Circadian Medicine: Pioneering Translational Applications from Human to Veterinary Clinical Care

by

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ABSTRACT

CIRCADIAN MEDICINE: PIONEERING TRANSLATIONAL APPLICATIONS FROM HUMAN TO VETERINARY CLINICAL CARE

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Tami Martino

Circadian rhythms underlie healthy physiology and are integral to recovery from disease as circadian disruption impairs healing and results in worse long-term outcomes. The field of circadian medicine applies concepts of circadian biology to clinical practice to improve the health and recovery of patients. Recent research has highlighted several applications of circadian medicine to human health. However, circadian medicine also has several applications to animal health. Modern hospital settings result in abnormal patient exposure to light and sound at night, disturbing circadian rhythms, particularly in the intensive care unit. This is also true of veterinary clinical care settings. This thesis investigates the application of circadian medicine in companion and agricultural animals, culminating in the field of circadian veterinary medicine. Additionally, this thesis investigates the potential impact of circadian misalignment on veterinary patients and possible strategies to ameliorate this impact. We show that inappropriate exposure to light at night in veterinary intensive care settings disrupts animal circadian rhythms thereby exacerbating disease and impairing recovery. The use of red light at night presents a possible solution, as demonstrated in our findings that patient rhythms were not impaired under this protocol.

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DECLARATION OF WORK PERFORMED

I declare that I have performed all of the work presented in this thesis as listed below:

Each section of chapter 2 was written in collaboration with the co-authors listed: James R. Templeman PhD, Charlene Hanlon PhD, Anna-Kate Shoveller PhD, Gregoy Y. Bedecarrats PhD, Lee Niel PhD, Jessica Joshua BSc, David Wilcockson PhD.

Wrote first drafts of thesis chapters and incorporated editorial suggestions from committee.

Pilot light and sound intensity data in the OVC ICU and in TAS were initially collected by Jessica Joshua. I analyzed and organized these data as they are currently presented.

Non-invasive Telemetry was performed on hounds provided by Dr. Shoveller with the assistance of Dr. James Templeman, Michelina Crosbie, Tammy Bittenhuis, and Chantelle Kuhn. I was responsible for collecting, analyzing and organizing these data as they are currently presented.

Radiotelemetry surgeries were performed in collaboration with Dr. Cristine Reitz. I assisted with the surgical implantation of the radiotelemeters, and collected, analyzed and organized these data as they are currently presented.

Isolated Heart Perfusion experiments were performed by me.

qRT-PCR experiments were performed in collaboration with Janan Shoja-Doost. Janan collected and purified the RNA from the hearts. I was responsible for collecting and fixing the hearts as well as analyzing and organizing these data as presented.

Statistical Analysis was performed in collaboration with Dr. Melissa Perreault.

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LIST OF ABBREVIATIONS

Abbreviation	Definition
12D	12 hours Dark
12L	12 hours Light
16L	16 hours Light
24D	24 hours Dark
24L	24 hours Light
8D	8 hours Dark
AANAT	Arkylamine N-acetyltransferase
ACE	Angiotensin Converting Enzyme
ACTH	adenocorticotropic hormone
AHP	Animal Health Partners
AKT	Protein Kinase A
AMP	Adenosine Monophosphate
AMPK	AMP-activated protein kinase
ANOVA	Analysis of Variance
APPA	American Pet Products Association
ATP	adenosine triphosphate
AVMA	American Veterinary Medicine Association
BENCH	Benazapril in Canine Heart Disease
bHLH-PAS	basic helix loop helix-per-ARNT-SIM
BMAL1	Brain and Muscle ARNT like 1
BP	blood pressure
BR	Breathing Rate
CAHI	Canadian Animal Health Institute
CHF	Chronic Heart Failure
CK1	Caseine Kinase 1
CLOCK	Circadian Locomotor Output Cycles Kaput
CREB	cAMP Response Element Binding Protein
CRP	C-reactive protein
CRY	Cryptochrome
CRY1	cryptochrome 1
CT	clock time
DBP	diastolic blood pressure
DCM	Dilated Cardiomyopathy
dLAN	dim Light at Night
ECG	Electrocardiography
FAO	Food and Agricultural Organization
FSH	follicle stimulating hormone

GIP	gastric inhibitory peptide
GLP	glucagon-like peptide
GnIH	Gonadotropin inhibiting hormone
GnRH	Gonadotropin releasing hormone
GSK3	Glycogen Synthase Kinase 3
HIOMT	hydroxyindole O-methyltransferase
HPG	Hypothalamus Pituitary Gonadal Axis
HR	Heart Rate
ICU	Intensive Care Unit
IFN	Interferon
IL	interleukin
IL1	interluekin 1
ipRGC	intrinisically photosensitive retinal ganglion cells
LD	Light-Dark
LED	Light Emitting Diodes
LH	leutinizing hormone
LL	Light-Light
LR	Light-red light
LVDP	Left ventricular developed pressure
MAP	mean arterial pressure
MBH	mediobasal hypothalamus
ME	median eminence
MHC	Major Histocompatibility complex
MI	myocardial infarction
MMVD	Myxatomous Mitral Valve Disease
MSH	Melatonin Stimulating Hormone
MT	Melatonin
MT1	Melatonin receptor 1
MT2	Melatonin receptor 2
MV	Minute Volume
NE	norepinephrine
NLRP3	NOD-.LRR-, and pyrin domain-containing protein 3
NPY	Neuropeptide Y
NR1D1	nuclear receptor subfamily 1 group D member 1
OPN4	Opsin 4
OPN5	Opsin 5
OVC	Ontario Veterinary College

PER	Period
PER1	period 1
POMC	proopiomelanocortin
PRC	Phase Response Curve
PT	pars tuberalis
PYY	Peptide YY
RAAS	renin-angiotensin-aldosterone system
RHT	retinohypothalamic tract
RIP	respiratory inductance plethysmography
RNA	ribonucleic acid
ROR	RAR related orphan receptor
RORE	RAR related orphan receptor element
ROS	Reactive oxygen species
RT-PCR	Reverse Transcriptase-Polymerase Chain Reaction
SBP	systolic blood pressure
SCN	suprachiasmatic nucleus
StAR	steroidogenic acute regulatory protein
T_3	triiodothyronine
T_4	thyroxine
TAS	Toronto Animal Shelter
TNF	Tumor Necrosis Factor
TOR	target of rapamycin
TPH	tryptophan hydroxylase
TRF	Time-restricted Feeding
TSH	thyroid stimulating hormone
TV	Tidal Volume
UNFAO	United Nations Food and Agricultural Organization
USA	United States of America
USDA	United States Department of Agriculture
VA	Verterbate Ancient Opsin
WHO	world health organization
WT	Wildtype
ZT	zeitgeber time

CHAPTER 1: INTRODUCTION

Circadian Rhythms

Life on earth evolved in the presence of rhythmic oscillations in the environment. The most notable rhythms occur over the course of the 24-hour day, and are known as circadian, a term coined by German physiologist Franz Halberg derived from the Latin *circa* meaning "around" and *diem* meaning "day"(Halberg, 1960). In 1960, Dr. Pittendrigh defined circadian rhythms as "endogenously produced, self-sustaining biological rhythms with a period of approximately 24 hours, and which are entrained by environmental cues or zeitgebers" (Pittendrigh, 1960). Dr. Jurgen Aschoff, coined the term zeitgeber from the German *zeit* meaning "time" and *geber* meaning "giver" for environmental cues that could reset intrinsic circadian rhythms, such as light, physical activity, social interaction, and food (Aschoff, 1954).

Circadian rhythms allow organisms to anticipate changes in their environment and coordinate physiological functions accordingly. For example, photosynthetic bacteria float to the surface of the water and produce components of the photosynthetic machinery at the end of the subjective night in anticipation of sunlight(Johnson et al., 2008). Richter provided the first evidence of mammalian circadian rhythms in 1922, when rats kept in constant darkness and temperature conditions demonstrated the persistence of daily activity rhythms(Richter, 1922). In 1965, Aschoff and Wever's landmark study subjected humans to temporal isolation in an underground bunker and found that sleep-wake cycles and temperature rhythms were maintained, albeit shifted compared to rhythms in a regular light dark cycle(Aschoff, 1965). These findings confirmed that sleep-wake and temperature rhythms were intrinsically regulated and use zeitgebers such as light to reset each day.

1.1 Intrinsically photosensitive retinal ganglion cells and the retino-hypothalamic tract

Light is the main zeitgeber of circadian rhythms(Aschoff, 1954; Pittendrigh, 1960). A critical study by Foster and colleagues in 1991 demonstrated that retinally degenerate mice (rd/rd) lacking functional rods and cones maintain circadian responses to light(Foster et al., 1991). Intrinsically photosensitive retinal ganglion cells (ipRGCs) containing the photopigment melanopsin are responsible for transmitting photic information to the brain region regulating circadian rhythms – the suprachiasmatic nucleus (SCN). Melanopsin is most sensitive to short wavelength light, particularly in the blue light range (460-480 nm) as demonstrated using light stimulation in whole-cell recordings from isolated rat retinae(Berson, 2002). The axons of ipRGCs form the retino-hypothalamic tract (RHT) and monosynaptically project to the SCN upon photostimulation of melanopsin evoking action potentials in the SCN(Moore et al., 1995).

1.2 Photic transduction and entrainment

In mammals, photic signals are projected to the SCN core, where glutamate, the neurotransmitter of RHT neurons, induces phosphorylation of Ca2+-cAMP response element binding (CREB) protein, resulting in the transcription of key genes involved in circadian regulation(Berson et al., 2002; Moore et al., 1995). Phase response curves (PRCs) describe the sensitivity of the circadian mechanism to light throughout the 24-hour day in terms of the phase of the circadian period(Shanahan et al., 1999). Accordingly, photostimulation in the early subjective night causes a phase advance, causing the animal to wake up earlier the next day, while photostimulation in the late subjective night causes a phase delay, causing the animal to wake up later the next day(Reebs & Mrosovsky, 1989). Interestingly, the shape of the PRC has been shown to be remarkably similar across all living organisms recorded thus far, which strongly suggests

that the circadian clock developed very early during evolution. The modulation of the circadian period is mediated by the endogenous circadian molecular mechanism.

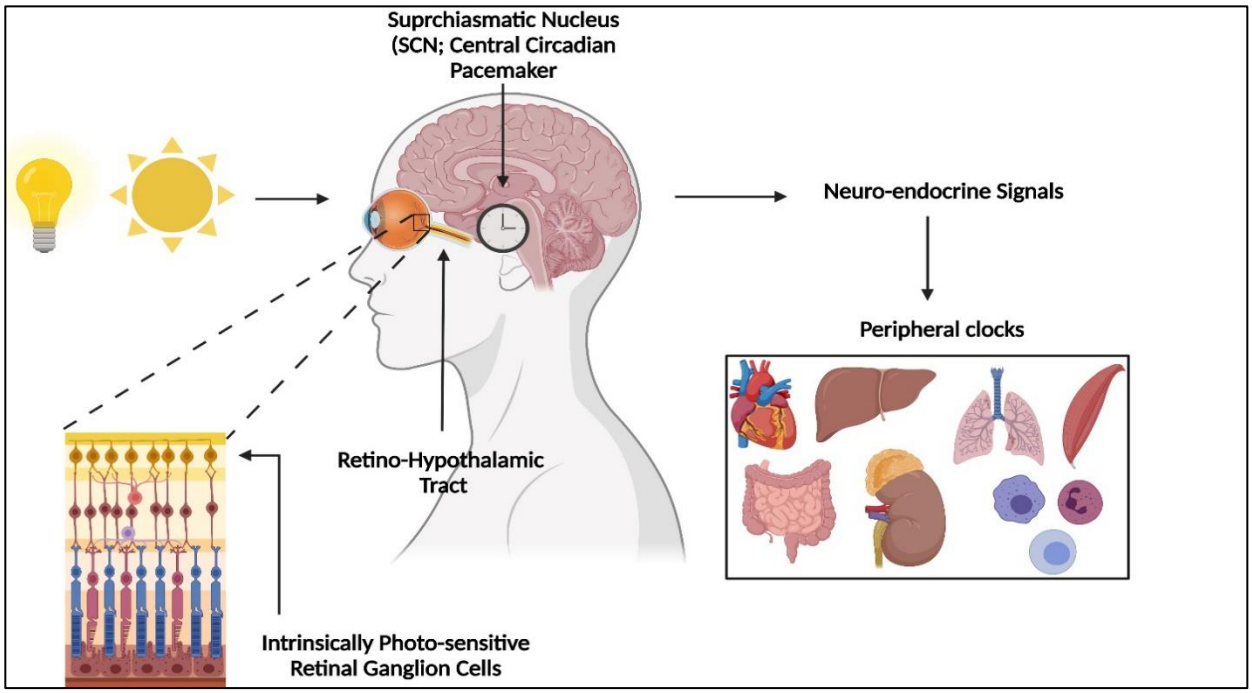


Figure 1.1 - Hierarchical Organization of the Circadian System.

Light produced by natural or artificial sources is a potent zeitgeber (time-giver) of circadian rhythms. Light information is received by intrinsically photosensitive retinal ganglion cells in the retina of the eye and transduced to the suprachiasmatic nucleus to reset the endogenous circadian mechanism. The central circadian pacemaker regulates rhythms of peripheral clocks in all tissues in the body via neuro-endocrine signals.

1.3 The circadian mechanism

The first known clock gene, *Clock*, which encodes a basic helix-loop-helix (bHLH) Period-Arnt-Sim (PAS)-type transcription factor, was cloned in 1997(King et al., 1997; Konopka & Benzer, 1971), followed by the cloning of the clock genes *Period1 (Per1)(Konopka & Benzer, 1971)* and *Brain and Muscle Arnt-like* (*Bmal1)(Hogenesch et al., 1997; Ikeda & Nomura, 1997).* BMAL1 and CLOCK proteins heterodimerize and bind to an E-box enhancer (CACGTG) site upstream of the *Per* gene, inducing *per* transcription. The PER protein translocates to the nucleus with Cryptochrome (CRY) 1 and 2(Kobayashi et al., 1998) and binds to the CLOCK/BMAL1

heterodimer inhibiting enhancer activity. Post-translational modifications by Casein Kinase 1 epsilon (CK1ε)(Camacho et al., 2001) and Glycogen Synthase Kinase 3 beta (GSK3β)(Iitaka et al., 2005) target PER and CRY for ubiquination, releasing their inhibition of CLOCK-BMAL1 transcriptional activity. One cycle of this negative feedback loop takes about 24 h. Other feedback loops have been identified to regulate circadian rhythms such as the *Bmal1* loop in which *Bmal1* expression is regulated by a retinoic acid receptor-related orphan receptor enhancer (RORE) site located upstream of the *Bmal1* gene. REV-ERBα(Dumas et al., 1994) and ROR(Giguere et al., 1994) compete to bind to this element where ROR binding activates gene expression, whereas REV-ERBα binding inhibits transcription. Each of the components of the circadian mechanism additionally induce gene expression to regulate various metabolic, transcriptional, and cellular functions(Reppert & Weaver, 2002).

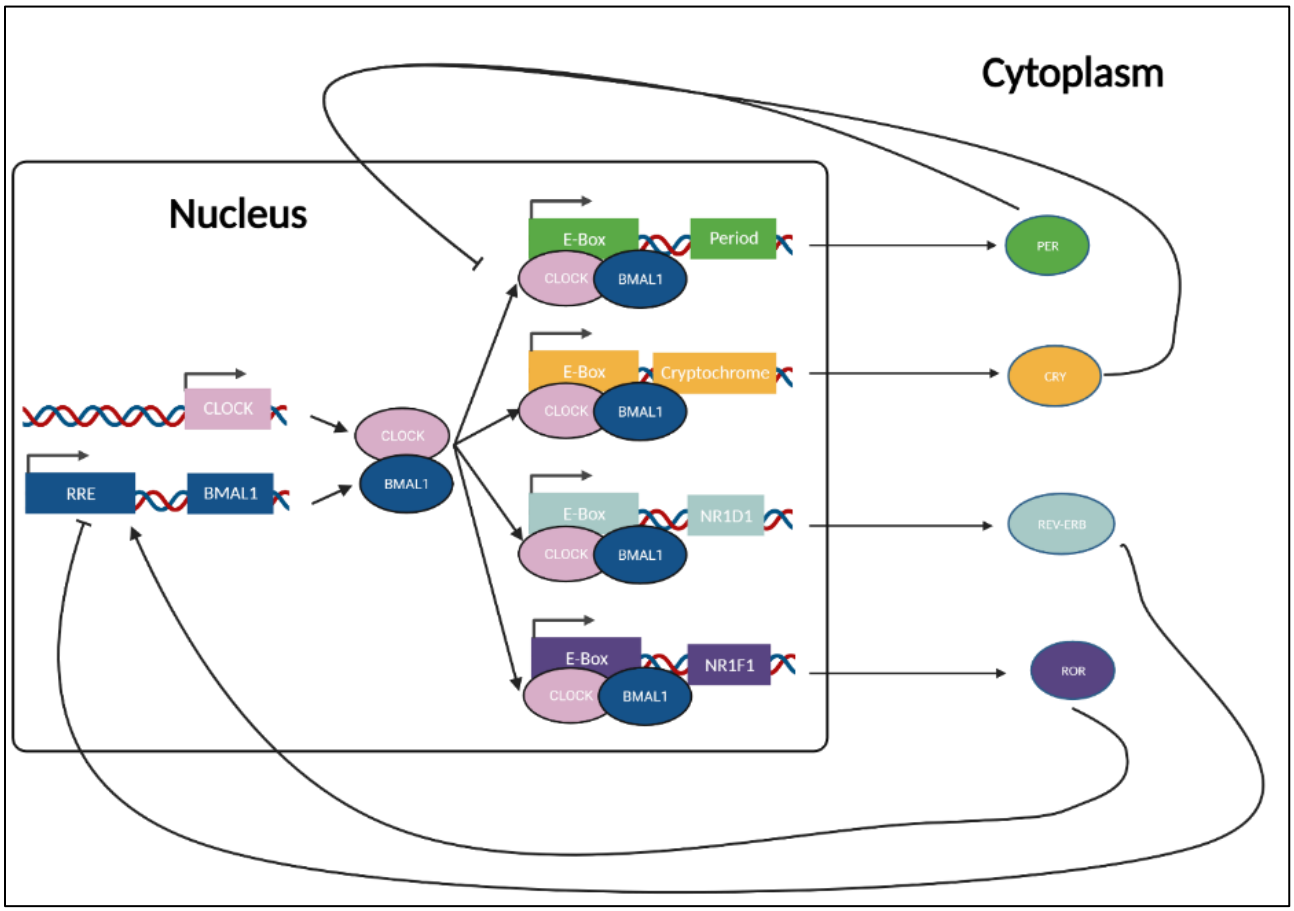


Figure 1.2 The Mammalian Molecular Circadian Mechanism

The endogenous circadian mechanism expressed in virtually all cells regulates the production of clock-controlled genes. CLOCK and BMAL1 form a heterodimer and bind to E-box sequences in the promoter regions of *period*, *cryptochrome*, *rev-erb*, and *ror*, genes to promote their transcription. PER and CRY proteins then translocate back to the nucleus to inhibit their own transcription by blocking CLOCK from binding to BMAL1. REV-ERB acts on a RORE sequence in the promoter region of *bmal1* gene to inhibit its transcription, whereas ROR acts to promote its transcription.

1.4 Peripheral clocks

The SCN plays an important role as conductor of a symphony of peripheral clocks, communicating with various tissues using neurohormonal signals. To investigate the SCN's ability to maintain circadian rhythms autonomously, Inouye and Kawamura demonstrated persistent

circadian rhythms in electrical activity from the SCN in vivo, even when surgically detached from surrounding brain areas(Inouye & Kawamura, 1979). The SCN's functional significance was further demonstrated by the ability of fetal SCN grafts to restore circadian activity rhythms in SCN-lesioned rats(Lehman et al., 1987). Cortisol and melatonin are two of the most widely studied hormones exhibiting circadian rhythms in mammals, and which act to synchronize peripheral clocks to the period of the SCN(Dibner et al., 2010; Shea et al., 2011). The circadian mechanism is ubiquitously expressed in virtually all cells investigated, each capable of endogenously producing rhythmic oscillations that are tissue specific (Florez, 1995; Ko & Takahashi, 2006; Takahashi, 2017). For example, Podobed and colleagues in 2014 described circadian rhythmicity in 7.8% of the murine circadian proteome, regulating structure and function across the 24h light-dark cycle (Podobed et al., 2014).

1.5 Circadian Rhythms in Physiology

1.5.1 Cardiovascular Physiology

Marked time-of-day dependent variations are observed in multiple cardiovascular parameters. Many of these rhythms mirror behavioural functions. Furthermore, cardiovascular function rhythms have been attributed to fluctuations in neurohormonal rhythms, although an underlying circadian component appears to play an important role. These rhythms persist when rodent hearts are perfused *ex-vivo*, suggesting that circadian variations in the neurohormonal axes act in concert with the intrinsic cardiomyocyte clock to produce rhythmic oscillations in function(Young et al., 2001). In rodents, heart rate (HR), contractility and cardiac output reach their peak during the dark or active phase when observed in vivo, or ex-vivo, consistent with sympathovagal tone. In the sarcomere, Myosin heavy chain (MHC), an important component of the contractile apparatus of cardiomyocytes, exhibits time-of-day oscillations in the rodent heart

at the transcript level in parallel with myosin ATP Ca^{2+} sensitivity, peaking during the dark phase(Wang et al., 1999; Young et al., 2001). Several other sarcomere components including MyBP-C, desmin, tropomyosin, troponins I and T, and titin cap also exhibit circadian rhythms driven by oscillation of Myosin ATP Ca^{2+} sensitivity(Podobed et al., 2014). Rhythms in Ca2+ and K+ transients have also been demonstrated in the rodent heart, with levels peaking during the light phase(Collins & Rodrigo, 2010; Yamashita et al., 2003). Collectively, these studies suggest that the cardiomyocyte clock plays an integral role in regulating cardiac function on the transcriptional, translational, and metabolic levels.

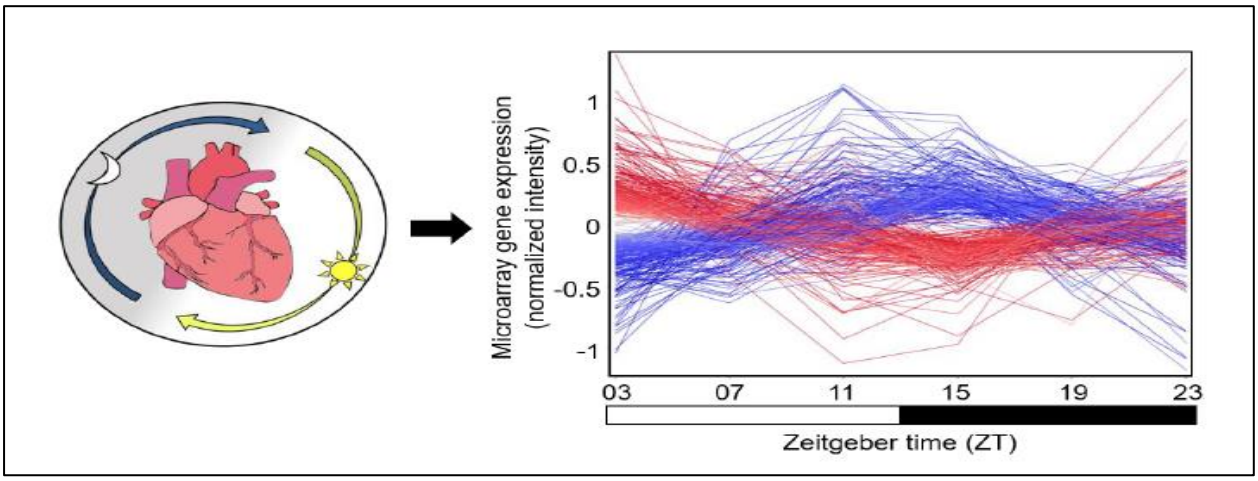


Figure 1.3 Time of day rhythms in cardiac gene expression

Hearts were collected and total RNA was isolated for downstream microarray experiments. Cardiac gene expression was assessed over 24 h using high-throughput microarrays and bioinformatics analysis. Red lines = genes up-regulated in the heart at ZT03, blue lines = genes down-regulated in the heart at ZT03.

