# THE INTERPLAY OF LIGHT AND MELATONIN IN BIOLOGICAL TIMEKEEPING:

## CONSERVATION, DIVERGENCE, AND TRANSLATION OF THE DROSOPHILA TIMEKEEPING NETWORK

By

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# Dedications

Dedicated with love to:

My parents Kathé and Dennis Dollish

Your constant encouragement for my love for science and love and support throughout my life, at the highest highs and lowest lows, are the reason this thesis exists.

&

My mentor Dr. Lynne Oland

The most dedicated, loving, passionate woman I have ever met. I don't know if I would be even a fraction of the woman I am today if I was not blessed enough to have been mentored by you.

Thank you all for every lesson you have taught me and every experience you gave me. If people were half as amazing as you this world would be a much better place.

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For your understanding, love, and support for 24 years, thank you for being my chosen sister. Phase one complete Pinky.

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**Abstract:**

The central pacemaker for biological rhythms in mammals is located in a 10,000-cell structure known as the suprachiasmatic nucleus (SCN) in the anterior hypothalamus (Hastings_2018, Meijer_1996). Clock cells in the suprachiasmatic nucleus work together to generate a coordinated and synchronous output that results in an intrinsic endogenous “ticking” of the biological clock at a set periodicity (Liu_1997). Circadian rhythms are generated by the daily fluctuation of a transcription-translation feedback loop between clock proteins and genes located in clock cells. Master pacemaker cells in the SCN project to peripheral pacemaker cells in various organs and tissues in the body. All biological rhythms oscillate at a set period known either as the intrinsic or endogenous period and is determined by the oscillatory speed of the transcription-translation feedback loop determined by the individual organism’s genetics (Jin_1999, Liu_1997).

The endogenous period is not typically aligned optimally with the external 24-hour period of the earth’s daily rotation about its axis. The amount of time it takes for an animal to return to the same phase of its endogenous rhythm without an external natural or artificial cycle to align to is known as the free running period.  Mammals do not have endogenous periods that cycle at exactly 24-hours but generally have free running periods ranging between 22 and 26 hours, with diurnal mammals typically exhibiting free running periods above 24-hours (Aschoff_1981). The difference in cycling between the endogenous period and the external 24-hour photoperiod (the period of light and dark denoting one complete rotation of the earth about its axis), eventually results in a drift and subsequent misalignment of important behaviors, which lead to negative and in some cases fatal health outcomes for living organisms (Aschoff_1981, Lewy_2009). In order to prevent misalignment and optimally time behaviors to the external photoperiod, the biological clock evolved a process known as entrainment. Entrainment of biological rhythms to an external zeitgeber is achieved through advancing or delaying internal rhythms based on the animal’s previous photic history, so that they optimally align with the 24-hour external period (Julius_2019).

The first chapter in this section will cover the anatomy and circuitry of the SCN, mechanisms of the mammalian molecular and biological clock, and explore the mechanisms of entrainment for optimally phase aligning biological rhythms to a 24-hour photoperiod. In the second chapter, I will describe the *Drosophila* biological time keeping network, discuss what mechanisms and functions are conserved or diverge between mammals and *Drosophila*, and where possible translational overlap between mammals and *Drosophila* can occur to further our understanding of entrainment of circadian and seasonal rhythms. The final chapter in this section will present published data on a comprehensive seasonal phase response curve (PRC) atlas of phase shifts in the activity rhythms of *Drosophila ananassae* under different seasonal photoperiods.

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Timing behavior and physiology to the 24-hour solar cycle has enabled life to persist and thrive on Earth. The most powerful time cue, light, is used for aligning endogenous rhythms to the day and night cycle; however, the clock also heavily relies on another internal rhythm, melatonin secretion, to help adjust circadian but primarily seasonal rhythms cooperatively with light input (Aschoff_1978, Ardent_2009). Melatonin and light work synergistically to entrain endogenous rhythms to the external photoperiod set by the solar cycle (Wood_2013, Wehr_1997). Together, both light and melatonin optimize interconnecting suites of behavior and physiology so that they are expressed during the most conducive parts of the day-and-year to enhance biological fitness. Regarding circadian/seasonal function, it is impossible to talk about melatonin without also understanding (1) the control that ambient light exerts over its secretion and (2) how melatonin feeding back onto the clock can work to adjust oscillatory behavior the clock makes in response to light exposure, especially during the subjective night.

Melatonin functions as an important biological imprint of night-length by supplementing daytime light information collected by the brain's circadian pacemaker. The suprachiasmatic nucleus (SCN) acts as the mammalian biological clock; and the Lateral and Dorsal Nuclei (LN and DN respectively) comprise the Drosophila clock network (Taghert_2006, Ardent_2009). Melatonin has its primordial role as an antioxidant that directly and indirectly – through metabolite cascades – sequesters free-radicals to help prevent oxidative stress (Zhao_2014, Manchester_2015).

Melatonin freely circulates throughout the body and passes through lipid membranes and the blood brain barrier (Pardridge_1980, Costa_1995). These two features contribute heavily to its potency as an antioxidant in both the plant and animal kingdoms. Timing of melatonin secretion is important to optimize its antioxidant properties and to phase-lock various biological behaviors to occur at the optimal phase of the day-night cycle. Its enhanced secretion at night tracks closely to periods of high oxidative stress that occur during the day when solar UV radiation and system energy demand are at their highest (Haldar_2010). This is thought to keep oxidative processes to a minimum during the day and time the bulk of them to occur during the evening when there is less demand on the system from UV-radiation and environmental conditions.

The history of melatonin's functionality, from just an antioxidant in simpler species to evolving more of a modulatory role for circadian and seasonal behaviors in higher invertebrates, avian species, and mammals, is an interesting one. It stemmed from melatonin's ability to orchestrate and participate directly in restorative processes that work best in the absence of light (such as DNA methylation and sequestration of free radicals) (Zhao_2019). More of its evolutionary history will be discussed later, but it is interesting to note that melatonin concentration is highest in mitochondria, where many reactive oxygen species (ROS) and reactive nitrogen species (RNS) are generated during the citric acid cycle.

Finally, data will be presented on a comprehensive seasonal study on melatonin secretion in *Drosophila ananassae* across different seasonal photoperiods. This data was collected in an attempt to draw a phylogenetic line between where melatonin took on its circadian and seasonal role and where it acts solely as an antioxidant. This data also sought to understand what, if any, circadian and seasonal properties of melatonin are conserved between the *Drosophila* and mammalian systems.

Using the gold standard of melatonin measurement, mass spectrometry (Markey_1981), and over 18,000 animals, we measured melatonin secretion in 4 photoperiods from high summer (16:8), equatorial (12:12), and high winter (8:16) photoperiods. Animals were also exposed to an extreme arctic summer photoperiod (20:4) to evaluate the potential suppressive effects of light on melatonin secretion in this species in addition to a physiologically extreme photoperiod.

# Part 1:

# Circadian and Seasonal Timekeeping

**Part 1: Introduction/Abstract**

The central pacemaker for biological rhythms in mammals is located in a 10,000-cell structure known as the suprachiasmatic nucleus (SCN) in the anterior hypothalamus (Hastings_2018, Meijer_1996). Clock cells in the suprachiasmatic nucleus work together to generate a coordinated and synchronous output that results in an intrinsic endogenous “ticking” of the biological clock at a set periodicity (Liu_1997). Circadian rhythms are generated by the daily fluctuation of a transcription-translation feedback loop between clock proteins and genes located in clock cells. Master pacemaker cells in the SCN project to peripheral pacemaker cells in various organs and tissues in the body. All biological rhythms oscillate at a set period known either as the intrinsic or endogenous period and is determined by the oscillatory speed of the transcription-translation feedback loop determined by the individual organism’s genetics (Jin_1999, Liu_1997).

The endogenous period is not typically aligned optimally with the external 24-hour period of the earth’s daily rotation about its axis. The amount of time it takes for an animal to return to the same phase of its endogenous rhythm without an external natural or artificial cycle to align to is known as the free running period.  Mammals do not have endogenous periods that cycle at exactly 24-hours but generally have free running periods ranging between 22 and 26 hours, with diurnal mammals typically exhibiting free running periods above 24-hours (Aschoff_1981). The difference in cycling between the endogenous period and the external 24-hour photoperiod (the period of light and dark denoting one complete rotation of the earth about its axis), eventually results in a drift and subsequent misalignment of important behaviors, which lead to negative and in some cases fatal health outcomes for living organisms (Aschoff_1981, Lewy_2009). In order to prevent misalignment and optimally time behaviors to the external photoperiod, the biological clock evolved a process known as entrainment. Entrainment of biological rhythms to an external zeitgeber is achieved through advancing or delaying internal rhythms based on the animal’s previous photic history, so that they optimally align with the 24-hour external period (Julius_2019).

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# Chapter 1

## Circadian and Seasonal Timekeeping in Mammals

## 1.1 Anatomy of the Suprachiasmatic Nucleus in Mammals

### 1.1.1 Circuitry and Projections of the Suprachiasmatic Nucleus

In mammals, the suprachiasmatic nucleus (SCN) is comprised mostly of GABAergic neurons that co-express different neuropeptides: arginine vasopressin peptide (AVP), vasoactive intestinal peptide (VIP), peptide histidine isoleucine (PHI), and gastrin releasing peptide (GRP) (Romijn_2006). The SCN can be anatomically segregated into 2 regions: the ventral core and the dorsal shell. The dorsal shell region of the SCN has predominately AVP neurons verses the area comprising the ventral core consisting of mainly VIP neurons. Interspaced between these regions are GRP and PHI neurons as well as other neuropeptide expressing cells; however, AVP and VIP neurons are by far the most prolific cell types within the SCN (Varadarajan_2018).

Light input from intrinsically photosensitive retinal ganglion cells (ipRGCs) is sent through the retinohypothalamic tract which has projections to VIP neurons in the ventral core. The ventral core is where light input to the SCN is integrated along with input from projections originating in the median raphe and intergeniculate leaflet in the thalamus (Moore_1973, Mieda_2019). VIP neurons send the majority of their projections to AVP neurons in the dorsal shell but some are sent to pacemaker cells the thalamus and basal forebrain and other areas outside the SCN (Leak_2001). The dorsal shell, along with receiving inputs from the ventral core, also receives afferent projections from the hypothalamus, thalamus, brain stem, and limbic regions (Leak_2001, Moga_1998). The main efferent projections of the SCN mostly come from the dorsal shell which sends projections to areas in the diencephalon, lateral septum, medial hypothalamic areas, and the paraventricular thalamic nucleus (Mieda_2019, Abrahamson_2001, Leak_2001).

