CIRCADIAN CHANGES IN CALCIUM UTILIZATION OF THE LAYING HEN

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by
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ABSTRACT

Calcium uptake in the duodenum, shell gland, and medullary bone of laying hen, and the effects of melatonin, serotonin, estradiol, and progesterone on this process were investigated at 0, 6, 12, and 18 hours after oviposition. Tissue was exposed to calcium for 2 minutes (time of active calcium uptake) or 5 minutes (time of calcium uptake saturation). Calcium uptake in the duodenum was greater at 12 and 18 hours than 0 or 6 hours after oviposition for 2 minute exposure, and was greater at 18 hours than 0, 6, or 12 hours after oviposition for 5 minute exposure. Calcium uptake in the shell gland was greater at 6 and 18 hours than at 12 hours after oviposition for both 2 and 5 minute exposures. Calcium uptake in medullary bone was highest at 12 hours and lowest at 6 hours after oviposition for 2 minute exposure, and was greater at 12 hours than 0, 6, or 18 hours after oviposition for 5 minute exposure. In vitro treatments of melatonin, serotonin, and estradiol resulted in increases in calcium uptake in medullary bone, but not in duodenum and shell gland. These results suggest that calcium uptake in duodenum, shell gland, and medullary bone in hen is under control of a biological clock. Furthermore, melatonin, serotonin, and estrogen may mediate medullary bone calcium uptake in part via fast-acting, membrane bound G-protein-coupled receptors.
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Introduction

Importance of the study

The avian egg consists of albumen (comprising ~ 60% of the total mass), the yolk (30 – 33%), and the shell (9 – 12%). Of these components, the shell is of particular importance because it not only forms a barrier protecting the egg from physical insults, but also a biological barrier, shielding contents from bacteria and other pathogens [1]. Consequently, shell quality is a determinant of egg quality in the industry. Broken and cracked egg shells present major economic losses to egg producers. According to a report by Anderson and Carter (1976), 1 billion out of 15 billion eggs produced in the United Kingdom were downgraded due to shell damage alone [2]. Roland (1977) suggested that 6.4% of the eggs in the US were lost due to inferior shells [3]. An increase in shell integrity, therefore, is a practical means of increasing egg production. If shell damage were to decrease by only 1% for 60,000 eggs produced daily, at a gain of 25 cents per egg, income per annum would increase by $4,500 [4]. Furthermore, a compromise in egg shell integrity can expose the inner content to pathogens, leading to outbreaks of diseases such as salmonellosis [5].

A major factor affecting shell integrity is the amount of calcium absorbed from chicken’s diet [6]. Each egg shell is estimated to contain 2.3 – 3 g of calcium, an amount equal to about 10% of the chicken’s total calcium stores [6, 7]. Although chickens are more
efficient than other animals in terms of calcium homeostasis, a prolonged calcium deficiency will still prove problematic.

Another problem arising from calcium insufficiency is osteoporosis, a progressive loss in the amount of structural bone [8]. Chickens affected by the disease known as cage layer fatigue could eat and drink normally, but could not stand [9, 10]. Young hens producing eggs at a high rate, as well as chickens housed in cages (due to the lack of space for normal ambulation and physical activity) were more susceptible to this condition. Bone brittleness and weakness were the major symptoms, and hens with this disease became emaciated and died if unassisted, but recovered if placed on the floor for several days. A determinant of whether hens will get this condition is the amount of dietary calcium, which is unsurprising because calcium, along with phosphate, is a major component of bone. Studies demonstrated the effects of decreased bone mineral content and increased bone fragility in laying birds [11, 12, 13]. However, this problem was partially improved simply by providing chickens with adequate calcium content in their diet [14]. In order to improve shell integrity and quality, and reduce the incidence of osteoporosis in the hen, a thorough understanding of avian calcium homeostasis and the factors affecting this process is required.
The oviposition cycle and calcium homeostasis in the laying hen

Oviposition cycle

The process of egg formation in the chicken has been extensively studied [15, 16]. Chickens lay egg in a sequence, with an ovulation – oviposition cycle slightly longer than 24 hours. As a result, the oviposition of the next egg in the sequence happens later than that of the previous egg until the sequence is terminated. After ovulation, the egg moves into the infundibulum, and the perivitelline membrane is added over the course of approximately 15 minutes. Afterwards, the egg moves into the magnum where albumen proteins are added to the egg over the course of approximately 3 hours. The egg then passes through the isthmus, taking about 1.5 hours, where the fibrous inner and outer shell membranes are added. Next, the egg moves to the uterus, also called the shell gland, where a calcarous egg shell is formed over the next 20 hours. In the first 5 hours, the egg is subjected to a process called “plumping” where water and electrolytes enter the albumen and albumen stratification occurs. The last 15 hours is when egg shell calcification takes place, beginning with the laying down of an organic shell matrix on top of the two fibrous membranes, followed by the deposition of calcium carbonate onto the matrix. Unlike in bone, this process is free from modulation by osteoblasts and osteoclasts. Subsequently, the egg passes through the cloacal opening and is oviposited to the outer environment.

Where does the calcium forming the egg shell come from? To answer this question, it is necessary to understand the homeostasis of calcium in the chicken.
Calcium homeostasis in the laying hen

Absorption of dietary calcium can happen in two ways: passive diffusion or active uptake. Passive diffusion happens in a paracellular fashion across the entire length of the intestine. This process is not saturable and is dependent on the concentration of calcium within the intestinal lumen [17]. Active calcium uptake, on the other hand, is saturable and dependent on the amount of calcium channels and transporters on the intestinal membrane, the amount of 1, 25–(OH)_{2}–D_{3} (the active metabolite of Vitamin D), and the abundance of calcium binding proteins. Active calcium uptake only occurs in the anterior portion of the intestine (especially the duodenum); most dietary calcium is taken up in this region of the small intestine [18].

