CIRCADIAN RHYTHM EVALUATIONS TO

DETERMINE BLOOD SAMPLE DEPOSITION TIME

By

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Abstract:

The use and applications of DNA and RNA analysis has become an invaluable forensic tool in criminal investigations. Recent research from this laboratory applied quantitative analysis of RNA as a means of estimating the age of crime scene samples. Linking the age of an evidentiary sample to the time a crime was committed can strengthen the prosecution of a perpetrator.

Organisms exhibit a circadian rhythm that is observed as 24-hour cyclic processes that can be detected through oscillating quantities of mRNA and protein. It has been estimated that ten percent of the human transcriptome demonstrates rhythmic expression patterns of mRNA. Thus, an opportunity exists to harness a natural biologic process through quantitative RNA analysis to perhaps refine our ability to estimate when a crime scene sample was created.

The aim of this current study was to determine if it is feasible to identify when, during a 24-hour day, a blood stain was created at a crime scene. In order to establish a reference pattern of gene expression that reflects a 24-hour day, it was necessary to assess the stability of the mRNA transcripts from cycling genes in dried stains as well as to explore the variability in the expression of these genes among members of the population. Results demonstrated that all transcripts studied were stable for the short period between production of a blood stain and the extraction and reverse transcription of RNA recovered from a stain. In addition, mRNA expression levels were compared between groups of blood donors of different age and also between the sexes. Evaluations of transcript levels were also performed to validate naturally occurring fluctuations in gene expression. Finally, blood stains collected every 4 hours for 24 hours were assessed using qPCR, for mRNA expression levels in order to identify genes exhibiting rhythmic patterns that could be referenced for time determination.

Overall, results from the current study validate the feasibility of using gene expression during the circadian rhythm to further refine our ability to determine when, during a 24-hour day, a blood stain was created at a crime scene.

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CHAPTER I

INTRODUCTION

Forensic Science is a multidisciplinary field that encompasses many areas of Natural Sciences and has become an essential part of the judiciary system. The discipline of Forensic Biology has traditionally revolved around human identification from the examination of DNA sequences that vary within the human population (Jobling & Gill, 2004). Although identifying the source of a crime scene sample is typically possible through DNA analysis, establishing the relevance of a biological sample(s) present at a scene to the crime that occurred there is presently not possible. For example, if the suspected perpetrator of a crime frequented the scene it might be expected that his/her DNA could be found there and not relevant to a crime whatsoever. Thus, correlation between the evidence and the crime can be critical in a criminal investigation. Being able to estimate the time a biological sample was created at a scene can link a crime to the evidence recovered at the site.

Circadian rhythm is a 24-hour cycle that our body undergoes that is reflected in a repeated cycle of changing levels of mRNA and proteins. The central control of these biologic oscillations occurs via light signals that are received in the suprachiasmatic nucleus(SCN) of the hypothalamus (Cassone, Speh, Card, & Moore, 1988). This circadian control center in the brain, synchronizes the rhythms that exist in peripheral tissues (through synchronization of peripheral clocks) (Yoo et al., 2004). Although peripheral clocks are synchronized by the brain, there are exogenous factors that influence their rhythmic cycles. The first evidence that circadian rhythm existed in peripheral tissue was documented fifty years ago in *Drosophila* (Konopka & Benzer, 1971) and in the years that followed, scientists characterized the interaction of the central clock (within the hypothalamus) and the peripheral clocks (in tissues throughout the body) in mammals (Richards & Gumz, 2012) Remarkably, though peripheral rhythms foster coordinated physiologic activity, transcripts that oscillate in one tissue may not oscillate in another(Akhtar et al., 2002; Panda et al., 2002). For this reason, it is necessary to characterize the circadian rhythm in a peripheral tissue if one wishes to understand the cyclic changes in physiology in that tissue. Thus, with respect to exploiting an understanding of the circadian rhythm for the estimation of the time of deposition of a blood stain, it is imperative to understand the characteristics of the rhythm in peripheral tissues.

While blood stains are a common biologic sample collected from a crime scene, defining circadian rhythm in blood stains is complicated by many factors. In addition to the fact one is analyzing a limited amount of dried blood, detecting a circadian rhythm of RNA quantity levels in blood is complicated by the presence of multiple cell lineages in the leukocyte population. The prospect that different cell types in the peripheral blood may

express circadian genes in different cyclic patterns presents a challenge to elucidating the circadian rhythm in blood. The possible existence of diverse patterns of gene expression in blood undoubtedly limits the sensitivity of any molecular assay designed to identify rhythmic pattern of gene expression that is not common to all lineages. In consideration of this confounder, researchers of circadian rhythm have isolated white blood cell populations in order to better define cyclic patterns. Published studies detail circadian rhythm data from research using isolated mononuclear or lymphocyte cell populations (Ackermann et al., 2012). However, supplementary information, such as core body temperature and melatonin and cortisol levels, was necessary to accurately identify a rhythmic pattern (Ackermann et al., 2012). Additionally, multiple blood samples from different collection times were needed for proper circadian assessment. Research reported in current literature use methods for data analysis for rhythmicity that involve complex equations incorporated with various normalizers for gene expression and graphic displays of data that suggest a rhythm from a chaotic collection of data points. Since physical separation of cell populations for analysis in a forensic blood stain is not feasible, an approach must be used that can either assess rhythmicity within the entire population of hematopoietic cells or is sensitive enough to detect rhythmicity in one cell line of interest.

The circadian rhythm studies published to date have also failed to identify variations in the circadian rhythm between the sexes, or age-related variations in the rhythm exhibited by whole blood without using additional information. Dried blood samples in the form of stains are typical types of crime scene evidence and therefore it is not possible to perform biochemical assays due to the increased rate of degradation of proteins. Core body temperatures can also not be obtained from a stain deposited as part of a crime. The ability to

define a circadian rhythm from a dried blood stain can provide important investigative information related to time that will assist with the reconstruction of a crime. In the study presented here, the quantification of RNA levels transcribed from a handful of genes that follow a predictable 24-hour pattern of expression holds the potential to estimate when, during the day, a stain was created.

CHAPTER II

REVIEW OF THE LITERATURE

There are many useful applications of DNA/RNA testing for human identification in Forensic Science. There is a growing body of knowledge to correlate the degradation rates of nucleic acids to the time a body fluid was deposited at a crime scene. Researchers strive to extract additional information from tissues and body fluid stains retrieved from a crime scene using molecular tools in an effort to develop as complete an understanding as possible about the nature of the crime. Although human identification is possible through DNA analysis of a biologic stain recovered from a crime scene, in some cases the stain cannot be conclusively attached to the crime. In such cases, the correlation of the time of deposition for the stain with the commission of the crime could validate the evidentiary nature of the sample to the criminal investigation. Circadian rhythm affords the possibility to identify a window of time in a biologic sample. Current research involving the circadian rhythm centers around disease identification and treatment. Studies by forensic scientists involving the circadian rhythm have focused no changes in circadian

rhythm genes that correlate with the biologic age of an unknown suspect in an unsolved case rather than on the time a sample was deposited at a crime scene. Nonetheless, the possibility to exploit our understanding of the genes associated with the circadian rhythm would provide a means to estimate the time of day a biologic sample was deposited at a crime scene. The studies presented here investigate the feasibility of identifying rhythmic changes in genes associated with the circadian rhythm and exploiting those changes to estimate the deposition time of blood stains; a common type of forensic evidence.

Forensic Science

Biologic evidence associated with a crime scene is often available in the form of trace amounts of dried samples deposited on a non-sterile matrix. It is the goal of the forensic scientist to associate the evidence with the crime in a way that advances an investigation and prosecution of a perpetrator. Analysis of forensic stains can proceed in different ways. For example, dried body fluid stains consist of numerous proteins, some of which represent genetic markers in the human population. However, analysis of specific proteins can be hampered by the small volume of body fluid composing the stain, as larger volumes may be required. In addition, analysis of proteins contained in a stain may be contingent on the matrix on which the sample is deposited. Environmental contaminants can also compromise the interpretation of data depending on the circumstances. Finally, proteins tend to be labile molecules and degradation due to localized proteases may prevent detailed analysis of the evidence.

Melatonin is a hormone that is sometimes used in time-of-death estimates and one whose expression varies with the diurnal day-night cycle of the body. Melatonin

levels expressed over a 24-hour span and detected in the pineal gland, urine and serum can provide time of death estimates in corpses (Mikami et al., 1994). Degradation of melatonin due to exogeneous factors in a biologic sample renders the results useless. Although estimates of time are important in the time of death, the ability to estimate the age of an evidentiary stain recovered from a crime scene can also be important for advancing an investigation.

Nucleic acids are generally more resilient to external conditions than proteins and the analysis of genetic markers in chromosomal DNA has become the mainstay technology for forensic laboratories to conclusively identify individuals involved with a crime. DNA profiling has gained more credibility in court cases since its initial applications and was deemed "the new gold standard in forensic science" in 2003 (Lynch, 2003).

The analysis of polymorphisms in human genomic DNA revolutionized Forensic Science. In the 1980's restriction fragment length polymorphism Analysis (RFLP) was applied to criminal investigations (Gill, Jeffreys, & Werrett, 1985). DNA polymorphisms that exist in human genomic DNA include single nucleotide polymorphisms and tandem repetitions of short nucleotide sequences that are collectively known as VNTRs (variable number tandem repeats). RFLP analysis employs the use of restriction enzymes to digest chromosomal DNA into a complex mixture of fragments. If the restriction enzyme used does not make cuts to the DNA within VNTR loci, DNA fragments will be produced whose size is proportional to the number of tandem repeats contained within the tandem array. Such fragments thus represent the alleles composing the DNA profile for the individual and, because of the high degree of variability exhibited by VNTR loci,

compelling evidence linking an individual to a crime can be produced. While RFLP-VNTR methods were powerful as investigative tools for the investigation of crime, large amounts of intact DNA were required for the test method and evidentiary samples frequently yielded inadequate amounts of DNA for analysis, or the sample was environmentally damaged. Current forensic applications of DNA analysis exploit short tandem repeats (STR). STRs are heritable loci composed of variable numbers of repeated tetranucleotide or pentanucleotide sequences. As with RFLP-VNTR methods, alleles comprising the DNA profile of a sample are of variable length (as determined by the number of repeats harbored by an allele) but STR analysis benefits from the ability to amplify alleles using PCR. Thus, the STR typing method requires minute amounts of DNA and the DNA can be partially degraded and still produce probative results. Finally, STR typing can be performed with many of the steps involved being automated. Combined DNA Index System (CODIS), a national reference data base of STR profiles, was established by the FBI in 1996. STR results are used to compare suspect and crime scene samples or to find an unknown assailant. Criminal DNA evidence is submitted to CODIS in search of a match for identification of an unknown assailant or submitted to the bank for future references.

In addition to needing tools to answer the question of "who" in criminal investigations, it is sometimes important to an investigation to be able to answer the question "when". For example, being able to estimate the age of an unknown perpetrator of a crime centers upon characterizing the lengths of telomers mapping to the ends of chromosomes that have been shown to shorten as one ages (Karlsson, Svensson, Marklund, & Holmlund, 2008). Another approach strives to evaluate changes in

methylation patterns that correlate with defined age groups (Hong et al., 2017; Horvath et al., 2012; Park et al., 2016). Methylation patterns for specific genes have also been associated with circadian rhythm particularly in brain tissue (Lim et al., 2014).

The determination of the length of time that has passed since a crime occurred can also be estimated through molecular testing. Fu and Allen (2019) developed a qPCR assay that characterized the degradation of several mRNA transcripts present in bloodstains stored over a span of 1 year and revealed that degradation proceeds in a predictable way that can be correlated with the age of the stain (Yu, Hazelton, Luebeck, & Grady, 2019). Using this assay, it is therefore possible to estimate the age of a blood stain such that its presence at a crime scene can be considered with respect to the time at which a crime occurred.

The ability to determine the time of day a crime occurred can also assist with an investigation. Genetic markers whose expression follows a circadian rhythm provide an opportunity to exploit and read the biological clock. For example, Kimura et al. (2011) quantified the expression of *Period 2* (*Per2*), *Brain and muscle ARNTL like protein-1* (*Bmal1*) and *Retinoic acid-related orphan nuclear receptor* (*Rev-Erbα*) genes in mice immediately following sacrifice and up to 48 hours after death in kidney, liver and heart tissues. Results confirmed that mRNA levels of core circadian rhythm genes cease to change upon death. In studies with human specimens, kidney, liver and heart samples from autopsied subjects that were less than 72 hours post-mortem were shown to harbor differing levels of RNA transcripts. Ratios of *PER2* and *BMAL1* mRNA were studied as possible markers for elapsed time. However, the rate of degradation for the two markers

differed and thus this approach was ineffective to accurately estimate time (Kimura, Ishida, Hayashi, Nosaka, & Kondo, 2011).

Current research strives to use circadian rhythm and DNA/RNA degradation rates to determine the time of death. In 2014 Lech et al. (2014) analyzed blood stains for microRNA (miRNA) degradation rates and tried to correlate rate changes with the deposition time for the stain. In this study miRNA 142-5 was analyzed and normalized to levels of miRNA 541. There was no correlation of degradation rates or rhythmicity detected (Lech et al., 2014). Martins et al. (2015) used quantification of nuclear DNA (nDNA) and mitochondrial DNA (mtDNA) in blood, hair and buccal swabs to estimate the time of day but failed to provide strong evidence that quantification of these DNAs was a viable approach to estimate time and stated "it is an expensive and more laborious methodology" and this approach could be used to "better predict if a STR analysis is viable" (Martins, Lima, Carvalho, Cainé, & Porto, 2015).

Circadian Rhythm History

Chronobiology is the study of a rhythmic cycle seen in organisms exhibited on a consistent daily schedule and commonly referred to as circadian rhythm. Circadian rhythm is revealed through repeated patterns of behavior and biochemical changes over a defined period of time. Biologic rhythms have been observed in microorganisms, plants, and animals, many sharing homologous control genes (Bjarnason, Jordan, & Sothern, 1999). Results from circadian rhythm research can be applied in numerous fields including cancer, psychological and sleep disorders, obesity, and forensic science. As

interest in this area increases so does the growing knowledge of the intricacies of the molecular clockwork of circadian rhythm.

The first evidence that circadian rhythm existed in peripheral tissue was documented in 1971 (Konopka & Benzer, 1971). These initial studies, using a mutant screening test in *Drosophila* flies, identified two genes (designated short period gene and long period gene) whose expression was regulated through light exposure during the day/night cycle (Konopka & Benzer, 1971). Subsequent research in mammals demonstrated a rhythmicity in gene expression in many peripheral non-neurologic tissues including mouse tongue, esophagus, stomach, jejunum, and rectal epithelium (Scheving, Burns, & Pauly, 1972). The bridge to human clock genes occurred when Bjarnason et al. (1999) analyzed levels of p53, cyclin-E, cyclin-A, cyclin-B1 and Ki-67 in human oral epithelium collected at 4-hour intervals. Their data revealed a detectable circadian rhythm in gene expression that correlated with the cell cycle (Bjarnason et al., 1999). Likewise, Akashi et al. (2010) reported that expression of clock genes in human hair follicles followed a predictable circadian rhythm when gene expression data were analyzed through a cosinar curve fitting analysis. (Akashi et al., 2010). Elucidation of circadian rhythms became plausible when expression of putative circadian markers was considered with additional clinical observations, such as core body temperature, and blood levels of melatonin and cortisol. For example, research elucidating elements of the circadian rhythm in human white blood cells, adipose tissue and skin epithelium has been possible considering core body temperature and melatonin levels that vary with light and dark cycles (Cermakian & Boivin, 2009). In peripheral blood, lymphocytes in particular, the rhythmic expression of cellular markers has been shown to be correlated with

cytokine production suggesting a link between the circadian rhythm and immune function (Born, Lange, Hansen, Mölle, & Fehm, 1997).

The demonstration of circadian rhythm gene expression in peripheral blood has made it feasible to easily study circadian rhythm in humans. The use of peripheral blood is less invasive, and samples are more readily obtainable when compared with tissue biopsies. Regrettably, the use of peripheral blood also introduces a confounder for data interpretation inasmuch as the different cell populations comprising the leukocyte fraction of blood could exhibit dissimilar levels of clock genes expression. Thus, scientists separated the different blood cell populations to more accurately analyze cyclic patterns. Peripheral blood mononuclear cells became more widely used in many research laboratories for circadian rhythm investigations. In 2005, one study evaluated male subjects in normal and altered light environments to imitate conditions of shift workers. Results were compared against core body temperature, melatonin and cortisol levels, whose values were normalized to the expression of the β actin gene. Significant variations in gene expression were observed between groups experiencing normal versus altered light environments (Takimoto et al., 2005). In another study, blood from young males was collected every 2 hours and the raw data were normalized with core body temperatures, melatonin and cortisol levels or by using relative levels of mRNA. The normalized results were analyzed using the cosine model mentioned previously. In this study, only 3 of 10 genes (*PERIOD 1*, *2*, and *3*) were found to exhibit a circadian rhythm. (Kusanagi et al., 2008).

There have been numerous studies employing a wide range of experimental approaches and sample types. It would be useful in forensic science and in medicine if a

circadian rhythm could be identified at the gene expression level in peripheral blood. Blood is a common type of forensic evidence recovered from crime scenes and blood is a typical sample for medical diagnostic tests. However, as it stands now, no well-defined circadian rhythm has been defined in peripheral blood, perhaps because of the complexity in cell populations comprising blood.

Circadian Rhythm Physiology

Multiple environmental cues (known as "zeitgebers") drive or maintain a consistent cyclic rhythm. Light acts as an external cue through the suprachiasmatic nucleus (SCN) in the anterior hypothalamus. Light is perceived by the retinal photosensitive pigments (i.e., melanopsin) that in turn transmit signals by the retinal ganglion cells (Albrecht & Eichele, 2003) via the retinohypothalamic tract to the SCN (Güler et al., 2008). The SCN contains more than 100,000 neurons (Cassone, Speh, Card, & Moore, 1988) which mediate the light signal through the levels of glutamate and pituitary adenylate (Albrecht & Eichele, 2003). The SCN is credited with directing and maintaining synchrony between peripheral oscillators throughout the body and is deemed the "master clock" (Okamura et al., 1989). Light, perceived in the SCN, controls the production of melatonin in the pineal gland. Melatonin levels in turn drive sleep cycles, which in turn affect the peripheral oscillators through melatonin receptors (MT1 and MT2) that modulate metabolism, cell cycle, and arterial vasoconstriction (Dubocovich & Markowska, 2005). Stability of the rhythm is supported by the natural resistance to changes in sleep and activity patterns (Scheer, Pirovano, Van Someren, & Buijs, 2005).

Peripheral oscillators are driven by activity, sleep patterns, eating and temperature but are kept in check by the SCN (Damiola et al., 2000). In addition to changes in the levels of gene expression, epigenetic modifications provide an additional level of control of rhythmic gene expression. The molecular control over gene expression exists in peripheral organs such as in the liver, kidneys and fibroblasts (Yamazaki et al., 2000). Scientific evidence supports the existence of tissue microenvironments with differing circadian rhythms controlled by multiple peripheral clocks in an organism (Liu, Cai, Sothern, Guan, & Chan, 2007). These peripheral rhythms vary from site to site and can be demonstrated in oral epithelium (Bjarnason et al., 1999), peripheral blood (Teboul et al., 2005), vitreous humor (Corradini et al., 2015), and liver (Cermakian & Boivin, 2009), but differ from rhythms in the various areas of the brain (Abe et al., 2002). Peripheral clocks also exhibit sensitivity to zeitgebers, such as changes in feeding schedules and temperature (Damiola et al., 2000; Hara et al., 2001). They can function independently of the SCN for a short period of time, as observed in tissue cultures (Balsalobre, Damiola, & Schibler, 1998). The plasticity of the peripheral oscillators adapts to the aforementioned zeitgebers, and changes in gene expression can be accompanied by post-translational modifications to fine-tune the expression pattern in a tissue specific manner. Although the patterns vary tissue to tissue, the master clock controller remains in the SCN.

Research utilizing microarray technology suggests that ten percent of all genes are expressed in a circadian pattern and are clock controlled (Masri & Sassone-Corsi, 2010). Transcription factors, ion channels, peptides, and metabolic enzymes are encoded by clock genes (Duffield, 2003) and are cyclically expressed. Core clock genes encode protein products that are necessary to generate and maintain the circadian rhythm. These

core clock genes are expressed in many tissue types, with higher levels of expression in the SCN. Core clock genes include *Brain and Muscle ARNTL-like protein-1* (*BMAL1*), *Circadian Locomotor Output Cycles Kaput* (*CLOCK*), *PERIOD1* (*PER1*), *PERIOD 2* (*PER2*), *PERIOD 3* (*PER3*), *CRYPTOCHROME 1 (CRY1*), and *CRYPTOCHROME 2 (CRY2)*.

CLOCK/BMAL1 **and E-boxes**

A complex, fine-tuned series of control mechanisms drive and maintain circadian physiology synchrony. There are two central transcription factors that initiate and sustain rhythmicity. Brain and Muscle ARNT-Like 1 (BMAL1) and Circadian Locomotor Output Cycles Kaput (CLOCK) proteins act as the central conductors of a circadian rhythm symphony. BMAL1 and CLOCK heterodimerize and bind to E-box activation sites on select genes (*PER1-3* and *CRY1-2* genes) to initiate transcription (Gekakis et al., 1998). A Per/Cry protein heterodimer forms in the nucleus and disrupts the BMAL1/CLOCK heterodimer in a negative feedback fashion, down regulating transcription (Lee, Etchegaray, Cagampang, Loudon, & Reppert, 2001). Additional regulators of the BMAL1/CLOCK heterodimer include nuclear receptor subfamily 1 Group D number 1 (REV-ERBα) and retinoic acid receptor-related orphan receptor alpha (RORα) which are also transcriptionally regulated by BMAL1/CLOCK heterodimers via E-box binding. REV-ERBα blocks transcription of BMAL1 whereas RORα activates BMAL1 transcription as it binds to *RORE* (retinoic acid-related orphan response elements) in the *BMAL1* promoter region (Guillaumond, Dardente, Giguère, & Cermakian, 2005).

CLOCK and BMAL1 exhibit a basic conformational structure known as basic helix-loop-helix (bHLH) (Hogenesch et al., 1997). Specific areas within these proteins exhibit 35% homology involving 60 amino acid residues in 3 domains (Huang et al., 2012). This complementarity of the amino acids between the two proteins (BMAL1 and CLOCK) allows for the formation of homodimers and heterodimers. [Figure 1] Several key amino acid residues within homodimer domains exhibit pronounced side-chain clashes that result in unstable structures. Within identical domains, Phe50 in CLOCK and His84 and Leu125 in BMAL1 promote these discordances (Wang, Wu, Li, & Su, 2013). This incompatibility creates a steric hinderance within these domains that reduces homodimer development and creates a preference for heterodimer formation. Hydrogen bonds form a stable connection between BMAL1 and CLOCK via five identified recognition sites within the helical structures. Among these five key positions, CLOCK His84 and BMAL1 Leu125 are the key recognition sites between bHLH structures of CLOCK and BMAL1 (Wang et al., 2013). The CLOCK/BMAL heterodimer is the primary transcriptional activator in the circadian clockwork.

A stable BMAL/CLOCK heterodimer can bind enhancer box (E-box) response elements in a variety of genes. Thus, the presence of E-box sites in DNA promoter regions provide strong evidence that these genes likely play a role in circadian rhythm. An E-box is a DNA response element that facilitates transcription upon activation. The 6bp recognition sequence often exhibits a nucleotide sequence palindrome of CACGTG (Bellet & Sassone-Corsi, 2010) and E-box sequences exhibit no appreciable observed degenerate recognition sites. Consequently, the lack of degenerate sequences provides evidence that each residue reacts with each site. Recent work by Wang et al.

demonstrates CLOCK Arg39, Glu43, Arg47 and BMAL1 His77, Glu81, Arg85 are the key residues that bind to the CACGTG E-box sequence for transcriptional activation (Wang et al., 2013).

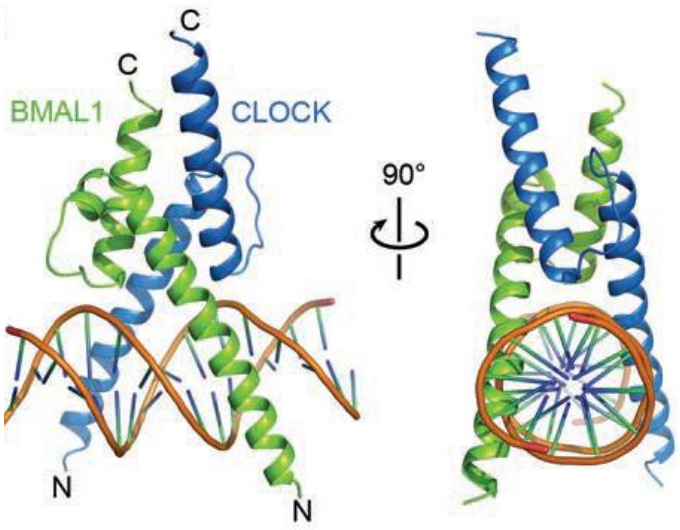


Figure 1. Human CLOCK/BMAL1 Heteroduplex. Both molecules contain interacting basic helix-loop-helix (bHLH) domains that interact with target DNA (Wang et al., 2013). Image with permission from Springer Nature Publishing.

PERIOD and *CRYPTOCHROME* Transcripts

In the circadian rhythm system, binding of the BMAL1/CLOCK heterodimer with an E-box provides accessibility to promoters to allow transcription of negative regulators of core clock genes (Gekakis et al., 1998). Thus, the BMAL1/CLOCK heterodimer reduces or eliminates expression of genes central to the circadian rhythm. These negative regulators include: *PERIOD 1* (*PER1*), *PERIOD 2* (*PER2*), and *PERIOD 3*(*PER3*) that are members of the *Period* family of genes (Sangoram et al., 1998). Other negative

regulators include *CRYPTOCHROME 1* (*CRY1*) and *CRYPTOCHROME 2* (*CRY2*), members of the *Crytpochrome* family of genes (Kume et al., 1999). These negative regulators accumulate in the cytoplasm of the cell and interact with components associated with circadian rhythm, metabolism and behavior. Period genes were the first circadian rhythm genes discovered and were identified in *Drosophila* (Konopka & Benzer, 1971). The expression of the *Drosophila* period genes *Period* and *Timeless* is activated in response to interaction of the genes with heteroduplex BMAL1/ CYCLE (Houl, Yu, Dudek, & Hardin, 2006). Mammalian homologs were soon identified as *Period 1-3*, *Cryptochrome 1-2* and the positive inducer BMAL/CLOCK heterodimer.

The complexity of the regulatory network surrounding the circadian rhythm is slowly emerging (Jagannath, Taylor, Wakaf, Vasudevan, & Foster, 2017). Period and cryptochrome proteins can exist singly or as heterodimers. These proteins accumulate in the cytoplasm and phosphorylation patterns determined their fate. Mammalian models provide evidence that core circadian rhythm proteins are phosphorylated via casein kinase 1 epsilon (CK1ε) and casein kinase 1 delta (CK1δ). Phosphorylation of a PER/CRY protein heterodimer facilitates reentry of the complex into the nucleus where the phosphorylated complex disrupts the BMAL1/CLOCK heterodimer and interferes with the subsequent binding of BMAL/CLOCK to E-box response elements. Following disruption of the binding of BMAL1/CLOCK, transcription of additional *Period* and *Cryptochrome* mRNA is halted (Gallego & Virshup, 2007). Unbound period proteins are targets for phosphorylation by the kinases described above and then become targets for removal from the cell through ubiquitination with ultimate proteasomal degradation in the cytoplasm (Zhou, Kim, Eng, Forger, & Virshup, 2015). Excess cytoplasmic

cryptochrome levels are controlled through a different ubiquitin protease-signaling pathway. Once the period and cryptochrome proteins decrease in the cytoplasm, transport of these proteins into the nucleus diminishes as well and the BMAL/CLOCK heterodimer is again available for transcriptional activation via E-box interactions. The symphony of gene expression patterns that support the circadian rhythm are diagrammatically summarized in Figure 2.

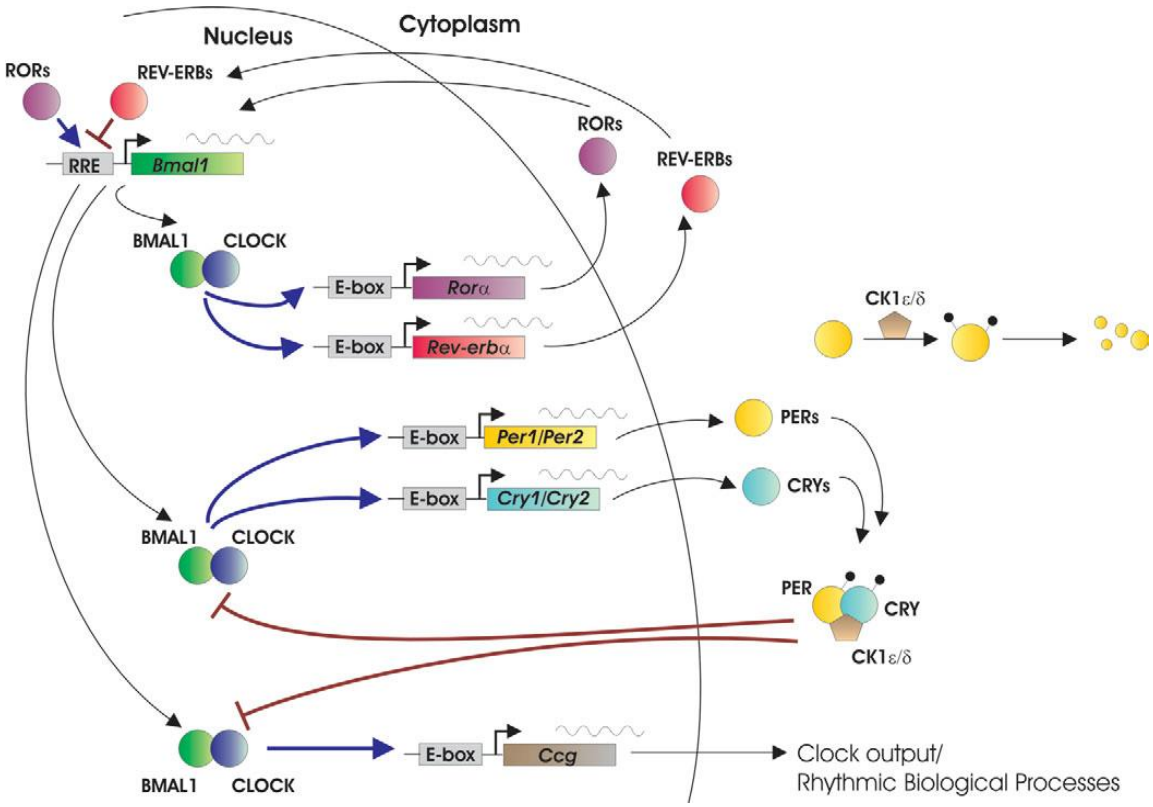


Figure 2. **Molecular Components of the Core Clock**. A network of transcriptional-translational feedback loops constitutes the mammalian circadian clock (Ko & Takahashi, 2006). Image used with permission from Oxford University Press.

Clock Controlled Markers

The core clock components (BMAL1, CLOCK, Period 1-3, Cryptochrome 1-2) are the first level of control in circadian rhythm. *CLOCK* and *BMAL1* are activated in the morning with subsequent activation of *Per1-3* and *Cry1-2*. Thus these genetic markers share very similar daily patterns of expression exhibiting peak levels of expression in the morning that slowly diminish throughout the day (Takahashi, 2017). Accurate assessment of circadian rhythm requires multiple markers that exhibit different expression peaks and troughs. It is expected that analysis of multiple markers on one sample could increase the sensitivity and accuracy of circadian rhythm determination. Thus, the use of additional markers associated with peripheral clocks allows a more dynamic circadian rhythm analysis. The circadian rhythm exhibited by peripheral organs is separate from the suprachiasmatic nucleus (SCN) but the rhythmicity is coordinated through the core clock genes. Additional exogenous stimuli contribute rhythmicity to various tissue types and molecular markers have been implicated in these processes. These supplementary rhythmic genes provide additional opportunities to determine time points in rhythmicity while remaining responsive to SCN oversight.

Cyclin E (*CYE*)

The eukaryotic cell cycle exhibits four phases that include gap 1(G1), DNA synthesis(S), gap 2(G2), and mitosis(M). The cell cycle is tightly regulated via cyclin proteins that fluctuate daily levels of proteins that coincide with observed cellular proliferation. Cyclin D, E, and A regulate progression from G1 to S phase while cyclin

B1 regulates G2 to M phase transitions (Sherr, 1994). (see Figure 3) Cyclin proteins are activated through phosphorylation by cyclin dependent kinases (cdk) (Morgan, 1997; Solomon, 1993) that in turn, are inhibited in their kinase activity by cdk inhibitors (Hengst, Dulic, Slingerland, Lees, & Reed, 1994). Circadian rhythm patterns have been observed in mouse gastrointestinal tissue, bone marrow and corneal epithelium (Scheving et al., 1972; Scheving, Burns, Pauly, & Tsai, 1978). Likewise, the association of the circadian rhythm involvement with the cell cycle has been documented in human rectal mucosa (Buchi, Moore, Hrushesky, Sothern, & Rubin, 1991), oral mucosa (Warnakulasuriya & MacDonald, 1993) and bone marrow (Smaaland et al., 1991).

The ability to identify and define a circadian rhythm as it relates to time and cell function provides a useful tool both in disease treatment and also perhaps in forensic studies. Bjarnason et al. attempted to predict the time of day based on expression levels of cyclin proteins A, B1, and E, p53 and Ki67 in human oral epithelium through a single cosinor method of analysis (Bjarnason et al., 1999). Their data reinforced the notion that detection of the circadian rhythm at the molecular level was feasible although their approach and sample types proved to be suboptimal.

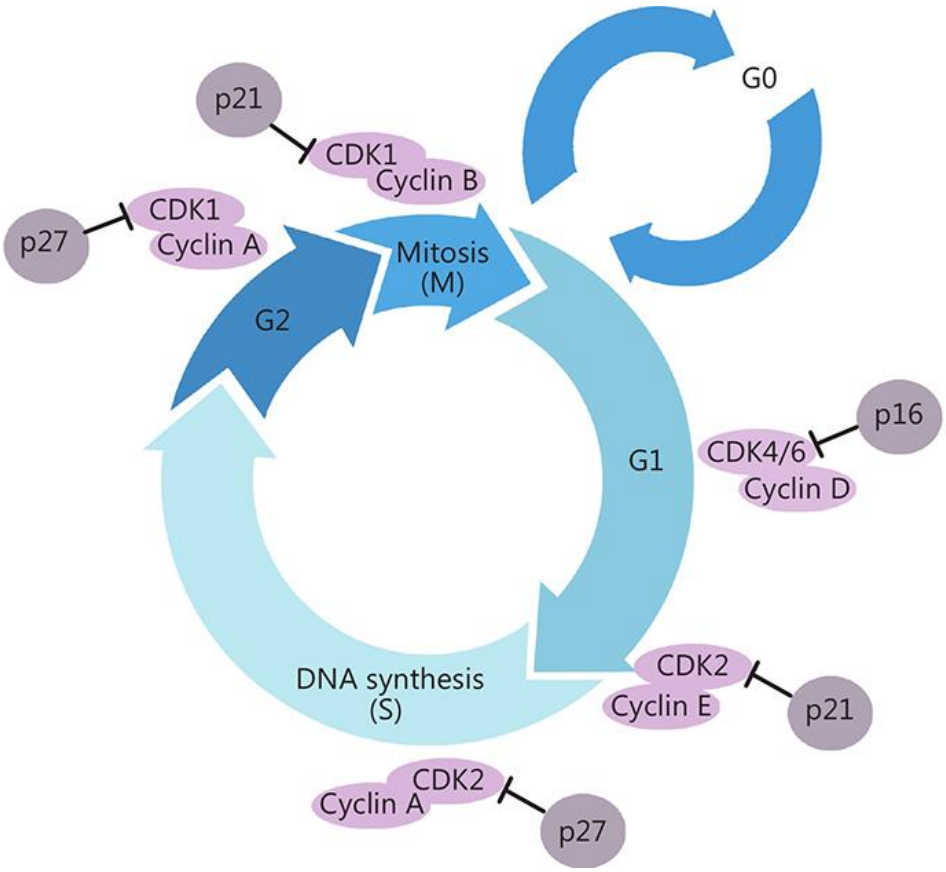


Figure 3. **Cyclin Proteins Involved in the Cell Cycle**. Cyclin proteins play an important role in each stage of the cell cycle. (http://www.cancerbiomed.org/index.php/cocr/article/view/1070/1194)

Map Kinase Interacting Serine/Threonine Kinase 2 (MKNK2)

MKNK2 is a calcium/calmodulin-dependent protein kinase that is activated (phosphorylated) by mitogen-activated protein (MAP) kinases (Waskiewicz, Flynn, Proud, & Cooper, 1997). MKNK2 appears to be influenced by exogenous factors first detected in studies where changes in sleep patterns did not alter expression levels (Lech et al., 2015). Subsequently, phosphorylated MKNK2 directly phosphorylates eukaryotic initiation factor-E (eIF4E) that in turn plays an important role in hematopoiesis (Gorentla et al., 2013) and cellular apoptosis (Chrestensen et al., 2007). Recent data has

demonstrated that peripheral blood mononuclear cells exhibited a rudimentary rhythmicity of *MKNK2* when normalized to levels of beta actin (*ACTB*) mRNA in the cells (Lech et al., 2015). Therefore, there is strong evidence that *MKNK2* in peripheral blood may prove to be a valuable marker for defining the circadian rhythm in hematopoietic cells.