### 1.1.2 Ventral Core and Vasoactive Intestinal Peptide (VIP) Neurons

VIP neurons are thought to be most important for synchronizing and maintaining individual clock neurons within the SCN (Herzog_2017). Mice without VIP or the VIP receptor gene *VIP2* show weaker behavioral rhythms due to lacking the VIP signaling needed to maintain circadian function (Harmar_2002). Loss of VIP or the VIP2 receptor abolishes circadian firing rhythms in about 50% of all SCN neurons and causes disruption of synchronous firing in rhythmic SCN neurons, with VIP2 agonists capable of restoring function (Aton_2005). Co-culturing cells with mutant periods with "host" wild-type SCN cells shows the host SCN taking on the period of the grafted mutant genotypes. VIP was the predominate neuropeptide that contributed to setting and maintaining the different periods of the mutant genotypes in the host SCN (Maywood_2011).

VIP secretion needs to be rhythmic in order for global synchronization by the ventral core to be effective. Over-production of VIP, mimicked by excessive application of VIP onto SCN slices, resulted in asynchronous firing of cultured SCN cells (Shinohara_1999). Paracrine signaling between VIP cells in the SCN is also needed for synchronous firing of SCN cells (Harmar_2003, Maywood_2011) as VIP is the predominate neuronal signal for pacemaking maintenance. However, other small molecules such as AVP and DRP are also needed for synchronization at both cellular and circuit level circadian pacemaking in the SCN (Maywood_2011, Ono_2016, 2021). Taken together it can be concluded that VIP and VIP neurons are the primary paracrine signal used to coordinate daily rhythms through maintaining and sustaining the rhythmicity of the broader SCN neuronal population.

VIP neurons are thought to exert their synchronous effect through GABAergic signaling (Aton_2006). In a summer photoperiod, where the daylight represents the larger portion of the photoperiod, rhythms between the dorsal and ventral regions are decoupled at the beginning of the summer photoperiod but gradually resynchronize as the photoperiod progresses (Evans_2013, Myung_2015). The resynchronization under summer conditions is blocked by a VIP antagonist which attenuates the advance or delay areas of the coupling curve between neurons (Myung_2015). $GABA_A$ antagonists also block resynchronization but only seem to affect the advance zone and GABA is able to restore synchronization in SCN cells lacking VIP but not WT SCN cells (Ono_2019).

There is growing and convincing evidence for entrainment of circadian and seasonal rhythms being one of, if not the, main role(s) of VIP neurons (An_2011). When applied to cultured cells, VIP rapidly resets Per2 rhythms in a temporal and dose dependent manor. VIP increases intracellular cAMP in SCN neurons and treatment of cells with daily VIP entrains the Per2 rhythm in several days. Simultaneous inhibition of adenylyl cyclase and phospholipase C activity blocks VIP-induced phase shifts. Thus, VIP neurons act to entrain rhythms in parallel with changes in adenylyl cyclase and phospholipase C activity (An_2011).

In another display of VIP mediation of entrainment, Jones et. al. preformed an in vivo photometry experiment in 2018 which showed spontaneous circadian calcium activity in VIP neurons in both Light:Dark (L:D) and constant dark (DD) conditions, but not in environments with continuous light (Jones_2018). Light pulses resulted in spikes of $Ca^{2+}$ activity during subjective dusk which peaked over the course of the subjective night. Hyperpolarization of VIP neurons attenuated light-induced locomotor behavior shifts (Jones_2018). Light induced phase shifting of the mammalian molecular clock is still under investigation; however, VIP neurons seem to play an important role in mediating entrainment in the SCN.

### 1.1.3 Dorsal Shell and Arginine Vasopressin Peptide (AVP) Nucleus

Aginine vasopressin peptide (AVP) neurons are mainly localized to the dorsal shell region of the SCN in mammals. The rhythmic fluctuation of AVP mRNA is highly circadian due to the E-box on the AVP promotor being a target of BMAL1/CLOCK transcription factors (Nakada_2021) which is the molecular driver of the circadian transcription-translation feedback loop in the SCN (Jin 1999).

AVP's main role is modulating the coupling of the SCN circadian network (Media_2015, Ono_2021). A study by Mieda et. al. looked at AVP's coupling role using a Cre-loxP genetic manipulation of AVP specific *Bmal1 knockouts (Avp-Bmal1*$^{-/-}$*)*. These mice were generated by crossing AVP-Cre mice with floxed Bmal1 alleles. Bmal1 was significantly reduced in AVP neurons as was verified using a Rosa26-tdTomato reporter for AVP neurons with anti-Bmal1 immunofluorescence, but neurons that contained Bmal1 were unchanged (Mieda_2015) when compared to controls.

BMAL1 AVP deficient mice showed a lengthening of the free-running period and activity time of behavior rhythms. This type of AVP deficiency also resulted in weaker coupling among SCN neurons. BMAL1 was also shown to be necessary for expression of key factors in AVP neurons, giving evidence that BMAL1 dependent oscillations of AVP neurons are used to stabilize and modulate circadian coupling of SCN network oscillations (Mieda_2015). AVP neurons are also thought to determine the circadian period at the network level (Mieda_2019). Knockout of casein kinase 1δ (CK1δ) through the SCN and Avp-CK1$\delta^{-/-}$ mice both show an increase in the free running period by roughly 40 minutes, indicating an important role for AVP neurons in determining the free-running period (van der Vinne_2018, Mieda_2019).

Interestingly, AVP mRNA levels have seasonal variations but little circadian fluctuation. In short photoperiods (8:16) morning rises in AVP mRNA occur later than under long photoperiods (16:8) (Jăč_2000). These data indicated a seasonal photoperiod effect on AVP mRNA levels that could contribute to the entrainment of seasonal rhythms.

### 1.2 The Mammalian Circadian Timekeeping System

The origin of behavioral genetics began with the discovery of clock genes in the 1960's and 70's, when Dr. Seymore Benzer proved that a single gene could control complex behaviors (Konopka & Benzer_1971). In this paper, Konopka and Benzer described the discovery of the per gene in *Drosophila* by noting that mutations in this gene resulted in behavioral changes, arrhythmic rhythms and either shortened or lengthened circadian periods, in the flies' normal 24-hour rhythms. This discovery was one of, if not the, most important finding to occur in neuroscience as it was the first study to show a direct correlation between genes and behavior.

Research on behavioral genetics, specifically how genes affected the biological timekeeping system, eventually lead to the Nobel Prize in Medicine being awarded in 2017 to Drs. Rosbash, Young, and Hall for their work in uncovering the molecular mechanisms underlying circadian rhythms and through their discovery and manipulations of the gene, *period*, in *Drosophila*. *Drosophila* is still a pioneering model of circadian and biological rhythms, and fortunately, findings were easily translatable into mammalian and avian species due to the high conservation of core clock mechanisms.

The molecular clock of all living organisms uses various zeitgebers, the most important and powerful being highly reliable environmental light cues, to synchronize internal rhythms to the exogenous environmental periodic cycling of light/dark transitions – photoperiod (Aschoff 1978, Hastings_2018). The mechanism of aligning internal rhythms to external zeitgebers is known as entrainment and one of the most important evolutionary processes for adaptation living organisms developed. Entrainment of internal rhythms to the external photoperiod ensures that physiological, cellular, and molecular processes and behaviors occur during the most optimal times in the solar cycle allowing species to occupy temporal niches in their environment.

For entrainment to work, the clock phase shifts endogenous behavioral and physiological rhythms to occur sooner (advance) or later (delay) to align to seasonal changes in photoperiod length. Whether or not the clock advances or delays biological rhythms is most dependent upon the previous day's photic history (Chang_2011). Another important factor in determining and advance or delay is if and where light is perceived during the night phase of a photoperiod, or the subjective night in free running, constant dark, conditions (Aschoff_1978, Aschoff 1981).

If an organism is exposed to light during the earlier part of the night the clock will interpret this as the new "sunset" and will delay the following day's rhythms to match this new photoperiod. Conversely, if light is perceived in the latter half of the dark/night phase the clock will interpret this as the new "dawn" and will advance the following day's rhythms to align with the now advanced dawn. When the clock "sees" light in the middle of the night phase, known as the singularity point, it is unable to determine if it is dawn or dusk and no phase shift will occur (Pendergast_2010, Glickman_2012). Another time where the clock will abstain from phase shifting is if light is seen during the day (at a higher intensity than background light) or during the subjective day in a free running DD condition, because it is already "day" and no adjustment to rhythmic phases needs to occur (Dollish_2022, Pendergast_2010).

In this section I will focus on how the molecular clock functions to produce behaviors and how light affects the speed at which the clock ticks. I will then discuss the possible mechanisms behind light-induced phase shifts of biological rhythms in mammals.

**1.2.1 Core Mechanisms of the Mammalian Biological Clock**

The core mechanism behind the mammalian molecular clock lies in a transcription-translation feedback loop between clock genes and their products (Figure 1). In mammals, CLOCK and Bmal1 genes encode basic helix-loop-helix on DNA E-boxes (Wang_2013). When CLOCK and *Bmal1* form the heterodimer CLOCK:BMAL1, it binds DNA elements on the E-box promotor regions on the DNA of *period* (PER) and *cryptochrome* (CRY) (Buhr_2013). Once attached, the CLOCK:BMAL1 complex transcribes mPER1 and mPER2 as well as mCRY1 and mCRY2 which then diffuse out of the nucleus into the cellular matrix where they are translated by organelles into PER and CRY proteins (Kwon_2011). In the cytoplasm PER and CRY form a stable dimer which then can reenter the nucleus to negatively inhibit their own production (Kwon_2011).

