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DETERMINING IF SERUM SHOCK AND DEXAMETHASONE INITIATE CIRCADIAN RHYTHMS IN BOVINE MAC-T MAMMARY EPITHELIAL CELLS

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ABSTRACT

Biological rhythms allow organisms to coordinate changes in the external environment with behaviors and physiological processes. Daily changes are orchestrated by endogenous circadian timekeepers, which create repeating cycles of about 24 hours. The regulation of circadian rhythms has not been well-studied in the mammary gland, and may be important to milk production. Serum shock and dexamethasone are known to initiate the circadian clock of fibroblasts, mesenchymal stem cells, peripheral blood mononuclear cells, and peripheral tissues of *Drosophilia* and zebrafish, but their effects have not been studied in mammary cells. The objective of this study was to determine if serum shock and dexamethasone treatment could re-initiate the biological clocks in bovine mammary epithelial (MAC-T) cells. MAC-T cells were cultured in DMEM medium, exposed to serum starvation for 30 hours, and treated with either dexamethasone (DEX), 50% serum media (serum shock, SS), or serum-free control media (CON). The cells were harvested and lysed 0, 4, 8, 12, 16, 20, and 24 hours after treatment. The relative expression of the core circadian clock genes *CLOCK*, *BMAL1*, *PER1&2*, and *CRY1* were measured using reverse transcriptase quantitative PCR (RT-PCR). Data were analyzed using the mixed model ANOVA procedure of SAS. Gene expression was normalized using the housekeeping gene *Rps9* as a covariate in the model. A zero-amplitude test was conducted to determine the fit of the cosine function. A cosine function fit the relative expression of *Per2*, *Cry1*, and *Clock* in DEX ($P<0.05$), but not CON ($P>0.10$), indicating that dexamethasone initiated a 24-hour circadian rhythm of the expression of these genes. Alternatively, dexamethasone did not initiate a rhythm of *Per1* or *Bmal1* expression. A cosine function fit the relative expression of *Cry1* in SS ($P<0.05$) but not in CON ($P>0.10$), indicating that serum shock initiated a 24-hour circadian rhythm of the expression of

this gene. Serum shock did not initiate a rhythm in *Clock*, *Per1*, or *Per2* gene expression. The results of this study suggest that dexamethasone re-initiates the damped biological clocks in cultured bovine mammary epithelial MAC-T cells, while serum shock appears unreliable for re-initiating rhythms of core clock gene expression.

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ABBREVIATIONS

BMALl1	Brain and Muscle ARNT-Like 1
cDNA	Complimentary DNA
CLOCK	Circadian Locomotor Output Cycles Kaput Protein
CON	Control Treatment
CRY1	Cryptochrome Circadian Clock 1
DEX	Dexamethasone Treatment
IGF-1	Insulin-like growth factor 1
mRNA	Messenger RNA
PAS	Period-Arnt-Single-Minded
PER1	Period Circadian Clock 1
PER2	Period Circadian Clock 2
RNA	Ribonucleic acid
RPS9	Ribosomal Protein S9
RT-PCR	Reverse Transcriptase Quantitative Polymerase Chain Reaction
SCN	Suprachiasmatic nucleus
SS	50% Serum Shock Treatment

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Chapter 1

Introduction

Circadian rhythms coordinate daily changes in the external environment with behaviors and physiological processes allowing organisms to anticipate external and environmental changes throughout the day (Dibner, Schibler, and Albrecht, 2010; Chi-Castaneda and Ortega, 2016). This time-keeping system occurs in almost, if not all, light-sensitive organisms and governs many aspects of physiology and behavior of most mammals (Doherty and Kay, 2010; Dibner et al, 2010). Examples of processes regulated by the circadian system include sleep-wake cycles, endocrine secretion, cardiovascular activity, body temperature fluctuations, appetite, GI function, and metabolism, among others (Gachon et al, 2004). It most likely evolved in single-celled organisms as a way to regulate and adapt cellular metabolism (Asher and Schibler, 2011). Crosstalk occurs between molecules responsible for circadian rhythms and molecules responsible for cellular metabolism as well as molecules responsible for cell cycle progression (Asher and Schibler, 2011; Borgs et al, 2009).