Thyroid Receptor Alpha 1 (*THRA1*)

THRA1 is a thyroid hormone receptor in a nuclear receptor superfamily that is involved with control of transcription (Flamant et al., 2006). THRA1 levels are exogenously affected by the effect food intake has on gene expression in the liver. *THRA1* expression therefore tends to be diurnal with higher levels existing during waking hours and the lowest levels during sleep. (Doulabi et al., 2004). The study of a rhythmic marker whose expression is influenced by something other than light offers the chance to study an alternate to light-controlled circadian rhythm. *THRA1* levels were found to exhibit a regular, consistent repeating pattern of abundance over a 24-hour period in peripheral blood mononuclear cells. In these studies, *THRA1* levels were normalized to the expression of the beta actin gene (Lech et al., 2015). Studies of rhythmic gene expression that are influenced by light and other stimuli affords the opportunity to expand the depth of our understanding of the circadian rhythm and to possibly exploit that knowledge for estimating time.

Tryptophan Hydroxylase (*TPOH*)

Melatonin is the primary hormone produced by the pineal gland that exhibits daily rhythms controlled by the SCN (Reiter, 1993). Levels of melatonin increase during

nocturnal phases and diminish during photoperiods resulting in the transmission of circadian information throughout the body (Goldman, 1999). Tryptophan Hydroxylase (TPOH) metabolizes tryptophan in a biochemical pathway that produces melatonin (Lovenberg, Jequier, & Sjoerdsma, 1967). The expression of the *TPOH* gene appears to play a pivotal role for the rhythmic expression of melatonin inasmuch as the TPOH protein exhibits a very short half-life. (Sitaram & Lees, 1978). Besancon et al. and Shibuya et al. showed parallel levels of *Tpoh* gene expression and enzyme activity during the day that peaked during the evening hours (Besançon, Simonneaux, Jouvet, Belin, & Fèvre-Montange, 1996; Shibuya, Toru, & Watanabe, 1977). Melatonin is a well-documented analyte exerting regulation of the circadian rhythm in concert with core body temperature and serum cortisol levels in living organisms (Skene & Arendt, 2006). The application of melatonin quantitation methods to biologic material studied in forensic investigations is limited because of marker stability and because of the inability to evaluate core body temperature. It is possible quantifying levels of *TPOH* mRNA may prove useful as a molecular marker with which to study the circadian rhythm.

Epigenetics and Molecular Modifications

Knowledge of the nucleotide sequence of a gene is not the only determinant affecting gene expression. Epigenetics roughly translates as “on top of genetics” meaning molecular interactions between genome and regulatory factors exist as an additional regulatory facet of gene expression. These epigenetic influences are heritable and do not alter the nucleotide sequence. Epigenetics has always been a possible source of control of the circadian rhythm in addition to the feedback described above that is related to factors

that interact with regulatory elements of select genes. Epigenetic modifications include biochemical modifications of DNA such as methylation and modifications of histone proteins through acetylation, methylation and phosphorylation. Modifications such as these can collectively be called acquired modifications. Inherited and acquired modifications of DNA sequence therefore control gene expression and alterations in the normal modification patterns are linked to a wide range of diseases.

DNA Modifications

Methylation is highly conserved and can be detected in organisms throughout eukaryotic organisms from *Neurospora* (Belden, Lewis, Selker, Loros, & Dunlap, 2011) to brain tissue of human cadavers (Nakatome et al., 2011). DNA methylation is a widespread heritable pattern of DNA modification whereby methyl groups are most commonly added to the C5 position of a cytosine residue by methyltransferases. This methylation most often occurs in CpG islands composed of 50% or greater of GC base pairs. CpG islands are typically found near promoter regions and thus methylation can alter transcription through promoter accessibility. Methylated DNA can also be found within coding sequences where alternate promoter regions may exist. (Gardiner-Garden & Frommer, 1987). Methylation is most often thought of in the context of X-chromosome inactivation or with regulation of gene expression although recent advances suggest the association of methylation changes with carcinogenesis and aging. Synchronous patterns of methylation also exist and produce transcriptional oscillation for genes involved with the cell cycle that is part of the circadian clock (Brown, Fraga, Weaver, Berdasco, & Szyf, 2007). Cai et al. showed that core circadian rhythm genes

exhibited methylation patterns in select E-box regions of mPer1 in mouse liver, thymus and testis (Cai et al., 2009). Similarly, Zhang, et al. analyzed clock genes in human leukocytes for methylation changes associated with aging (L. Zhang, Lin, Ding, Wang, & Cai, 2012). Since methylation changes have been verified for core clock genes that exhibit expression rhythmicity, it is possible these waves of gene expression can be used forensically to learn about biological evidence recovered from a crime scene.

Histone Modifications

A nucleosome is a fragment of genomic DNA approximately 146 base pairs in length wound around a histone octamer involving pairs of core histone proteins, i.e., H2A, H2B, H3, and H4 (Luger, Mäder, Richmond, Sargent, & Richmond, 1997). A nucleosome functions to compact and repress gene expression and is often associated with heterochromatin. The unwinding of the DNA from a nucleosome converts heterochromatin to euchromatin that has more relaxed and accessible structure compatible with gene expression. Histone modifications assist with controlling the conversion of heterochromatin to euchromatin and back again and provide an additional layer of transcriptional control. There are various types of chemical modification to histones that have been described but those modifications thought involved with circadian rhythm include acetylation, phosphorylation, and methylation (Crosio, Cermakian, Allis, & Sassone-Corsi, 2000; Etchegaray, Lee, Wade & Reppert, 2002; Masri & Sassone-Corsi, 2010; Ripperger & Schibler,2006).

The type of histone acetylation that has been extensively examined involves the addition of an acetyl group to a lysine residue in the N-terminal tail of histones

H2A,H2B, H3 and H4 (Kuo & Allis, 1998). The addition of an acetyl group removes the positive charge from the histone which in turn decreases the interaction with the negatively charged phosphate group of the DNA backbone and allows genes to assume a more relaxed structure that is more accessible for transcription (Mutskov et al., 1998). Within the circadian rhythm controls, the CLOCK polypeptide exhibits histone acetyltransferase activity (HAT) when bound to an E-box as a heterodimer with BMAL1 (Doi et al., 2006; Etchegaray et al., 2002). The acetylation of H4 histones frees up the promoters at the E-box genes thus increasing transcription (Frank, Schroeder, Fernandez, Taubert, & Amati, 2001). Additionally, acetylation of BMAL1 enables CRY-dependent repression of the CLOCK/BMAL1 heterodimer (Hirayama, et al., 2008). The histone deacetylase activity (HDAC) of BMAL1 reverses the effects of an acetyl group thus allows the repressor to bind and reduce transcription. SIRT1 (silent mating type information regulation type 2 homolog 1) also acts as a HDAC to circadian rhythm machinery. The oscillating characteristic of SIRT1 is linked to NAD+/NADH levels that link cellular energy metabolism to circadian rhythm (Asher et al., 2008; Imai, Armstrong, Kaeberlein, & Guarente, 2000).

Histone phosphorylation is achieved through an array of different kinases that target serine, threonine or tyrosine residues in the polypeptide chain. Phosphate groups are removed from histones through the action of phosphatases. Phosphorylation of histones initiates remodeling of the chromatin which, of course has consequences for gene expression. Light is one of the main driving forces in the circadian rhythm and the connection between a light stimulus and the rhythmic cycling of cell physiology provides an important connection between the environment and circadian rhythm. Light-directed

histone remodeling was discovered in the suprachiasmatic nucleus (SCN), the circadian pacemaker, where exposure to light caused rapid phosphorylation of histone 3 on serine residue #10 associated with the *c-fos* and *Per1* genes (Crosio, Cermakian, Allis, & Sassone-Corsi, 2000). The histone remodeling response to light exposure provides support for the notion of light entrainment. Phosphorylation acts as an intermediate modification in the histone code and functions to condense the chromatin during cell division, regulate transcription and serve as a recruiting point for DNA repair mechanisms (Lowndes & Toh, 2005; Rossetto, Avvakumov, & Côté, 2012).

Histones can also be methylated which involves the addition of a methyl group to a lysine or arginine in the N- terminal tail of histones H2A, H2B, H3, and H4. The residue that is methylated determines if transcription is blocked or enhanced. Histones can be mono-,di- or tri-methylated that in turn determines the effect on transcription (Y. Zhang & Reinberg, 2001). There is no consistent effect of any specified histone methylation pattern on transcription, the effect is defined by the residues modified and the presence of other modifications on that histone. The enzyme histone methyltransferase transfers methyl groups to histones while histone demethylases remove the methyl group (Shi et al., 2004).

The amalgamation of these biochemical modifications constitutes the histone code. The histone code reflects the combination of modifications that dictate the final transcriptional state of a genomic region. Histone modifications are assessed through mass spectrophotometry and immunoprecipitation assays. Although histone modifications exhibit a cyclic pattern and are connected to light stimuli, metabolism and

cell cycle, the ability to assess histone modifications in a forensic sample is not feasible due to sample quality and volume limitations.

Non-Coding RNA

Non-coding RNAs (ncRNA) are untranslated transcripts that exhibit regulatory functions at the posttranscriptional level. There is increasing evidence that ncRNAs play a large role in epigenetic control (Wei, Huang, Yang, & Kang, 2016). Non-coding RNAs are classified by function into housekeeping or regulatory categories. The regulatory ncRNAs can be further classified as short chain non-coding RNA (including small interfering RNA [siRNA], microRNA [miRNA], and piRNA [piwi interacting RNA] and long non-coding RNA [lncRNA]. Non-coding RNAs interact directly with mRNA or indirectly through epigenetic mechanisms to alter expression through binding or recruitment of acetyl and methyl groups for histone modifications (Peschansky & Wahlestedt, 2014). Diseases are linked to alterations in gene expression that may be due, in part, to quantitative changes in ncRNA. Tani et al. observed variable expression of ncRNA in human clonal cultures when exposed to different environmental factors (Tani & Torimura, 2015) thus linking pathogenesis to exogenous influences.

Vast differences exist in research pertaining to ncRNA applications. Short interfering RNAs are useful as a knockout method in cell culture. Vollmers et al. used siRNA to knockout BMAL1 and Cry1 in human cell cultures to further investigate the core clock interactions (Vollmers, Panda, & DiTacchio, 2008). Differentiating night from day in a forensic study was published by Lech, et al (2015). In this study, RNA was extracted from 400 µl of whole blood. Markers that had previously shown differences in

levels in victims that had died during the day or nighttime were assessed (miRNA 142-5 and miRNA 541). Results were normalized to miRNA 222 abundance. No discernable pattern of ncRNA expression was detected in this study although in vitro miRNA stability was observed (Lech et al., 2014). Later research attempted to correlate miRNA with circadian rhythm using 92 miRNA markers from fresh plasma samples (Heegaard et al., 2016). Rhythmicity in abundance was detected in 26 of the miRNAs when the data was analyzed using the cosinor method without an established normalizer (Heegaard et al., 2016). Results from this study provided evidence of miRNA oscillation but the lack of a normalizing marker and the possible instability of miRNA retrieved from a crime scene limits the utility of ncRNA assessment in forensic evidence at this time.

Circadian Rhythm Disorders

An understanding of the circadian rhythm can be powerful knowledge for applications in many scientific disciplines. The complexity of the rhythmic physiology that is the circadian rhythm creates opportunities to apply an understanding of the process to the diagnosis and treatment of a pathologic condition or perhaps even identifying a moment in time. Physiological changes that accompany some diseases result in an abnormal circadian rhythm. Changes in sleep patterns and variations in light exposures can disrupt normal rhythmicity and also promote an increased incidence of disease. Genetic mutations occurring in genes whose expression cycles, or molecular modifications to those gene products can also contribute to an abnormal circadian rhythm. Changes in the circadian rhythm can negatively impact normal functioning including sleep cycles, metabolism, hormone levels and cell cycles.

Light acts as an external influence on circadian rhythm both in the core clock and in peripheral clocks. Light perceived through specialized ganglia in the retina dictates the level of melatonin that circulates and influences oscillations throughout the body. Melatonin levels serve to synchronize the peripheral systems. Alterations in the length and timing of light exposure affects normal circadian rhythm (Wright et al., 2013). Disruptions to the normal rhythm have been associated with disorders such as cardiovascular disease due to atherosclerosis (Schilperoort et al., 2020). Metabolic disruptions (Skinner, Rizwan, Grattan, & Tups, 2019), and atypical emotional conditions (Stephenson, Schroder, Bertschy, & Bourgin, 2012) are also linked to circadian rhythm abnormalities. Furthermore, energy consumption is linked to circadian rhythm through metabolic sensors such as SIRT1, a histone deacetylase, that acts as an energy sensor via NAD+ levels. When NAD+ levels are increased SIRT1 activity is increased that deacetylates histones at clock promoters interacting with BMAL1 and PER2 thus affecting circadian rhythm (Asher et al., 2008; Imai et al., 2000; Nakahata, Sahar, Astarita, Kaluzova, & Sassone-Corsi, 2009). Alterations in these core clock components can have a wide-spread downstream effect on physiologic homeostasis.

Nucleotide sequence polymorphisms in clock genes are also associated with metabolic abnormalities, mood disorders, atypical sleep cycles, and schizophrenia. The transcriptional activity of clock genes and the action of their gene products influence all areas of the body. So, deviations from the norm that alter circadian rhythm can have wide ranging physiological effects.

There are numerous disorders that are linked to aberrant rhythms. One such example is Familial Advanced Sleep Phase Syndrome (FASPS) that results from a serine

to glycine amino acid change in a phosphorylation site on the Per 2 gene (Gekakis et al., 1998). Characteristics of FASPS include life-long accelerated sleep cycles that typically begin at 7:30pm and end at 4:30am.

Recent studies have shown that shifted sleep patterns are also sometimes associated with cancer. Sleep cycles and cell cycles exhibit a circadian rhythm. Thus, if a circadian rhythm abnormality is present, it is likely that other peripheral circadian rhythm (CR) systems are affected as well. In cancer, malignant cells exhibit a high mitotic activity that is associated with an increase in the population of abnormal cells. In 2002, researchers successfully correlated circadian rhythm disturbances in cell cycle genes with cancer in mice (Filipski et al., 2002). Further research connected atypical sleep patterns with an increased incident of metabolic (Sahar & Sassone-Corsi, 2009) and breast cancers (Schernhammer et al., 2001; Wegrzyn et al., 2017). P53, a tumor suppressor gene, regulates cell division and plays a critical role in detection and repair of damaged DNA. P53 also exhibits a circadian rhythm in its expression and so it might be expected that abnormalities in P53 expression are associated with over 50% of human cancers (Schmitt et al., 2002).

Atypical epigenetic modifications of key clock genes have also been implicated in pathologic conditions such as the abnormal activity of methyl transferase on the cytosines in the promoter region of per1, per2, and per3 (Chen et al., 2005). Abnormal methylation results in unregulated cell division in breast cancer.

There is an abundance of literature that demonstrates it is possible to detect and quantify levels of gene expression that accompany progression through the circadian rhythm. The question arises as to whether it might be possible to use rhythmic gene

expression as a forensic tool to estimate the time of day biologic evidence was created and deposited at a crime scene. In previous work from this laboratory, Fu and Allen (2019) demonstrated that it is possible to estimate the age of a body fluid stain to within 2-4 weeks of its actual age. Extending this research, it might be possible to use changes in gene expression, based on circadian rhythm, to determine the time of day a body fluid stain was created. Two mRNA transcripts from among many studied followed a predictable 24-hour cyclic pattern of expression in blood stains that show promise as useful circadian rhythm markers for time determination in forensics.

CHAPTER III

METHODS

Institutional Review Board (IRB)

IRB approval #2018010 April 24, 2018.

IRB protocol was followed for the collection and handling of all venous and capillary samples collected for this research. (Appendix A)

METHYLATION STUDIES

Donor Selection for Methylation Study

This study was designed to determine if a 24-hour rhythmic pattern of DNA methylation could be detected in peripheral blood. Individual donors over the age of 18 were randomly chosen with one contingent; volunteers were required to have normal sleep patterns. A normal sleep pattern was defined as routine sleep cycles beginning after 8:00 pm and ending after 5:00 am with a minimum of 6 hours of sleep within that time frame. Donors exhibiting a "normal sleep pattern was defined as routine sleep cycles beginning after 8:00 pm and ending after 5:00 am with a minimum of 6 hours of sleep

within that time frame. Donors exhibiting a "normal sleep pattern" were chosen in the hope of establishing reference methylation patterns in a normal population. Once normal sleep cycles were confirmed in a donor, volunteers signed consent forms (Appendix B) that clarified collection details with possible complications, had the testing protocols explained and were subjected to de-identification practices. Each volunteer also completed a survey (Appendix C) with questions pertaining to normal waking hours to support their selection for the study. Daily exposures to natural and artificial light, eating schedules and any use of sleep aides were also investigated for each donor.

Blood Collection for Methylation Study

All capillary and venous blood samples were collected from volunteers. Collections were performed by trained and/or certified medical professionals. Fingerstick capillary blood was collected every 4 hours over a 24-hour period. Sample collection began at 8:00 am and was completed at 4:00 am. Collecting the final sample at 4:00 am established the least expected interference with normal circadian routines during the collection period. Fingerstick samples for capillary blood were performed with SurgiLance™ 2.8 mm 21-gauge safety lancets (MediPurpose™, Duluth, GA). Capillary samples were spotted in a minimum of a 20 mm circumference onto sterile and nuclease-free 705™ Classic Specimen Cards (Fitzco®, Spring Park, MN). Although there is no literature to support complications, anticoagulant was avoided to circumvent possible interference with methylations studies. Specimen cards were stored in the dark at room temperature in the laboratory. Collection and extraction times were documented for each sample.

DNA Extraction for Methylation Study

Extractions were performed after 6 samples, representing a 24-hour day, were collected from each donor. Extractions were performed within 48 hours of the first fingerstick. Dried blood stains from specimen cards were cut into small 1-2 mm strips and placed into microfuge tubes. Cellular and nuclear membranes were lysed, and proteins degraded using an extraction buffer containing 100 µg/mL of proteinase K, 0.5% (v/v) sodium dodecyl sulfate in TNE Buffer *(*50 mm Tris–HCl (pH 7.4) with 200 mM NaCl and 0.1 mM EDTA). Following lysis, samples were subjected to extraction with phenol/chloroform/isoamyl alcohol (9:0.96:0.04 v/v/v respectively) to remove non-nucleic acid cellular macromolecules from the sample thereby cleaning up the DNA extract. DNA was recovered from the sample by binding to silica in spin columns using the Zymo DNA Clean and Concentrator-5 Kit™ (Zymo Research, Irvine, CA) following manufacturer's instructions. DNA bound to the spin columns was eluted using TE-4 (10 mM Tris-Cl, pH 8.0 containing 0.1 mM EDTA).

DNA Quantification

DNA was quantified in extracts spectrophotometrically on a Nanodrop® ND1000 (Thermo Fisher, Waltham, MA). Concentration and purity were determined using ND1000 V3.8.1 software. DNA yield from the above protocol averaged 1µg per extraction.

Bisulfite Treatment

Cytosine methylation occurs naturally throughout the genome of eukaryotic and prokaryotic organisms. A methyl group is added to the fifth carbon position of a guanine/cytosine site via endogenous methyltransferase activity. Characterization of methylation sites in genomic DNA provides insight into gene regulation that has implications in aging and disease. Frommer et al. showed that bisulfite treatment of DNA chemically modifies unmethylated cytosines resulting in their conversion to uracil (Frommer et al., 1992). Once the DNA is converted through bisulfite treatment, oligonucleotide primers can be designed to differentially amplify methylated or unmethylated CpG targets. Thus, it is rather straightforward to amplify genomic DNA that has been treated with bisulfite and assess potential methylation differences based upon which of the oligonucleotide primers mediate amplification of a product.

Twenty microliters containing approximately 1 µg of extracted DNA were subjected to bisulfite treatment using the EZ DNA Methylation-Lightening™ Kit (Invitrogen, Carlsbad, CA) to enable the differentiation of methylated and unmethylated CpG sites. The protocol provided with the kit was followed to convert unmethylated cytosines into uracil while methylated cytosines remained unchanged. Lightening™ Conversion Reagent containing 45% ammonium bisulfite was mixed with the DNA sample and conversion occurred in an Applied Biosystems 2700 Thermocycler (Life technologies, Carlsbad, CA) programmed to reach a temperature at which unmethylated cytosines become deaminated. Thermocycler conditions consisted of 8 minutes at 98˚C and 60 minutes at 54˚C. Bisulfite treated samples were mixed with M-Binding Buffer and centrifuged in an EZ DNA Methylation-Lightening™ Conversion column and washed

with M-Wash Buffer. Addition of L-Desulphonation Buffer converted all deaminated cytosines to uracil. Two additional washes were performed, and the samples were eluted with DNase/RNase free water. Samples were stored at -20° C until testing.

Primer Design for Methylation Study

Primers were designed to target identical loci in genomic DNA but differed biochemically in 2 ways (L. Zhang et al., 2012). The primers intended to target unmethylated cytosines terminated in A residues at GC sites and thus were complementary to the uracil residues converted from unmethylated cytosine. In this way, forward primers contained thymine and reverse primers contained adenine in the unmethylated cytosine positions. Furthermore, amplified PCR product lengths differed in that amplicons produced from DNA containing methylated cytosines were between 15 and 20 base pairs longer than amplicons produced from the same, but unmethylated target. This primer design allowed easy visual differentiation between methylated and unmethylated products using agarose gel electrophoresis of amplified products. Primers for methylated sequences were complimentary to available GenBank sequences. Primers sequences for methylated and unmethylated *PER1*, *PER2*, *CRY1*, *CRY2*, *BMAL1*, NPAS2 and *CLOCK* were taken from a previously published study (L. Zhang et al., 2012). Methylated and unmethylated primers for *PER3* were designed in house following the same strategy for primer design as discussed above. Primer sequence accuracy and specificity were determined using the Basic Local Alignment Search Tool (BLAST) for nucleotides on The National Center for Biotechnology Information (NCBI) site (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Primer self-complementarity and dimer

formation were assessed using the PrimerBlast tool (https://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi) on the NCBI site. Primers were commercially produced by Invitrogen (Invitrogen, Carlsbad, CA).

Table 1. Methylated and Unmethylated Primers. This table contains primer sequences, expected product sizes of each amplicon and the location of the amplified target in regards of the start site (origin = 0).

GENE NAME	PRIMER	5' – SEQUENCE – 3'	SIZE (bp)	LOCATION
PER1	MForward	ATTTAGGTTTACGTGCGTTC	298	-1879 to -1860
	MReverse	CGACTCAAAAACGAAAATCG		-1582 to -1601
	UForward	TAGTATTAGTATTTAGGTTTATGTGTGTTT	318	-1889 to -1860
	UReverse	AACAACAATCCAACTCAAAAACAAAAATCA		-1572 to -1601
PER2	MForward	GCGGTTTCGTTGCGGTTTAC	140	-178 to -159
	MReverse	GCCGACGCCGTTTCAAACCG		-39 to -58
	UForward	GTGGTGTGGTGTGGTTTTGTTGTGGTTTAT	160	-188 to -159
	UReverse	ACACCCCCACACCAACACCATTTCAAACCA		-29 to -58
PER3	MForward	CGGGAGTTTTGGGTATTCGC	182	-453 to -444
	MReverse	CGACCCGACTAACTAAAACG		-281 to -301
	UForward	TGGGTGGTTGGGTGGGAGTTTTGG	194	-465 to -444
	UReverse	AATCCAACACCAACAACCCAACTA		-269 to -301
CRY1	MForward	TCGTTTTGTTTTTAGGGGTC	166	-420 to -401
	MReverse	GCAACCGCCTAAAAACGACG		-255 to -274
	UForward	ATTTTGGGTGGGTTGTTTTGTTTTTAGGGGTT	187	-432 to -401
	UReverse	ACACCCACCACAACCACCTAAAAACAACA		-246 to -274

CRY2	MForward	GTTTATTTTCGGTATTTCGC	149	-308 to -289
	MReverse	TAACGATTAACCCAAAAACG		-159 to -178
	UForward	TTGAGATTTGGTTTATTTTTGGTATTTTGT	170	-318 to -289
	UReverse	TACCTTCCACCTAACAATTAACCCAAAAACA		-149 to -178
CLOCK	MForward	GTTTTTTATTCGATTAGGTTTC	168	-454 to -433
	MReverse	CGTTAAACAACACGAAACCG		-290 to -309
	UForward	GTTGGTTGGTTGTGTTTTTTATTTGATTAGGTTTT	188	-467 to -433
	UReverse	CAACTTACCCCATTAAACAACACAAAACCA		-279 to -309
NPAS2	MForward	GGTTTAGTTCGCGTTCGGTTTC	140	-84388 to -84366
	MReverse	CCACGCTAACGAACAAATAACCG		-84349 to -84342
	UForward	TGTGTTTTTTTGGTTTAGTTTGTGTTTGGTTTTG	158	-84399 to -84365
	UReverse	ACATCCTCCACACTAACAAACAAATAACCA		-84261 to -84342
BMAL1	MForward	GGAGGTGTTTGTTTATTCGC	138	30 to 39
	MReverse	AAATACGCGAAATCGCGTCG		153 to 167
	UForward	TAGGTTAGGGATGGAGGTGTTTGTTTATTTGT	161	18 to 39
	UReverse	AACCCCCAACAAAATACACAAAATCACATCA		153 to 178

Note: MForward: forward primer directed specifically toward methylated sequences; MReverse: reverse primer directed specifically toward methylated sequences; UForward: forward primer directed specifically toward unmethylated sequences; UReverse: reverse primer directed specifically toward unmethylated sequence.

Methylation Controls:

CpG methylase attaches a methyl group to all cytosine residues in a DNA sample containing the dinucleotide sequence 5' ….. CpG ….3'. One microgram of a control DNA sample (supplied with STR typing kits) was methylated with a CpG methylase isolated from *Escherichia coli* expressed *Spiroplasma* sp. strain MQ1 methyltransferase available commercially (Zymo Research, Irvine, CA). The methylated product was used

as a positive methylation control with all primer pairs. Methylated reactions consisted of a mix of CpG reaction Buffer, S-adenyosymethionine (SAM) acting as the methyl donor, CpG methylase, and extracted DNA. The methylase mix was incubated at 30°C for 2 hours. Negative controls were represented by non-bisulfite treated DNA.

Polymerase Chain Reaction (PCR) for Methylation Study

Each PCR reaction consisted of 10 ng of DNA combined with 1 µl of a 1X primer mix (forward and reverse, 1 µM final concentration), and 5 µl GoTaq® Hot Start DNA Polymerase (Promega Corporation, Madison, WI) in a total volume of 10 µl. Positive and negative methylation controls were included in each reaction. Optimized PCR settings were determined for methylated and unmethylated reactions using temperature gradient conditions on an MJ Mini Personal Thermocycler (BioRad, Hercules, CA). Once optimal cycling conditions were determined, amplifications were routinely performed on an Applied Biosystems GeneAmp®PCR System 2700 Thermocycler (Applied Biosystems, Inc., Foster City, CA). Final optimized cycling conditions varied in annealing temperature alone and are listed in Table 2.

Table 2. Optimized Cycling Conditions for Methylated and Unmethylated Primer Sets.

Temperature	Time	Number of cycles	Primers used per specified annealing temperature
95°C	2 minutes	1 cycle	
95°C	30 seconds	30 cycles	
72°C	30 seconds		
55°C	30 seconds		UPER1, MPER1, MCRY1, UCLOCK, MCLOCK, MCRY1
57°C			UPER2, MPER2, UPER3, UPER3, UBMAL1
59°C			UNPAS1, MNPAS1, MBMAL1, UCRY1

U(unmethylated), M(methylated)

Agarose Gel Electrophoresis

One microliter of loading dye was added to 5 µl of each amplification product and added to sample wells of a 2.5% agarose gel. A 100 bp DNA ladder (Thomas Laboratories, Swedesboro, NJ) was also electrophoresed with amplifications for size comparisons. Agarose gel electrophoresis was performed in TAE buffer (.04M tris acetate, .001M EDTA, pH 8.3) and visualization of DNA fragments was made possible with GelRed® Nucleic Acid Stain (Thomas Laboratories, Swedesboro, NJ). Samples were separated at 500mA current. Syngene InGeniusL Syngene Bio Imaging System (Synoptics Ltd, Cambridge, England) was used to capture and save gel images.

EXPRESSION STUDIES

Donor Selection for Expression Studies

Donor selection guidelines were identical to those used in the methylation study.

Donors for the Stability Study: Three volunteers were randomly selected to participate in the stability study.

Donors for the Age Comparison Study: Donors were selected and categorized by age. Group 1 consisted of volunteers between the ages of 20 and 29 years old. Group 2 involved volunteers that ranged in ages from 30 to 39 years old. Group 3 included volunteers between the ages of 40 to 49 years old and group 4 volunteers were between 50 to 59 years old. A minimum of three male and three female volunteers from each age group were selected.

Donors for the 24-Hour Expression Pattern Study: Volunteers selected were screened for sleep patterns as discussed previously and additionally were required to be trained in blood collections and safety practices involved in biohazardous material handling as well as sharps safety.

Blood Collection for Expression Study

General Venipuncture Blood Collection: The experiments designed to assess the stability of mRNAs expressed by circadian rhythm genes required larger blood volume sample sizes to accommodate multiple RNA extractions from each blood stain stored in the lab over a period spanning 8-days. Venipunctures were performed by certified medical professionals to acquire 3-5 mL of peripheral blood. Venous blood samples were collected using 22-gauge needles fitted onto 5 cc anticoagulant-free syringes, and

immediately spotted on nuclease-free 705™Classic Specimen Cards (Fitzco®, Spring Park, MN). Collection dates and times were documented for each sample and specimen cards were stored in the dark at room temperature prior to extractions.

General Fingerstick Blood Collection: Fingerstick samples for capillary blood were performed with SurgiLance™ 2.8 mm 21-gauge safety lancets (MediPurpose™, Duluth, GA) by a certified medical professional. Capillary samples were spotted in a 20mm circumference onto nuclease-free 705™ Classic Specimen Cards (Fitzco®, Spring Park, MN). Specimen cards were stored in the dark at room temperature. The times and dates for both collection and extraction of blood stains were documented for each sample.

Capillary blood was collected for the study of physiologic variability in gene expression among each blood donor via a fingerstick at approximately 1:00 pm +/- an hour for a minimum of 3 collections in the span of 3 weeks.

Age Comparison Study: Forty capillary blood samples were collected via a fingerstick at approximately 1:00 pm +/- an hour from volunteers from different age groups.

24-Hour Expression Patterns Study: Each volunteer received a collection kit that consisted of 7 SurgiLance™ 2.8mm 21-gauge safety lancets (MediPurpose™, Duluth, GA) , 10 alcohol pads, 10 sterile gauze squares, and one nuclease-free 705™ Classic Specimen Cards (Fitzco®, Spring Park, MN). A biohazard bag was included to deposit used supplies for return and proper disposal. Instructions were included with each kit (Appendix D). Capillary blood was collected via a fingerstick every 4 hours over a 24-hour period. Sample collection began at 8:00 am and was completed by 4:00 am. Volunteers were instructed to allow the samples to air dry and to return the specimen

cards no later than 24 hours after the last sample was collected. Additionally, biohazard and sharps safety were discussed. Appropriate safety material was provided, and all used and unused material was returned for proper handling. Specimen cards were stored in the dark at room temperature for no more than 48 hours prior to extraction of total RNA from each sample. Collection time and RNA extraction times were documented for each sample.

Venipuncture and fingerstick needle handling were in line with current sharps safety (OSHA regulation 29 CFR 1910.1030(d)(2)(i)) and disposal guidelines (OSHA requirements 29 CFR 1910.1030(d)(4)(iii)(A)(2)(i)).

RNA Extractions for Expression Study

Stability Study: A portion of the dried blood sample specimen card was excised for extraction each day for 8 days. Day 0 was extracted within 2 hours of collection to allow the sample to completely dry prior to processing. All subsequent RNA extractions were performed within +/- 1 hour of the time at which the time zero extraction was performed. A 20 mm x 20 mm portion of the sample card was cut into 1-2 mm strips and placed into 2.0 ml microfuge tubes.

General RNA Extraction Protocol

One milliliter of Trizol® (Sigma Aldrich, St Louis, MO) was added to the blood stain and the constituents were agitated at low speed on a Phenix Benchmix 1000 (Thomas Laboratories, Swedesboro, NJ) for 30 minutes at room temperature. Following the 30-minute agitation, 200 µl of chloroform was added to the blood/Trizol mixture that

was inverted continuously by hand for 30 seconds. The microfuge tube was placed into a rack at room temperature for 3 minutes followed by centrifugation for 15 minutes at 12,000 g at 4°C. After centrifugation, the aqueous (upper) layer containing the cell-free nucleic acids was transferred to a new 1.5 ml microfuge tube. The addition of 550 µl 100% ethanol precipitated the nucleic acids. The ethanol mixture was transferred to a Zymo Spin Column seated in a reservoir and centrifuged for 30 seconds at 10,000 g. Total RNA was recovered from the extract using the Zymo RNA Clean and Concentration Kit™ (Zymo Research, Irvine, CA). The manufacturers protocol was followed for cleaning and purification of the sample. Nucleic acids were eluted using 15 µl of DNase/RNase-free water.

RNA Quantification

The Qubit RNA HS Assay Kit (Invitrogen, Carlsbad, CA) was utilized for RNA quantification. One microliter of undiluted nucleic acid eluate was mixed with 199 µl of Qubit™RNA HS Buffer and 1 µl Qubit™RNA HS Reagent 200X concentrate in a Axygen 0.5 ml tube. The use of the RNA HS reagents assures measurement or RNA with minimal interference of dsDNA and ssDNA. Tubes were briefly vortexed and RNA concentration was quantified using a Qubit™ Fluorimeter 2.0 (Invitrogen, Carlsbad, CA).

DNase Treatment

All quantified RNA extracts underwent DNA digestion with the Invitrogen™ EZ DNase™ System (Invitrogen, Carlsbad, CA) per the manufacturer's protocol to remove potential contaminating residual genomic DNA that might interfere with mRNA

quantitation assays. The entire RNA extract (~14µl remaining) was combined with 1.5 µl 10X ezDNase™ buffer and 1µl ezDNase™ enzyme and placed in a 37°C water bath for 2 minutes. The sample was placed on ice immediately following incubation.

Reverse Transcription

Reverse transcription with Invitrogen SuperScript IV™ VILO RT reverse transcriptase (Invitrogen, Carlsbad, CA) was performed following supplier protocols for all RNA extracts immediately following ezDNase™ treatment. The Superscript IV™ VILO master mix kit uses a combination of random and oligo dT primers for reverse transcriptase with a recombinant reverse transcriptase engineered to exhibit reduced RNase H activity and high processivity (Thermo Fisher, Waltham, MA). The entire RNA extract was transferred to a clean 0.2 ml PCR amplification tube and 4 µl of SuperScript IV™ VILO RT master (5X) mix was added. The reaction was placed in a MJ Mini Personal Thermocycler (BioRad, Hercules, CA) for 1 cycle of 25°C for 10 minutes, 50°C for 10 minutes and 85°C for 5 minutes.

Primer Design for Expression Study

Stability Study – Primer Design

PERIOD 1, PERIOD 2, PERIOD 3, CRYPTOCHROME 1, CRYPTOCHROME 2 and *CLOCK* genes that were discussed in Chapter 2 were selected for this study. Publications of previous research involving these markers served as a guide for target locations for qPCR amplification within the mRNA and for primer selection (Liu et al., 2007; Yang et al., 2006). *S100A12-241* primers were designed and supplied by the

Forensic DNA lab of Dr. Robert W. Allen (Fu and Allen 2019) The *S100A12* mRNA transcript is expressed at constant levels in blood and was quantified to normalize qPCR results obtained for the circadian rhythm markers. The *S100A12* protein belongs to a superfamily of Ca^{2+} binding proteins. *S100A12* protein is released by neutrophils (Vogl et al., 1999) and is involved in a proinflammatory pathway that facilitates leukocyte rolling and extravasation (Foell et al., 2013). The 241 region of the *S100A12* mRNA lies within a coding region of the transcript distant from the 3' end of the transcript and exhibits stability in dried blood stains (Fu & Allen, 2019). The abundance of the *S100A12-241* mRNA fragments in dried blood stains produced Cq values during qPCR in the 18 to 20 range that was deemed appropriate for normalization of clock mRNA levels whose Cq values ranged from 27 to 32 depending upon the particular sample. Amplicon target lengths between 100 and 150 base pairs were favored for qPCR targets and amplicons with similar lengths allowed for possible later applications in PCR multiplexing.