Regulation of PER and CRY levels in mammals is done through casein kinase 1 epsilon (CK1ε) and the F-box proteins FBXL3 and FBXL21. PER levels are regulated by CK1ε which phosphorylates PER and targets it for degradation (Blau_2008), and also changes its confirmation to prevent reentry into the nucleus (Vielhaber_2000). CRY levels are regulated by F-box proteins. FBXL3 promotes CRY degradation in the cytoplasm and is translocated with it into the nucleus (Yoo_2013). Interestingly, CRY degradation in the nucleus by FBXL3 is inhibited by another F-box protein FBXL2 (Yoo_2013). Regulation of endogenous period is partially due to these F-box proteins as mutations in FBXL21 shorten the endogenous period while mutations in the FBXL3 protein result in period lengthening (Yoo_2013).

In addition to the primary CLOCK:BMAL1 PER/CRY loop, there is a secondary feedback loop involving the REV-ERBα and RORα which regulate BMAL1 activity to affect the speed of the primary loop (Figure 1). REV-ERBα and RORα proteins bind their respective retinoic acid related orphan nuclear receptors (Crumbley_2012) and compete with each other to bind the same receptor on BMAL1 in the nucleus at the retinoic acid-related orphan receptor response elements (ROREs) (Guillaumond_2005).

Through ROREs, RORα and REV- ERBα are able to regulate BMAL1 by either activating it in the case of RORα binding (Akashi_2005) or repressing it through REV- ERBα binding the ROREs, Preitner_2002). This feedback loop with the CLOCK:BMAL1 dimer controlling RORα and REV- ERBα expression and RORα and REV- ERBα feeding back on the BMAL1 to regulate it is known as the secondary stabilizing loop (Ko_2006, Kwon_2011).

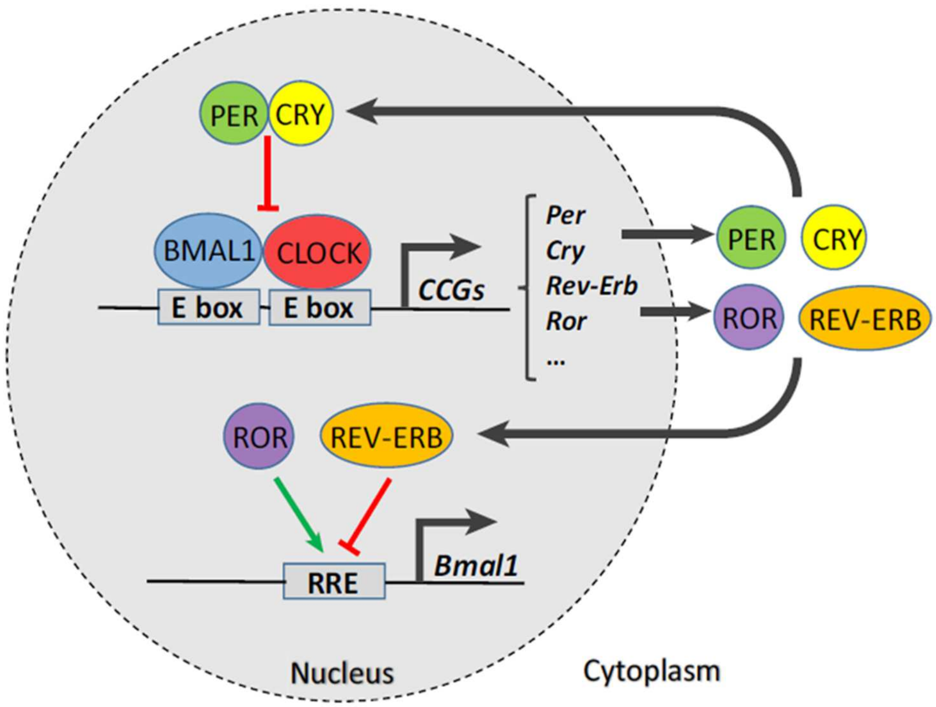



**Figure 1:** The core mechanism of the molecular clock functions as a transcription-translation feedback loop between CLOCK and BMAL1 transcription factors and PERIOD (Per) and CRYPTOCHROME (Cry) proteins. CLOCK and BMAL1 form a heterodimer and bind to the E-box region on Per and Cry genes to transcribe mRNA for Per and Cry that then translocate into the cytoplasm. In the cytoplasm Per and Cry proteins are translated from their respective mRNAs. Per and Cry then form a dimer complex which allows for reentry into the nucleus where they bind CLOCK:BMAL1 and negatively inhibit their production. The rate of cycling of Per and Cry levels sets the endogenous period. A secondary feedback loop between CLOCK:BMAL1 and ROR and REV-ERB influences the endogenous cycling of Per and Cry by affecting the rate of BMAL1 transcription. ROR and REV-ERB mRNA are transcribed by CLOCK:BMAL1 and translated into ROR and REV-ERB proteins in the nucleus. RORs feedback onto BMAL1 to stimulate BMAL1 transcription but REV-ERBs inhibit BMAL1 transcription. Figure taken from a paper in Trends in Cell Biology by Gaucher, Montellier and Sassone-Corsi (2018).

**1.2.2 Phase Shifting Biological Rhythms in Mammals in the SCN**

Understanding how endogenous periods, in the absence of zeitgebers, are generated within the biological clock, leads to the discussion of how the clock uses external cues to entrain the non-24 hour endogenous period to that of the external photoperiod. Given that light is the most powerful time cue, understanding how light is able to entrain the endogenous period to the external world is highly desirable in researching circadian and biological rhythms. Light is able to phase shift the molecular clock in the SCN by speeding up or slowing down the circadian cycling of its molecular feedback loop. As stated before, light information enters the SCN through the retinohypothalamic tract (RHT) to inform the clock about the external photoperiod. Light at night is especially important, as transient light exposure during this phase of the cycle will result in a rapid resetting of the biological clock and its behavioral outputs (Aschoff_1981).

Levels of PER and CRY accumulate in the cytoplasm during the night, forming heterodimers that then enter the nucleus to turn off their RNA synthesis by inhibiting BMAL1:CLOCK transcription of PER and CRY. This leads to a cell-wide decrease in mRNA for PER and CRY and subsequently their protein products during the day (Maywood_2003). Once there are no longer enough proteins to form stable dimers capable of entering the nucleus, BMAL1:CLOCK can begin transcribing mRNA for PER and CRY and levels begin to rise during the night (Maywood_2003, Buhr_2013, Kwon_2016). The rate of cycling between the degradation and production, determined by genetics, of PER and CRY and mRNA for PER and CRY determines the internal period of the living organism (Emery_1998, Rosbash_1995).

To alter the endogenous period, light changes the speed of PER and CRY production to adjust the internal period to phase align to the external photoperiod (Emery,1998, Zylka_1998, Aschoff_1981). Although light is known to have this effect, the mechanism behind its phase shifting properties on the mammalian biological clock has yet to be fully elucidated. However, it is thought that mechanisms driving photic resetting are due in part to rapid upregulation and downregulation of various aspects of different circadian gene expression (Buhr_2013, Michael_2017).

**1.2.2a Mechanisms for Phase Shifting in Mammals: Period**

One proposed mechanism is photic entrainment through mitogen stress activated protein kinase 1 (MKS1). Light activates MSK1 (mitogen stress activated protein kinase 1) via PACAP-ERK/MAP kinase mechanism which couples to a cAMP response element-binding protein (CREB) on the gene used to transcribe mRNA for PER1 (Butcher_2005). CREB goes on to activate cAMP response elements (CREs), specifically the promotors of Fos and PER1 (Travnickova-Bendova_2002). Research shows that the gene for the PER1 promotor responds to the co-activation of both MSK1 and cAMP pathways only when CREs were intact. These results taken with the fact that activation of PER1 gene promotors by CLOCK:BMAL1 heterodimers would occur even in the absence of CREs, show that signaling dependent activation of mPER1 and mPER2 are separate from CLOCK:BMAL1 driven transcription (Travnickova-Bendova_2002).

Another potential mechanism for light controlling PER and CRY protein levels through mRNA translation, is through the mitogen-activated protein kinase/MAPK-interacting serine/threonine-protein kinases pathway, or MAPK/MNK pathway. One branch of the MAPK/MNK pathway ends in the phosphorylation of eukaryotic translation initiation factor 4E (eIF4E) (Bianchini_2008). eIF4E is a translation factor whose activities are regulated by mTOR eIF4E binding proteins (4E-BPs) which prevent the formation of the eIF4F complex (Sonenberg_2009). It was found that when light activates the MAPK/MNK pathway it leads to the phosphorylation of eIF4F in the mouse SCN. eIF4E activity has been shown to have circadian rhythmicity in its activity in the SCN. Photo-phosphorylated eIF4E promoted translation of PER1 and PER2 mRNAs and increased the amount of basal and inducible PER proteins. This provides a means for photic phosphorylation of eIF4E to modulate PER synthesis and help to fine tune circadian resetting to new photoperiods in the SCN (Cao_2015).

**1.2.2b Mechanisms for Photic Phase Shifting in Mammals: Cryptochrome**

The control of cytochrome expression is important for proper timing of rhythms by the SCN (Michaels_2017) and are present in the inner retina (Miyamoto & Sancar_1998). Mice with a depletion of retinaldehyde, which activate opsins, have SCNs that are still photo responsive. Cryptochrome is a flavoprotein which does not depend on retinaldehyde as a chromophore and could explain the photosensitivity in the absence of opsins in the SCN (Rollag_2003).

Initially it was thought that cryptochrome was the main photosensitive protein in mammals as it was in *Drosophila*; however, understanding CRY involvement in photoentrainment is difficult. Since CRY is needed for endogenous cycling of the biological clock, genetic knockouts of CRY, one would think, will naturally exhibit a lack of self-sustaining rhythms; however, mice do show a reduced photic response visualized through *c-fos* expression (Selby_2000). The idea of cryptochrome being the main photosensitive protein was dispelled as KO experiments using Bmal1$^{-/-}$;Opn4$^{-/-}$and Per1$^{-/-}$; Per2$^{-/-}$;Opn4$^{-/-}$ mice showed the same phenotypes as CRY knockout (rd1/rd1:Cry$^{-/-}$;Cry$^{-/-}$) mice (Owens_2012).

Later, a photopigment originally discovered in dermal melanocytes of clawed frogs (*Xenopus*) (Provencio_1998) was found to be present in a small population of retinal photoreceptors (ipRCGs) in mice (Panda_2002). Melanopsin is photosensitive and is an essential part of the phototransduction cascade as loss of melanopsin abolishes intrinsic photosensitivity in mice (Rollag_2003). In addition, melanopsin-knockout mice do not show as much period lengthening in constant light conditions as wild-type counterparts (Rollag_2003). Melanopsin is not the sole photosensitive opsin, however, as knockouts do not show circadian deficiencies in activity rhythms in constant darkness (Rollag_2003).