1, 25–(OH)_{2}–D_{3} mediates its action in the duodenum via a genomic mechanism, by increasing transcription of genes responsible for calcium uptake, such as calcium channels and calcium-dependent ATPases [19, 20, 21]. Also, 1, 25–(OH)_{2}–D_{3} increases the concentration of calcium binding protein (CaBP) in duodenal cells. No CaBP is present in the duodenum of vitamin D deficient chicks, and the amount of intestinal nuclear localization of 1, 25–(OH)_{2}–D_{3} is highly correlated with the concentration of intestinal CaBP [22]. Calcium binding protein sequesters ionic calcium in duodenal cells, preventing a high concentration of free calcium [23]. After calcium is absorbed by the duodenum, it travels through the vasculature, which serves as a transient reservoir for 20 – 25 mg of calcium at any given time [24]. Depending on the time of the day and the phase of the egg formation cycle, calcium will either be transported to medullary bone for storage or to the shell gland for egg shell deposition.
During the day, calcium is primarily stored in medullary bone. Medullary bone is unique to birds (and some egg laying reptiles). It is sandy in appearance and resides in the marrow cavity of the femur and tibia of laying birds [25]. Medullary bone is composed of hydroxyapatite, as is cortical bone. Unlike in cortical bone, where hydroxyapatite is distributed with respect to the underlying organic matrix, the distribution of hydroxyapatite in medullary bone is random. The formation of medullary bone is mediated by osteoblasts, and the resorption of medullary bone is mediated by osteoclasts. During the day, when no shell calcification is occurring, the activity of osteoblasts predominates, and medullary bone is built up. During the night, when egg shell calcification is actively taking place, medullary bone is broken down by the action of osteoclasts [25]. Calcium resorbed from medullary bone has quick access to the vasculature via the vessels inside the marrow cavity, and thus can be quickly and easily transported to the shell gland, where active shell calcium deposition is taking place. Therefore, medullary bone provides a reservoir of calcium for egg calcium formation. Tracer experiments have determined that medullary bone calcium supplies as much as 37% of the calcium needed for shell calcification [26, 27].

At night, calcium from medullary bone is mobilized for shell calcification in the shell gland. Ionic calcium is secreted with bicarbonate (HCO$_3^-$) by the epithelial cells into the shell gland lumen, where they mix to form calcium carbonate, which constitutes the majority of shell mineral [28]. Calcium secretion onto a wax surrogate egg by epithelial cells has been shown to proceed slowly at first, acquiring maximum calcification midway through the process, and slows down to baseline 2 hours before the egg is laid [29].
Consequently, calcium transport in the body of the laying chicken is correlated with the oviposition cycle. As this cycle has a duration of approximately 24 hours, and is repeated every day in a sequence, it exhibits a circadian rhythm, although one that is only loosely synchronized to the light/dark cycle.

**Circadian rhythms and biological clocks**

Most animal behaviors, such as the sleep and wakefulness, reproduction, and calcium homeostasis follow circadian rhythms, and therefore are regulated by an internal biological clock. In mammals, the biological clock which regulates circadian rhythms is the suprachiasmatic nucleus (SCN) of the hypothalamus. In non–mammalian vertebrates such as birds, this central clock is a system comprised of the pineal gland, retina, and SCN. The hormone melatonin is the main hormone produced by the pineal gland, and acts as a timing signal to various organs. In addition to the central clock, peripheral clocks are located in most organs in the body.

Many gastrointestinal processes such as gut motility, maintenance and restoration of the protective mucosal barrier, production of digestive enzymes, and nutrient transport show circadian rhythms [30]. The duodenum produces its own serotonin under control of a biological clock, with concentrations being greater at night and less during the day [31]. Due to the cyclical pattern of calcium homeostasis and its close association with the oviposition cycle, it is hypothesized that the functions of tissues involved in calcium homeostasis are controlled by biological clocks.
In vitro studies revealed a circadian rhythm of calcium uptake in the digestive systems of the rat and the frog. Under an *ad libitum* diet and 12:12 LD, calcium uptake in the duodenum of rats displayed a circadian rhythm that was highest near the middle of the scotophase, and then decreased until it reached the lowest point near the middle of photophase [32]. In the frog, *Rana pipiens*, the circadian rhythm of calcium absorption also followed the same pattern as that of the rat, with greatest uptake points during the night and lowest during the daytime [33]. Additionally, food access has been determined to be an important entrainment factor of the biological clock in the GI tract. This behavior only exists if animals were fed cyclically, and is abolished under *ad libitum* feeding [34, 35].

**Hormones of interest**

In addition to investigating the circadian effects on calcium uptake, we also wanted to investigate the effects of different hormones on this process. These hormones were chosen based on their circadian patterns of synthesis and release [31, 39, 57] and their potential effects upon calcium homeostatic related processes. They include melatonin, serotonin, estradiol, and progesterone.

**Melatonin**

Melatonin (N–acetyl–5–methoxytryptamine) is an indole hormone produced primarily by the pineal gland of vertebrates, although the retina also rhythmically regulates
melatonin synthesizing enzymes [36], indicating that the retina produces melatonin under control of a clock. Also, the gastrointestinal tract produces melatonin independently of the pineal gland [37].

Melatonin is synthesized from its precursor, serotonin, in two steps, mediated by the enzymes aralkylamine (serotonin) N–acetyl transferase (AANAT), which converts serotonin into N–acetyl serotonin, and hydroxyindole–O–methyltransferase (HIOMT), which converts the resulting N–acetyl serotonin into melatonin [38]. AANAT is the rate-limiting enzyme in this process, and its synthesis and activity are inhibited by light during the day. Consequently, melatonin is low during the day and elevated at night, leading to melatonin being coined “the hormone of darkness” [39].