Primer location is a critical consideration for primer design. The environment in which a forensic sample is recovered from a crime scene can promote degradation of biomolecules contained within a stain and thus, in the case of mRNA, the region to be amplified must be located within a stable area in order to avoid degradation issues and yield accurate transcript quantity estimates. Previous research concerning selected mRNA (*PER1, PER2, PER3, CRY1, CRY2, CLOCK*) were referenced to aid in the strategy regions targeted for amplification (Liu et al., 2007; Yang et al., 2006). Primers were designed to reduce primer dimers and self-complementarity and to contain approximately 50% guanine/cytosine residues. A melting point target for the primers of 55-60° C allowed optimization of primer sets for each target to facilitate planned multiplexing. Primer

specificity to the targeted area of interest was evaluated using Basic Local Alignment Search Tool (BLAST) for nucleotides on The National Center for Biotechnology Information (NCBI) site (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Primer self-complementarity and dimer formation was assessed using the PrimerBlast tool (https://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi) on the NCBI site. Characteristics of the primers designed are shown in Table 3 below.

Table 3. Primer Characteristics for Expression Study.

Primer	Primer Sequence	Tm	Product Length
PER1 F	5' GAATACTACCAGCAGTGGAG 3'	55°C	119 bp
PER1 R	5' GTATCCTGGTTCTGAAGTGTG 3'	56°C	
PER2 F	5' GAAATCCGCTACCACCCCT 3'	57°C	121 bp
PER2 R	5' GCTTCATAACCAGAGTGCAC 3'	56°C	
PER3 F	5' GAGACGCAATAAACCAAGCA 3'	57°C	115 bp
PER3 R	5' CTGAGGTGCTCCATTCTGAC 3'	58°C	
S100A12 F	5' TCCAAGGCCTGGATGCTAATC 3'	60°C	93 bp
S100A12 R	5' TGTGGTAATGGGCAGCCTTC 3'	60°C	

In an extension of our studies on the stability of RNA transcripts in dried stains, PCR primers were designed to amplify larger segments of a handful of circadian rhythm transcripts, the notion being that successful amplification of longer pieces of each transcript would confirm the degree of intactness of the mRNA in dried stains. In order to assess that minimal degradation existed in the mRNA, additional primers flanking the *CLOCK*, *PER3* and *TPOH* genes were designed. Since these primers were designed to amplify longer fragments and are not intended to be used in later applications in multiplexing, melting temperature and fragment length were not restrictive.

Table 4. Primers for Larger Products to Refute Degradation. Primers designed to 5' and 3' flanking regions of initial primer sets for *PER3* and *TPOH*. Intact mRNA was assessed with combinations of the new long(L) primers and the original primer sets from this study.

Primer	**Sequence**	**TM (C)**	**Location on mRNA (bp)**	**Product with new L reverse primer (LR)**	**Product with initial forward primer (F)**	**Product with initial reverse primer (R)**
LCLOCK F	5' CCAACCCCTTCTGCCTCTTC 3'	64°	671-692	1411 bp		730 bp
LCLOCK R	5' AAATGCTGCCTGGCTGGAG 3'	60°	2091-2111		800 bp	
LPER3 F	5' TGTTGGAGGGAAAAGCTCCT 3'	60°	82-100	1436 bp		504 bp
LPER3 R	5' ACGTGAACTGGCTGTAAGAGA 3'	62°	1497-1517		1047 bp	
LTPOH F	5' ACCATTGTGCCAACAGAGTTC 3'	62°	402-423	1403 bp		663 bp
LTPOH R	5' GTAACAATATGAGTCAGCGCCA 3'	60°	1780-1804		843 bp	

Physiologic Variation Study - Primer Design

The purpose of this study was to analyze the biological variability in the quantities of mRNA transcripts for rhythmicity expressed genes among our pool of blood donors consisting of unrelated males and unrelated females. Primers targeting core clock gene cDNA, as described previously in the Stability Study, were used. Primers for markers targeting additional clock genes and supplementary rhythmic genes were added for this study. The addition of gene markers beyond the core clock genes provided increased sensitivity to detect additional, time-dependent patterns of gene expression.

In order to develop an assay for estimating the time of day a stain was created, it is imperative that a reference pattern of gene expression exhibiting peaks and troughs over the course of a 24-hour period be available to estimate the deposition time for an unknown. With multiple gene markers exhibiting varying patterns of gene expression, time determination should be more accurately estimated within a narrow window of time associated with a crime. The additional rhythmic genes selected have been reported to exhibit oscillating patterns of expression in various peripheral tissues (Ebert-Zavos, Horvat-Gordon, Taylor, & Bartell, 2013; Lech et al., 2015; Masri, Cervantes, & Sassone-Corsi, 2013). Publications of previous research using the genes of interest were reviewed (Champier et al., 1997; Kusanagi et al., 2008; Lech et al., 2015; Salani et al., 2007) and primer sequences and target positions within each transcript were considered for this study. Primer sequences generally targeted a central position in the mRNA sequence and we have confirmed for many transcripts that this area of the mRNA is the most resistant to degradation (Fu & Allen, 2019). Primers were synthesized to direct amplification of

BMAL1 from the core clock mechanism and *CYE*, *MKNK*, *THR*A1, and *TPOH* from the secondary line of circadian rhythm. Table 5.

Table 5. Additional Primers for Expression Study.

Primer	**Primer Sequence**	**Tm**	**Product Length**
BMAL1 F	5’ CCCATTGAACATCACGAGTAC 3’	62°C	116 bp
BMAL1 R	5’ GAGCCTGGCCTGATAGTAG 3’	60°C	
CLOCK F	5’ CCAACCCCTTCTGCCTCTT 3’	59°C	121 bp
CLOCK R	5’ GTAAATGCTGCCTGGGTGG 3’	59°C	
CRY1 F	5’ TTGGAAAGGAACGAGACGC 3’	58°C	125 bp
CRY1 R	5’ CGGTTGTCCACCATTGAGTT 3’	58°C	
CRY2 F	5’ GTGTGGAAGTAGTGACGGA 3’	56°C	113 bp
CRY2 R	5’ GCTGATGATGGCCTGAAAG 3’	56°C	
CYE F	5’ GTTGCACCAGTTTGCGTATG 3’	60°C	122 bp
CYE R	5’ GCCAGGACACAATAGTCAG 3’	54°C	
MKNK2 F	5’ CATAACAAAGGCATCGCCC 3’	58°C	120 bp
MKNK2 R	5’ GTTGAGTTTGATGCCGCTG 3’	58°C	
THRA1 F	5’ GTGTGGGGACAAGGCAAC 3’	58°C	120 bp

THRA1 R	5’ CTGTCATATTTGCAGGAATAG 3’	56°C	
TPOH F TPOH R	5’ GCTGTTCAAAAACTGGCAAC 3’ 5’ GAAGAAAGTAAGCCAGCACC 3’	58°C 60°C	101 bp

Primers were designed to reduce primer dimers and self-complementarity and to contain approximately 50% guanine/cytosine residues. A melting point target for the primers of 55-62° C allowed for optimized activities of both primer sets and later applications. Primer sequence accuracy and specificity to amplify only the target of interest was evaluated using the Basic Local Alignment Search Tool (BLAST) for nucleotides on The National Center for Biotechnology Information (NCBI) site (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Primer self-complementarity and dimer formation was determine using the PrimerBlast tool (https://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi) on the NCBI site. Melting curves for each primer set were performed to verify that only one amplified product would be produced. Efficiency tests for select primers provided evidence that amplifications were at optimal capacity and shared similar efficiencies. This assured that variations in amplification quantities reflected mRNA amounts and eliminated variations due to inhibitors.

Polymerase Chain Reaction (PCR)

Stability Study PCR

PCR reactions for longer amplicons contained 10 ng of cDNA from Sample 2, day 0 and Sample 2, day 2 from the stability study described above. Additionally, 5 µl GoTaq® Hot Start DNA Polymerase (Promega Corporation, Madison, WI) and 1 µl of a 1X (10 µM final concentration) of primer mix ((Long forward(LF)/Long Reverse(LR) F/LR, F/R and LF/R as described in Table 2) was added for a 10 µl final volume. Amplification was performed on a GeneAmp® PCR System 2700 (Life Technologies, Carlsbad, CA) as described in Table 6.

Table 6. Thermocycler Conditions Used for Variable Length Products.

98°C 2 minutes	35 cycles			4°C hold
	98°C 30 seconds	58°C 30 seconds	72°C 30 seconds	

Quantitative PCR (qPCR)

Quantitative PCR was used to quantify mRNA levels in blood stains stored for varying periods to assess RNA degradation in a stored sample. All qPCR reactions were analyzed in MicroAmp®Fast 96-well reaction plates (0.1 mL) (Life Technologies, Carlsbad, CA). Each reaction consisted of 10 ng of cDNA with nuclease free water to a total of 4 µl and 1 µL of an 8 µM forward and reverse primer mix (final primer concentration was 0.8 µM) . Five microliters of PowerUp™SYBR™Green Master Mix (Life Technologies, Carlsbad, CA) was added to the cDNA/primer mix to a total 10 µL volume reaction. All reactions were performed in triplicate to distinguish preparational,

technical and random errors. Each reaction plate was analyzed on a 7500 Real Time PCR System (Life Technologies, Carlsbad, CA). qPCR reaction conditions are listed in Table 7.

Table 7. Quantitative PCR Conditions.

Denaturation		Amplification – 40 cycles			Disassociation	
50°C	95°C	95°C	58°C	72°C	95°C	60°C
2 min.	2 min.	15sec.	15 sec.	1 min.	15 sec.	1 min.

Disassociation curves for each primer set were performed to verify that only one amplified product was produced, and thus that data acquired from amplification accurately reflected quantities of the targeted mRNA. Efficiency tests for primers demonstrated that all primers were optimally efficient, that each primer set shared similar efficiencies and that cDNA preparations did not contain endogenous or exogenous inhibitors.

Agarose Gel Electrophoresis for Stability Study

Agarose gel electrophoresis was performed to visualize PCR amplicons from long primer sets directing amplification of long amplicon products. One microliter of loading dye was added to 5 ul aliquots of each amplified product and then added to sample wells of a 2.5 % agarose gel containing Phenix GelRed®Nucleic Acid Stain (Thomas Laboratories, Swedesboro, NJ). A 100 bp reference ladder was included in the gel for

size determination. TAE buffer (0.4M tris acetate, 0.01M EDTA, pH 8.3) was the running buffer for electrophoresis using 500 mA current. Gel images were captured with a Syngene InGeniusL Bio Imaging System (Synoptics Ltd, Cambridge, England). Agarose gels were assessed for expected length amplicons in order to verify that mRNA was intact in the samples tested.

Data Analysis General

Results from qPCR analysis were performed using 7500 Applied Biosystems Software version 1.2.3. A quantification cycle (Cq) reflects the point in amplification when the product signal crosses the threshold and higher Cq results indicate lower concentrations of mRNA. Instrument default settings were used to establish threshold values. Cq levels of each triplicate assay were averaged and standard deviation values greater than 0.5 were reviewed for stochastic events. Normalization of qPCR results is important in order to reduce experimentally induced variation. Therefore, Cq values of *S100A12* or *CLOCK* were subtracted from each transcript Cq for accurate quantitative levels or expression.

Stability Study Data Analysis

All Cq data produced for each marker quantified with qPCR in RNA extracts produced from stains stored for the different time points (incorporating data from 24 samples) were combined and plotted as a line graph using Microsoft® Office Excel (US Microsoft Corporation, Redmond, WA). A one-way ANOVA was used to determine if the time elapsed from sample collection to the time the RNA was extracted differed

significantly in terms of Cq values. Cq values for transcripts extracted from stains stored for varying periods that did not differ significantly from freshly prepared stains were accepted to reflect intact mRNA with minimal degradation. Results produced using qPCR were supported by results produced using agarose gel electrophoresis demonstrating the presence of larger PCR products amplified from RNA extracts stored for the short periods elapsing from blood collection to RNA extraction.

Physiologic Variation Study Data Analysis

Samples were normalized to *S100A12* and *CLOCK*. Normalized Cq values for triplicate qPCR reactions for each volunteer and for each primer set were averaged. Coefficient of variation (CV) results were calculated using Microsoft® Office Excel to determine the normal amount of variation that can be expected within an individual whose blood was collected at the same time on different days. Data from this study defined experimental variation in terms of the individual markers among biological replicates of RNA extracts from a single individual and ultimately these experiments allowed for the identification of circadian rhythm markers that exhibit a narrow range of gene expression over a 24-hour period. A narrow range of variation in gene expression is preferred for establishing reference patterns of rhythmic gene expression to be used forensically.

Age Comparison Study Data Analysis

Another possible source of variation in qPCR results relates to the age of the blood stain sample donor. The abundance of gene transcripts in stains created for each

age group were normalized to expression levels of *S100A12* and *CLOCK*. Mean values, standard deviations and standard errors were calculated using Microsoft® Office Excel. A one-way Anova, with a post hoc Tukey Test with Prism 8™ GraphPad (GraphPad Software, San Diego, CA), was used to determine if there was a significant difference in the expression of circadian genes between age groups (20's vs 30's, 30's vs 40's, etc). Subsequently, data from men and women were separated to determine if there was a significant sex-related difference in donor age groups. The same statistical approach was used to determine if there was a significant difference between men and women regardless of age.

24-Hour Expression Pattern Study Data Analysis

Triplicate qPCR values for each time point were averaged and normalized to *CLOCK* and *S100A12* levels. Male and female data was separated prior to further data analysis. Data from each time point was combined and mean values, standard deviations and standard errors were calculated using Microsoft® Office Excel. Combined data for each time point is shown as a line graph representing mean values with standard errors for every 4-hour time point over a 24-hour day. Each marker was assessed in this way. Graphs for male and female data were displayed separately. The most reliable markers for time determination were considered those that exhibited rhythmic patterns that demonstrate the least amount of standard error between individuals.

CHAPTER IV

METHYLATION STUDY

Optimization of Methylated and Unmethylated Primer Sets

In order to accurately quantify DNA through PCR amplification it is imperative that reaction conditions are optimized for each set of primers. In this way, varying levels of amplicon products observed after amplification accurately reflect their physiologic levels. This was the rationale for experiments aimed at optimizing PCR conditions to detect the relative proportions of methylated and unmethylated DNA associated with genes whose expression is linked to the circadian rhythm. The DNA sample used in these experiments was the same in order to provide accurate comparisons and more accurately identify optimal amplification conditions. Experimental amplification cycles were performed with each primer set and amplification products were electrophoresed on agarose gel for visual comparisons and relative quantification. The preferred PCR conditions were those that exhibited the brightest targeted bands that unique to amplifications directed by specific pairs of primers (i.e. were not non-specific amplification products). The principal variable evaluated in this study was the annealing

temperature whose effect manifested itself in the intensity of the amplicon band in the agarose gel and allowed the most effective amplifications conditions to be determined. Annealing temperatures ranged from 55°C to 61°C with all other conditions unchanged.

Effective amplification of genomic DNA with primers specific for methylated CpG sites required methylation positive DNA and thus methylation of random DNA was performed for a 2-hour period as described in the CpG Methylase protocol (Zymo Research, Irvine, CA). These methylated DNA samples were bisulfite treated (bisulfite treatment was performed to include each step that would be used for random samples) with subsequent amplification with methylated primer sets. Bisulfite treated random DNA was also used to program amplification using the unmethylated primer sets. Gradient temperature increases for the PCR reactions were performed in a MJ Mini Personal Thermocycler (BioRad, Hercules, CA). Five microliters of the PCR products were separated by agarose gel electrophoresis and the intensities of amplicon bands were evaluated. Images of the gel can be seen in Figures 4 and 5. Tables 8 and 9 provide the details about the amplicons in each well, their expected size and the temperature of each PCR reaction per sample. One hundred base pair ladders are included for size comparisons.

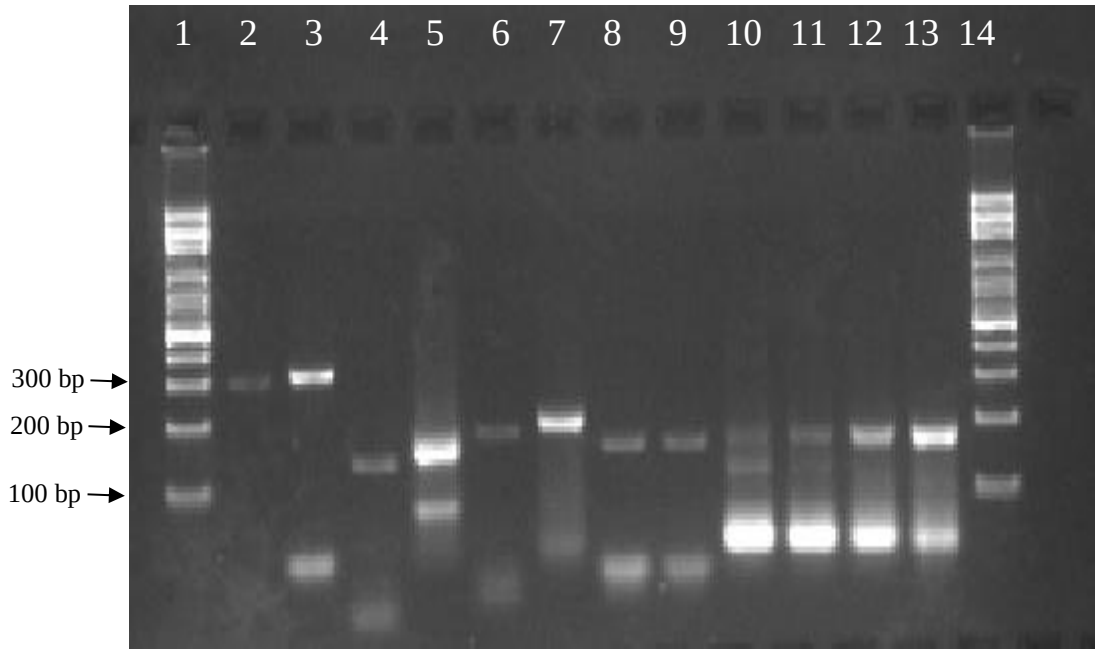


Figure 4. Conditions for Methylation Primers PER1-3 and CRY1. Bands ranging from 140 bp to 318 bp represent specific target amplicons. Bands below 100 bp represent excess primers and primer dimers. Lane 1 and 14 contain 100 base pair ladders. Table 8 describes the lane specifics for each reaction.

Table 8. Conditions for Methylation Primers PER1-3 and CRY1. The table reflects the contents of each lane of the gel in figure 4, the expected band sizes of each amplicon and the annealing temperatures used in each reaction. Interpretations of the products have been noted in the comments section of the chart and the temperature selected for subsequent experiments are indicated with an asterisk ”*”.

LANE	Sample	EXPECTED SIZE	ANNEALING TEMPERATURE	COMMENTS
1	100 bp ladder			
2	PER1 methylated	289 bp	55°C	Acceptable *
3	PER1 unmethylated	318 bp	55°C	Acceptable *
4	PER2 methylated	140 bp	57°C	Acceptable *
5	PER2 unmethylated	160 bp	57°C	Acceptable *
6	PER3 methylated	182 bp	57°C	Acceptable *
7	PER3 unmethylated	207 bp	57°C	Acceptable *
8	CRY1 methylated	166 bp	55°C	Acceptable *
9	“ “ “ “	“ “	57°C	Acceptable
10	CRY1 unmethylated	187 bp	55°C	Non-specific band
11	“ “ “ “	“ “	57°C	Non-specific band
12	“ “ “ “	“ “	59°C	Acceptable *
13	“ “ “ “	“ “	61°C	Acceptable
14	100 bp ladder			

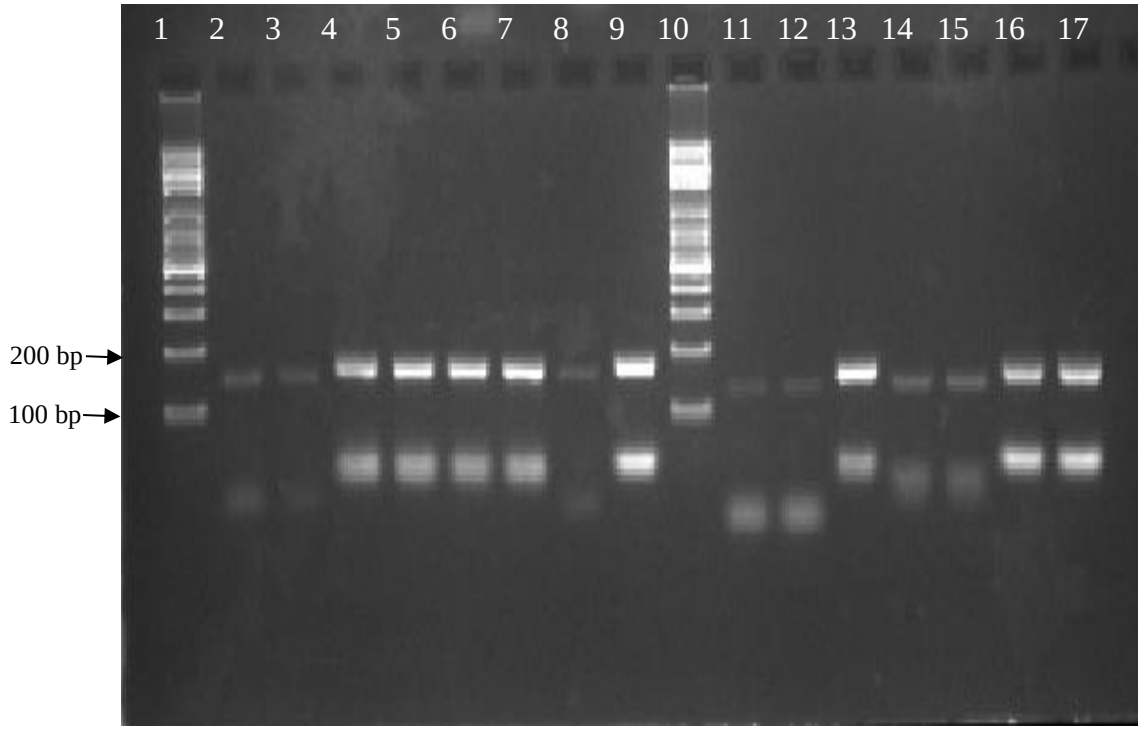


Figure 5. **Conditions for Methylation Primers CRY2, CLOCK, BMAL and NPAS2).** Optimization of annealing temperature for primers directing amplification of methylated and unmethylated genes (CRY2, CLOCK, BMAL1, and NPAS2) Amplicons produced with varying annealing temperature. Lanes 1 and 10 are 100 bp ladders. Details of each reaction is described in Table 9.

Table 9. **Conditions for Methylation Primers CRY2, CLOCK, BMAL1 and NPAS2**. Expected sizes of amplicons and annealing temperatures for each reaction are included within this table. Interpretations of the PCR products have been noted in the comments section of the chart. An asterisk ”*”indicates the temperature selected for subsequent PCR reactions with each specific primer set.

LANE	Sample	EXPECTED SIZE	ANNEALING TEMPERATURE	COMMENTS
1	100 bp ladder			
2	CRY2 methylated	149 bp	55°C	Acceptable *
3	“ “ “ “	“ “	57°C	Weaker
4	CRY2 unmethylated	170 bp	55°C	Acceptable *
5	“ “ “ “	“ “	57°C	Acceptable
6	“ “ “ “	“ “	59°C	Acceptable
7	“ “ “ “	“ “	61°C	Acceptable
8	CLOCK methylated	168 bp	55°C	Acceptable *
9	CLOCK unmethylated	188 bp	55°C	Acceptable *
10	100 bp ladder			
11	BMAL1 methylated	138 bp	57°C	Weaker
12	“ “ “ “	“ “	59°C	Acceptable *
13	BMAL1 unmethylated	161 bp	57°C	Acceptable *
14	NPAS2 methylated	140 bp	59°C	Acceptable *
15	“ “ “ “	“ “	61°C	Acceptable
16	NPAS2 unmethylated	158 bp	59°C	Acceptable *
17	“ “ “ “	“ “	61°C	Acceptable

Band intensity was used to determine the optimal amplification conditions with each primer set. In some cases, one temperature was used to verify the conditions used in the publications from which the primer sequences were derived. Based on the gradient annealing experiments from above, a 55°C annealing temperature for primers, PER1 M(methylated), U(unmethylated); CRY1 M; CRY2 M, U; and CLOCK M, U was selected for all future experiments. An annealing temperature of 57°C was selected for PCR reactions using PER2 M, U; PER3 M, U; and BMAL1 U primer sets while 59°C was selected for PCR reactions involving CRY1 U; BMAL1 M; and NPAS2 M, U primers.

CpG Methylase Verification

Amplicon bands produced with primers targeting methylated DNA appeared weaker than products amplified from unmethylated DNA. The CpG Methylase Protocol (Zymo Research, Irvine, CA) states an incubation time of 2 hours for methylation but allows up to 24 hours for complete methylation to occur. A new sample of DNA was methylated with the CpG Methylase Reagents (Zymo Research, Irvine, CA) but to optimize methylase activity, a 24-hour incubation time was used. It is likely that weaker bands in the previous protocol were due to lesser amounts of methylated products. Thus, an annealing temperature of 55°C was used with primers for methylated PER1, CLOCK, CRY1, and CRY2. Primers for methylated PER2 and PER3 had annealing temperatures of 57°C while methylated primer sets for BMAL1 and NPAS2 were exposed to annealing temperature of 59°C. The amplicon products were separated and visualized on an agarose gel (Figure 5). Results indicate an improved amplicon quantity with DNA template that was incubated for 24 hours in the methylase protocol. Additionally, PCR conditions used in this experiment appear adequate for assessment of methylation patterns.

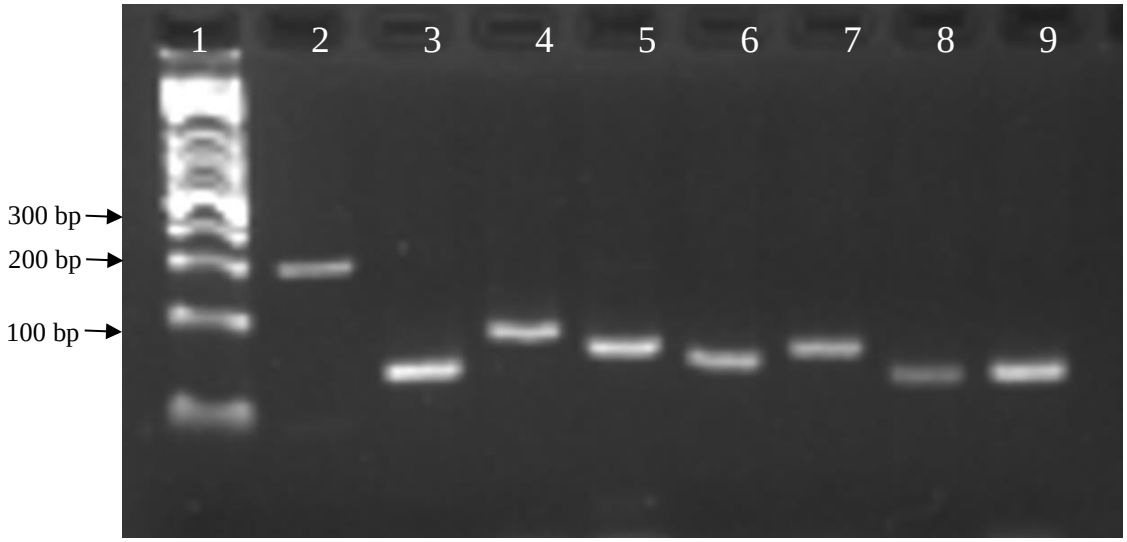


Figure 6. Methylation Amplicons from Methylase Treated DNA. Amplicon products using methylation specific primer sets with random DNA that was CpG Methylase treated over a 24-hour period. Lane 1 contains a 100 bp ladder. Lane 2 PER1M (289 bp), lane 3 PER2M (140 bp), lane 4 PER3M (182 bp), lane 5 CRY1M (166 bp), lane 6 CRY2M (149 bp), lane 7 CLOCKM (168 bp), lane 8 BMAL1M (138 bp) and lane 9 NPAS2M (140 bp). All results were produced using a single sample of genomic DNA (a positive control DNA supplied as part of an STR typing kit.

24-Hour Methylation Pattern Assessment

Detectable methylation has been described previously (Parle-Mcdermott & Harrison, 2011) and epigenetic modifications have been identified in circadian rhythm machinery (Sahar & Sassone-Corsi, 2013) . Characterization of an identifiable and predictable methylation pattern in dried blood stains provides the opportunity to identify a timeframe during the biologic 24-hour day in which the stain was created and possibly associated with a crime. The optimized PCR conditions described in the previous paragraphs were applied to investigate the involvement of DNA methylation in the circadian expression of selected genes. Twenty-four capillary blood samples were

collected from 3 volunteers every 4 hours for 24 hours. DNA was extracted from the samples within 48 hours of collection. All DNA samples were bisulfite treated in order to convert any unmethylated CpG sites to uracil. Those CpG sites that were methylated remained unchanged by bisulfite treatment. Aliquots of DNA from the donors was amplified with optimized conditions specific to each primer set. PCR products were electrophoretically separated in an agarose gel and band intensity was visualized (Figures 7 – 12).

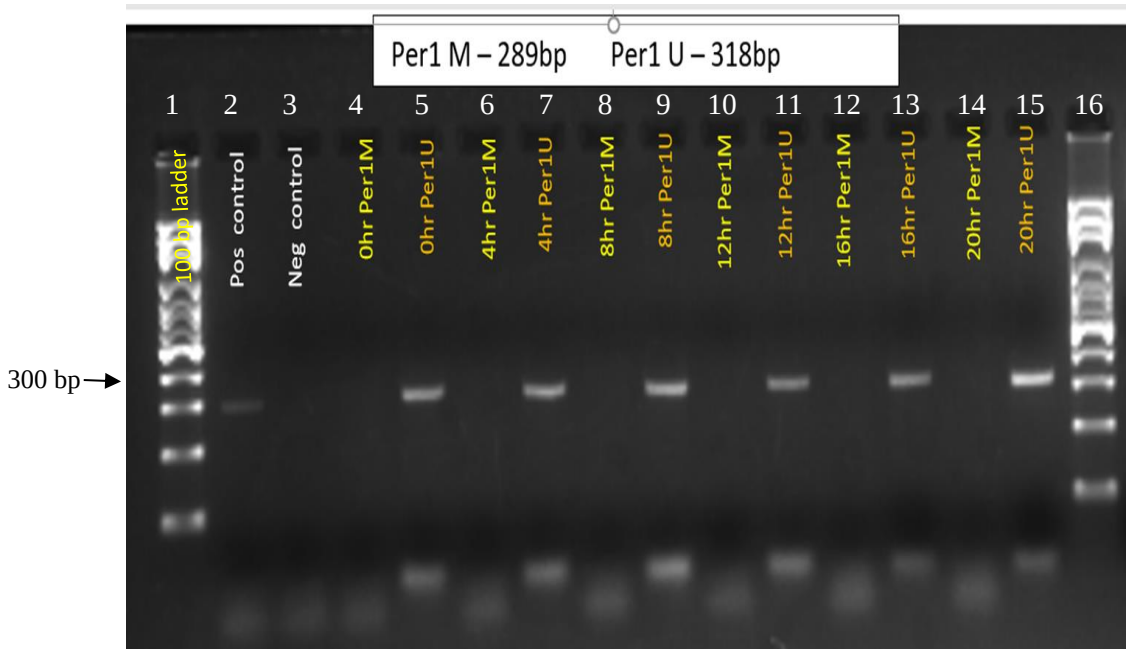


Figure 7. **Methylation Assessment of the *PERIOD 1* Gene over 24-Hours.** Lanes 1 positive control, Lane 2 negative control. All time references are in relation to midnight. Zero hour refers to midnight while 4hr refers to 4 hours after midnight or 4:00 am. Samples amplified with primers for methylated sites are designated M while those amplified with primers for unmethylated sites are labeled with U. The expected size for each amplicon product is noted at the top of the figure.

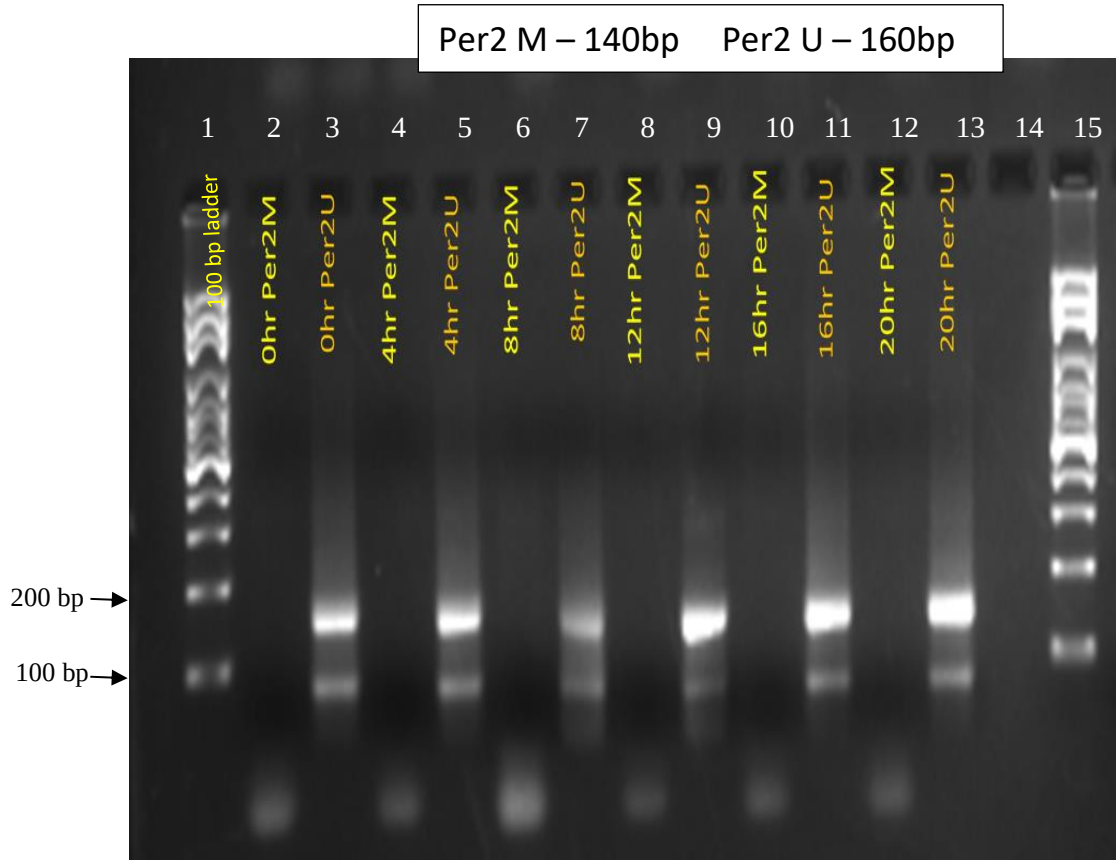


Figure 8. Methylation Assessment of the *PERIOD 2* Gene over 24-Hours. Methylated *PERIOD 2* (*PER2*) amplicon expected length is 140 bp while unmethylated *PER2* amplicons are 160 bp. One hundred base pair ladders are in lanes 1 and 15. The time is designated as the number of hours after midnight (i.e. 16hr = 4:00 pm).

