RESULTS	26
Rhythmic gene expression	26
Serotonin concentrations in duodenum and plasma	32
DISCUSSION.....	35
LITERATURE CITED	40

LIST OF FIGURES

Figure 1: The hypothesized “Neuroendocrine Loop”	3
Figure 2: Transcription of clock genes	5
Figure 3: Diagram of tissue harvest times	19
Figure 4: Graphs of average fold changes of <i>bmal1</i> , <i>bmal2</i> , and <i>clock</i>	28
Figure 5: Graphs of average fold changes of <i>per2</i> , <i>per3</i> , and <i>cry1</i>	29
Figure 6: Graph of average fold changes of <i>VIP</i>	30
Figure 7: Graphs of average fold changes of <i>TPH</i>	31
Figure 8: Graph of serotonin concentrations in the duodenum.....	33
Figure 9: Graph of serotonin concentrations in the plasma	34

LIST OF TABLES

Table 1: Forward primer sequences for qRT-PCR.....	22
Table 2: Reverse primer sequences for qRT-PCR.....	22
Table 3: Average fold changes of clock gene and clock-controlled gene expression.....	26
Table 4: Probability values for statistics on clock gene expression.....	27

ACKNOWLEDGEMENTS

I would like to thank Dr. Bartell, Dr. Ott, and Dr. Maria Horvat-Gordon for their support and guidance. Additionally, I would like to thank to the Schreyer Honors College, the College of Agricultural Sciences, and the Department of Poultry Science for providing funds for this project.

INTRODUCTION

Circadian Rhythms

All organisms organize their biological processes according to the cycles found within their environment, and the temporal control of these processes is regulated by biological clocks found within the organism. Biological clocks generate rhythms that are repeated at regular intervals throughout the life cycle of the organism. The period of the rhythm (cycle length) is defined as the time between two consecutive identical phases of the rhythm, which can range in length from minutes to months (Bell-Pederson et al., 2005). The most commonly studied biological clocks in vertebrates are those that control rhythms that recur on a daily (circadian; *circa*= about, *dies*= day) or a yearly (circannual; *circa*=about, *annum*=year) basis (Kumar et. al, 2010).

Endogenous circadian rhythms have several defining properties. First, they are repeated daily (over an approximately 24 hour period) without the influence of external environmental stimuli. The length of this unsynchronized, or unentrained, rhythm is referred to as the free-running period (τ). Second, circadian rhythms are able to entrain to cyclical environmental stimuli. Synchronizing cues are known as Zeitgebers (German for “time giver”) and the dominant Zeitgeber for most species is the daily cycle of light and dark. Lastly, circadian rhythms must exhibit temperature compensation; that is, the period length of the rhythm does not change significantly when the temperature of the natural environment varies within the organism’s physiological range. Temperature compensation demonstrates that biochemical or

physiological buffering exists within the organism to compensate for the typical changes in rates of biochemical reactions with changes in temperature (Bell-Pederson et al., 2005).

The circadian system includes a central pacemaker in the brain, which dictates the phasing and speed of clocks in peripheral tissues (Herzog et al., 2001). An endogenous circadian pacemaker is a specialized oscillator that operates independently to drive rhythmic outputs, either directly or indirectly, and typically works by influencing clocks in peripheral tissues or organs. The phase of the pacemaker is adjusted daily by environmental cues (Bell-Pederson et al., 2005). In mammals, the central pacemaker is found in the neuronally-dense suprachiasmatic nucleus (SCN) of the hypothalamus. Because these cells control biological processes by firing in rhythmic patterns over the course of the day, the removal of the SCN results in arrhythmicity (Herzog et al., 2001). In avian species, central pacemakers are found within the avian homolog of the suprachiasmatic nucleus, within the pineal gland, and within the retina. These clocks function together to form the avian circadian clock system. It has been proposed that the avian circadian system is a “Neuroendocrine Loop”, which functions through the interactions of the SCN, pineal, and retina (Figure 1; Cassone and Menaker, 1984). The three individual components are hypothesized to contain coupled circadian oscillators which vary in their innate circadian periods. Within this system, multiple sets of photoreceptors exist that enable entrainment of the system as a whole (Cassone and Menaker, 1984).

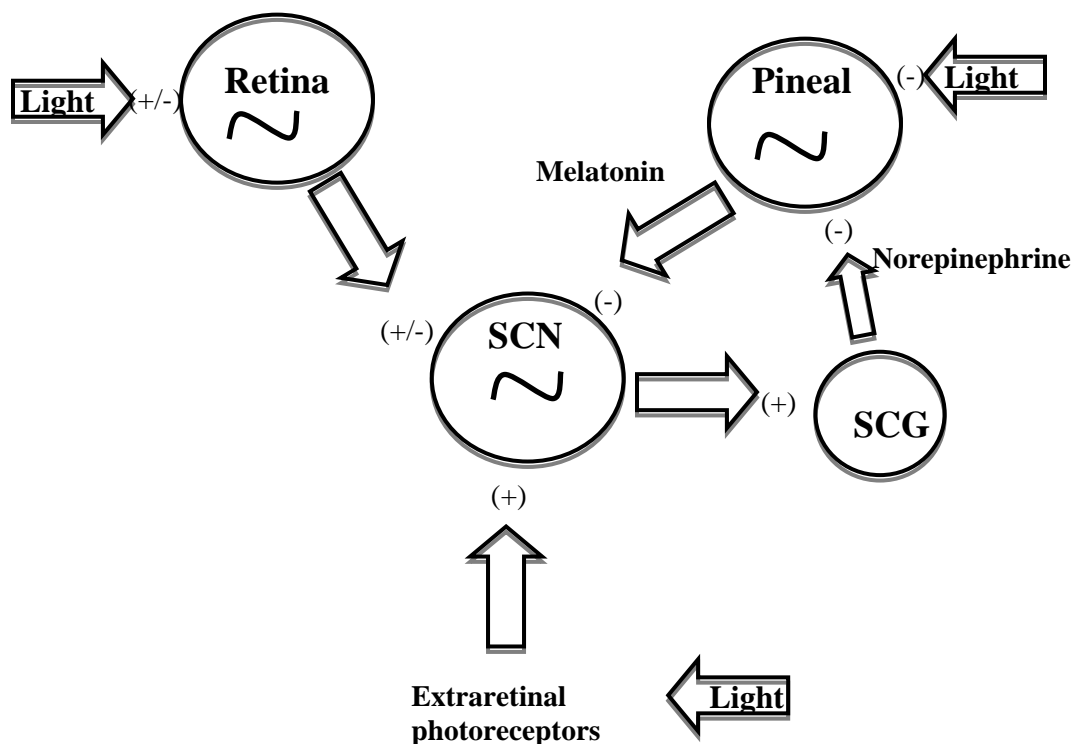


FIG 1. The proposed “Neuroendocrine Loop” for the circadian system. The pineal and possibly the retina synthesize and release melatonin into the bloodstream during the night. This melatonin inhibits the activity of the SCN. During the daytime, light acts on the pineal and retina to inhibit melatonin secretion, which enables the SCN to resume functioning. The retina also affects the SCN via direct retinal innervation (Cassone and Menaker, 1984). The superior cervical ganglion (SCG) inhibits the production of melatonin during the day through the release of norepinephrine from the pineal gland (Zatz, 1991).

Clock genes were discovered first in *Drosophila* in the early 1970s. The *period* (*per*) gene was identified as a clock gene because the knockout of *per* abolished rhythms in constant darkness. Modifications of *per* altered the length of the free-running period (Konopka and Benzer, 1971). A τ mutant with a shorter period in constant darkness was discovered within an in-bred line of golden hamsters used as research models (Ralph and Menaker, 1988). SCN transplants from wild type hamsters restored rhythmicity and transferred period length, indicating that τ can be transferred among individuals and that gene products in the SCN are responsible for production and features of circadian rhythms (Miller, 1989).

At the cellular level, circadian clocks function through the auto-regulatory actions of interlocking positive and negative feedback loops of clock genes. The basic helix-loop-helix-PAS transcription factors *circadian locomotor output cycles kaput* (*clock*) and *brain and muscle ARNT-like protein* (*bmal*) heterodimerize and activate E-box (5'-CACGTG-3')-mediated transcription of the negative elements of the loop, particularly *period* (*per*) and *cryptochrome* (*cry*; Herzog et al., 2001). The protein products of *per* and *cry* translocate into the nucleus and repress *bmal* and *clock*-mediated E-box activation, thereby completing the cycle (Herzog et al., 2001). Genes that are controlled by molecular circadian clocks have E-boxes in their promoter regions and are regulated at the level of transcription by *clock* and *bmal* activation in a rhythmic fashion (Figure 2). The positive and negative feedback loops are linked by REV-ERB α (also known as nuclear receptor subfamily 1, group D, member 2 or Nr1d2), a protein that acts as a transcriptional repressor. In addition

to the expression of *per* and *cry*, the expression of REV-ERB α is also driven by *clock* and *bmal*. REV-ERB α also inhibits *bmal* transcription via a retinoic-acid-related-orphan-receptor response element located within the *bmal* promoter. The negative feedback elements (*per* and *cry*) are able to repress expression of REV-ERB α , allowing for renewed expression of *bmal* and/or *clock* (Fu et al., 2005).