The dominant regulation and synchronization of the circadian clock occurs due to the functioning of the central circadian oscillator in the brain, specifically the neurons in the suprachiasmic nucleus (SCN) of the hypothalamus (Dibner et al, 2010). The SCN of mammals is primarily entrained by photic cues (Balsalobre, Damiola, and Schibler, 1998). In addition to signals from the central clock, there are also peripheral timekeeping mechanisms in most tissues and cells throughout the body, including the liver, heart, kidney, adipose, and mammary tissues, to name a few (Dibner et al, 2010). Processes regulated by peripheral clocks include, but are not limited to,

carbohydrate and lipid metabolism, detoxification, urine production, blood pressure, and heart rate. The SCN uses humoral and neuronal signals to communicate with the peripheral clocks, and they receive additional entrainment signals from environmental cues such as feeding and temperature. In fact, research suggests that the primary timing cue of peripheral clocks is feeding time (Nagoshi et al, 2004). Additionally, glia cells found in other structures throughout the brain have been shown to express *PER*-based circadian rhythmicity which is dependent on the SCN (Chi-Castaneda and Ortega, 2016). The SCN and peripheral clocks are similar in that they have comparable molecular components and both include transcriptional-translational feedback loops (Ko and Takahashi, 2006). Furthermore, both have found to be cell-autonomous and self-sustained *in vivo* as well as *in vitro*, although to varying degrees.

Circadian rhythms are regulated by the expression of the core circadian clock genes, which include, among others, *CLOCK, BMAL1, CRY1, CRY2, PER1,* and *PER2* (Amano et al, 2010). These genes encode transcription regulation factors in most organs, tissues, and cells of mammals, regulating the central and peripheral clocks. These genes are divided into two primary groups: transcription-promoting factors and transcription-suppressing factors (Amano et al, 2010). The transcription-promoting factors make up the 'positive arm' of the molecular clockwork and include the genes *CLOCK* and *BMAL1*, which transcribe PAS domain helix-loop-helix proteins, which then bind to enhancer box (E-Box) domains on clock-controlled genes (Amano et al, 2010; Asher and Schibler, 2011). These 'positive arm' core clock genes positively control the expression of the 'negative arm' of the molecular clockwork, which includes the transcription-suppression factors *CRY1, CRY2, PER1,* and *PER2* (Amano et al, 2010). The expression of *BMAL1* and *CLOCK* is subsequently inhibited by PER and CRY proteins, generating an indirect negative feedback loop

that creates the circadian clock. These genes fluctuate over 24-hour cycles and the clock genes in different organs coordinate oscillation throughout the body (Nebzydoski et al, 2010).

The use of *in vitro* cell culture has many advantages over *in vivo* methods including ease of use and more readily accessible molecular genetic tools (Izumo et al, 2006). However, studying circadian rhythms in culture is challenging because many peripheral oscillators often desynchronize between cells and their rhythms dampen due to the removal of signaling from the SCN, which results in a lowered amplitude of clock gene expression (Izumo et al, 2006; Schibler, 2003). One exception is fibroblast cells, which have been shown to have self-sustained circadian rhythms in culture (Nagoshi et al, 2004). In a study done by Yamazaki et al. (2000), rat cells from the SCN exhibited a rhythm for up to 32 days *in vitro*, while the expressed rhythms of peripheral tissues *in vitro* were dampened after a few days.

The glucocorticoid hormone analog dexamethasone has been used to phase shift peripheral clocks and induce circadian gene expression in various cell cultures (Balsalobre et al, 2000a). Balsalobre et al. (2000a) reported that dexamethasone elicited strong expression of *PER1*, *PER2*, *PER3*, and *CRY1* in cultured rat-1 fibroblasts. Also, cultured peripheral blood mononuclear cells of canines treated with dexamethasone have exhibited entrained rhythms (Ohmori et al, 2013). It is speculated that the SCN may utilize various chemical messengers, including glucocorticoids, to regulate peripheral clocks *in vivo* (Balsalobre, et al. 2000b). Mechanistically, dexamethasone is thought to play a role in this *in vivo* entrainment of peripheral oscillators, while eliciting no effect on SCN neurons, due to a lack of glucocorticoid receptors on those cells (Balsalobre et al, 2000a). Serum shock has also been shown to re-initiate circadian gene expression of cells cultured *in vitro*, including immortalized rat-1 fibroblasts (Balsalobre et al, 1998), mesenchymal stem cells (Huang et al, 2009), and isolated peripheral tissues from *Drosophilia* and zebrafish (Izumo et al, 2006).

Very little circadian rhythm research has been done in ruminants (Nebzydoski et al, 2010). The regulation of daily rhythms in the bovine mammary gland has been especially poorly characterized, but it may be important to milk production in dairy cows. For example, the timing of food intake can desynchronize the central clock from peripheral clocks, such as those of mammary cells. In humans, circadian dyssynchrony can cause numerous disorders, including several metabolic diseases and obesity (Bass and Takahashi, 2010). The disorganization of the core circadian clock can also result in cells that are more prone to cancer and accelerate the rate of tumor growth (Borgs, 2009). The accurate synchronization of the central and peripheral clocks may improve milk yield and efficiency by timing nutrient availability with the metabolic ability of the mammary gland to efficiently utilize nutrients.