Note: Adapted from Reitz. C.J. (2020). Circadian Medicine: The Role of the Circadian Clock Mechanism in Cardiovascular Health and Disease [Doctoral dissertation, University of Guelph].

1.5.2 Hormones

1.5.2.1 Melatonin

Melatonin is one of the most widely studied downstream targets of the circadian system due its ubiquitous functions in physiology. In mammals, the rhythmic secretion of this serotonin-

derived indole hormone from the pineal gland is driven by the circadian clock in the suprachiasmatic nucleus (SCN) of the hypothalamus (Korf & von Gall, 2006). Regardless of their diurnal or nocturnal tendencies, all mammals synthesize and release melatonin into the bloodstream at night in response to norepinephrine (NE) signals originating in the SCN in response to darkness (Challet, 2007). Light, in turn, acts to decrease the release of NE in the pineal gland, leading to the cessation of melatonin biosynthesis by N-acetyltransferase (AANAT) (Klein & Weller, 1970). Melatonin acts upon G-protein coupled receptors MT-1 and MT-2 expressed in various tissues throughout the body (Dubocovich, 2007; Slominski et al., 2012; Williams et al., 1989). Both of these receptors are expressed in the SCN, suggesting a regulatory feedback role for melatonin (Dubocovich, 2007; Hunt et al., 2001; Reppert et al., 1988). Jet lag experiments with mice that lack either MT1, MT2 or both receptors show that endogenous melatonin signals act to facilitate re-entrainment of the locomotor activity by acting on the MT2 receptor (Pfeffer et al., 2018). In addition, melatonin acts as an immune modulator, comprising both pro- and anti-inflammatory effects. Namely, the release of pro-inflammatory cytokines including several interleukins, TNFα, and IFNγ have been widely observed in monocytes, monocyte-derived cells and T-helper cells. In contrast, suppression of NLRP3 inflammasome activation has been observed under various conditions, resulting in an anti-inflammatory profile (Hardeland, 2018). Importantly, disruption of circadian rhythms, by shift-work or constant light exposure for example, result in nocturnal melatonin levels being suppressed and a loss of melatonin rhythm in rats (Rumanova et al., 2020). Overall, melatonin is a potent biomarker and effector of circadian rhythms as its production and secretion are very tightly coupled with the central circadian mechanism.

1.5.2.2 Cortisol

Cortisol, most commonly known as a stress hormone, is another important downstream target of the circadian system and is a potent biomarker of circadian rhythms. The steroid hormone, secreted from the adrenal cortex in response to stimulation by adrenocorticotropic hormone (ACTH) from the anterior pituitary, serves a number of important functions in the human body (Nicolaides et al., 2015). In addition to its role in regulating metabolic (Brillon et al., 1995), immune (Norbiato et al., 1997), muscle (Holmäng & Björntorp, 1992) and brain (de Leon et al., 1997) function, cortisol transmits the circadian message from the SCN to peripheral clocks, acting as a synchronizer (Dickmeis, 2009). In diurnal mammals, cortisol levels decrease across the waking day and are lowest near bedtime, after which they increase across the night period, reaching a peak at the time of waking (Dickmeis, 2009; Timmermans et al., 2019). Notably, exposure to light at night results in the loss of daily rhythms in cortisol production and secretion in mouse and rat models (Waite et al., 2012). However, it is still unclear if this is a result of alterations to the central clock or the peripheral clock in the adrenal cortex due to the influence of feeding rhythms (Rumanova et al., 2020). Further investigations are underway and promise to provide further insight on this phenomenon and its impact on the immune system and metabolism. In sum, cortisol is an important component of the circadian system, regulating its downstream effects and keeping peripheral clocks in tune with the central clock.

1.5.3 Inflammation

An integral component of the body's response to tissue injury or infection is the recruitment, activation and action of inflammatory cells. Several inflammatory cells including macrophages (Early et al., 2018), natural killer cells (Labrecque & Cermakian, 2015), T lymphocytes (Sutton et al., 2017), and eosinophils (Baumann et al., 2013), exhibit autonomous

clocks and display diurnal rhythmic activity. In addition, local and systemic pro-inflammatory cytokines, and inflammatory markers display rhythmic activity (Hand et al., 2016). The circadian regulation of the innate immune system suggests that susceptibility to infection may be time gated and that inflammatory responses may be affected by circadian disruption.

As mentioned in the previous section, the steroid hormone cortisol, acts as a link between the central circadian mechanism and peripheral clocks, including the immune system. In a recent study, one night of total sleep deprivation increased early morning and early evening cortisol levels as compared to participants with normal sleep. In contrast, when participants were subjected to a 24.6-hour day length for 25 days to induce circadian misalignment, cortisol levels were lower across the day while levels of the anti-inflammatory cytokine IL-10 and the pro-inflammatory cytokines TNF-α and CRP were increased (Wright et al., 2015). Previously our group showed that disruption of diurnal rhythms for as little as 5 days following a myocardial ischemia in a murine model resulted in an exacerbated inflammatory response and consequently impaired recovery (Alibhai et al., 2014). More recently, our group showed that SR9009, a REV-ERB agonist, administered for one day following myocardial ischemia-reperfusion abated the NLRP3 inflammasome and prevented progression to heart failure (Reitz et al., 2019). Therefore, in both murine and human models, circadian disruption results in inflammatory misalignment. Furthermore, targeting the circadian mechanism regulated the inflammatory response providing further evidence for the link between the circadian system and the innate immune system.

1.5.4 Autophagy

Mitochondrial dysfunction results in the formation and accumulation of reactive oxygen species (ROS) (Santos et al., 2011), which activate the NOD-, LRR- and pyrin domain-containing

protein 3 (NLRP3) inflammasome and induce NLRP3-dependent lysosomal and mitochondrial damage(Cadenas, 2018). ROS-mediated mitochondrial dysfunction also results in the release of cardiolipin(Paradies et al., 2014) which, in combination with an ineffective autophagic clearance of damaged mitochondria, leads to NLRP3-mediated activation of caspase-1 and subsequent production of IL1-β (Tschopp & Schroder, 2010). *Clock* transcriptionally coordinates the efficient removal of damaged mitochondria by directly controlling transcription of genes required for mitochondrial fission/fusion and autophagy as shown by transcriptome and gene ontology mapping in CLOCKΔ19/Δ19 mice(Rabinovich-Nikitin et al., 2021). Additionally, recent evidence suggests that circadian clock and autophagy reciprocally regulate one another (Juste et al., 2021).

While much is known about autophagy during ischemia, the role of autophagy activation during reperfusion is largely unclear. Autophagy is stimulated during reperfusion through Beclin 1-dependent mechanisms(Matsui et al., 2007). Accordingly, mice with systemic heterozygous *Beclin 1* gene deletion displayed a significant reduction in ischemic injury suggesting that *Beclin 1*-dependent upregulation of autophagy is deleterious during reperfusion(Matsui et al., 2007). Ma and colleagues reported that autophagosomes accumulate in the heart in response to reperfusion injury because of the concomitant impairment in autophagic flux and increased autophagosome formation due to Beclin 1 induction(Ma et al., 2012). Interestingly, CLOCK Δ19/Δ19 mice exhibited greater myocardial cell injury and dysfunction following ischemia-reperfusion, which is attributed to impaired activation of several genes involved in mitochondrial dynamics and autophagy (Rabinovich-Nikitin et al., 2021). In sum, autophagy in response to mitochondrial dysfunction is regulated by the circadian mechanism, and one's disruption affects the other bidirectionally.

1.6 Circadian Disruption in Human Hospitals

1.6.1 Light

The impact of the hospital environment on patients' circadian rhythms, particularly in the intensive care setting, has been clinically appreciated for some time now. As a result of around the clock care, patients in critical condition are exposed to light at night from overhead lights, monitors and other exogenous sources. One study demonstrated that between the hours of 10 pm and 6 am, lights were on for at least 30 minutes of each hour, most significantly at the beginning and end of nurses' shifts (Dunn et al., 2010). Hu et al. (2010) investigated the impact of simulated ICU light levels on healthy volunteers and found that nocturnal urinary melatonin levels were decreased, while nocturnal urinary cortisol levels were increased. Interestingly, the use of earplugs and eye-masks resulted in increased nocturnal urinary melatonin levels, but did not affect levels of urinary cortisol (Hu et al., 2010). Moreover, to assess the impact of the ICU environment on clock gene expression, a recent study studied the rhythm of *clock, bmal1*, *cry1*, and *per2* genes after 1 day and 1 week of admission to the ICU respectively. This study demonstrated that after just 1 week of ICU care, rhythmicity of all 4 genes was completely abolished (Diaz et al., 2020). While this study did not specifically measure light levels, they did mention that minimal light is allowed at night in their unit. Taken together, these studies indicate that light, an important zeitgeber, significantly impacts circadian rhythms in the ICU environment and could have consequences on patients' sleep and overall health.

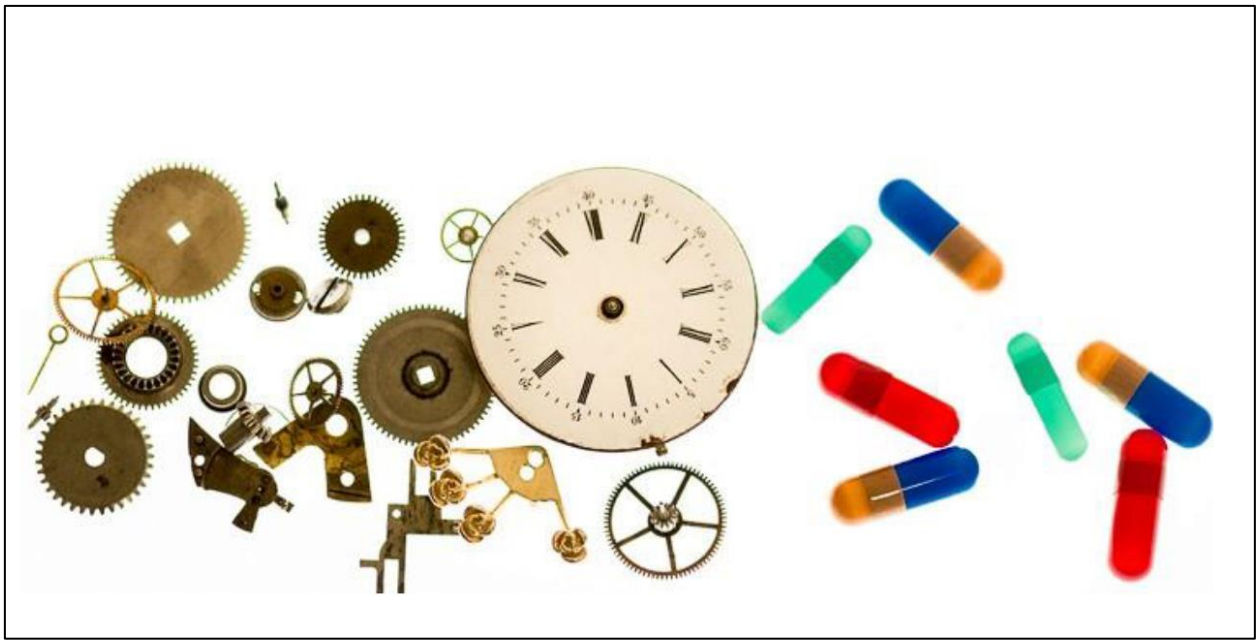
1.6.2 Noise

In addition to light, noise is a potent disruptor of sleep and circadian rhythms of patients being cared for in hospital settings. Hospitals in urban centers are exposed to noise from traffic and airplanes (Griefahn & Robens, 2008), as well as monitor alarms (Solet & Barach, 2012),

among others contributing to an environment not conducive for sleep. The noise contributed by these sources often exceeds the threshold of 35 dB recommended for healthcare settings by the WHO (Berglund et al., 1999). Presentation of common hospital noises during various sleep stages in healthy volunteers resulted in arousal as well as elevation of heart rate, particularly relevant in certain critical care settings (Buxton et al., 2012). Interestingly, even in non-critical settings, such as a child cancer ward, sound intensity levels were on average 45 dB at night, adversely affecting patients' sleep quality (Linder & Christian, 2012). Sleep and circadian rhythms are important factors for healthy recovery, and their disruption by hospital noise impacts patients in critical and acute care settings alike.

1.7 Circadian Medicine

The emerging field of circadian medicine aims to apply concepts of circadian biology clinically to benefit patient health and recovery from disease. In the past decade, our group and others have developed innovative techniques to target the circadian mechanism underlying healthy



physiology. Circadian medicine encompasses several therapeutic approaches including chronotherapy, circadian lighting and chrono-nutrition.

Figure 1.4 Circadian Medicine

Circadian medicine is an emerging field that aims to apply our basic science understanding of the role of circadian rhythms in physiology and pathophysiology into how we practice medicine, in order to develop new approaches to benefit patients.

Photo by Dr. Tami Martino, August 2019.

1.7.1 Chronotherapy

The term chronotherapy refers to timing therapies and treatments to correspond with the endogenous circadian clock to maximize efficacy (Sulli et al., 2018). By considering the circadian rhythms of drug pharmacokinetics and of the target tissue, the effects of the drug may be more or less potent at certain times of the day. Several studies have highlighted the translational benefit of this concept in the treatment of cardiovascular disease. For example, evening administration of short-acting angiotensin converting enzyme (ACEi) quinapril, a common drug for the treatment of hypertension, was shown to be more effective at reducing 24 h blood pressure as compared to morning dosing (Palatini et al., 1992). Additionally, administration of aspirin at bedtime showed greater efficacy in reducing morning platelet reactivity in healthy subjects, suggesting that aspirin being taken in the evening may mitigate the observed increased cardiovascular event risk in the morning (Bonten et al., 2014). These clinical findings have been supported by data from experimental rodent models demonstrating the benefits of chronotherapy. Martino *et al.* demonstrated that sleep time administration of short-acting ACEi captopril led to less adverse cardiac remodeling as compared to wake-time administration independent of any differences in

blood pressure reduction using a murine pressure overload-induced cardiac hypertrophy model (Martino et al., 2011)

Chronotherapy could potentially be applied to several other drug classes, considering over 50% of the top 100 best-selling drugs in the United States target circadian gene products (Zhang et al., 2014). Surprisingly however, chronotherapy is not widely applied clinically, as demonstrated by a large scale investigation of hospital treatment which found that drugs were largely administered at specific times of day that did not correspond to any clinical benefit but rather to operational rhythms (Ruben, Francey, et al., 2019). Collectively, these studies show that chronotherapy is an important consideration in the treatment of cardiovascular diseases, as well as other pathophysiology. **Chapter 2** of this thesis discusses the implementation of chronotherapy to current drug treatments for companion animals.

1.7.2 Circadian Lighting

Artificial lighting, a ubiquitous feature of our modern world, exposes us to light well beyond the natural cycle of day and night. While this has allowed for a more productive society, it has also imposed significant consequences on our health. One example of this is in shift-workers, a group which encompasses 1 in 6 Americans (Archer et al., 2014) and approximately 30% of Canadians (Shields, 2002). Shift-workers have unpredictable schedules and are often required to work night shifts and day shifts in the same week, resulting in circadian misalignment. Simulated night shift work protocols revealed that circadian misalignment reduces the percentage of rhythmic transcripts in the blood of healthy non-shift workers, and reduces the amplitude of rhythmic transcripts from peripheral blood mononuclear cells (Resuehr et al., 2019). Moreover, as mentioned in the previous section, nocturnal light is a prevalent problem affecting patient recovery

in the ICU (Craig & Mathieu, 2018; Knauert et al., 2019). Previously we demonstrated that just 5 days of circadian desynchrony following a myocardial infarction in a murine model resulted in adverse cardiac remodeling and worse long-term outcomes (Alibhai et al., 2014). Several investigations in human clinical settings aim to implement circadian lighting to support patient recovery (Engwall et al., 2017; Fan et al., 2017; Katrina N. Leyden & Smolensky, 2015; Knauert et al., 2019; Patel et al., 2014). **Chapter 2** of this thesis discusses several applications of circadian lighting to improve companion and agricultural animal health and welfare. **Chapter 3** of this thesis discusses the implementation of circadian lighting protocols in veterinary clinical care settings.

1.7.3 Chrono-nutrition

In addition to extending the hours of the day in which we can stay active, our modern world extends the timeframe of eating and reduces fasting time. Recent observations have suggested that this lengthened eating period may contribute to the onset of chronic diseases including diabetes, obesity and heart failure. Recently, loss of food intake diurnal rhythm was shown to coincide with non-dipping blood pressure (BP) in diabetic *db/db* mice, and that imposing a food intake diurnal rhythm using a time-restricted feeding protocol, prevented the development of non-dipping BP, and restored BP dipping in mice that already have developed non-dipping blood pressure (Hou et al., 2021). Non-dipping BP is prevalent in type-2 diabetic patients (Mahabala et al., 2013) and is associated with left-ventricular hypertrophy (Cuspidi et al., 2012). Our group recently demonstrated that disrupting circadian rhythms of gut microbiota using a $Clock^{\Delta 19/\Delta 19}$ murine model resulted in impaired cardiac repair following myocardial infarction. Interestingly, imposing time-restricted feeding prior to myocardial infarction resulted in improved cardiac healing (Mistry et al., 2020). Clinically, these studies could address the pandemic of obesity and diabetes, which

often result in cardiovascular diseases. **Chapter 2** of this thesis discusses the application of chrono-nutrition to address these chronic diseases in companion animals.

1.8 Study Rationale

Intensive care units are an integral part of modern healthcare, caring for patients in critical conditions. Around-the-clock care is necessary for proper care in ICUs, inadvertently resulting in patients being exposed to light and sound at night. Previous studies demonstrate the impact of circadian misalignment on recovery from disease using genetic or environmental models. Recently, human hospitals have begun implementing systemic changes to lighting systems in the hospital to limit disruption of patient rhythms. However, few studies investigate circadian disruption in veterinary hospitals. In my thesis, I investigate i) whether there is light and sound at night in veterinary hospitals, ii) whether exposure to light at night disrupts circadian rhythms in diurnal and nocturnal animal models, and iii) whether disruption of circadian rhythms affects recovery from disease.

Hypothesis

Light and sound at night in veterinary clinical care settings disrupt patient circadian rhythms and adversely affect recovery from disease.

Objectives

1. *To determine whether patients in veterinary clinical care settings are exposed to abnormal levels of light and sound at night.*

Patients in human hospitals are known to be exposed to light and sound at night as a result of frequent patient-staff interactions, monitoring systems and computers. To determine if veterinary clinical care settings are exposed to light and sound at night, I measured light and sound intensity levels over a 2-week period in several areas throughout the intensive care unit and general wards

of 2 veterinary hospitals and an animal shelter. I compared the levels of light and sound intensity levels at night to a control area.

2. *To investigate the impact of light at night on circadian rhythms of key physiological parameters.*

The circadian system drives 24 h rhythms in gene expression and plays a critical role in regulating circadian activity, heart rate, blood pressure and breathing rate rhythms. To determine the impact of light at night on circadian rhythms of a diurnal animal, I exposed dogs to 3 different lighting conditions; 12 h L: 12 h D (LD), 24 h L (LL), 12 h L: 12 h red L (LR). To measure the impact of these conditions, I used electrocardiography, and respiratory inductance plethysmography for 48 hours. To confirm these effects, I repeated these conditions in mice and measured activity using running wheel actigraphy and monitored heart rate and blood pressure using implanted radiotelemetry.

3. *To determine whether circadian misalignment impacts recovery from disease.*

Circadian rhythms underlie healthy physiology and regulate responses to disease. To determine the impact of circadian disruption by light at night on recovery from disease, I exposed mice to each of the above lighting conditions for 2 weeks before extracting their hearts and subjecting them to hypoxia-reoxygenation injury using an isolated perfused heart system in langendorff mode. To investigate the impact of these lighting conditions on gene expression of the autophagy pathway involved in myocardial healing, I used quantitative real time polymerase chain reaction on the hearts after reoxygenation. Mice were used for these experiments as we could not perform

them using patients in the OVC ICU, nor could we extract the hearts of the hounds used in the previous experiment.

CHAPTER 2: CIRCADIAN DISRUPTION BY LIGHT AT NIGHT IN VETERINARY INTENSIVE CARE UNITS IMPAIRS RECOVERY FROM DISEASE

Based on the publication:

FARAG HI, JOSHUA J, REITZ CJ, SHOJA-DOOST J, PERREAULT ML, SHOVELLER AK, BERSENAS A, NIEL L, MARTINO TA. Circadian Disruption by Light at Night in the Veterinary Intensive Care Unit Impairs Recovery from Disease. In preparation for Science Translational Medicine, 2022.

Background

The concept of intensive care units (ICUs) arose from the 1952 polio epidemic in Copenhagen, when over 300 patients required artificial ventilation for several weeks. As a result, mortality from polio in Copenhagen dropped from 80% to 40%(Lassen, 1953). By the 1960's and 1970's, ICUs had been established in the UK. They were beginning to take hold in the United States, thanks to Max Harry Weil, who is widely considered to be the "father of modern intensive care"(Kelly et al., 2014). In the past decade, the ability to temporarily support the function of multiple organ systems in critical condition has become the cornerstone of intensive care medicine. As a result, medical interventions for intensive care patients with fragile physiology are more numerous and invasive than those in general ward settings(Kelly et al., 2014). Critically ill patients are therefore increasingly susceptible to sleep deprivation and disturbances than their counterparts in general wards as a result of increased noise(Xie et al., 2009), patient care interactions(Gao & Knauert, 2019), mechanical ventilation(Frisk et al., 2004), artificial light(Olofsson et al., 2004), and stress(Dunn et al., 2010). Moreover, exposure to persistent light and sound disrupt the link between environmental rhythms and patients' endogenous circadian rhythms(Diaz et al., 2019).