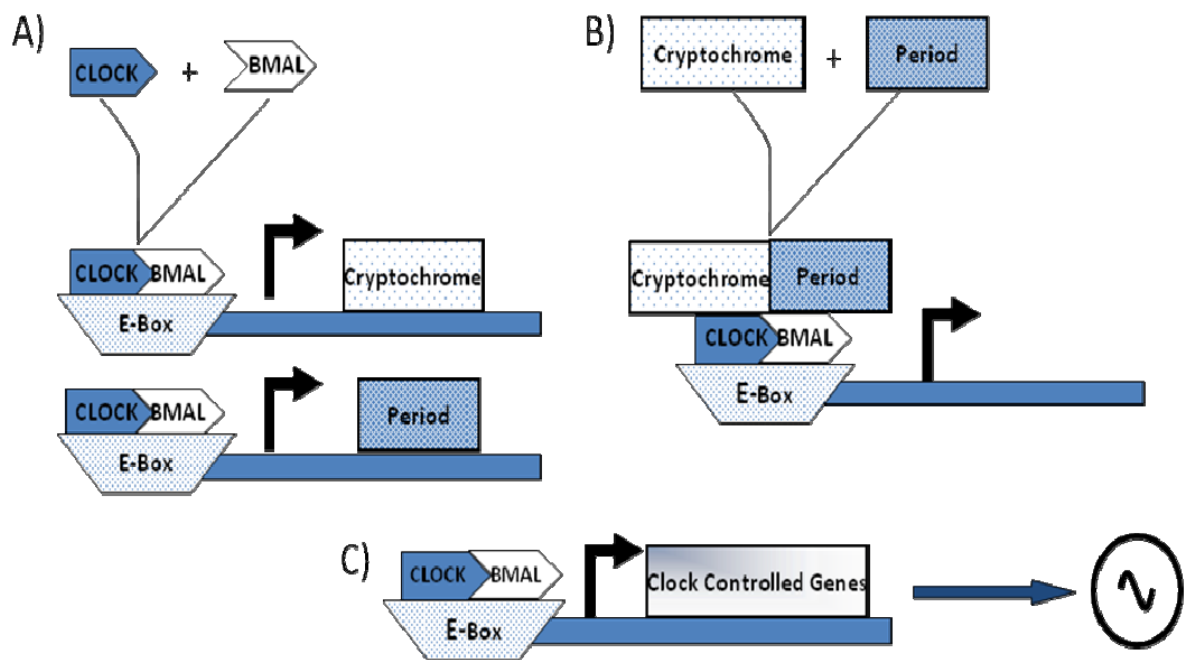


FIG 2. A) *Clock* and *bmal* heterodimerize and bind to genes with E-boxes in their promoter regions, such as *cry* and *per*, to turn on transcription. B) Negative feedback elements *cry* and *per* dimerize, enter into the nucleus, and repress *clock/bmal*-mediated E-box activation. C) Genes regulated by the circadian clock contain E-boxes in their promoter regions and can thus be activated by *clock/bmal*. Transcription of these genes occurs rhythmically (Figure taken from Bartell, 2010).

In birds, several homologs of *Drosophila* clock genes have been found in the retina, pineal, and SCN, including *per* genes (*per2* and *per3*), *cry* genes (*cry1* and *cry2*), *bmal* genes (*bmal1* and *bmal2*), and *clock* (Kumar et al. 2010; Larkin et al. 1999; Chong et al. 2000, 2003; Yoshimura et al. 2000, 2001; Brandstätter et al. 2001a; Bailey et al. 2002; Fu et al. 2002; Haque et al. 2002; Yasuo et al. 2002, 2003). Avian homologs of *bmal1*, *bmal2*, and *clock* are rhythmically expressed in the pineal gland with levels highest later in the day. However, in the retina, only *bmal1* expression is rhythmic. Similarly, *per2*, *per3*, *cry1*, and *cry2* are all rhythmic in the pineal whereas only *per3* and *cry1* are rhythmic in the retina (Bell-Pederson et al., 2005). The avian clock system therefore differs from that of mammals as indicated by the lack of an avian *per1* homolog and variations in the phasing of some of the clock gene constituents (Abraham et al., 2002; Brandstatter et al., 2001; Yoshimura et al., 2000; Abraham et al., 2003).

Vasoactive intestinal polypeptide (VIP) is a neuropeptide correlated with circadian rhythms that has been shown to be involved in the regulation of circadian output from the pineal gland (Faluhelyi et al, 2006). In particular, VIP triggers the release of melatonin from the pineal in adult chickens without affecting the properties of the intrinsic molecular circadian clock (Faluhelyi et al, 2006). Besides influencing melatonin release, VIP and its receptors are found in areas of the brain responsible for regulating feeding (Jozsa et al, 2006). In the avian digestive tract, VIP functions as a neurotransmitter in the intestinal wall neurons to stimulate glandular

organ secretions and inhibit enteric smooth muscle movement (Sundler et al, 1979; Liu et al, 2009; Epstein and Poulsen, 1991; Suzuki et al, 1996; Liu et al, 2009).

Serotonin

Serotonin, also known as 5-hydroxytryptamine (5-HT), is a bioamine derived from tryptophan. Besides being a neurotransmitter associated with mental disorders, such as depression, serotonin is also synthesized in the gut and has receptors in bone, indicating this neurotransmitter has widespread physiological functions beyond those typically associated with neurobiological function (Rosen, 2009). Serotonin is produced in brainstem neurons and enterochromaffin cells of the duodenum (Yadav et al., 2008). Tryptophan hydroxylase 1 (*TPH1*) is the key enzyme involved in the synthesis of serotonin in the gut whereas tryptophan hydroxylase 2 (*TPH2*) is the predominant enzyme for serotonin synthesis in the brain (Yadav et al., 2008).

Gut-derived serotonin is unable to cross the blood-brain barrier and, as a result, serotonin has varying functions depending on the location of its production. In the duodenum, serotonin production, specifically *TPH1*, is regulated by low-density lipoprotein receptor-related protein 5 (LRP5). Once produced, serotonin can enter circulation and inhibit the formation of osteoblasts by binding to Htr1b, a serotonin receptor on preosteoblasts, and inhibiting the intracellular transcription factor, cyclic AMP response element (CREB; Rosen, 2009). CREB is also involved in the control of several circadian genes, including *per* genes and *clock*, suggesting that there is a correlation between bone regulation and circadian rhythms (Rosen,

2009). Humans taking selective serotonin reuptake inhibitors (SSRIs), drugs that increase extracellular serotonin concentrations in the body, for treatment of depression and other affective disorders have been shown to have decreased bone mass (Yadav, 2008).

TPH1 is activated by the mucosal stimulation in the gut that occurs after meals and catalyzes the production of serotonin from tryptophan (Rosen, 2009). Serotonin then diffuses into enteric nerve endings to promote peristalsis, e.g. the movement of food through the digestive system, or enters circulation (Rosen, 2009). When in the blood, serotonin attaches to 5-hydroxytryptophan transporters (5-HTT) and is inactivated via reuptake by platelets (Rosen, 2009; Yadav et al., 2008). In the gut, 5-HTT is found in epithelial cells (Rosen, 2009). The portion that remains in blood plasma may act as a hormone which, after binding to receptors found on target cells, could potentially provide timing cues to target tissues involving the digestive system and nutritional status (Yadav et al., 2008).

In some tissues, such as the eye and pineal, it has been demonstrated that serotonin is produced under the control of the molecular circadian clock by activating transcription of Tryptophan Hydroxylase (*TPH*) (Thomas and Iuvone, 1989). Additionally, it has recently been determined that bone remodeling is directly regulated by blood-borne serotonin and that the primary source of plasma serotonin is the duodenum, as serotonin cannot cross the blood-brain barrier (Yadav, 2008). This, coupled with the findings that serotonin is synthesized under

the direct control of a circadian clock in the brain and eyes, suggests that a circadian clock could control bone remodeling.

In addition to its role in bone remodeling, serotonin also has an effect on gut motility. Neurotransmitters, such as tachykinins and acetylcholine, are known to stimulate motility whereas peptides from the vasointestinal protein (VIP) family, such as VIP, and gas transmitters, such as carbon monoxide and nitric oxide, inhibit motility (Velarde, 2010). Serotonin can be both depending on the species and position in the digestive tract (Velarde, 2010). In the chicken, serotonin has been shown to cause gut contractions (Velarde, 2010).

Serotonin is a precursor to endogenous melatonin production. Interestingly, in the gut and in bone remodeling, serotonin and melatonin have opposite effects. Serotonin causes gut contraction and is involved in bone breakdown whereas melatonin induces gut relaxation and is thought to be involved in fracture repair.

Melatonin

Melatonin is the major hormone involved in the maintenance of circadian rhythms. The pineal gland, and in some cases the retina, influence the avian circadian system by secreting the neurohormone at night. Because the pineal gland (and retina) are light sensitive, melatonin production can vary according to the light-dark cycle with peak synthesis and release occurring during darkness. This rhythm provides temporal information to the organism and, in doing so, helps regulate physiological functions such as locomotor activity, feeding, and seasonal timing of reproduction (Natesan et al., 2002).

It has been suggested that the avian pineal and retina inhibit SCN activity during the night through the secretion of melatonin while the SCN inhibits the synthesis of melatonin in the pineal during the day (Karaganis et al., 2009). This hypothesis is based on the proposed model that the three pacemakers in the avian circadian system—the pineal gland, the retina, and the avian homolog of the SCN—contain dampened oscillators that interact within the “Neuroendocrine Loop” to maintain rhythmicity over multiple cycles (Karaganis et al., 2009; Cassone and Menaker, 1984). Besides inhibiting the functioning of the SCN, melatonin synthesized by the pineal gland is secreted into the bloodstream to control peripheral clocks that possess melatonin receptors (Bell-Pederson 2005).

In most vertebrate species, the primary source of endogenous blood-borne melatonin is the pineal gland. Melatonin, however, can also be synthesized in the gut and plays a role in the digestive process (Bubenik, 2002). Melatonin is produced in the vertebrate retina and pineal gland through the following pathway: dietary tryptophan → 5-hydroxy-L-tryptophan → 5-hydroxytryptamine (serotonin) → N-acetyl-serotonin → melatonin (Klein et al., 1981; Nesharse et al., 1988; Cahill and Besharse, 1990). Serotonin is acetylated by the enzyme arylalkylamine-*N*-acetyltransferase (AA-NAT) and the product subsequently methylated by hydroxyindole-*O*-methyl transferase (HIOMT) to melatonin. Control of the production and activity of these enzymes are key mechanisms by which molecular clocks regulate melatonin biosynthesis (Natesan et al., 2002).

In mammals, a circadian clock located in the SCN regulates the production of melatonin by the pineal through indirect input by the postganglionic fibers of the superior cervical ganglia (SCG) and is entrained to the light/dark cycle by light perceived through the eyes (Miller, 1989). The release of norepinephrine from the SCG induces the production of melatonin at night by stimulating expression of the enzyme involved in the rate-limiting step in melatonin synthesis, aralkylamine N-acetyltransferase (AA-NAT; Zatz, 1991). In contrast, in avian species, melatonin synthesis and release is directly regulated by oscillators found in the pineal gland and indirectly by oscillators in the avian homolog of the SCN through innervation by the SCG. The SCG inhibits the production of melatonin during the day by releasing norepinephrine onto the pineal gland (Zatz, 1991). Pineal cells in the chicken are also intrinsically photosensitive and can thus independently entrain the pineal circadian clock to light dark cycles (Bernard et al., 1997).