Although cryptochrome likely plays an important role in photic entrainment, it is more likely that there are various proteins and opsins that act as a photo regulating network of circadian entrainment in mammals. Opsins that might be included in this network are: pinopsin, vertebrate ancient opsin, parapinopsin, ERrod-like opsin and teleost multiple tissue opsin (Rollag_2003). Research into their circadian functions still needs to be done and their presence and function in mammalian photoreceptors is still indeterminate.

## 1.3 Seasonal Rhythms in Mammals

The internal clock evolved in parallel to circadian variations in photoperiod length and adaptive response to seasonal variations as well. Seasonal rhythms such as breeding, hibernation, and migratory rhythms are less commonly thought of but still under the control of the internal timekeeping system. The clock not only calculates day-to-day changes in daylength but also week-to-week and month-to-month changes in the day-night cycle. The attunement of the clock to the external phase relative to internal rhythms is achieved through irradiance detection of environmental light and the rate of intensity change and differences in wavelength around sunrise and sunset (Bedrosain_2013, Bonmati-Carrion_2017, Usui_2000).

To entrain rhythms on a seasonal scale, the SCN network exhibits robust plasticity (Coomans_2015). The temporal distribution of single-cell activity that together drives the coherent waveform of global SCN output differs between short (winter) and long (summer) photoperiods (Vanderleest_2007, Rohling_2006). Seasonal activity in the SCN also seems to be somewhat localized to the pars tuberalis (PT) which may act as the "seasonal" center for the biological clock in mammals (Guilding_2009). Interestingly, seasonality is not only differentiated on a ventral-dorsal axis, but also a rostral-caudal one with evidence for caudal and rostral SCN encoding dusk and dawn information respectively (Coomans_2015). Seasonal and circadian rhythms seem to have anatomically district regions, at least for seasonal rhythms in the SCN of mammals.

SCN plasticity is heavily involved in adjustments to daylength, especially with seasonal changes to the day/night length. The electrical activity of the SCN is dependent on the photoperiod. Long day photoperiods show an increase in the duration of electrical activity periods at the population level compared to short days (Mrugala_2000). The phase distribution between SCN neurons is also important for seasonal entrainment. Phase distribution between subpopulations of SCN neurons show a functional significance for encoding seasonal photoperiods (VanderLeest_2007). Short days resulted in a relatively synchronized firing pattern between these subpopulations while long days resulted in the activation of these subpopulations at different phases of the day/night cycle (VanderLeest_2007).

Phase synchronization and desynchronization of SCN cells are different on short verses long days which underly photoperiodic modulation of tissue clock genes (Meijer_2010). In long photoperiods, cells in the rostral and caudal SCN are desynchronized with rostral cells having a bi-modal pattern of activity with one activity peak at dawn and the other at dusk and the caudal end only having activity locked to dusk (Inagaki_2007).

**Chapter 1 Summary**

The core of the mammalian molecular clock is fairly well understood but mechanisms of entrainment, especially by light, are still uncertain. The generation of endogenous rhythms by the transcription-translation primary and secondary feedback loops provide the bulk of our understanding of the molecular and genetic clock in mammals. Mechanisms of entrainment, specifically where light is able to entrain rhythms by affecting the speed of PER and CRY degradation, is most likely to occur by altering BMAL1 transcription or translation or degradation of PER mRNA and PER, respectively. The identification of photo-sensitive proteins and enzymes that degrade PER and/or TIM or affect BAML1:CLOCK transcription of clock proteins would go a long way in furthering our understanding of entrainment processes.

Seasonal timekeeping in mammals is interesting as research shows a segregation of seasonal rhythms along a rostral caudal axis in the SCN. The differential firing pattern of SCN cells at a network level also indicates that the ratio of day-to-night in a photoperiod is important for entraining biological rhythms. In later chapters dealing with melatonin in the mammalian system we further explore the seasonal timekeeping system in mammals.

# Chapter 2

## Circadian and Seasonal Timekeeping in Drosophila

## 2.1 Anatomy of the Drosophila Clock Network

Unlike mammals with the centralized pacemaking structure of the SCN, the *Drosophila* biological clock is segregated into roughly 150 cells between the lateral and dorsal nuclei (Nitabach_2008). The core *Drosophila* clock proteins, *period* (PER) and *timeless* (TIM), the fly homolog of CRY; and clock genes *CLOCK* and *CYCLE,* the mammalian homolog of *BMAL1*, still function in a relatively conserved feedback loop that generates, synchronizes, and entrains biological rhythms (Schubert_2017). Clock neuron cell bodies of *Drosophila* are located in the lateral and dorsal nuclei which are distributed throughout the brain. Lateral neurons (LNs) and dorsal neurons (DNs) interact with the accessory medulla (aME) the structure which acts as the central pacemaker for insect species (Hermann-Luibl_2014). Output sites for the aME include the dorsal protocerebellum (Tomioka_2010, Helfrich-Förster_2007), mushroom bodies (Joiner_2006) and pars intercerebralis (Velasco_2007). The dorsal protocerebellum mediates and controls locomotion and sleep behaviors, whereas the pars intercerebralis is involved with modulating activity rhythms (Hermann-Luibl_2014).

However, LNs and DNs are not the sole inputs to the aME. Aminergic (Shang_2011), glutamatergic (Collins_2012), acetylcholinergic and GABA-ergic (Gmeiner_2013) non-clock neurons also send inputs to the aME (Muraro_2013). Along with these inputs, the aME receives light input from the HB-eyelets and eye structures which contribute heavily to aME entrainment of endogenous rhythms to the external photoperiod (Helfrich-Förster_2020). Another source of light input is directed toward large ventral LN (l-LNv). Light input to l-LNvs come largely from monopolar cells in the L2 lamina of the retina. There is heavy l-LNvs innervation to the aME that assists with receiving and transferring light information which is transferred to aME projection sites (Schlichting_2016, Helfrich-Förster_2020).

### 2.1.1 Drosophila Clock Neurons: Lateral Neurons (LNs)

Lateral neurons (LNs) can be subdivided into 3 groups: dorsal lateral neurons ($LN_d$), large ventral lateral neurons (l-LNv), and small ventral lateral neurons (s-LNv) (Helfrich-Förster_2007) all located in the anterior brain of *Drosophila*. Pigment dispersing factor (PDF), a photo-sensitive protein that is essential for neuromodulation of the Drosophila clock network (Helfrich-Förster_2014, 2015) is expressed in 4 s-LNvs and one s-LNv is PDF negative (Helfrich-Förster_2007, 2014). The $LN_d$ group contains 6 neurons with 3 cryptochrome-positive neurons and 3 cryptochrome-negative or reduced cryptochrome neurons (Taghert_2006, Helfrich-Förster_2007).

Functions of l-LNvs are largely for mediating arousal and sleep rhythms in Drosophila. A 2008 study by Sheeba et. al. found that flies with hyperexcited LNvs showed a reverse day-night firing pattern with l-LNvs having increased firing and along with PDF mediated enhancement of nocturnal locomotor activity and reduced sleep from this population. Flies also lacking s-LNvs but retaining l-LNvs still showed robust nocturnal locomotor behavior (Sheeba_2008).

In comparison to l-LNvs, s-LNvs are more involved in generating circadian pacemaking behavior and modulating peripheral pacemakers (Taghert_2006). s-LNvs show a daily cycling of PDF within terminals of s-LNv axonal projections that contribute to the generation of circadian rhythms. The post-translational rhythms of PDF are eliminated with PER and TIM null-mutations thus disrupting circadian behavioral rhythmicity (Park_2000, Shafer_2002, Lin_2004). Research has also shown that s-LNv are necessary for generating rhythms under constant dark conditions as well as modulating the free-running period of other peripheral pacemaker cells (Taghert_2006, Stoleru_2004).

### 2.1.2 Drosophila Clock Neurons: Dorsal Neurons (DNs)

Dorsal neurons ($DN_{1-3}$) consist of 3 groups located in the superior brain (Kaneko_1997, Yoshii_2005) and are essential for circadian pacemaking in the Drosophila clock network. They seem to also have a special role in the regulation of the evening activity peaks and driving evening oscillators which are described by Pittendrigh-Daan's 2-oscillator model (Pittendrigh-Daan_1976). The $DN_1$ group consist of roughly 17 neurons spread throughout the dorsal brain. Interestingly the second group, $DN_2$s, are found in close proximity to vLNs but their function and neurochemistry are poorly understood. The last group, $DN_3$s, are also poorly understood, however, they account for 80 neurons out of the 150 total

Drosophila clock neurons (40 per hemisphere), comprise the majority of the Drosophila clock network (Taghert_2006), and vary in size and projection patterns (Shafer_2006, Taghert_2006).

$DN_1$s can be further separated into two subgroups. $DN_1$s that express the transcription factor *glass* are light sensitive and contribute to the photosensitivity of the pacemaking network (Klarsfeld_2004). The second subgroup is small with 2 *Glass*-negative $DN_1$s. *Glass*-negative $DN_1$s are distinguishable from *glass*-positive, not just by lacking *glass*, but because they express IPNamide (IPNa) and are anatomically segregated anterior relative to the posterior position of *glass*-positive $DN_1$s (Klarsfeld_2004, Taghery_2006, Helfrich-Förster_2007).

They are further segregated from the rest of the DN population in the anterior position of the adult brain and are thus labeled $DN_{1anterior}$ to denote their unique characteristics from other $DN_1$s posterior to their location but central with other DNs ($DN_{1posterior}$). Both $DN_{1a}$ and $DN_{1p}$ send projections to the ipsilateral aME (Klarsfeld_2004, Helfrich-Förster_2007).

## 2.2 Core Mechanisms of the *Drosophila* Molecular Clock Network

The *Drosophila* molecular clock operates under the same relatively conserved transcription-translational feedback loop described in mammals (Section 1.2a). The transcription factor CYCLE forms a dimer with CLOCK and binds to the E-box region of clock protein DNA and begins transcribing mRNA for the proteins *period* (PER) and *timeless* (TIM). PER and TIM protein levels accumulate in the cytoplasm and form dimers which then translocate back into the nucleus and inhibit CLOCK:CYCLE transcription to shut off their own production (Tataroglu_2015).