Serum shock and dexamethasone are known to initiate the circadian clocks of many peripheral cell types, but their effects have not been studied in mammary cells. The goal of this experiment was to examine if serum shock and dexamethasone treatment could re-initiate the peripheral clocks in bovine mammary epithelial cell culture (MAC-T; Huynh et al, 1991) grown *in vitro*. The predicted outcome is the acute modification of core clock gene expression leading to re-initiation of peripheral clock rhythms in MAC-T cells by dexamethasone and serum shock treatment. This experiment hopes to provide a better mechanistic understanding of the peripheral clocks in mammary epithelial cells in bovine cell lines.

Chapter 2

Materials and Methods

Cell Culture, Treatment, Harvesting, and Lysing

Dexamethasone

MAC-T cells were grown in 6 well plates to 80% confluence in DMEM (Dulbecco's modified Eagle's) medium at 37°C. Cells were then exposed to serum starvation incubation in serum-free DMEM medium for 30 hours, with a medium change after 24 hours. The cells were treated with either 10 μg/ml dexamethasone (DEX) or a serum-free control media (CON). This experiment was done in triplicate. The cells were harvested and lysed at 0, 4, 8, 12, 16, 20, and 24 hours after treatment. Each well was lysed in a chloroform-phenol RNA extracting reagent (RNAsolv RT; Omega Bio-tek Inc., Norcross, GA) and placed in autoclaved 1.5 ml microcentrifuge tubes. The samples were stored at -80°C prior to RNA extraction.

Serum shock

MAC-T cells were grown in 6 well plates to 80% confluence in DMEM (Dulbecco's modified Eagle's) medium at 37°C. Cells were then exposed to serum starvation incubation in serum-free DMEM medium for 30 hours, with a medium change after 24 hours. The cells were

treated with either 50% serum media (serum shock, SS) or serum-free control media (CON). This experiment was done in triplicate. The cells were harvested and lysed at 0, 4, 8, 12, 16, 20, and 24 hours after treatment. Each well was lysed in a chloroform-phenol RNA extracting reagent (RNAsolv RT; Omega Bio-tek Inc., Norcross, GA) and placed in autoclaved 1.5 ml microcentrifuge tubes. The samples were stored at -80°C prior to RNA extraction.

RNA Extraction

Samples were thawed over ice and then incubated at room temperature for 5 minutes. Next, 200 µl of chloroform was added and samples were shaken vigorously for 15 seconds. The samples were incubated at room temperature for 2 to 3 minutes and then centrifuged at 12,000 x *g* for 15 minutes at 4°C. Five-hundred µl of the top clear aqueous layer of each sample was transferred to new 2ml flat-bottom tubes and 70% ethanol was added in a 1:1 ratio with the supernatant. The samples were vortexed and 700 µl of each sample were transferred immediately to spin columns (Omega Bio-tek Inc.). The samples were spun at 8,000 x g at room temperature for 30 seconds. The flow-through was discarded. Wash Buffer 1 (350 µl RNA) was added to each column and the samples were spun at 8,000 x *g* for 30 seconds at room temperature. The flow through was discarded. On column DNA digestion was accomplished by adding 75 µl of DNase 1 incubation mix (1050 µl Sigma DNase digestion buffer, 150 µl DNase 1 stock solution) directly to each column membrane and the samples were incubated at room temperature for 12 minutes. The samples were then placed in new 2 ml collection tube. After the incubation, 350 µl RNA Wash Buffer 1 was added to each column and the samples were spun at 8,000 x g for 30 seconds at room temperature. The flow through was discarded and 500 µl RNA Wash Buffer 2 was added to each

column and the samples were spun at 8,000 x g for 30 seconds. The flow through was again discarded and this step was repeated again. The columns were placed in new 2 ml collection tubes and spun at 11,000 x g for 1 minute at room temperature. The columns were placed in new autoclaved 1.5 ml microcentrifuge tubes. Finally, RNA was eluted by addition of 40 µl of DEPC water added directly to the center of each column membrane. The samples sat at room temperature for 1 minute and were then spun at 8,000 x g for 1 minute at room temperature. The eluate was pipetted back onto the column membranes and spun again at 8,000 x g for 1 minute at room temperature. The resulting RNA was quantified with a spectrophotometer (Nanodrop-1000, ThermoFisher Scientific, Waltham, MA) and stored at -80°C.

Gene Expression Analysis

RNA samples were diluted to a concentration of 10 ng/ul. RNA was denatured by incubating at 65°C for 5 minutes. Complimentary DNA (cDNA) was generated from RNA using the High Capacity cDNA Reverse Transcriptase kit (Applied Biosystems Inc., Foster City, CA). The reactions were incubated at 25°C for 10 min; 37°C for 2 hr; 85°C for 2 sec in a thermocycler (Bio-Rad C1000, Hercules, CA). A negative reverse transcriptase control was also generated.