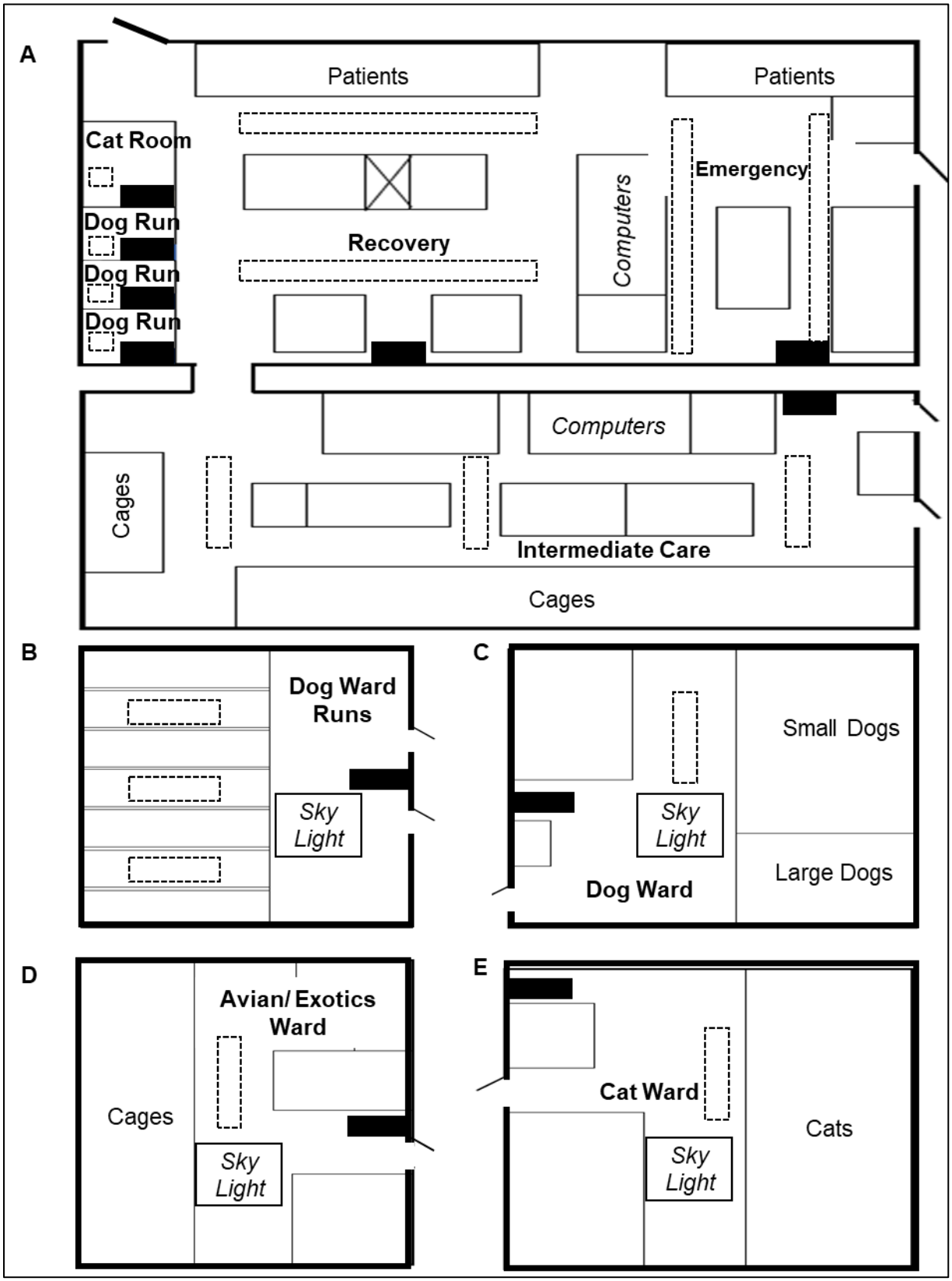
The discovery of the circadian mechanism was recognized by the Nobel Prize in Physiology or Medicine in 2017. Research is now focused on the translation of circadian biology to clinical medicine as circadian rhythms are integral for healthy physiology and recovery from disease. The circadian mechanism is a molecular transcription/translation feedback loop driven by discrete 24-h oscillations between CLOCK and BMAL1 (positive arm), PERIOD and CRYPTOCHROME (negative arm), and others (Aschoff, 1989; Ko & Takahashi, 2006; Reppert & Weaver, 2002). The circadian mechanism integrates external cues or zeitgebers to synchronize the suprachiasmatic nucleus (SCN), or "central clock" to the environment(Wood et al., 2020). In

the retina of the eye, intrinsically photosensitive retinal ganglion cells (ipRGCs) expressing the photoreceptor melanopsin, which is most sensitive to blue-spectrum light(Panda et al., 2005), are responsible for transmitting environmental light information to the SCN via the retino-hypothalamic tract(Moore et al., 1995). The master clock regulates physiological rhythms by synchronizing peripheral clocks around the body(Honma, 2018). In the last two decades, evidence for circadian oscillations of components of the immune system has emerged, potentially impacting disease onset and therapies. Previously, we demonstrated that diurnal rhythm disruption immediately after myocardial infarction impaired healing and exacerbated maladaptive cardiac remodelling(Alibhai et al., 2014).

Several studies have documented the presence of light at night in ICU and general ward areas in human hospitals and its impact on endogenous circadian rhythms(Diaz et al., 2020; Engwall et al., 2017). Namely, light at night results in disrupted expression of the clock mechanism (Diaz et al., 2020). While the effects of circadian disruption on human patients have been evaluated extensively in recent years, little attention has been given to its effects on companion animal patients. Given that veterinary hospitals operate in a similar manner to human hospitals, we hypothesize that veterinary patients in critical condition may have analogous reactions to stimuli disrupting their circadian rhythms. Here, we demonstrate the presence of abnormal light and sound at night in veterinary care areas and investigate the impact of circadian disruption on healthy responses in dogs, and disease physiology in a mouse model.

2.1 Results

To investigate the presence of abnormal light and sound at night in veterinary care settings, we measured the levels of light intensity (lux) and sound intensity (dB) using dataloggers over the span of two weeks. To best support physiology, sleep and wakefulness, a recent study has recommended indoor light exposure levels of 250 lux during the day, 10 lux during the evening and 1 lux at night (Brown et al., 2022). Similarly, the WHO has recommended that sound intensity levels do not exceed 35 dB at night in hospital settings (Berglund et al., 1999). Dataloggers were placed at patients' eye level throughout several areas in the Ontario Veterinary College (OVC) intensive care unit (ICU) **(Fig 2.1A)**, and wards **(Fig 2.1B-D)**, Animal Health Partners (AHP) veterinary hospital **(Fig 2.1F-G)** and the Toronto Animal Shelter **(Fig. 2.1H-I)**. For each of these clinical settings, a control room was chosen based on the recommended light and sound exposure parameters above. The control areas in each of the settings had maximum light intensity levels of 10 lux and maximum sound intensity levels of 15 dB between 8PM – 6 AM.Services Shelter **(Fig. 2.1H-I)**. In addition to the light provided by fluorescent white light fixtures in all areas, extraneous light was emitted by computer monitors, and sky lights. Sources of noise included staff conversations, monitor alarms, and ventilation systems.


A
Patients
Patients
Cat Room
Dog Run
Dog Run
Dog Run
Recovery
Computers
Emergency
Computers
Cages
Intermediate Care
Cages
B
Dog Ward Runs
Sky Light
C
Small Dogs
Sky Light
Large Dogs
Dog Ward
D
Avian/ Exotics Ward
Cages
Sky Light
E
Cat Ward
Cats
Sky Light

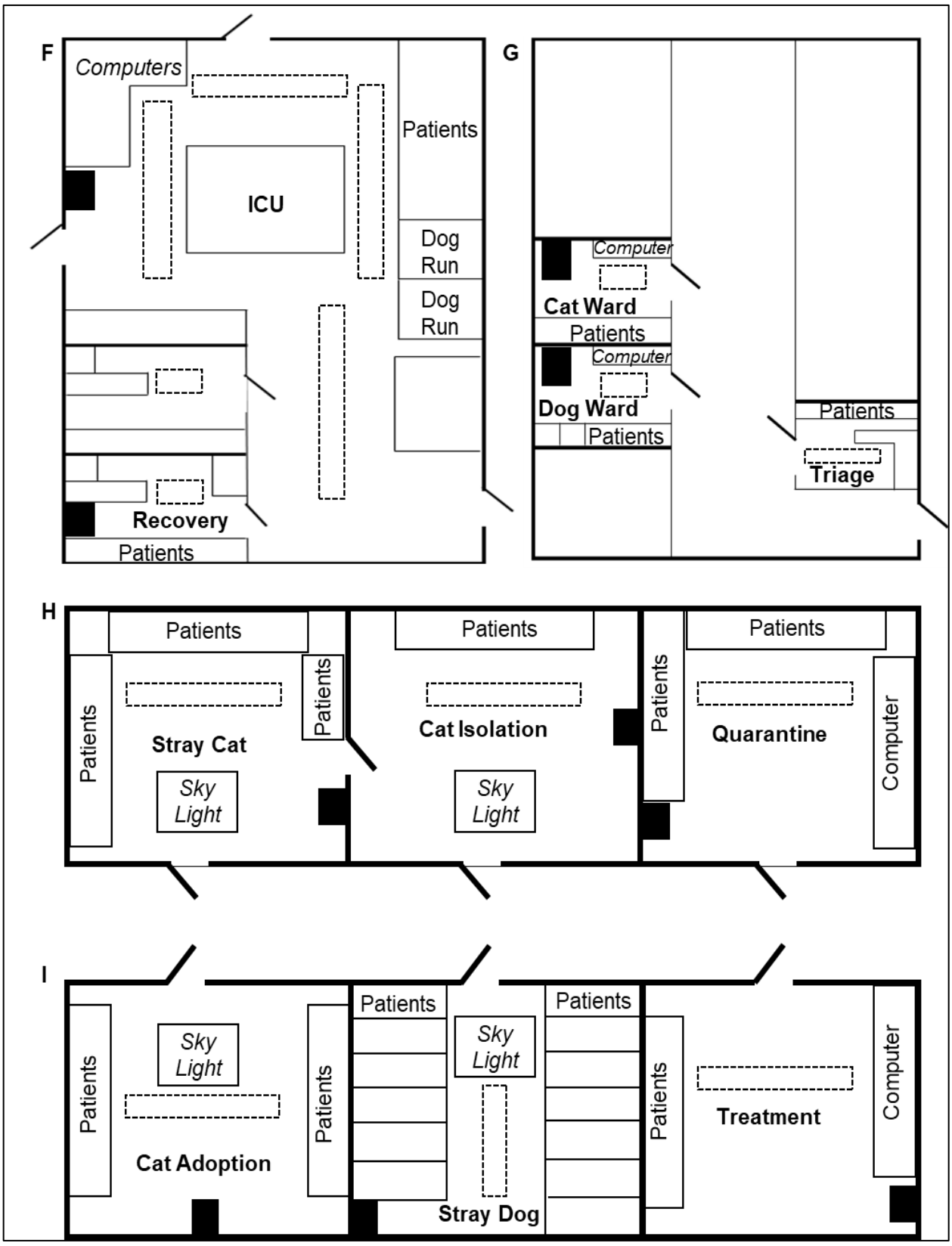

F
Computers
Patients
ICU
Dog Run
Dog Run
Recovery
Patients
G
Computer
Cat Ward
Patients
Computer
Dog Ward
Patients
Patients
Triage
H
Patients
Patients
Stray Cat
Sky Light
Patients
Patients
Cat Isolation
Sky Light
Patients
Patients
Quarantine
Computer
I
Patients
Sky Light
Cat Adoption
Patients
Patients
Sky Light
Stray Dog
Patients
Patients
Treatment
Computer

Figure 2.1 Schematic Layout of Veterinary Clinical Care Centres

Layout of Ontario Veterinary College (**A)** Emergency, Recovery and Intermediate care areas in the intensive care unit. **(B)** Dog ward runs. **(C)** Dog ward. **(D)** Avian/Exotics Ward. **(E)** Cat Ward. (**F) Layout of Animal Health Partners Hospital** including Intensive care unit and recovery area, and **(G)** Triage, dog and cat ward. **(H) Layout of Toronto Animal Shelter including** Cat isolation and stray housing, and quarantine rooms, and **(I)** Cat adoption, stray dog and treatment rooms. Black squares denote location of data loggers, which are placed at the animals' eye level. Dotted lines denote sources of light.

First, we looked at light intensity levels from the hours of 8 pm to 6 am. In the OVC ICU, all areas demonstrated significantly different levels of light intensity at night throughout the week in comparison to one another by repeated measures ANOVA ($F(5,63)=22.4$, $p<0.001$). The areas with the greatest fold-change of light at night compared to control were the recovery (8.54 ± 1.06**,** $p<0.001$) and intermediate care (7.21 ± 0.82**,** $p<0.013$) areas, where patients in critical condition are kept for observation (**Fig. 2.2A*ii*)**. In the OVC wards, all areas demonstrated significantly different levels of light intensity at night throughout the week by in comparison to one another by repeated measures ANOVA ($F(4,89)=2.8$, $p<0.001$). The cat ward (1.83 ± 0.30**,** $p<0.001$) and ward dog runs (2.04 ± 0.32, $p<0.001$) demonstrated the greatest fold change of light at night compared to control **(Fig. 2.2B*ii*)** during the weekend. In the AHP, most areas demonstrated significantly increased levels of light at night throughout the week in comparison to one another by repeated measures ANOVA ($F(5,138)=43.0$, $p<0.001$). The area with the greatest fold-change of light at night compared to control was the triage area (11.60 ± 0.45**,** $p<0.001$) **(Fig. 2.2C*ii*)**. Finally, in the TAS, no areas investigated demonstrated light exposure at night throughout the week. This is likely because the shelter is only staffed from 8 AM to 5 PM each day **(Fig. 2.2D*ii*)**.

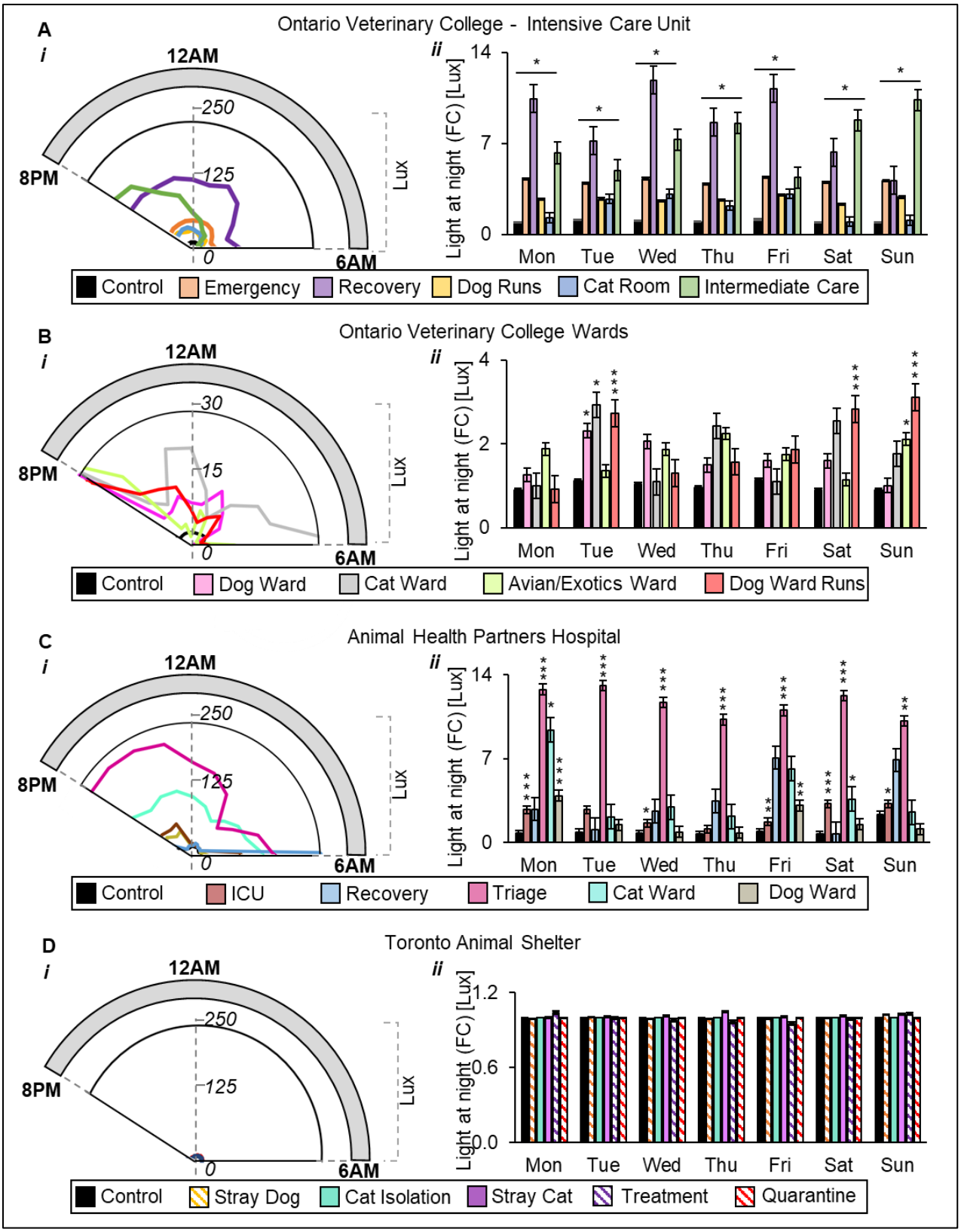

A
Ontario Veterinary College - Intensive Care Unit
i
12AM
250
125
0
8PM
6AM
Lux
ii
Light at night (FC) [Lux]
14
7
0
Mon
Tue
Wed
Thu
Fri
Sat
Sun
Control
Emergency
Recovery
Dog Runs
Cat Room
Intermediate Care
B
Ontario Veterinary College Wards
i
12AM
30
15
0
8PM
6AM
Lux
ii
Light at night (FC) [Lux]
4
2
0
Mon
Tue
Wed
Thu
Fri
Sat
Sun
Control
Dog Ward
Cat Ward
Avian/Exotics Ward
Dog Ward Runs
C
Animal Health Partners Hospital
i
12AM
250
125
0
8PM
6AM
Lux
ii
Light at night (FC) [Lux]
14
7
0
Mon
Tue
Wed
Thu
Fri
Sat
Sun
Control
ICU
Recovery
Triage
Cat Ward
Dog Ward
D
Toronto Animal Shelter
i
12AM
250
125
0
8PM
6AM
Lux
ii
Light at night (FC) [Lux]
1.2
0.6
0.0
Mon
Tue
Wed
Thu
Fri
Sat
Sun
Control
Stray Dog
Cat Isolation
Stray Cat
Treatment
Quarantine

Figure 2.2 Abnormal Light at Night in Veterinary Clinical Care Centres

(A) *i)* Radar plot of light intensity (lux) levels of 5 areas in the Ontario Veterinary College (OVC) Intensive Care Unit (ICU) between 8 PM and 6 AM, *ii)* bar graph of fold change of light intensity at night compared to control of each area throughout the week (right). **(B)** *i)* Radar plot of lux levels of 4 areas in the OVC wards between 8 PM and 6 AM, *ii)* bar graph of fold change of light intensity at night compared to control of each area throughout the week. **(C)** *i*) Radar plot of light intensity (lux) levels of 5 areas in the Animal Health Partners hospital between 8 PM and 6 AM, *ii)* bar graph of fold change of light intensity at night compared to control of each area throughout the week. **(D)** *i)* Radar plot of light intensity (lux) levels of 5 areas in the Toronto Animal Shelter between 8 PM and 6 AM, *ii)* bar graph of fold change of excess light intensity at night compared to control of each area throughout the week. Each area is represented by a different colour as denoted by the legends. Underlined * represents significant difference ($p<0.05$) across all groups on that day by mixed-effects ANOVA. * over a bar represents $p<0.05$, ** over a bar represents $p<0.01$, *** over a bar represents $p<0.001$ as compared to control by mixed effects ANOVA.

Next, we investigated sound intensity levels from the hours of 8 pm to 6 am throughout all the areas in which the study was conducted as displayed in **(Fig. 2.3A-D*i*)**. In the OVC ICU, all areas demonstrated significantly different levels of sound intensity at night throughout the week in comparison to one another by repeated measures ANOVA ($F(5, 63)=532.5$, $p<0.001$). On average, all areas had sound intensity levels 1.5 times greater than control at night **(Fig. 2.3A*ii)***. In the OVC wards, all areas demonstrated significantly different levels of sound intensity at night throughout the week in comparison to one another by repeated measures ANOVA ($F(4,89)=82.6$, $p<0.001$). On average, sound intensity levels were 1.4-2.1 times greater than control at night **(Fig. 2.3 B*ii)*.** In the AHP, all areas demonstrated significantly different levels of sound intensity at night throughout the week in comparison to one another by repeated measures ANOVA ($F(5,140)=464.1$, $p<0.001$). Except for the dog and cat wards, sound intensity levels were on average 1.5 times greater than control at night **(Fig. 2.3C*ii)*.** Finally, in the TAS, all areas demonstrated significantly different levels of sound intensity at night throughout the week in comparison to one another by repeated measures ANOVA ($F(5,66)=247.1$, $p<0.001$).

Interestingly, sound intensity levels were higher at night in all areas compared to control **(Fig. 2.3D*ii)*.**

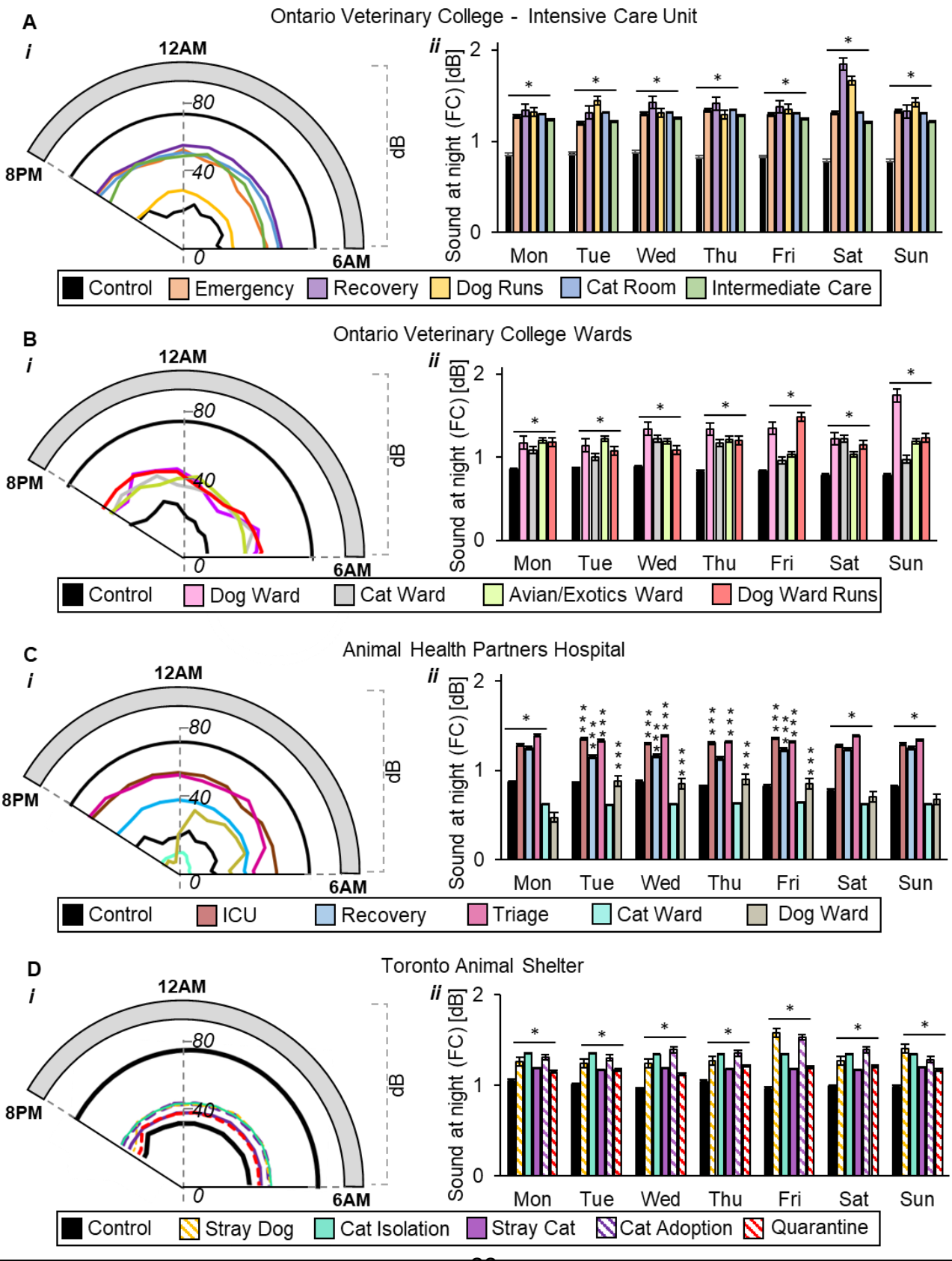

A
Ontario Veterinary College - Intensive Care Unit
i
12AM
8PM
6AM
80
40
0
dB
ii
Sound at night (FC) [dB]
2
1
0
Mon
Tue
Wed
Thu
Fri
Sat
Sun
Control
Emergency
Recovery
Dog Runs
Cat Room
Intermediate Care
B
Ontario Veterinary College Wards
Control
Dog Ward
Cat Ward
Avian/Exotics Ward
Dog Ward Runs
C
Animal Health Partners Hospital
Control
ICU
Recovery
Triage
Cat Ward
Dog Ward
D
Toronto Animal Shelter
Control
Stray Dog
Cat Isolation
Stray Cat
Cat Adoption
Quarantine

Figure 2.3 Abnormal Sound at Night in Veterinary Clinical Care Centres.