Melatonin increases calcium concentrations inside rat enterocytes in vitro [41]. In birds, iodomelatonin binding was detected in the duodenum, suggesting a direct role of melatonin in endocrine control of the tissue [42]. In the shell gland, long term melatonin administration lowered egg shell weight [43]. This finding, combined with the knowledge that melatonin receptor 1c gene was differentially expressed in the shell gland of a strain of chicken [44] opened up the possibility that this hormone can play a direct role in shell gland calcium regulation. Melatonin increases expression of rat bone sialoprotein (an important component for osteoblast differentiation and matrix mineralization signaling) in vitro [45]. Melatonin enhances rat osteoblast cell differentiation by increasing Runx2, osteocalcin, BMP2 and BMP4 [46]. Also, melatonin, through RANKL down-regulation, suppresses bone resorption by reducing osteoclast formation and activation [47]. In developing chicks, pinealectomy (a procedure that abolishes plasma melatonin) results in an increased incidence of scoliosis [48]. It is worth noting that all of these studies addressed
relatively long term (days to weeks) effects of melatonin on the proliferation and development of bone cells, and did not address short term effects of melatonin on these cells.

Short term melatonin effects can be mediated by melatonin receptors (Mel1a, Mel1b, and Mel1c). These are membrane bound, G-protein-coupled receptors that (in the case of Mel1a and 1b) inactivate adenylyl cyclase, thereby decreasing the concentration of cyclic AMP and protein kinase A (PKA) [40]. Decreasing PKA causes a potassium ion efflux and subsequently a calcium influx. Therefore, it is possible that melatonin will cause an increase in calcium uptake in the duodenum, shell gland, and medullary bone if melatonin receptors 1a or 1b are present in these tissues, even though these melatonin receptors have not been specifically documented in these tissues. Little evidence is available on Mel1c signaling, and many suspect it to be a null receptor, mediating no direct effect when bound.

**Serotonin**

Serotonin (5-hydroxytryptamine), a precursor of melatonin, is a hormone generated by brainstem neurons as well as enterochromaffin cells of the duodenum. The synthesis of serotonin involves two steps, the first step being the addition of a hydroxyl group on carbon number 5 to form 5-hydroxytryptophan, which is mediated by the enzyme tryptophan hydroxylase (Tph1). This is the rate limiting step in this process. The second step is the removal of the carboxyl group to form serotonin, mediated by aromatic amino acid decarboxylase [49, 50]. Serotonin also displays a circadian rhythm in abundance. In the
chicken, plasma concentrations of serotonin are decreased during the day and elevated at night [31].

Serotonin exerts its effects via serotonin receptors, which are classified into 7 subfamilies (5HT,7). Except for 5HT, these are G-protein-coupled receptors, with activities ranging from a decrease in cyclic AMP and an increase in IP3/DAG to an increase in cyclic AMP depending on receptor type [51].

Serotonin performs seemingly contradictory functions in bone. Lrp5, a member of the low density lipoprotein, can inhibit Tph1 and increase bone formation in mammals. Furthermore, serotonin inhibits osteoblast proliferation through Htr1b receptor and CREB [52]. In contrast, brain derived serotonin increases bone formation and decreases bone resorption. Because serotonin can’t cross the blood brain barrier, the effects of brain-derived serotonin are hypothesized to be mediated through the control of food intake, energy expenditure, and sympathetic tone [53].

In chicken, serotonin receptors of type 5HT2 are present in osteoblasts and osteocytes [51]. Bound 5HT2 receptors activate phospholipase C, leading to an increase in intracellular diacylglycerol (DAG) and IP3. IP3 can then activate the release of calcium from the reserve in the ER, and also the influx of calcium from the extracellular environment via L-type membrane calcium channels [54]. So, it is reasonable to hypothesize that if medullary bone cells also contain 5HT2 receptors, calcium uptake will increase upon addition of serotonin. Furthermore, in smooth muscle cells of the vasculature of rats, serotonin activates TRPV4 channels on the membrane, allowing an increase in intracellular concentration of calcium by means of endoplasmic reticulum calcium release and extracellular calcium entry [55].
In contrast with the role of serotonin in bone, which has been quite thoroughly elucidated, the role of serotonin in calcium homeostasis in the GI tract and the shell gland has not been investigated, even though it is worth noting that serotonin has been detected to be present in the shell gland of chickens using spectrofluorometry [56].

**Estradiol and Progesterone**

Estradiol and progesterone are synthesized from cholesterol and typically exert their physiological effects via nuclear receptors, leading to changes in gene expression. In birds, concentrations of estrogen and progesterone are highest 4 – 7 hours before ovulation [57]. Estrogen receptors are localized to osteoblasts [58], and estrogen can induce the formation of medullary bone in both female [58] and male birds [59]. In mammals, regardless of reproductive state, progesterone reduces bone loss following ovariectomy. Administration of medroxyprogesterone reduces resorptive bone loss [60]. Progesterone’s effects are mostly reported to ameliorate the bone-destructing effects of glucocorticoids [61]. A direct effect of progesterone on bone cells, including medullary bone, has not yet been elucidated.

In the GI tract, estrogen increases the formation of 1, 25-(OH)₂-D₃, the hormone most potently associated with calcium uptake in the duodenum [62, 63]. Estrogen, combined with progesterone and testosterone maximized the increase of renal 25–hydroxyvitamin D₃-1α–hydroxylase, a key enzyme in 1, 25-(OH)₂-D₃ production in the chick. However, progesterone alone could not accomplish this feat [64].
Estrogen is an important regulator of shell gland growth; estrogen implants enhance the development of the epithelial cell layer responsible for calcium transport [67]. Administration of estrogen to immature chicks also results in a greater concentration of calcium binding protein (CaBP) in the shell gland [68]. Progesterone may also play a role in calcium transport in the shell gland, as progesterone receptors are present in epithelial cells [69].