In the cytoplasm, PER is degraded when it is phosphorylated by the kinase doubletime (DBT) and when not enough TIM protein is present to stabilize it. When TIM and PER dimerize, DBT is still attached and translocated into the nucleus with the Per:Tim dimer (Price_1998). Once PER and TIM release from their dimer form, DBT can then phosphorylate PER in the nucleus where it is then degraded (Sabado_2017).

Light also influences the rate of PER and TIM dimerization in the cytoplasm. In the presence of light, the protein *cryptochrome* (CRY) binds TIM in the cytoplasm. TIM is sequestered from PER upon the formation of the CRY:TIM complex, preventing TIM from protecting against the phosphorylation of PER by DBT. This eventually leads to an increase PER degradation in the cytoplasm, adjusting the output period of the clock network. There is also a blue-light sensitive receptor for Cry which activates it (Benito_2008). Blue-light activated Cry interacts with TIM at the F-box of the protein JETLAG (Koh_2006). Once this interaction with JETLAG occurs, TIM is able to be phosphorylated and subsequently degraded. Thus, light is able to directly affect the speed of PER and TIM accumulation by adjusting the rate of dimerization. This is the primary mechanism by which the Drosophila clock network achieves entrainment and phase shifting of biological rhythms (Emerson_2009).

## 2.3 Phase Shifting of Biological Rhythms in the Drosophila Clock Network

### 2.3.1 Phase Shifting Rhythms: Period, Timeless, and Cryptochrome

Almost all *Drosophila* cells in the clock network either directly detect light or receive light input from compound eye structures and HB-eyelets (Helfrich-Förster_2020, Hardin_2011). However, unlike the SCN, whose synchronous firing of pacemaker neurons entrain the internal period, *Drosophila* have a network of asynchronous firing of LNs and DNs that generate a representation of the external 24-hour period (Liang_2014, Hardin_2011). Pacemaker LNs and DNs show daily changes in rhythmic activity that are capable of entrainment to the external period with $Ca^{2+}$ rhythms in these neurons occurring roughly 4 hours prior to morning and evening bouts of locomotor activity (Liang_2014). The neuropeptide pigment dispersing factor (PDF) was shown to be required to modulate the $Ca^{2+}$ rhythm of LNs and DNs. Mutations to the PDF receptor in flies with the hypomorphic mutation $han^{5304}$ show unimodal and arrhythmic behaviors in constant dark conditions (Hyun_2005). Loss of PDF resulted in an increase in synchronous $Ca^{2+}$ rhythms, ultimately disrupting the timing of the Drosophila clock network (Liang_2014).

As discussed in section 2.2, light affects levels of PER and TIM by regulating the degradation rate of these proteins. In the presence of light, TIM is degraded in as little as 30 minutes (Hunter-Ensor_1996). This rapid degradation of TIM by light causes either a phase delay or advance by the network, which is determined by the timing of a light stimulus during an entrained 24-hour period. As in mammals, light presented during the early part of the night is seen as the new "sunset" and induces a phase delay, whereas light presented during the latter half of the night is seen as the new "sunrise" and induces a phase advance in the following day's rhythms (Aschoff_1981).

Delaying or advancing a rhythm is tied to mRNA levels of TIM being high during the early part of the night and low during the latter half. When a light stimulus reduces TIM levels during the early part of the evening (when mRNA of TIM is high), TIM levels can easily be replenished; however, where in the latter half of the night mRNA of TIM is low and the premature loss of TIM induces a phase advance (Hardin_2011). The ability of the clock to replenish TIM determines if an advance or delay needs to occur to correct and entrain endogenous rhythm to align with the newly perceived photoperiod (Hardin_2011, Niadoo_1999).

### 2.3.2 Phase shifting  Rhythms: The H-B eyelet and Compound Eye

CRY is important for degradation of TIM. However, it is only responsible for light resetting by acute exposure to light and is not required for sustained light exposure or entrainment (Hardin_2011). Light input to the clock nuclei comes from compound eye structures and Hofbauer–Buchner (H–B) eyelets that help supplement CRY mediated photic phase shifting (Helfrich- Förster_2001, Rieger_2003). Loss of phototransduction in the compound eyes, in addition to loss of CRY, further weakens phase shifting capabilities but does not abolish entrainment to a new photoperiod (Stanwisky_1998) indicting another source of light input, in this case the H-B eyelet. Indeed, when all external photoreceptors in the eye, CRY, and the H-B eyelets are removed, *Drosophila* are unable to entrain to a new photoperiod (Helfrich-Förster_2001).  Thus, CRY in addition to information from the eyes and H-B eyelet are necessary to entrain biological rhythms in *Drosophila*.

Interestingly, the H-B eyelet and compound eyes are now thought to govern morning, evening, and siesta peaks of activity independently by splitting interpretation of low (LI), medium (MI) and high (HI) intensity light between the structures. In a model proposed by Schlichting et. al., MI light, is perceived by histamine in the compound eyes where it is sent to the optic lobe and l-LNvs to control morning and evening oscillations in behavior. They also propose that HI light is perceived by the H-B eyelets where it is sent to morning oscillatory cells that either inhibit evening oscillator activity or project to DN siesta cells in the dorsal brain (Schlichting_2019).

**2.3.3 Phase Shifting Rhythms: Lateral and Dorsal Neurons**

TIM is degraded by LNs only when light stimuli are perceived during the latter half (phase advancing) of the subjective night, as do all oscillating neurons, but do not degrade TIM when a light is presented in the first half (phase delaying) of the subjective night (Tang_2010). In s-LNvs there is no change in TIM degradation with light pulses given in the early subjective night (Tang_2010). DNs, however, are involved in phase shifting, specifically producing phase delays. The subgroup, DN1, is a good candidate for facilitating phase delays. Certain DN1s are CRY-positive and degrade TIM when light pulses are applied in the delay zone (early night) (Hardin_2011, Tang_2010). They also receive light input from LNvs via PDF signaling and send information to LNv cell bodies (Hardin_2011).

Drosophila, under LD cycles, display anticipatory peaks in morning and evening locomotor behaviors and the phases of these peaks shift with both seasonal temperature and photoperiod changes. Under summer conditions, where days are longer and the temperature is higher, there is an increase in morning anticipatory and post-evening locomotion, whereas under winter conditions the reverse is true with more evening anticipatory and post-morning locomotion (Majercak _1999, 2004). Light induced transcription of TIM when the temperature is low is thought to play a role in winter variation of phase differences in morning and evening anticipatory and locomotor behavior (Chen_2006). This integration of photoperiod and temperature inputs is thought to occur in DNs and lateral posterior LNs. The seasonal change in the anticipatory phase is thought to be due to temperature sensitive intron splicing of PER and TIM 8 and their final introns respectively (Boothroyd_2007, Collins_2004, Majercak_1999, 2004).

In flies housed under L:D cycles combined with temperature cycles (advanced 6 hours relative to the light cycle), DNs and lateral posterior neurons (LPNs) showed phasic shifts of TIMELESS expression, but the lateral protocerebral neurons ($LN_p$s) showed no phasic changes. This indicates a preference in $LN_p$s for photic only entrainment, while the DNs and LPNs are preferentially entrained by temperature (Miyasako_2007). This functional difference of entrainment to temperature and light cycle information might indicate a role of LPNs and DNs for seasonal entrainment in *Drosophila*.

**Chapter 2 Conclusions**

Despite neuroanatomical differences, the core mechanism behind the molecular circadian clock is highly conserved between *Drosophila* and mammals. As opposed to mammals however, much more is known about the underlying molecular mechanisms of entrainment and light-induced phase shifting in *Drosophila*. How LNs and DNs encode, process, and influence different circadian behaviors needs to be further elucidated, but the genetic and molecular tools coupled with *Drosophila* being a much more time efficient model organism lends optimism for faster discoveries in this area.

Much less is known about seasonal timekeeping in *Drosophila*. Although *Drosophila* do not live through multiple seasons, typically, they do show seasonal behaviors such as delayed eclosion in the winter months, changes in ovary development and seasonal changes in electrical activity in the clock neuron network. This next chapter will summarize findings that describe how seasonal photoperiods affect light induced phase shifting of activity rhythms in *Drosophila ananasae*.

# Chapter 3

## The Drosophila Circadian Phase Response Curve to Light: Conservation across seasonally relevant photoperiods and anchorage to sunset

The work contained within this chapter has been published under Dollish, H. K., Kaladchibachi, S., Negelspach, D. C., & Fernandez, F. X. (2022). The Drosophila circadian phase response curve to light: Conservation across seasonally relevant photoperiods and anchorage to sunset. *Physiology & behavior*, *245*, 113691. The work summarized in this chapter is contained in Appendix A of this thesis.

**3.1 Study Significance**

The ability of organisms to time endogenous rhythms and behaviors to the external photoperiod is critical for survival. The biological clock evolved a mechanism to align our endogenous periods with the external solar cycle in a process known as entrainment. However, there is a gap in understanding of how the biological clock differentially integrates light information to entrain both circadian and seasonal rhythms to daily seasonal changes in photoperiod length.

Many studies focus on entrainment of circadian rhythms, whose changes take place on a 24-hour scale. However, much less is known about how biological rhythms change on a yearly, or seasonal scale. Photoperiods are not static across the year, they dynamically change across seasons with variation dependent on latitude. In fact, much of the world's population resides at +/- 30° latitude, where photoperiods range from 8:16 at the peak of winter and 16:8 at the height of summer. Rhythms and behaviors are shifted to match the optimal timepoint for both the day and night phases of the photoperiod. This might account for some of the variation in efficacy of chronobiotic therapies such as light therapy for seasonal affective disorder (SAD) and co-administration of chronobiotics, such as melatonin, with chemotherapy in cancer patients.

Studying the effect of seasonality on the biological clock network can be time consuming and burdensome to laboratory finances when conducted in mammals and humans. Preliminary research using *Drosophila* allow us some insight into the mechanisms that are behind seasonal entrainment without a heavy time or monetary investment compared to rodent and human research. As discussed in Part 1 of this thesis, the *Drosophila* and mammalian molecular and genetic clock has a fair degree of conservation, allowing for inferences about interspecies mechanisms of timekeeping.