Melatonin has recently been linked to fracture repair in rats (Halici et al., 2010). The antioxidant activity of melatonin prevents the negative effects of free oxygen radicals on fracture healing. Neutrophils, a type of white blood cell, are produced in the early stages of fracture repair and release reactive oxygen metabolites, such as superoxide, which can cause tissue injury and damage. Free oxygen radicals promote the formation and activation of osteoclasts. Melatonin also stimulates several anti-oxidative enzymes, further enhancing its antioxidant properties (Halici et al., 2010). Consequently, melatonin has been thought to have an inhibitory effect on bone resorption. Additionally, melatonin has also been shown to

stimulate osteoblastic proliferation and mineralization of bone matrix *in vitro* (Halici et al., 2010). Based on its effects on osteoblasts and osteoclasts, melatonin levels are positively correlated with fracture healing.

Bone Remodeling

Bone remodeling refers to the physiological process in which vertebrates renew their bone matrix during adulthood. This process takes place in two stages. First, pre-existing mineralized bone matrix undergoes resorption by a specialized cell type known as an osteoclast. Next, *de novo* bone formation occurs via another specialized cell type known as an osteoblast (Yadav, 2008). An important regulator in bone remodeling is LDL-receptor related protein 5 (LRP5). LRP5 is a protein found on the cell surface and is a co-receptor for Wnt proteins, which are the vertebrate homologs of Wingless, a growth factor in *Drosophila* (Rosen, 2009; Bhanot et al., 1996; Yadav et al., 2008). The Wnt signaling pathway is involved in the regulation of bone mass and bone regeneration. LRP5 is also involved in the regulation of *TPH1* and, consequently, of serotonin production in the gut. In LRP5 deficient mice, *TPH1* expression in the gut and levels of serotonin in plasma increased (Yadav et al., 2008; Yadav et al., 2010). Mice deficient in LRP5 that were fed low tryptophan diets to decrease plasma serotonin concentration were shown to have normal bone mass and normal bone formation (Yadav et al., 2008). These findings suggest that serotonin plays a role in bone remodeling by promoting bone resorption.

Bone remodeling is considered to be a homeostatic function, since bone resorption and formation must occur sequentially and in a balanced manner to keep bone mass constant (Fu, 2005). Because most homeostatic functions occur in a circadian fashion, the circadian clock could control bone remodeling (Fu, 2005). The daily oscillation in the production of type 1 collagen and osteocalcin, the two main biosynthetic products of osteoblasts, supports this hypothesis (Fu, 2005). Mutations in clock genes have been shown to affect bone remodeling, further supporting this hypothesis. In *per1*, *per2*, *cry1*, and *cry2* knockout mice, there was a significant increase in the number of osteoblasts (Fu, 2005). Mice lacking *per* genes or possessing a mutated *per2* PAS domain exhibited a high bone mass, particularly in vertebrae and long bones (Fu, 2005). This high bone mass phenotype was also seen in *cry1* and *cry2* knockouts (Fu, 2005). These observations suggest that the molecular clock regulates bone mass and that high bone mass is a loss-of-function phenotype in circadian mutants (Fu, 2005).

An imbalance between the bone resorption and formation can lead to osteoporosis or other bone degenerative diseases (Yadav 2008). Osteoporosis is the increased reduction in the amount of mineralized structural bone over time that leads to bone fragility and increased incidence of fractures. Bone weakness, primarily due to osteoporosis, can lead to paralysis in laying hens. The paralysis seen in layers can sometimes be credited to spinal degeneration, but is most likely due to inhibition of muscular activity due to irregularities in intracellular calcium metabolism (Whitehead, 2004). These abnormalities can also deplete calcium

reserves in bone and increase the severity of osteoporosis (Whitehead, 2004). About 30% of all laying hens experience fractures, a statistic that can be attributed to the prevalence of osteoporosis (Whitehead, 2004). Consequently, understanding the roles of clocks and bone regulation could be important biologically.

Medullary Bone

Medullary bone is a significant (about 30-40%) source of calcium for shell formation. Medullary bone is a type of bone found only in birds and crocidilians on the surfaces of structural bone and in spicules within the medullary cavities (Whitehead, 2004). Medullary bone is produced by osteoblasts starting at the onset of reproductive maturity and it differs from cortical bone in that it contains a fewer collagen fibrils and has a higher degree of calcification (Whitehead, 2004). In comparison to structural bone, medullary bone has a rapid turnover rate. Calcium is needed in the highest amount during the time the egg is in the shell gland, the site of shell formation. This period usually occurs at night when there is limited availability of dietary calcium. When there is not enough calcium in the diet, the skeleton, primarily medullary bone, becomes an essential source of calcium (Whitehead, 2004). Thus, a significant portion of the calcium for shell production comes from osteoclastic resorption of medullary bone. However, osteoclasts do not act only on medullary bone during this period. These cells also breakdown exposed structural bone when medullary bone stores are insufficient, leading to bone loss characteristic of osteoporosis (Whitehead, 2004). Medullary bone is rapidly formed during the early stages of egg laying and can continue to build up slowly over the

rest of the laying period (Whitehead, 2004). During the time when medullary bone is being produced, structural bone formation is reduced (Whitehead, 2004). However, bone resorption via osteoclasts still proceeds when calcium is needed. This breakdown can lead to a loss in structural bone and ultimately cause osteoporosis and bone fracture even though total bone content may remain constant or even increase due to the presence of medullary bone. Medullary bone is much weaker than structural bone because it is woven with an irregular arrangement of collagen fibrils and most calcium is contained in spicules (Whitehead, 2004). Once the hen stops laying, the formation of structural bone recommences and medullary bone is fully resorbed (Whitehead, 2004). Thus, structural bone loss and regeneration is cyclical based on the hen's current stage in the egg laying process. The genetic selection for longer clutches in laying hens is thought to lead to increased susceptibility for osteoporosis due to negative calcium balance.

Eggshell Formation

Domestic hens are the most common model for studying eggshell formation due to their lack of photorefractoriness and sequential pattern of laying eggs (Bar, 2009). Additionally, their high reproduction rate and economic importance make hens an important focus of study. Industrialization and environmental contamination have compromised egg shell integrity (Bar, 2009). Eggshell quality has become particularly important because humans consume 10^{12} eggs per year (Bar, 2009).

Egg laying involves an extra calcium pathway, withdrawal through the shell gland. This calcium pathway seems to exist without the control of the three main calcium-regulating hormones, the parathyroid hormone, the hormonal form of vitamin D3, and calcitonin, and is therefore different from calcium transport in the intestine, bone, and kidney (Bar, 2008 and 2009).

During the period of egg laying and shell calcification, calcium homeostasis in the hen is challenged because shell calcification utilizes more calcium than that which is present extracellularly (Bar, 2009). The calcium content of successive eggs in a clutch has been shown to decrease (Bar, 2009). In the laying hen, 2 to 3 grams of calcium, or about 10% of the total body calcium, is secreted during the 11-14 hour period (out of the 24-25.5 daily cycle) of shell formation (Bar, 2009). Laying hens take in approximately 4.2 to 4.6 grams of calcium per day, which is based on a 115 grams per day diet with the recommended calcium content of 3.6 to 4% (Bar, 2009).

Calcium absorption from the gut to plasma occurs primarily when calcium is provided by the diet. The rate of calcium absorption from the diet is greater than the theoretical 50% due to diffusion because calcium moves from the gut to circulation (Bar, 2009). Absorption only occurs during daylight and the early evening because eating stops at dark; after 4 to 5 hours of darkness, the intestinal lumen is devoid of calcium (Bar, 2009). Because shell calcification occurs -1 to 13 hours after the lights are out, a significant portion of calcium deposited in the shell is not derived straight from the diet (Bar, 2009). Additional calcium must come from reserves in the bone, primarily medullary bone, and from blood borne calcium. Bone formation can

recommence during the following period of daylight when dietary calcium is present, but the process is often incomplete (Bar, 2009). Due to the decreased calcium availability, eggshells become increasingly thinner until soft-shelled eggs are laid or laying is disrupted (Whitehead, 2004).

Calcium concentrations in SCN neurons are rhythmic, suggesting that mechanisms involved in calcium homeostasis may be under the control of the circadian molecular clock (Ikeda, 2004). Shell calcification may be controlled via the circadian regulation of gonadal hormones because the concentrations of these hormones fluctuate in temporal relation to shell formation during the egg laying cycle and may influence calcium transport and activity (Bar 2009). Progesterone concentrations peaked shortly before termination of shell calcification and the injection of a small amount of progesterone while the egg was in the magnum inhibited shell formation, suggesting that progesterone acts as the “turn-off” signal for shell calcification (Bar, 2009). The “turn-on” signal is unclear, but may also be dependent upon gonadal hormones (Bar 2009).

Objectives

The main goals of this series of experiments were to determine if in the chicken: 1) the duodenum contains a functional molecular circadian clock; 2) the molecular circadian clock regulates genes that control serotonin biosynthesis; 3) the amount of serotonin in the duodenum varies across the day; and 4) serotonin profiles in blood are rhythmic. In doing so, we aimed to shed insight on the relationship between melatonin, serotonin, and bone health. The circadian regulation of plasma serotonin by the duodenum may represent an additional biochemical signal in the blood encoding time and could be used by target tissues to indicate the status of nutrient absorption, in particular calcium.

MATERIALS AND METHODS

Duodenum collection

Duodenum was harvested from chickens (*Gallus gallus*) 3, 9, 15, and 21 hours after the lights came on, also known as Zeitgeber time (Figure 2). Zeitgeber time, abbreviated ZT, refers to the standard of time based on the 24-hour period with ZT0 defined as lights on. ZT3, 9, and 15 were during daytime and ZT21 was during nighttime. Four chickens from each time point were humanely euthanized by cervical dislocation (IACUC #29982). Tissues were frozen on dry ice and stored at -80°C.