The expression of *CLOCK*, *BMAL1*, *PER1&2*, *CRY1* and *RPS9* were quantified using Real-Time quantitative PCR (qPCR; ABI 7900HT, Applied Biosystems Inc.) with a SYBR Green reporter and ROX passive dye (PerfeCTa SYBR Green SuperMix, Quanta Biosciences Inc., Beverly, MA). Previously validated forward and reverse primers for each gene were used for quantification (TABLE 1). These genes were chosen to represent both the positive and negative arm of the circadian clock, with *RPS9* serving as a housekeeping gene.

Statistical Analysis

Data were analyzed using the mixed model ANOVA procedure of SAS 9.4 SAS 9.4 (SAS Institute, Inc. Cary, NC, USA). Fixed effects in the model included the treatment, the cosine parameters, and their interaction. The preplanned contrast tested the difference between treatments at each time point. Data were normalized using RPS9 as a housekeeping gene, which was included as a covalent in the model. A zero amplitude test was conducted to determine the fit of the cosine function and resulting *P*-values were examined, with a *P*-value < 0.05 indicating an initiated rhythm, a *P*-value between 0.05 and 0.10 indicating a trend (tendency towards rhythm), and a *P*-value > 0.10 indicating no initiation of a rhythm.

Table 1. **Validated forward and reverse primers used to quantify selected core clock gene expression**

Target[1]	**5'-sequence**[2]	**T_a**[3]
RPS9	f-CCTCGACCAAGAGCTGAAG	60
	r-CCTCGACCAAGAGCTGAAG	
CLOCK	f-TCAGTCTCAAGGAAGCGTTG	60
	r-GGAGTGCTAGTATCTGTTGGG	
BMAL1	f-CAGCTCTCCTCCTCCGAACAC	60
	r-TGGCCCAGGGGTTATATCTG	
PER1	f-CCAGGAGTTCTACCAGCAATG	60
	r-GAGACAGCCACGGAGAAG	
PER2	f-ACGTTTTCCAGTCTCGCTG	60
	r-CGTTTGCACTTCAGTTTTCGG	
CRY1	f-TGCAAGTTGTACTCAAGGGAG	60
	r-CAATACTCTGCGTGTCCTCTTC	

[1]Circadian Locomotor Output Cycles Kaput Protein (*CLOCK*), Brain and Muscle ARNT-Like 1 (*BMAL1*), Period Circadian Clock 1 (*PER1*), Period Circadian Clock 2 (*PER2*), and Cryptochrome Circadian Clock 1 (*CRY1*) were the target genes examined.

[2]Selected forward (f) and reverse (r) primers for validated for genes encoding the mRNA target

[3]Annealing temperature (°C)

Chapter 3

Results & Discussion

Dexamethasone Treatment

According to the the zero-amplitude test (Table 1), a cosine function fit the relative expression of *PER2*, *CRY1*, and *CLOCK* in DEX ($P < 0.05$), but not CON ($P > 0.10$), indicating that dexamethasone initiated a 24-hour circadian rhythm of the expression of these genes. Alternatively, dexamethasone did not initiate a 24-h rhythm of *PER1* expression, where both DEX and CON did not fit the relative expression of these genes ($P > 0.10$). Both DEX and CON exhibited a rhythm of *BMAL1* expression, as both fit the relative expressions of these genes and therefore a 24-hour rhythm ($P < 0.05$). This result is surprising because it suggests that a *BMAL1* rhythm is expressed constitutively in these cells, which contradicts with research in other models and with the results from the serum shock experiment presented in this paper (Table 2). The results could also be attributed to the necessary media change performed before treatment, which may have re-started the clocks in the CON treatment. DEX treatment did, however, shift the time at peak of the cosine rhythm of *BMAL1* from 1411 h (2:11 PM) to 744 h (7:44 PM) ($P<0.05$). Therefore, dexamethasone treatment is clearly differentially affecting the expression and regulation of *Bmal1* transcription.

This data indicates that dexamethasone initiated gene expression of both the negative arm (via *Per2* and *Cry1* gene expression) and the positive arm (via *CLOCK* gene expression) of the molecular clockwork, indicating that dexamethasone can be used to re-initiate dampened rhythms of *in vitro* bovine mammary MAC-T epithelial cells. This finding supports other research that has also used dexamethasone to re-initiate the damped clocks of other cultured cells, including cultured

rat-1 fibroblasts (Balsalobre et al, 200a) and peripheral blood mononuclear cells of canines (Ohmori et al, 2013). Dexamethasone, a potent glucocorticoid, is a high affinity activator of the steroidal signaling pathway of peripheral tissues, which may explain why it re-initiates the circadian rhythms of several types of cultured cells (Dickmeis, 2009). Therefore, it is not surprising that dexamethasone also re-initiated the clocks of bovine mammary epithelial (MAC-T) cells, especially given that glucocorticoids have been shown to be primary stimulators of mammary cell differentiation (Akers, 2006). Additionally, it is speculated that the re-initiation of circadian gene expression in serum shock treated cultures is due to the presence of glucocorticoids (Dickmeis, 2009).