Effects of estradiol and progesterone are traditionally thought to occur via changes in gene expression. This process takes time to occur, therefore changes are not detected for hours or days. However, recent evidence suggested there were actions of estradiol that were transcription-independent and rapid. One effect of note is the increase in intracellular calcium in various tissues, including rat osteoblasts and duodenum, both by the release of calcium from internal (ER) calcium stores and uptake of calcium from outside sources [65, 66]. These non–genomic actions were mediated through a whole new class of estrogen receptors that had characteristics of action similar to G–protein-coupled receptors. Membrane bound progesterone receptors have been characterized in the ovary of the spotted sea trout [70]. In 2006, the first non–genomic effect of progesterone via the membrane bound receptor was characterized. Membrane bound progesterone receptors display characteristics similar to G-protein-coupled receptors, and decrease cAMP concentration [71].
Current experiment description and hypotheses

The goal of the experiment is to determine the calcium uptake in chicken duodenum, shell gland, and medullary bone throughout the day. In addition, the effects of hormones such as melatonin, serotonin, estradiol, and progesterone on this process are investigated. During the daytime, calcium uptake in the duodenum should be less than that at night, because at this time the chicken is actively consuming feed, and thus duodenal cells will have been absorbing dietary calcium, decreasing the gradient for calcium transport into the duodenal cells. I also expect medullary bone to accrue calcium during the day, peaking at about ZT12, the point when shell calcification is increasing, but has not reached its maximum rate. During the night, when shell calcification is most active, medullary bone releases calcium for shell formation. In contrast, the shell gland should have a greater uptake of calcium during the night to keep up with calcium deposition, and less during the day when shell calcification has completed. I hypothesize that all three tissues will show circadian rhythms for calcium uptake throughout the 24 hour light cycle.

Melatonin should increase medullary bone and duodenal calcium uptake, due to activation of G-protein-coupled melatonin receptors, increasing calcium entrance via membrane bound calcium channels. In contrast, melatonin should decrease shell gland calcium uptake in accordance to reports indicating a negative relationship between a melatonin receptor gene expression and shell calcification [44]. Serotonin, estradiol, and progesterone should increase calcium uptake in all tissues by activating their respective
G-protein-coupled receptors to increase calcium entrance via membrane bound calcium channels. No interaction effect between time of day and hormone treatment is expected.

Methods

White leghorn chickens (W33, Hyline, n = 6 per time point) raised under a 16:8 LD cycle were used for the study. Oviposition times were determined for all chickens, and only chickens that had oviposition times between 2 and 4 hours after lights on were selected for the study. The day after oviposition, chickens used for the study were sacrificed by cervical dislocation at one of four time points: ZT0 (time of oviposition), ZT6, ZT12, and ZT18. Only chickens that already laid eggs were sacrificed. Tissues were immediately extracted as described below. This process was repeated until 6 chickens were examined at each of the four time points selected. All experiments were performed in strict accordance with and approval from The Pennsylvania State University Animal Care and Use Committee (IACUC #35091).

Tissue preparation

Medullary bone was extracted from femur and tibia of animals. The ends of each bone were removed using rongeurs, and the bone was then cracked open longitudinally. The bone cavity was washed thoroughly with ice cold sterile PBS (pH = 7.5) using 10 ml syringes with 16 G 1/2 needles. Medullary bone was then scraped from the cavity using a stainless steel curette, placed in a Petri dish with PBS, and crushed with a stir bar.
Medullary bone spicules were then placed in a 50 ml centrifuge tube with PBS and centrifuged at 100 rpm for 1 min (Beckman Coulter, Allegra X-22R). PBS was then decanted, and the process was repeated until the bone was free of lipid, connective tissue, and blood. Approximately 100 mg of medullary bone was used in each individual incubation.

Duodenum was removed and the associating pancreas was excised. Duodenum was then placed in a Petri dish containing ice cold sterile PBS, and incised longitudinally. The interior of the duodenum was washed with PBS until all digesta had been removed and then transferred to a Petri dish containing oxygenated M199 media (M-3469, Sigma) at 37.5°C, where it was cut into 7mm x 7mm pieces for incubation. A 7mm x 7mm tissue size was chosen based on results from a previous pilot study, which demonstrated a decreased variability in calcium uptake in both duodenum and shell gland at this size relative to other sizes (2mm x 2mm and 5mm x 5mm).

Shell gland was extracted from the same animal, washed with ice cold sterile PBS, and transferred to a Petri dish containing oxygenated M199 media at 37.5°C. Only the epithelial layer of the shell gland was used for the study. Pieces (7mm x 7mm) from this layer were carefully excised from the rest of the shell gland and put in M199 media at 37.5°C for incubation.

Tissue pieces from duodenum and epithelial shell gland were subjected to Trypan Blue staining. Visual inspections were done to determine the viability of the epithelial cells from each tissue, as the epithelial layer was involved in calcium uptake in both the duodenum and shell gland. Dead cells were identified as cells with a blue stain; most cells in the preparation were found to be viable.
**Tissue incubation**

Incubations (42 or 45 minutes long) were performed at 37.5°C with 5% CO₂ in an incubator (NuAire, NU-5200). Tissues were incubated for 30 minutes in M199 media containing either 0.1 mM melatonin, 0.1 mM serotonin, 0.01 mM estradiol, 0.01 mM progesterone, or DMSO (control), then transferred into a Calcium Transport Buffer (CaTB) containing 0.5 mM CaCl₂ and the same hormone concentrations as described earlier to acclimate the tissues for calcium uptake for 10 minutes. Tissues were then transferred into CaTB containing 7.17 x 10⁻³ mg of ⁴⁵Ca/ml (an equivalent of 25,000 dpm/100 µl) for either 2 minutes or 5 minutes. These two exposure times (2 minutes and 5 minutes) were chosen based on a previous pilot study, which indicated that active calcium uptake happened at 2 min, but did not reach saturation until 5 minute. Calcium uptake was terminated by placing the tissue into 150 mM mannitol.