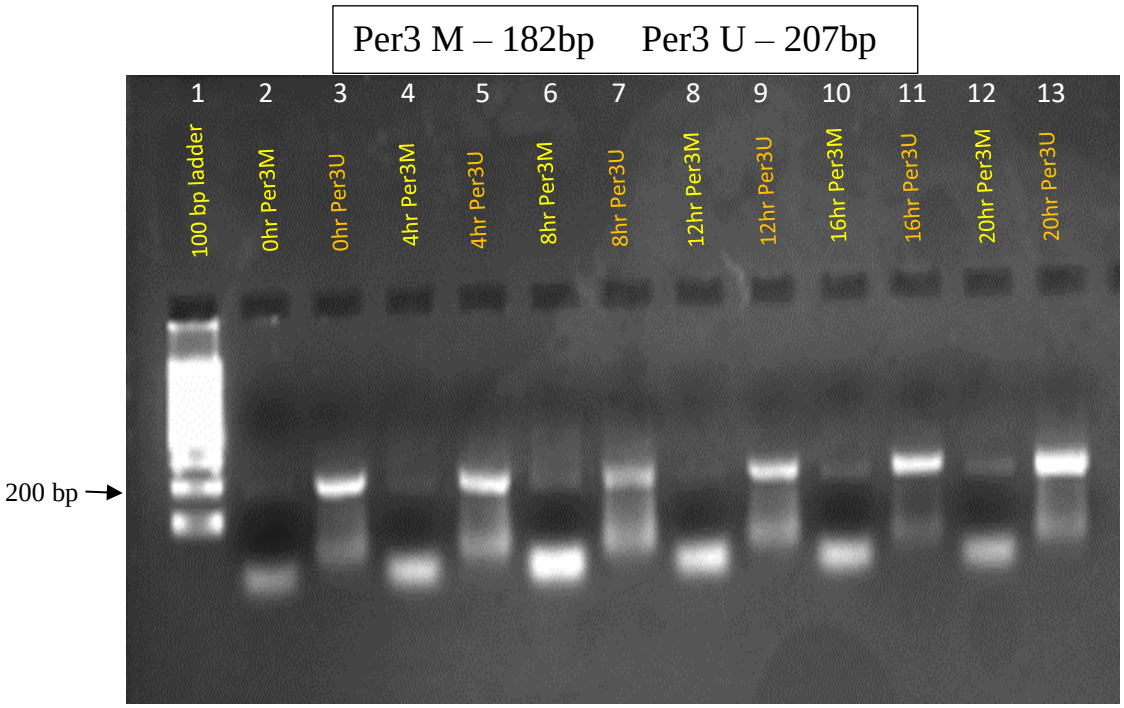


Figure 9. **Methylation Assessment of the *PERIOD 3* Gene over 24-Hours.** Lane 1 contains a 100 base pair ladder. The expected length of amplicons from the methylated (M) primer set is 182 bp while the unmethylated primer set should have products of 207 bp in length. Military time is applied to time references (i.e. 4hr = 4:00 am).

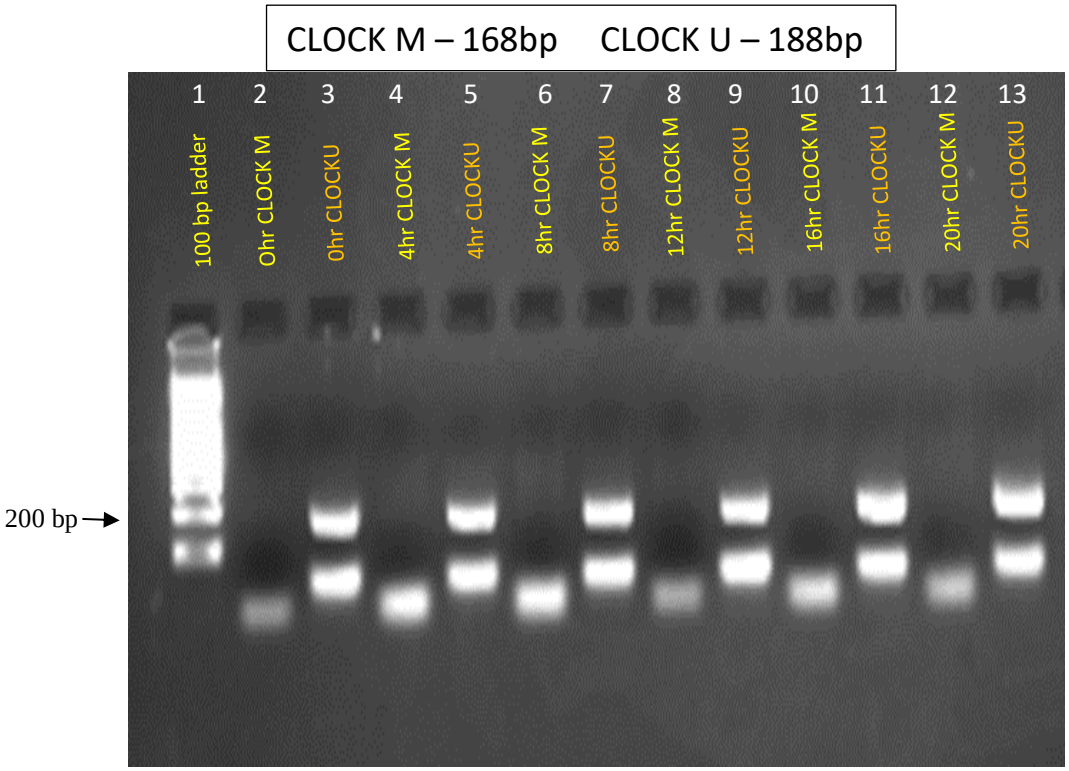


Figure 10. **Methylation Assessment of the *CLOCK* Gene over 24-Hours.** Lane 1 contains a 100 bp ladder. Methylated primer products should be 168 bp in length while unmethylated primer products are 188 bp. Time is based on military time (i.e. 8hr = 8:00 am).

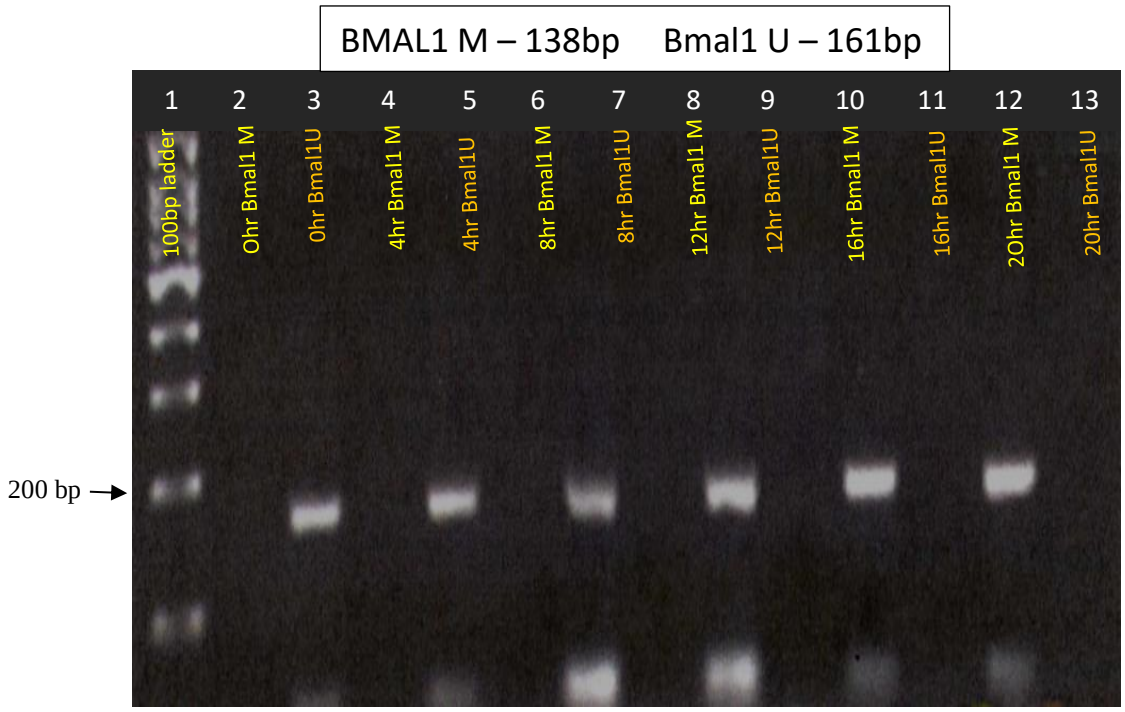


Figure 11. **Methylation Assessment of the *BMAL1* Gene Over 24-Hours**. Lane 1 contains the 100 bp ladder. The expected length of methylated (M) amplicons is 138 bp while the unmethylated amplicons should be 161 bp. Time points are expressed in military time (i.e. 12hr = 12:00 pm).

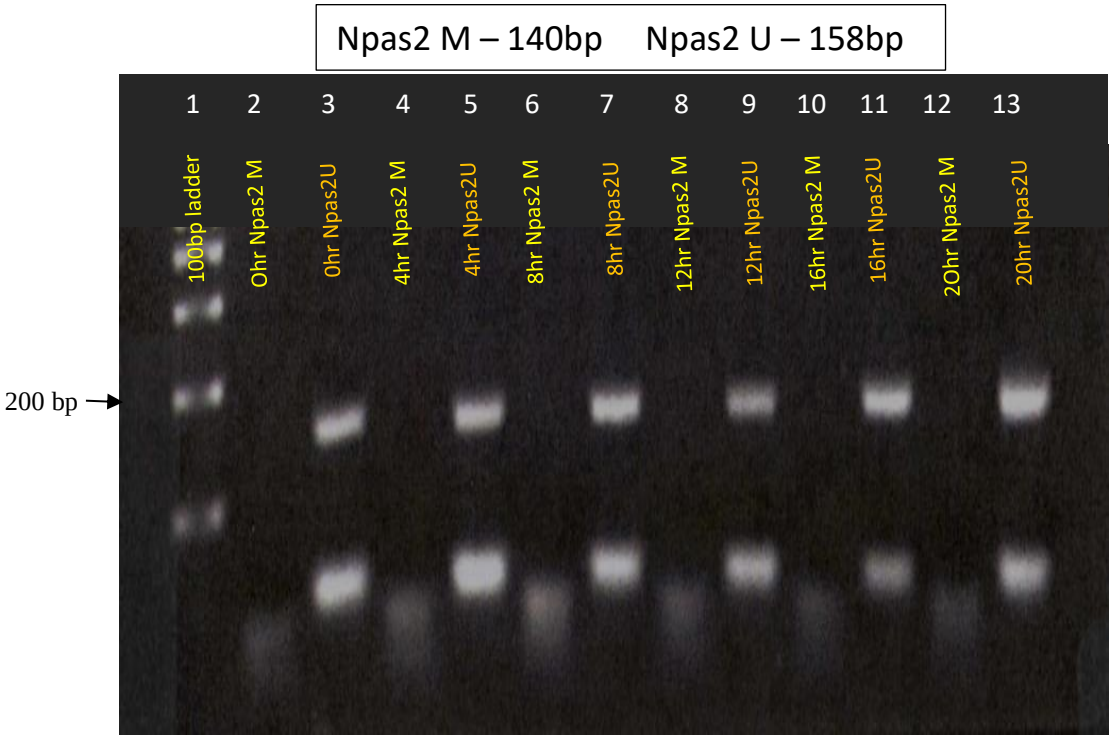


Figure 12. **Methylation Assessment of the *NPAS2* Gene over 24-Hours.** Lane 1 contains the 100 bp ladder for size comparison. The expected product size of a PCR reaction using the methylated set of primers for NPAS2 is 140 bp while the unmethylated primer set is expected to produce a product of 158 bp in length. Time designations are based on military time (i.e. 16hr = 4:00 pm).

The gel images were prepared using DNA extracted from blood from one of three volunteers used in this initial study. However, results shown for individual donors were reflective of results from all individuals who provided blood samples for the study. Amplicons of expected length were visualized at all time points in all reactions that targeted unmethylated target sequences. Extracted DNA from blood samples using methylated primer sets for *PER1-2*, *CRY1-2*, *BMAL1*, *CLOCK*, and *NPAS2* yielded no visible amplification at any of the time points tested whereas positive controls exhibited

bands of methylated genes contained no products with the exception of the *PERIOD 3* gene which showed very weak bands in all time points. Fragment sizes for all amplified products were consistent with products of expected size. There were no non-specific bands present in any of the observed PCR reactions beyond residual primers and primer dimers below the 100 bp marker.

Conclusion

Detection and correlation of epigenetic modifications of the genes involved with the circadian rhythm has gained momentum in the past decade. Applications of microarray technology provides evidence that rhythmic daily methylation patterns exist. In one such experiment, regions of DNA methylation in whole blood were reported with possible applications for the detection of senescence (Bell et al., 2012). Similarly, Day et al. evaluated methylated DNA among various tissues to correlate methylation and age, also using microarray technology (Day et al., 2013). Methylation of specific genes has also been investigated for forensic applications where Freire-Aradas et al. (2016) used microarray technology to correlate the age of an individual with the extent of the methylated DNA (Freire-Aradas et al., 2016). Microarray technology yields evidence of rhythmic methylation but fails to pinpoint the specific genes involved. Correlation of DNA methylation with specific genes and implications with circadian rhythm will require a complex molecular understanding of genomics. It is reasonable to assume that if methylation changes are associated with a 24-hour cyclic pattern, these chemical modifications may be involved with circadian rhythm. Joska et al. reported detection of

DNA methylation patterns involving circadian rhythm in *Neurospora*, mice and cancer cells (Joska, Zaman, & Belden, 2014).

In this study, methylation patterns were assessed over a 24-hour period to detect changes in genes that drive circadian rhythm. Primers specific to methylated or unmethylated sequences were used to determine if there are discernible methylation differences at multiple time points of the day. This study revealed that all clock genes analyzed, at the targeted sights, remained unmethylated in every sample tested. Additionally, there was evidence that *PERIOD 3* remained weakly methylated in every sample, continuously throughout the day. The atypical presentation of *PERIOD 3* that differs from *PERIOD 1* and *PERIOD 2* may be explained in work reported by Shearman et al. where a *Period 3* knockout mouse retained its rhythmicity while a knockout of *Period 1* or *Period 2* exhibited disrupted day/night cycles(Shearman, Jin, Lee, Reppert, & Weaver, 2000). The information gleaned from this finding suggests that although PERIOD 3 show interactions with core clock genes, it may not be necessary to drive the circadian rhythm. Theories exist that *PERIOD 1* and *PERIOD 2* represent redundant drivers of rhythmicity in case one gene is lost. Additional functions of *PERIOD 3* are currently under investigation. Given the information gained from this experiment, differential rhythmic methylation patterns cannot be used to determine the time of day using this methodology and the markers selected.

It should also be noted that the tissue used in this study (i.e. blood stains) represents a complex mixture of different cell types and it is possible that rhythmic methylation of the genes studied here actually exist in some differentiated cell types but that the differential methylation patterns are obscured by the other cell types present.

Future experiments might be warranted in which blood cell subpopulations are separated and the experiments repeated.

CHAPTER V

mRNA STABILITY STUDY

Results and Discussion

In experiments that aim to accurately quantify levels of gene expression during the daily cycle in dried blood stains, it is crucial to assure that the amount of mRNA detected at a particular time point represents the physiologic amounts in the donor. RNA degradation must be avoided as Fu and Allen (2019) correlated degradation in dried blood stains over time. Degradation of mRNA that might occur can falsely suggest decreased expression levels and confuse the interpretation of data. Since blood stains used in this study could be collected on one day and not subjected to RNA extraction for 24-48 hours, because of weekends and other interfering circumstances, we assessed the effect of storage for short periods on the stability of the rhythm markers in dried blood stains. Dried blood stains were prepared from 3 volunteers and stored for up to 8 days in the laboratory before extraction and reverse transcription of total RNA. Extractions were performed immediately after spotting and drying of blood stains and every 24 hours for 8

days in the laboratory before extraction and reverse transcription of total RNA. Extractions were performed immediately after spotting and drying of blood stains and every 24 hours for 8 days. The specified clock gene markers *PER 1-3* were quantified via qPCR. Raw data and normalized results produced from each time point were compared to results from Day 0 stain to determine if a significant difference in the quantity of each targeted mRNA from each storage time point. Genes of different expression levels were selected to account for possible accelerated degradation with low mRNA levels and to improve the sensitivity of the assay.

The Cq values for all markers were analyzed in triplicate and the raw data averaged. The mean Cq values of *PER1-3* from each day were also normalized to the corresponding *S100A12* Cq values. The *S100A12* transcript was previously shown to be stable for the 8-day maximum storage period in this study (Fu & Allen, 2019). Data from each volunteer was reviewed individually. Additionally, Cq values from all volunteer samples were averaged from the raw and normalized data. Table 9.

Table 10. Stability Results for *PER1-3* Raw Data and Normalized to *S100A12*.
Average of triplicate Cq values for *PER1-3* and *S100A12*. Raw data represents the average of the triplicate run of each marker for each of 3 volunteers over 8 days. Each day is represented by a mean value for *PERIOD 1-3* and an *S100A12* mean values. Normalized values were obtained by subtracting the mean *S100A12* value from the corresponding period marker mean value.

	Sample 1 Raw Data	Sample 1 normalized to *S100A12*	Sample 2 Raw Data	Sample 2 normalized to *S100A12*	Sample 3 Raw Data	Sample 3 normalized to *S100A12*
	PER1		PER1		PER1	
0day	31.7	12.61	33.20	12.57	30.0	9.81
1day	31.1	10.54	34.14	12.5	29.8	9.55
2day	31.5	12.03	33.94	12.3	30.0	9.64
3day	31.0	11.57	31.28	10.3	30.0	10.10
4day	33.7	14.20	34.66	12.4	30.5	9.88
5day	32.4	12.58	31.74	10.9	30.5	10.26
6day	34.2	13.46	32.82	12.0	31.4	10.85
8day	33.6	13.48	33.50	12.2	33.6	12.23
	PER2		PER2		PER2	
0day	29.4	10.24	31.2	10.60	28.7	8.56
1day	31.1	10.54	31.9	10.27	28.6	8.29
2day	28.9	9.47	31.2	9.63	28.8	8.51
3day	27.6	8.17	28.2	7.29	28.6	8.70
4day	28.0	8.51	29.6	7.34	29.5	8.81
5day	29.3	9.49	29.7	8.88	29.1	8.92
6day	31.3	10.57	30.0	9.20	30.1	9.52
8day	29.7	9.56	30.0	8.72	32.1	10.70
	PER3		PER3		PER3	
0day	31.4	12.3	33.0	12.4	31.3	11.1
1day	33.4	12.8	33.9	12.3	31.2	10.9
2day	31.5	12.0	33.5	11.9	31.5	11.2
3day	31.1	11.7	32.6	11.6	31.4	11.5
4day	33.1	13.6	34.2	11.9	32.2	11.5
5day	31.7	11.9	32.2	11.5	31.8	11.6
6day	33.7	13.0	32.5	11.7	32.5	12.0
8day	31.8	11.6	32.9	11.6	35.3	14.0
	S100A12		S100A12		S100A12	
0day	19.1		20.6		20.2	
1day	20.6		21.6		20.3	
2day	19.5		21.6		20.3	
3day	19.4		21.0		19.9	
4day	19.5		22.3		20.7	
5day	19.8		20.8		20.2	
6day	20.7		20.8		20.6	
8day	20.2		21.3		21.4	

All raw Cq values were compared to day zero to determine if a significant difference existed during the 8-day extraction period (Figures 13-15). Subsequently, Cq values for *PERIOD 1-3* were normalized to *S100A12* and evaluated for evidence of degradation (Figures 16-18).

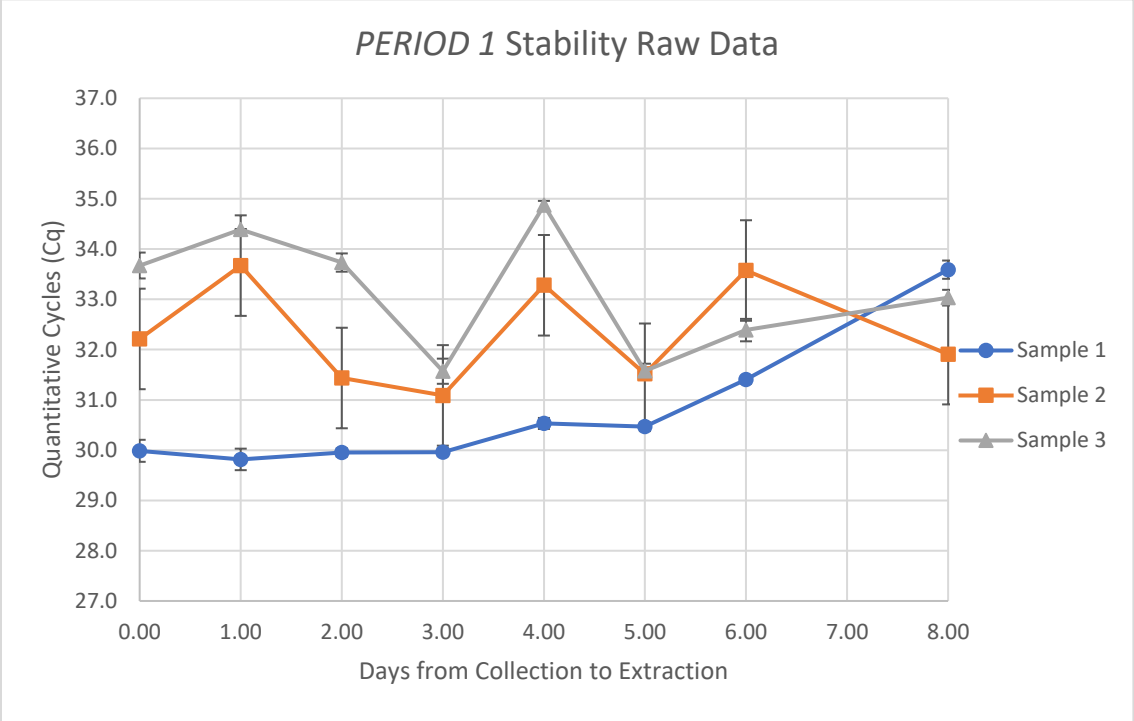


Figure 13. *PERIOD 1* Stability Raw Data. *PERIOD 1* Cq data of three samples tested for degradation. Extractions of mRNA were performed over 8 days and qPCR assays performed. Quantitation of mRNA, expressed as Cq values, was observed and compared to Day 0. The error bars represent the standard deviation of each time point.

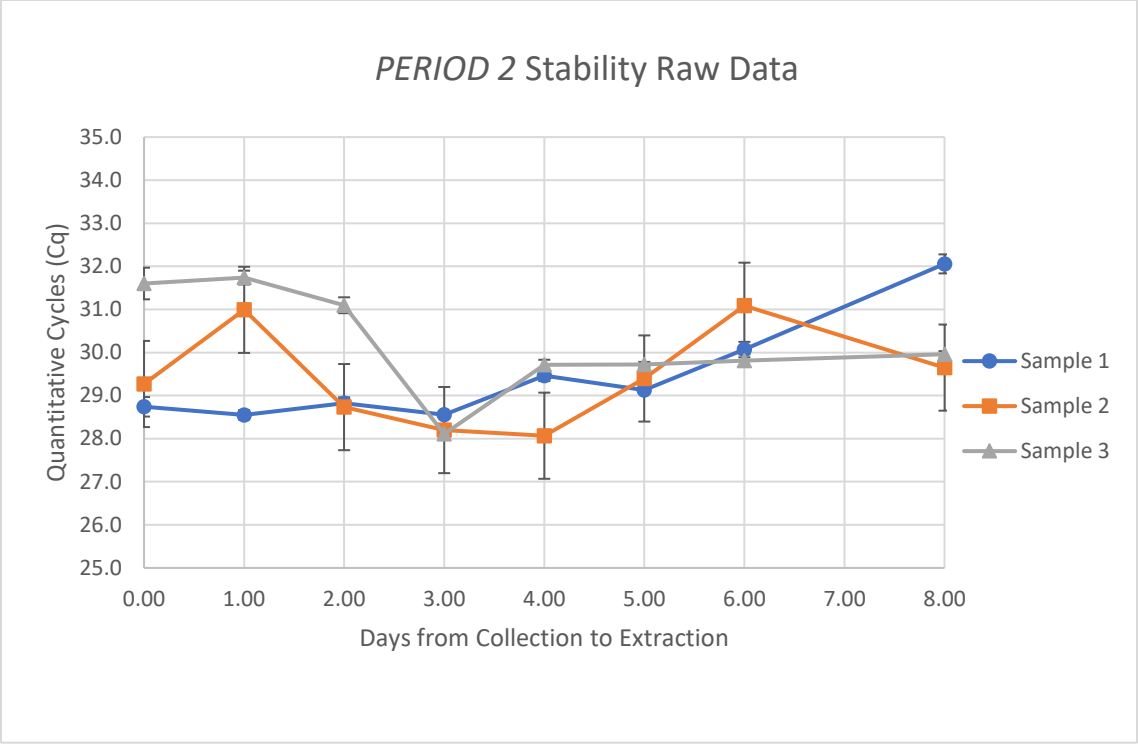


Figure 14. *PERIOD 2* Stability Raw Data. *PERIOD 2* Cq data of the three samples tested. Extractions of mRNA were performed over 8 days and qPCR assays performed. Quantitation of mRNA, expressed as Cq values, was observed and compared to Day 0. The error bars represent the standard deviation for each time point.

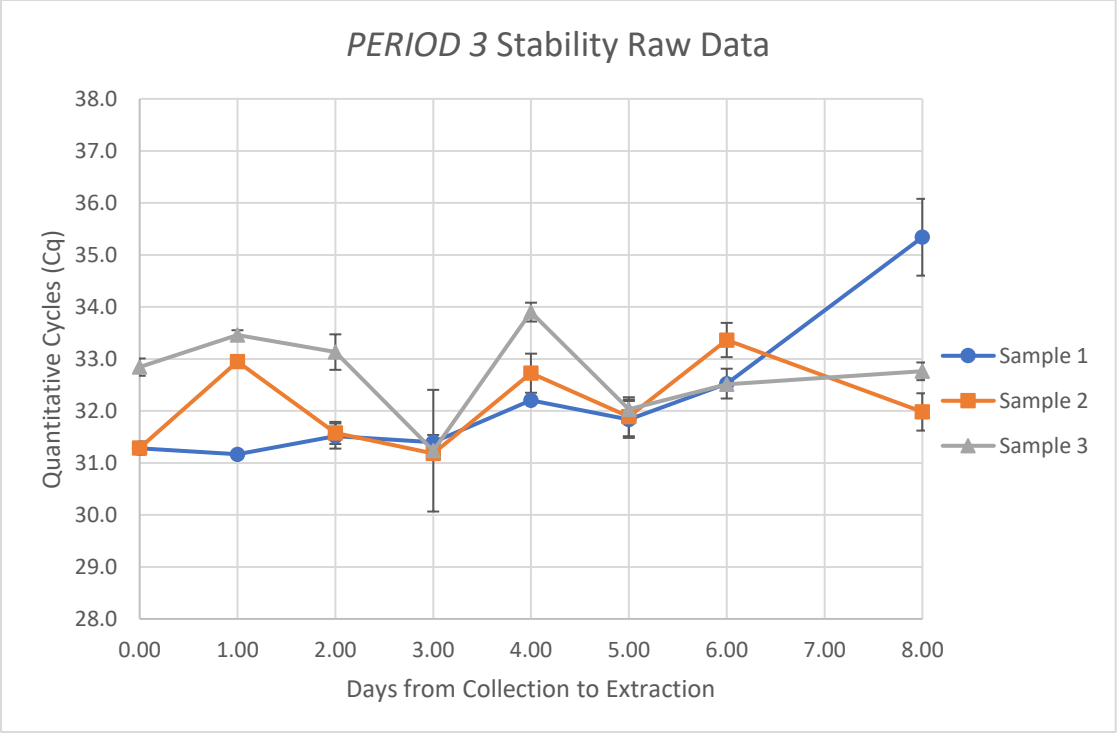


Figure 15. *PERIOD 3* Stability Raw Data. Cq data of the three samples evaluated for degradation. Extractions of mRNA were performed over 8 days and qPCR assays performed. Quantitation of mRNA, expressed as Cq values, was observed and compared to Day 0. The error bars represent the standard deviation from each time point.

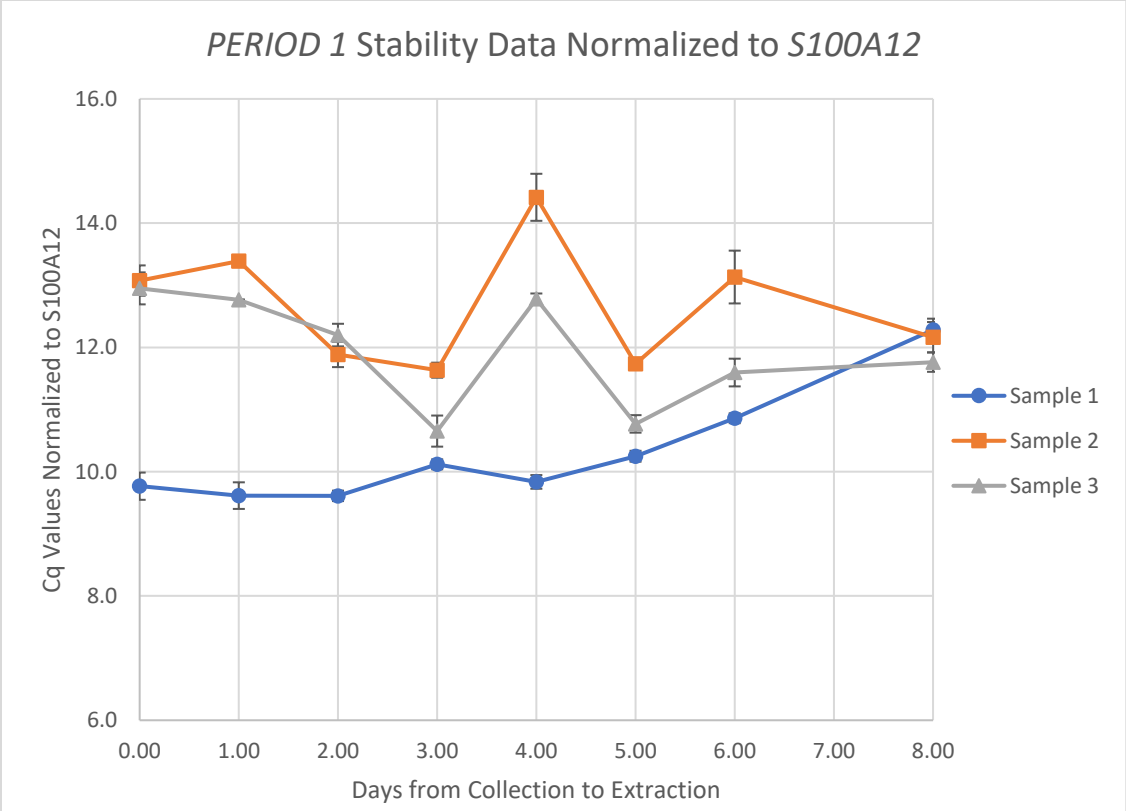


Figure 16. *PERIOD 1* Stability Data Normalized to *S100A12*. *PERIOD 1* Cq values normalized to *S100A12*. Blood samples of 3 donors that were extracted over an 8-day period and normalized. The error bars represent the standard deviation for each time point.

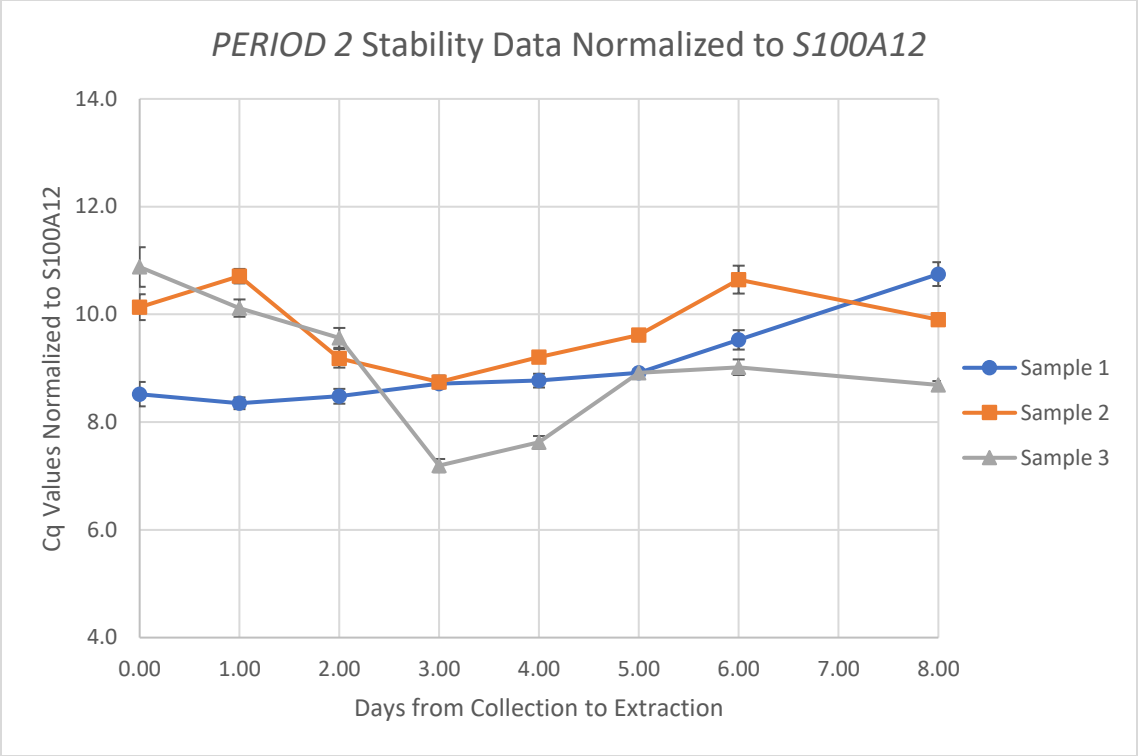


Figure 17. *PERIOD 2* Stability Data Normalized to *S100A12.* *PERIOD 2* Cq values normalized to *S100A12* over an 8-day extraction period. Quantitative PCR Cq levels were normalized to corresponding *S100A12* Cq levels for 8 consecutive days. Each normalized Cq value is plotted for the appropriate extraction day. The error bars represent the standard deviation for each time point.

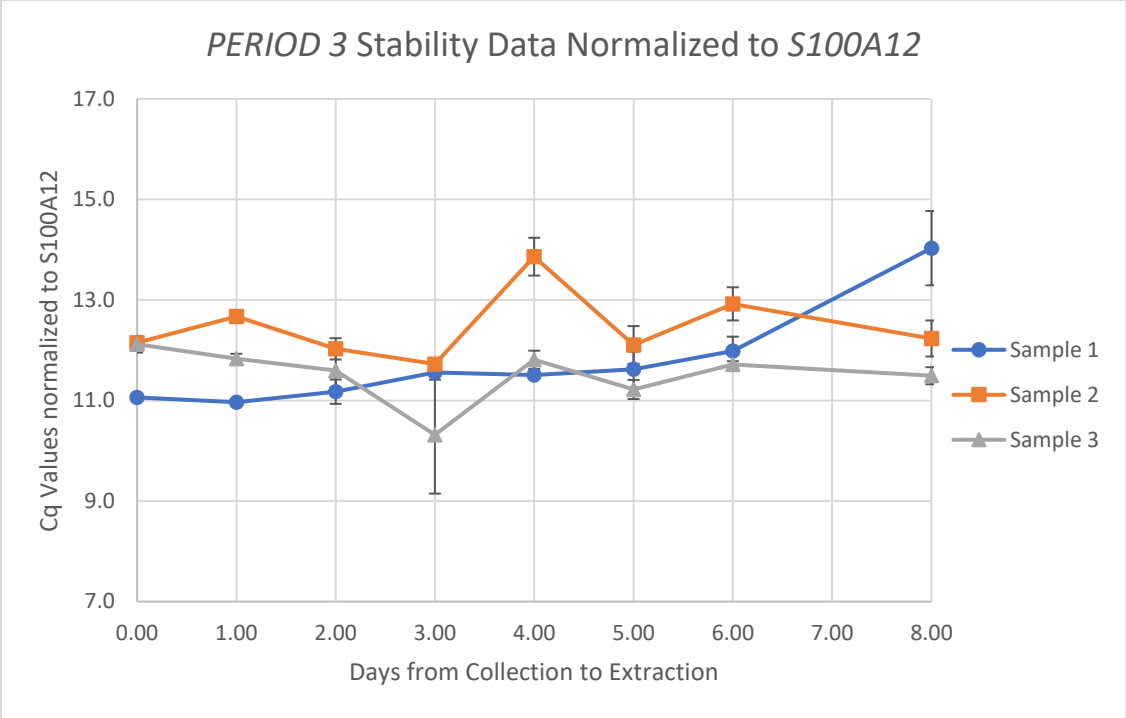


Figure 18. *PERIOD 3* Stability Data Normalized to *S100A12*. *PERIOD 3* Cq values normalized with corresponding *S100A12* results. *PERIOD 3* qPCR results (Cq) for an 8-day period were compared to day 0 for degradation determination. The error bars represent the standard deviation for each time point.

The variability in mRNA abundance among individual sample donors and the combined average abundance for all three donors at each time point was unexpected. Combining male and female results could be one reason for the variability in individual versus combined data. Later experiments showed that differences may exist between sexes. Although sample collection at differing times during the day may result in variable levels, data was normalized and all samples averaged together. Combining data in this way should allow detection of shifts from day 0 as an overall trend. Instrument sensitivity may also be a contributing factor as Cq levels greater than 30 represent very low levels of target. Average Cq levels of *PER1* ranged from 30-34, *PER2* ranges from 27-31 while

PER3 ranged from 31 to 35. Cq levels above 30 introduce the potential for a larger range in variability. It is possible that higher Cq values introduced some random error at different time points that muted the data when averaged.