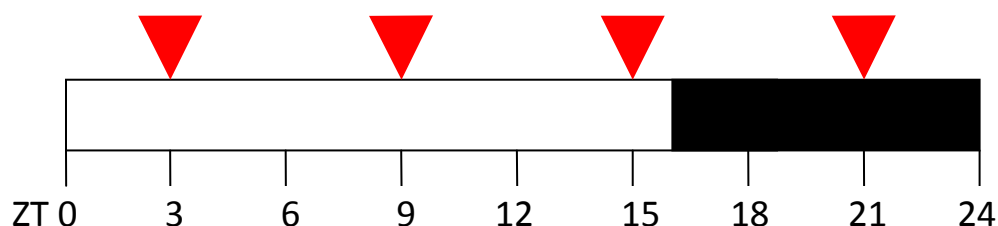


FIG 3: The artificial light-dark cycle of the chickens and time points at which tissue was harvested. Chickens were raised under a 16:8 light:dark cycle and tissue was collected 3, 9, 15, and 21 hours after the lights came on (ZT).

RNA extraction

Messenger RNA (mRNA) was extracted from 0.1 g of tissue in 1 mL of Trizol by homogenizing with a roto-stator for approximately 1 minute. Homogenized samples were centrifuged at 12,000 x g at 4°C for 5 minutes and the mRNA was removed using an RNeasy kit (Qiagen; Valencia, CA). The supernatant was removed and added to 0.32 mL of chloroform (0.2 mL of chloroform per 1 mL of Trizol). The

tubes were then shaken vigorously by hand for 15 seconds, incubated at room temperature for 3 minutes, and centrifuged at 12,000 x g for 15 minutes at 4°C. The upper (aqueous) phase was carefully transferred to a fresh 2 mL tube. An equal quantity of 70% ethanol was added to the sample and vortexed. The sample (700 µL) was then transferred to the Qiagen RNeasy column and centrifuged for 30 seconds at 9,000 x g. The liquid from the collection tube was discarded and the previous step repeated using the remaining sample. DNaseI solution (10 µL DNaseI and 70 µL RDD buffer) was added to the column and allowed to sit for 15 minutes at room temperature. RWI buffer (700 µL) was then added to the column. The column was centrifuged for 30 seconds at 9,000 x g at room temperature, transferred to a new 1.5 mL tube, and centrifuged again at 15,000 x g for 1 minute to remove any remaining ethanol. The RNA was eluted into a 1.5 mL collection tube using 35 µL RNase free water by centrifugation for 1 minute at 9,000 x g. The sample was checked for concentration and quality of RNA with a spectrophotometer (Nanodrop; Waltham, MA) and then stored at -80°C until needed.

cDNA synthesis

Complementary DNA (cDNA) was synthesized from mRNA using Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT; New England Biolabs; Ipswich, MA). To create cDNA, RNA (1 µg) and enough water to make the total volume of 13 µL were added to a clean centrifuge tube. To this mixture, 1 µL RNase Out (Invitrogen; Carlsbad, CA) and 1 µL random hexamers (New England Biolabs; Ipswich, MA) were added. Tubes were heated to 70°C for 10 minutes in a

thermocycler (BioRad; Hercules, CA) and then immediately cooled on ice. To each tube, 2 μ L 10x reaction buffer, 2mM deoxyribonucleotidetriphosphate (dNTP) mix, and 1 μ L M-MLV Reverse Transcriptase were added to make the total reaction volume 20 μ L. Solutions were mixed by pipetting and then lightly centrifuged. Samples were incubated at 42°C for 60 minutes, followed by heating at 70°C for 15 minutes. After heating, 30 μ L of nuclease free water was added to bring the volume to 50 μ L and then stored at -80°C until used.

qRT-PCR

Real time PCR (qRT-PCR) was performed to determine the amount of mRNA of *per2*, *per3*, *CLOCK*, *BMAL1*, *BMAL2*, *cry1*, *VIP* and the genes that encode for *TPH1* and *TPH2*. A standard reaction volume of 20 μ L was comprised of 10 μ L SYBR Green Super Mix (Quanta; Houston), 6.3 μ L water, 0.6 μ L of forward primers (Invitrogen; Carlsbad, CA), 0.6 μ L reverse primers (Invitrogen), and 2.5 μ L of cDNA (about 50 ng/reaction).

Forward Primer	Sequence (5'-3')
Cyclophilin	GCAAGCAGATCACCATTTCCA
Per 2	CCCCAGTAGTTGGTGCTCACTT
Per 3	CAGAATGGAAACGATCAGCCTAT
BMAL 1	TTCCCACAGCTTGCAGCTT
BMAL 2	GAAGCAGAAGTTCTGGAGACTTCAG
CLOCK	CGTGTGGAGCGGTAATGGT
Cry 1	CCGGGAAACGCCAAA
VIP	CGAAGCCAGGAAGAGTTAAATCCA
TPH1	TGCAAGCAAGAGGGACAGCTTA
TPH2	ACAGTGAGACCGTTGCTGGAT

Table 1: Sequences of the forward primers used in qRT-PCR.

Reverse Primer	Sequence (5'-3')
Cyclophilin	CGGAATGTCAGGCGTTAAGAC
Per 2	GACTGGTGAGCGATACAACACTTT
Per 3	TCGGGAGAAAACAGGAAGCA
BMAL 1	TTTTGGGCCCGCCTTCTC
BMAL 2	CCACCGAGGAGAGGCTCAT
CLOCK	GGGCAGCAAAAGTGGGATAA
Cry 1	TGCTCTGCCGCTGGACTT
VIP	CGTGGCTCAGCAGTTCATCTACA
TPH1	TGCAGGTGACCTTTGGATCA
TPH2	GATCCGAGCCGTGTCGTACATA

Table 2: Sequences of the reverse primers used in qRT-PCR.

Control samples without template and samples without reverse transcriptase were included in each qRT-PCR experiment. Each sample was added to a 96-well plate on ice, covered, and briefly centrifuged at 200 rpm at 4°C. Samples (minus controls) were run in duplicate on the plates using an Opticon II thermocycler (BioRad). The $\Delta\Delta C_t$ method of relative quantification using cyclophilin as an endogenous control gene was used to determine fold changes. Cyclophilin was used as the control because it is constantly expressed across the day in all of the tissues investigated. For each sample, a cycle threshold (C_t) was estimated from the number of cycles required for fluorescence to exceed the background level. These levels are inversely related to the amount of the target DNA within the sample. The formula used was: $\Delta\Delta C(t) = \{C(t)_{\text{target gene}} - C(t)_{\text{Cyclophilin}}\} \times \text{time} - \{C(t)_{\text{target}} - C(t)_{\text{Cyclophilin}}\} \times \text{time zero}$. Fold changes were normalized to levels at ZT3, which were arbitrarily set at 1.

HPLC

We used high-pressure liquid chromatography (HPLC) to determine concentrations of serotonin in the duodenum and plasma over the course of the day. Blood samples were collected from chickens 3, 9, 15, and 21 hours after the lights came on from the brachial vein using capillary tubes with heparin sulfate (125 IU/mL) as an anticoagulant. Blood was placed in heparinized microcentrifuge tubes on ice, centrifuged at 300x g at 4°C for 20 minutes, and the plasma removed and stored at -80°C until analysis. Samples were thawed on ice, vortexed and 200 μL was removed to a clean centrifuge tube. To the plasma, 160 μL of 0.1M HClO_4 , 20 μL

of 2.22 mM of butylated hydroxytoluene (BTH; the antioxidant) and 20 μ L of 0.07 mg/mL methyl-serotonin (the internal standard) were added and mixed. Methyl-serotonin is degraded in a similar manner as serotonin, but has a slightly different molecular weight. Consequently, the extraction efficiencies and degradation of endogenous serotonin in the samples can be determined by ratios of remaining methyl-serotonin. The solution was then kept on ice for 1 hour to precipitate the protein from the mixture. The mixture was then centrifuged for 12000 x g for 20 minutes at 4°C, and the supernatant was removed and filtered through a 0.22 μ Spin-X filter (Corning; Corning, NY) at 2500 x g for 5 minutes at 4°C.

Extracted samples (10 μ l) were separated using a Hypersil Gold a Q column (150 x 4.6 mm, 5 μ m particle size) at a flow rate of 0.8 ml/minute. A Hypersil Gold a Q drop-in guard cartridge (10 x 4.6 mm, 5 μ m particle size; Thermo Scientific; Waltham, MA) was used as a pre-filter. Samples were detected using an electrochemical detector (Antec; Fremont, CA) with a glass fiber electrode set at +400 mV. The mobile phase consisted of: A) 20 mM KH_2PO_4 and 20 μ M Na_2EDTA with a pH of 3.0 ± 0.05 and B) MeOH. A and B were mixed together at a ratio of 97: 3. The column and electrochemical detector were kept at a constant temperature of 30°C. The serotonin signal from each sample was compared to the signal from the standard curve to determine the amount of serotonin in the sample. The standard curve was constructed by measuring the peak area under the curve in the chromatograms of known concentrations of serotonin.

Statistical Analysis

Differences among clock gene expression at different time points were evaluated using the Rayleigh test. A Rayleigh test analyzes the significance of the phase in a cycle (period) histogram (Rhode, 1976).

RESULTS

PCR Experiment

The expression of all the clock genes (*PER2*, *PER3*, *CLOCK*, *BMAL1*, *BMAL2*, and *CRY1*) and the clock-controlled genes (*VIP*, *TP1*, and *TPH2*) varied over the course of the day in the duodenum (Table 3, Figures 4, 5, 6, and 7).

Gene	Time point			
	ZT3	ZT9	ZT15	ZT21
VIP	1	1.840162	-1.4238	-1.19458
PER2	1	1.2773	-4.11887	1.032399
PER3	1	-1.22596	3.246758	2.214913
CLOCK	1	2.958433	4.060057	3.74444
BMAL1	1	2.999731	2.773101	3.015192
BMAL2	1	-1.29026	2.395372	3.033535
CRY1	1	1.304578	1.016775	-1.02207
TPH1	1	2.998685	3.751584	2.420408
TPH2	1	1.037477	2.378414	1.71297

TABLE 3. Average fold changes of the clock genes and clock-controlled genes at time points ZT3, ZT9, ZT15, and ZT21.

Significant changes were found for all genes examined (Table 4).

Gene	P values
VIP	0.008
PER2	0.00219
PER3	0.02
CLOCK	0.0085
BMAL1	0.02
BMAL2	0.035
CRY1	0.01
TPH1	0.04
TPH2	0.045

TABLE 4. Probability values for clock genes and clock-controlled genes as determined by the Rayleigh test.