**Tissue solubilization, decoloration, and radioactive counting**

Tissues were transferred from mannitol solution into pre-weighed scintillation vials containing 1 ml of Solvable (6NE9100, Perkin Elmer). Scintillation vials were weighed again to determine the mass of tissue within. For medullary bone, contents of scintillation vials were dissolved in a water bath (SHEL LAB) at 60°C for 3 days with constant shaking and frequent vortexing at 2 hour intervals. For duodenum and shell gland, the scintillation vials were left in the water bath at 60°C overnight with constant shaking.

To decolorize the solution, 200 µl of 30% H₂O₂ was added to each scintillation vial. The vials remained at room temperature overnight to allow for complete offgassing.
Subsequently, 6 ml of scintillation fluid (Ultima Gold LLT, Perkin Elmer) were added to each scintillation vial. Cpm values of the vials were counted in a beta scintillation counter (LKB Wallac, Model # 1209). From the concentration of $^{45}\text{Ca}$ obtained from the producer, the amount of calcium (in milligrams) of the radioactive calcium transport buffer solution was calculated. A standard curve was constructed from cpm values obtained in the counter for solutions containing 0, 10, 50, 100, and 200 µl of radioactive CaTB and concentration of calcium in these solutions, and an equation with calcium concentration as the independent variable and cpm values as the dependent variable was obtained. Using this equation, the amounts of $^{45}\text{Ca}$ taken up by the tissues were calculated, and the values normalized to the weight of tissue.

**Statistical analysis**

Two way analysis of variance was conducted with time of day (ZT0, ZT6, ZT12, and ZT18) and treatment (blank, melatonin, serotonin, estradiol, and progesterone) as factors. In the case of the medullary bone data, normality assumption was not satisfied, so the data were log transformed prior to performing the ANOVA. A Tukey post–hoc analysis with 95% confidence interval was performed for all significant observations. All statistical analyses were done using Minitab Version 17 (Minitab, Inc.).
Results

Calcium uptake in the duodenum

Calcium uptake results for duodenum after 2 and 5 minute exposures to $^{45}$Ca are shown in Figure 1A and 1B, respectively. Values are displayed as mean ± standard error.

For the 2 minute $^{45}$Ca exposure time, there was a significant increase in calcium uptake by the duodenum at ZT12 and ZT18, compared to that at ZT0 and ZT6 ($p < 0.01$). No significant differences in calcium uptake were observed between ZT12 and ZT18, or between ZT0 and ZT6. There were no significant differences detected between control and hormone-treated groups in the duodenum ($p = 0.961$). No significant interaction effects between time of day and hormone treatment were observed ($p = 0.884$), and thus time of day effect did not depend on hormone treatment, and vice versa.

For the 5 minute $^{45}$Ca exposure time, calcium uptake in the duodenum at ZT18 was significantly greater than those of ZT0, ZT6, and ZT12 ($p < 0.01$). No significant differences were detected in calcium uptake between ZT0, ZT6, and ZT12. No significant differences were detected between control and any of the hormone-treated groups ($p = 0.290$) and no significant interaction effects between time of day and hormone treatment were observed ($p = 0.829$).
Calcium uptake in the shell gland

Results for shell gland calcium uptake are shown in Figure 2A (2 minute $^{45}$Ca exposure) and 2B (5 minute $^{45}$Ca exposure). Values are displayed as mean ± standard error.

For the 2 minute $^{45}$Ca exposure, calcium uptake at ZT12 was significantly less than at ZT18 ($p < 0.01$), and calcium uptake at ZT18 was significantly greater than at ZT0 ($p < 0.01$). However, there were no significant differences detected between calcium uptake at ZT12 and ZT6, or at ZT12 and ZT0. There were no significant differences in calcium uptake between ZT18 and ZT6. There were no significant effects of hormone treatment ($p = 0.845$) and no interaction effects between time of day and hormone treatment ($p = 0.731$).

For the 5 minute $^{45}$Ca exposure, calcium uptake was significantly different across the day ($p < 0.01$). Specifically, calcium uptake was greater at ZT6 and ZT18 compared to that at ZT12. However, calcium uptake was not significantly different between ZT6 and ZT18, or between ZT6, ZT18 and ZT0. There were no significant effects detected as a result of the different hormone treatments ($p = 0.847$). There were also no significant interaction effects between time of day and hormone treatment ($p = 0.947$).

Calcium uptake in medullary bone

Data for calcium uptake amount in medullary bone did not follow normality assumptions, so they were transformed by base 10 log before analysis. Because the original data points have values larger than zero but smaller than one, the log of these
calcium amounts are negative, leading to negative means. The more negative the mean, the less calcium uptake.

After 2 minute incubation in $^{45}$Ca, significant differences in calcium uptake for both time of day ($p < 0.01$) and hormone treatment ($p < 0.01$) were observed. Specifically, medullary bone calcium uptake was highest at ZT12 and lowest at ZT0. Calcium uptake at ZT6 and ZT18 were both greater than at ZT0, less than at ZT12, but were not different from each other. For hormone effects, calcium uptake was significantly elevated after treatment with melatonin, serotonin, or estradiol compared to vehicle-treated (control) or progesterone groups. Calcium uptake was not different between control and progesterone groups, nor was calcium uptake different between melatonin, serotonin, or estradiol-treated groups. Also, a significant interaction effect was observed between time of day and hormone treatment ($p = 0.011$, Figure 3A) such that at ZT12, uptake in the untreated and progesterone conditions were significantly greater than uptake for the untreated and progesterone-treated conditions at other times of day (ZT0, ZT6, or ZT18), yet calcium uptake at ZT12 for melatonin, serotonin, or estradiol treatment were the same as those at other times.