Quantitative PCR results support the belief that little mRNA degradation occurs (at least for the 100 to 150 bp qPCR amplicons quantified) between when a sample is collected, and RNA is extracted and converted to cDNA. However, in an effort to more completely understand any degradative processes existing in dried blood stains, primers were designed to target flanking regions of the initial *CLOCK*, *PERIOD 3* and *TPOH* targets that would direct the amplification of larger segments of each cDNA. The rationale of this approach was that if the degradation occurred anywhere within a transcript, amplification of a larger portion of the mRNA would be detectable. cDNA produced from the Stability Study sample #2 from day 0 and from day 2 was amplified using mixed primer combinations (see Table 10). PCR products were assessed via agarose gel electrophoresis. Figure 19.

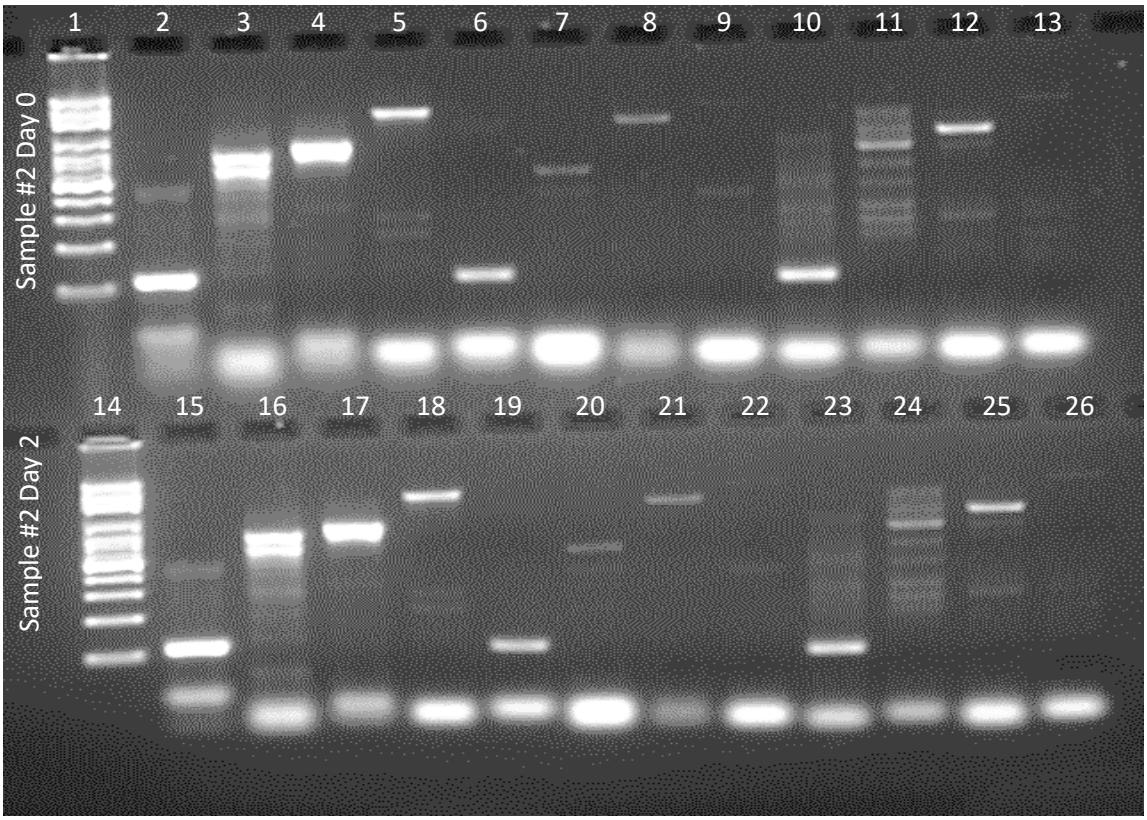


Figure 19. Amplification with Flanking Primers for Intact Determination. The primers used and expected products are as described in Table 10. Reactions using the flanking primers (L) resulting in larger sized fragments reinforces the presence of intact cDNA and refute that the products from qPCR merely represent fragments of degraded mRNA.

Table 11. Agarose Gel Contents for mRNA Intact Determination. The contents of each well and expected product length for each reaction from the agarose gel in Figure 19. Lanes 2 through 13 represent the day 0 extraction from sample 2 in the stability study while lanes 15 through 26 represent the day 2 extraction from sample 2.

Location on gel	Lane contents/ primers used	Expected band size	Location on gel	Lane contents/ primers used
Lane 1	100bp ladder		Lane 14	100 bp ladder
Lane 2	CLK F/ CLK R	119 bp	Lane 15	CLK F/CLK R
Lane 3	LCLK F/ CLK R	730 bp	Lane 16	LCLK F/ CLK R
Lane 4	CLK F/ LCLK R	800 bp	Lane 17	CLK F/ LCLK R
Lane 5	LCLK F/ LCLK R	1411 bp	Lane 18	LCLK F/ LCLK R
Lane 6	Per3 F/ Per3 R	115 bp	Lane 19	Per3 F/ Per3 R
Lane 7	LPer3 F/ Per3 R	504 bp	Lane 20	LPer3 F/ Per3 R
Lane 8	Per3 F/ LPer3 R	1047 bp	Lane 21	Per3 F/ LPer3 R
Lane 9	LPer3 F/ Lper3 R	1436 bp	Lane 22	LPer3 F/ Lper3 R
Lane 10	TPOH F/ TPOH R	101 bp	Lane 23	TPOH F/ TPOH R
Lane 11	LTPOH F/ TPOH R	663 bp	Lane 24	LTPOH F/ TPOH R
Lane 12	TPOH F/ LTPOH R	843 bp	Lane 25	TPOH F/ LTPOH R
Lane 13	LTPOH F/ LTPOH R	1403 bp	Lane 26	LTPOH F/ LTPOH R

Bands of expected lengths were visible in all PCR products amplified with the different primer mixes although nonspecific bands were also sometimes present. A high number of cycles in PCR is necessary for successful amplification of low copy targets but often results in non-specific amplification. To assure non-specific products were absent for quantitative analysis, melting curves were performed following amplification in qPCR. Results from the melting curves showed that no non-specific amplification was produced for the primer sets used in time assessment studies.

Conclusion

The aim of this study was to determine if our experimental plan that included possible delays from collection to RNA extraction and reverse transcription would have an effect on the accuracy of qPCR results reflecting levels of gene expression in circadian clock genes. ANOVA comparison of means of samples extracted over an 8-day period of storage showed no statistically significant difference in the quantity of mRNAs markers studied. Additional evaluations with amplification of larger segments of the *CLOCK*, *PER3* and *TPOH* genes confirmed that the mRNA remains largely intact and therefore extraction of RNA from a stain prepared 2 days earlier will not be significantly degraded. The existence of larger intact amplicons provides evidence that the qPCR data are reliable as indicators of the actual abundance of the transcripts and hence an accurate reflection of the level of gene expression. Although this study validates mRNA stability in dried blood stored up to 8 days prior, all extractions were performed within 48 hours of collection.

CHAPTER VI

PHYSIOLOGIC VARIATION STUDY

Results and Discussion

The levels of many mRNA are expected to vary during the day due to exogenous and endogenous influences. For any study of gene expression involving the abundance of transcribed mRNA, it is important to know the amount of variation in RNA levels that is due to technical causes as well as the variation that may exist when testing the pool of unrelated subjects that provide the biological samples. Knowing the amount of variation that exists in a study allows an investigator to confidently detect changes in gene expression that result from environmental conditions, or changes that are the result of daily oscillations in gene expression that characterize the circadian rhythm. Accurate quantification of mRNA levels associated with rhythmic gene expression allows for the possibility of estimating time throughout a 24-hour day. In order to establish the normal range of expression for each circadian rhythm marker, blood samples were collected, on up to 4 occasions, from 8 volunteers at the same time of day over 3-week periods. Quantification or mRNA levels for the rhythmic markers was determined with qPCR and

Cq results were normalized to *S100A12* and to *CLOCK* mRNA expression levels. Male and female data were analyzed separately in consideration of possible sex-related differences in gene expression. Mean values, standard deviations and coefficients of variation were calculated to determinate the limits of variation in gene expression that an average individual may exhibit. Table 12. A gene that exhibits less variability may act as a more sensitive indicator of naturally changing levels. Thus, one purpose of these experiments was to identify those markers of circadian rhythm that are better able to meet our needs for estimating the time of day a blood stain was created.

Table 12. Physiologic Variations Normalized to *S100A12* and to *CLOCK*. Observed physiologic variation in mRNA levels quantified in blood stains obtained from an individual sample over three weeks' time. Blood samples from eight volunteers were collected at the same time of day, three to four times over a period of up to three weeks for a total of 26 blood stains. Mean values were calculated from normalized (*S100A12* and *CLOCK*) Cq values and coefficients of variation were determined. Results in this table represent the observed amount of variation within the sample population of each marker for men and women.

mRNA	Females(n=4) CV(%) from samples normalized to *S100A12*	Females(n=4) CV(%) from samples normalized to *CLOCK*	Males(n=4) CV(%) from samples normalized to *S100A12*	Males(n=4) CV(%) from samples normalized to *CLOCK*
PER1	14.8	10.2	8.5	15.2
PER 2	18.9	17.1	10.1	27.3
PER 3	14.2	11.0	8.1	7.3
BMAL1	37.2	29.4	18.2	24.7
CLOCK	27.6	0	13.4	0
CRY 1	20.7	22.2	10.7	24.2
CRY 2	18.0	14.1	9.5	21.5
Cye	11.5	12.5	11.1	17.4
MKNK2	40.6	66.0	23.8	29.2
THRA1	15.9	25.5	13.5	44.0
TPOH	11.0	9.4	10.8	15.3

Decreased variation and reliable gene expression levels define an optimal marker for time estimation. Ideal markers of circadian rhythm are those that exhibit a predictable pattern of abundance over a 24-hour period and whose mRNA levels fall within a narrow range of expression at defined points in time. It is possible that mRNA levels that exhibit a higher amount of variation may be influenced by factors outside of circadian rhythm and therefor may not be optimal for our purposes. In some cases, the excessive CV's obtained in this study may be due to analytical sensitivities in the testing protocol or in the instrumentation used. Cq levels of greater than 30 represent sparse target mRNA thus reproducibility may be problematic. Decreased amounts of mRNA may also exhibit a higher sensitivity to degradation and to stochastic effects.

Additional sample analysis could reduce CV values although this study was performed in order to screen markers for later applications. The data from this study represents one time point in a 24-hour day, therefore this approach does not generate peaks and troughs of gene expression during the 24-hour cycle. Based upon this analysis, no markers were dropped from this study. Information generated here contributed to the overall understanding of rhythmic marker expression and detection in blood samples.

Conclusion

Optimal markers for time determination exhibit lower variations of predictable rhythmic expression and therefore have the potential to be reliable markers for time estimation. Expression levels of *BMAL1* and *MKNK*2 in men and women normalized to *CLOCK* and *S100A12* appear too variable for a reliable indicator of time. Additionally, *CRY1* normalized to both *CLOCK* and *S100A12* and *THRA1* normalized to *CLOCK*

exhibit a wide range of expression at one time point in women. Furthermore, *PER2*, *CRY1*, *CRY2*, and *THRA1* normalized to *CLOCK* all exhibit large ranges of expression that consequently may reduce the efficacy in time determination applications. Although some of the markers exhibited a large variation of expression at one time point, mRNA peak and trough levels at different points in the 24-hour period may have a large enough difference to overcome the variability. These observations will be considered as additional information about the selected markers is revealed.

CHAPTER VII

AGE-RELATED VARIATION STUDY

Results and Discussion

In order to reduce variability in mRNA abundance levels between samples, experiments were performed to identify sources of variability that affect the reproducibility of mRNA abundance quantitation and establish the thresholds of significance for the cyclic expression of markers during the circadian rhythm. Accurate estimates of the time of day a stain was created will depend upon comparing RNA levels in a crime scene sample against a reference curve that plots RNA abundance versus the time of day a stain was created. Since age-related changes in rhythmic gene expression levels is well documented (Hood & Amir, 2017; Yu, Hazelton, Luebeck, & Grady, 2019), we sought to evaluate the age related differences in expression of our panel of circadian markers. Volunteer blood donors were divided into 4 age categories (20-29, 30-39, 40-49, and 50-59) to assess whether or not differences exist between age groups in the rhythmic expression of genes of interest. Forty samples were collected at 1:00pm +/- 30 minutes and assessed for age-related variations in the expression of eleven genes. Rhythmic

markers included *PER1-3, CRY1-2, BMAL1, CLOCK, CYE, MKNK2, THRA1* and *TPOH*. *S100A12* or *CLOCK* were used to normalize data. Quantitative PCR was performed in triplicate for each marker and normalized to either *S100A12* or *CLOCK*. Mean values, standard deviations and coefficients of variation were calculated for each age group (Tables 13 and 14). A one-way ANOVA with a post hoc Tukey Test was performed using Prism 8™ GraphPad (GraphPad Software, San Diego, CA). No significant differences between age groups was observed.

Table 13. Comparison of Expression Levels Normalized to *S100A12* Based on Age. Cq values were normalized to *S100A12* and were compared using a one-way ANOVA analysis and post hoc Tukey's Test on Prism™ GraphPad (GraphPad Software, San Diego, CA). Observed variations in each marker and age group is expressed as the coefficient of variation (CV). No significant difference was observed between age groups when expression levels were normalized to *S100A12*. (average p-value = 0.9528)

	20's		30's		40's		50's	
	Mean Cq	CV	Mean Cq	CV	Mean Cq	CV	Mean Cq	CV
PER1	9.30	13.9%	10.42	10.1%	8.91	10.3%	9.55	17.7%
PER2	7.05	14.3%	8.48	12.1%	6.79	9.8%	7.60	18.2%
PER3	9.80	10.1%	10.38	8.0%	9.17	9.1%	9.77	10.0%
BMAL1	3.10	15.2%	3.73	19.6%	2.50	32.2%	3.27	28.4%
CLOCK	5.05	13.4%	5.87	13.1%	4.15	20.4%	5.25	21.9%
CRY1	5.98	14.5%	7.03	12.8%	5.59	14.0%	6.96	22.8%
CRY2	6.82	9.7%	8.05	11.8%	6.84	15.9%	6.96	12.4%
CYE	9.21	13.9%	10.17	11.9%	9.37	11.6%	7.68	6.6%
MKNK2	3.53	44.7%	4.32	26.7%	3.72	18.1%	10.41	16.5%
THRA1	6.96	15.9%	7.79	16.2%	6.83	10.5%	8.33	8.3%
TPOH	9.75	11.7%	10.37	13.0%	9.48	8.9%	10.55	9.8%

Table 14. Comparison of Expression Levels Normalized to *CLOCK* Based on Age. Cq values normalized to *CLOCK* and were compared for differences between age groups with a one-way ANOVA test using Prism™ GraphPad (GraphPad Software, San Diego, CA). Comparison of means between groups was performed using a post hoc Tukey's Test to detect differences between groups. No significant difference was observed between age groups in the mRNA tested when expression levels were normalized to *CLOCK*.

	20's		30's		40's		50's	
	Mean Cq	CV	Mean Cq	CV	Mean Cq	CV	Mean Cq	CV
PER1	4.24	21.0%	4.54	12.6%	4.73	8.6%	4.52	18.3%
PER2	2.00	34.3%	2.54	17.4%	2.68	10.7%	2.66	24.4%
PER3	4.74	10.7%	4.70	8.1%	4.96	6.9%	5.06	5.1%
BMAL1	-1.96	18.4%	-2.16	14.4%	-1.67	19.8%	-1.98	17.5%
CRY1	0.92	28.8%	1.19	19.8%	1.37	13.5%	1.28	23.4%
CRY2	1.86	19.7%	1.85	14.1%	2.03	7.9%	1.28	15.2%
CYE	4.15	14.9%	4.22	13.6%	4.61	11.9%	2.01	15.5%
MKNK2	-1.53	72.9%	-1.70	40.1%	-1.58	60.4%	4.46	80.4%
THRA1	1.96	30.7%	1.71	35.1%	2.53	42.0%	2.23	42.4%
TPOH	4.61	18.1%	4.23	14.6%	4.97	14.5%	4.78	14.4%

Although no significant differences were observed between age groups, differences between sexes were suspected. Therefore, it was important to re-evaluate data to determine if different expression levels exist between males at different ages as well as in females of different age. This question was analyzed using the data collected for the age-related study described above (Table 13 and 14) thus data were further separated by sex. Once separated by sex, age related differences were again determined using a one-way ANOVA with a post hoc Tukey's Test on Prism™ GraphPad (GraphPad Software, San Diego, CA). Significant differences between age groups in females as well as in males were not observed. (Tables 15 and 16). Subsequently, all female data was combined, regardless of age, and compared to combined male data for each marker tested

to examine if significant sex-related differences in mRNA levels exist between males and females (Tables 15 and 16).

Table 15. **Age Comparisons Between Males and Females Normalized to *S100A12*.** Mean values and coefficients of variation of each age group that were separated by sex are listed. The total column indicates the overall mean of combined ages in female and the overall mean of combined ages in males for each marker. The coefficients of variation (CV) for males and females are included.

	20's		30's		40's		50's		Total	
PER1	**Mean**	**CV**	**Mean**	**CV**	**Mean**	**CV**	**Mean**	**CV**	**Mean**	**CV**
Female	9.74	15%	11.03	8%	9.42	17%	9.53	16%	9.98	14%
Male	8.9	13%	9.81	12%	8.39	2%	9.56	19%	9.29	14%
PER2										
Female	7.36	16%	9.03	10%	7.02	21%	7.07	18%	7.69	18%
Male	6.73	0.13	7.92	14%	6.55	1%	7.51	18%	7.31	15%
PER3										
Female	9.93	13%	11.12	9%	9.44	17%	9.19	11%	9.98	14%
Male	9.67	8%	9.63	7%	7.51	15%	10.34	9%	9.73	9%
BMAL1										
Female	3.32	11%	3.92	21%	1.91	65%	2.82	23%	3.04	36%
Male	2.88	19%	3.53	18%	3.09	12%	3.50	22%	3.31	19%
CLOCK										
Female	4.74	15%	5.84	16%	3.82	38%	4.83	20%	4.86	25%
Male	5.36	12%	5.90	11%	4.48	5%	6.31	22%	5.66	19%
CRY1										
Female	5.77	16%	7.09	14%	5.40	25%	6.20	13%	6.16	19%
Male	6.18	13%	6.97	11%	5.78	4%	7.76	26%	6.85	20%
CRY2										
Female	7.03	13%	8.45	11%	7.18	27%	7.65	12%	7.62	17%
Male	6.60	6%	7.64	13%	6.49	4%	7.74	8%	7.26	12%
CYE										
Female	8.72	7%	10.49	15%	9.92	21%	10.80	9%	9.86	16%
Male	9.69	20%	9.84	8%	8.82	1%	10.02	4%	9.73	10%
MKNK2										
Female	3.67	46%	4.69	30%	4.36	30%	5.15	9%	4.38	31%
Male	3.38	44%	3.94	23%	3.08	2%	4.07	26%	3.76	27%
THRA1										
Female	6.80	12%	8.13	18%	7.73	17%	8.77	2%	7.68	16%
Male	7.12	20%	7.44	15%	5.93	2%	7.89	16%	7.32	17%
TPOH										
Female	9.04	7%	10.88	19%	10.21	15%	11.28	9%	10.35	15%
Male	10.4	16%	9.9	7%	8.75	2%	9.81	10%	9.81	10%

Table 16. Age Comparisons Between Males and Females Normalized to *CLOCK*.

Mean values of qPCR Cq results were normalized to *CLOCK* for each age group in males and females. The total column indicates the average normalized Cq values in all age categories in females and the combined normalized Cq values in all ages of males for each marker tested. The coefficients of variation for males and females are included.

	20's		30's		40's		50's		Total	
PER1	**Mean**	**CV**	**Mean**	**CV**	**Mean**	**CV**	**Mean**	**CV**	**Mean**	**CV**
Female	4.99	16%	5.17	7%	5.51	7%	5.12	12%	5.21	11%
Male	3.5	28%	3.91	19%	3.91	11%	3.91	27%	3.82	19%
PER2										
Female	2.62	23%	3.05	10%	3.28	11%	2.97	10%	2.99	15%
Male	1.37	0.55	2.02	29%	2.07	10%	2.35	43%	1.99	36%
PER3										
Female	5.18	16%	5.30	6%	5.49	9%	5.14	2%	5.28	9%
Male	4.30	4%	4.09	10%	2.35	36%	4.98	8%	4.44	10%
BMAL1										
Female	-1.41	-37%	-1.94	-20%	-1.94	-26%	-2.01	-22%	-1.83	-27%
Male	-2.49	-8%	-2.37	-10%	-1.39	-11%	-1.94	-13%	-2.08	-22%
CRY1										
Female	1.02	27%	1.30	17%	1.44	21%	1.37	18%	1.29	23%
Male	0.82	31%	1.07	23%	1.30	5%	1.19	30%	1.10	26%
CRY2										
Female	2.29	17%	2.63	11%	2.75	9%	2.82	9%	2.62	13%
Male	1.42	24%	1.74	23%	2.01	1%	2.11	41%	1.83	30%
CYE										
Female	3.98	8%	4.49	19%	4.83	20%	4.53	21%	4.49	18%
Male	4.32	36%	3.94	8%	4.38	4%	4.39	10%	4.23	16%
MKNK2										
Female	-1.08	-105%	-1.44	-63%	-0.70	-182%	-1.00	-66%	-0.98	-107%
Male	-1.98	-55%	-1.95	-23%	-2.46	-26%	-1.40	-91%	-1.88	-47%
THRA1										
Female	2.08	10%	2.01	34%	2.69	42%	2.73	21%	2.51	17%
Male	1.76	51%	1.54	22%	1.58	10%	1.72	49%	1.64	17%
TPOH										
Female	4.32	14%	4.71	16%	5.19	13%	5.12	17%	4.83	15%
Male	5.1	21%	4.0	9%	4.40	3%	4.44	6%	4.40	15%

One-way ANOVA assays were again performed to compare male versus female qPCR (Cq) values for all markers that were normalized to *S100A12* or *CLOCK*. No significant differences were observed in *S100A12* normalized data between males and females, but significant differences were revealed between males and females in some of the markers observed when normalized to *CLOCK*. Figure 20 and 21.

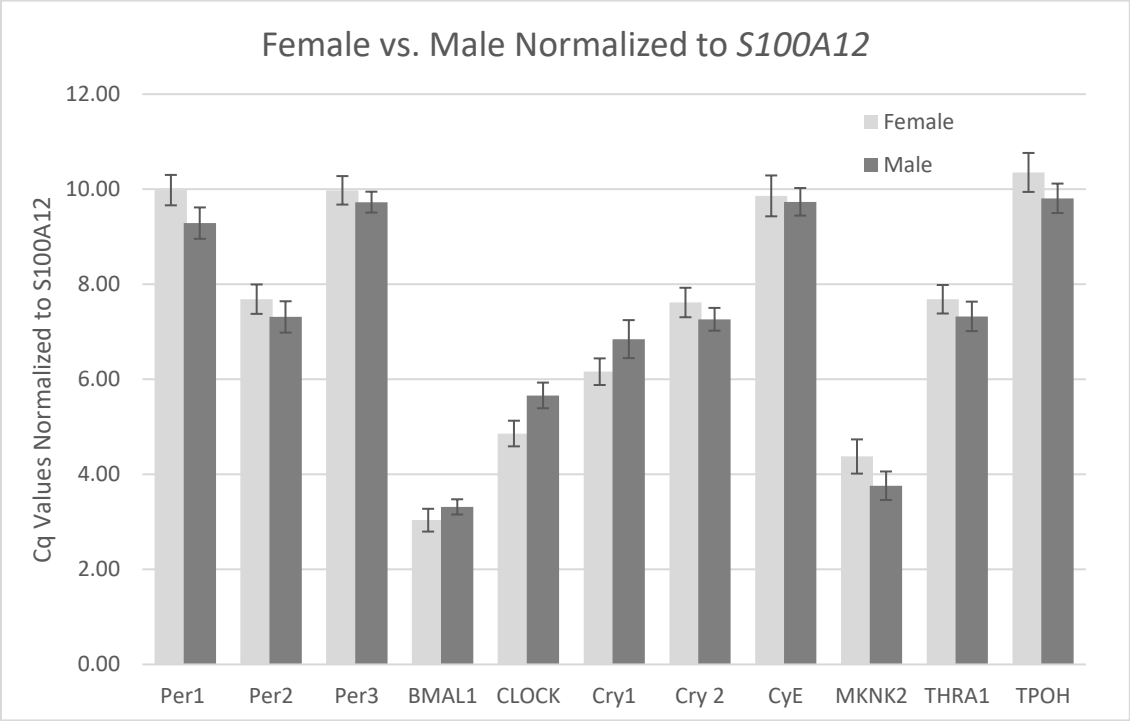


Figure 20. Female and Male Data Normalized to *S100A12*. qPCR Cq values for males and females were normalized to *S100A12*. Each column represents the combined mean of all male or female data regardless of age. Error bars represent the standard error for each marker. Significant differences were not observed in the markers tested when qPCR Cq values were normalized to *S100A12*.

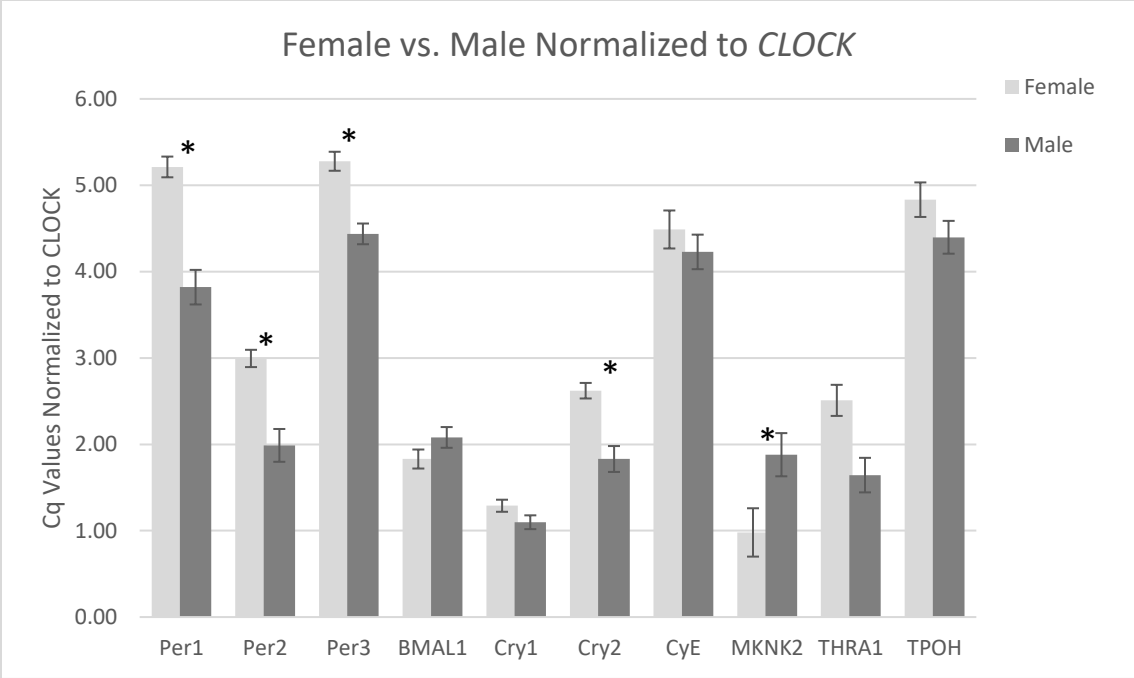


Figure 21. Female and Male Data Normalized to *CLOCK*. Columns represents the average of all *CLOCK* normalized Cq values in females and in males for each gene tested. Error bars denote the standard error for each marker. Significant differences were observed between males and females when Cq values were normalized to *CLOCK*. Statistically significant differences are denoted with an asterisk (*) and p values listed in Table 17.

Table 17. Male and Female Differences in *CLOCK* Gene Expression Levels. One-way ANOVA analysis was calculated using Prism™ GraphPad (GraphPad Software, San Diego, CA) to compare between means and a post hoc Tukey's Test was used to compare between groups. No statistical difference was observed between male and female data when Cq values were normalized to *S100A12*. ANOVA analysis of *CLOCK* normalized mean exhibited several significant differences (p-values <0.5 and denoted by "*").

mRNA evaluated for Female vs Male expression	p-value normalized to *S100A12*	p-value normalized to *CLOCK*
PER1	0.9910	<0.0001 *
PER2	>0.9999	0.006 *
PER3	>0.9999	0.160
BMAL1	>0.9999	.9998
CLOCK	0.9519	NA
CRY1	0.9922	>0.9999
CRY2	>0.9999	0.0363*
CYE	>0.9999	0.9998
MKNK2	0.9992	0.0112 *
THRA1	>0.9999	0.0539
TPOH	>0.9999	0.8912

Conclusion

There was no significant difference in the circadian rhythm markers between age groups (20's to 50's) when Cq values were normalized to *S100A12* or to *CLOCK*. Additionally, there was no significant differences in data normalized to *CLOCK* or *S100A12* observed between age groups in women or in men when compared separately. Thus, no significant difference was observed in data normalized to *S100A12* or *CLOCK* in the genes tested based on age.

There was, however, a significant difference between men and women in expression levels of several of the circadian rhythm genes tested. Therefore, male and female data must be interpreted independently for accurate estimates of time.

CHAPTER VIII

24 HOUR EXPRESSION PATTERN STUDY

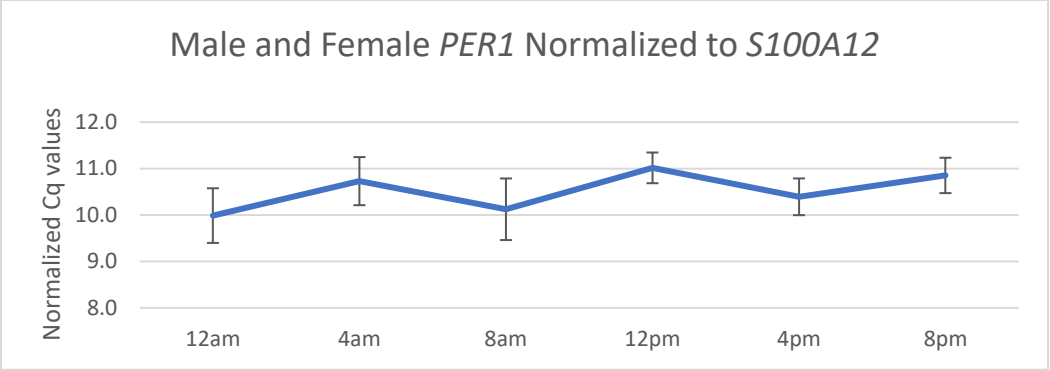
Results and Discussion

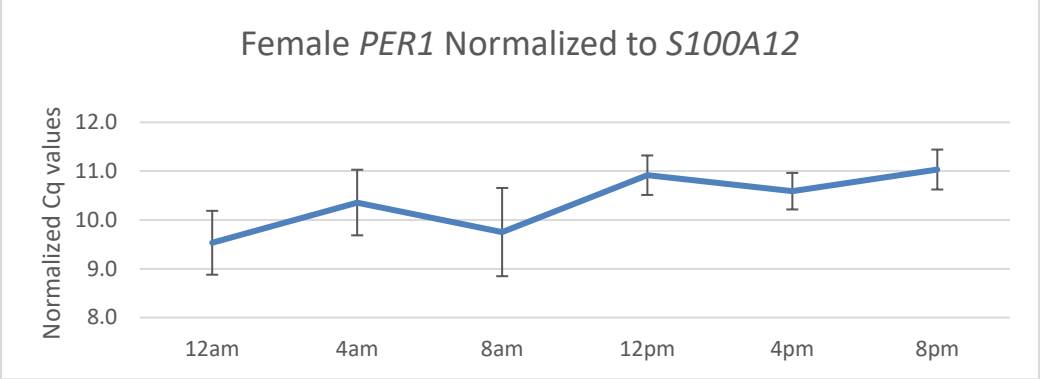
Eleven genes associated with circadian rhythm have been characterized for stability, and for differences in expression among age groups and sexes during a 24-hour period. Messenger RNA levels for each gene were quantified using qPCR and expression levels were normalized against the abundance of mRNA transcribed from either the *S100A12* or *CLOCK* genes whose expression appeared stable throughout the 24-hour time period. To establish the expression of a gene varied in a rhythmic way, it was necessary to quantify the variation that might exist in our RNA quantity estimates originating from gene expression differences among members of our blood donor population (i.e. individual variations in gene expression or variation in gene expression in males versus females). The understanding gained from our analyses of RNA quantification data allowed for the creation of a reference pattern of gene expression range for each marker analyzed. This reference range allowed for the confident

identification of genes rhythmically expressed over the day and also can allow for estimates of when during the day a forensic blood stain was created.

Blood samples were collected every 4 hours for a 24-hour period. Defined collection times included 4:00 am, 8:00 am, 12:00 pm, 4:00 pm, 8:00 pm, and midnight. Blood samples were collected within 30 minutes of these target collection times. Dried blood stains were submitted to the laboratory within 24 hours of the collection and stain preparation. All blood samples (6) from each collection time period were extracted simultaneously. Reverse transcription and DNAse treatment immediately followed extraction in all cases. The cDNA samples were immediately frozen at -20°C until being analyzed using qPCR.

Quantitative PCR for each collection time was performed for each marker in triplicate. Triplicate results were averaged and normalized to expression of the *S100A12* and/or *CLOCK* for prior to downstream analysis of the quantification data. There are 6 figures representing the relative expression data for each gene analyzed. The first 3 graphs of each marker represent data normalized to *S100A12* in which expression data in males and females is combined and also data separated by sex. The second set of three graphs for each marker represent the same analysis but the data have been normalized to *CLOCK* gene expression. A One-way ANOVA and a post hoc Tukey's Test were performed to compare the means of each time point and determine if a significant difference exists at any observed time point in the day.





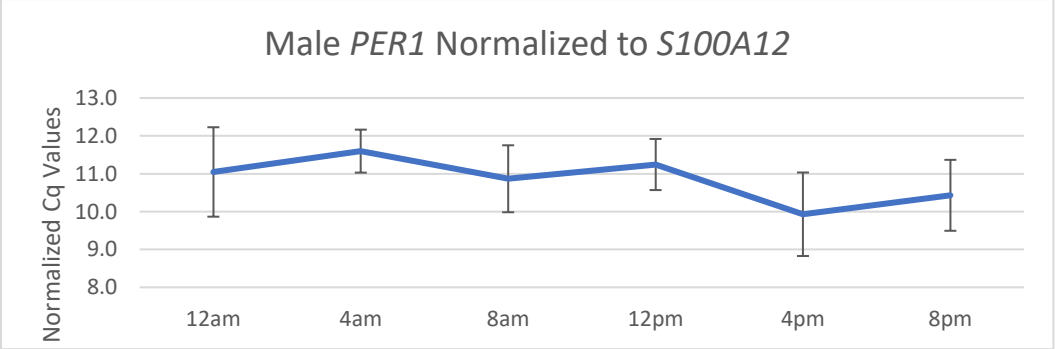
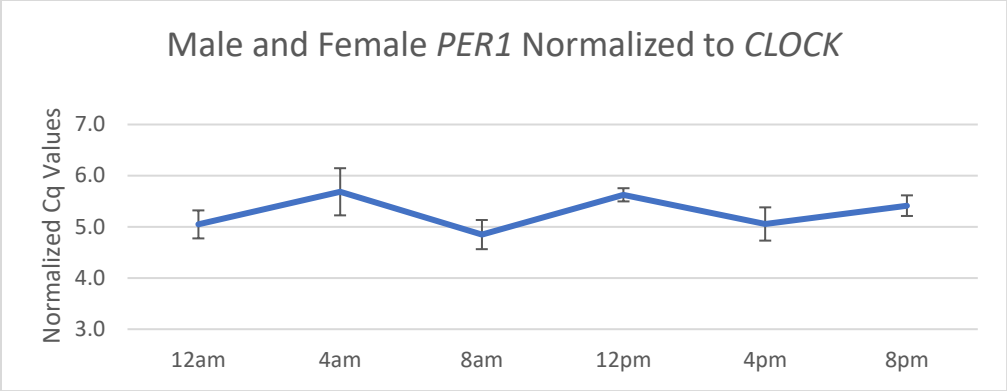
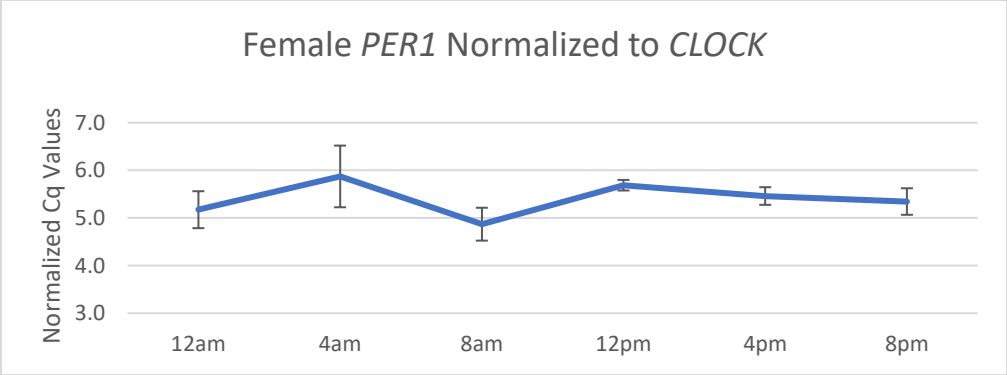


Figure 22. *PERIOD 1* 24-Hour Expression Normalized to *S100A12*. Twenty-four expression levels of *PERIOD 1* normalized to *S100A12* in all samples and separated into male and female samples. Standard error bars indicate the large variation in expression levels at each time point. There is no discernible rhythmicity observed when normalizing *PER1* to *S100A12* in either male, female or combined populations. There are no significant differences between any time point. *PERIOD 1* mean Cq values ranged from 28.8 to 33.8 and *S100A12* results ranged from 21.5 to 25.9.