Serum Shock Treatment

According to the zero-amplitude test (TABLE 2), a cosine function fit the relative expression of *CRY1* in SS ($P < 0.05$). but not CON ($P>0.10$), indicating that serum shock initiated a 24-hour circadian rhythm of its expression. However, serum shock did not initiate a rhythm in *CLOCK* or *BMAL1* gene expression, where both DEX and CON did not fit the relative expression ($P > 0.10$). Surprisingly, a cosine function fit the relative expression of *PER1* in CON ($P < 0.05$), initiating a rhythm, while SS did not ($P > 0.10$). Also, *PER2* had a tendency for rhythm in CON ($0.05 < P <0.10$), but did not initiate a rhythm in SS ($P > 0.10$).

While serum shock did initiate gene expression of one of the components of the ‘negative arm’ (*CRY1)* of the molecular clock, it failed to initiate gene expression of any other clock gene. The experiment also provided surprising results pertaining to *PER1 and PER2* gene expression, which need to be further studied to determine if the gene expression observed in this experiment

is due to experimental error or a mechanism not yet understood. While it is possible that a *PER1* and *PER2* rhythm was constitutively expressed in these cells and was desynchronized by the addition of 50% serum media, it seems unlikely, especially since no rhythm was seen in the *PER1* and *PER2* CON treatment of the dexamethasone experiment, which utilized the same cell line. The results could also be attributed to the necessary media change performed after treatment. This media change may have re-started the clocks of the cells, resulting in rhythmicity of CON cells, while subsequent addition of a 50% serum shock to SS cells may have modified this response, causing the cells to once again to become desynchronized.

The results of this study suggest that serum shock is not a reliable treatment to re-initiate the circadian rhythms of bovine mammary MAC-T epithelial cells. Serum shock is high in a complex mix of growth factors, which is why it is thought to induce rhythms in many cultured cell types, such as immortalized rat-1 fibroblasts (Balsalobre et al, 1998), mesenchymal stem cells (Huang et al, 2009), and isolated peripheral tissues from *Drosophilia* and zebrafish (Izumo et al, 2006). Due to these previous findings, it was thought that serum shock would cause a similar response in bovine mammary epithelial (MAC-T) cells. Serum shock treatment may not have induced a rhythm in these cells for many reasons, including a low concentration of growth factors in the specific lot of serum used in the experiment. Serum shock exhibits batch to batch variation and the particular lot used in this experiment may be low in growth factors necessary to restart the clocks of these cells (van der Valk, 2010). Another possibility is that the cells used in this experiment may not have receptors for the growth factors found in serum, and it may be beneficial to test these methods using other growth factors already known to regulate mammary cells, such as prolactin and insulin-like growth factor 1 (IGF-1) (Akers, 2006). It is also possible that a 50% serum shock is simply not a high enough concentration to restart the clocks, and

bovine mammary epithelial (MAC-T) cells may need to be exposed to a higher concentration serum shock, such as a 100% serum shock treatment.

It is important to keep in mind that not only is the response of cell cultures to treatments variable, but the rhythms themselves often exhibit unstable baseline shifting that can change among experiments as well as among samples and may result in rhythms with different phase relationships (Izumo et al, 2006). Cells may also be at different levels of synchrony within the culture before any treatment is administered, causing variability in the results. These reasons may have been responsible for the appearance of rhythms in control cells for *BMAL1* in the dexamethasone experiment and *PER1* and *PER2* in the serum shock experiment.