CLOCK was the most highly expressed before darkness with levels remaining high during lights out. *BMAL1* had the lowest expression three hours after lights on with the levels remaining constant after that time point. *BMAL2* dipped in expression midday and increased in expression right before and during darkness.

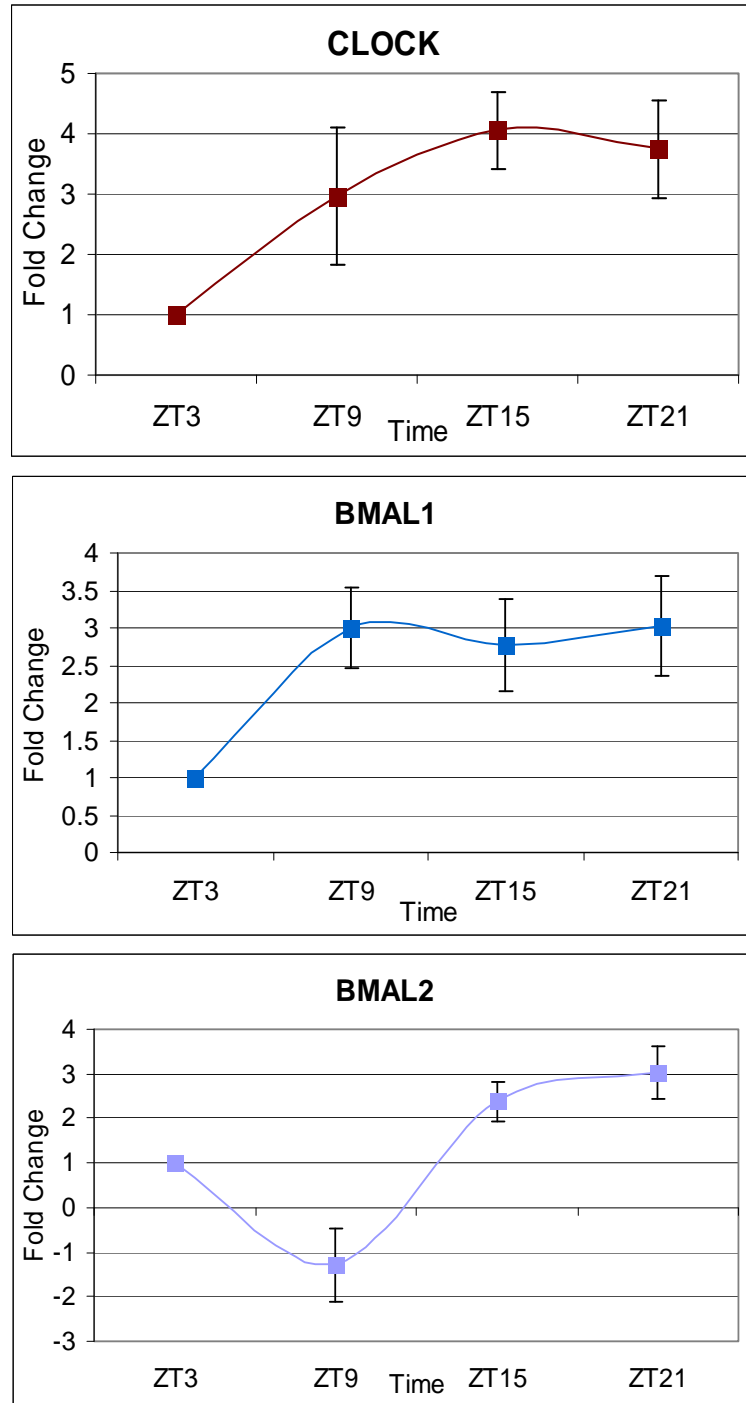


FIG 4. Fitted curves of average fold changes in expression of positive feedback elements *clock* (top), *bmal1* (middle), and *bmal2* (bottom) at different times throughout the light-dark cycle, known as Zeitgeber time (ZT). *Clock*, *bmal1*, and *bmal2* gene products were determined to be rhythmic across the day (p values were 0.0085, 0.02, and 0.035, respectively).

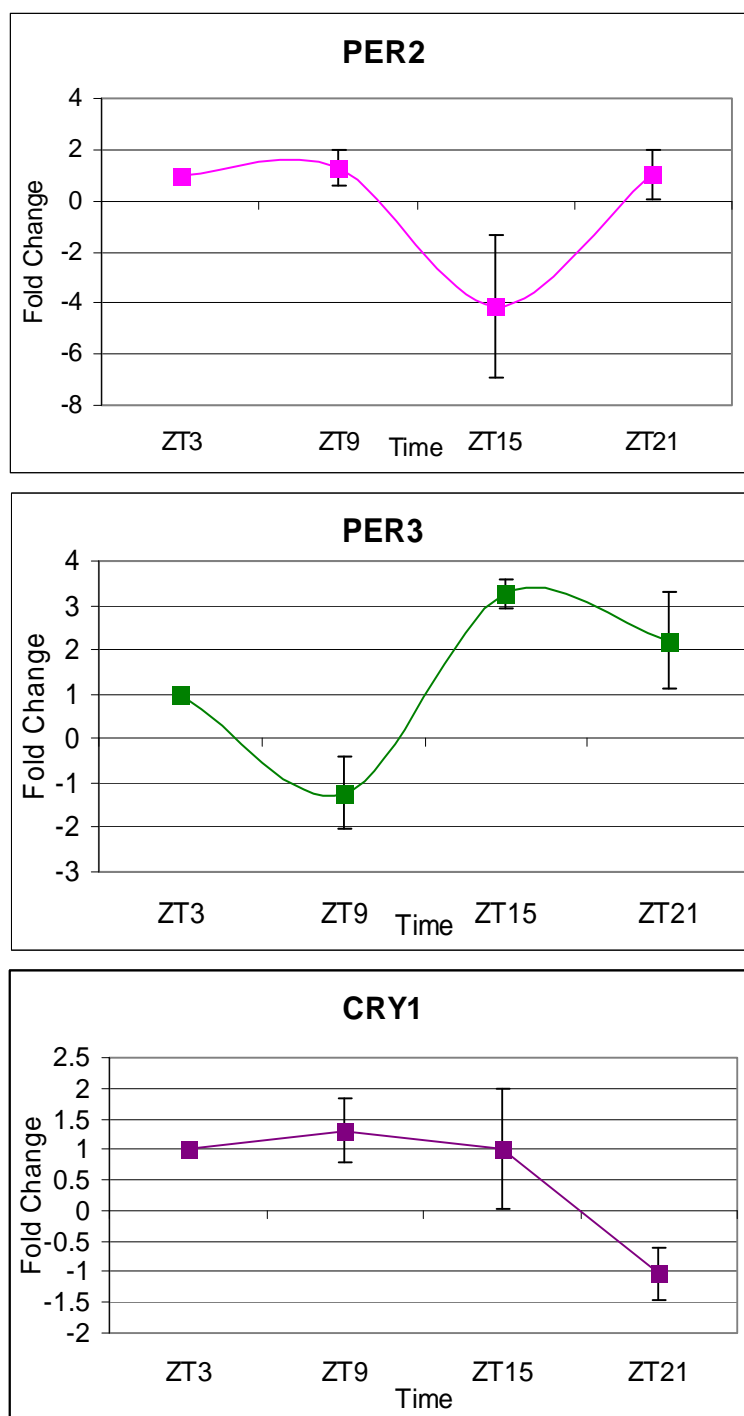


FIG 5. Fitted curves of average fold changes in expression of the negative feedback elements *per2* (top), *per3* (middle), and *cry1* (bottom). *Per2*, *per3*, and *cry1* gene products were determined to be rhythmic across the day (p values were 0.00219, 0.02, and 0.01, respectively).

Per2 greatly decreased in expression (4x less than at time point ZT3) right before lights out while remaining relatively constant throughout the rest of the 24-hour period. *Per3* expression fluctuated over the course of the day with the highest expression before the onset of darkness. Expression was 3x and 2x as great later in the day in comparison to ZT3. *Cry1* expression did not exhibit much variation through the 24-hour period. *VIP* was lowest in expression right before and shortly after lights off, with the lowest expression right before lights off.

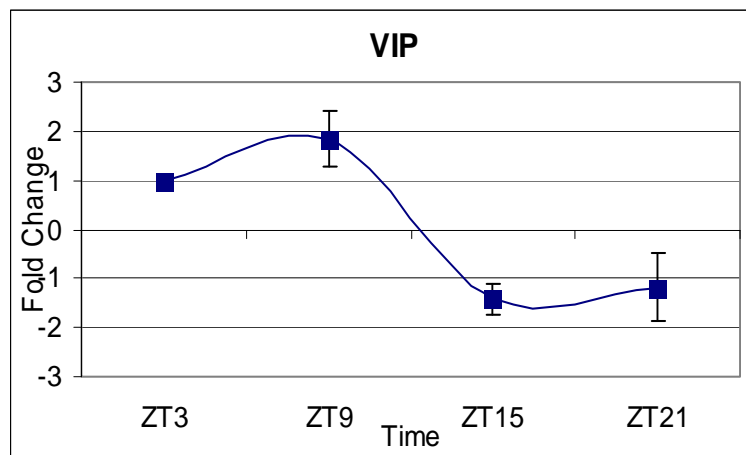


FIG 6. Fitted curve of average fold changes in expression of the clock-controlled gene *VIP*. *VIP* gene products were determined to be rhythmic across the day (p value was 0.008).

The levels of expression of Tryptophan Hydroxylase 1 and 2 (*TPH1* and *TPH2*) were also rhythmic in the duodenum, indicating that the transcription of the rate-limiting step controlling serotonin production in the gut is rhythmic (Table 3,

Figure 7). For both genes, expression was highest right before darkness and lowest three hours after lights on.