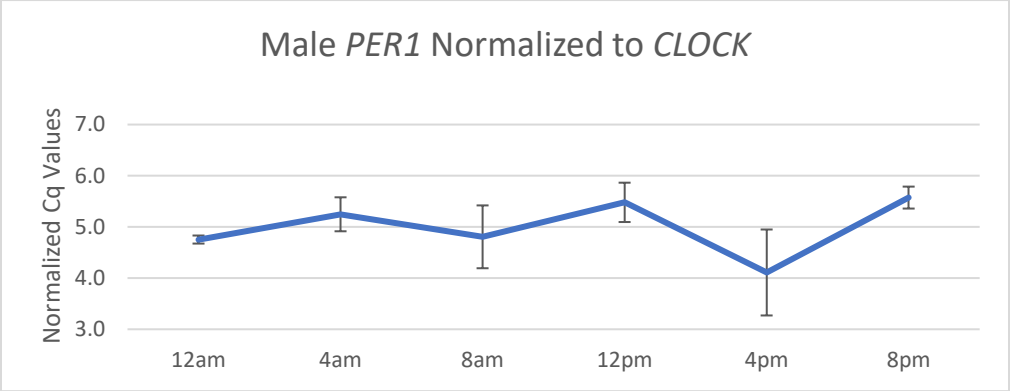
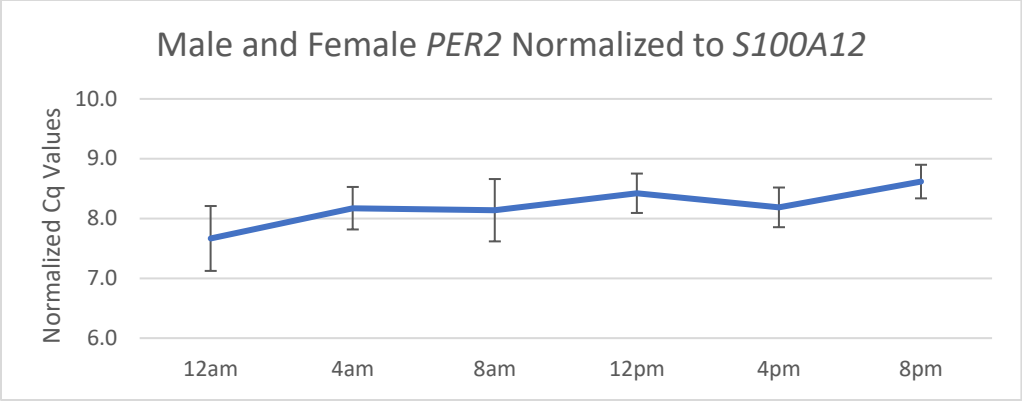
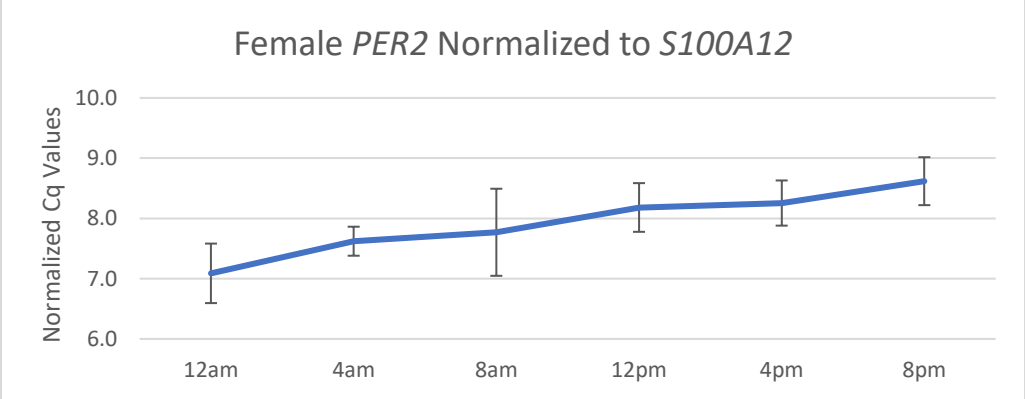


Figure 23. *PERIOD 1* 24-Hour Expression Normalized to *CLOCK*. *PER1* data normalized to *CLOCK* combined and separated by sex. Standard error bars are reduced compared to Period 1 results normalized to *S100A12*. Rhythmicity is not observed in any of the data although error bars are reduced compared to *S100A12* data. There are no statistically significant differences between any time point observed. *PER1* mean Cq values ranged from 28.8 to 33.8 and *CLOCK* results ranged from 25.0 to 31.2.





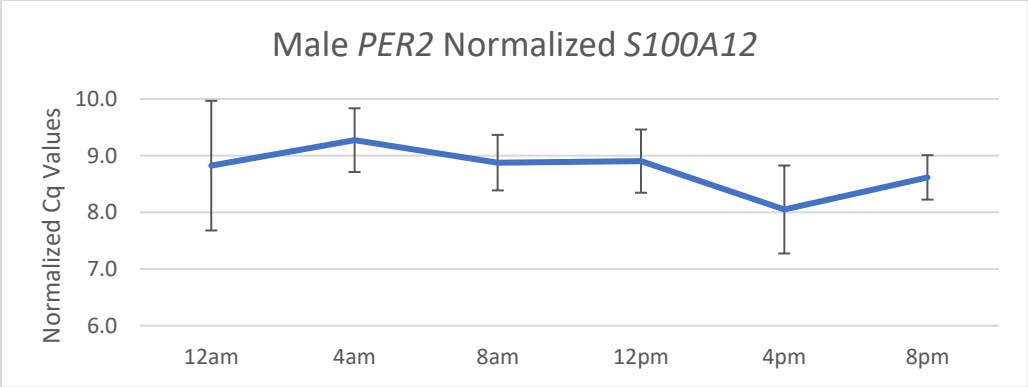
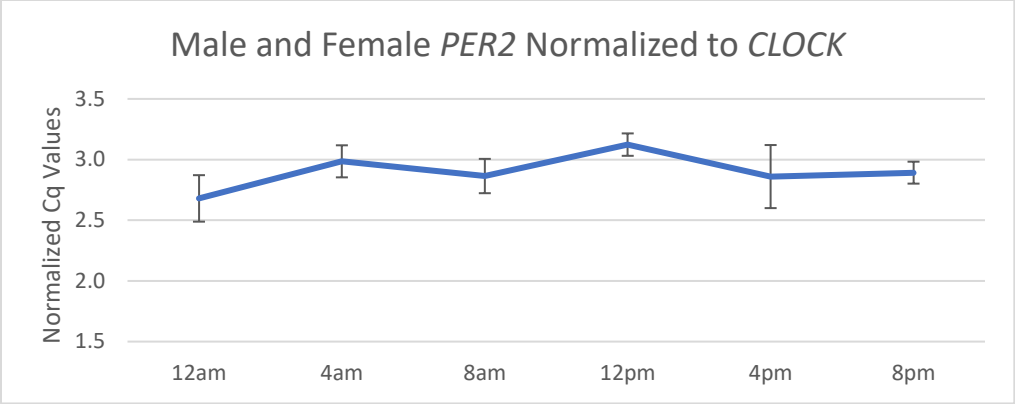
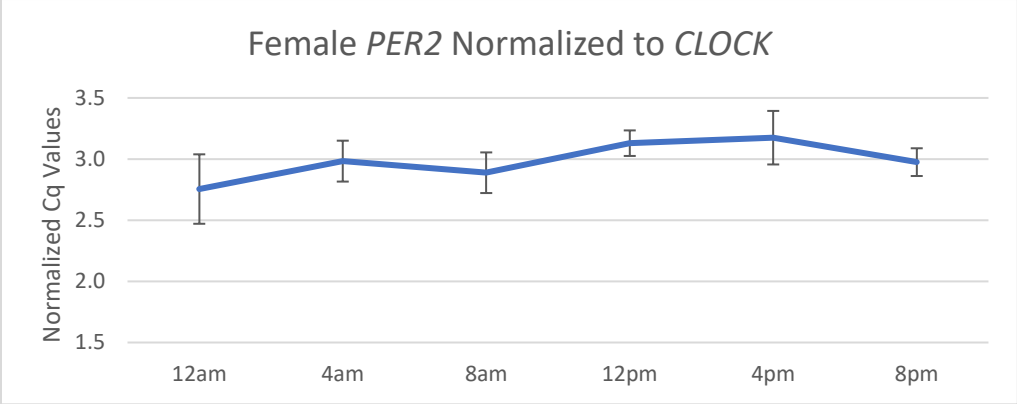


Figure 24. *PERIOD 2* 24-Hour Expression Normalized to *S100A12*. *PER2* Cq values normalized to *S100A12* in all samples combined and separated by sex. Rhythmicity appears neutralized by the sizeable standard errors at each time point in males and females. Additionally, the trend is reduced when the data is combined. There is no statistically significant difference between any time points in the time points observed in *PER2* normalized to *CLOCK*. *PERIOD 2* mean Cq values ranged from 27.2 to 31.5 and *S100A12* results ranged from 21.5 to 25.9.





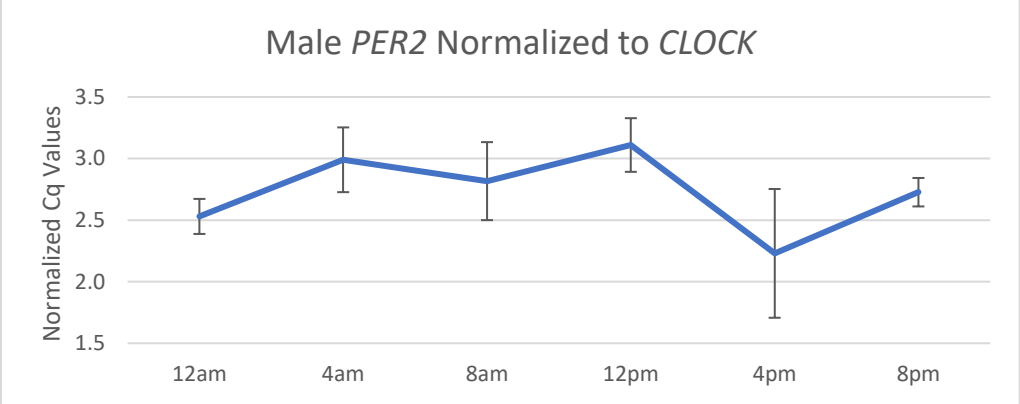
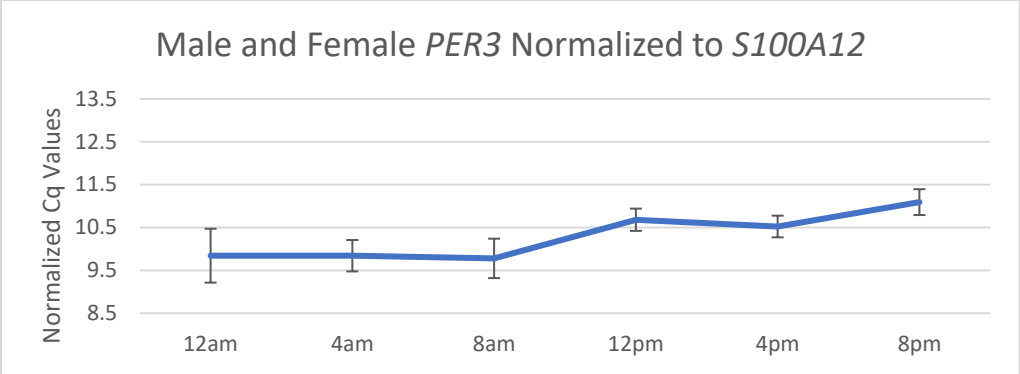
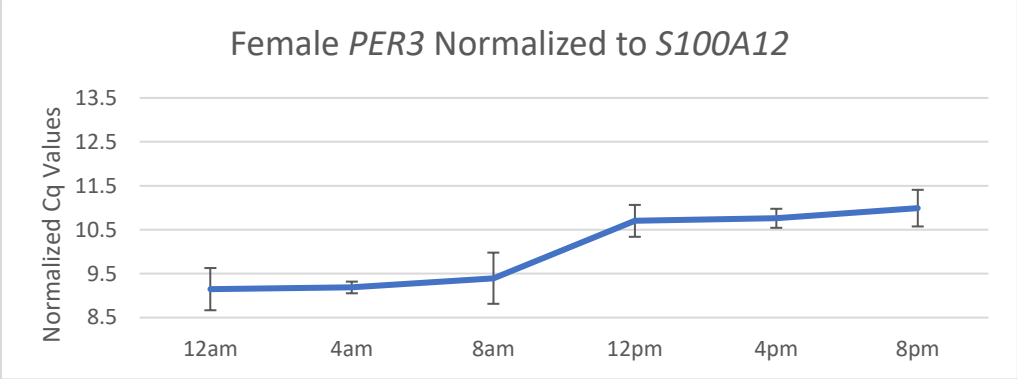


Figure 25. *PERIOD 2* 24-Hour Expression Normalized to *CLOCK*. *PER2* Normalized Cq data was observed combined and separated into male or female samples. Standard error bars are reduced from those observed when normalized to *S100A12* although there is still no evident rhythmicity observed in *PER2* normalized to *CLOCK*. There is no statistical significance between any time point in the 24-hour samples observed of *PER2* normalized to *CLOCK*. *PER2* mean Cq values ranged from 27.2 to 31.5 and *CLOCK* results ranged from 25.0 to 31.2.





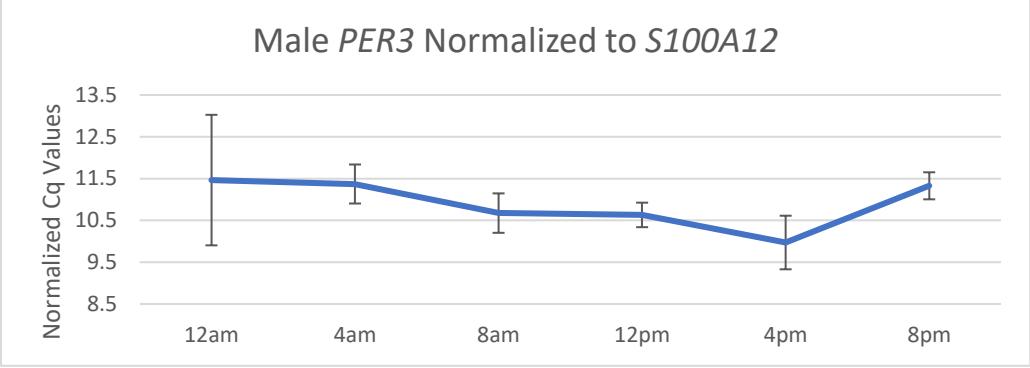
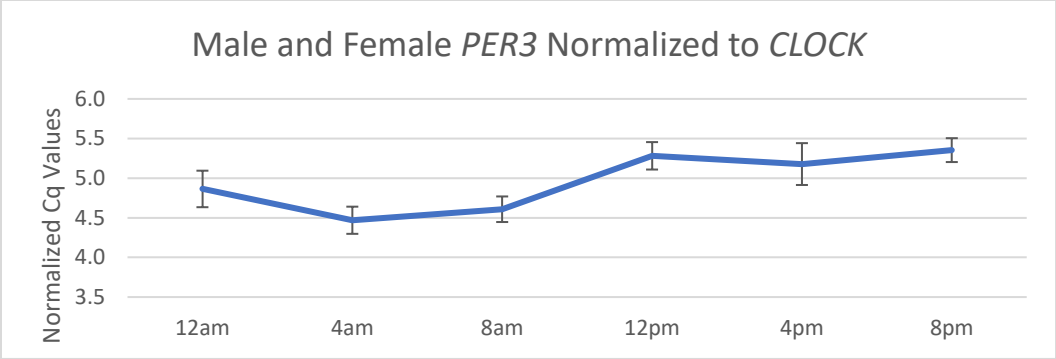
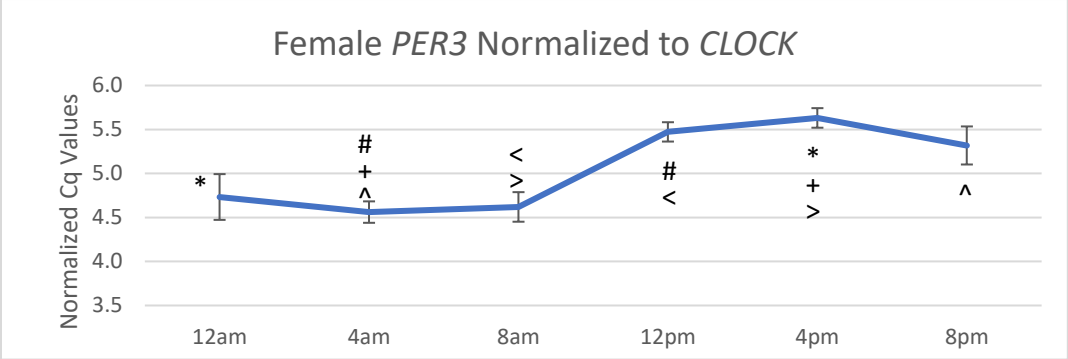


Figure 26. *PERIOD 3* 24-Hour Expression Normalized to *S100A12*. Combined male and female data exhibit a subtle trend in expression from 8:00am to 12:00pm. The change in expression is more pronounced when observed in female samples alone. Smaller standard error bars represent reduced variability in expression levels. There are no statistically significant differences between time points when male and female data are combined or in male data alone. There are statistically significant differences in female *PER3* data normalized to *S100A12* between 12:00 am and 8:00 pm (p=0.0250) and 4:00 am and 8:00 pm (p=0.0297). *PERIOD 3* mean Cq values ranged from 28.7 to 34.8 and *S100A12* results ranged from 21.5 to 25.9.





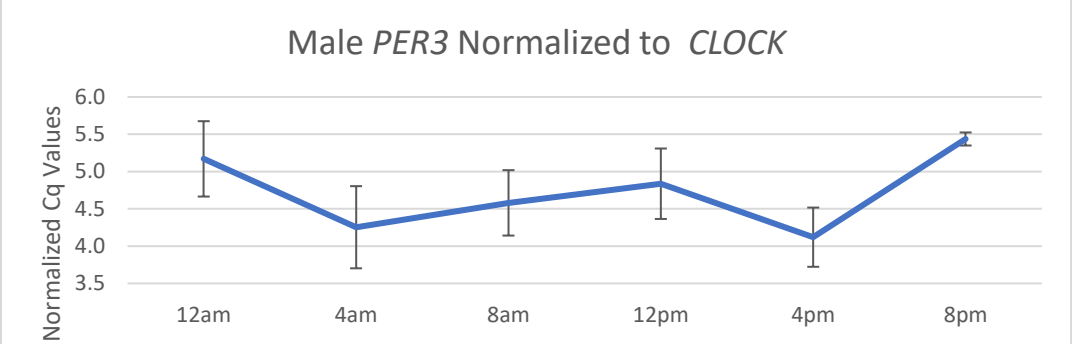


Figure 27. *PERIOD 3* 24-Hour Expression Normalized to *CLOCK*. *PERIOD 3* normalized to *CLOCK* in all samples combined, and in separated male or female samples. There is no observed pattern in male samples normalized to *CLOCK* and standard error bars indicate a large variation in expression at most time points. Combined results exhibit a rhythmic pattern although when observed separately, the female sample exhibit rhythmicity with reduced variance throughout the collection period. There is a statistically significant difference in combined data between 4:00 am and 8:00 pm (p=0.0271) although most likely attributed to female data. Rhythmicity is observed in female *PER3* Cq values normalized to *CLOCK* with a statistical significant differences between 12:00 am and 4:00 pm(*) (p=0.0099), 4:00 am and 12:00 pm# (p=0.0086), 4:00

am and 4:00 pm (+) (p=0.0014), 4:00 am to 8:00 pm (^) (p=0.0427), 8:00 am to 12:00 pm (<) (p=0.0162), and 8:00 am to 4:00 pm (>) (p=0.0028) *PER3* mean Cq values ranged from 28.7 to 34.8 and *CLOCK* results ranged from 25.0 to 31.2.

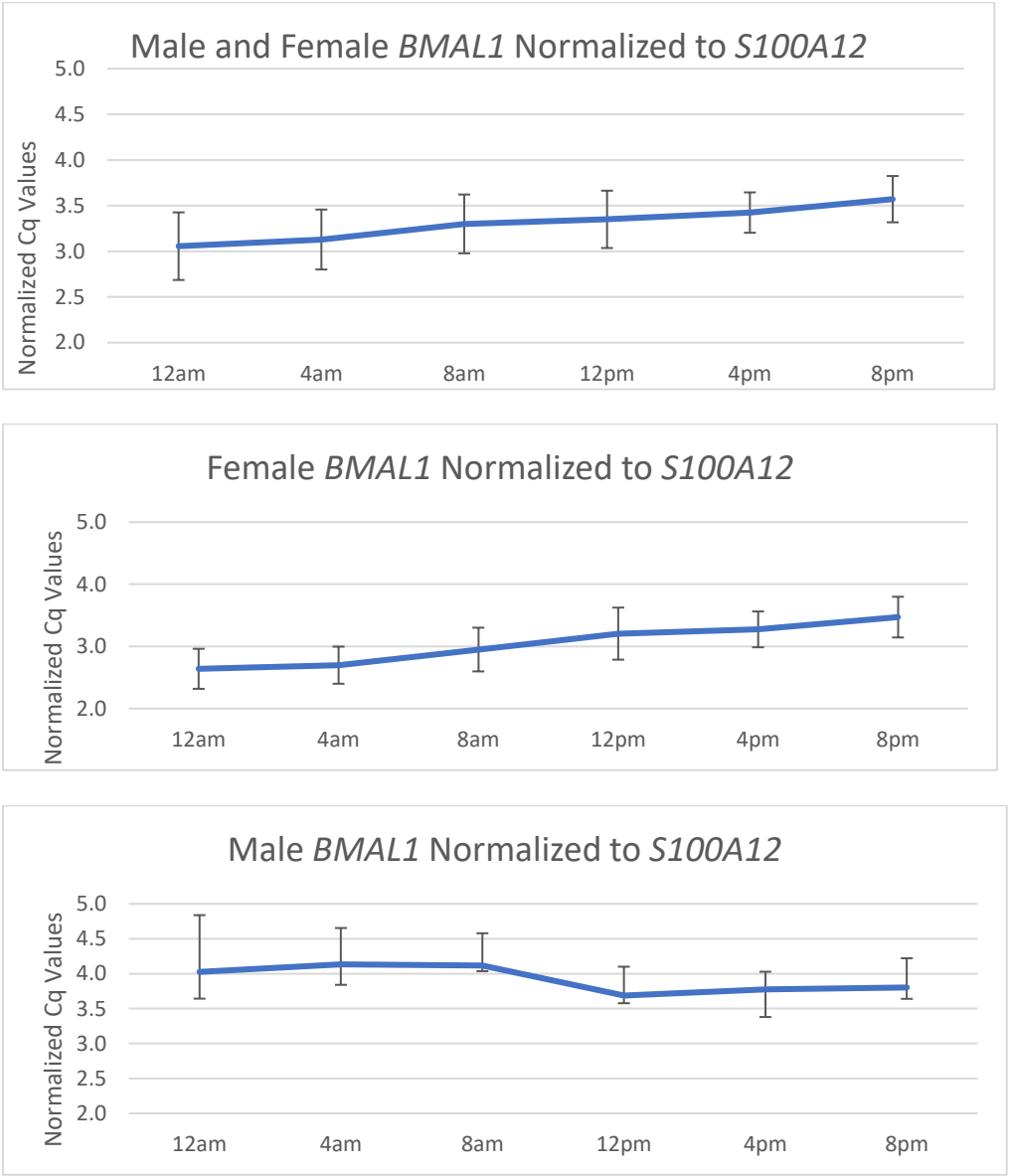
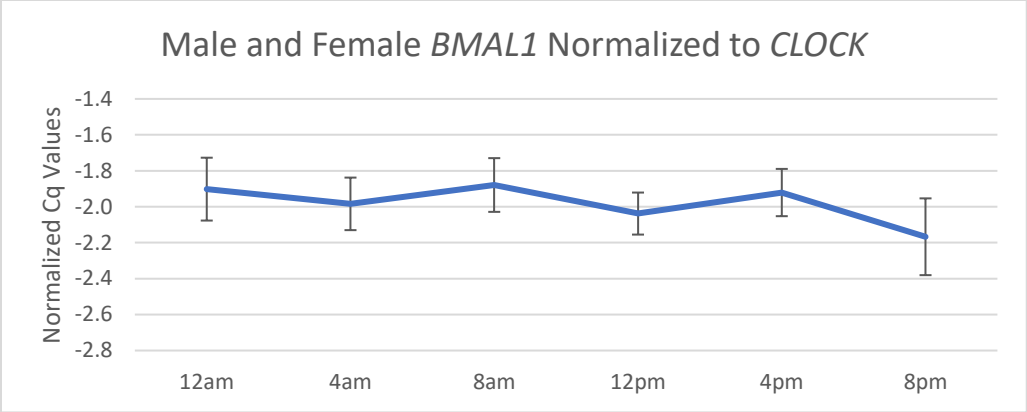
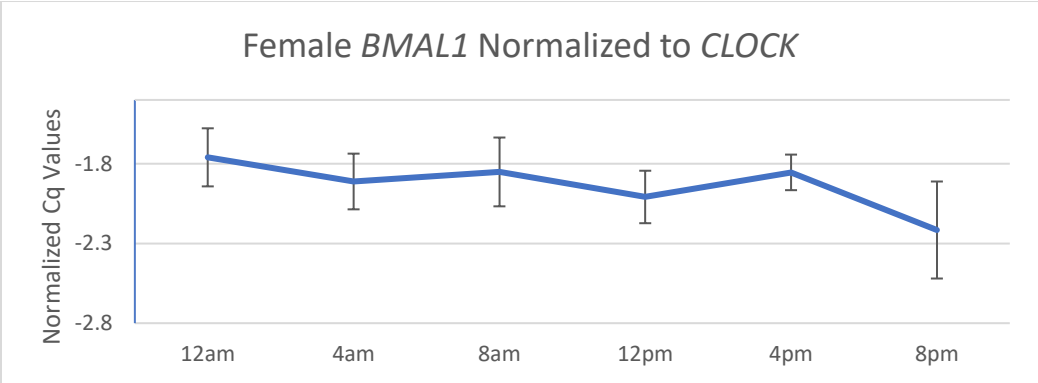


Figure 28. *BMAL1* 24-Hour Expression Normalized to *S100A12*. *BMAL1* Cq values normalized to *S100A12* in all samples combined and in male and female separated samples. Enlarged standard error bars indicate a large variation in expression in all sample groups. There is no statistically significant difference between time points when *BMAL1* is normalized to *S100A12*. *BMAL1* mean Cq values ranged from 23.8 to 29.8 and *S100A12* results ranged from 21.5 to 25.9.





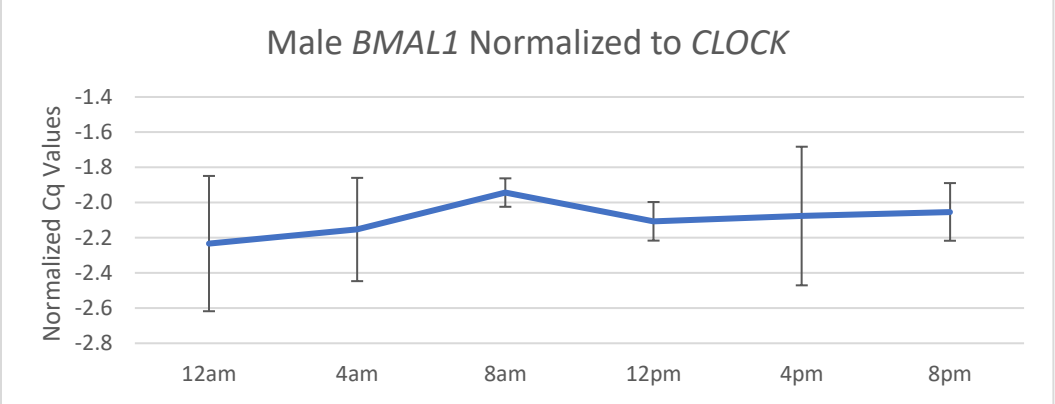
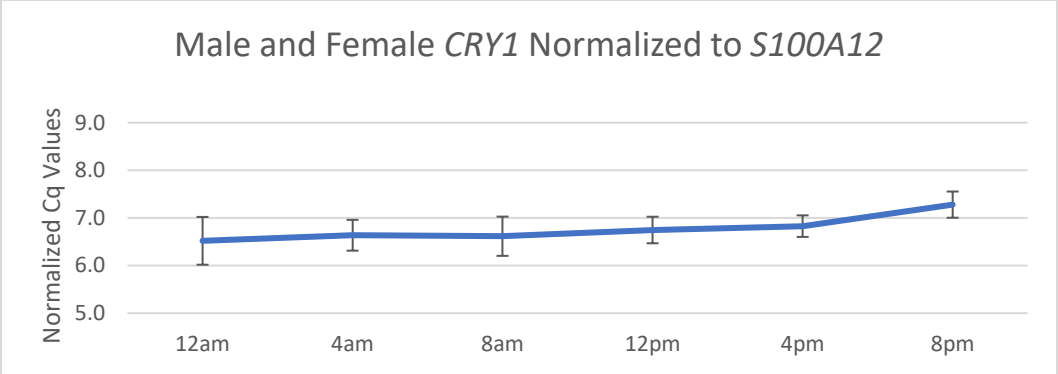
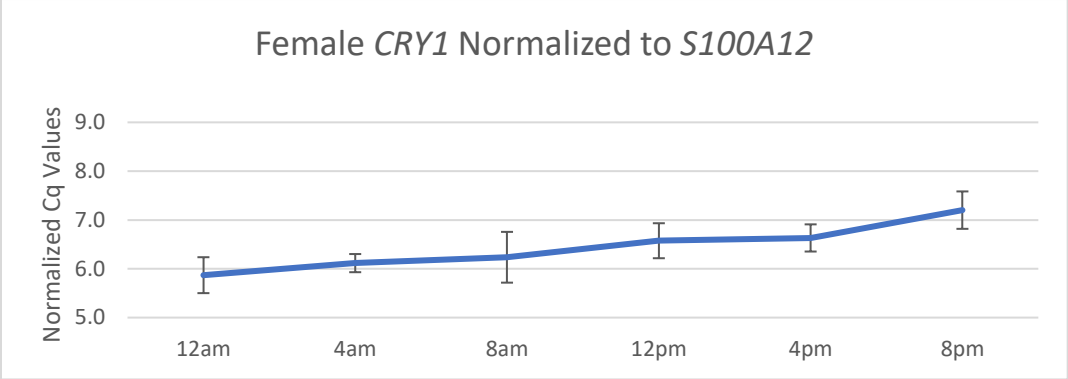


Figure 29. *BMAL1* 24-Hour Expression Normalized to *CLOCK*. *BMAL1* Cq values were normalized to *CLOCK* and all data were observed together and separated by sex. Standard error bars indicate a high level of variance while no apparent rhythmic pattern is observed. Additionally, there are no statistically significant differences between time points. *BMAL1* mean Cq values ranged from 23.8 to 29.8 and *CLOCK* results ranged from 25.0 to 31.2.





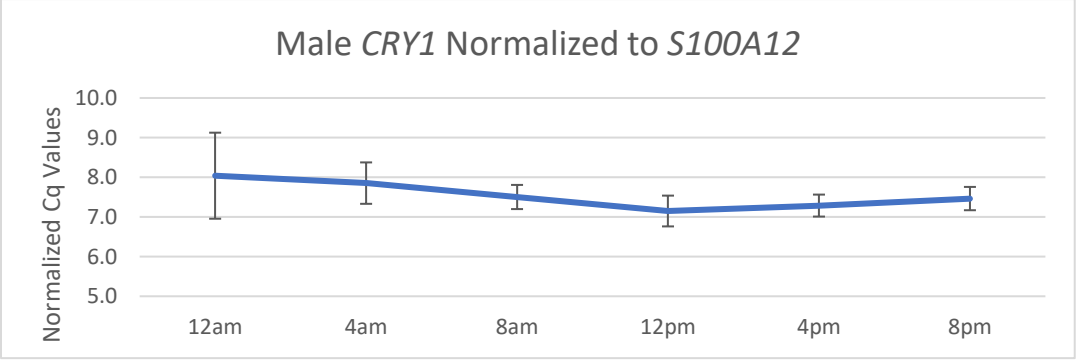
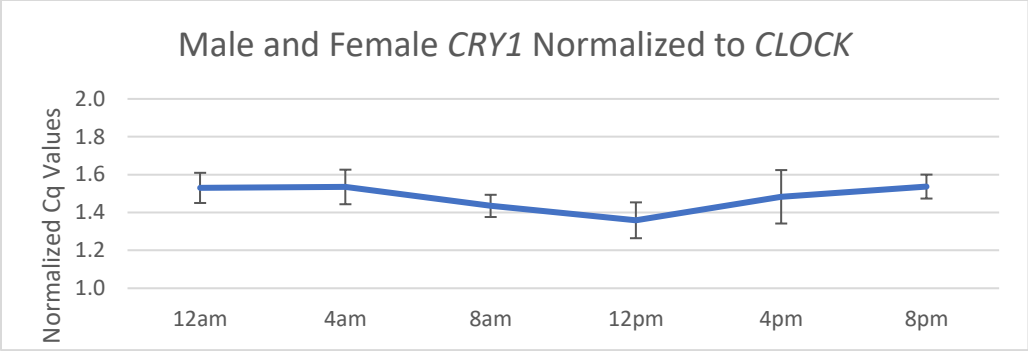
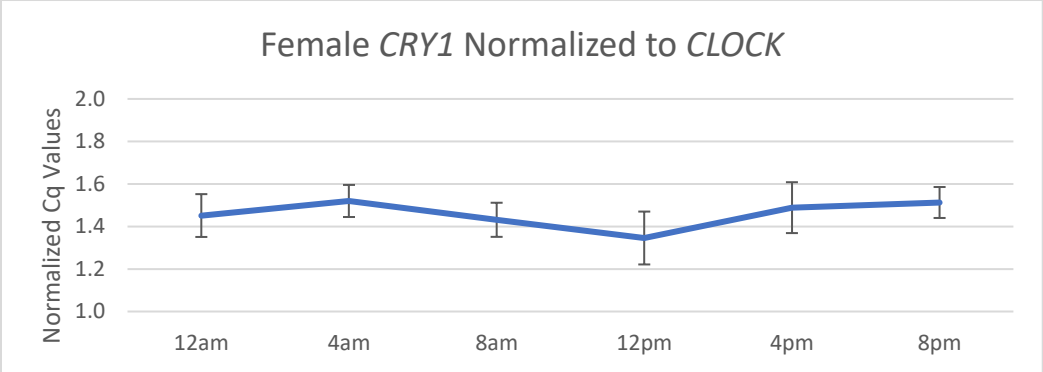


Figure 30. *CRYPTOCHROME 1* 24-hour Expression Normalized to *S100A12*. *CRY 1* Cq values were normalized to *S100A12* and assessed together and then reassessed separately by sex. Combined data appear flat with large variations indicated by the standard error bars. When the data is observed separately, trends are present although standard error bars, representing variation at each time point, remains elevated. There are no statistically significant differences in expression levels between time points. *CRY1* mean Cq values ranged from 26.5 to 33.3 and *S100A12* results ranged from 21.5 to 25.9.