For the 5 minute exposure, there were significant differences in medullary bone calcium uptake for both time of day ($p < 0.01$) and hormone treatment ($p < 0.01$), but there were no significant interaction effects between time of day and hormone treatment ($p = 0.053$) (Figure 3B). For time of day effects, medullary bone calcium uptake was greater at ZT12 than any other times of day. Calcium uptake at ZT0, ZT6, or ZT18 were not different from each other. For hormone treatments, calcium uptake was again significantly elevated for melatonin, serotonin, and estradiol treatment groups compared
to untreated and progesterone-treated groups. Calcium uptake was not different between untreated group and progesterone, as well as between melatonin, serotonin, and estradiol-treated groups.

Figures
Figure 1. Duodenal calcium uptake after oviposition with hormone treatments. Values are means ± SEM. For time of day, ZT0 was the time of oviposition. Hormone treatments are shown as separate columns for each time of day, and labeled by color. From lightest to darkest: blank (media without hormone, Blk), melatonin (MLT), serotonin (5HT), estradiol (E2), and progesterone (P4). Lower case letters above each cluster of columns at each ZT represent the result of Tukey test: calcium uptake at two ZTs were significantly different if they do not share a letter with each other. 1A: calcium uptake (mg Ca/g tissue) from duodena exposed to $^{45}$Ca for 2 minutes. 1B: calcium uptake (mg Ca/g tissue) from duodena exposed to $^{45}$Ca for 5 minutes.
Figure 2. Shell gland calcium uptake after oviposition with hormone treatments. Values are means ± SEM. For time of day, ZT0 was the time of oviposition. Hormone treatments are shown as separate columns for each time of day, and labeled by color. From lightest to darkest: blank (media without hormone, Blk), melatonin (MLT), serotonin (5HT), estradiol (E2), and progesterone (P4). Lower case letters above each cluster of columns at each ZT represent the result of Tukey test: calcium uptake at two ZTs were significantly different if they do not share a
Figure 3. Medullary bone calcium uptake (log transformed) after oviposition and with hormone treatments. 1A: log calcium uptake from medullary bone exposed to $^{45}$Ca for 2 minutes. 1B: log calcium uptake from medullary bone exposed to $^{45}$Ca for 5 minutes. For time of day, ZT0 was the time of oviposition. Hormone treatments are shown as separate columns for each time of day, and labeled by color. From lightest to darkest: blank (media without hormone, Blk), melatonin (MLT), serotonin (5HT), estradiol (E2), and progesterone (P4). Hormone treatments
not sharing any letters are different from each other, and times of day not sharing any number are different from each other.

**Discussion**

Calcium uptake in the duodenum displayed a pattern of being highest at night (ZT18 in both 2-minute and 5-minute exposures), which was expected. Chickens are diurnal eaters, only consuming food during the day. Therefore, calcium concentrations inside the duodenal lumen would be high during this time. There are two calcium absorption methods in the duodenum: paracellular passive diffusion, which is not saturable and dependent on the calcium concentration in the gut [17], and active transport, which is saturable and not exclusively dependent on calcium concentration in the gut. Active transport can be altered by the active metabolite of Vitamin D, 1–25 (OH)\(_2\)–D\(_3\), which induces an increase in calcium uptake by means of increasing calcium binding protein inside the duodenal cytoplasm, calcium channels (such as ECaC and CaT1), and calcium-dependent ATPases on the cell membrane [20, 21, 22]. ECaC and CaT1 are calcium channels that are present on the apical membrane of the duodenal cells, and accommodate calcium moving down a concentration gradient into these cells [75, 76]. Calcium-dependent ATPases, on the other hand, are present on the basolateral membrane, and require ATP for calcium transport against its concentration gradient [91]. Calcium binding protein sequesters ionic calcium inside the duodenal cells and reduce its concentration, allowing more calcium to be absorbed [22]. It is likely that both passive diffusion and active transport are involved in calcium uptake in this experiment. Calcium
taken up via passive diffusion would go through tight junctions between duodenal cells into the interstitial space, while calcium taken up by active uptake would be present inside the duodenal cells.

At night (ZT18), the calcium concentration inside the duodenum is less than during the day, resulting in the amount of calcium inside duodenal cells as well as the interstitial space between cells at that time being less compared to that during the day. Furthermore, concentration of 1, 25-(OH)$_2$–D$_3$ is highest at night during maximal shell calcification [77], likely leading to an increase in the number of calcium channels, calcium-dependent ATPases and calcium binding protein, resulting in more calcium absorbed. Therefore, it was unsurprising to see the amount of calcium uptake increased at night rather than during the day, and further indicated the circadian control of duodenal calcium transport via active transport.

The gastrointestinal tract has an intrinsic biological clock. Most GI activities display a circadian rhythm, and these include gut motility, gastric acid secretion, and digestive enzyme secretion [30]. The expression of clock genes, such as per2, are found in the myenteric plexus, a part of the enteric nervous system, and it is speculated that per2 is involved in circadian regulation of the gut [84]. Other components of the molecular clock were discovered in the GI tract as well [85, 86]. In birds, the duodenum expresses components of the molecular clock such as Cry1, Bmal1, Bmal2, Clock, Per2, and Per3 [31]; all but Cry1 displaying rhythmic expressions throughout the light – dark cycle. Tryptophan hydroxylase, the rate-limiting enzyme involved in serotonin production, is also expressed in a daily rhythm in the duodenum, indicating that the duodenum has a biological clock controlling serotonin formation. Results indicate that
the duodenal clock in hens also control calcium uptake. It is likely that the molecular clock modifies abundance of calcium transporter and calcium binding protein to alter transport of calcium.