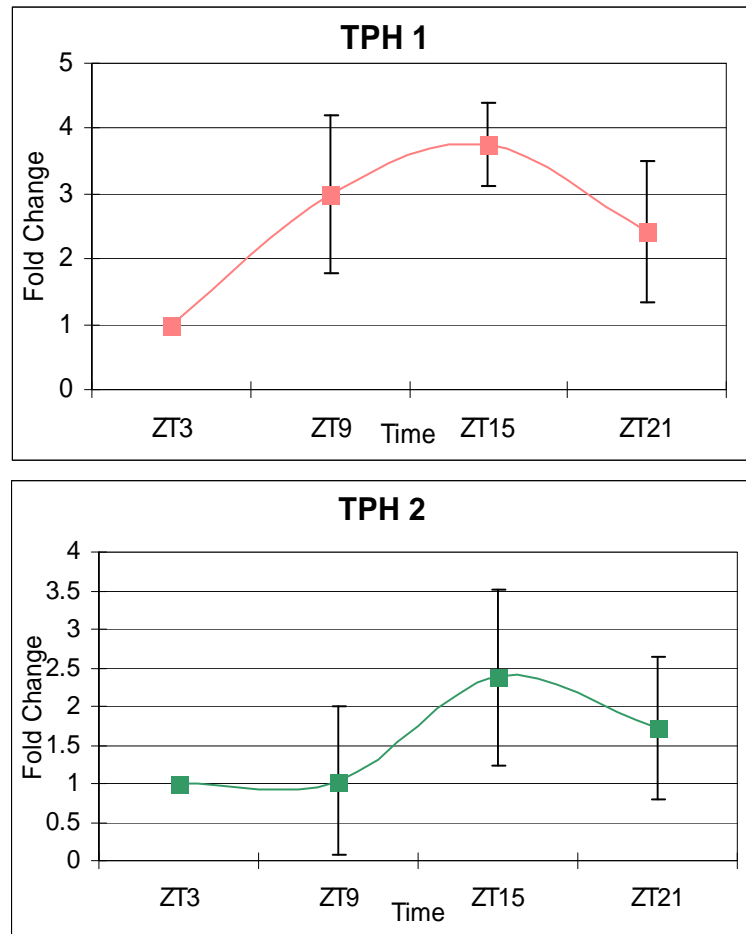


FIG 7. Fitted curves of average fold change values of *TPH1* (top) and *TPH2* (bottom) throughout the light-dark cycle. *TPH1* and *TPH2* gene products were determined to be rhythmic across the day (p values were 0.04 and 0.045, respectively).

Serotonin Concentration in the Duodenum and in Plasma

Serotonin concentrations in the duodenum and in plasma as determined by HPLC were similarly found to fluctuate over the course of the day (Figures 7 and 8). In the duodenum, levels of serotonin remained constant 3 hours and 9 hours after the lights came on. Serotonin concentration increased to almost 2.5 ng serotonin/ng tissue right before lights on at ZT15 and then decreased to below 1 ng serotonin/ng tissue 3 hours after lights off.

In plasma, serotonin concentrations were similar 3 hours, 9 hours, and 21 hours after the lights came on while the concentration was increased 15 hours after lights on. In both plasma and duodenum, the highest levels of serotonin occurred 15 hours after the lights came on, just to lights off. Concentration at the time point during nighttime was lowest in the duodenum and also lowest in plasma.

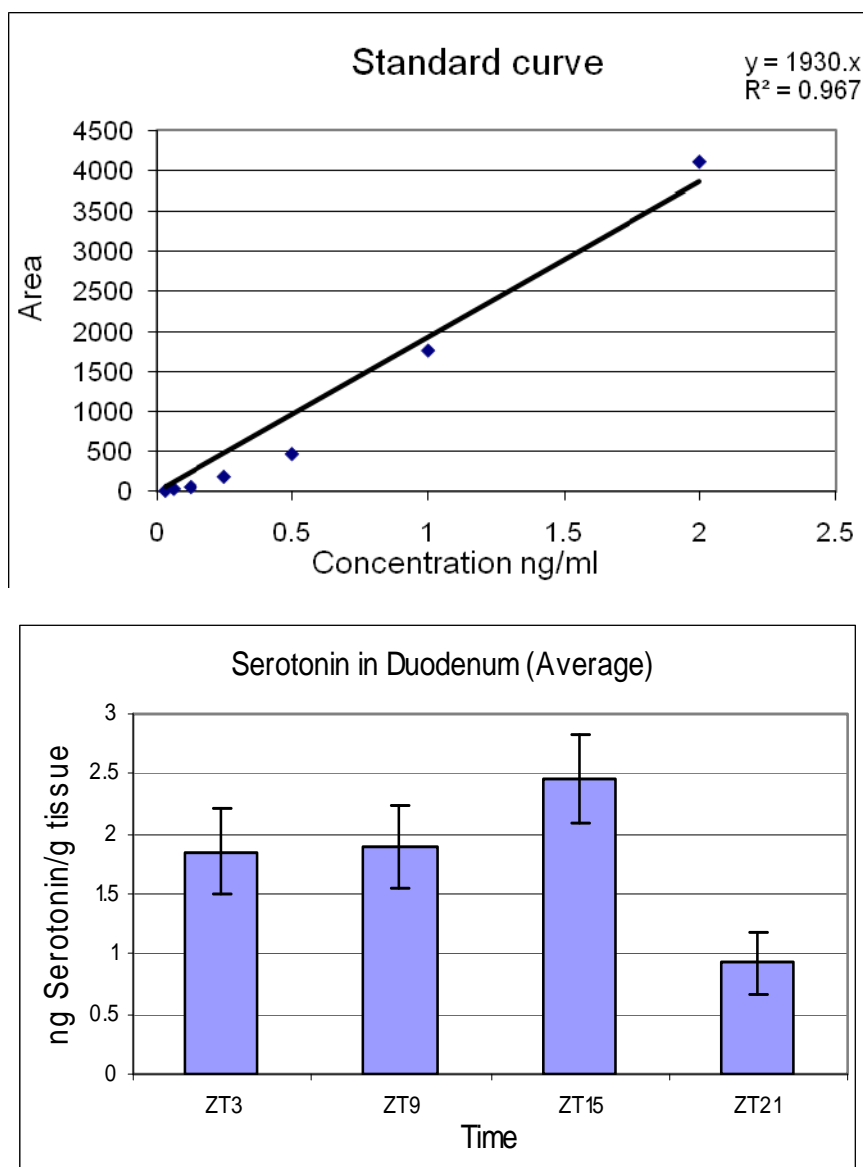


Fig 8. Mean levels of serotonin in the duodenum as determined by HPLC. The standard curve was constructed using known concentrations of the internal standard and measuring the peak area under the chromatogram. The signals from the samples were then compared to those from the standard curve to obtain the concentrations. Serotonin in the duodenum was found to exhibit rhythmicity across the day.

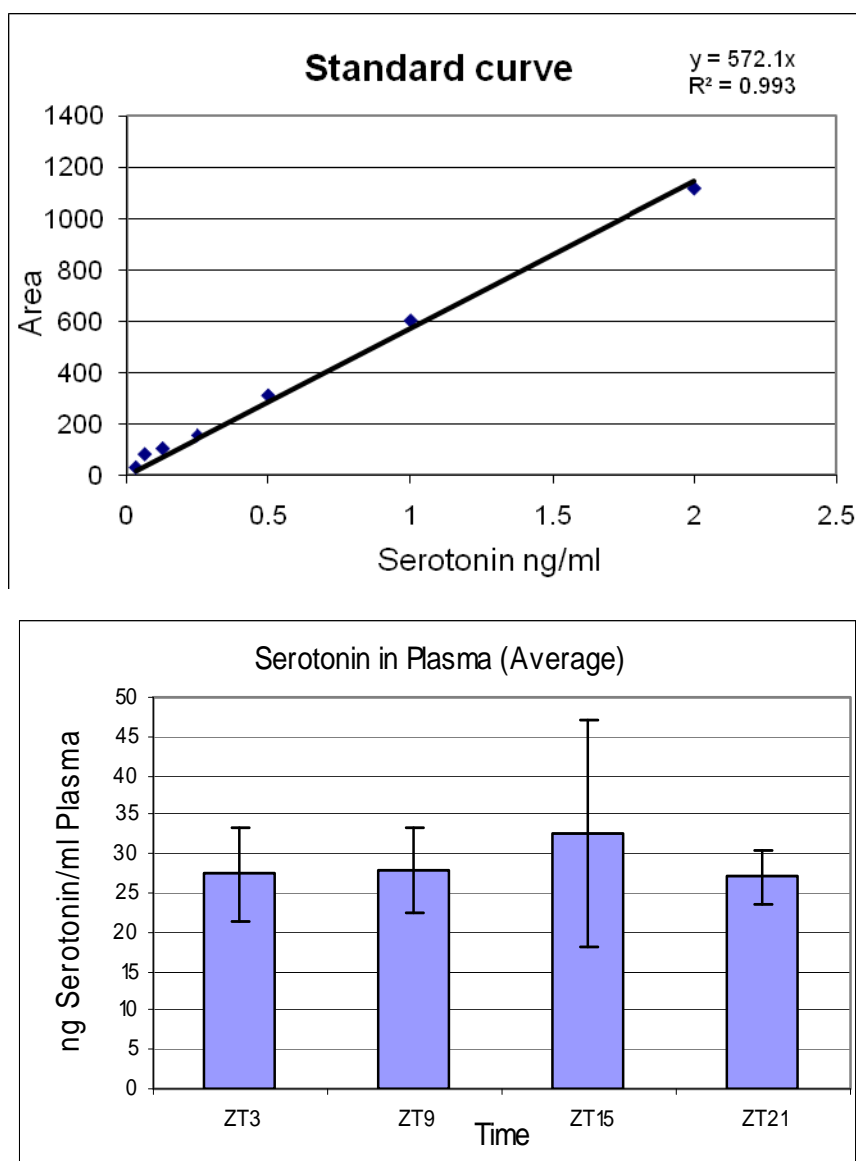


Fig 9. Mean levels of serotonin in the plasma as determined by HPLC. The standard curve was constructed using known concentrations of the internal standard and measuring the peak area under the chromatogram. The signals from the samples were then compared to those from the standard curve to obtain the concentrations. Serotonin in plasma was found to exhibit slight rhythmicity across the day.

DISCUSSION

The expression of clock gene products, as determined by estimating mRNA abundance using qPCR, was rhythmic across the light/dark cycle. The positive feedback elements in the circadian system, *bmal* and *clock*, followed a similar pattern of expression, with transcription increasing as the day progressed. As expected, the negative feedback element *cry* followed a pattern opposite that of *bmal* and *clock*. However, the *per* genes did not exhibit a pattern that corresponded to the feedback loops of the proposed circadian system, suggesting additional differences in regulation of avian circadian clocks in comparison to the mammalian and *Drosophila* models. Interestingly, it was documented that mRNA transcripts for *per3* and *cry1* are expressed 180° out of phase, with *per3* peaking at night and *cry1* peaking in the day (Bell-Pederson et al., 2005). Results presented here for these genes are consistent with this finding.