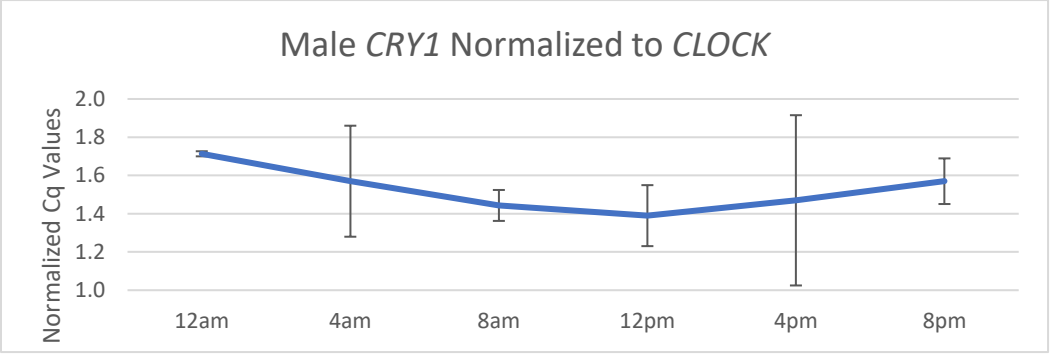
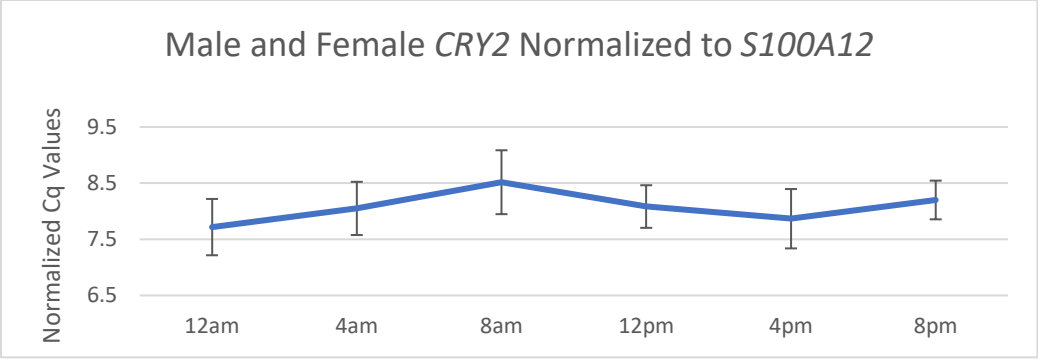
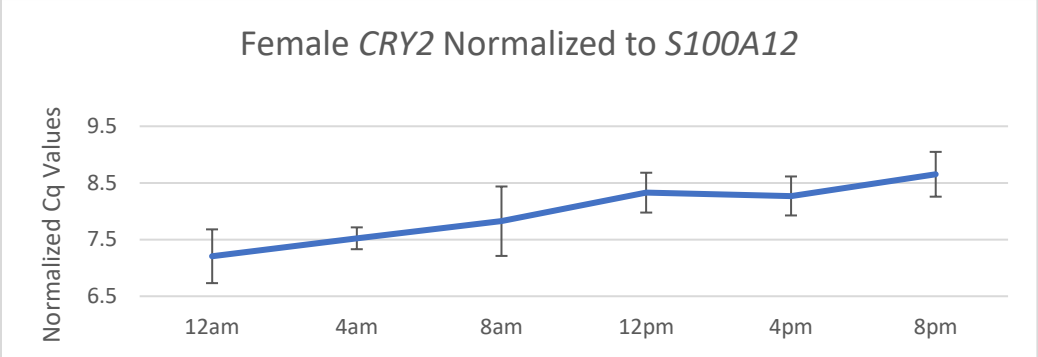


Figure 31. *CRYPTOCHROME 1* 24-Hour Expression Normalized to *CLOCK*. *CRY 1* qPCR data normalized to *CLOCK* in males, females and when all data was combined. There is no rhythmicity observed and there is no statistically significant difference between time points in any *CLOCK* normalized *CRY1* time points evaluated. Standard error bars represent the amount of variation at each time point. *CRY1* mean Cq values ranged from 26.5 to 33.3 and *CLOCK* results ranged from 25.0 to 31.2. It is interesting to speculate that the large range of expression of *CRY1* in males may reflect individual differences among male donors in the expression gene. There is no reason to suspect a different level of technical variation in the qPCR assays for this transcript.





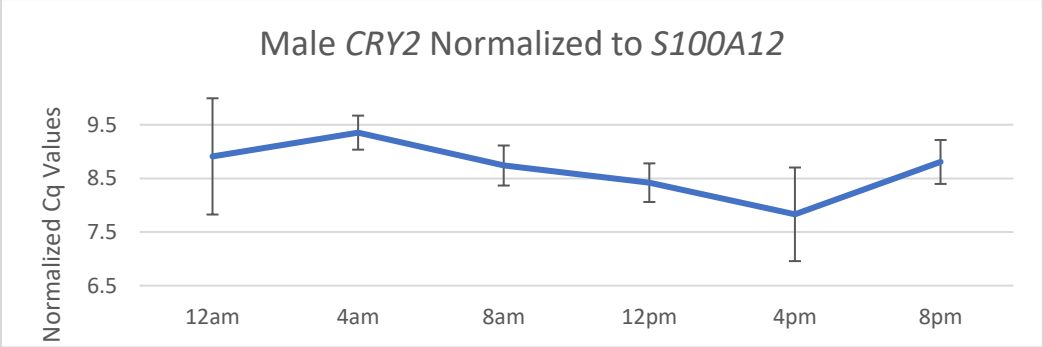


Figure 32. *CRYPTOCHROME 2* 24-Hour Expression Normalized to *S100A12*. *CRY2* was normalized to *S100A12* and the data was platted on a line graph Female, male and combined data were evaluated for rhythmic trends, variability and statistically significant differences between time points. An increase in *CRY2* was observed from 12am to 8pm in female samples although the higher standard error values reduce the statistical significance between points. Combined data and male only data exhibit highly variable nonrhythmic patterns. There are no statistical significance differences between time points. *CRY2* mean Cq values ranged from 26.9 to 32.0 and *S100A12* results ranged from 21.5 to 25.9.

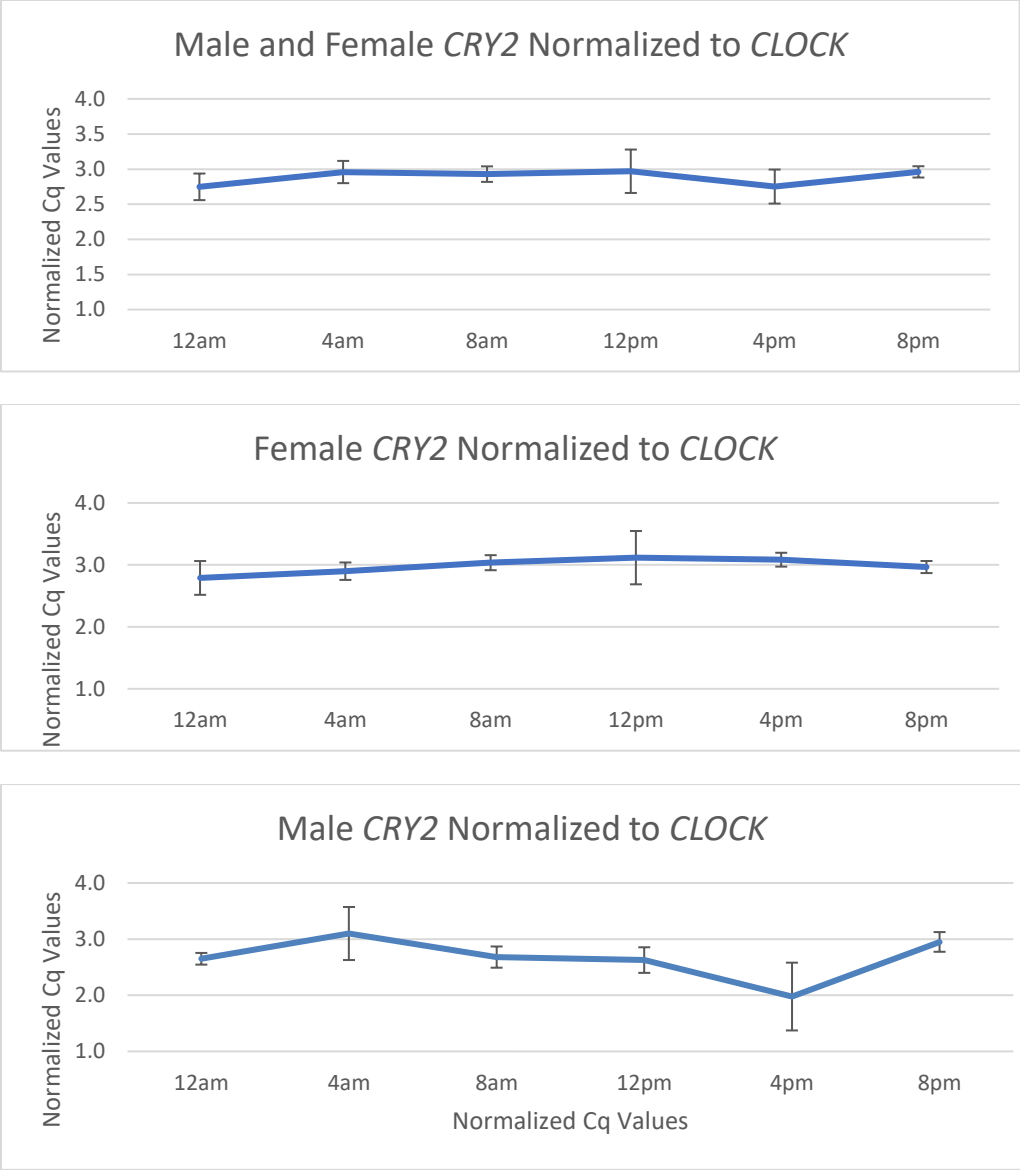
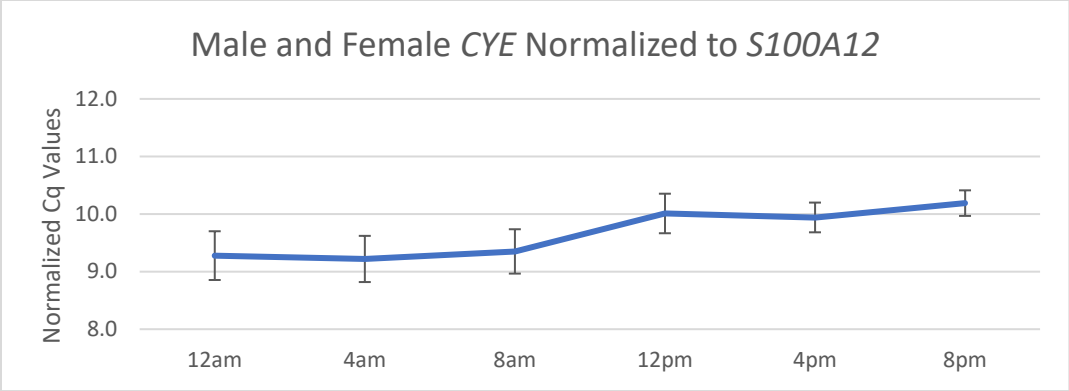
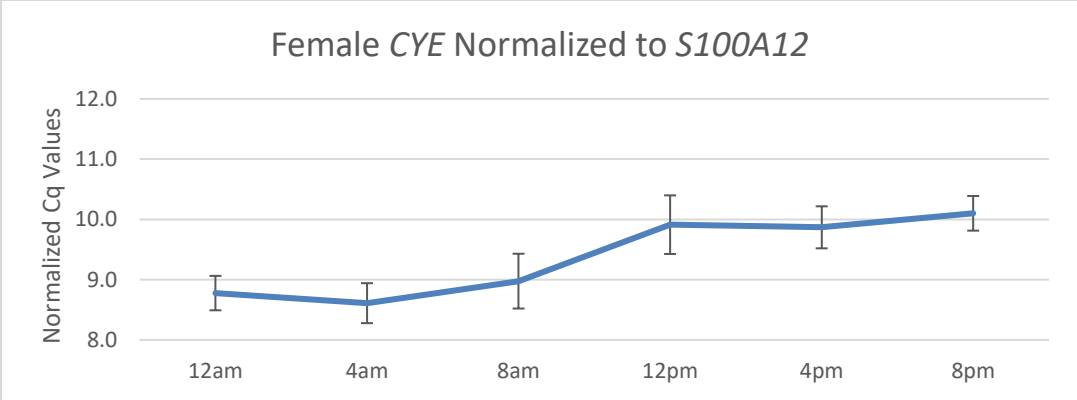


Figure 33. *CRYPTOCHROME 2* 24-Hour Expression Normalized to *CLOCK*. *CRY2* Cq values normalized to *CLOCK* were observed together and after separation in male and female groups. There is no observed rhythmic pattern over the 24-hour collection period although the variability is reduced, as seen by the smaller error bars. There are no statistically significant differences between time points. *CRY2* mean Cq values ranged from 26.9 to 32.0 and *CLOCK* results ranged from 25.0 to 31.2.





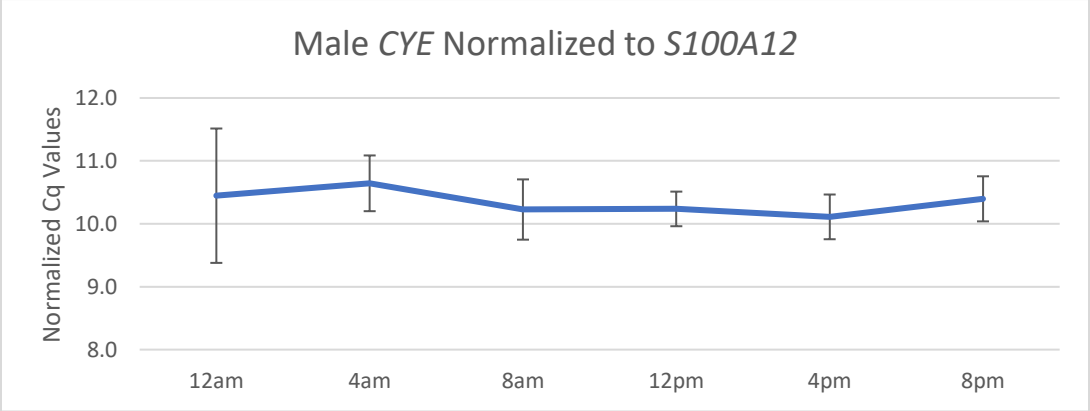
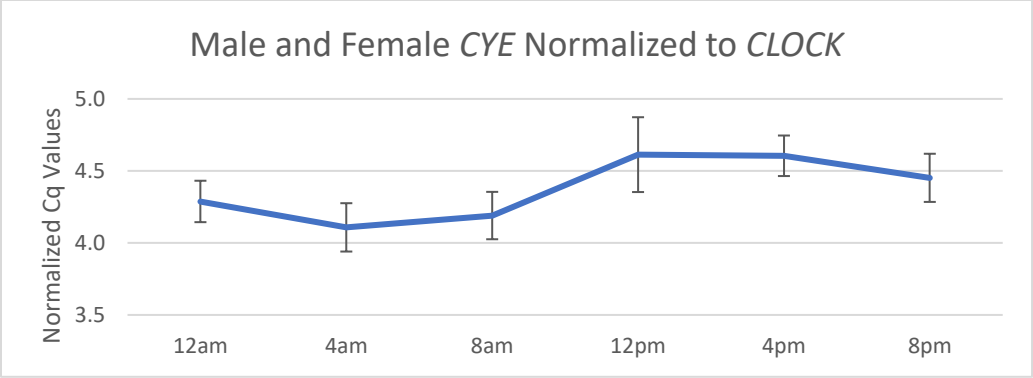
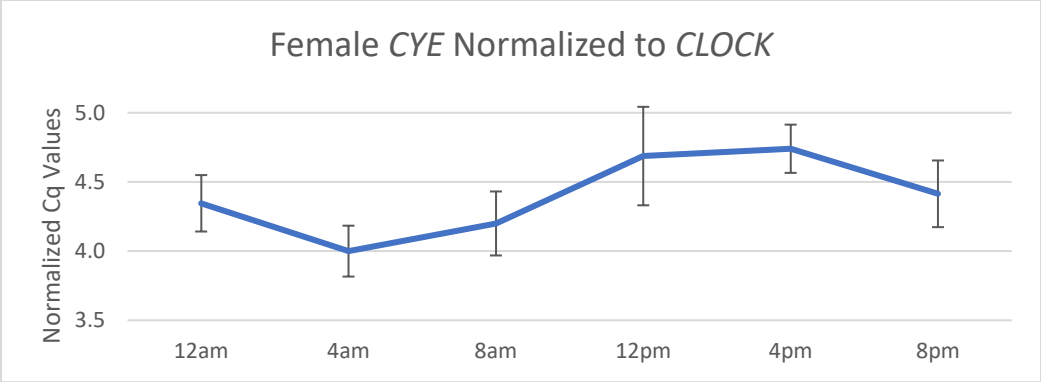


Figure 34. *CYE* 24-Hour Expression Normalized to *S100A12*. *CYE* normalized to *S100A12* in male, female and in all samples combined. Larger standard error bars represent an increase in variance at a specific time point. Possible rhythmic expression is observed in female *CYE* that is normalized to *S100A12*. Male data shows no rhythmicity thus the combined data exhibit a muted rhythmic expression when males and females are combined. There are no statistically significant differences between time points. *CYE* mean Cq values ranged from 29.2 to 35.3 and *S100A12* results ranged from 21.5 to 25.9.





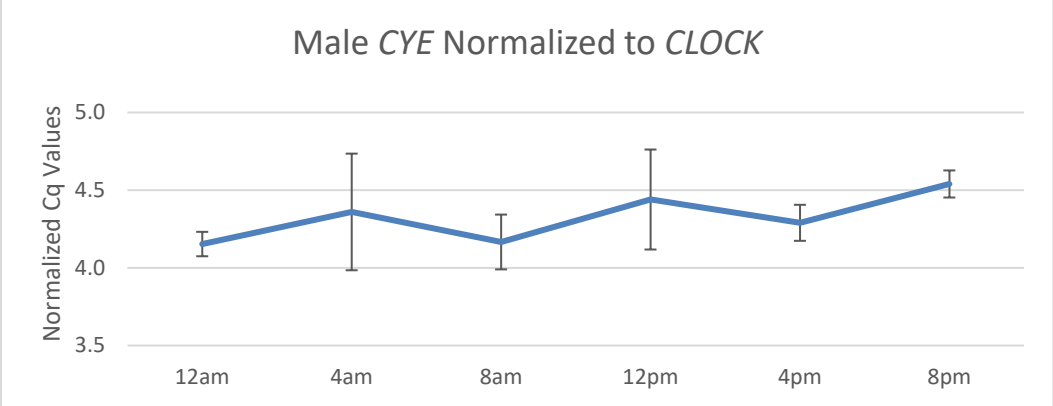
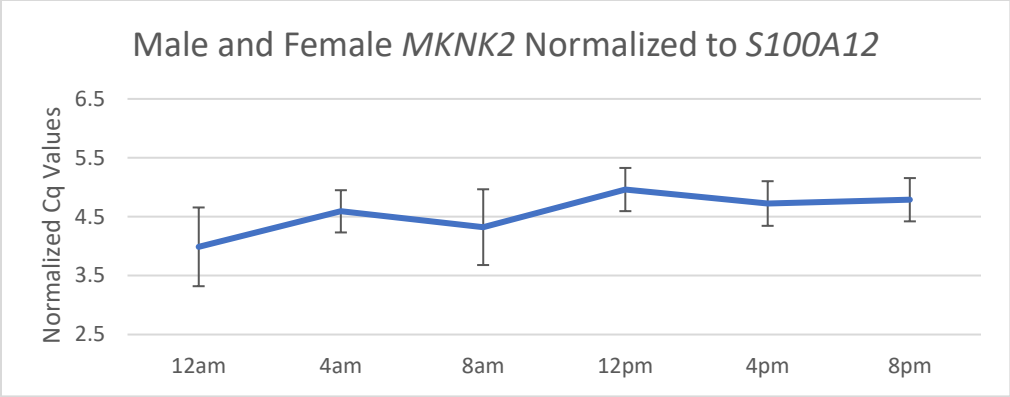
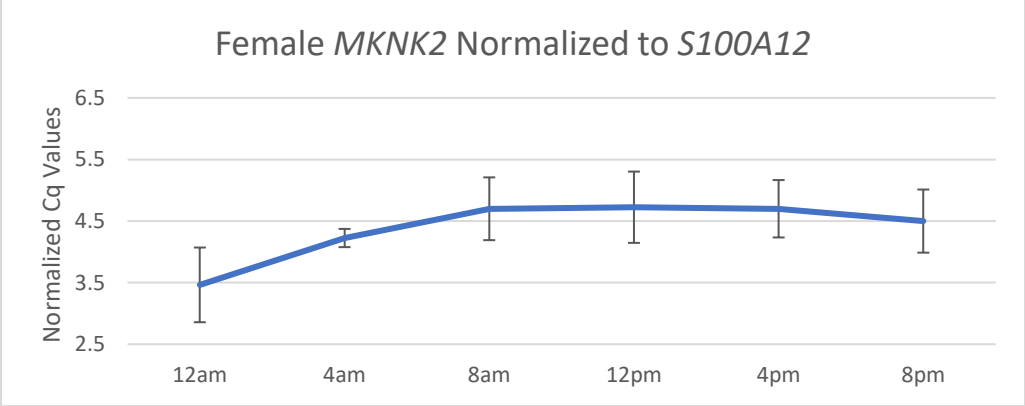


Figure 35. *CYE* 24-Hour Expression Normalized to *CLOCK*. *CYE* normalized to *CLOCK* exhibits a rhythmic pattern of expression over a 24-hour period in females. No rhythmic pattern of expression was observed in male samples thus combined data exhibits a muted rhythm. Standard error within time periods nullifies the difference observed at each time point. There was no statistically significant difference between time points observed. *CYE* mean Cq values ranged from 29.2 to 35.3 and *CLOCK* results ranged from 25.0 to 31.2.





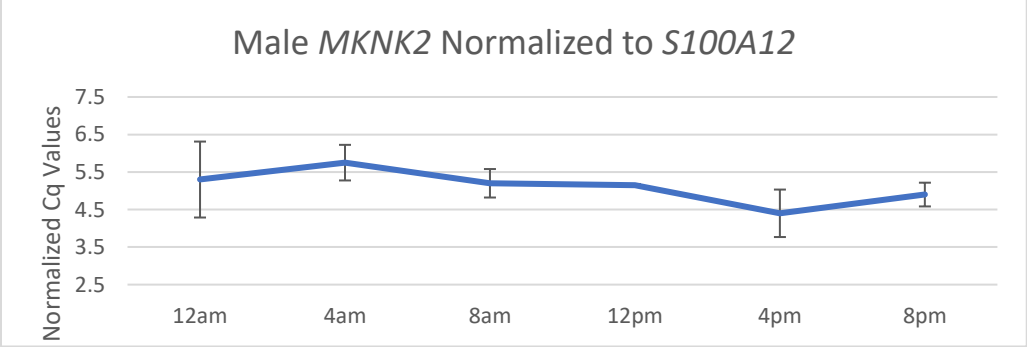
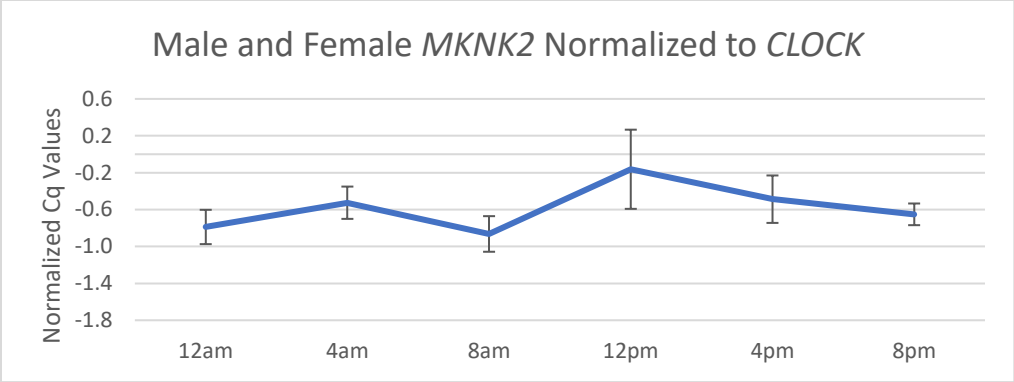
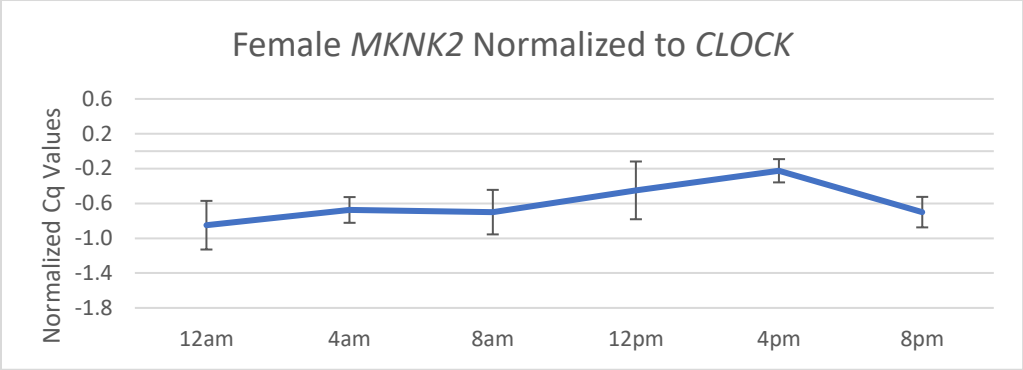


Figure 36. *MKNK2* 24-Hour Expression Normalized to *S100A12*. *MKNK2* normalized to *S100A12* in male, female and combined data. Rhythmicity is observed although extensive variability, indicated by the standard error bars, reduces the difference between time points. There are no statistically significant differences between time points. *MKNK2* mean Cq values ranged from 23.9 to 29.3 and *S100A12* results ranged from 21.5 to 25.9.





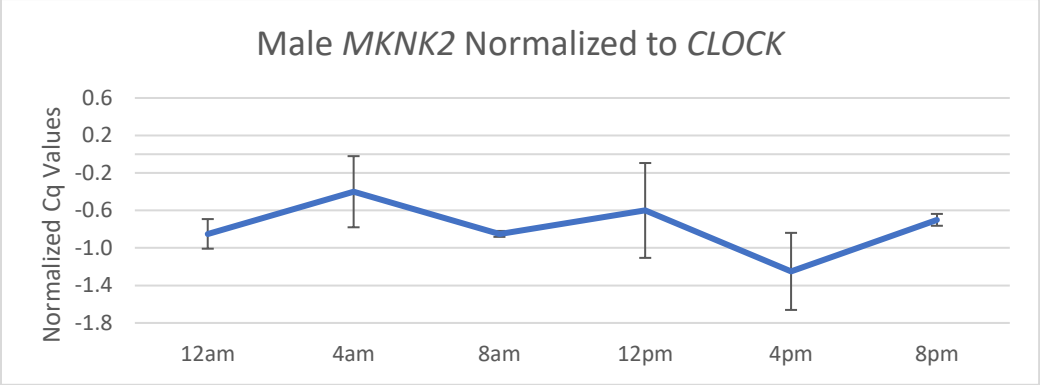
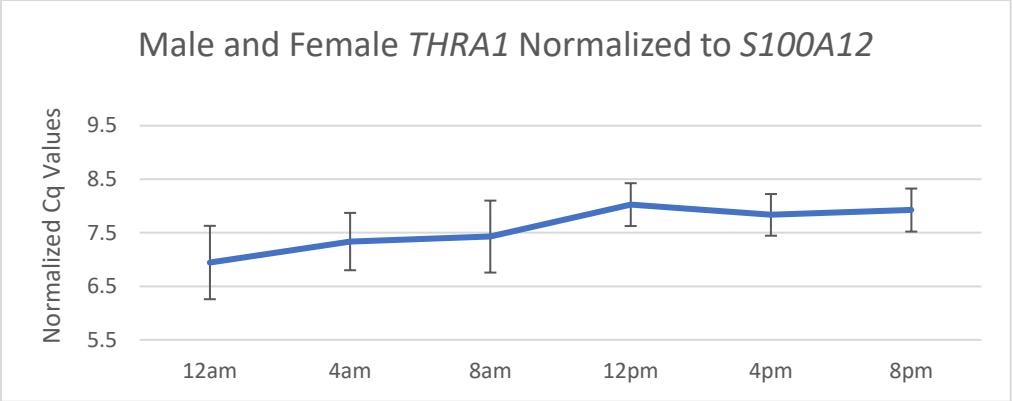
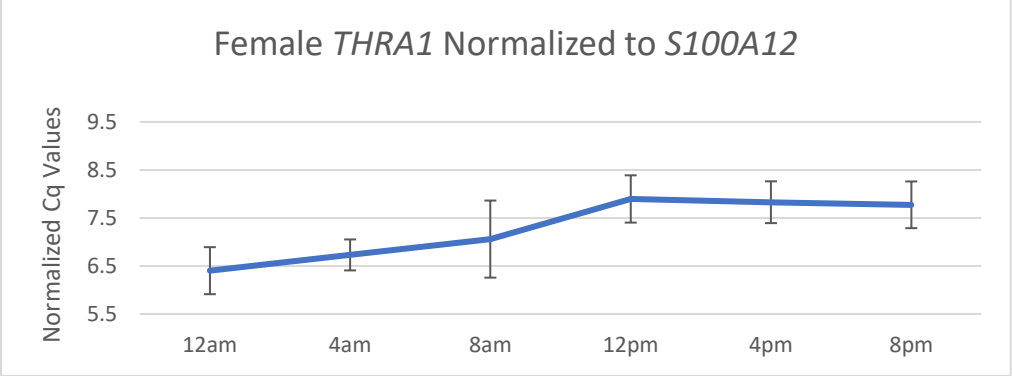


Figure 37. *MKNK2* 24-Hour Expression Normalized to *CLOCK*. *MKNK2* normalized Cq values were assessed together and after separation into groups consisting of males, and females. Rhythmicity is not observed in any group when normalized to *CLOCK*. The large standard error bars indicate a wide range of expression at most time points. There is no statistically significant difference between time points. *MKNK2* mean Cq values ranged from 23.9 to 29.3 and *CLOCK* results ranged from 25.0 to 31.2.





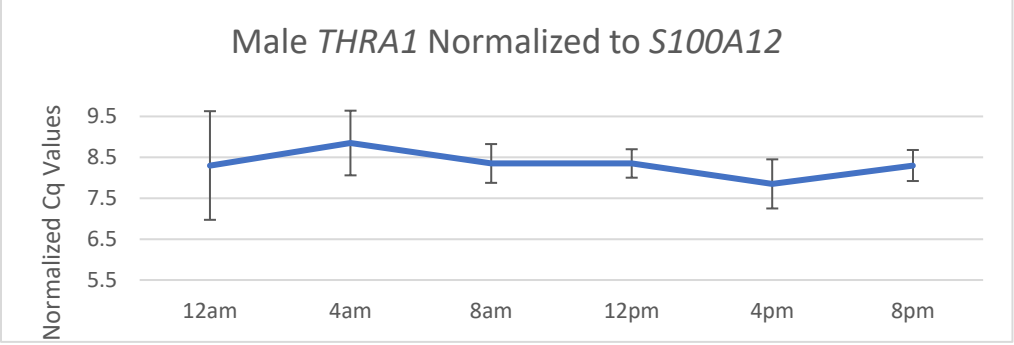
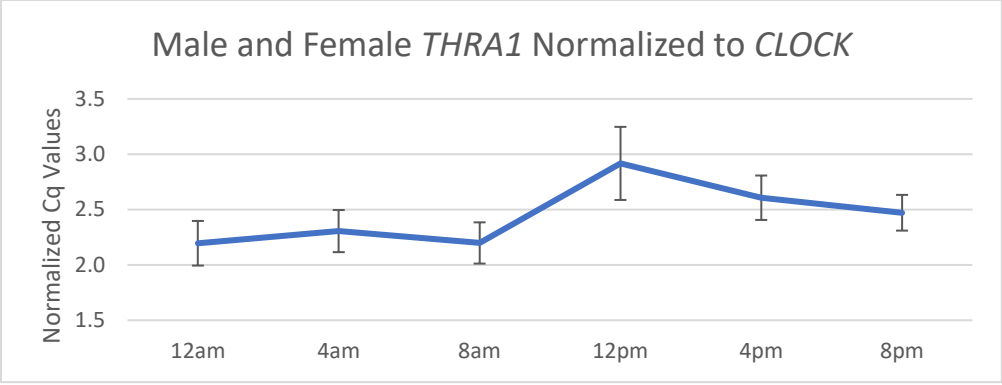
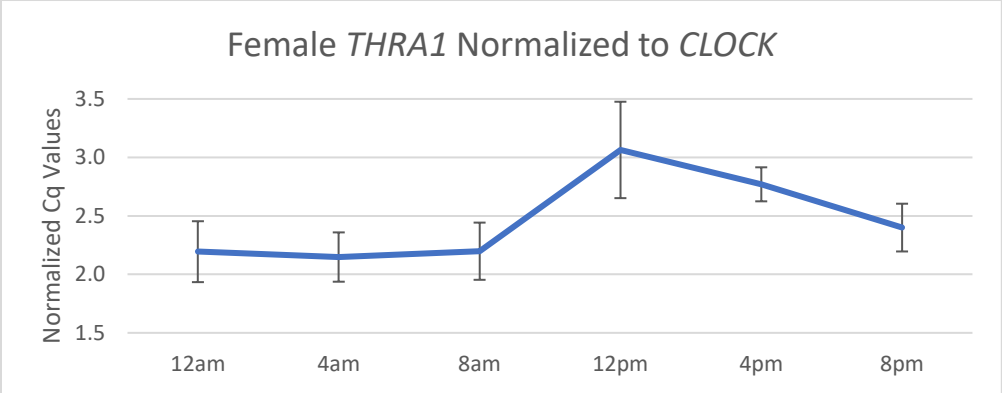


Figure 38. *THRA1* 24-Hour Expression Normalized to *S100A12*. *THRA1* qPCR Cq values normalized to *S100A12* were assessed together and after separation into male and female groups. Standard error bars reflect the variability in expression present at each time interval. A pattern of expression is observed in females although the variability observed at each time point reduces the efficacy of *THRA1* as an accurate indicator of time. Data from males and combined male and female data lack rhythmic expression when normalized to *S100A12*. There is no statistically significant difference between time points when THRA1 Cq values are normalized to *S100A12*. *THRA1* mean Cq values ranged from 27.0 to 32.9 and *S100A12* results ranged from 21.5 to 25.9.





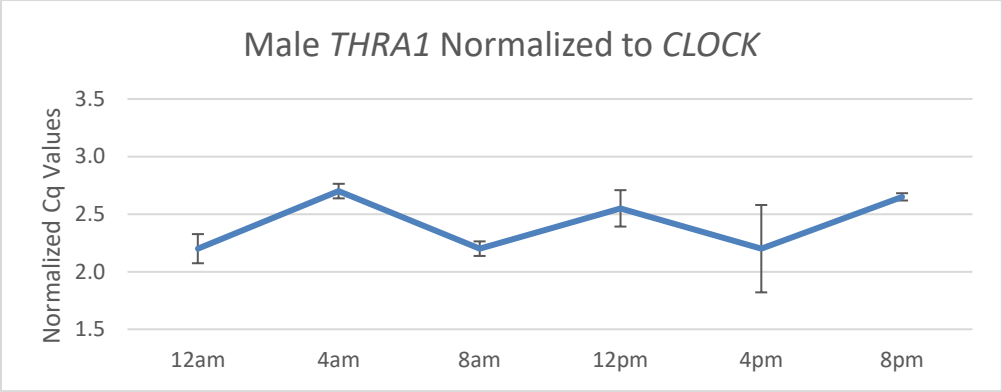
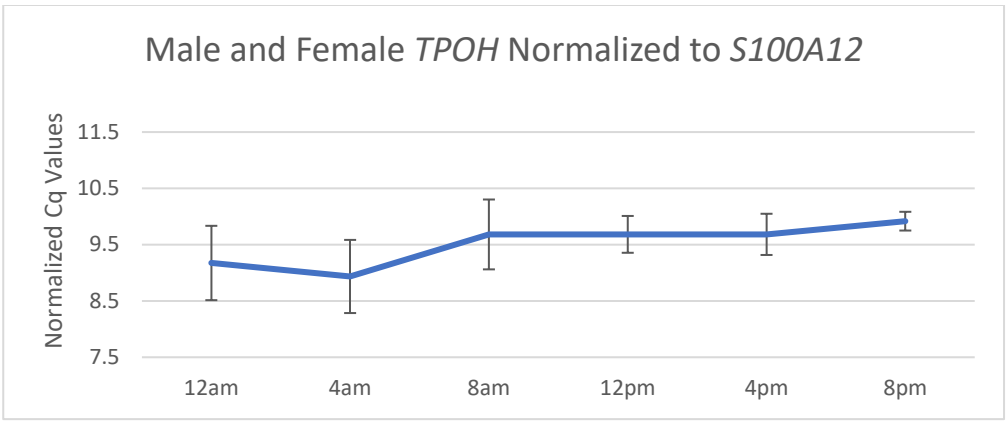
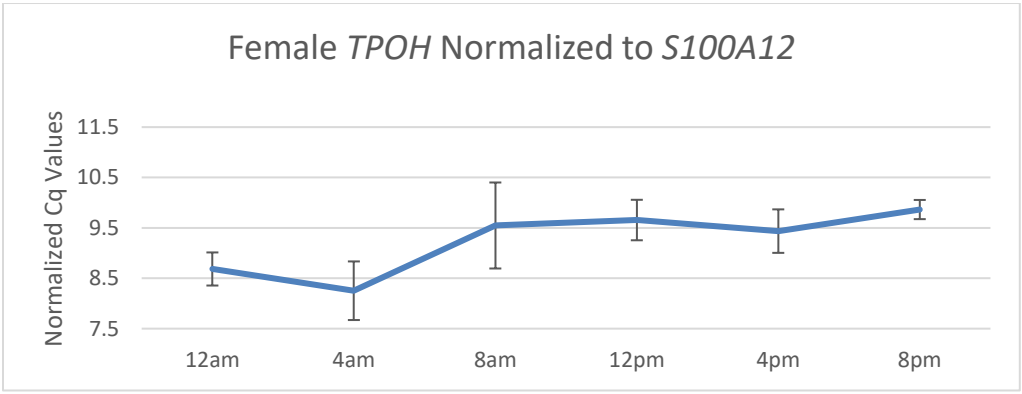


Figure 39. *THRA1* 24-Hour Expression Normalized to *CLOCK*. *THRA1* Cq values normalized to *CLOCK* in males, females and in combined data. No discernible 24-hour pattern of expression in males is observed while females exhibit a rhythmic pattern of expression. Variability seen in the standard error bars reveals extensive variability in female samples consequently there are no statistically significant differences between time points in *THRA1* Cq values normalized to *CLOCK*. *THRA1* mean Cq values ranged from 27.0 to 32.9 and *CLOCK* results ranged from 25.0 to 31.2.