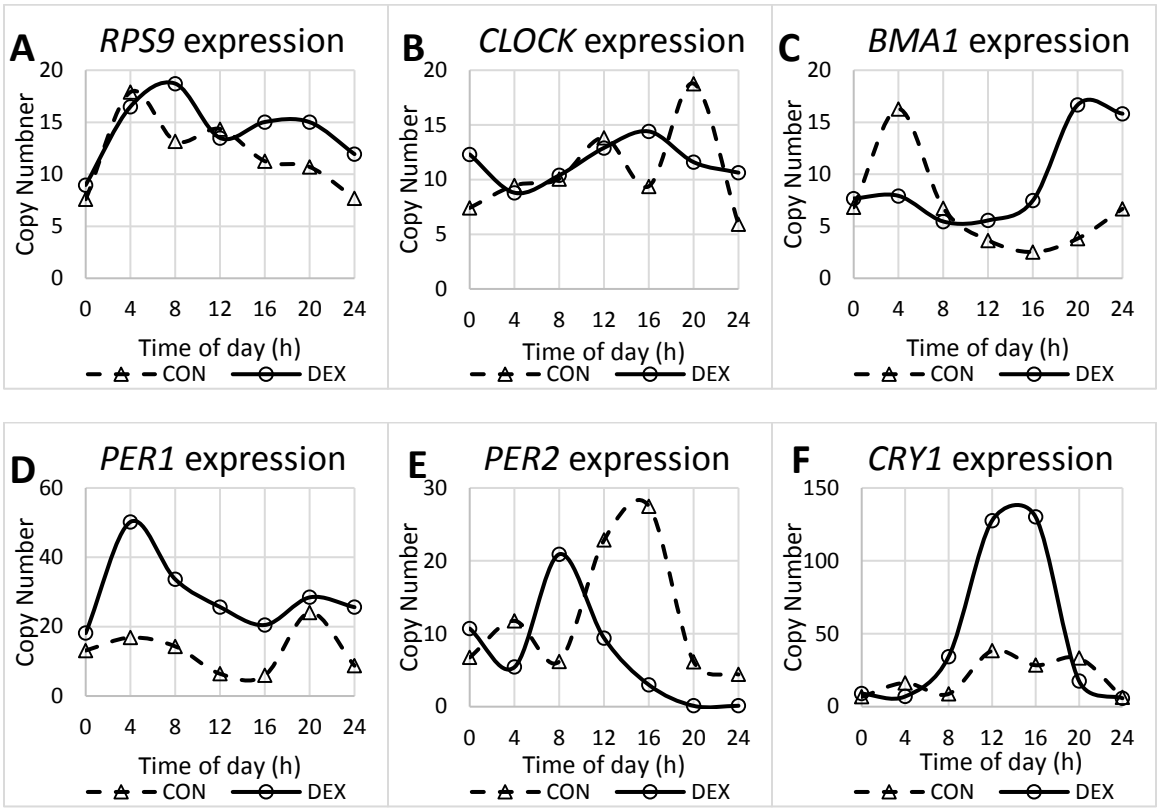

Figure 1. Expression of dexamethasone on regulation of circadian genes in bovine mammary epithelial (MAC-T) cell culture

Cells were grown to 80% confluence in DMEM medium and treated with either 10 μg/ml dexamethasone or a serum-free control media after 30 hours of serum starvation. Cells were harvested every 4 hours (n = 3). Expression of the genes Ribosomal Protein s9 (*RPS9*, Panel A), Circadian Locomotor Output Cycles Kaput Protein (*CLOCK*, Panel B), Brain and Muscle ARNT-Like 1 (*BMAL1*, Panel C), Period Circadian Clock 1 (*PER1*, Panel D), Period Circadian Clock 2 (*PER2*, Panel E), and Cryptochrome Circadian Clock 1 (*CRY1*, Panel F) were examined. *RPS9* was used as a housekeeping gene. A 24-hour rhythm was tested by fit of a cosine function using a zero-amplitude test (Table 1).