(A) *i)* Radar plot of sound intensity (dB) levels of 5 areas in the Ontario Veterinary College (OVC) Intensive Care Unit (ICU) between 8 PM and 6 AM, *ii)* bar graph of fold change of sound intensity at night compared to control of each area throughout the week (right). **(B)** *i)* Radar plot of sound intensity levels of 4 areas in the OVC wards between 8 PM and 6 AM, *ii)* bar graph of fold change of sound intensity at night compared to control of each area throughout the week. **(C)** *i*) Radar plot of sound intensity (dB) levels of 5 areas in the Animal Health Partners hospital between 8 PM and 6 AM, *ii)* bar graph of fold change of sound intensity at night compared to control of each area throughout the week. **(D)** *i)* Radar plot of sound intensity (dB) levels of 5 areas in the Toronto Animal Shelter between 8 PM and 6 AM, *ii)* bar graph of fold change of excess sound intensity at night compared to control of each area throughout the week. Each area is represented by a different colour as denoted by the legends. Underlined * represents significant difference ($p<0.05$) across all groups on that day by mixed-effects ANOVA. * over a bar represents $p<0.05$, ** over a bar represents $p<0.01$, *** over a bar represents $p<0.001$ as compared to control by mixed effects ANOVA.

Since several areas in the ICU and wards demonstrated significantly high levels of light at night, we investigated the effect of these lighting conditions experimentally on healthy dogs using non-invasive telemetry. **Fig. 2.4A** shows Freddie (dog *1)* equipped with the complete EMKA telemetry system including electrocardiography (ECG) leads and respiratory inductance plethysmography (RIP) band covered by a jacket carrying a transmitter, Ringo (dog *2)* wearing the ECG leads and Elton (dog *3)* wearing the full EMKA telemetry system in addition to a cervical collar while on a walk. First, we demonstrated the circadian rhythmicity of a highly rhythmic physiology parameter, heart rate, under normal light: dark (LD) conditions. However, when dogs were kept under light at night, circadian rhythms in heart rate were disrupted. For dog 1, exposure to light at night resulted in an early increase of heart rate at the end of the first night, higher heart rate during the day and a delayed decrease in heart rate at the beginning of the second night **(Fig. 2.4B).** For dog 2, exposure to light at night resulted in a later increase of heart rate at the end of the second night, a delayed acrophase during the day, and a delayed decrease of heart rate at the

beginning of the second night **(Fig 2.4B)**. For dog 3, exposure to light at night resulted in an early increase of heart rate at the end of the first night, higher heart rate during the day and an attenuated decrease in heart rate at the beginning of the second night **(Fig. 2.4B).** Next, to investigate strategies to mitigate circadian disruption by light at night, we used red light during the dark period, as melanopsin expressing retinal ganglion cells are least sensitive to light in the red-light spectrum. For dog 1, this resulted in a delayed increase in heart rate at the end of the second night, lower heart rate during the day and throughout the second night compared to LD **(Fig. 2.4B*).*** For dog 2, red light at night resulted in an early increase in heart rate at the end of the second night and an early decrease in heart rate at the beginning of the second night compared to LD **(Fig. 2.4B).** Lastly, for dog 3, red light at night resulted in a diurnal rhythm of heart rate mirroring the rhythm under LD conditions **(Fig. 2.4B).** Moreover, light at night resulted in a 25 beats/min (bpm) increase in peak heart rate as compared to LD during the light period (**Fig. 2.4C).** Moreover, red light at night seems to be effective at normalizing day-night rhythms of heart rate to those observed under normal light: dark conditions. Additionally, light at night resulted in a 1-hour phase shift in peak heart rate as compared to LD **(Fig. 2.4D).**

Next, we investigated the effect of light at night and red light at night on a second rhythmic physiological parameter, breathing rate. For dog 1, light at night resulted in increased breathing rate throughout the second night of recording as compared to LD**,** whereas under red light at night, this effect was ameliorated **(Fig. 2.4E).** For dog 2, light at night also resulted in increased breathing rate throughout the second night as compared to LD**,** whereas under red light at night, breathing rate remained low **(Fig. 2.4E).** Lastly, for dog 3, light at night resulted in increased breathing rate throughout the second night as compared to LD, whereas red light at night resulted in slightly lower breathing rate as compared to LD **(Fig. 2.4E).** Moreover, light at night resulted in a

significant ($p<0.001$) increase of 16% in breathing rate at night compared to LD, while red light at night returned breathing rate at night to normal as measured by area under the curve **(Fig. 2.4F).**

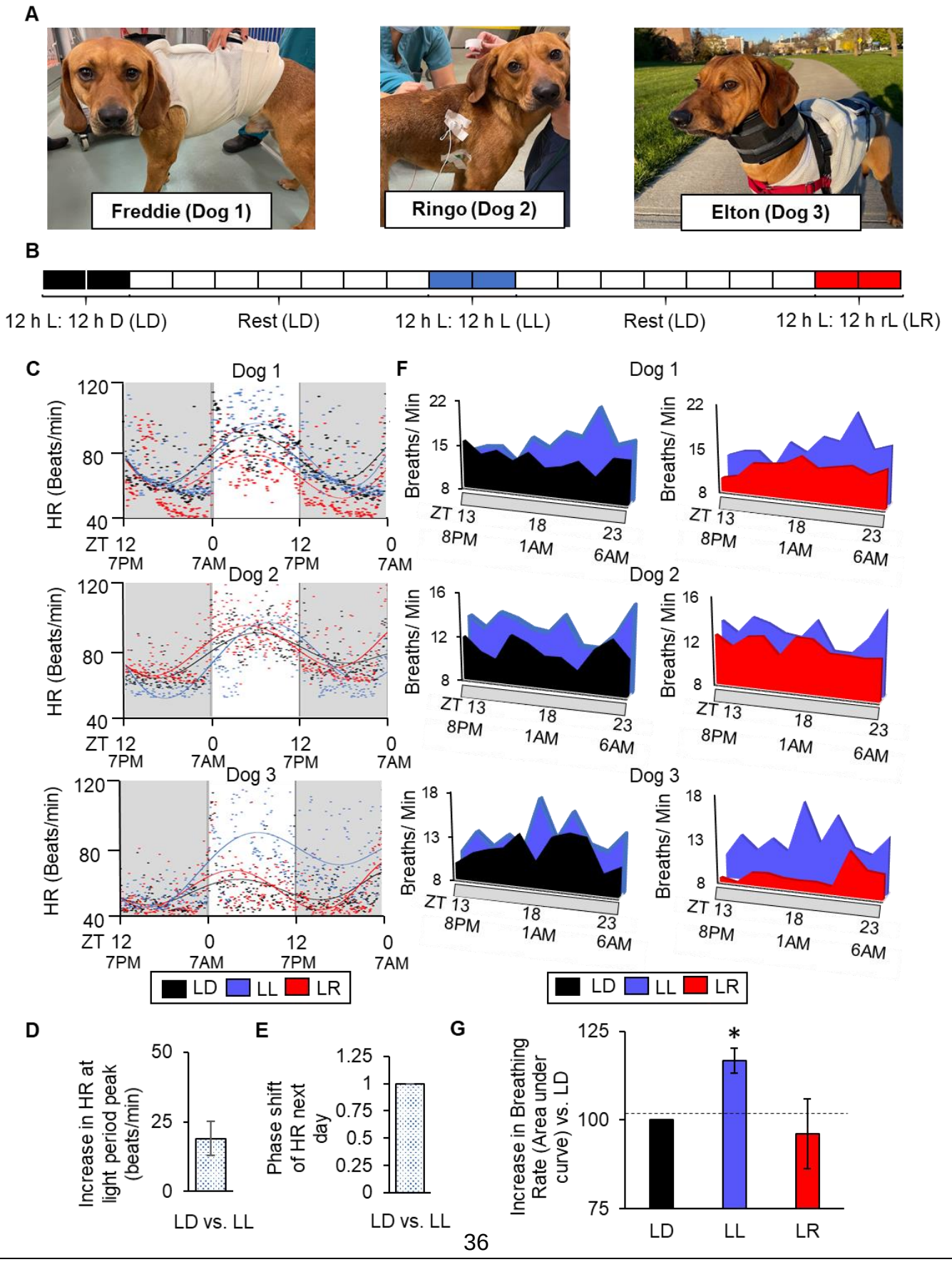

A
Freddie (Dog 1)
Ringo (Dog 2)
Elton (Dog 3)
B
12 h L: 12 h D (LD)
Rest (LD)
12 h L: 12 h L (LL)
Rest (LD)
12 h L: 12 h rL (LR)
C
Dog 1
Dog 2
Dog 3
HR (Beats/min)
120
80
40
ZT 12
0
12
0
7PM
7AM
7PM
7AM
LD
LL
LR
F
Dog 1
Dog 2
Dog 3
Breaths/ Min
ZT 13
18
23
8PM
1AM
6AM
D
Increase in HR at light period peak (beats/min)
50
25
0
LD vs. LL
E
Phase shift of HR next day
1.25
1
0.75
0.5
0.25
0
LD vs. LL
G
Increase in Breathing Rate (Area under curve) vs. LD
125
100
75
*
LD
LL
LR

Figure 2.4 Physiological responses to circadian disruption by light at night in a diurnal animal.

(A) Pictures of 3 hound dogs used for this study, Freddie (left) wearing full EMKA telemetry equipment in the hound colony room, Ringo (middle) with electrocardiography (ECG) leads adhered to the skin surrounding his thoracic cage, and Elton (right) wearing EMKA telemetry equipment and cervical collar while out on a walk. **(B)** Heart rate (beats/min) of Dog 1, 2 and 3 measured over a 36-hour period in 3 different lighting conditions: 12 hours(h) Light(L): 12 h Dark (D) (LD); 12 h L: 12 h L (LL); and 12 h L: 12 h red L (LR) **(C)** Change in amplitude of heart rate peak during the light period under LD and LL conditions. **(D)** Phase shift of HR peak under LL conditions as compared to LD conditions. (**E)** Breathing rate of Dog 1, 2 and 3 under LD, LL and LR conditions recorded during the dark/rest period from ZT13 (8 PM) to ZT23 (6 AM). **(E)** Area under curve of breathing rate during the dark/rest period under LD, LL and LR conditions.

Given that light at night disturbs the circadian physiology of dogs, we sought to investigate the effect of light at night on the circadian physiology of nocturnal mice, as an additional animal model allowing more invasive manipulations. **Fig. 2.5A** shows representative running wheel actigraphy under normal light: dark (LD) for 7 days and constant light (LL) conditions for 14 days. Consistent with previous findings, constant light conditions result in desynchrony of the endogenous circadian rhythm from environmental rhythms and a significant increase in circadian period as compared to LD conditions (24.03 ±0.06 vs. 26.65 ± 0.04 hours) **(Fig. 2.5B).** We then used running wheel actigraphy to demonstrate the effect of red light during the rest period (LR) and found that red light during the rest period did not disturb endogenous circadian rhythms and resulted in similar activity profiles to LD conditions **(Fig. 2.5C).** Accordingly, the circadian period of activity for mice kept in LD and LR conditions was not significantly different (24.03 ± 0.06 vs. 24.01 ± 0.02 hours) **(Fig. 2.5D)**. Next, to investigate the effect of light during the rest period on circadian rhythms of heart rate and blood pressure, we used implanted radio-telemeters. We found that under constant light conditions, the amplitude of heart rate rhythms was blunted as compared to under normal light-dark conditions. In contrast, red light during the rest period resulted in a

normalization of rhythms, resembling those observed under light-dark conditions **(Fig. 2.5E)**. In contrast to the dogs which are diurnal mammals, light at night resulted in a 127 bpm and 165 bpm decrease in peak heart rate as compared to LD during the first and second light (sleep) periods respectively (**Fig. 2.5F).** Additionally, light at night resulted in a 7.75- and 12.75-hour phase shift in peak heart rate, during the first and second day of LL, respectively, as compared to LD **(Fig. 2.5D).** Similarly, under constant light conditions, the amplitude of mean arterial pressure rhythms was blunted as compared to under normal light-dark conditions. In contrast, red light during the rest period resulted in a normalization of rhythms, resembling those observed under light-dark conditions **(Fig. 2.5H).** Light at night resulted in a 15.5 mmHg and 18 mmHg decrease in peak mean arterial pressure as compared to LD during the first and second light periods respectively (**Fig. 2.5I).** Additionally, light at night resulted in a 5- and 11.25-hour phase shift in peak mean arterial pressure, during the first and second day of LL, respectively, as compared to LD **(Fig. 2.5J).**

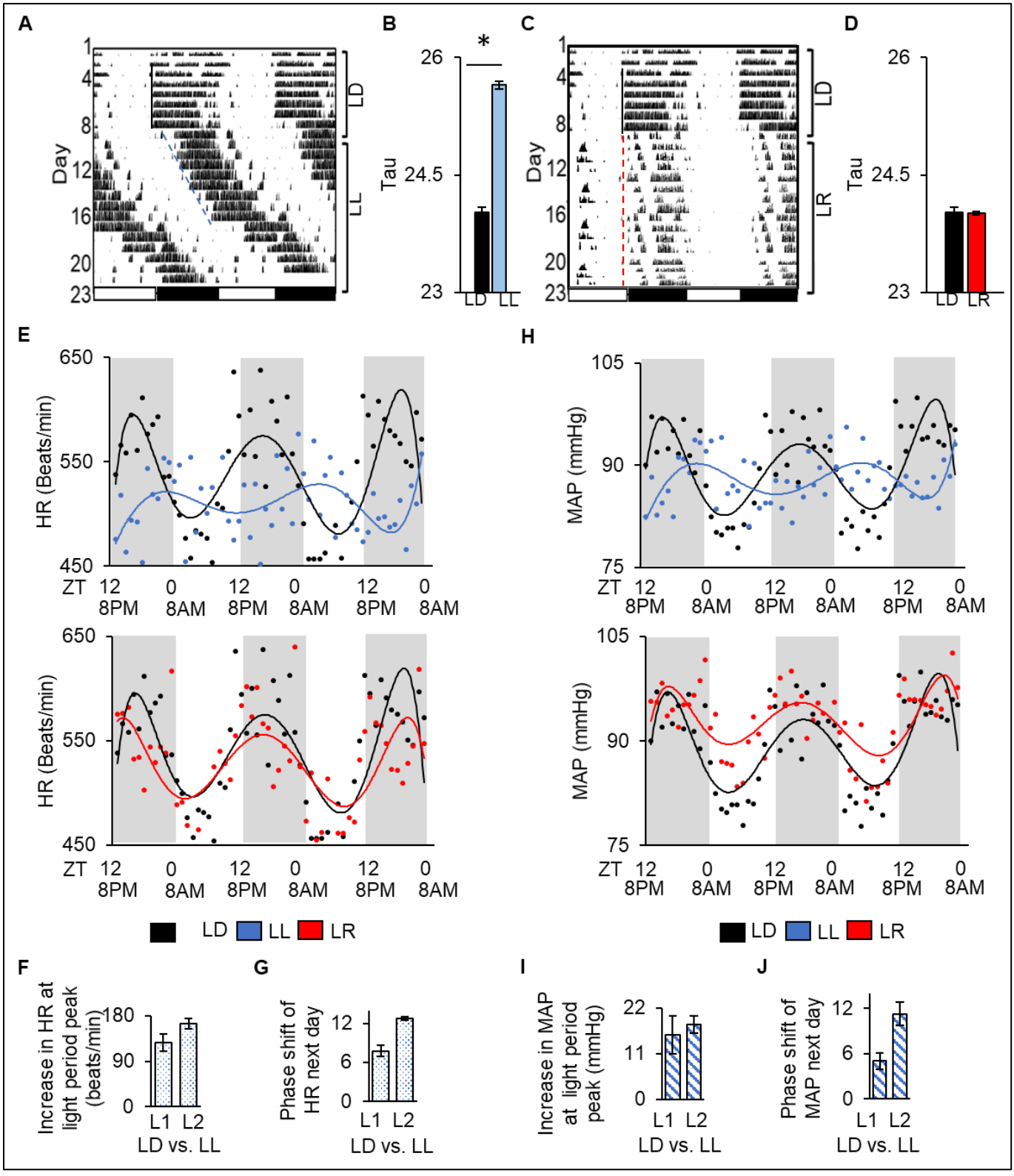

A
B
C
D
E
H
F
G
I
J
Day
LD
LL
LR
Tau
*
LD LL
LD LR
HR (Beats/min)
MAP (mmHg)
ZT 12 0 12 0 12 0
8PM 8AM 8PM 8AM 8PM 8AM
LD LL LR
Increase in HR at light period peak (beats/min)
Phase shift of HR next day
Increase in MAP at light period peak (mmHg)
Phase shift of MAP next day
L1 L2
LD vs. LL

Figure 2.5 Physiological responses to circadian disruption by light at night in a nocturnal animal

(A) Running wheel actigraph of mice (n=4) kept in 12 h Light (L): 12 h Dark (D) conditions for 7 days followed by constant light (LL) conditions for 14 days. (**B)** Circadian period (Tau; T) of mice kept in mice kept in LD and LL conditions using running wheel actigraphy. (**C)** Running wheel actigraph of mice (n=6) kept in 12 h Light (L): 12 h Dark (D) conditions for 7 days followed by 12h L: 12 h Red L (LR) conditions for 14 days. (**D)** Circadian period (Tau; T) of mice kept in mice kept in LD and LR conditions using running wheel actigraphy. (**E)** Heart Rate (beats/min) measured over 72 hours using telemeters implanted in mice kept in LD, LL and LR conditions (n=4). (**F)** Change in amplitude of heart rate peak during the light period under LD and LL conditions. (**G)** Phase shift of heart rate peak under LL conditions as compared to LD conditions. (**H)** Mean arterial pressure (mmHg) measured over 36 hours using telemeters implanted in mice kept in LD, LL and LR conditions (n=4). (**F)** Change in amplitude of peak mean arterial pressure during the light period under LD and LL conditions. (**G)** Phase shift of mean arterial pressure peak under LL conditions as compared to LD conditions.

To investigate the effects of circadian disruption by constant light conditions on recovery from disease, we used an *ex-vivo* isolated heart perfusion model in Langendorff mode. First, we measured left ventricular developed pressure (LVDP) at baseline in hearts from mice housed on a normal LD cycle and from those housed under LL conditions to mimic the increased light at night observed in the ICU environment. Hearts were collected between ZT09 and ZT12 as this is the transition time from sleep to wake and where differences between lighting conditions were most evident in activity, heart rate and blood pressure . Baseline measurements were collected over a 20-minute period. Ischemia was then initiated and sustained for 20 minutes, followed by reperfusion for a minimum of 40 minutes **(Fig. 2.6A-B)**. Normal hearts recovered significantly ($p=0.0002$) better than LL hearts after 40 minutes of reperfusion (39.43 ± 1.50 % vs. 14.53 ± 1.99 %) **(Fig. 2.6A, C)**. Next, we investigated the effect of red light during the rest period on recovery from hypoxia and found that hearts extracted from mice kept in red light during their rest period recovered to a similar degree as normal hearts (39.43 ± 1.50 % vs. 39.05 ± 0.52 %) **(Fig. 2.6B, C)**. To investigate the mechanisms responsible for the attenuated recovery of LVDP in mice exposed

to constant light, we performed quantitative polymerase chain reaction on the reperfused hearts, to assess changes in gene pathways critical for myocardial healing responses post-ischemia. We found that LL conditions resulted in significantly ($p<0.0001$) increased expression of the core circadian mechanism gene *clock* (46.97 ± 1.48 vs. 40.73 ± 2.02), as well as autophagy related genes *ampk* (42.22 ± 0.47 vs. 40.88 ± 0.55), *becn1* (42.55 ± 4.92 vs. 34.66 ± 2.18), and *atg5* (47.03 ± 1.88 vs. 44.93 ± 2.05), as compared to LD conditions (**Fig. 2.6D**). LR conditions resulted in significantly ($p<0.05$) different expression of *clock* (42.80 ± 2.04 vs. 40.73 ± 2.02) and *becn1* (29.30 ± 0.83 vs. 34.66 ± 2.18), while the expression of *ampk* and *atg5* was comparable to that of LD hearts **(Fig. 2.6D)**. These results demonstrate the importance of limiting exposure to light at night to maintain normal circadian rhythms, as hearts from LL mice clearly do not recover from ischemic insult as well as hearts from mice kept in normal conditions.