**3.2 Methods Summary**

Virgin female *Drosophila ananassae*, a *Drosophila* subspecies, were aged in a 12:12 photoperiod for 3 days post-eclosion before being entrained under an Aschoff II paradigm to either an 8:16, 10:14, 12:12, 14:10 or 16:8 photoperiod for 3 days. On the eve of the 3$^{rd}$ day, animals were placed in constant dark

(DD) conditions and a 15-minute broad spectrum 600 lux light stimulus was administered at hourly intervals for the next 24-hours. Animals were left to free run for 4-5 days post-pulse. Activity rhythms were used to measure photic-induced phase shifting with activity onset as our phase marker. (see Appendix A).

Phase response curves were used to measure the direction and magnitude a stimulus is able to phase shift rhythms. Photic phase response curves (pPRCs) were used to measure how light shifted activity rhythms in seasonal photoperiods. The x-axis indicates the time of subjective day/night a light pulse was administered, and the y-axis indicates the direction and magnitude of shift. Negative y-axis values indicate a phase delay with the numbers indicating how many hours the rhythms are delayed, and a positive y-axis value indicates a phase advance with the numbers indicating how many hours the rhythms were advanced. pPRCs generally take on a horizontal sigmoidal shape. (Appendix A)

**3.3 Results Summary:**

Results show that pPRCs in different seasonal photoperiods show no significant change in their overall shape or wave form (Appendix A Figure 2). The width and the amplitudes of seasonal pPRCs remained constant and showed no significant deviation between photoperiods. Interestingly, pPRCs are phase locked to lights-off, the experimental representation of "sunset" (Appendix A Figure 3). Why anchoring to sunset might be preferred over anchoring to sunrise might be due to *Drosophila* being a diurnal species. It would be interesting to generate a pPRC atlas for a nocturnal insect species to see if phase-anchoring to sunset is conserved or if it would instead anchor to sunrise.

Seasonality seems to have the most effect, at least in *Drosophila*, on circadian photoresponsivity during the summer months. The pPRCs for high and low summer (16:8 and 14:10) show a phase advance in their pPRCs during the subjective day. When light stimuli are administered during the subjective day under 12:12 conditions – where almost all traditional experiments are conducted – it will not illicit a phase shift in either direction. This area is known as the dead zone and photic stimuli at this time were thought to be unable to illicit a phase advance or delay. This idea is at least upended in Drosophila where photic stimulation at least 1-2 hours into the subjective day is capable of producing a phase advance in activity rhythms, which are also tied to phase shifts in other rhythms such as core body temperature, cortisol, and melatonin secretion.

These findings show a greater need to understand the effects of seasonality on biological rhythms in mammals and other living organisms. Special attention should be paid to how seasonal photoperiods might reduce or enhance the effectiveness of light therapies and chronobiotics for medical use. Also, in a world with ever increasing light pollution, understanding how long photoperiods affect the output of the biological clock will help us adapt to this newly lit world.

# Part 2: Melatonin

**Introduction:**

Timing behavior and physiology to the 24-hour solar cycle has enabled life to persist and thrive on Earth. The most powerful time cue, light, is used for aligning endogenous rhythms to the day and night cycle; however, the clock also heavily relies on another internal rhythm, melatonin secretion, to help adjust circadian but primarily seasonal rhythms cooperatively with light input (Aschoff_1978, Ardent_2009). Melatonin and light work synergistically to entrain endogenous rhythms to the external photoperiod set by the solar cycle (Wood_2013, Wehr_1997). Together, both light and melatonin optimize interconnecting suites of behavior and physiology so that they are expressed during the most conducive parts of the day-and-year to enhance biological fitness. Regarding circadian/seasonal function, it is impossible to talk about melatonin without also understanding (1) the control that ambient light exerts over its secretion and (2) how melatonin feeding back onto the clock can work to adjust oscillatory behavior the clock makes in response to light exposure, especially during the subjective night.

Melatonin functions as an important biological imprint of night-length by supplementing daytime light information collected by the brain's circadian pacemaker. The suprachiasmatic nucleus (SCN) acts as the mammalian biological clock; and the Lateral and Dorsal Nuclei (LN and DN respectively) comprise the Drosophila clock network (Taghert_2006, Ardent_2009). Melatonin has its primordial role as an antioxidant that directly and indirectly – through metabolite cascades – sequesters free-radicals to help prevent oxidative stress (Zhao_2014, Manchester_2015).

Melatonin freely circulates throughout the body and passes through lipid membranes and the blood brain barrier (Pardridge_1980, Costa_1995). These two features contribute heavily to its potency as an antioxidant in both the plant and animal kingdoms. Timing of melatonin secretion is important to optimize its antioxidant properties and to phase-lock various biological behaviors to occur at the optimal phase of the day-night cycle. Its enhanced secretion at night tracks closely to periods of high oxidative stress that occur during the day when solar UV radiation and system energy demand are at their highest (Haldar_2010). This is thought to keep oxidative processes to a minimum during the day and time the bulk of them to occur during the evening when there is less demand on the system from UV-radiation and environmental conditions.

The history of melatonin's functionality, from just an antioxidant in simpler species to evolving more of a modulatory role for circadian and seasonal behaviors in higher invertebrates, avian species, and mammals, is an interesting one. It stemmed from melatonin's ability to orchestrate and participate directly in restorative processes that work best in the absence of light (such as DNA methylation and sequestration of free radicals) (Zhao_2019). More of its evolutionary history will be discussed later, but it is interesting to note that melatonin concentration is highest in mitochondria, where many reactive oxygen species (ROS) and reactive nitrogen species (RNS) are generated during the citric acid cycle.

Finally, data will be presented on a comprehensive seasonal study on melatonin secretion in *Drosophila ananassae* across different seasonal photoperiods. This data was collected in an attempt to draw a phylogenetic line between where melatonin took on its circadian and seasonal role and where it acts solely as an antioxidant. This data also sought to understand what, if any, circadian and seasonal properties of melatonin are conserved between the *Drosophila* and mammalian systems.

Using the gold standard of melatonin measurement, mass spectrometry (Markey_1981), and over 18,000 animals, we measured melatonin secretion in 4 photoperiods from high summer (16:8), equatorial (12:12), and high winter (8:16) photoperiods. Animals were also exposed to an extreme arctic summer photoperiod (20:4) to evaluate the potential suppressive effects of light on melatonin secretion in this species in addition to a physiologically extreme photoperiod.

# Chapter 4

## Melatonin: Antioxidant and Chronobiotic in Mammals and *Drosophila*

## 4.1 Melatonin Synthesis and Secretion

### 4.1.1Melatonin Synthesis

Melatonin synthesis from L-tryptophan takes place namely in the brain, gut, and in mitochondria (Wurtman_1963, Chen_2011, Reiter_2020). The first step in melatonin synthesis is the hydroxylation of L-tryptophan to 5-hydroxytryptophan which is then decarboxylated into serotonin. Once serotonin is synthesized, it is either acetylated to form N-acetylserotonin (NAS) or methylated to form 5-methoxytrypamine (5-MT), both of which are precursors to melatonin (Zhao_2019, Bernard_1999). The catalyst in the conversion of serotonin to NAS is serotonin N-acetyltransferase (NAT) also known as arylalkylamine N-acetyltransferase (AANAT). N-acetylserotonin O-methyltransferase (ASMT formerly HIOMT) is the catalyst enzyme to produce melatonin from NAS (Lui_2005, Axelrod_1960). A secondary pathway for melatonin synthesis is first O-methylating serotonin to 5-MT by AMST; then N-acetylating 5-MT through AANAT activity to produce melatonin (Zhao_2019).

Melatonin secretion, especially in the pineal gland, is regulated by AANAT (Ganguly_2002). The amount of freely circulating melatonin is thought to be determined by the degree of AANAT phosphorylation and binding to 14-3-3 proteins (Ganguly_2002, 2004). AANAT activity and gene transcription has been shown to be light dependent, with light inhibiting melatonin synthesis by preventing PKA phosphorylation of AANAT (Zawilska_2007, Valdez_2012, Olcese_1989).

In darkness, cAMP levels in photoreceptors are high which activate the PKA pathway and modulates AANAT gene transcription (Ho_2009). Cre binds to the AANAT promotor and initiates transcription roughly 150-fold within 2-hours of dark onset. AANAT activity increases with a similar magnitude to AANAT transcription rate, with the peak of activity occurring roughly 9-hours after dark onset (Roseboom_1996). AANAT is also phosphorylated by the PKA pathway, enabling its association with 14-3-3 proteins to stabilize AANAT and promote synthesis of NAS (Ganguly_2004). This complex results in an increase in NAS production which can be converted to melatonin by HIOMT (Pozdeyev_2006, Ganguly_2004).

When light is present, cAMP levels are reduced and AANAT is dephosphorylated and rapidly degraded since it cannot associate with 14-3-3 proteins in a dephosphorylated state (Pozdeyev_2006, Iuvone_2005). As light intensity increases so does the degradation of AANAT, thus generating an environment that favors the dephosphorylated over the phosphorylated state of AANAT (McIntyre_1989, Nathan 2000, Bayarri_2002). Because of light's influence on AANAT activity and the tight regulation of mAANAT and AANAT degradation pathways, AANAT is canonically thought to be the rate-limiting enzyme in melatonin production.

Interestingly, Liu et. al. in 2005 showed that AANAT might not be the canonical rate-limiting enzyme of melatonin synthesis as previously thought. Instead, they propose, at least for evening synthesis of melatonin, that ASMT/HIOMT is the rate-limiting enzyme in darkness. Using pineal night-specific ATPase (PINA) KO rats and a combination of molecular and in vivo measurement of pineal melatonin production, they observed that NAS concentration was present in excess during the night. They also showed an increase in AANAT [NAT] levels during the late night that did not produce a reciprocal or proportional increase in melatonin secretion by the pineal gland. The most convincing aspect of their data is that increases in NAS in the same animal over multiple circadian cycles still did not show a corresponding increase in melatonin concentration (Liu_2005).