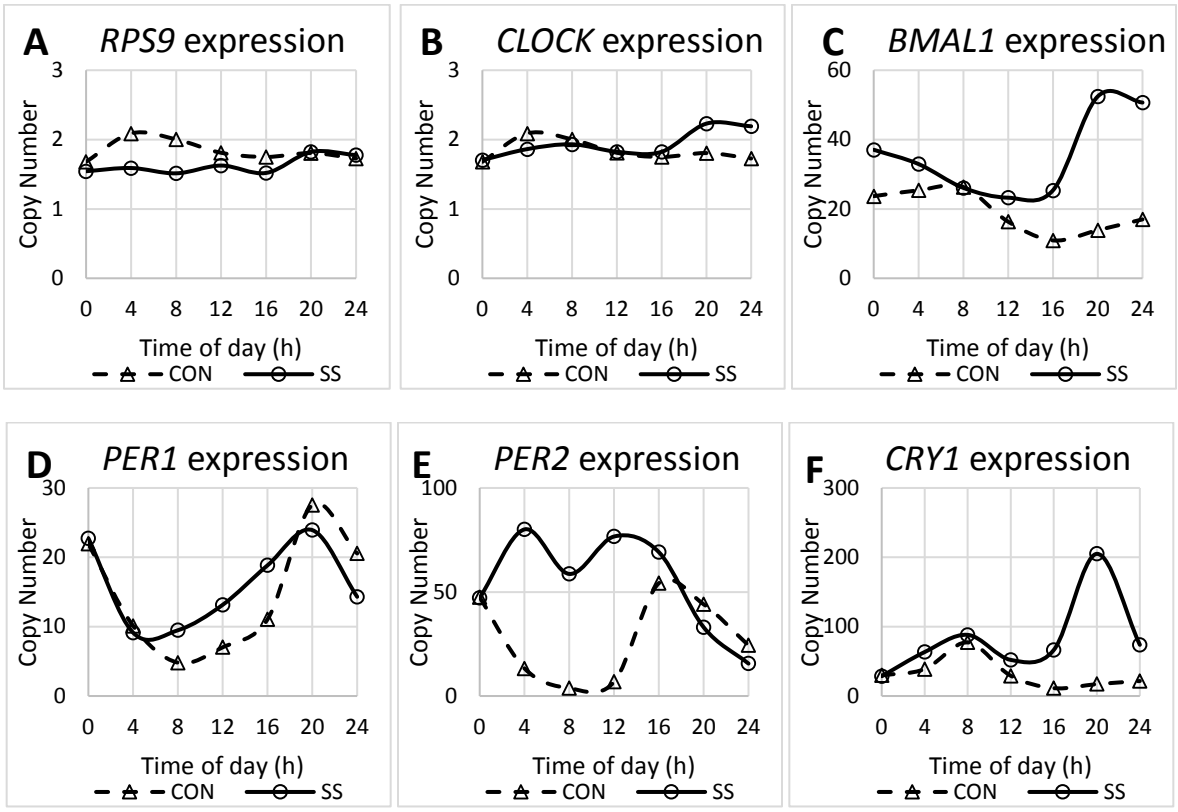

Figure 2. Expression of serum shock on regulation of circadian genes in bovine mammary epithelial (MAC-T) cell culture

Cells were grown to 80% confluence in DMEM medium and treated with either a 50% serum shock or a serum-free control media after 30 hours of serum starvation. Cells were harvested every 4 hours (n = 3). Expression of the genes Ribosomal Protein s9 (*RPS9*, Panel A), Circadian Locomotor Output Cycles Kaput Protein (*CLOCK*, Panel B), Brain and Muscle ARNT-Like 1 (*BMAL1*, Panel C), Period Circadian Clock 1 (*PER1*, Panel D), Period Circadian Clock 2 (*PER2*, Panel E), and Cryptochrome Circadian Clock 1 (*CRY1*, Panel F) were examined. *RPS9* was used as a housekeeping gene. A 24-hour rhythm was tested by fit of a cosine function using a zero-amplitude test (Table 2).

Table 2. Zero-amplitude test to determine the fit of a cosine function with a 24-hour period for dexamethasone treatment

Gene[1]	*Treatment*[2]	*F value*	*P value*[1]
Per1	CON	1.5739	0.24
	DEX	0.1506	0.99
Per2	CON	2.2516	0.14
	DEX	3.7908	0.04
Cry1	CON	0.3167	0.92
	DEX	4.2992	0.02
Clock	CON	0.4286	0.66
	DEX	4.5885	0.03
Bmal1	CON	8.8191	<0.001
	DEX	8.4265	0.001

[1] Circadian Locomotor Output Cycles Kaput Protein (*CLOCK*), Brain and Muscle ARNT-Like 1 (*BMAL1*), Period Circadian Clock 1 (*PER1*), Period Circadian Clock 2 (*PER2*), and Cryptochrome Circadian Clock 1 (*CRY1*) were examined.

[2] Cells were grown to 80% confluence in DMEM medium and treated with either dexamethasone (DEX) or a serum-free control media (CON) after 30 hours of serum starvation. Cells were harvested every 4 hours (m=3).

[3]A *P*-value < 0.05 indicates that a cosine function fit the relative expression of the gene, indicating a 24-hour circadian rhythm expression. A *P*-value between 0.05 and 0.10 indicates a tendency towards rhythm. A *P*-value > 0.10 indicates that a cosine function did not fit the relative expression of the gene, indicating no initiation of a 24-hour circadian rhythm

Table 3. Zero-amplitude test to determine the fit of a cosine function with a 24-hour period for serum shock treatment

Gene[1]	*Treatment*[2]	*F value*	*P value*[3]
Per1	CON	6.0959	0.005
	SS	0.6062	0.66
Per2	CON	2.4118	0.098
	SS	1.8514	0.18
Cry1	CON	0.8751	0.50
	SS	3.3998	0.04
Clock	CON	0.5629	0.69
	SS	0.3569	0.83
Bmal1	CON	0.6111	0.66
	SS	0.4185	0.79

[1]Circadian Locomotor Output Cycles Kaput Protein (*CLOCK*), Brain and Muscle ARNT-Like 1 (*BMAL1*), Period Circadian Clock 1 (*PER1*), Period Circadian Clock 2 (*PER2*), and Cryptochrome Circadian Clock 1 (*CRY1*) were examined.

[2]Cells were grown to 80% confluence in DMEM medium and treated with either 50% serum shock (SS) or a serum-free control media (CON) after 30 hours of serum starvation. Cells were harvested every 4 hours (m=3).

[3]A *P*-value < 0.05 indicates that a cosine function fit the relative expression of the gene, indicating a 24-hour circadian rhythm expression. A *P*-value between 0.05 and 0.10 indicates a tendency towards rhythm. A *P*-value > 0.10 indicates that a cosine function did not fit the relative expression of the gene, indicating no initiation of a 24-hour circadian rhythm.