There were no effects of any of the hormones tested upon calcium uptake in the duodenum. Melatonin receptors exists in the avian duodenum [42]. Melatonin receptors 1a and 1b are G\textsubscript{i} coupled, and can reduce the amount of intracellular cAMP and PKA and typically increase the amount of calcium entering the cells via activation of calcium channels on the membrane [78]. However, besides from Mel1a and Mel1b, there are nuclear melatonin receptors that mediate genomic, long term effects of melatonin [79]. Also, the signaling pattern of Mel1c receptors is not clear, and many considered it to be a null receptor, mediating no effects [78]. It is possible that the receptor detected was a nuclear melatonin receptor, which had genomic effects and therefore would not have been able to be detected in just a mere 40 minute incubation or the null receptor Mel1c.

Calcium uptake in the shell gland exhibited a bimodal pattern, being highest at ZT6 and ZT18. Calcium deposition of egg shell initially starts at a low rate, increasing to a maximum rate at about ZT15, before decreasing to a minimum 2 hours before laying [7]. It was unsurprising, therefore, to see the peak in calcium uptake corresponded to ZT18. A high rate of calcium deposition at ZT18 would deplete calcium inside the epithelial cells, and lead to greater uptake by these cells when exposed to calcium. Also, 1, 25–(OH)\textsubscript{2}–D\textsubscript{3} concentration is highest at this time [77], and could increase active transport of calcium by increasing activity of calcium channels, calcium-dependent ATPases, and calcium binding protein within the shell gland [28, 89]. Calcium uptake continued to be high at ZT0 and ZT6. At ZT6, the egg was still in the isthmus, and had
not entered the shell gland, so high calcium uptake at ZT0 and ZT6 seemed counterintuitive. One possible explanation for this is the concentration of calcium inside the epithelial cells at these two time points. At these times, calcium inside these cells would have reach their lowest concentrations, as shell calcification would use a great amount of calcium. Therefore, the difference in concentration gradients for calcium between the epithelium and the lumen at these two times are highest, and thus the cells would be able to absorb more calcium.

At ZT12, calcium uptake was lowest. Shell calcification started at about ZT6 and increased to maximum at about ZT15 [7]. Therefore, at ZT12, shell calcification would be active, but would not have reached its maximum rate, and calcium concentration inside epithelial cells would still be relatively high. Also, active calcium uptake, mediated by 1, 25–(OH)₂–D₃ was increasing at this time [77]. Therefore, at ZT12, the concentration of calcium inside the epithelial cells would have likely reached their daily highest. Because of the increased calcium concentration, a need for further calcium uptake was not present, and thus the amount of calcium absorbed was less.

This study showed that there were differences in calcium uptake in the epithelial cells of the shell gland across the day. The existence of a peripheral biological clock for the shell gland remains unknown, although findings suggest that a shell gland biological clock is likely, and it would be a good direction for future research to find out if components of the molecular clock play a role in shell gland physiology.

Unexpectedly, no hormonal effects on calcium uptake in the shell gland were observed at any time of the day. Previous research showed that melatonin, given orally for one year could increase the strength of long bones but simultaneously decrease shell
weight [43]. Furthermore, melatonin 1c receptor expression had previously been identified as a candidate for egg shell thickness modification [44]. Given these previous findings, I hypothesized that a short duration of melatonin exposure could affect calcium uptake in the shell gland. However, the data did not support this hypothesis. It is likely that melatonin’s actions on egg shell are long term effects secondary to those observed in bone. It is likely that an increase in bone formation decreased the amount of calcium available for depositing in the shell in the previous experiments. Estradiol increases calcium binding protein in the shell gland [67]. Administration of diethylstilbestrol (a synthetic non-steroidal estrogen) via silastic capsules increases the growth of epithelial cells in the shell gland [68]. Progesterone receptors were reported to be expressed in the nuclei of the surface epithelial cells [69]. In this study, no effect of either estradiol or progesterone on calcium uptake in the shell gland was observed. In this study, a 40 minute hormone exposure was used. This exposure time is sufficient only for inducing non-genomic actions, unlike the effects reported from long-term studies. Membrane bound, G-protein-coupled estrogen and progesterone receptors exist and are characterized by fast acting responses to the respective hormones [70, 71, 72, 73]. However, in this study, these types of receptor did not appear to be mediating the hormone effects to regulate calcium uptake in the shell gland.

In medullary bone, calcium uptake was rhythmic, being highest at ZT12 and lowest at ZT0. There are three cell types in medullary bone: osteoblast, osteoclast, and osteocyte [25]. In medullary bone, bone mineralization is mediated by osteoblasts, and bone resorption is mediated by osteoclasts. Bone is a highly dynamic tissue, always being remodeled by the actions of osteoblasts and osteoclasts. However, unlike cortical bone
where bone remodeling occurs at a slower rate, medullary bone remodeling happens very quickly over the course of a day in order to provide the calcium for calcification at night, and build up a calcium reserve at day [25]. Osteoblasts mediate bone formation by laying down osteoid, which is a collagenous scaffold for mineralization. Osteoclastic activity is predominant at the time of shell calcification (throughout the night) and osteoblastic activity is predominant during the day [25, 88]. It is likely that at night, osteoblastic activity is reduced, producing fewer osteoid for mineralization. As a result, less calcium is absorbed by the bone up until the time of oviposition. In contrast, during the day, osteoblastic activity is increased, producing more osteoid, and resulting in a gradual increase in calcium absorption for mineralization.

The results of this study are consistent with previous findings, showing increased medullary bone calcification during the day, and decreased medullary bone calcification at night [25, 88]. It is likely that medullary bone cells have their own circadian rhythm. Exploring the role of clock genes in medullary bone osteoblasts and osteoclasts, is worthy of further investigation.