### 4.1.2 Melatonin Secretion

The rhythmic secretion of melatonin is under the control of an area of the hypothalamus in avian species and mammals known as the suprachiasmatic nucleus (SCN) (Cardinali_1998, Natesan_2002). The SCN exerts its circadian influence over melatonin secretion through a polysynaptic circuit that runs from the SCN to the center of melatonin production in the pineal gland (Klein_1983, Ganguly_2002). Pineal cells that receive this input and regulate melatonin secretion mainly through AANAT expression and do so via the cone-rod homeobox (CRX) transcription factor. These cells also facilitate cAMP response element-based circadian melatonin production in the pineal gland (Rohde 2014).

Secreted melatonin then feeds back on the SCN to modulate CLOCK gene expression. One model for melatonin feedback on the SCN by Vriend et. al. proposed melatonin's influence over the expression of various clock genes is achieved by inhibiting the proteasome in SCN neurons during the night (Vriend_2014, Vriend_Reiter_2014). In their model, melatonin inhibits the proteosome, which degrades transcription factors like the BMAL1 protein. This regulation of the proteosome by melatonin may adjust SCN output in seasonal photoperiods by interfering with the PER/CRY and REV-ERBα feedback loops on BMAL1-dependent transcription at night (Chapter 1, Vreind_2014). This model provides evidence for the "time-of-day" effect seen by melatonin injections on the reproductive status of seasonal breeders (Vreind_2014).

Melatonin synthesis is regulated by light-mediated suppression in an intensity dependent manor (Illnerova_1979). During the day, light inhibits the production of melatonin by preventing phosphorylation of AANAT thereby preventing the enzyme from being activated and stabilized by 14-3-3 proteins (Bayarri_2002). At night, melatonin production is high, but can be rapidly suppressed by administering a light stimulus during the natural and subjective night (Wright_2008, Illnerova_1979). The recovery rate of melatonin post-pulse is species-dependent with rodents showing periods of longer suppression post-pulse (Illnerova_1979, Brainard_1984) compared to humans (Zeitzer_2000) and rams (Ardent_1988) which exhibit nearly immediate post-pulse recovery of melatonin levels and production. These data show that the effect of light on melatonin secretion and the secretion profile is variable between different species (Spitschan_2022).

Photic information to suppress melatonin secretion comes from the SCN and travels through descending hypothalamic projections, through the intermedial lateral cell column to the superior cervical ganglia, then ascends to its final destination in the pineal gland (Klein_1983). Noradrenaline G-protein coupled receptors in the pineal gland trigger a signaling cascade to increase melatonin synthesis via cAMP and PKA pathways (Mikkelsen_2001, Ferreira_2004). Light-input reduces noradrenaline production in an intensity dependent manner, thus reducing/suppressing the magnitude of pineal melatonin production (Redlin_2009).

### 4.2 Melatonin Receptors Structure, Mechanism, and Function

Melatonin is a freely circulating hormone whose signaling is transduced through at least 3 melatonin receptors discovered in mammals named Melatonin Receptor1, 2, and 3 (MT1, MT2 and MT3 respectively) (Yan-Li_2013). Research on MT1 and MT2 receptors is more comprehensive and prolific compared to MT3 (Lopez-Canul_2019). The main functions of MT1 and MT2 appear to mediate signaling processes related to circadian and seasonal rhythmicity (Wu_2013, Yen-Ng_2017) while MT3 has mainly an auxiliary role for melatonin's antioxidant effects (Tan_2007). This section will focus on the MT1 and MT2 receptor types controlling circadian and seasonal rhythmicity.

#### 4.2.1 Cloning and Resolving the Structure and Signaling Pathways of Melatonin Receptors 1 and 2

The first melatonin receptor was cloned in 1994 by Ebisawa and colleagues from *Xenopus laevis* (frog) dermal melanophores. This initial effort laid the groundwork that eventually resulted in the cloning of the mammalian receptor MT1 first in sheep, then in humans by the same group (Ebisawa 2010). Resolution of the MT1 sequence yielded a protein that was 350 amino acids in length and weighing 39 kDa. The MT1 receptor is coupled to a toxin-sensitive guanine nucleotide-binding protein (G-protein coupled receptor) (Song 1997, Carlson 1989, Liu 2016). Roughly a year later, Reppert and colleagues (1996) cloned the MT2 receptor from human pituitary tissue. MT2 is 60% homologous with MT1 but is a bit larger (40 kDa); the protein is comprised of 362 amino acids and is also a G-protein coupled receptor (Reppert_1996). Both MT1 and MT2 inhibit adenylyl cyclase and soluble guanylyl cyclase pathways (cAMP and cGMP) (Petit 1999). In doing so, both activate downstream PKA and CREB pathways, as well as phospholipase-C pathways when the MT1/MT2 heterodimer and pertussis sensitive G-protein$_q$ ($G_q$) are activated (Jockers 2008). Interestingly MT2, but not MT1, stimulates cGMP, PKG, and GC signaling when the pertussis toxin-sensitive $G_i$-protein is activated (Petit 1999).

### 4.2.2 Identifying Melatonin Receptors in the Brain and Periphery

MT1 and MT2 receptors are localized to specific areas of the brain and periphery. Past research to identify sites of MT receptor expression was done using the non-selective radioligand2-[1251]Idomelatonin to identify high-affinity melatonin binding sites using autoradiography (Weaver_1989, Vanecek_1987). Although autoradiography is commonly used there are some experimental caveats, namely, autoradiography being unable to discriminate between the 3 types of melatonin receptor, especially MT1 and MT2 (Adamah-Bassai_2014). The issue is the potential dimerization shown to occur in this receptor class is also an issue as it can reduce binding affinity to radioligand2-[1251]Idomelatonin (Yen Ng 2017).

More recent attempts at determining melatonin receptor localization and identifying specific receptor subtypes were conducted with BAC C3H/HeN transgenic mice (Adamah-Biassi 2014). This model utilizes a red fluorescent protein that emits a fluorescent signal when the MT1 promotor region is activated. Oddly, their experiment failed to reveal the presence of MT2 receptors in the SCN or striatum, which were present during autoradiography experiments, however, taking into account the issues with MT receptor differentiation with autoradiographical approaches they may indeed be absent (Dubocovich_1998).

A reliable antibody that binds melatonin receptors with high affinity would go a long way in furthering understanding of MT receptors, the functions and localization in the brain and periphery. Until then, a reliable means of detecting and identifying MT receptors continues to hamper research in the melatonin field. Currently, a combination of PCR and immunoreactive hybridization methods are being looked at as approaches that may eventually become "best practices" for localizing melatonin receptors *in vivo* (Yen Ng 2014).

### 4.3 Localization of Melatonin Receptors 1 and 2 in the Brain and Periphery

MT1 and MT2 receptors are not only found in various regions of the brain but also beyond the blood brain barrier (BBB). This isn't surprising given that melatonin readily passes the BBB and is synthesized *de novo* in the gut (Naji_2004, Chen_2011), bone marrow, and some to all mitochondria (Reiter_2020, Conti_2008). Areas of the brain and periphery where MT1 and MT2 receptors can be found in higher densities are the retina, cerebral cortex, reticular thalamic nucleus, habenula, hypothalamus, pituitary gland, periaqueductal grey, dorsal raphe nucleus, the midbrain, and the cerebellum (Yen-Ng_2017). The following section will discuss the distribution of MTs in these areas and their functions.

### 4.3.1 Melatonin Receptors in the Retina

Part of melatonin's role in the circadian and seasonal timing and entrainment is deduced via receptor expression in the retina. MT1 mRNA and protein are localized to the inner and outer plexiform layers, ganglion, and both types of photoreceptors in the retina (Fujieda_1999). MT1 receptors are also localized to amacrine and horizontal cells which provide lateral inhibition to bipolar and ganglion cells in controlling center-surround receptive field inhibition (Fujieda_1999).

MT2 are found on the inner nuclear layer and photoreceptor cell segments as well as cellular processes in the inner and outer plexiform layer (Yen-Ng_2017). Melatonin synthesis does occur in the retina, specifically in photoreceptors. Retinal melatonin as acts as both a paracrine signaling hormone for the regulation of amacrine cells and lateral inhibition (through MT1 activation) as well as a local antioxidant through MT3 activation (Weichmann_2013,Marchiafava_2007, Gonzalez-Iglesias_2014).

### 4.3.2 Melatonin Receptors in the Cerebral Cortex

Melatonin receptors are also found in the cerebral cortex (Stankov_1991). MT1 receptors seem to be the predominate receptor subtype present here with the highest density present in the retrosplenial cortex (Comai_2019). The retrosplenial cortex is an area involved in episodic memory, navigation, processing of visual scenes and imagining future events, hinting melatonin's involvement through MT1 activation, in some of these processes (Vann 2009). MT2 receptors are less represented in cerebral cortex but are expressed in some tissues in the entorhinal and piriform cortices. Interestingly, where MT1 receptors are located, MT2 appear to be lacking and vice versa (Comai_2019, Yen-Ng_2017), indicating functional differences between these two receptor subtypes in cerebral cortex.

Although melatonin synthesis primarily occurs in the pineal gland, astrocytes in the cerebral cortex have been shown to be capable of producing and secreting melatonin (Acuña-Castroviejo_2014). N-acetyltransferase (NAT) and hydroxyindole-O-methyltransferase (HIOMT) are present in rat cortical astrocytes (Liu_2007), both of which are needed for melatonin synthesis. One idea for the independent synthesis of melatonin by cortical astrocytes, instead of sourcing melatonin from the pineal gland, could be due to melatonin's role as an antioxidant and its neuroprotective effects after traumatic brain injury in the cerebral cortex (Şenol_2014, Wang_2019).

The cerebral cortex has a heavy cognitive load for receiving, integrating, processing, and distributing information and signals from and to the rest of the brain. The high energy demand, paired with an increase in the production of ROS and RNS as mitochondria work to produce the required energy for these processes, results in an environment where oxidative stress is high (Wang_2010).

Glial fibrillary acidic protein content (GFAP) is synthesized by astrocytes in response to physical and metabolic damage to the CNS to help repair damage (Eng_1994). ROS and RNS species rapidly degrade GFAP and inhibit repair and cell-cell communication (Ortiz-Rodriguez_2020). Melatonin is able to reverse this damage and reduce degradation of GFAP by ROS and RNS in rodents as well as reducing neural tissue peroxidation (Baydas_2002). Taken together, melatonin receptors can act both as signaling receptors and as cites of antioxidant activity in cerebral cortex.