In addition to the canonical clock genes, the clock-controlled gene *VIP* also exhibited rhythmicity with expression being lowest just before and continuing during lights off. This expression pattern differs from previous work showing that *VIP* is released rhythmically in the SCN with higher concentrations at night (Irwin and Allen, 2010). Our results, however, correspond to the intake of feed because feeding ceases shortly before dark in the chicken. Melatonin concentrations in the gut have been positively linked to the intake of feed and *VIP* triggers the release of melatonin from the pineal gland, suggesting that our results correspond to previous findings in chicken at least within duodenal tissue (Bubenik, 2002). Additionally, *VIP*

was shown to reduce the concentration of calcium in the SCN in a manner dependent on the organism's activity level and changes in cytosolic calcium concentrations within the SCN exhibit rhythmicity throughout the day (Ikeda, 2004; Irwin and Allen, 2010). As a result, calcium homeostasis may be under the control of the circadian molecular clock and calcium may be an important intracellular messenger conveying time.

In addition to the clock genes and clock-controlled gene *VIP*, *TPH1* and *TPH2* also showed rhythms of expression. Because these genes code for Tryptophan Hydroxylase, the enzyme involved in the rate-limiting step in serotonin biosynthesis, transcriptional regulation could be responsible for the rhythmic expression of serotonin in the duodenum. This hypothesis is supported by the HPLC results that demonstrate that concentrations of plasma serotonin oscillate over the course of the day in chickens. The qPCR results for the expression of *TPH1* and *TPH2* correspond to the HPLC data for serotonin concentration in the duodenum and in plasma. *TPH1* and *TPH2* were most highly expressed right before darkness at time point ZT15 while serotonin was also found in the highest amount at this time.

The oscillation of clock genes and clock-controlled gene expression in the duodenum of the chicken along with the rhythmic production of serotonin suggests the presence of a functional molecular clock in this tissue. A duodenal peripheral clock could function independently of the central molecular clocks in the avian SCN, pineal, and retina or could be driven by central clocks. Thus, in the future, we would like to examine if clock genes oscillate in the duodenum *in vitro* and examine how

the molecular clock in the duodenum regulates serotonin synthesis and release. This would allow determination of whether the biological clock controlling the production of serotonin in duodenum is self-sustaining without rhythmic input.

It would be interesting to examine the role of serotonin as a signal to other tissues indicating the status of nutrient absorption, such as calcium. Feeding exists as an entrainment mechanism independent of the central clock system as exemplified by increased locomotor activity in rats with bilateral SCN lesions in the hours prior to a fixed meal even when fasted for 3 days (Stephan et al., 1979a; Blum et al., 2011). This occurrence demonstrated the presence of an endogenous circadian food-entrainable clock (Stephan et al., 1979b; Blum et al., 2011). Synchronization to meal-time guarantees that organisms consume the necessary amounts of energy and nutrients, even when food is only given at a certain time of the day. Thus, food metabolites and hormones linked to feeding/starvation, such as serotonin, could provide temporal cues to peripheral tissues.

Because serotonin affects duodenal absorption of nutrients, the circadian molecular clock could control calcium availability. For the hen, synchronizing circadian rhythms to the egg laying cycle is beneficial for reasons similar to that of feeding entrainment. Shell formation places a high demand for calcium on the animal, which often results in bone degradation because there is not enough calcium available in the diet or within blood plasma. Thus, entraining food intake to periods of high caloric or nutrient demand enables the hen to more efficiently fulfill its own nutrient requirements and still produce high quality eggs.

In addition to its role in the digestive system, serotonin is also involved in the control of ovulation. In quail, *per* and *clock* are rhythmically expressed in the ovary, suggesting a relationship between clock genes in the ovary and ovulation (Yoshiumura et al., 2000). An increase in the activity of serotonin within the hypothalamus increases prolactin secretion, which blocks ovulation through the inhibition of follicle stimulating hormone (FSH) and gonadotropin releasing hormone (GnRH; Sharp et al, 1984). Brain-derived serotonin is also involved in the regulation of the preovulatory release of lutenizing hormone (LH) in the hen, which is supported by the presence of a class of serotonin binding sites in the anterior hypothalamus of the laying hen that are not seen in out-of-lay or broody hens (Macnamee and Sharp, 1989a; Sharp et al., 1989). LH is produced by the anterior pituitary gland and a surge in LH concentration triggers ovulation. Additionally, serotonin was detected within the ovary in White Leghorn laying hens with no eggs in the shell gland (Paczoska-Eliasiewicz, 1990). Because serotonin cannot cross the blood-brain barrier and most circulating serotonin is produced within the gut, duodenal serotonin could affect reproductive tract function. This finding, combined with our results showing that serotonin is rhythmically produced in the gut under the regulation a circadian molecular clock, suggests that a circadian molecular clock controls ovulation. In conclusion, if ovulation and the clock responsible for calcium availability can be regulated through the production of serotonin, we could help laying hens produce eggs with thicker, stronger eggshells and also reduce skeletal problems, such as osteoporosis.

In summary, results presented in this thesis demonstrate that: 1) A functional molecular clock is present in the duodenum 2) The rate-limiting enzyme in serotonin biosynthesis, Tryptophan Hydroxylase (*TPH*), is rhythmically expressed in the duodenum and 3) Concentrations of plasma serotonin are rhythmic in chickens. These findings suggest that the circadian regulation of plasma serotonin could represent an additional biochemical signal in the blood encoding time and as a result, serotonin could be used by target tissues to integrate timing cues derived from the digestive system.

LITERATURE CITED

- Abraham, U., U. Albrecht, and R. Brandstatter. "Hypothalamic Circadian Organization in Birds II. Clock Gene Expression." *Chronobiology International* 20 (2003): 657-669.
- Abraham, U., U. Albrecht, E. Gwinner, and R. Brandstatter. (2002). "Spatial and Temporal variation of passer Per2 gene expression in two distinct cell groups of the suprachiasmatic hypothalamus in the house sparrow (*Passer domesticus*)." *European Journal of Neuroscience* 16 (2002):429-436.
- Bailey, M., N. Chong, J. Xiong, and V. Cassone. "Chicken's cry2: molecular analysis of an avian cryptochrome in retinal and pineal photoreceptors." *FEBS Letter* 513 (2002): 69-72.
- Bar, A. "Calcium Transport in Strongly Calcifying Laying Birds: Mechanisms and Regulation." *Comparative Biochemistry Physiology* 152 (2009): 447-469.
- Bartell, Paul. "The Role of Biological Clocks in Avian Reproduction." Research Seminar. Department of Dairy and Animal Science, Pennsylvania State University. 29 March 2010.
- Bégay, V., J. Falcon, G. Cahill, D. Klein, and S. Coon. "Transcripts Encoding Two Melatonin Synthesis Enzymes in the Teleost Pineal Organ: Circadian Regulation in Pike and Zebrafish, but not in Trout." *Endocrinology* 139 (1998): 905-912.
- Bell-Pedersen, D. V. Cassone, D. Earnest, S. Golden, P. Hardin, T. Thomas, and M. Zoran. "Circadian Rhythms from Multiple Oscillators: Lessons from Diverse Organisms." *Nature Reviews Genetics* 6 (2005): 544-556.
- Bernard, M., D.C. Klein, and M. Zatz. "Chick pineal clock regulates serotonin N-acetyltransferase mRNA rhythm in culture." *Proceedings of the National Academy of Sciences of the USA* 94 (1997): 304-309.
- Besharse, J., P. Iuvone, and M. Pierce. "Regulation of rhythmic photoreceptor metabolism: a role for post-receptor neurons." *Progress in Retinal Research* 7 (1988): 21-61.
- Bhanot, P., M. Brink, C. Samos, J. Hsieh, Y. Wang, J. Macke, D. Andrew, J. Nathans, and R. Nusse. "A new member of the frizzled family from *Drosophila* functions as a Wingless receptor." *Nature* 382 (1996): 225-230.

- Blum, I., E. Waddington Lamont, and A. Abizaid. "Competing clocks: metabolic status moderates signals from the master circadian pacemaker." *Neuroscience and Biobehavioral Reviews* (2011).
- Brandstätter, R., U. Abraham, and U. Albrecht. "Initial demonstration of rhythmic per gene expression in the hypothalamus of a non-mammalian vertebrate, the house sparrow." *Neuroreport* 12 (2001): 1167-1170.
- Bubenik, G. "Gastrointestinal Melatonin: Localization, Function, and Clinical Relevance." *Digestive Diseases and Sciences* 47 (2002): 2336-2348.
- Cahill, G. and J. Besharse. "Retinal melatonin is metabolized within the eye of *Xenopus laevis*." *Proceeding of the National Academy of Science in the USA* 86 (1989): 1098-1102.
- Cahill, G. and J. Besharse. "Circadian regulation of melatonin in the retina of *Xenopus laevis*: limitation by serotonin availability." *Journal of Neurochemistry* 54 (1990): 716-719.
- Cassone, V. and M. Menaker. "Is the Avian Circadian System a Neuroendocrine Loop?" *Journal of Experimental Zoology* 232 (1984): 539-549.
- Chong, N., M. Bernard, and D. Klein. "Characterization of the chicken serotonin N-acetyl transferase gene: activation via clock gene heterodimer/E box interaction." *Journal of Biological Chemistry* 275 (2000): 32991-32998.
- Chong, N., S. Chaurassia, R. Haque, D. Klein, and P. Iuvone. "Temporal-spatial characterization of chicken clock genes: circadian expression in retina, pineal gland, and peripheral tissues." *J Neurochem* 85 (2003): 851-860.
- Delmas, P. "Biochemical Markers of Bone Turnover." *Journal of Bone and Mineral Research* 8 (1993): 549-555.
- Epstein, M. L., and K. T. Poulsen. "Appearance of somatostatin and vasoactive intestinal peptide along the developing chicken gut." *Journal of Comparative Neurology* 311(1991):168-178.
- Faluhelyi, N., D. Reglodi, and V. Csernus. "The Effects of PACAP and VIP on the *in vitro* Melatonin Secretion from Embryonic Chicken Pineal Gland." *Annals of the New York Academy of Sciences* 1070 (2006): 271-275.
- Fu, L., M. Patel, A. Bradley, E. Wagner, and G. Karsenty. "The Molecular Clock Mediates Leptin-Regulated Bone Formation." *Cell* 122 (2005): 803-15.