In contrast with the duodenum and shell gland, calcium uptake in medullary bone increased after exposure to pharmacological doses of melatonin (0.1 mM), serotonin (0.1 mM), and estradiol (0.01 mM). Osteoid is made up of collagen, and therefore contains no hormone receptors. Because hormones are unable to affect osteoid, it is more likely that they increased the calcium uptake of bone cells (osteocytes or osteoblasts) via membrane calcium channels [75, 76] and calcium-dependent ATPases [90, 91]. This calcium uptake would not automatically contribute to bone mineral, as only calcium taken up by osteoid would contribute to hydroxyapatite in bone.
Melatonin is a potent activator of osteoblast activity and inhibitor of osteoclast function [45, 46, 47]. This experiment was the first to illustrate a direct and quick acting effect of melatonin on medullary bone cells. Due to the fast nature of the effect, it was likely that melatonin’s actions in medullary bone were mediated by the conventional membrane bound, G-protein-coupled receptors as opposed to the nuclear melatonin receptors of type ROR/RZR [78, 79].

In mammals, serotonin exerts two opposing effects upon bone, depending on the serotonin source. Brain–derived serotonin increases bone formation and decreases bone resorption due to secondary effects on food intake, energy expenditure, and sympathetic tone. Gut–derived serotonin inhibits osteoblast proliferation, causing a decrease in bone formation [53] via the Htr1b receptor, expression of which has not been detected in chickens. There are seven types of G-protein-coupled, membrane bound serotonin receptors, each with different subtypes, and all with different signaling patterns. Only 5HT2 receptors had previously been detected in chicken bone cells [51]. This receptor activates PLC, resulting in an increase of IP3 and DAG, and potentially inducing the uptake of calcium from the surrounding environment into the cell [54]. This is still a speculation, and further research in the future should focus on elucidating the quick acting action of serotonin in bone cells.

Estrogens were traditionally believed to have only genomic effects, however, the discovery of G-protein-coupled estrogen receptors changed that view, and explained the quick acting effects of estrogen [72, 73]. Later, G-protein-coupled progesterone receptors and their effects were discovered [70, 71]. G-protein-coupled estrogen receptors had been detected in osteoblasts [73] so it is tempting to speculate that they may be present on
chicken medullary bone cells as well. These results coincide with previous findings, suggesting the presence of G-protein-coupled estrogen receptors in hen medullary bone cells. Estradiol induces major effects upon medullary bone, propagating its formation [62, 63, 64], but this was the first time that a quick acting effect of estradiol in medullary bone had been documented. In contrast to the effects of estradiol, I could not detect any effects of progesterone upon calcium uptake in medullary bone. This could be due to G-protein-coupled progesterone receptors in medullary bone being uncoupled to calcium uptake, or being absent all together.

An interaction effect was discovered between time of day and hormone effects for calcium uptake of medullary bone that was exposed to $^{45}$Ca for 2 minutes. Specifically, at ZT12, calcium uptake of medullary bone exposed to melatonin, serotonin, and estradiol was not different from those at ZT0, ZT6, and ZT18. However, at ZT12, medullary bone calcium uptake of the control and progesterone conditions were greater than those at ZT0, ZT6, and ZT18. It was expected that at ZT12, calcium uptake for control condition would be greater than that at ZT0, ZT6, and ZT18 because at this time, osteoblastic activity would be at its peak, producing the most osteoid for mineralization. It was also expected that melatonin, serotonin, and estradiol would increase calcium uptake in osteoblasts and osteocytes via their G-protein-coupled receptors. However, the observation that the calcium uptake by tissues exposed to melatonin, estradiol, and progesterone at ZT12 did not show any different than those at ZT0, ZT6, and ZT18 was unexpected, because it implied that calcium uptake mediated by hormones was less at this time than at ZT0, ZT6, and ZT18 (as calcium uptake of bone in the control condition increased at this time). Future studies should try to elucidate this matter.
There were some limitations of this study. Firstly, exposure to hormones were only done for 40 minutes. It is possible that melatonin, estradiol, and progesterone could all be affecting calcium uptake via changes in gene expressions of proteins involving in the process of calcium uptake, and a 40 minute exposure time is not enough to detect these effects. Secondly, the concentrations of the hormones used were high relative to the typical physiological concentrations of these hormones. This can result in non-specific binding of the hormones to receptors with lesser affinity, which may lead to the changes observed in calcium uptake of the current study. Furthermore, estradiol and progesterone, being steroid hormones, can aggregate in the plasma membrane and influence its fluidity, which may also lead to the increase in calcium uptake observed. However, if this were the case, we would expect to observe similar effects for both progesterone and estradiol, which did not happen in this study.

Taken together, these results indicate that calcium uptake by duodenum, shell gland, and medullary bone is under the control of a biological clock that is correlated with the daily ovulation - oviposition cycle. As the construction and mechanisms of the molecular clock have not been discovered in the shell gland and medullary bone, this would be useful for future exploration. Also, exploration of the mechanisms by which melatonin, serotonin, and estradiol regulate calcium uptake in medullary bone cells deserves more attention in further research.
Figure 4. Medullary bone calcium uptake (without log transformation) after oviposition and hormone treatments. For time of day, ZT0 was the time of oviposition. Different hormone treatments are shown as separate columns for each time of day, and labeled by...
color. From lightest to darkest: blank (media without hormone, Blk), melatonin (MLT), serotonin (5HT), estradiol (E2), and progesterone (P4). 4A: calcium uptake (mg Ca/g tissue) from medullary bone exposed to $^{45}$Ca for 2 minutes. 4B: calcium uptake (mg Ca/g tissue) from medullary bone exposed to $^{45}$Ca for 5 minutes.
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