Table 1 - Absolute Values of qPCR expression of Circadian and Autophagy pathway genes

	LD	LL	LR
clock	40.73 ± 2.02	46.97 ± 1.48	42.8 ± 2.04
ampk	40.88 ± 0.55	42.22 ± 0.47	40.77 ± 0.37
becn1	34.66 ± 2.18	42.55 ± 4.92	29.3 ± 0.83
atg5	44.93 ± 2.05	47.03 ± 0.49	44.88 ± 0.42

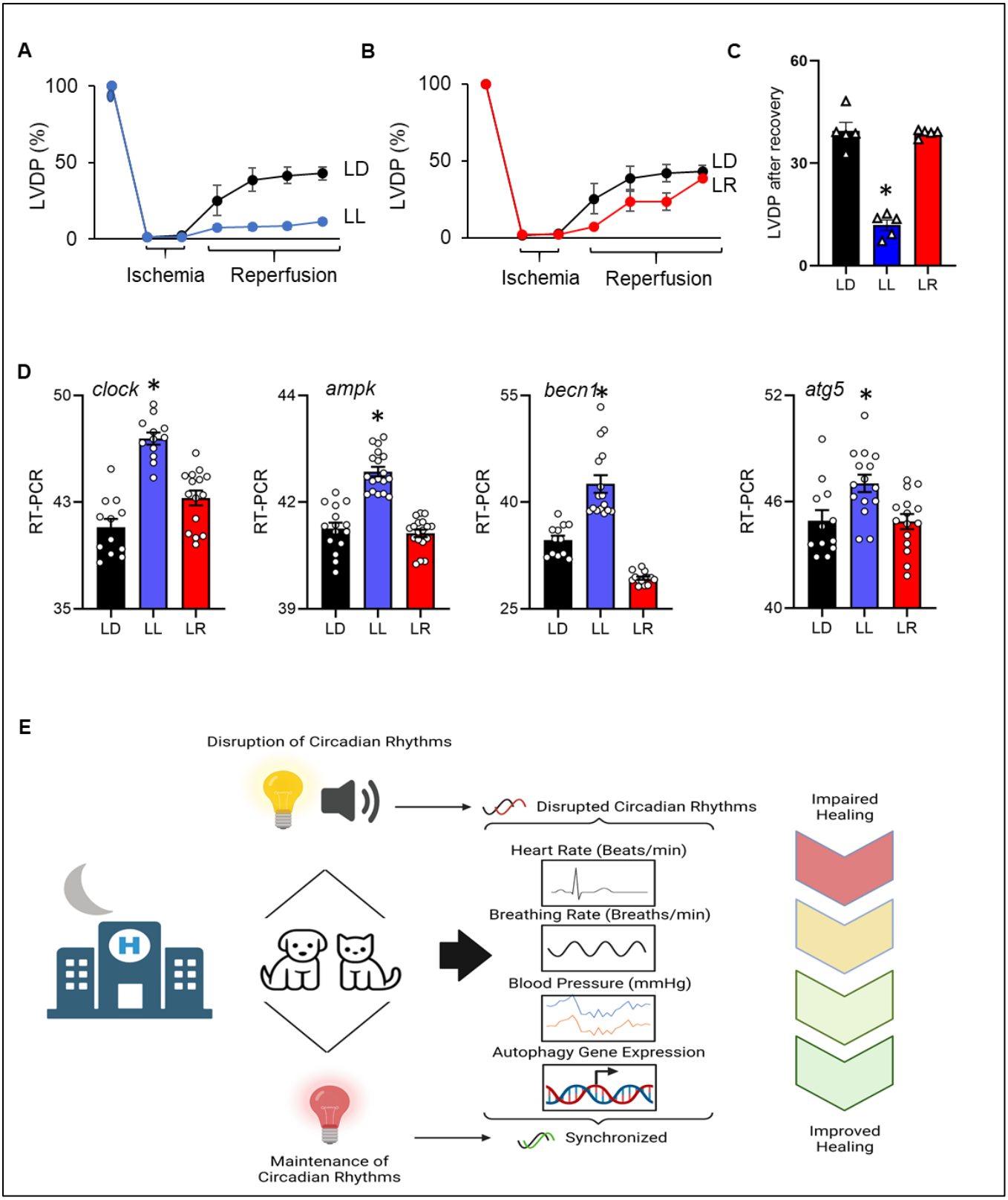

A
100
50
0
LVDP (%)
LD
LL
Ischemia
Reperfusion
B
100
50
0
LVDP (%)
LD
LR
Ischemia
Reperfusion
C
60
30
0
LVDP after recovery
LD
LL
LR
D
clock
RT-PCR
50
43
35
LD
LL
LR
ampk
44
42
39
becn1
55
40
25
atg5
52
46
40
E
Disruption of Circadian Rhythms
Disrupted Circadian Rhythms
Heart Rate (Beats/min)
Breathing Rate (Breaths/min)
Blood Pressure (mmHg)
Autophagy Gene Expression
Synchronized
Maintenance of Circadian Rhythms
Impaired Healing
Improved Healing

Figure 2.6 Recovery following circadian disruption by light at night

(A) % of baseline left ventricular developed pressure (LVDP) of isolated mouse hearts (n=5/group) collected from mice at ZT09 kept in LD and LL conditions for 2 weeks prior to sacrifice. (**B)** % of baseline LVDP of isolated mouse hearts (n=6/group) collected from mice at ZT09 kept in LD and LR conditions for 7 days prior to sacrifice. (**C)** % recovered LVDP of isolated LD, LL and LR mouse hearts after 40 minutes of recovery (right). (**D)** RT-PCR data depicting the expression of 4 genes, *clock, ampk, becn1, and atg5* in hearts exposed to LD, LL and LR conditions and hypoxia-reoxygenation. (**E)** Summary image depicting the veterinary hospital environment at night, which results in patients being exposed to excess light and sound at night. Excess light and sound at night lead to desynchronized rhythms in heart rate, breathing rate, and blood pressure, which impair healing. Providing red light allows staff to carry out routine monitoring and procedures without disturbing circadian rhythms, therefore improving healing overall. * denotes a significant difference ($p<0.05$) by student's t-test vs. LD.

2.2 Discussion

In these series of studies, we first demonstrated the presence of excess light and sound at night in two veterinary hospitals and an animal shelter. Next, we investigated the effect of light at night on healthy dogs and found that it disturbed circadian rhythms of heart rate and breathing rate. Conversely, we found that red light at night did not disrupt rhythms of these parameters and mirrored those seen under a normal light-dark schedule. To confirm our findings, we investigated the effect of light during the rest period on circadian rhythms of activity, heart rate, and blood pressure in mice using running wheel actigraphy and implanted radiotelemeters. We found that light during the rest period resulted in disrupted circadian rhythms of all three physiological parameters and that red light at night restored these rhythms, similar to the effects observed in the dogs. Lastly, we investigated the effect of circadian disruption by light at night on recovery from disease using an *ex-vivo* isolated mouse heart model of hypoxia-reoxygenation. We found that hearts from mice subjected to light at night did not recover from hypoxic conditions as well as hearts from mice kept under regular light-dark conditions or under red light during the rest period. These findings demonstrate the effect of circadian disruption by light at night on healthy and diseased physiology and confirm our hypothesis **(Fig. 4E).**

Patients in the ICU have severe medical conditions and require constant monitoring. Several studies have documented the presence and impact of light at night on circadian rhythms in humans (Diaz et al., 2020; Engwall et al., 2017). While the effects of patients in human clinical settings has been well established. In contrast, little data exists surrounding the presence of stimuli at night in veterinary hospitals and their effects on the circadian rhythms of animal patients. A prospective, observational study performed in two academic veterinary ICU settings found that noise levels were comparable to ICUs in human hospitals(Dornbusch et al., 2020), and exceed

recommendations by the World Health Organization (WHO) of a maximum of 35 dB at night in human ICUs(Fullagar et al., 2015).(Fullagar et al., 2015). More recently, another study confirmed these findings and found that average decibel (dB) levels between 6 PM and 9 PM exceeded 76.97 dB, equivalent to the noise level of a vacuum cleaner or an average radio(Dornbusch et al., 2020). Sources of noise in the ICU included alarms, heating and cooling systems, patient vocalization and staff conversations. All areas in the ICU and wards of both veterinary hospitals that we studied displayed significant levels of light **(Fig. 1)** and sound **(S. Fig. 1)** exposure at night. Consistent with previous findings, decibel levels in all areas throughout both hospitals exceeded the WHO recommendation of 35 dB and were on average 1.5 times greater **(S. Fig. 1).** These findings suggest that, like human patients, veterinary patients are exposed to abnormal levels of light and sound at night in ICU areas.

Although no studies have investigated the presence and effect of light at night in veterinary hospitals, several studies have demonstrated the effect of circadian disruption in companion animal models. Healthy male and female dogs displayed robust rhythmicity of intraocular pressure, a routine investigation in eye examinations, under 12 h light: 12 h dark, a reverse light cycle of 12 h dark: 12 h light, and constant dark conditions(Piccione et al., 2010). However, under constant light conditions, no rhythmicity was observed, suggesting that this parameter is under diurnal regulation and is disrupted by constant light(Piccione et al., 2010). Furthermore, low light intensity (50 lux) at night promotes improved sleep in dogs, whereas strong illumination (1600 lux) during this time has a negative influence on sleep behavior(Fukuzawa & Nakazato, 2015). Similarly, cats exposed to 12 h light: 12 h dark schedules displayed robust circadian rhythms in cerebral spinal fluid concentrations of vasopressin and melatonin(Reppert et al., 1982). However, under constant light conditions, the rhythms of both hormones were found to be free running, maintaining a 24-

hour period but no longer following diurnal rhythms. We demonstrated that when healthy dogs are exposed to constant light conditions, the rhythmicity of heart rate and breathing rate, two parameters that exhibit robust circadian rhythms, is disrupted **(Fig. 2)**. Not only light intensity, but the spectrum of light that ipRGCs are exposed to has an impact on circadian rhythms(Fonken et al., 2019). Specifically, light in the range of 485-480 nm, seen as blue-cyan light, is the strongest activator of the melanopsin photopigment found in ipRGCs(Souman et al., 2018). Monochromatic fluorescent white light, commonly used in hospital and office settings, have emission spectral profiles with significant amounts of radiance in the blue light spectrum, from 430 – 500 nm(Elvidge et al., 2010). A recent study outlined the recommended levels of light intensity to support healthy physiology, sleep and wakefulness in humans; 250 lux during the day, 10 lux during the evening, and 1 lux at night(Brown et al., 2022). The effect of light at night was ameliorated when red light was applied at night instead of white light, resulting in normalized circadian rhythms in both parameters **(Fig. 2)**. A potential caveat presented with the use of red light is the practicality in monitoring patients at night. Normally, white light is used as it allows the observer to clearly see any signs of bruising, bleeding or otherwise abnormal findings. Future studies should investigate the possibility of utilizing light with the blue spectrum diminished but balanced by other wavelengths in order to maintain its white appearance.

As a proof of concept, we repeated the conditions experienced by the dogs using mice and evaluated effects on locomotor activity using running wheel actigraphy, as well as heart rate and blood pressure using implanted radiotelemetry. We found that constant light conditions resulted in circadian disruption in all 3 parameters, as in dogs, while red light applied during the rest period restored rhythmicity **(Fig. 3).** These findings suggest that constant light conditions, like those found in veterinary ICU settings, result in circadian disruption of heart rate and blood pressure,

two parameters that exhibit robust circadian rhythmicity. Furthermore, this suggests that peripheral clocks regulating other physiological functions, such as immune function, which is critical for healing, may be disrupted as well.

Previously, a study by our group evaluated the effect of the ICU environment, mimicked by exposing mice to 5 days of a 10-hour light: 10-hour dark schedule, following myocardial infarction (MI) on cardiac healing. Our data showed that the ICU model led to worse cardiac remodeling and long-term outcomes due to the dysregulation of the inflammatory response following MI(Alibhai et al., 2014). More recently, it has been shown that mice exposed to dim light at night (dLAN) following cardiac arrest and resuscitation experienced attenuated recovery as compared to regular light-dark schedule mice(Fonken et al., 2019). We investigated the effects of circadian disruption by constant light conditions, as found in the ICU setting **(Fig. 1)** on recovery from disease using an *ex-vivo* isolated mouse heart perfusion model of hypoxia-reoxygenation. We found that hearts collected from mice kept under constant light conditions at ZT09-ZT12, the end of the sleep period, experienced attenuated recovery of left ventricular developed pressure as compared to hearts collected from mice kept under normal light-dark schedules. Accordingly, exposure to constant light resulted in an increase in the expression of genes involved in the autophagy pathway, *ampk, becn1,* and *atg5* and the key circadian mechanism gene *clock*. Mitochondrial damage during myocardial ischemia activates autophagy to dispose of damaged mitochondria. *Clock* transcriptionally coordinates the efficient removal of damaged mitochondria during myocardial ischemia by directly controlling transcription of genes required for mitochondrial fission/fusion and autophagy as shown by transcriptome and gene ontology mapping in CLOCKΔ19/Δ19 mice(Rabinovich-Nikitin et al., 2021). Additionally, recent evidence suggests that circadian clock and autophagy reciprocally regulate one another(Juste et al., 2021).

Therefore, disruption of *clock* gene expression, as seen under constant light conditions results in disrupted transcription of downstream autophagy pathway genes, leading to increased myocardial damage and impaired recovery. Moreover, hearts collected from mice kept in red light during the rest period displayed similar levels of recovery to those from mice kept under normal light-dark conditions. Mirroring these results, exposure to red light at night resulted in no significant difference in the expression of *ampk* and *atg5* while the expression of *clock* was significantly greater and *becn1* was significantly lower as compared to LD **(Fig. 4)**. Intriguingly, the restoration of CLOCK activity, rescued autophagic gene expression and mitophagy during hypoxia (Rabinovich-Nikitin et al., 2021). Therefore, restoration of *clock* gene expression, as seen under red light during the rest period conditions results in restored transcription of downstream autophagy pathway genes, leading to decreased myocardial damage, and improved recovery. These findings suggest that circadian disruption by light at night have an effect on healing and recovery, consistent with previous findings.

While it is possible to decrease interventions and monitoring at night in more stable ward patients, ICU patients are often in more severe conditions, and reducing attentiveness at any time of day would be detrimental to their recovery. Therefore, interventions that reduce circadian disruption while not interfering with physicians' ability to perform their duties are clearly warranted. Several studies have suggested the implementation of an integrated "chrono-bundle" of interventions to entrain faltering circadian rhythms in critically ill human patients(Hu et al., 2010; McKenna et al., 2018; Patel et al., 2014; Scotto et al., 2009; Xie et al., 2009). One solution might be the implementation of a circadian lighting system, with bright, blue-enriched light during the day and dim, blue-poor light during the night, to minimize the disruptive effect of the light while allowing the physician to observe the patient with proper lighting conditions. By minimizing

power density between 450 and 500 nm and adding an extra spectral “peak” around 420 nm to maintain color temperature, Souman et al. (2018) were able to show a significant reduction in melatonin suppression as compared to normal monochromatic white light, indicating the potential utilization of blue-poor light for medical settings during the rest period(Souman et al., 2018). This approach has been implemented in several major human hospitals and has displayed significant results including reduced sleep disruption and subsequent delirium(Engwall et al., 2015; Engwall et al., 2017; Ruben, Francey, et al., 2019; Ruben, Hogenesch, et al., 2019). Other interventions include reducing exposure to computer or tv screens at night, which emit blue-rich light as well. Reducing noise exposure by separation of emergency triage areas from recovery areas in the ICU may also prove to be beneficial and reduce distress in recovering animals. While our results were able to demonstrate disrupted rhythms in dogs under a simulated light cycle, future studies should evaluate the impact of continuous lighting and sound on the disruption of diurnal companion animal circadian rhythms directly in the hospital environment. Physiological parameters including blood pressure, heart rate, temperature, and activity as well as humoral rhythm biomarkers including melatonin and cortisol should be measured in ICU patients and compared with animals maintained in optimized ICUs. The use of non-invasive telemetry for measuring these parameters could be implemented and could be translated into clinical settings to evaluate the effect of circadian disruption on critically ill patients in the veterinary hospital.

The findings of this study serve as evidence that the concern of abnormal light and sound exposure, which are disruptive to patient circadian rhythms, are as pervasive in veterinary medicine as they are in human medicine. Further, interventions currently being implemented in human medicine can be as effective in veterinary patients in reducing circadian disruption, leading to improved healing and recovery.

2.3 Materials and Methods

Study Design

The objectives of this study were to demonstrate the presence of light and sound at night in veterinary hospitals and investigate its effect on endogenous circadian rhythms of companion animal patients. Healthy dogs used in this study were housed in the Central Animal Facility at the University of Guelph. These dogs were actively involved in studies prior and following this investigation, involving altered micronutrient diets, however, feeding times were kept consistent throughout so as to not disturb peripheral circadian rhythms. Individual dogs were chosen to participate in the study based on their tolerance to the required telemetry equipment following approximately 2 months of acclimation. Mice used in this study were obtained from Charles River Laboratory and housed in the Central Animal Facility at the University of Guelph. All procedures involving animals were authorized by the Animal Care Committee at the University of Guelph (AUP 4090, 4667) and complied with provincial and federal regulations governing care and use of research animals.

Samples sizes were determined based on previous experiments (Reitz et al., 2019) showing that this size could guarantee good reproducibility and emergence of statistically significant differences. No statistical methods were used to predetermine sample size. No statistical methods were used to determine outliers.

Measuring Light and Sound Intensity

Light and sound intensity were monitored using the HOBO U12-012 data logger (Temp/RH/Light/External channel, Onset, MA)(Linder & Christian, 2011, 2012) in lux and decibels (dB), the conventional SI unit for illuminance and sound, respectively. The light sensor

measures light intensity between 10.7 lux to 32, 291 lux. The data logger has a time accuracy of ±1 minute per month at 25°C and a 12-bit resolution which enables detection of variability within the recorded data and is capable of storing 43,000 measurements. The data logger's dimensions are 2.3×2.9×0.85 inches. Sound levels were monitored using the digital sound level meter (Extech Instruments, US) model 407736 in volts, and later converted into– decibels (dB). Light and sound were recorded continuously at 5-minute intervals for 14 days and stored in each data logger's internal memory. The data loggers and sound meters were attached separately to the wall with hook and loop tape in each area. They were attached using a 2.5mm stereo cable (0-2.5V). The sensors were not directly exposed to natural light sources to prevent the data logger from monitoring outdoor conditions. The positions of the devices were determined in consultation with facility staff and were maintained in a constant location and did not interfere with any hospital activities.

Light and sound intensity data were exported from the data logger using HOBO ware Pro Version 3.7.14 software. Data were then moved into MS excel. Since sound intensity was measured in volts, these data were converted to A-weighted decibels (dB) by multiplying the values by 100. Hourly averages were then calculated for both light and sound intensity data. Data were analyzed by weekdays, weekends, nightly averages for each day of the week were calculated by averaging values between 7 PM and 7 AM. 24-hour light and sound intensity in each area were represented using radar plots as well as histograms (MS Excel). Only times when the light was designated "on", or above 11.8 lux, were used to calculate the fold change compared to control in each area. Fold changes in light and sound intensity at night compared to control were represented using bar charts.

Non-Invasive Telemetry of Dogs

For this experiment, 3 hound mix dogs were selected from a colony of 10 dogs generously provided by Dr. Shoveller, housed in the Central Animal Facility at the University of Guelph, in accordance with the Animal Care Committee's Guidelines. These 3 dogs were selected based on their tolerance for the telemetry equipment used in this experiment (EMKApack4G; EMKA technologies etc.) after approximately 2 months of acclimation. The equipment consists of 4 Electrocardiography (ECG) leads adhered directly to the skin surrounding the thorax, secured with adhesive bandages to prevent movement, a Respiratory Inductance Plethysmography (RIP) band measured to fit each individual dogs' thoracic cage both connected to a transmitter as well as a protective jacket and cervical collar to prevent dogs' from removing equipment. The ECG leads and RIP band transmit information to a receiver connected to a computer, data are then acquired by IOX software. The following parameters are collected and calculated by the software: R-R interval (RR), QRS interval (QRS), Heart Rate (HR), Breathing Rate (BR), Tidal Volume (TV), and Minute Volume (MV). These measures were then analyzed in 5 minute bins and plotted over 48 hours on Microsoft excel. Lights were turned on at 7 AM (ZT0) each morning and turned off at 7 PM (ZT12) each evening. Animal care technicians cleaned pens from 7 AM (ZT0) to 8 AM (ZT1), then fed the dogs at 8 AM (ZT1). To investigate the effect of circadian disruption in a diurnal animal, the EMKApack4G non-invasive telemetry system was used to measure rhythms of heart rate and respiratory rate in 3 different lighting conditions: 12 hours (h) light (L): 12 h dark; 12 h L: 12 h red L; 24 h L. Dogs were equipped with the EMKApack4G apparatus starting at ZT4 (11 AM) on the first day of each lighting condition, ending at ZT4 on the day after the end of the lighting condition. During measurement periods, dogs' schedules were maintained as normal. Each condition was maintained

for 48 hours, with a washout period of 7 days in between. The order of conditions was as followed: LD, LL, LR.

Running Wheel Actigraphy

Actigraphy experiments were performed as previously described (Alibhai et al., 2017; Alibhai et al., 2018; Reitz et al., 2020; Reitz et al., 2019). 6 Healthy C57BL/6 Mice (Charles River) were individually housed under 12:12 LD cycle conditions for 2 weeks in running wheel cages in order to acclimatize prior to the experiment. 1) first, measurements of locomotor activity were collected over 7 consecutive baseline days, 2) followed immediately by 2 weeks where mice were kept in either LL or LR conditions. Continuous recordings of diurnal locomotor activity were collected and analyzed in 10-minute bins. Binned running wheel revolution counts displayed as actograms were generated with ClockLab (Actimetrics). Circadian period was determined by periodogram analysis on ClockLab software and represented using bar graphs generated using MS excel.

Implanted Telemetry in Mice

Diurnal cardiovascular hemodynamic responses to different lighting conditions were measured using PA-C10 murine telemetry probes (Data Sciences International) to collect *in vivo* BP recordings from conscious, freely moving healthy WT mice as described previously (Alibhai et al., 2017). At 8 weeks old, mice were anesthetized with 4% isoflurane, intubated, ventilated (model 687; Harvard Apparatus) and were maintained under anesthesia with 2.5% isoflurane during the surgery. The carotid artery was isolated, and the telemeter catheter was implanted and advanced to the aortic arch. The transmitter unit of the telemeter was then inserted into a subcutaneous skin pouch, then the neck incision was closed with a silk 6-0 suture 40 (Covidien). Mice were administered buprenorphine (0.1 mg/kg) analgesia immediately upon awakening, at 8 h, and 24 h

post-operation. All radiotelemetry recordings were initiated in mice at 1-week post-implantation. Mice were kept in LD conditions for 3 days, followed by LL conditions for 3 days. Mice were then switched back to LD conditions for a washout period of 1 week before being switched to the LR condition for 3 days. SBP and DBP were recorded, and MAP was calculated by (SBP + (2 x DBP))/3. Parameters were measured every 5 minutes over a 30 second time interval, averaged into 1 h time bins based on ZT time, and analyzed using Data Quest IV system (Data Sciences International). These 1h time bins were averaged across all mice yielding 12 h plots of absolute BP. These time bins were also used to yield a delta change in BP from experimental days vs. baseline.

Ex-Vivo Langendorff Isolated-Heart Perfusion

The isolated heart for small rodents (IH-SR, type 844, Harvard Apparatus) system was used to measure left-ventricular pressure before, during and after hypoxia is induced using methods previously described (Podobed et al., 2014). Briefly(Podobed et al., 2014). Briefly, following euthanization by carbon dioxide overdose, hearts were excised, mounted, and perfused with Krebs-Henseleit buffer (118 mM NaCl, 25 mM $NaHCO_3$, 1.2 mM KH_2PO_4, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgSO_4$, 11 mM glucose (95% O_2-5% CO_2, 37°C and 80-mmHg perfusion pressure). A balloon attached to a pressure transducer (Harvard Apparatus) was inserted into the left ventricle and inflated to give end-diastolic pressures of 10-15 mmHg. Left ventricular developed pressure (LVDP) was determined after 20 min of stabilization (Isoheart W). Function was assessed in hearts collected in the light period (murine sleep time) at ZT9 after 2 weeks in LD, LL or LR conditions. 6 mice were used for each group, and were euthanized at 10 weeks old. After 40 minutes of reoxygenation, hearts were snap frozen using liquid nitrogen and kept in -80°C for further analysis.

Quantitative Polymerase Chain Reaction (qPCR) of Autophagy and Circadian Mechanism Pathway genes.