Chapter 4

Conclusion

Dexamethasone treatment has been shown to initiate a 24-hour circadian rhythm for several core clock genes (*Per2*, *Cry1*, and *Clock*), and can therefore be used to re-initiate the dampened biological clocks in *in vitro* bovine mammary epithelial MAC-T cells, while serum shock has proven unreliable in reinitiating the core clock genes.

The serum shock data should be further examined and the treatment repeated in order to further understand the results obtained in this experiment, since serum shock appeared to initiate a rhythm in *Per1* CON ($P < 0.05$) and tended to cause a rhythm in *Per2* CON ($P < 0.05$), but not in SS of either *Per1* or *Per2* ($P > 0.10$). These unexpected results need further examination and testing to determine whether the results found in this study are a function of the treatment on the control of these core clock genes, or if it was a source of human experimental error.

Dexamethasone and serum shock treatment could be further examined in different mammary epithelial cell lines, including those of other species, such as mouse HC-11 mammary epithelial cells, to determine the effect of serum shock and dexamethasone on the initiation of circadian rhythms in other mammary epithelial cells. This data can further validate the use of dexamethasone to initiate 24-hour circadian rhythms in cell cultures and provide researchers with the opportunity to conduct research using various types of mammary epithelial cells with re-initiated rhythms.

The validation of this cell culture model and use of dexamethasone to re-initiate dampened circadian rhythms in this cell line allows for its use in future experiments. The results

of this experiment can be applied to further study how circadian clocks and hormone signaling pathways interact in bovine mammary epithelial cells in relation to applications involving increasing the efficiency of milk synthesis. This includes examining the ability of nutrient and hormone treatments to modify these rhythms and core clock gene expression, which would result in a greater mechanistic understanding of the regulation of circadian rhythms in the mammary gland. These findings could then be extrapolated to dairy cows and used to increase yield and efficiency of milk production.

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ACADEMIC VITA

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Education

Major: Veterinary and Biomedical Sciences
Minor: International Agriculture
Honors: Veterinary and Biomedical Sciences

Research Experience

Schreyer Honors thesis 2016-2017

- Thesis Title: Determine if Serum Shock and Dexamethasone Initiate Circadian Rhythms in Bovine MAC-T Mammary Epithelial Cells and Mouse HC-11 Mammary Epithelial Cells
- Thesis Supervisor: Dr. Kevin Harvatine

Avian Virology Department, Animal Diagnostic Laboratory 2015

- Student Intern

Braithwaite Behavioral Cognitive Ecology Research Laboratory 2013-2015

- Research Assistant

Work Experience

Pennsylvania State University 2016-2017

- Resident Assistant (RA)

Shaver's Creek Environmental Center 11/2015- 9/2016

- America Reads Literacy Mentor, Conservation Educator, Animal Caretaker

Grace Lutheran Preschool and Kindergarten 11/2015-5/2016

- America Reads Literacy Mentor, Teacher's Assistant

Pennsylvania Governor's School for the Agricultural Sciences 7/2015- 8/2015

- Governor's School Mentor

Mountain Shadow Veterinary Hospital 5/2015- 1/2016

- Pet Care Attendant

Professional Experience

Veterinary Experience

- USDA APHIS Animal Care Extern 2016
- PA Game Commission, Dr. Justin Brown 2016
- APVMA National Symposium Attendee 2014-2017
- Mountain Shadow Veterinary Hospital, Job Shadow 2014-2015
- All-Pets Veterinary Wellness and Spay/Neuter Clinic 2013-2015

Animal Experience

- Susquehanna Service Dogs, Intern 2016
- Susquehanna Service Dogs, Puppy Raiser 2016-2017
- Red Creek Wildlife Rehabilitation Center 2014

Association Membership/ Activities

Penn State Dance Marathon (THON)
- Rules and Regulations Event Safety Committee Member 2014-2015

Penn State Pre-Vet Club
- APVMA Symposium Chair 2016-2017
- THON Chair 2014-2016

Relay For Life of Penn State
- Entertainment Overall Chairperson 2014-2015
- Luminaria Committee Captain 2013-2014

International Experience

Guangxi Veterinary Research Institute
- International Summer Research Intern Summer 2015

International Agricultural Animal Industry Studies in Switzerland Spring 2017
- ANSC 499B Embedded Course

Scholarships, Honors and Awards

Pennsylvania State University Grants
- College of Agricultural Sciences Undergraduate Student Research Award

Pennsylvania State University Scholarships
- Harry R. and Kathleen E. Ulrich Scholarship in Agricultural Sciences
- Rosie and Stuart Kahan Scholarship in Pre-Veterinary Sciences In Memory of Lawrence Kahan
- Shigley Memorial Pre-Veterinary Scholarship
- Gertrude B. Paschall Memorial Scholarship in Agricultural Sciences
- Provost Award

Presentations

Gamma Sigma Delta Undergraduate Research Exposition April 2017