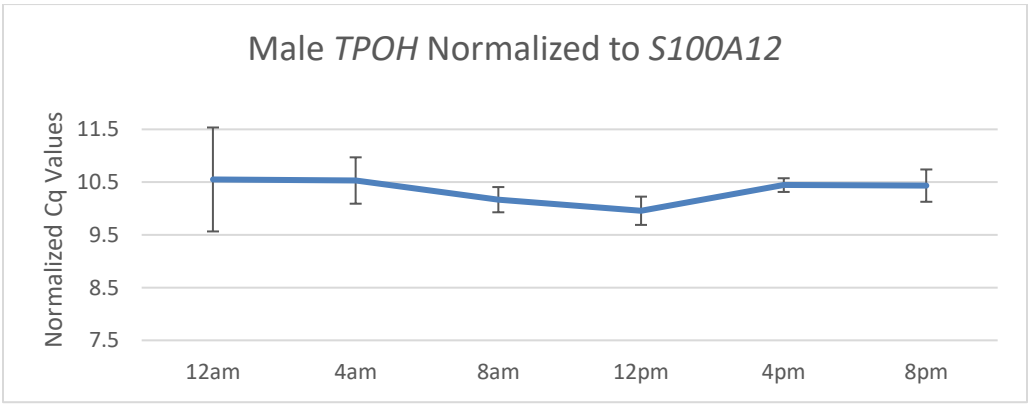
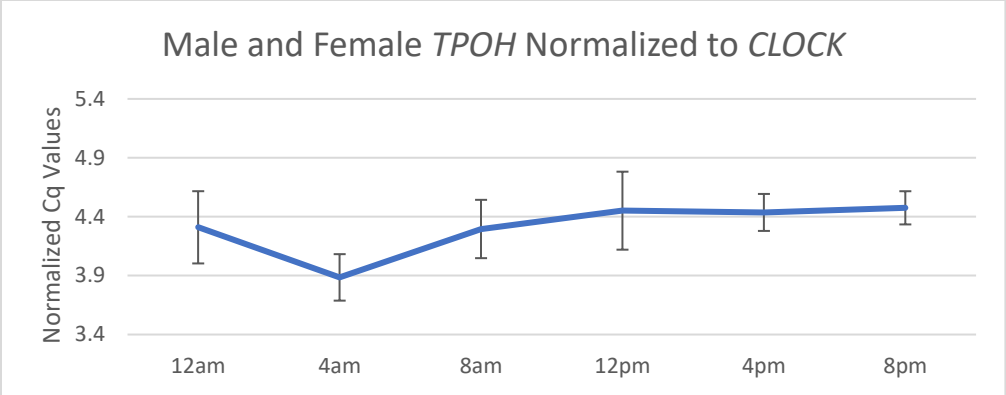
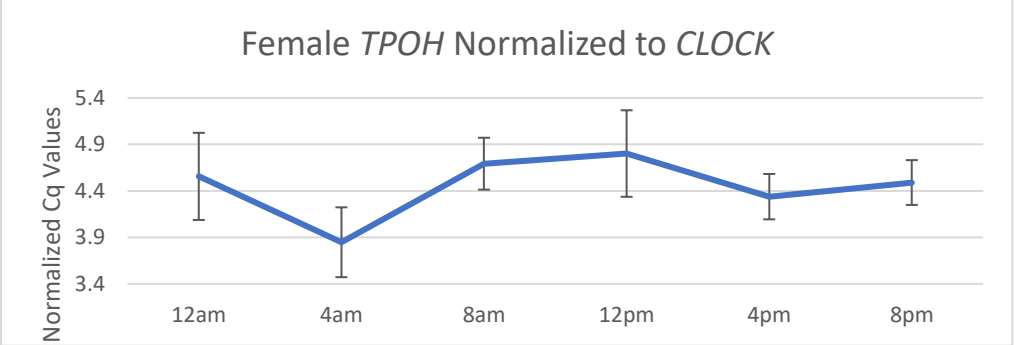


Figure 40. *TPOH* 24-Hour Expression Normalized to *S100A12*. Quantitative PCR Cq values for *TPOH* normalized to *S100A12* exhibit a subtle pattern of expression over 24 hours in female data. Combined and male data exhibit constant levels of expression throughout the day when standard error results are considered. There are no statistically significant differences between time points in Cq values normalized to *S100A12*. *TPOH* mean Cq values ranged from 29.2 to 36.0 and *S100A12* results ranged from 21.5 to 25.9.





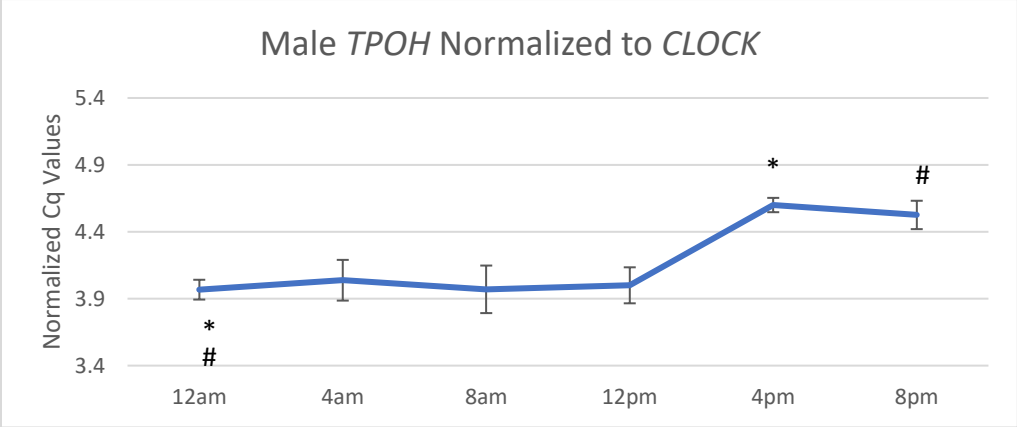
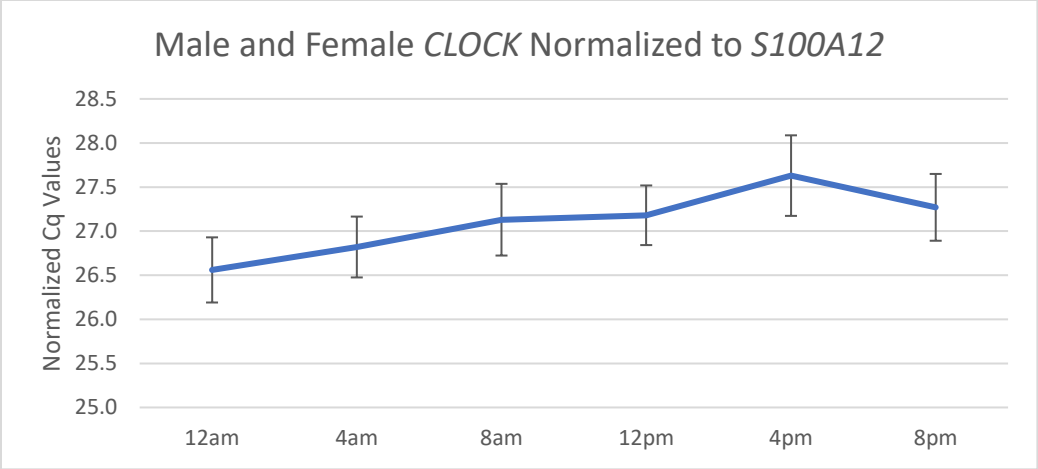
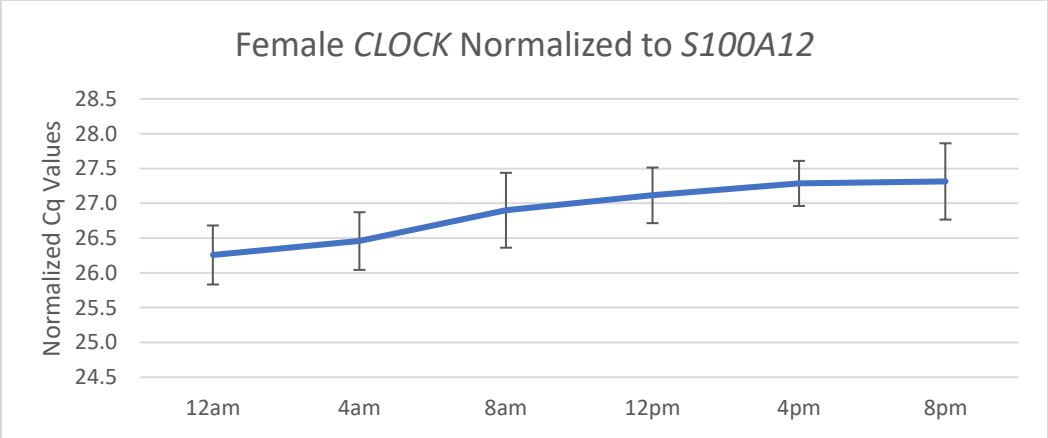


Figure 41. *TPOH* 24-Hour Expression Normalized to *CLOCK*. *TPOH* Cq values were normalized to *CLOCK* and observed together and separated by sex. A rhythmic pattern is observed in the combined data and in male only results. Female data contains a large amount of variation per time point as indicated by the standard error bars. Expression variability in male data exhibits minimal variation of expression at each time point and statistically significant differences were observed between 12:00 am and 4:00 pm (*) (p=0.0278) and 12:00 am to 8:00 pm (#) (p=0.0304). *TPOH* mean Cq values ranged from 29.2 to 36.0 and *CLOCK* results ranged from 25.0 to 31.2.





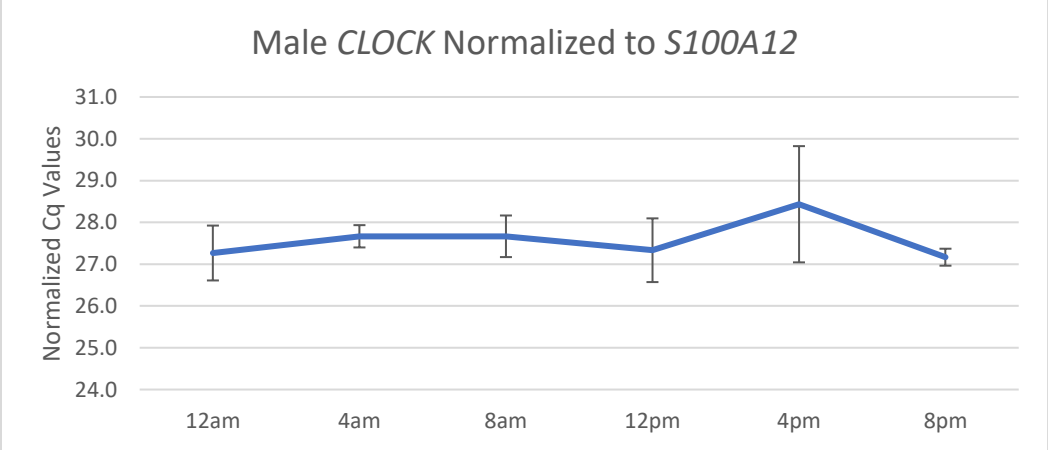


Figure 42. *CLOCK* 24-Hour Expression Normalized to *S100A12*. Comparison of *CLOCK* and *S100A12* expression appear stable throughout the 24-hour collection period with a standard amount of variation at many time points.

Earlier research established that *S100A12* and *CLOCK* are expressed at constant levels throughout the day (Bjarnason et al., 2001) although normalizing with *S100A12* versus *CLOCK* frequently resulted in different outcomes in rhythmic patterns and standard error. *S100A12* is a Ca+ binding protein involved in proinflammatory responses and expression is normally associated with neutrophils. Neutrophils normally constitute 55% to 65% of the white blood cells in the peripheral blood but may increase to 75% or more during bacterial infections and inflammatory conditions.

In contrast, CLOCK is a key component in the heteroduplex of the BMAL/CLOCK heterodimer that drives circadian rhythm. Since the peripheral blood is composed of multiple cell lineages (red blood cells, platelets, neutrophils, lymphocytes, monocytes, eosinophils and basophils) it is possible that expression levels for both circadian rhythm genes and the genes their expression is normalized against may vary in complex ways among the blood cell lineages. While previous studies of circadian rhythm investigated different tissue types, often those tissues consisted of a histologically similar populations of cells with the expected homogeneity of gene expression. In extensions of this work, it will be important to connect the gene of interest with the hematopoietic cell lines that express it.

Cell specific expression of circadian rhythm genes was available from GeneCards® The Human Gene Database (LifeMap Sciences Inc. Carlsbad, CA). Expression levels for each gene studied is shown in whole blood, white blood cells and in lymph node (representative of the lymphocyte population) samples in Table 16. From this information, it appears there is a greater amount of *S100A12* in white blood cells than in the lymph node. This data correlates with the findings of Hou (2015) that showed

S100A12 in peripheral blood is expressed by neutrophils, monocytes and macrophages (Hou, 2015). Thus, when more target mRNA is found in lymph nodes than in white blood cells, those differences can be attributed to a larger expression in lymphocytes over other white blood cells. Conversely, when a larger amount of expression is seen in the white blood cells than in lymph nodes, lymphocytes most likely demonstrate a lower level of expression.

Table 18. Expression Levels of Selected Genes by Cell Population. Whole blood contains red blood cells, platelets and white blood cells (neutrophils, lymphocytes, monocytes, eosinophils and basophils). The lymph node represents mainly lymphocytes with a smaller population of monocytes/macrophages. Fragments per kilobase of exon per million fragments mapped (FPKM) normalizes the amount of target RNA detected. (*)Fragments Per Kilobase of exon per million fragments mapped (*FPKM*).

Marker	Whole Blood $(110x\ FPKM)^{1/2}$ *	White Blood Cells $(110x\ FPKM)^{1/2}$ *	Lymph Node $(110x\ FPKM)^{1/2}$ *
S100A12	495	264	47
CLOCK	7	19	18
PER1	65	18	34
PER2	10	14	12
PER3	4	10	11
CRY1	11	27	31
CRY2	20	27	31
CYE	7	8	12
MKNK2	84	82	74
THRA1	16	25	37
TPOH	2	1	3
Data from Gene Card (https://www.genecards.org)			

The differences in gene expression levels observed in samples normalized to *S100A12* versus *CLOCK* can be attributed to the cell population that expresses the marker of interest and to the appropriate marker used to normalize those expression levels. The distribution of cells in an individual adds an additional caveat to the complexities

involved. Quantification of mRNA that is expressed in lymphocytes but normalized to a neutrophilic marker may not only be ineffective, but in the presence of an inflammatory condition, exaggerates the error. Additionally, neutrophils exhibit a diurnal pattern in cell numbers that results in a state of pseudoneutropenia in the morning due to a rhythmic expression of granulocyte colony stimulating factor (GCSF) stimulating neutrophil release from the bone marrow (Jilma et al., 1999). The circadian cycles of neutrophils introduce an additional complication to 24-hour data interpretation. Therefore, the difference in expression patterns and standard errors when normalizing to *S100A12* or to *CLOCK* can be attributed to normalization of Cq values for the rhythmic marker to a constitutively expressed gene in a cell of the same closely related cell lineage. Regardless of the complexities of our experimental system, it appears that a reference range of expression for *PER3* in females and *TPOH* in males can be defined with expression levels in morning and night that are statistically different. If the sex of a sample is known, the expression profiles for *PER3* (females) and *TPOH* (males) could distinguish between a stain created in the morning versus one created at night.

Conclusion

Ideal candidates for time determination are those markers that have minimal variation at each time point assessed and exhibit a 24-hour rhythmic expression. Of the 11 circadian genes assessed, *PERIOD 3* normalized with *CLOCK* shows minimal variation and statistically significant differences between time points. *PERIOD 3* normalized to *CLOCK* has the potential to indicate a 12-hour window of time in which a female blood stain was created. In males, the expression of *TPOH*, normalized to

CLOCK, exhibits a 24-hour rhythmic pattern of expression with very little deviation from each time point assessed. *TPOH* can potentially determine an 8-hour time period from a male blood stain.

CHAPTER IX

FINAL CONCLUSIONS

Circadian rhythm is a continuous regularly oscillating pattern of gene expression and behavior exhibited by living organisms. This rhythm is stimulated by both exogenous and endogenous factors and can be observed through cyclic biologic processes. Because biologic processes often involve interactions with proteins translated from mRNA that are transcribed from cyclically expressed genes, the timely fluctuations of RNA levels provide a means to identify points in circadian 24-hour cycles.

One means of regulating gene expression is through epigenetics modifications of nucleic acids and proteins that can act to control mechanisms influencing both transcription and translation. Since epigenetic effects can be observed through the chemical modification of DNA, the time of day may be correlated with modifications such as methylation and may offer an alternative approach to estimate time. *PERIOD 1-3*, *CRYPTOCHROME 1-2*, *NPAS2* and *BMAL1* methylation patterns were assessed using DNA extracted from blood stains collected every 4 hours for a 24-hour period.

Comparisons of likely methylation targets in the *PER1-2, CRY1-2, NPAS2* and *BMAL1* genes were assessed using a PCR protocol designed to distinguish between methylated and unmethylated sequences. *PERIOD 3* was the only gene target that exhibited methylation and the methylation detected was nominal and unchanging at every time point. All other markers were negative for methylation at all time points tested. Results from the methylation study revealed that methylation of the selected DNA markers in dried blood stains is not correlated with the circadian rhythm and therefore this approach is not useful to estimate the time of day a blood stain was created.

Because there are a multitude of factors contributing to the complex orchestration of transcription and translation during the circadian rhythm, the study of mRNA abundances as a measure of rhythm during the daily cycle was logical as one approach for defining the cycle throughout the day. Messenger RNAs transcribed from genes shown previously to vary in expression in a cyclic manner in dried blood stains were analyzed at defined time points during a 24-hour period. In order to accurately assess and compare levels of mRNA it was first necessary to confirm that the target mRNA did not degrade during the time that passes between sample collection and RNA extraction from dried stains. Because the mRNA levels of the targeted genes tended to be low, there was concern that lower amounts of mRNA would be susceptible to even small amounts of degradation. Blood stains were collected and stored for varying periods before extracting RNA that was subsequently quantified for transcripts from several rhythmically expressed genes. Results showed no significant degradation within an 8-day period of storage. Transcript quantities for all of the studies reported here were obtained using RNA extracts prepared within 48 hours of sample collection.

In order to establish the fluctuations of mRNA quantities during the daily cycle with validity, it was important to establish the extent to which the expression of targeted genes fluctuate naturally. To investigate naturally occurring variation in transcript levels, blood samples were collected at the same time of day from donors over several weeks. Extractions were performed within 48 hours of collection and qPCR was used to quantify mRNA levels. Most of the markers exhibited less than a 15% variation in abundance when mRNA levels were normalized to *S100A12*. This value rose to ~20% variation when mRNA levels were normalized to *CLOCK*. Observations from this study help to define reference profiles of gene expression during the circadian rhythm. Such reference profiles will be useful to estimate the time of day biological evidence was created at a crime scene.

The ability to estimate the time a blood stain was deposited at a crime scene requires a reference pattern of gene expression that correlate with defined times throughout a 24-hour period. For this study, it was important to explore the feasibility of creating a reference pattern of gene expression using blood stains created at known times during the day using blood donors whose daily habits are representative of those of the population general. Thus, the reference pattern of gene expression created here was from blood donors whose sleep habits and daily routines were somewhat known. The question arose as to whether age differences among blood donors might affect RNA levels. Donor groups selected by age were evaluated in the Age Comparison Study. Volunteers were separated into age categories and blood was collected from each volunteer at the same time of day. Quantitative PCR was performed and Cq values were normalized. Mean values were compared to determine if age effected mRNA expression levels. There was

no significant difference observed in the abundance levels of the transcripts tested among the age categories. There was, however, significant difference in gene expression between males and females regardless of age. These findings demonstrate that, although there is no age-related difference in gene expression for the circadian markers studied, there is a need for separate reference patterns in males and females for the accurate assessment of time.

Eleven rhythmic genes associated with circadian rhythm were characterized for stability, and variability within an individual, and between sexes and age groups. This information was incorporated into the development of our experimental approach to defining a reference pattern of gene expression during the circadian rhythm. In these experiments, an attempt was made to determine if 24-hour expression patterns can be detected in blood stains. Blood stains were collected every 4 hours for 24 hours, RNA was extracted from the stains and converted to cDNA. The abundance of a group of selected mRNAs was determined using qPCR and Cq values were normalized to the transcripts of *S100A12* and/or *CLOCK* genes. Mean Cq values, standard deviations and ANOVA analysis with a post hoc Tukey Tests was performed on all data. Transcript abundance values for each marker was assessed for male and female donors together and separately.

Statistically significant differences in abundance between rhythm time points were observed for *PERIOD 3* mRNA levels in females when normalized to *CLOCK*. The reference pattern of *PERIOD 3* gene expression in female blood stains has the potential to define a 12-hour window of time during the day. Likewise, statistically significant differences in *TPOH* gene expression in males were observed when *TPOH* mRNA levels

were normalized to *CLOCK*. Using this reference pattern, it may be possible to define an 8-hour window of time during the day in male blood stains.

The goal of this research project was to explore if it is feasible to determine when during a 24-hour day a blood stain was created at a crime scene. The results of this study are encouraging as to the feasibility of using the cycling patterns of gene expression during the day as a forensic tool for the furtherance of a criminal investigation. With a sufficient number of circadian rhythm markers included in the analysis, it could be possible to identify a narrow time point within the day using the mRNA levels of select genes.

Future directions

More cyclic genes exhibiting low variability need to be identified to increase the sensitivity of time determination and narrow the window of time that can be identified. Because approximately 10 % of mRNA is expressed rhythmically (Doherty & Kay, 2010) and eleven genes were targeted in this study, there is a great opportunity to further this research. The ability to identify time from a single blood stain could improve the breadth of knowledge surrounding criminal investigations.

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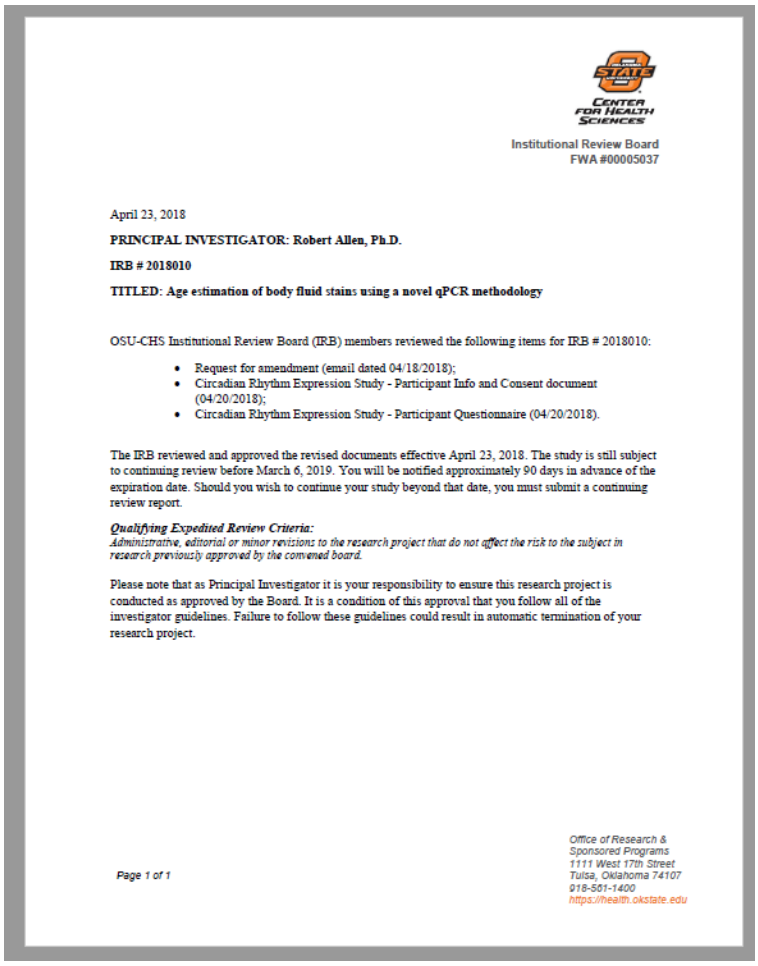
APPENDICES

APPENDIX A: IRB Approval.



Institutional Review Board
FWA #00005037

April 23, 2018

PRINCIPAL INVESTIGATOR: Robert Allen, Ph.D.

IRB # 2018010

TITLED: Age estimation of body fluid stains using a novel qPCR methodology

OSU-CHS Institutional Review Board (IRB) members reviewed the following items for IRB # 2018010:

- Request for amendment (email dated 04/18/2018);
- Circadian Rhythm Expression Study - Participant Info and Consent document (04/20/2018);
- Circadian Rhythm Expression Study - Participant Questionnaire (04/20/2018).

The IRB reviewed and approved the revised documents effective April 23, 2018. The study is still subject to continuing review before March 6, 2019. You will be notified approximately 90 days in advance of the expiration date. Should you wish to continue your study beyond that date, you must submit a continuing review report.

Qualifying Expedited Review Criteria:
Administrative, editorial or minor revisions to the research project that do not affect the risk to the subject in research previously approved by the convened board.

Please note that as Principal Investigator it is your responsibility to ensure this research project is conducted as approved by the Board. It is a condition of this approval that you follow all of the investigator guidelines. Failure to follow these guidelines could result in automatic termination of your research project.

Page 1 of 1

Office of Research & Sponsored Programs
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APPENDIX B: Participant Information and Consent Form

PARTICIPANT INFORMATION AND CONSENT FORM

Title of Project: **Age estimation of body fluid stains using a novel qPCR methodology**

Investigator: Robert W. Allen, PhD, School of Forensic Sciences, 918-561-1292

You are being asked to participate in a research study by donating small samples of blood. If this consent form contains any words you do not understand, please ask the investigator to explain these words so that you understand them. This consent form contains important facts to help you decide if it is in your best interest to take part in this study.

In order to contribute a sample, you must:

- Be at least 18 years old
- Have (or be in the process of obtaining) a basic knowledge of molecular biology or a related field such that the overall goals of this project could be understood.
- Be willing to provide a blood sample with which to prepare dried stains that can be used as a source of RNA for analysis.

No identifying information (your name, address, social security number, etc.) will remain with the biological sample(s) collected from you. All samples upon collection will be assigned an identification number that will remain with the sample throughout analysis. The decision of whether to participate or not is completely up to you. If you decide not to sign this form, there will be no consequences for your decision.

I. What is the purpose of this research?

The investigation of a crime strives to answer the questions of who, what, where, and when so that the events surrounding a crime can be reconstructed. Of the questions posed above, the question of when is often the hardest to answer. The question of when has relevance in trying to determine when someone died (the postmortem interval) and when a biological sample of evidence was left at a crime scene.

In earlier research performed in this laboratory, we have been able to estimate the age of blood stains using a molecular assay involving quantitative PCR (qPCR) in which the degradation of specific mRNA transcripts correlates well with sample age. The purpose of this research is to determine the time of day a sample is collected based on cyclic expression of specific genes.

In addition, in preliminary studies, stains were prepared on inert bloodstain cards often used for the long-term preservation of biological fluids. Thus, the substrate upon which body fluid stains were prepared in our preliminary studies was also not representative of the substrates bearing stains at crime scenes and therefore the effect of the substrate on mRNA degradation is also to be assessed in this project.

II. How many people will participate?

This project will involve the collection of small blood samples (i.e. 05-1.0 cc) from 20-30 male and female donors (10-15 for each sex).

III. What will you ask me to do if I want to participate in this study?

If we find that you are eligible to give a sample, we will collect the sample type(s) that you have agreed to provide. Blood will be collected in the OSU CHS Human Identity Lab sample collection room under sterile conditions by a certified phlebotomist.

- Blood: We will collect 0.5-1.0 cc's of blood from your arm or by fingerstick. Sallie Ruskoski will perform the venipuncture collections. Sallie is a Medical Technologist (ASCP) and has taught phlebotomy for the TCC Med Lab Tech program. She is currently the Director for the Medical Laboratory Sciences program at Northeastern State University.

IV. What will happen with the sample I give you?

The sample will be processed and stored at OSU-CHS. The only information included with the sample will be the sample identification number assigned to that sample upon collection that reflects the date and time of the collection and spotting of the samples to prepare stains.

Processing and storage of the sample will include:

- Deposition of biological samples onto paper cards.
- Isolation of RNA from fresh blood samples followed by reverse transcription of the RNA into cDNA.

Interrogation of each cDNA library for the quantity of specific mRNA transcripts that are associated with cyclic expression.

V. What are the costs and payments associated with this study?

Individuals willing to contribute blood samples for this study will receive no compensation for donating small blood samples.

VI. What are the risks of participating in this study?

You may have some brief physical pain and bruising when we collect your blood. There is a small chance that you may get an infection, have excess bleeding, become dizzy, or faint from the blood draw.

VII. Are there any benefits to participating in this study?

There are no known direct benefits to you for participating in this study. Your participation will help us contribute knowledge to the field of forensic science. Specifically, we are attempting to strengthen the investigative capabilities of a forensic scientist to reconstruct the events surrounding a crime. This enhancement will lead to more effective prosecution of criminals and therefore enhanced public safety.

VIII. How will you protect my privacy?

We will protect your privacy in several ways:

- We will store your signed consent form in a locked file, only Dr. Robert Allen will have access to this file.
- We will not label your sample with your name or other identifying information (such as address, birth date, or Social Security number). Upon collection, samples will be given a 10-digit identification code that will remain with the sample throughout the analysis process. The sample and data associated with that sample will only ever be referred to with that 10-digit code.
- We will keep a record of each sample, the name of the person who donated the sample, the code associated with that sample, and the date and time of sample collection. This record will be kept in a locked file, unassociated with the samples or sample data that only Dr. Robert Allen will have access too.
- Samples will be stored in a card-key access, secured storage room that only Dr. Robert Allen (principal investigator) and Dr. Jun Fu will have access too. However, the only identifiers on each sample Dr. Fu has access to will be the identification codes discussed above that identify the sex of the donor and the time a sample was collected and spotted onto a stain card of other solid substrate.
- Samples that are not used will be destroyed upon completion of the study.

The duration of the study will be no longer than 3 years and upon completion of the study, all biological samples will be disposed of in biological waste. The key connecting individual biological samples to donors will also be destroyed upon completion of the study by shredding of the document in a secure document shredder.

IX. Can I change my mind after I decide to participate?

Your participation in this research study is voluntary. You are free to refuse to participate in any procedure and to refuse to answer any question at any time, and are free to withdraw consent, and to withdraw from the research at any time during the study without penalty, including your relationship with OSU. Note that if you withdraw from the study as a semen donor, you will not be compensated.

Any specimen(s) obtained for the purposes of this study will become the property of the School of Forensic Sciences, OSU CHS. Once you have provided the specimens, you will not have access to them. The specimen(s) and the key connecting individual biological samples with a donor will be destroyed following completion of the study using an appropriate means for destroying biological samples. The sample key will be destroyed via shredding with a secure shredder.

If you wish to withdraw from the study, contact Robert W. Allen, PhD, at 918-561-1292. If you wish to withdraw from the study after the completion of the study, all information and data obtained from your biological sample(s) will remain as a part of the study even after the biological sample(s) are destroyed.

If you have any questions about your rights as a research participant while in this study, please contact the Director of Institutional Review Board, Amber Hood, MS, at 918-561-1413.

CONSENT TO PARTICIPATE

Please read the information below, think about your choice and sign if you agree.

I agree to donate the following sample type (please circle all applicable sample types)

Venous Blood Capillary Blood

I have read and been given information about this research study and the risks involved have been explained to me. Any questions I may have had were answered to my satisfaction, and I have been told who to contact should additional questions arise. As a result, I give my informed consent to participate in this research. I will receive a copy of this consent form.

_____ _____

Participant Signature Date

Copy given to participant: _____Yes

*Funding for this research is solicited from the National Institute of Justice Grant; Research and Development in Forensic Sciences for Criminal Justice Purposes.

APPENDIX C: Volunteer Survey

Circadian Rhythm Expression Study

Sample Identifier #_____ Gender (circle one) M F

Age (circle one) 18-24 25-30 31-35 36-40 41-45 46-50 51-55 56-60 61-70 71+

When time do you usually start your sleep cycle? _____

What time do you usually end your sleep cycle? _____

What is your average hours of sleep per night? _____

Has your normal sleep routine been interrupted in the last 4 days? yes____ no ____

Do you sleep in a dark or lighted room? _____

Do you wake frequently (>5 times) during your sleep cycle? yes____ no ____

Have you used a sleeping aid (ie. cpap, medication) in the past week? yes____ no ____

Do you have a consistent schedule (work, school)? yes____ no ____

What time is your typical breakfast _____lunch_____ and dinner _____?

Thank you for volunteering for this project.

Research information:

Specimen type_____ Specimen collection date_____ time_____

Collector initials_____

RNA Extraction/RT date and time_____ Lab procedures initials_____

APPENDIX D: Blood Sample Collection and Handling Instructions

Thank you for your willingness to do this to yourself and/or others.

As I am typically brain challenged I thought some directions might be helpful for those that do not have an identic memory.  I am researching circadian rhythm, in that different transcripts will exhibit different levels throughout the day. It is my hope those levels are reliable and repeat within a sensitive range. In this way, we will be able to predict time of a sample.

Directions: I need a sample of blood every 4 hrs (I'm sorry)  Collection times need to be **4am, 8am, 12pm, 4pm, 8pm, and 12am(midnight)**. I have provided sample cards for which to spot samples. Try not to touch the paper that you will be spotting as fingers contain RNase and will destroy your samples. This goes for spotting sampling and holding cards. The blood spot  should be about the size of a nickel. Try to keep collection times as close to the designated time as possible 

Note the date/time near the spot if possible. Do not use the defined circles because we need 6 samples. After collecting blood allow spot to air dry at room temperature. Do not place damp spotted card into plastic bag as this will cause increased degradation and possible contamination.

I have included an alcohol swab and a spring-loaded lancet.  Clean your finger with the alcohol pad, like you know we do, uncap the lancet, just place the it upright and press down. You can dispose of the lancets in sharps or send them back to me for disposal.

Thanks tons 

and please call me anytime (even 4am) if you have any questions.

Dianne

(918)510-0195

VITA

Dianne E. Kirk

Candidate for the Degree of

Doctor of Philosophy

Dissertation: CIRCADIAN RHYTHM EVALUATIONS TO DETERMINE BLOOD SAMPLE DEPOSITION TIME

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Biographical:

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Completed the requirements for the Doctor of Philosophy/Education in Biomedical Science at Oklahoma State University Center for Health Sciences, Tulsa, Oklahoma in May 2020.

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Experience:
Instructor of Medical Laboratory Science, Northeastern State University, Broken Arrow, Oklahoma (August 2014 to present).
Instructor of Clinical Pathology for Veterinary Technology Program, Tulsa Community College, Tulsa, Oklahoma (August 2000 to present).
Medical Technologist, Supervisor, Cancer Treatment Centers of America, Tulsa, Oklahoma (November 2001 to August 2011).
Medical Technologist Generalist/Hematology, Summa Health Systems, Akron, Ohio (September 1987 to August 1997).

Professional Memberships:
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