Hearts used for ex-vivo Langendorff isolated heart perfusion were kept in -80°C to investigate mRNA expression of autophagy and circadian pathway genes using methods previously described (Alibhai et al., 2017). The total RNA was isolated from hearts collected at ZT09 using the RNeasy kit (Qiagen). To determine mRNA expression profiles we used the Power SYBR Green RNA-to-CT one Step Kit (Life Technologies) on a ViiA7 real time PCR system (Applied Biosystems) using primers found in **Table 2**. Relative expression was determined by normalizing the CT values of genes to histone. qRTPCR was performed using n = 4 individual hearts/group, and n = 3 technical replicates.

Table 2 - Primer sequences used for qPCR of Autophagy and CLOCK genes

Primer	Sequence (5' – 3')
Mouse ATG5 Forward	TGTCCTCCTCGCTAGATG
Mouse ATG5 Reverse	CTGTTGCCTCCACTGAAC
Mouse BECN1 Forward	ACTCCGTCCTCACTTGTAG
Mouse BECN1 Reverse	CACGTCGCACACAGTATC
Mouse CLOCK Forward	GCCTCAGCAGCAACAGCAGC
Mouse CLOCK Reverse	ACCGCATGCCAACTGAGCGA
Mouse AMPK Forward	CTCAGTTCCTGGAGAAAGATGG
Mouse AMPK Reverse	CTGCCGGTTGAGTATCTTCAC

Statistical Analysis

All values are represented as mean ± SEM. The data were assessed for normal distribution and similar variance between groups by Levene's test using SPSS software (IBM). For the light and sound intensity study, we used a repeated measures ANOVA with room type as the between subjects variable and day of data acquisition as the within subjects variable, and with Games

Howell post hoc test. For analysis on each day a two-way ANOVA was used. Data with $p \leq 0.05$ were considered significant.

CHAPTER 3: CIRCADIAN MEDICINE APPLICATIONS TO VETERINARY PRACTICE

In addition to my bench work in the previous chapter, I worked on a collaborative review highlighting current and future applications of circadian medicine to benefit veterinary patients.

Based on the Publication:

FARAG HI, TEMPLEMAN JR, HANLON C, SHOVELLER AK, BEDECARRATS GY, NIEL L, JOSHUA J, WILCOCKSON D, MARTINO TA. Circadian Medicine Applications to Veterinary Practice. In preparation for Journal of Biological Rhythms, 2022.

Background

Circadian clocks are endogenous time-keeping systems that adapt the physiology and behaviour of most living organisms to anticipate changes in their environment. Several environmental cues also known as zeitgebers (time-givers) can entrain the circadian system, the most potent of which is light as reviewed by (Albrecht, 2012; Dibner et al., 2010; Foster et al., 2020; Golombek & Rosenstein, 2010; Hastings et al., 2019; Lowrey & Takahashi, 2004; McWatters et al., 1999; Piggins & Loudon, 2005; Roenneberg & Merrow, 2016; Yan et al., 2020). Accordingly, some organisms have evolved to partition their activity to the daytime (diurnal), or to the nighttime (nocturnal), while yet others display peaks of activity at twilight (crepuscular) (Bennie et al., 2014). Regardless of chronotype, light works as a zeitgeber by stimulating photoreceptors which relay photic signals to the central circadian pacemaker, the suprachiasmatic nucleus (SCN), located on the hypothalamus of the brain as reviewed by (Takahashi, 2017).

We have known for some time about the molecular underpinnings of our circadian biology, the 24 hour oscillating levels of proteins that drive our cellular circadian mechanisms including Circadian Locomotor Output Cycles Kaput (CLOCK) (King et al., 1997; Vitaterna et al., 2001), Brain and Muscle Arnt-like Protein 1 (BMAL1) (Ikeda & Nomura, 1997), Cryptochromes 1 and 2 (CRY1/2) (Kobayashi et al., 1998), Periods 1 and 2 (PER1/2) (Tei et al., 1997), Casein Kinase 1 Epsilon, Delta (CK1ε/δ) (Camacho et al., 2001; Lowrey, 2000), nuclear receptor subfamily 1 group D member 1 (NR1D1/REV-ERBα/β) (Enmark et al., 1994; Preitner et al., 2002) and retinoic acid-related orphan receptor alpha (RORα) (Sato et al., 2004). These too have been extensively reviewed (Buhr & Takahashi, 2013; Florez, 1995; Goldman, 2001; Hastings, 2000; King, 2000; Takahashi, 2017). Furthermore, the SCN regulates peripheral clocks, found in every tissue of the body to coordinate critical physiological functions (as reviewed by (Honma, 2018).

Our understanding of the underlying mechanisms of the circadian system enable us to apply concepts discovered in model organisms to clinical practice to improve health and healing from disease, culminating in the creation of the field of circadian medicine. Circadian medicine is an emerging frontier of circadian biology research that has the potential to influence health and longevity of multiple species. However, much of the previous circadian medicine research by our group and others has focused on experimental rodent models of human disease, and translation in pre-clinical and clinical settings relevant to human medicine. As such, very little investigation has been directed towards circadian veterinary medicine.

In North America, approximately 35% of households have a dog, while 38% have a cat (AVMA, 2018a, 2018b; CAHI, 2021). This trend extends globally, with approximately 471 million dogs and 373 million cats kept as pets worldwide (Purina, 2019). This translates to approximately $31.4 billion spent on veterinary care each year (APPA, 2020), representing increasing opportunities for circadian medicine to improve companion animal veterinary care. With regards to agriculture, over 70 billion animals are managed in a farm setting every year, including 25 billion chickens, 1.5 billion cows, and 59 million horses, representing a significant sector of the world economy (FAO, 2021; UNFAO, 2017). For example, Canadian livestock farms spend $21 billion in operating expenses per year, with up to 5% of that cost spent on veterinary services (Lachapelle, 2014). Similarly, American livestock farms spend $357 billion in operating costs per year, with up to 8% of costs spent on veterinary services (USDA, 2019). Thus, there are considerable opportunities to apply circadian medicine to animal settings to improve health and welfare of animals as well. This review focusses on the nascent and growing appreciation of circadian medicine applications that improve the health and wellbeing of our companion and

agricultural animals, an important dialogue regarding additional broad benefits to veterinary medicine.

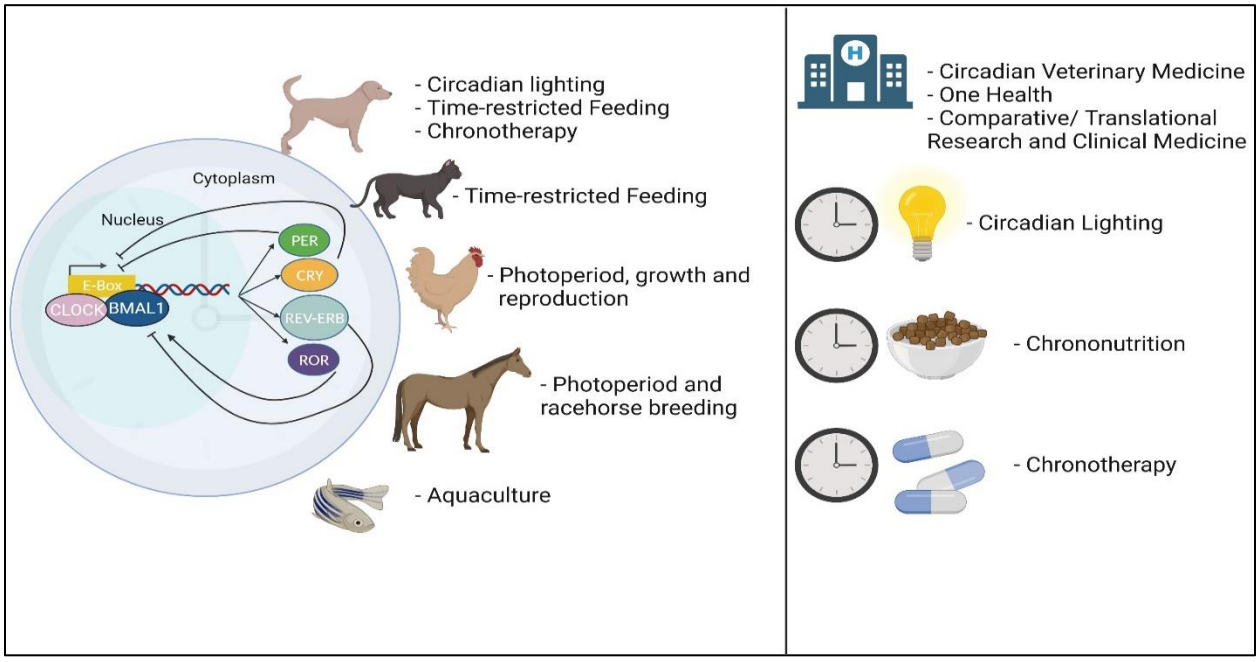


Figure 3.1 Circadian Medicine applications to veterinary practice

The intracellular circadian mechanism drives diurnal behaviour in most living organisms and synchronizes their physiology with the external environment using cues or zeitgebers. Circadian biology concepts have been applied experimentally to several animal models including dogs, cats, chickens, horses and fish. Here, we demonstrate how these concepts can be applied clinically and introduce the field of Circadian Veterinary Medicine. In the hospital, circadian lighting, chrono nutrition and chronotherapy can be used to improve healing and recovery of veterinary patients.

3.1 COMPANION ANIMALS - DOGS

3.1.1 Circadian rhythms in dogs

Dogs are the descendants of wolves and were domesticated by humans over 100,000 years ago. Several studies examined the circadian rhythms of wolves and characterized them as crepuscular, with nocturnal preferences. In contrast, healthy adult domesticated dogs exhibit diurnal locomotor activity/rest patterns and show a complex relationship between locomotor activity, age, and housing environment (Nishino S., 1997; Zanghi et al., 2012). Similar to humans, canine aging has been associated with disrupted locomotor activity patterns and sleep patterns (Zanghi et al., 2016; Zanghi et al., 2012). Furthermore, experimental and clinical studies have revealed robust diurnal circadian rhythms in several parameters of healthy physiology including heart rate (Ashkar, 1979; Matsunaga T., 2001), blood pressure (Ashkar, 1979), body temperature (Refinetti & Piccione, 2003), respiratory rate (Ashkar, 1979), bone metabolism (Liesegang A., 1999), heat dissipation (Besch E. L., 1977) similar to other diurnal mammals. In contrast, data supporting daily rhythms in endocrine hormones such as ACTH, cortisol and thyroxine remain controversial in dogs as results differ between studies (Kemppainen & Sartin, 1984; Palazzolo & Quadri, 1987), even though these hormonal patterns are clearly evident in humans and other diurnal mammals (Gamble et al., 2014). Collectively, these studies suggest that circadian rhythmicity is fundamentally important for normal canine physiology. In this section, we will characterize circadian medicine strategies to improve the health and disease outcomes of companion dogs.

3.1.2 Circadian Medicine Applications

a) Circadian lighting and sleep

Circadian and sleep disruption are often unavoidable in our modern society due to artificial lighting lengthening our days (photoperiods). For instance, in mice, prolonged circadian and sleep disruption by constant light exposure led to impaired immune tolerance (Mizutani et al., 2017). Pet dogs are also exposed to these extended photoperiods, and therefore sleep deprivation. Interestingly, in dogs, just one night of total sleep deprivation significantly reduced insulin sensitivity to a similar degree as 9 months of chronic high-fat feeding (Brouwer et al., 2020). This effect has also been observed in humans (Broussard et al. 2012, Buxton et al. 2010, Donga et al. 2010, Broussard et al. 2016). However, this effect is unlikely to persist chronically as other studies in humans demonstrated insulin sensitivity can be recovered with just two nights of recovery sleep (Broussard et al. 2016). Studies that apply chronic sleep deprivation to dogs and follow measures of insulin sensitivity long-term are required to further elucidate the impact of circadian and sleep disruption. The mechanisms by which sleep loss impairs insulin sensitivity are not yet known; however, factors related to the sympathetic nervous system, the hypothalamic-pituitary-adrenal axis, and increased inflammation have been proposed (Reutrakul & Van Cauter, 2018).

Dogs express intrinsically photosensitive retinal ganglion cells (ipRGCs) responsible for phototransduction and entrainment of the SCN (Yeh et al., 2017). These ipRGCs are most sensitive to short wavelength light, particularly the blue light range (450-485 nm) (McDougal & Gamlin, 2010). Furthermore, low light intensity (50 lux) at night promotes improved sleep in dogs, whereas strong illumination (1600 lux) during this time has a negative influence on sleep behaviour (Fukuzawa & Nakazato, 2015). In humans, exposure to high levels of light intensity in the evening is known to inhibit melatonin production by the pineal gland (Gooley et al., 2011), while for dogs, melatonin concentration in peripheral blood appears to be rhythmic and peaks at night (Stankov et al., 1994), consistent with the concept of improved sleep under low light

conditions. In humans, exposure to polychromatic white light spectrally tuned to reduce short blue light wavelengths in the evening resulted in reduced melatonin suppression (Souman et al., 2018); therefore, in dogs, exposure to white light deficient in the blue light spectrum may enable improved sleep conditions both at home and in clinical environments. Furthermore, circadian lighting is a major area of therapeutic development in human medicine. For example, patients in intensive care units exposed to light at night benefited from circadian lighting systems conducive to their circadian rhythms with bright light during the day and darkness at night (Engwall et al., 2015; Engwall et al., 2017). Together these data suggest that circadian lighting profiles that expose dogs to high intensity blue rich light during the day and low intensity blue poor light in the evening can also be utilized to benefit sleep homeostasis and overall health in dogs.

b) Time-restricted feeding for the treatment of obesity and associated comorbidities

The prevalence of veterinarian-assessed overweight and obesity in dogs in the United States is reported to be 34% and 5%, respectively (AVMA, 2018a). Canine overweight and obesity are associated with a number of comorbidities and an overall reduced lifespan (Adams et al., 2015; Kealy et al., 2002). Risk factors associated with increased body weight in dogs include aging, neutering, decreased exercise, and inappropriate feeding practices (Perry et al., 2020).

Like other mammals, dogs' circadian systems are highly sensitive to timing of food intake. When food is available for only a limited amount of time per day, mammals display food anticipatory activity characterized by an increase in locomotor activity, body temperature, adrenal secretion of corticosterone, gastrointestinal motility, and digestive enzyme activity in anticipation of food 2-4 hours before it is available (Comperatore & Stephan, 1987; Mistlberger, 1994; Stephan, 2002; Zanghi et al., 2012). Food intake is regulated by the incretins ghrelin and leptin, stimulating

hunger and satiety, respectively. In dogs, ghrelin levels peak in anticipation of feeding (Yokoyama et al., 2005) and decrease postprandially, while leptin levels peak 5-8 hours after food intake (Ishioka et al., 2005). Interestingly, ghrelin and leptin rhythmicity appear to be entrained by food intake, as shifts in time of feeding cause shifts in the peaks of both hormones (Ishioka et al., 2005; Yokoyama et al., 2005).

The circadian clock intimately interacts with nutrient sensing pathways. During feeding, activation of the insulin-pAKT-mTOR pathway drives downstream gene activities, promoting protein synthesis and cell growth (Fonseca & Proud, 2009). In contrast, periods of fasting activate adenosine monophosphate kinase (AMPK), promoting catabolism. AMPK also inhibits mTOR activity, ensuring separation of catabolic and anabolic processes (Inoki et al., 2011). The mTOR pathway phosphorylates several anabolic targets, including casein kinase 1 and glycogen synthase kinase 3, both of which phosphorylate the circadian clock component PER, marking it for degradation, thereby lengthening the circadian period (Zheng & Sehgal, 2010). In contrast, during fasting, AMPK phosphorylates CRY, destabilizing it, thereby shortening the circadian period (Lamia et al., 2009).

In humans, time-restricted feeding, a form of intermittent fasting wherein food intake is partitioned to less than 12 h a day, improves insulin sensitivity, β cell responsiveness, blood pressure, oxidative stress and appetite in men with prediabetes (Sutton et al., 2018). Time-restricted feeding has not been extensively investigated in dogs; however, application of alternate day intermittent fasting in dogs for one week with a high fat diet resulted in improved insulin sensitivity and lower fasting glucose concentrations compared to dogs fed daily. Additionally, dogs who were intermittently fasted and fed a low fat, high carbohydrate diet consumed less

calories overall and lost more weight than those fed daily or fed a high fat diet (Leung et al., 2020). As such, feeding dogs early in the day and imposing a fasting period in the evening and night-time may improve insulin sensitivity in obese, pre-diabetic dogs, potentially leading to weight loss and an improvement in overall health.

c) Chronotherapy for Treatment of Cardiovascular Disease

Circadian Medicine holds significant promise to benefit the cardiovascular health of dogs. Indeed, it is estimated that 7.8 million dogs in the USA, 10% of the population, have some degree of heart disease (Atkins et al., 2009; Häggström et al., 2008; Lombard et al., 2006; O'Grady et al., 2008; O'Grady et al., 2009). Congestive heart failure (CHF) is a primary cause of morbidity and mortality with an increasing prevalence in human and canine populations (Guglielmini, 2003). In dogs, CHF most often develops consequent to myxomatous mitral valve disease (MMVD) (Borgarelli & Buchanan, 2012). MMVD is characterized by thickening and shortening of the atrioventricular valves, and affects about 75% of dogs over the age of 16 (Guglielmini, 2003). Moreover, dilated cardiomyopathy (DCM), a cardiovascular disease predominantly affecting large breeds (Dukes-McEwan et al., 2003; McCauley et al., 2020) can also lead to CHF (O'Grady et al., 2009). CHF results in decreased blood pressure and triggers renin release from the juxtaglomerular apparatus of the kidney. This is a common compensatory mechanism to counteract reduced cardiac output observed in the symptomatic stages of CHF in humans and dogs (Hall, 1991; Watkins et al., 1976).

From a clinical perspective, the renin-angiotensin-aldosterone system (RAAS) is a critical regulator of outcomes related to cardiovascular disease, such as high blood pressure and adverse cardiac remodeling (Ferrario & Strawn, 2006; Jia et al., 2018; Orsborne et al., 2017; Pacurari et

al., 2014). Renin release is stimulated by decreased renal perfusion as a result of inefficient cardiac function. Renin cleaves a peptide bond in angiotensinogen, converting it into angiotensin I which is subsequently converted to angiotensin II (AngII) by the angiotensin-converting enzyme (ACE). AngII works to constrict peripheral blood vessels thereby increasing systemic vascular resistance and subsequently, blood pressure (Unger & Li, 2004). ACE inhibitors (ACEi) are a class of medications used for the treatment of high blood pressure and heart failure. Mechanistically, ACEi inhibit ACE activity, and subsequent production of AngII, a potent vasoconstrictor (Brown & Vaughan, 1998). By decreasing systemic vascular resistance, ACEi are known to improve cardiac hemodynamics in humans and dogs (Lefebvre et al., 2007; Levine, 1984; Uretsky et al., 1988). Interestingly, RAAS peptides have been shown to oscillate with day-night differences in dogs, demonstrating a robust circadian rhythm (Mochel et al., 2013). Chronotherapy of ACEi is a novel circadian approach for the treatment of cardiovascular disease. We found in a murine model of pressure overload, that captopril administered to mice, only at sleep time, provides protection against cardiac remodeling, whereas captopril given at wake time had no measurable benefit and was identical to placebo (Martino et al., 2011). The beneficial effects of administering ACE inhibitors at sleep time may be mediated, at least in part, by interfering with the peaking actions of RAAS on cardiovascular remodeling. (Alibhai et al., 2015; Martino et al., 2011; Sole, 2009; Tsimakouridze et al., 2015). In dogs, renin activity peaks at the beginning of the rest period (night-time), suggesting that ACEi administration would be most effective at this time (Mochel et al., 2013).Benazepril, enalapril, imidapril, and ramipril are the currently approved ACEi for use in dogs with CHF (Lefebvre et al., 2007). Benazepril Hydrochloride (BH) is a non-sulfhydryl prodrug which is converted by esterases into its active metabolite, benazeprilat, a highly potent and selective inhibitor of ACE (Webb et al., 1990). BH

has well-documented effectiveness in the treatment symptomatic canine CHF (King et al., 1995; Lefebvre et al., 2007) and occult dilated cardiomyopathy in Doberman pinschers (O'Grady et al., 2009). In the BENCH (BENazepril in Canine Heart Disease) study, the mean survival time of benazepril-treated dogs with mild to moderate CHF was improved by a factor of 2.7 compared with the placebo group (BENCH, 1999). Importantly, dogs with congestive heart failure may benefit from administering benazepril hydrochloride at bedtime that likely supresses the RAAS cascade and ultimately improves survivorship and quality of life (Mochel and Danhof, 2015). Further clinical investigations are clearly warranted for chronotherapy of drugs targeting RAAS, and the benefits of night-time coverage for dogs with heart disease. Taken together, lighting conditions, feeding practices, and chronotherapy of drugs are important circadian medicine applications that can be used to improve health and disease outcomes in dogs.

3.2 COMPANION ANIMALS - CATS

3.2.1 Circadian rhythms in cats

Literature characterizing circadian rhythms in domestic cats is scarce and contradictory. Early reports suggested a lack of rhythmicity in activity and body temperature (Hawking F, 1971; Sterman et al., 1965); however, research has since demonstrated circadian fluctuations in total sleep time and brain temperature indicating a bimodal pattern of wake-fullness at dusk and dawn, supporting the notion of crepuscular rhythms under artificial light: dark cycles (Kuwabara et al., 1986). These rhythms are endogenously produced and not merely a response to light-dark cycles, as cats were observed to have free running circadian organization of activity and feeding behaviour when kept in constant darkness and arrhythmicity when kept in constant light (Randall W., 1985). These results are somewhat contradictory to the definition of circadian rhythms, which describes free-running rhythms in any constant condition (Vitaterna et al., 2001). Although more recently,

Parker and colleagues demonstrated circadian rhythms in domestic cat locomotion using automatic recording technologies, confirming the previously observed bimodal profile (Parker et al., 2019). Moreover, cats appear to display similar bimodal circadian rhythms in endocrine hormones including norepinephrine (Reis, 1969), melatonin (Reppert et al., 1982), and blood pressure (Mishina M., 2006). In contrast, rhythms in plasma cortisol, adrenocorticotropic hormone (ACTH), alpha-melanin stimulating hormone (α-MSH), thyroxine (Kemppainen & Sartin, 1984), and aldosterone (Yu & Morris, 1998) are absent, while they are evident in humans and other mammals.

Characterizing the daily rhythms of the domestic cat has proven difficult, as recent studies have demonstrated that cats exhibit different chronotypes according to their housing conditions (Piccione et al., 2013). In addition, as cats are considered symbionts to humans, their activity and feeding behaviours have been shown to be affected by human interaction (Randall W., 1985). Collectively, these studies describe the presence of a bimodal profile of circadian rhythms with crepuscular peaks associated with twilight. Ultimately, by improving our understanding of feline circadian rhythms, we may be able to further develop nutritional and housing guidelines to support health and recovery from disease.

3.2.2 Circadian medicine applications

a) Feeding frequency for the prevention and treatment of obesity and diabetes

Similar to dogs, obesity and its associated comorbidities in cat populations are a growing concern worldwide (Chandler et al., 2017; German, 2006, 2010; Ward, 2019). The percentage of overweight and obese cats was recently estimated to be between 22 and 52%, respectively, depending on study parameters and country of origin (Colliard et al., 2009; Öhlund et al., 2018;

Prevention, 2017; Rowe et al., 2017). Clinically, obesity is considered a low-grade inflammatory disease, resulting from positive energy balance due to increased food intake, often a result of feeding regimen mismanagement by the owners, or reduced energy expenditure (German, 2006, 2010; Laflamme, 2006). However, many other factors contribute to obesity, including activity, breed, gender, neutering status, and age (Wall et al., 2019). Obese cats are also 3.9 times more likely to develop diabetes mellitus (Nelson & Reusch, 2014), a health conditioned characterized by insulin resistance, decreased glucose tolerance and glucosuria.