- Fu, Z., M. Inaba, T. Noguchi, and H. Kato. "Molecular cloning and circadian regulation of cryptochrome genes in Japanese quail (*Coturnix coturnix japonica*)."
Journal of Biological Rhythms 17 (2002): 14–27.
- Gupta, B., R. Spessert, and L. Vollrath. "Molecular components and mechanism of adrenergic signal transduction in mammalian pineal gland: regulation of melatonin synthesis." *Indian Journal of Experimental Biology* 43 (2005): 115-149.
- Halici, M., M. Oner, A. Guney, O. Canoz, F. Narin, and C. Halici. "Melatonin promotes fracture healing in the rat model." *Joint Diseases and Related Surgery* 21 (2010): 172-177.
- Haque, R., S. Chaurasia, J. Wessel, and P. Iuvone. "Dual regulation of cryptochrome 1 mRNA expression in chicken retina by light and circadian oscillator." *NeuroReport* 13 (2002): 2247–2251.
- Herzog, Erik and G. Tosini. "The Mammalian Circadian Clock Shop." *Cell and Developmental Biology* 12 (2001): 295-303.
- Ikeda, M. "Calcium Dynamics and Circadian Rhythms in Suprachiasmatic Nucleus Neurons." *Neuroscientist* 10 (2004): 315-324.
- Irwin, R., C. Allen. "Neuropeptide-mediated calcium signaling in the suprachiasmatic nucleus network." *European Journal of Neuroscience* 32 (2010): 1497-1506.
- Jozsa, R., J. Nemeth, A. Tamas, T. Hollosy, A. Lubics, B. Jakab, A. Olah, I. Lengvari, A. Arimura, and D. Reglodi. "Short-Term Fasting Differentially Alters PACAP and VIP Levels in the Brains of Rat and Chicken." *Annals of the New York Academy of Sciences*. 1070 (2006): 354-358.
- Karaganis, S. P. Bartell, V. Shende, A. Moore, and V. Cassone. "Modulation of metabolic and clock gene mRNA rhythms by pineal and retinal circadian oscillators." *General and Comparative Endocrinology* 161 (2009): 179-92.
- Karsenty, Gerard. "Lrp5 Controls Bone Formation by Inhibiting Serotonin Synthesis in the Duodenum." *Cell* 135 (2008): 825-37.
- Klein, D. D. Auerbach, M. Namboodiri, and G. Wheler. "Indole metabolism in the mammalian pineal gland." *The Pineal Gland, Vol. 6: Anatomy and Biochemistry* (1981): 199-227.

- Konopka, R. and S. Benzer. "Clock mutants of *Drosophila melanogaster*." *Proceedings of the Academy of Science of the USA* (1971): 2112-2116.
- Kumar, V., J. Wingfield, A. Dawson, M. Ramenofsky, S. Rani, and P. Bartell. "Biological Clocks and Regulation of Seasonal Reproduction and Migration in Birds." *Physiological and Biological Zoology* 83 (2010): 827-835.
- Larkin, P., W. Baehr, and S. Semple-Rowland. "Circadian regulation of iodopsin and clock is altered in the retinal degeneration chicken retina." *Molecular Brain Research* 70 (1999): 253-263.
- Macnamee, M., P. Sharp, R. Lea, and R. Sterling. "Evidence that a VIP-like peptide is a prolactin releasing factor in the bantam hen." *Proceedings of the 3rd Joint Meeting of the British Endocrine Society* (1984): 76.
- Miller, J. "Clockwork in the brain." *BioScience* (1989): 75-78.
- Moore, R. "Neural control of pineal function in mammals and birds." *Journal of Neural Transmission Supplement* 13 (1978): 47-58.
- Natesan, A., L. Geetha, M. Zatz. "Rhythm and Soul in the Avian Pineal." *Cell Tissue Research* (2002): 309: 35-45.
- Paczoska-Eliasiewicz, H. "Histochemical demonstration of the presence of serotonin in the hen (*Gallus domesticus*) reproductive tract". *Folia biologica (Warszawa)* 38(1990): 27.
- Patel, M. S., and F. Elefteriou. "The New Field of Neuroskeletal Biology." *Calcified Tissue International* 80 (2007): 337-47.
- Prockop, O. and Kivirikko, K. "Hydroxyproline and the metabolism of collagen." In: Gould BS editors. *Treatise on Collagen*. New York: Academic Press. (1968): 215-246.
- Ralph, M. and M. Menaker. "A mutation of the circadian system in golden hamsters." *Science* 241 (1988): 1225-1227.
- Rhode, W.S. "A test for the significance of the mean direction and the concentration parameter of a circular distribution." (1976) Web.
<www.neurophys.wisc.edu/comp/docs/not011/not011.html>
- Roenneberg, Till and M. Merrow. "Circadian Clocks-the Fall and Rise of Physiology." *Nature Reviews* 6 (2005): 965-970.

- Rosen, Clifford J. "Serotonin's Secrets." *Nature* 15 (2009): 145-146.
- Sharp, M., M. MacNamee, R. Talbot, R. Sterling, and T. Hall. "Aspects of the Neuroendocrine Control of Ovulation and Broodiness in the Domestic Hen." *Journal of Experimental Zoology* 232 (1984): 475-483.
- Stephan, F., J. Swann, and C. Sisk. "Anticipation of 24-hr feeding schedules in rats with lesions of the suprachiasmatic nucleus." *Behavioral Neural Biology* 25 (1979a): 346-363.
- Stephan, F., J. Swann, and C. Sisk. "Entrainment of circadian rhythms by feeding schedules in rats with suprachiasmatic lesions." *Behavioral Neural Biology* 25 (1979b.): 545-554.
- Sundler, F., J. Alumets, and J. Fahrenkrug. "Cellular localization and ontogeny of immuno-reactive vasointestinal polypeptide (VIP) in the chicken gut." *Cell Tissue Research* 196 (1979): 193-201.
- Suzuki M., Y. Ohmori, and T. Watanabe. "Projections of neurons in the intestinal nerve of Remak to the chicken intestine." *Journal of the Autonomic Nervous System* 61 (1996): 79-86.
- Thomas, K. and P. Iuvone. "Tryptophan hydroxylase activity in chicken retina and pineal gland displays circadian rhythmicity." (Abstr) *Society for Neuroscience Abstract* 15(1989): 1396.
- Whitehead, C. "Overview of Bone Biology in the Egg-Laying Hen." *Poultry Science* 83 (2004): 193-199.
- Velarde, E. M. Delgado, and A. Alonso-Gomez. "Serotonin-induced contraction in isolated intestine from a teleostfish (*Carassius auratus*): characterization and interactions with melatonin." *Neurogastroenterology and Motility* 22 (2010): 364-373.
- Yadav, V., J. Ryu, N. Suda, K. Tanaka, J. Gingrich, G. Schutz, F. Glorieux, C. Chiang, J. Zajac, K. Insogna, J. Mann, R. Hen, P. Ducy, and G. Karsenty. "Lrp5 Controls Bone Formation by Inhibiting Serotonin Synthesis in the Duodenum." *Cell* 135 (2008): 825-837.
- Yasuo S., M. Watanabe, N. Okabayashi, S. Ebihara, and T. Yoshimura. "Circadian clock genes and photoperiodism: comprehensive analysis of clock gene expression in the mediobasal hypothalamus, the suprachiasmatic nucleus, and the pineal gland of Japanese quail under various light schedules." *Endocrinology* 144 (2002): 3742-374

- Yasuo S., T. Yoshimura, P. Bartell, M. Iigo, E. Makino, N. Okabayashi, and S. Ebihara. "Effect of melatonin administration on *qPer2*, *qPer3* and *qClock* gene expression in the suprachiasmatic nucleus of Japanese quail." *European Journal of Neuroscience* 16 (2002): 1541–1546.
- Yoshimura, T., Y. Suzuki, E. Makino, T. Suzuki, A. Kuroiwa, Y. Matsuda, T. Namikawa, and S. Ebihara. "Molecular analysis of avian circadian clock genes." *Molecular Brain Research* 78 (200): 207-215.
- Yoshimura, T., S. Yasuo, Y. Suzuki, E. Makino, Y. Yokota, and S. Ebihara "Identification of the suprachiasmatic nucleus in birds." *American Journal of Physiology* 280 (2001): R1185-R1189.
- Zatz, M. "Light and norepinephrine similarly prevent damping of the melatonin rhythm in cultured chick pineal cells: regulation of coupling between the pacemaker and overt rhythms?" *Journal of Biological Rhythms* 6 (1991): 137-147.

Elizabeth Ebert-Zavos

6760 Covenant Court
Frederick, MD 21702
epe5006@vt.edu

Education The Pennsylvania State University, University Park
B.S. in Animal Sciences, Science Option with Honors
Expected date of graduation: August 2011

Experience January 2009-Present: **Undergraduate Research Assistant**
The Pennsylvania State University, Department of Poultry Science

- Harvest tissue, bone, and blood samples from chickens at various times in a 24 hour period
- Run real-time PCR using the cDNA from the samples

May 2008-Present: **Veterinary Assistant**
Frederick Veterinary Center, LLC

- Assist with appointments, surgery, and other procedures
- Attend to animals in for boarding
- Maintain facilities
- Perform front-desk duties

Summer 2007: **Student Intern**
Kingsbrook Animal Hospital

- Shadowed the veterinarians
- Cared for in-patients
- Maintained facilities

June 2006-June 2007: **Student Intern**
The National Cancer Institute of the National Institute of Health, Lab of Gene Regulation and Chromosome Biology

- Used E. Coli as a model to study RNA polymerase

Honors

- Academic Excellence Scholarship (August 2007-Present)
Schreyer Honors College
- Academic Scholarship (August 2008-Present)
The College of Agricultural Sciences, Pennsylvania State University
- Undergraduate Research Grant (Summer 2009)
Schreyer Honors College
- Undergraduate Research Grant (Fall 2009-Spring 2010)
The College of Agricultural Sciences, Pennsylvania State University