In the wild, the cat is an opportunistic carnivorous hunter, feeding on small prey who in themselves have different circadian rhythms, such as diurnal birds and nocturnal rodents, suggesting flexibility in the wild cats' feeding patterns (Konecny, 1987). However, feeding rhythms may differ in domestic cats, which largely rely on humans for food provision. To investigate rhythms in food intake, domestic cats kept in light: dark cycles of 10: 14, 15: 9, and 17: 7, with ad libitum access to food and water, displayed rhythms in feeding behaviour, albeit with a wide range of interindividual variability in nocturnal vs. diurnal preference (Randall W., 1985). With regard to nocturnal feeding, there was a strong association with simulated nocturnal starlight as well as with human presence (Randall W., 1985). As this study sought to determine percent nocturnality in cat food intake and activity, it was not clear whether the animals displayed multiple peaks in food intake throughout the day. Recently, Parker et al. (2019) investigated rhythms in food intake in a colony of 14 cats with ad libitum access to food, 7 of which showed a tendency towards bimodality, 4 being unimodal, and 3 being arrhythmic with peaks occurring between 4 AM – 10 AM (dawn) and 5 PM – 9 PM (dusk) (Parker et al., 2019). While these studies appear to report contradicting results regarding cat feeding rhythms, they also reiterate that cats display interindividual variability and flexibility in their food intake, adapting to the

environment around them. Further studies investigating the factors that determine diurnal vs. nocturnal preference and bimodality are required.

In 2018, a consensus statement by the American Association of Feline Practitioners recommended frequent small meals throughout the day to support healthy body composition and weight, despite a lack of empirical evidence to support this regimen (Sadek et al., 2018). Ad libitum access to food permits a cat to eat in excess of its energy requirements and may lead to increased body weight. Furthermore, ad libitum feeding relies on presentation of dry food, which generally has a greater inclusion of carbohydrates compared to wet foods, since wet cat food cannot be reserved for extended periods of exposure at room temperature (Michel et al., 2005). As cats are obligate carnivores, diets that are high in carbohydrates result in longer periods of postprandial hyperglycemia, which may lead to insulin resistance, a major risk factor for obesity and diabetes (Farrow et al., 2013). Therefore, strategies to reduce body weight that rely less on caloric restriction are clearly warranted.

As discussed in the previous section, time-restricted feeding also can impart important cardiometabolic benefits. In humans, early time-restricted feeding reduces mean levels of ghrelin and leptin, thereby reducing subjective appetite and increasing fat oxidation, without affecting energy expenditure in overweight adults (Ravussin et al., 2019). Therefore, time-restricted feeding may be an effective strategy for decreasing body weight in cats by imposing less frequent feeding schedules. To investigate the effect of decreased feeding frequency on metabolic and satiety hormone rhythms, Deng et al. (2013) fed cats either twice or four times daily. Cats fed commercial dry food twice daily displayed more variable concentrations of glucose and insulin over 24 hours and maintained higher concentrations of insulin as compared to cats fed four times. Moreover,

total ghrelin remained below baseline throughout the 24 h period in cats fed four meals daily, but concentrations remained above baseline during the light period in cats fed two meals daily, while the opposite was true for leptin concentrations. These results suggest that cats fed commercial dry food more frequently are more satiated than cats fed less frequently (Deng et al., 2013). In contrast, a recent study showed that cats fed wet commercial food once a day were more satiated than cats fed four times a day. Cats fed once a day had greater postprandial levels of appetite-regulating hormones gastric inhibitory polypeptide (GIP), glucagon-like peptide-1 (GLP-1) and peptide YY (PYY) than those fed four times and had lower postprandial respiratory quotients, which suggest greater fat oxidation occurring (Camara et al., 2020). Furthermore, increasing feeding frequency has been shown to decrease diurnal fluctuations in glucose, insulin, leptin, and ghrelin in cats (Deng et al., 2013) as well as in humans (Scheer et al., 2009), likely due to a more chronic exposure to dietary nutrients; however, nutrient content of those diets is certainly a variable that also contributes to the physiological response. Over time, the response that characterizes multiple feedings will result in increased glucose tolerance, decreased insulin sensitivity and eventual weight gain. Furthermore, in mice, circadian disruption by $CLOCK^{\Delta 19/\ \Delta 19}$ mutation results in hypercholesterolemia, hyperglycaemia, and hyperinsulinemia, ultimately leading to the development of an obese phenotype (Reitz et al., 2020; Turek, 2005). This suggests that feeding cats multiple meals throughout the day may result in a loss of entrainment by food, subsequently leading to disruption of circadian rhythms and possibly hyperglycaemia and hyperinsulinemia. Ultimately, these studies indicate that strategies to align feeding practices with cats' circadian rhythms by providing food less frequently throughout the day could be useful in the prevention and management of feline obesity and diabetes especially when combined with appropriate lighting.

3.3 AGRICULTURAL ANIMALS – CHICKENS

3.3.1 Circadian Rhythms in Chickens

Chickens and other avian species have a highly complex and diversified circadian system. While the retinal circadian clock initially identified in mammals (Tosini and Menaker, 1996) has been conserved across the avian species (McMillan et al., 1975; Ebihara et al., 1984; Barrett and Underwood, 1991), additional self-sustaining circadian oscillators have been identified in the pineal gland and hypothalamus of birds (Ebihara et al., 1984; Zatz and Mullen, 1988; Okano et al., 1994; Natesan et al., 2002). In fact, the avian retina is not essential to the entrainment of circadian rhythms (Menaker, 1968; Menaker et al., 1970), as enucleated house sparrows maintained their biological rhythms under standard daylength periods due to the presence of these extra-retinal oscillators. This was further supported by the arrhythmicity developed in birds when light was unable to penetrate the skull, but the eyes remained intact, demonstrating the necessity of these encephalic circadian oscillators for entrainment (Menaker et al., 1970; Foster and Follett, 1985). Incidentally, avian photoreceptors also reside in these three photoreceptive organs, including rhodopsin and melanopsin (OPN4) in the retina, pinopsin, OPN4, and vertebrate-ancient opsin (VA-Opsin) in the pineal gland, and VA-Opsin, OPN4, and neuropsin (OPN5) in the hypothalamus (Foster et al., 1985, 1994; Chaurasia et al., 2005; Halford et al., 2009; Kang et al., 2010; Nakane and Yoshimura, 2010; Davies et al., 2012; Ohuchi et al., 2012). Together, the localization of these photoreceptors within the encephalic tissues acts to sustain biological rhythms through the capture and transduction of photons through the skull and relays this information to the circadian oscillators in peripheral tissues (Ebihara and Kawamura, 1981; Takahashi and Menaker, 1982; Cassone and Moore, 1987).

In birds, the retina and pineal gland detect changing daylengths (Gaston and Menaker, 1968; Binkley et al., 1971; Ebihara and Kawamura, 1981; Lu and Cassone, 1993; Wang et al., 2012) via elevations in tryptophan hydroxylase (TPH; Chong et al., 1998), aralkylamine N-acetyltransferase (AANAT; Bernard et al., 1997), and Hydroxyindole-O-methyltransferase (HIOMT) during the dark phase (Hamm and Menaker, 1980; Thomas and Iuvone, 1991), resulting in increased melatonin (MEL) production and release from the pineal gland (Deguchi, 1979; Hamm and Menaker, 1980; Takahashi et al., 1980). In response to light stimulation, MEL production is downregulated, demonstrating a circadian rhythm under 24-h light: dark (L:D) cycles. Arrhythmic responses have been observed under continuous 24L or continuous 24D photoperiods, with melatonin rhythmicity absent under both conditions (Saito et al., 2005; Özkan et al., 2012; Honda et al., 2017; Ma et al., 2019). Thus, light stimuli serves as primary zeitgeber in avian species.

Earlier studies in pigeons and house sparrows investigated feeding schedules as an additional possible zeitgeber (Phillips et al., 1993; Rashotte and Stephan, 1996), yet little advancement has been made in this field. It has been established that food-entrainable oscillators can be desynchronized from the photic response. This has been demonstrated as an earlier onset of photophase altered the rise of core body temperature and oxygen consumption, typically identified as anticipatory feeding behaviours, with the birds gradually re-entraining their feeding schedule within days (Rashotte and Stephan, 1996). This served as a weak zeitgeber (Hau and Gwinner, 1992), with desynchronized birds also able to demonstrate 'masking' behaviour, adjusting their feeding behaviour under these conditions (Hau and Gwinner, 1996). Predictably, Honda et al. (2017) showed constant feed intake of male broiler chicks throughout a period of 24-h continuous light and increased feed intake prior to and following the dark period of a 12L:

12D photoperiod. However, despite differences in feed intake, the expression of an appetite-stimulating peptide, neuropeptide Y (NPY), and an appetite suppressing peptide, pro-opiomelanocortin (POMC), did not differ between these photoperiods (Honda et al., 2017). This suggests there is little interaction between the circadian rhythm and the melanocortin system, with these peptides demonstrating no rhythmic pattern, and further investigation is required into the mechanisms behind the control of feed intake and photoperiods.

3.3.2 Current Industry Practices

Over the last century, there has been a substantial increase in the production capacity of meat (broilers) and table eggs (laying hens), shifting production from a dual-purpose backyard flock model to larger-scale farms with specific, divergent breeding goals (Lawler, 2012). Within these larger indoor systems, producers are able to control the environmental conditions, including temperature, humidity, ventilation, and lighting, allowing for year-round production in the temperate zones. Specifically, producers often manipulate the length of photoperiodic exposure to promote health in broilers (reviewed by: Classen et al., 1991) and maximize the reproductive efficiency of laying hens and broiler breeders (reviewed by: Sharp, 1993).007A

Since photoperiod is the primary zeitgeber in poultry, several lighting programs have been utilized over the previous decades, each with benefits and challenges. Current industry standards vary between broilers and laying hens based on the breeding objectives. Broilers are typically reared under longer daylengths to allow for extended feed access and promote growth (Cobb-Vantress, 2020). Meanwhile, to optimize reproductive performance, laying pullets are typically maintained under short-day lengths of less than 10 h, stepping up to 16L: 8D at the time of

maturation, which occurs at approximately 18 and 22 weeks of age in layers and broiler breeders, respectively (Lohmann-Tierzucht, 2015; Aviagen, 2016).

3.3.3 Implications of Spectrum Lighting on the Circadian Rhythm

In addition to photoperiodic manipulation, the application of spectrum lighting has been extensively studied for commercial purposes, especially with the phasing out of inefficient lighting system such incandescent bulbs and the introduction of new technology such as light-emitting diode (LED) sources. It is largely understood that hens housed under red wavelengths will demonstrate improved reproductive capacity (Mobarkey et al., 2010; Min et al., 2012; Hassan et al., 2013; Baxter et al., 2014), and hens under green wavelengths will display elevated proliferation of skeletal muscle cells, contributing to improved growth (Halevy et al., 1998; Rozenboim et al., 1999). However, more recent studies have provided evidence that these wavelengths can directly impact the circadian rhythm.

Under green light, the positive clock genes (clock and Bmal1) have been shown to elevate (Jiang et al., 2016, 2020; Cao et al., 2017; Yang et al., 2020) and activate AANAT (Cao et al., 2017; Jiang et al., 2020; Yang et al., 2020), while the negative clock genes (Per and Cry) are downregulated (Jiang et al., 2016; Yang et al., 2020). This leads to an elevation in MEL production under green wavelengths (Jiang et al., 2016, 2020; Cao et al., 2017; Bian et al., 2020; Yang et al., 2020). Meanwhile, under red wavelengths, the opposite effect was observed, with negative clock genes upregulated (Cao et al., 2017; Yang et al., 2020), while positive clock and AANAT were downregulated in the hypothalamus (Cao et al., 2017; Jiang et al., 2020; Yang et al., 2020). Interestingly, Bmal1 was found to be elevated in the pituitary gland of females only, but no further studies have been conducted (Wang et al., 2015). The shortest wavelength, blue

light, was observed to negatively impact the expression of both positive and negative clock genes (Yang et al., 2020), inhibiting melatonin secretion (West et al., 2011). In fact, maintaining male broiler chicks under a lighting program of 12h white: 12h blue maintained clock gene expression (Honda et al., 2017), as the bird perceived blue light in a similar manner to scotophase. The implications of these altered circadian patterns will be further discussed in terms of their applications in growth and lay.

3.3.4 Photoperiodic control of reproduction in laying hens and broiler breeders

As chickens are seasonal breeders, their reproductive system is heavily regulated by the circadian and circannual systems, with short and long photoperiods inhibiting and stimulating the hypothalamic-pituitary-gonadal (HPG) axis, respectively (Tsutsui et al., 2000; Bentley et al., 2003; Ubuka et al., 2005). To maintain the immature state, pullets are exposed to a short day (less than 10 h of light) to promote the release of MEL (Ubuka et al., 2005). This hormone then upregulates the expression of gonadotropin-inhibitory hormone (Gn**IH**), which upon binding to its receptor (GnIH-R) directly inhibits the release of gonadotropin-releasing hormone (GnRH) and gonadotropins (Follicle-Stimulating Hormone; FSH and Luteinizing Hormone; LH), effectively shutting down the HPG axis. At the time of photostimulation, reducing the length of the dark phase results in a decline in MEL production, and therefore downregulates the expression of GnIH. The mediobasal hypothalamus (MBH), containing a molecular clock (Yasuo et al., 2003), then integrates the changes in photic information to stimulate thyrotrope cells in the pars tuberalis (PT) of the pituitary gland to produce thyroid-stimulating hormone (TSH). This elevation in TSH stimulates tanycytes on the base of the third ventricle to upregulate type 2 deiodinase (dio2), an enzyme upregulated under longer day lengths (Yoshimura et al., 2003) to promote the conversion of thyroxine (T_4) to triiodothyronine (T_3) (Bernal, 2002; Nakao et al.,

2008). Elevated levels of T_3 within the MBH act on the median eminence (ME), allowing GnRH nerve terminals to interact with the basal lamina and release this stimulatory neuropeptide to activate the remainder of the HPG axis (Prevot et al., 1999; Yamamura et al., 2004, 2006). While this downstream process has been well established, the receptor responsible for responding to light and signalling this cascade of events remains unclear. Working hypotheses currently propose that a single 'breeding opsin' is responsible for initiating the HPG axis activation, with the proposed deep brain photoreceptors including VA-Opsin (Hankins et al., 2008; Halford et al., 2009; García-Fernández et al., 2015), OPN4 (Foster et al., 1987; Bailey and Cassone, 2005; Hankins et al., 2008), and OPN5 (Tarttelin et al., 2003; Halford et al., 2009; Nakane and Yoshimura, 2010). The plausibility of these photoreceptors being involved in the seasonal response has been recently reviewed by Hanlon et al. (2020).

Upon sexual maturation of the hen, the reproductive tract will also work to control the timing of the ovulatory cycle through circadian oscillators and clock genes (Fahrenkrug et al., 2006; Karman and Tischkau, 2006; Nakao et al., 2007; Yoshikawa et al., 2009). This cycle can be entrained to photoperiods between 21 and 30 h, depending on the species. In the domesticated laying hen under 16L:8D, this process will slightly exceed a 24 h rhythmic cycle, as it has been demonstrated that a second rhythm controlling the growth and maturation of the follicle is not synchronized with the ovulatory process. When both ovulation and growth of the follicle coincide, this results in a sequence of eggs laid on consecutive days, also referred to as a clutch (reviewed by: Bahr and Johnson, 1984). These eggs will be laid within a 6-10 h window of time referred to as the 'open period.' Once these two rhythms become desynchronized from this period, a pause day will reset the cycles (Etches et al., 1984).

The preovulatory LH surge conveys a signal to the ovary, acting as a zeitgeber under the control of clock genes (Tischkau et al., 2011). Specifically, LH acts via steroidogenic acute regulatory (StAR) protein and Bmal1 (Nakao et al., 2007), leading to an elevation in progesterone production immediately preceding ovulation (Tischkau et al., 2011). The importance of this ovarian clock is highlighted in ovariectomized hens, in which core body temperature becomes arrhythmic (Underwood et al., 1997; Zivkovic et al., 2000) and in hens maintained under 24L cycles, in which hormonal profiles were able to maintain the ovulatory cycle (Kadono et al., 1981). Interestingly, it has been determined that only the largest follicle (F1) to F3 exhibit oscillatory responses, while less mature preovulatory follicles exposed to LH did not display rhythmic expression of these clock genes (Zhang et al., 2017), indicating the rhythmic interaction is acquired as the follicle develops. This rhythm is hypothesized to be controlled by the central clocking system, as hens under 16L: 8D supplemented with exogenous MEL laid eggs with significantly larger yolks due to the disrupted timing of ovulation leading to an extended rapid growth phase for lipid deposition (Taylor et al., 2013). Thus, photoperiods can be utilized to manipulate the weight of the egg and internal components.

3.3.5 Lighting Applications for Reproduction.

Light duration and spectrum have been widely studied regarding improvements in reproductive capacity. Following the discovery that longer day lengths can stimulate the reproductive axis, studies aimed to take advantage of this phenomenon with continuous 24-h lighting. However, mature hens exposed to this continuous light displayed disrupted synchronization between follicular maturation, the LH surge, ovulation, and oviposition (Dawson and Goldsmith, 1997), resulting in a lower production rate over the entire cycle (Callenbach et al., 1944; Wilson et al., 1956). To avoid the limitations of the ‘open period,’ ahemeral lighting

programs were developed, with the total hours of light exceeding 24 h in an effort to match the natural ovulatory rhythms observed (Byerly and Moore, 1941). However, this was unsuccessful, as eggshell quality and weight improved at the expense of the production rate (Leeson and Summers, 1988). This is likely due to the variation of the open window period within individuals in a flock. Thus, the photostimulatory periods currently used in the industry take advantage of the physiological benefits of 24-h LD cycles while maintaining circadian rhythmicity.

Regarding spectrum lighting, red wavelengths have demonstrated earlier sexual maturation and a greater cumulative production (Min et al., 2012; Hassan et al., 2013; Baxter et al., 2014), despite its role in downregulating the positive clock genes, AANAT, and ultimately MEL production (Jiang et al., 2016; Bian et al., 2020; Yang et al., 2020). This may indicate that red light has the ability to better synchronize the follicular maturation and ovulatory processes, and further studies are required to investigate the role of red wavelengths in circadian processes. Since green light is known to upregulate MEL production, the delayed onset of lay is consistent with the interaction between MEL and GnIH (Min et al., 2012; Hassan et al., 2013; Baxter et al., 2014), and reduced production rate is consistent with the ability of MEL to delay the initiation of each clutch (Greives et al., 2012). However, it is interesting to note that while green light significantly delayed maturation, this was further exacerbated when the retina remained intact, compared to hens with retinal degeneration, indicating the retina plays a substantial role in the production of MEL under green light (Baxter et al., 2014).

3.3.6 Photoperiodic Control of Bone Growth and Development

Early studies in layers demonstrated the requirement for MEL synthesis to maintain the skeletal structure and prevent bone disorders, such as scoliosis (Machida et al., 1995; Wang et

al., 1998). While a pinealectomy contributed to anywhere between 52-100% of birds developing scoliosis, MEL treatment was able to reduce the severity, if not completely inhibit the etiology of this disease (Machida et al., 1995). Further investigation has revealed that this is due to the stimulatory effect MEL has on osteoblastic proliferation and differentiation (Nakade et al., 1999; Cardinali et al., 2003; Park et al., 2011) while simultaneously inhibiting the activation and formation of osteoclasts (Koyama et al., 2002). Additionally, MEL promotes Tyle I collagen production (Nakade et al., 1999), and in the absence of MEL, endochondral ossification is interrupted at the epiphyseal plate (Aota et al., 2013). Thus, manipulating the length of the dark period will greatly impact the growth and development of bones. This has been primarily studied regarding broiler chicks and layer hens.

3.3.7 Broiler Leg Health

Broilers have been intensively selected for increased growth rate and feed efficiency, and in order to optimize feed intake to achieve these goals, these birds are primarily housed under 18 to 23-h photoperiods to provide access to feed (Cobb-Vantress, 2020). However, while extended photoperiods promote increased feed intake, short scotophases demonstrate an abolishment of the circadian rhythmicity, decreasing the locomotor activity. In combination, this results in a rapid weight gain leading to the development of leg abnormalities, including but not limited to lameness and tibial dyschondroplasia (Classen and Riddell, 1989; Sørensen et al., 1999), impacting the ability to access feed and water and often ending with on-farm culling. Thus, this is a major economic and welfare concern within the industry. In fact, broilers demonstrating leg weakness preferentially select feed supplemented with an analgesic agent (McGeown et al., 1999; Danbury et al., 2000), indicating a significant level of pain associated with these disorders (Sørensen et al., 1999). However, shortening the photoperiod has been shown to be effective in reducing the

incidence of leg abnormalities through the maintenance of rhythmic MEL production (Taylor et al., 2013; van der Pol et al., 2017, 2019).

3.3.8 Laying Hen Bone and Shell Development

Laying hens require 2.2-g of calcium for deposition on the shell of each egg produced, equating to ~10% of their total calcium content on a 24-h basis (Bouvarel et al., 2011). This means that calcium homeostasis is critical to their health, welfare, and the quality of the consumer product. In the case of breeder layers, this shell quality becomes even more important due to its role in providing calcium to the growing embryo for cartilage formation (Qi et al., 2016; Torres and Korver, 2018). In order to accommodate the demands of eggshell deposition during scotophase, hens maintain a specialized, readily labile source of bone within the endosteal surface of long bones (Bloom et al., 1941; Mccoy and Reilly, 1996), referred to as medullary bone. This bone source provides ~30% of total required calcium, in addition to the dietary source, to prevent the breakdown of structural bone (Driggers and Comar, 1949; Mueller et al., 1964). However, the incidence of osteoporosis, a common disease in laying hens resulting in a disorder termed cage layer fatigue, remains prevalent and is major causes of poor health and welfare (Whitehead and Fleming, 2000).

It has been well-established that circadian oscillators are able to be entrained by the timing of calcium consumption, acting as a potential zeitgeber for the pacemakers in the kidney of hens (Damiola et al., 2000; Lin et al., 2018). When the same levels of calcium were fed in the morning and evening, the clock genes in the jejunum and kidney demonstrated a normal circadian rhythm. However, when the calcium provided was higher in the morning and lower in the evening, these clock genes became arrhythmic (Lin et al., 2018). In fact, serum calcium has been determined to

exhibit a circadian rhythm (Sloan et al., 1974) which could be altered with photoperiod manipulations (Pablos et al., 1995). This is likely due to the altered production rates in these hens under varying photoperiods, which impacts the timing of eggshell formation. In addition to calcium intake, calcium deposition and transport to the shell gland also occur rhythmically via the circadian expression of 1, 25-dihydroxyviatmin D_3 (1,25 $(OH)_2D_3$) (Abe et al., 1979; Frost and Roland, 1991). It was previously hypothesized that 1,25 $(OH)_2D_3$ rhythms were controlled by sex hormones involved in the circadian rhythm of the ovulatory cycle (Peterson and Common, 1972; Abe et al., 1979). However, this theory was dismissed since hens laid shell-less eggs when 1,25 $(OH)_2D_3$ lacked rhythmicity, despite normal circadian patterns of sex hormones (Nys et al., 1986).

3.3.9 Lighting Applications for Improving Bone Growth and Development.

Recent studies have considered the impact of photoperiods during incubation to improve early bone development. Studies using continuous light during embryonic growth negatively impacted not only MEL synthesis but also reealed weaker bones with higher rates of tibial dyschondroplasia. Under continuous dark photoperiods, traditionally used in hatcheries, there were no detrimental effects. In fact, earlier ossification was observed in the tibia and femur of these chicks. However, using 12L:12D during incubation demonstrated further benefits, with increased osteoblast activity by embryonic day 13 (van der Pol et al., 2019), suggesting that photoperiods inducing circadian patterns can be perceived during embryonic growth, and these benefits have been shown to be carried over in the chick. While studies are now shifting focus to spectrum lighting during this period, there is no data on the impact on the circadian rhythm to date. Considering the previous data, if MEL production is beneficial to bone development and promoted under green light, we would hypothesize that there would be an advantage to using this spectrum during incubation and early growth. Furthermore, most of these studies have been conducted in

broiler chicks, but we suspect these benefits could lead to similar improvements in laying hens, which would help alleviate the incidence of osteoporosis later in the life of the hen. In addition to these beneficial effects on bone development, as an anti-inflammatory, MEL is inhibited by inflammatory mediators through the downregulation of AANAT and clock gene expression (Majewski et al., 2005b; a; Carrillo-Vico et al., 2013). Thus, constant light also negatively impacts health by promoting inflammatory markers (Majewski et al., 2005b; Shini et al., 2010).

3.4 AGRICULTURAL ANIMALS - HORSES

3.4.1 Circadian rhythmicity in horses

Since their domestication over 6000 years ago, horses have been an important part of human life and have served in different roles including agriculture, transportation, and racing. However, the domestication of horses has changed their environment from lush grasslands where they were exposed to natural photoperiods and continuous grazing and activity to confined indoor housing with regimented feeding, exercise, and social interaction. Horses maintained on pasture under a natural photoperiod exhibited ultradian rhythms in activity with multiple bouts equally distributed over day and night, while horses maintained in the barn exhibited circadian rhythms with significant diurnal variation (Martin et al., 2010). This makes the distinction of horses being diurnal or nocturnal more difficult, as they display different rhythms under different conditions. While melatonin levels are diurnally rhythmic in horses, the rhythm is not considered circadian as it is absent under constant dark conditions, suggesting that horses are very sensitive to light entrainment (Murphy et al., 2011). Melatonin acts as the hormonal hand of the circadian clock, working to entrain peripheral clocks in all tissues by binding to melatonin receptors (Pandi-Perumal et al., 2008). Several tissues across equine physiology express melatonin receptors and are responsive to its binding including the adrenal gland, which controls the circadian rhythm of

glucocorticoid production, which in turn control the rhythmic production of steroid hormones (Son et al., 2008).

3.4.2 Effect of light schedules on mare breeding

The equine racing industry operates with universal birthdays for foals in since race entries are tied to age in many instances. The birthday varies between breeds and hemisphere with January 1st being the birthday for Thoroughbred and Standardbred horses in North America. In order to have mares' foal as close to this birthday as possible, breeders use artificial lighting schedules to advance the breeding season in mares by extending the photoperiod to mimic spring conditions. Shorter durations of melatonin reduce the inhibition of gonadotrophin releasing hormone. Historically, light from a 100-watt bulb in a 12-foot by 12-foot stall has been used. Mares exposed to an artificially extended photoperiod during anoestrus responded to exogenous GnRH administration within 6 weeks, while mare exposed to natural photoperiod responded within 12 weeks (Nequin et al., 1990).

In 2011, a study demonstrated the threshold level of blue light required to inhibit circulating concentrations of melatonin in the horse and found it to be within the range of 10-50 lux, much lower than the 250 lux illumination produced by 100-watt bulbs in stalls (Murphy et al., 2011). Moreover, blue light directed at a single eye achieved melatonin suppression equal to that of blue light directed at both eyes. In a second, multi-institutional study, low level light was directed at a single eye from a head-worn light mask in non-pregnant mares maintained outdoors starting December 1st. 80% of this group indicated ovulation, as compared to 87.5% of another group housed indoorsunder 250 lux conditions until 11 PM and 21% of the control group, housed outdoors under natural photoperiod. Therefore, low-level blue light from mobile light masks can

successfully advance the breeding season in mares by mimicking the photoperiod of summer and has implications for improving efficiency of equine breeding management (Murphy et al., 2014). Furthermore, administration of artificially extended photoperiods pre-partum in another study resulted in reduced gestation lengths, increased birth weight, and improved coat condition as compared to control mares bred early in the year (Nolan et al., 2017). Equilume Performance Lighting, a company based in Ireland, has developed this unique blue light mask system, allowing racehorse breeders to improve conditions for their animals (Lutzer et al., 2022).

3.5 NEW FRONTIERS

3.5.1 Aquaculture

Aquaculture is the fastest growing food sector (G D Stentiford et al., 2022; Grant D. Stentiford et al., 2017) and intensification of the industry poses significant health and welfare challenges, and wider environmental issues. In fish, it is now common practice to use highly extended daylengths or even constant light (LL) in order to increase growth (Boeuf & Le Bail, 1999) and manipulate maturation (Strand et al., 2018) and reproduction (Wang et al., 2010). Whilst in some species these extreme light regimes appear to have no observable behavioural or physiological effects (Fang et al., 2019; Hamilton et al., 2022; Hines et al., 2019), in others they elicit markers of stress and/or alter immune profiles (Leonardi & Klempau, 2003; Melingen et al., 2002). However, the extent to which manipulation of circadian biology in cultured aquatic species influences susceptibility to disease – arguably the greatest challenge to aquaculture (G D Stentiford et al., 2022; Grant D. Stentiford et al., 2017)- is largely unknown. In common with other vertebrates, fish have cycling immunity (Lazado et al., 2016; Onoue et al., 2019; Zhang et al., 2020). However, they have 'decentralised clocks' (Frøland Steindal & Whitmore, 2019) and perturbation of these peripheral clocks and consequent disruption of rhythmic immunity could expose vulnerabilities in the host fish to pathogenic attack or parasitism. Indeed, it has recently been shown that LL disrupts immune gene expression in fish skin and has negative impacts on resistance to lice infestation (Ellison et al., 2021). Intriguingly, in the same study, skin microbiome profiles which also showed daily modulation in abundance and diversity are concomitantly perturbed by LL. Given the association of microbial communities and immunological status in other models (Thaiss et al., 2016) it is likely that LL might have

detrimental consequences not yet revealed with more routine physiological or behavioural analyses.

A recognition and deeper appreciation of the nuances of circadian biology on fish health could yield considerable benefits from a productivity and welfare perspective, contributing to a more sustainable industry. For example, chronotherapeutic approaches to pathogen control and disease mitigation could benefit the producer in terms of efficacious dosing and have positive ecological impacts as have been shown in other vertebrate and even plant systems (Belbin et al., 2019). Moreover, time restricted feeding (TRF) strategies are gaining interest in terms of fish growth and feed efficiencies (the single greatest cost to producers (Asche & Oglend, 2016). While TRF is known to affect immunity and other health parameters in mammalian models (Di Francesco et al., 2018; Geiger et al., 2017; Zheng et al., 2020), this is largely unexplored in cultured aquatic species. Vitally, chronotherapeutic and chrononutrition practices must consider inter-specific differences; matching conditions to chronotypes to enhance welfare, growth, and viability as a whole.

3.5.2 Circadian medicine in veterinary hospitals

Medical interventions for intensive care patients with fragile physiology are more numerous and invasive than those in a general ward settings (Kelly et al., 2014). Critically ill patients are therefore more susceptible to sleep deprivation and disturbances (Pisani et al., 2015) than their counterparts in general wards as a result of increased noise (Xie et al., 2009), patient care interactions (Gao & Knauert, 2019), mechanical ventilation (Frisk et al., 2004), artificial light (Olofsson et al., 2004) and stress (Dunn et al., 2010). Moreover, exposure to persistent light and sound disrupt the link between environmental rhythms and patients' endogenous circadian rhythms

(Diaz et al., 2019). Specifically, light in the range of 485-480 nm, seen as blue-cyan light, is the strongest activator of the melanopsin photopigment found in ipRGCs (Souman et al., 2018). Monochromatic fluorescent white light, commonly used in hospital and office settings, have emission spectral profiles with significant amounts of radiance in the blue light spectrum, from 430 – 500 nm (Elvidge et al., 2010). Several studies have documented the presence of light at night in ICU and general ward areas in hospitals and its impact on endogenous circadian rhythms (Diaz et al., 2020; Engwall et al., 2017). Evidence for the detrimental effects of ICU-type circadian disruption on healing and recovery is robust in studies using experimental rodent models. For example, a study by our group evaluated the effect of the ICU environment, mimicked by exposing mice to 5 days of a 10-hour light: 10-hour dark schedule following myocardial infarction (MI) on cardiac healing. Our ICU model led to worse cardiac remodeling and long-term outcomes due to the dysregulation of the inflammatory response following MI (Alibhai et al., 2014). More recently, mice exposed to dim light at night following cardiac arrest and resuscitation, experienced attenuated recovery as compared to regular light-dark schedule mice (Fonken et al., 2019). While the effects of circadian disruption on human patients have been evaluated extensively in recent years (Diaz et al., 2020; Telias & Wilcox, 2019), little attention has been given to its effects on companion animal patients.

Veterinary critical care units operate in a similar manner to human hospitals, providing intensive 24-h observation and care, using similar equipment for treatment and monitoring, working with multiple patients in shared common areas, and keeping housing and treatment areas well-lit to accommodate these activities. As a result, animal patients in these critical care units are subject to many of the same disturbances as human patients and likely have analogous reactions to stimuli disrupting their sleep and circadian rhythms. Exposure to constant light is anticipated to

have the greatest impact on sleep disturbance in this context. However, given the behavioural biology and standard management of domestic cats and dogs during veterinary visits, it is possible that other non-photic disruptions are also particularly relevant with a greater impact on animal patients than they do on human patients. Key differences between the species that might impact circadian disruptions include enhanced sensory sensitivity, particularly to auditory and olfactory stimuli, and a high prevalence of fear-related issues related to clinic visits and veterinary handling and procedures.

Both cats and dogs have a wider range of hearing than humans (Hefner, 1983; Heffner and Heffner, 1985), with increased sensitivity to high frequency noises that are common in veterinary settings including electronics (e.g., monitoring equipment, computer screens) and mechanical noise (e.g., kennel doors opening and closing, equipment being moved around). In addition, both species show obvious disturbance and stress responses during exposure to loud noises in general (Beerda et al., 1998; Eagan et al. 2020; Gruner, 1989; Haverbeke et al., 2008) and during veterinary visits (Stellato et al 2019; Furgala et al, submitted), and are prone to noise phobia (Blackwell et al, 2013; Gates et al 2019; Storengen and Lingaas, 2015). Thus, sound is an important disruptor to consider in veterinary critical care units. A prospective, observational study performed in two academic veterinary ICU settings found that noise levels were comparable to ICUs in human hospitals, and exceed recommendations by the World Health Organization (WHO) of a maximum of 35 dB at night in human ICUs (Fullagar et al., 2015). Further research has confirmed these findings and found that average decibel (dB) levels between 6 PM and 9 PM exceeded 76.97 dB, equivalent to the noise level of a vacuum cleaner or an average radio (Dornbusch et al., 2020). In addition to the impact of background noise, domestic cats and dogs also have a highly sensitive olfactory systems Kokocińska-Kusiak et al, 2021; Vitale Shreve and

Udell, 2017) with the potential for fear elicitation and general disturbance from exposure to olfactory stimuli that go largely unnoticed by less-sensitive humans (e.g., pungent cleaning supplies, odours from natural predators, pheromones). To date no research has examined the impact of these olfactory cues on animals in veterinary settings, and it is an important area requiring further investigation. Overall, the potential impact of disturbance from these types of stimuli is likely to be greater in companion animals than in human patients and requires careful consideration.

In recent years there has been increasing recognition that many animal patients experience high levels of fear and stress during veterinary visits; rodent studies suggests that fear experiences can impact circadian rhythms (e.g., Amir and Stewart, 1998; Pellman et al 2015), highlighting the potential for stressors to exacerbate circadian disruptions in veterinary critical care units. Animal patients are exposed to a range of stressors in clinic including unfamiliar environments, noises, odours, people and other animals, as well as handling and restraint and uncomfortable and painful procedures. While these experiences are also common for humans during hospital visits, animals do not have the same understanding of the necessity for their visit and generally lack predictability and control of their experiences during care. As a result, a majority of cats and dogs show increased signs of fear and stress during standard clinic visits and during routine handling and procedures (Döring et al ; Glardon et al., 2010; Moody et al 2020; Stanford, 1981), and this can lead to increased disturbance during routine monitoring and procedures, as well as ongoing hypervigilance during undisturbed periods.

Although no studies have investigated the specific impacts of high light levels and persistent disturbance on cats and dogs undergoing care in veterinary hospitals, there is some

evidence to suggest that these species are, in fact, physiologically impacted by circadian disruption. Low light intensity (50 lux) at night promotes improved sleep in dogs, whereas strong illumination (1600 lux) during this time has a negative influence on sleep behaviour (Fukuzawa & Nakazato, 2015). Furthermore, constant light conditions have been found to result in disruption of rhythmicity in measures such as intraocular pressure in dogs (Piccione et al., 2010), and cerebral spinal fluid concentrations of vasopressin and melatonin in cats (Reppert et al., 1982).

While it is possible to decrease interventions and monitoring at night in more stable ward patients, ICU patients are often in more severe conditions, and decreasing attention at any time of day would be detrimental to their recovery. Therefore, interventions that reduce circadian disruption while not interfering with monitoring and care are clearly warranted. Cycled lighting and light-blocking at night are two interventions that have shown promising results in mitigating the adverse effects of light exposure at night in humans. Several studies have suggested the implementation of an integrated "chrono-bundle" of interventions to entrain faltering circadian rhythms in critically ill human patients (Hu et al., 2010; McKenna et al., 2018; Patel et al., 2014; Scotto et al., 2009; Xie et al., 2009). One solution might be the implementation of a circadian lighting system, with bright, blue-enriched light during the day and dim, blue-poor light during the night, to minimize the disruptive effect of the light while allowing care-providers to observe the patient with proper lighting conditions. By minimizing power density between 450 and 500 nm and adding an extra spectral "peak" around 420 nm to maintain color temperature, Souman et al. (2018) were able to show a significant reduction in melatonin suppression as compared to normal monochromatic white light, indicating the potential utilization of blue poor light for medical settings during the rest period (Souman et al., 2018). This approach has been implemented in several major human hospitals and has displayed significant results including reduced sleep

disruption and subsequent delirium (Engwall et al., 2015; Engwall et al., 2017; Ruben, Francey, et al., 2019). Other interventions include reducing exposure to computer or tv screens at night, which emit blue-rich light as well. Reducing noise exposure by separation of emergency triage areas from recovery areas in the ICU may also prove to be beneficial and reduce distress in recovering animals.

Taken together, these studies demonstrate the detrimental effects of circadian disruption by constant light and noise in ICU settings for both human and animal patients. While several human care settings have begun implementing solutions to mitigate circadian disruption, veterinary hospitals have been left out of the picture. Strategies to implement circadian lighting, chronotherapeutics and align environmental rhythms with patient endogenous rhythms may improve healing and recovery of our companion animals.

3.6 CONCLUSION

This review introduces the field of circadian veterinary medicine by highlighting and discussing current practices in veterinary medicine and applications of circadian biology to improve the welfare, health and recovery of our companion and agricultural animals. To date, most efforts have not advanced past the description of circadian physiology in companion and agricultural animals, and for the most part, to be translated to human medicine. Further research pertaining to the application of circadian lighting, chronotherapy and time-restricted feeding, to name a few, have enormous promise to improve both veterinary and human medicine.

CHAPTER 4: GENERAL DISCUSSION

Summary

In this thesis, I explored applications of circadian biology to veterinary medicine, highlighting a novel field of circadian veterinary medicine. Chapter 1 discussed the current literature supporting the rationale of this research. Chapter 2 demonstrated that several circadian biology concepts can be readily applied to benefit the health and welfare of companion and agricultural animals. Chapter 3 presented evidence to support my hypothesis, that light and sound at night in veterinary clinical care settings disrupt patient circadian rhythms and adversely affect recovery from disease. To test this hypothesis, I had 3 objectives to investigate; **Objective 1**) Are patients in veterinary clinical care settings exposed to abnormal levels of light and sound at night? This was shown by measuring levels of light and sound intensity in veterinary clinical care settings for 2 weeks. **Objective 2)** Does exposure to light at night impact circadian rhythms of key physiological parameters in dogs? This was shown by measuring the physiological response to circadian disruption by light exposure during the rest period, and circadian realignment by red light exposure during rest using non-invasive telemetry in dogs and running wheel actigraphy as well as invasive telemetry in mice. **Objective 3)** Does circadian rhythm misalignment affect recovery from disease? This was shown by measuring the recovery of left ventricular function of hearts isolated from mice subjected to circadian misalignment following ischemia-reoxygenation using *ex-vivo* isolated heart perfusion in langendorff mode as well as by measuring differences in gene expression of autophagy pathway genes *ampk, becn1 and atg5* and the central circadian gene *clock.* In this section, future directions and potential applications of this research will be discussed.

4.1 Circadian Medicine in a Veterinary Hospital

4.1.1 Cancer Models

Recently, the longevity of our companion animals has increased, owing to improvements in veterinary treatment, housing, and nutrition. However, this has also resulted in an increased incidence of malignant cancers. At present, the largest cause of death in older companion dogs is malignant cancer in countries including Sweden, the United States, the United Kingdom and Japan (Bonnett et al., 2005; Fleming et al., 2011; Inoue et al., 2015; O'Neill et al., 2013). In fact, between 15-30% of mortalities in older companion dogs were due to cancer depending on breed and country (Inoue et al., 2015). The impact of circadian rhythms in cancer pathophysiology has been thoroughly investigated using murine models. For example, circadian disruption has been shown to promote tumor immune microenvironment remodeling and increase tumor cell proliferation (Mul, 2020). Recently, several reviews have identified potential circadian targets for cancer therapy, focusing on human cancers (Puppala et al., 2021; Ruan et al., 2021; Ruben et al., 2018). Future studies could apply the methods I used to investigate the effects of circadian disruption and realignment of circadian rhythms and identify their effects on cancer pathophysiology clinically.

4.1.2 Monitoring patients in veterinary clinical settings

In this thesis, I demonstrated the presence of light and sound at night in veterinary clinical care settings, and further demonstrated their disruptive effect on the circadian rhythms of healthy dogs by simulating the ICU environment. Previous studies in human clinical care settings measured the effect of circadian disruption by hospital settings on patients in clinic (Diaz et al., 2020). The next step to implementing circadian lighting strategies in veterinary hospitals would be to measure circadian rhythms of veterinary patients in the ICU using the non-invasive telemetry techniques I used. This would allow for a greater understanding of the effect of circadian disruption on sick

animals and how to best mitigate the effects of light and sound at night. Alternatively, patients in ICU commonly have several parameters being measured at all times, including blood pressure, heart rate and temperature. These parameters could be used to monitor the effect of the hospital environment on patient rhythms as they are highly regulated by the circadian system.

4.1.3 Operational rhythms in veterinary hospitals

In this thesis, I demonstrate that veterinary hospitals operate in a similar manner to human hospitals with regards to light and sound exposure. Previous studies have identified operation rhythms in human hospitals, which affect the timing of drug prescription and administration (Ruben, Francey, et al., 2019). Since veterinary hospitals also operate on a 24-h schedule and utilize shift-working conditions, it is presumable that operational rhythms exist here as well. Identifying these rhythms would require studying a large database of patient records over several years. These rhythms are important as timing of drug administration, or chronotherapy, has been suggested to improve drug efficacy (Cederroth et al., 2019; Ruan et al., 2021; Ruben, Francey, et al., 2019; Sulli et al., 2018).

Like many other industries, modern healthcare operates night and day year-round, creating the need for shiftwork. Shift-working conditions in hospital settings impact the health of patients as well as healthcare workers. For example, night-shift nurses exhibited significantly dampened rhythms in melatonin and cortisol levels as compared to day-shift nurses, in addition to misaligned rhythmic transcripts (Resuehr et al., 2019). Previously, our group showed that shift-working conditions applied following myocardial infarction result in impaired recovery and worse long-term outcomes (Alibhai et al., 2014). In addition, time of medication administration is an important aspect of treatment. While many medications should be administered at a certain time, a recent

study found that administration time exhibits a 24-h rhythm characterized by a morning surge and overnight lull consistent with rounding times (Ruben, Francey, et al., 2019). The systemic bias towards treatment at a certain time of day is problematic as medications should be administered when they are needed to provide the maximal benefit. Overall, shift-working conditions present a systemic challenge for healthcare workers as it affects both their own and their patients' health.

4.2 Circadian Applications to Veterinary Medicine

4.2.1 Scientists

This thesis introduces the field of circadian veterinary medicine, which aims to apply established circadian medicine approaches including chronotherapy, circadian lighting and chrono-nutrition to benefit companion and agricultural animal health. In order to expand this field and provide novel circadian medicine therapies to benefit veterinary patients, more groups should aim investigations to have translational value to veterinary as well as human patients. This would be especially efficient as several animal models are already used in translational medicine studies. For example, Poliquin et al. used a non-human primate model, specifically rhesus macaques, to investigate the effects of prolonged intensive care (Poliquin et al., 2017). Similarly, Leyden et al. used a swine model to assess the effect of intensive care on biological rhythms (Katrina N. Leyden & Smolensky, 2015). These studies hold immense value for translation to human clinical medicine, however, their value in veterinary care settings is often overlooked. In sum, communicating the scale of veterinary care and the importance of circadian biology to veterinary patient health and recovery to translational medicine groups aimed towards human health would greatly benefit circadian veterinary medicine. The review paper discussing the potential applications of circadian

medicine to veterinary medicine could serve as a valuable resource to groups unsure of how their circadian medicine approaches could improve companion and agricultural animal health and recovery from disease.

4.2.2 Veterinarians

The importance of circadian rhythms to patient health has been increasingly appreciated in human clinical settings (Chan et al., 2012; Diaz et al., 2020; Gao & Knauert, 2019; Telias & Wilcox, 2019). In contrast, literature discussing the clinical relevance of circadian biology in veterinary medicine is sparse. For example, a recent review discusses the causation and implications of psychogenic stress in hospitalized veterinary patients (Lefman & Prittie, 2019). Apart from this review, I was unable to find any other comprehensive literature highlighting circadian biology in veterinary medicine. This means that veterinarians are unable to access information regarding circadian rhythms and circadian medicine with regards to their patient populations and are thus unable to apply these therapies to benefit their patients. More investigations applying circadian biology to veterinary patients clinically will add to the literature base of circadian veterinary medicine, making information more easily accessible. Additionally, integrating circadian rhythms into veterinary education will make veterinarians more aware of the importance of circadian biology to their patients' health and well-being. My review serves as a valuable resource to veterinarians to understand the potential applications of circadian medicine to their patients and will spur more clinical trials to investigate further.

4.3 Conclusions

Collectively, this work provides new understanding of the circadian system in veterinary settings, pioneering unique applications of circadian medicine to companion and agricultural

animals in clinical and industrial environments. These studies provide a strong base for the field of circadian veterinary medicine to improve the lives of animal and human patients alike.

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