### 4.3.3 Melatonin Receptors in the Hippocampus

MT1 and MT2 receptors are both found, albeit different locations, in the hippocampus (Klosen_2019, Yen-Ng_2017). MT1 receptors are diffusely distributed across hippocampal subregions, while the MT2 receptor class is concentrated mainly within the pyramidal layers of CA1 and CA3 (Klosen_2019). Again, this segregated pattern of expression suggests a differential role of MT1 and MT2 receptors in the hippocampus. What their roles are, however,  is not well understood. One thought is that melatonin facilitates dentate neurogenesis via signaling by MT2 receptors in CA3 as shown by the inhibition of cell death in disease models associated with high fat diets such as dementia and Alzheimer's (Manda 2010, Wang_2006).

Insulin resistance is also starting to gain traction as a key player in the intersection between metabolic and cognitive disorders with hippocampal involvement such as Alzheimer's disease (Song_2017, Shen_2022). Insulin receptors are present in the hippocampus and cerebral cortex and may be subject to modulation by melatonin (Shen_2022, Kamsrijai_2020). In STZ-induced hyperglycemia rats, melatonin improved memory by restoring insulin signaling pathways independent of its effects on blood glucose and insulin levels (Kamsrijai_2020). Melatonin was also found to attenuate insulin resistance in the periphery and lower hippocampal oxidative stress in rats fed a high-fat diet. (Xu 2019). Melatonin not only affects insulin resistance and helps prevent oxidative damage, but also reverses the cognitive decline typically seen in high-fat diet models, along with normalizing hippocampal LTP and restoring hippocampal BDNF levels (Shen_2022, Xu 2019).

### 4.3.4 Melatonin Receptors in the Hypothalamus

The hypothalamus is of interest when considering melatonin's role in circadian and seasonal rhythms. MT1 is the main receptor subtype populating this area in humans, with the highest content found in the SCN (Waly_2015, Klosen_2019). The MT2 receptor exhibits relatively lower expression in the SCN sometimes not even detectable (Waly_2015, Klosen_2019) compared to MT1, with most MT2 receptors localized to other areas of the hypothalamus such as the paraventricular nucleus (PVN) and supraoptic nucleus (SON) (Wu 2013). However, more recent work and improvements in melatonin receptor detection and differentiation has placed MT2 receptors in the SCN as well as other areas (Klein_2019).

The concentration of melatonin receptors in the SCN provides an avenue by which circulating melatonin can feedback onto the SCN to autoregulate its secretion. Despite its overall lower expression, MT2—not MT1—receptors are the probable gatekeepers for this feedback. MT2 receptors are capable of advancing biological rhythms through activation in the SCN at both dusk and dawn (Hunt_2001). Melatonin feedback on MT2 receptors in the SCN desensitizes them, providing a temporal sensitivity profile for the SCN to a melatonin signal (Gerdin_2004).

MT1 receptors may still play important part in regulating SCN circadian output. The receptor density of MT1 in the SCN exhibits circadian fluctuations with two peaks of mRNA expression for MT1 detected at dusk and dawn, two important transition points and time cues for SCN entrainment of biological rhythms (Poirel_2002, Waly_2015). However, it should be noted that neither of these studies detected MT2 in the SCN despite replication studies showing its presence. This highlights the importance of establishing a reliable method of melatonin receptor detection for future experiments.

### 4.4: Light and Melatonin: Circadian and Seasonal Rhythms

#### 4.4.1 Light and Melatonin Suppression

Light and melatonin work together not unlike a metaphorical “sun” and “moon” for the internal milieu of biological processes and behaviors. Both have means of feeding back onto each other as well to regulate and suppress their respective signals. The interplay between light and melatonin provides the backbone of endogenous and circadian entrainment of internal rhythms to each other and the external environment.

Light is capable of suppressing melatonin in an intensity-dependent fashion with illuminance directly correlating to magnitude of melatonin suppression by light (Arendt_1998, Redlin_2001). The suppressive effect of light occurs at the molecular level through modulation of AANAT phosphorylation and AANAT transcription factors. As stated above, AANAT is a rate-limiting enzyme that converts serotonin to N-acetylserotonin, melatonin’s precursor. Light prevents AANAT from becoming phosphorylated, leaving it in an unstable readily degradable state (Pozdeyev_2006) as well as inhibiting its transcription factors, dualistically suppressing its stability and formation. (Rohde_2014).

Inhibition of the rate limiting enzyme, however, is typically not sufficient to result in the drastic level of suppression of a molecule or hormone that we see with melatonin (Arendt_1998). An explanation for this will come later with AANAT potentially not being the “nighttime” enzyme for AANAT secretion. Light when presented during melatonin’s usual secretion time (night) suppresses melatonin immediately (Arendt_1998), and total elimination of melatonin (in humans) is achieved in 40-60 minutes (Kovacs_1999). This is the result of melatonin’s rapid degradation in the body; in some species it takes as little as 6 minutes to reduce melatonin levels. This rapid degradation is highly regulated due to melatonin’s dual roles as both a powerful antioxidant and a timer of night.

Rapid degradation is needed for the system to quickly adjust to a changing environment and ensure that behaviors are efficiently and quickly aligned to the new photoperiod. Melatonin is involved in many processes that need or are most efficient at night. As soon as the system perceives potential daylight, melatonin is likely initiating or terminating a signal for many seasonal and circadian behaviors that need to be highly regulated in regard to seasonal and circadian photoperiods. If the signal took hours to be received, the organism might be 2-3 hours into its new photoperiod before behaviors or processes are affected, leading to potentially fatal consequences.

### 4.4.2 Melatonin phase shifts biological rhythms through MT1 and MT2 activation

Melatonin may influence phase shifting via MT1 and MT2 receptor activation. MT2 receptors in the SCN are capable of eliciting phase shifts in SCN generated biological rhythms (Yen Ng_2017). When 4-phenyl-2-propionamidtetralin (4P-PDOT), a MT2 antagonist, is given in vitro, phase shifting was decreased. When MT2 was activated, circadian phase advanced were observed (Hunt_2001) MT1 also also seems to play a role in phase shifting but not as much as the MT2 receptor (Yen_Ng_2017, Poirel_2002).

A recent study by Wang et. al. found that melatonin could curtail the increase in the activity period (wheel running behavior in rodents) was seen when NMDS (N-methyl-D-aspartate) receptors are blocked by the NMDAR antagonist MK-801. Melatonin regulated homeostasis in the ventral lateral preoptic area (VLPO) via MT2 receptors rather than directly affecting the SCN circadian response. EEG recordings showed further the effects of melatonin for VLPO regulation of sleep rhythms. The first latency of NREM sleep was recovered from the delaying effects of MK-801 by melatonin increasing intracellular VLPO Ca2+ signaling (Wang_2020).

Despite VLPO involvement, the SCN is still the main regulator of circadian processes, an important one being core body temperature rhythms. The SCN along with melatonin rhythms modulate core body temperature and decrease in the thermoregulatory threshold of heat production during the day and heat loss at night in diurnal species. Along with this, the melatonin peak signals the reduction in core body temperature roughly 80-120 minutes before onset and a decline in melatonin results in an increase in core body temperature (Lopez-Canul_2019, Cagnacci_1997). Further studies with supplemental melatonin given during daylight hours have confirmed a decrease in total body temperature with a rise in internal melatonin concentration (Ardent_2005, Lockley_1997). In this case, melatonin exerts its circadian functions  through both MT1 and MT2 receptors. Findings by Lopez-Canul et al. in 2019 showed that both MT1 and MT2 need to be activated simultaneously and in a time-dependent manner to reduce total body temperature during the dark-phase. (Lopez-Canul_2019).

Melatonin receptors also are involved in regulating the secretion of various hormones of the pituitary. MT1 is present in high amounts in the pars tuberalis (PT) in humans (Adamah-Baissi_2014). MT1 seems to colocalize with vasopressin, oxytocin, and corticotropin-releasing hormone secreting neurons in the PT (Wu_2006). Melatonin is also heavily involved in prolactin secretion, mediating its effects on puberty, and reproductive functions such as milk secretion and breast growth (Weber_1985, Wilson_1989, Yellon_1992, Anderson_2017). Reproductive changes need to be timed to occur during proper times in the seasonal photoperiod so that birth can be timed to occur when resources are high and environmental risks, such as predation and temperature changes, are low (James-Shine_1985).

**4.4.3 Melatonin's role as a circadian zeitgeber**

Melatonin's role in circadian entrainment has been described as modulatory, acting to supplement photic information about day and night length. In mammals this modulation could occur in the SCN where MT1 and MT2 are present. MT1 and MT2 may act as locations for melatonin to feedback on and regulate its own rhythm, or possibly play a more important role in circadian entrainment than previously thought. Evidence for melatonin's entrainment properties, or at least effects on the sleep/wake rhythm can be seen in experiments looking at supplemental dosing of melatonin.

In free-running rodents (constant dark conditions), .01- 1mg doses of melatonin can re-entrain the activity rhythm to a 24-hour period, with injection time marking the beginning of the "active" period (Oakley_1993). In humans, melatonin (2mg) given regularly over a monthly period in the early afternoon (17:00h) can advance the endogenous melatonin rhythm along with evening fatigue and modulates anterior pituitary prolactin release (Arendt_1998, 2005, Falcon_2003, Lincoln_2002). However, melatonin had no effect on phase advancing or delaying any other anterior pituitary hormonal rhythms (Arendt_1998).

Melatonin supplementation is also able to advance feelings of "sleepiness" when administered at 17:00 hr for 3-4 weeks as well as advance the timing and extended the endogenous melatonin secretion profile (Arendt_2005). Melatonin given during the "morning" phase of a photoperiod when endogenous melatonin is declining results in a delay of sleep rhythms (Arendt_2005). However, melatonin administration and dosage are highly individualized with some humans experiencing the expected advances and delays while others seem to be unaffected, especially with oral dosing (Arendt_2005). One hypothesis for individual variability is the rate at which each person clears the exogenous dose as well as slower endogenous clearance around delaying and advancing times (Arent_2005).

There is a clear role for the entrainment of the melatonin rhythm by light, but it is less clear if melatonin itself, despite feedback onto the SCN, is able to directly entrain biological rhythms. Recent evidence for this was in a recently published study by Marqueza et. al. in 2021 where in a double-blind, randomized, placebo-controlled study they showed exogenous melatonin (3mg) was able to reduce circadian misalignment in 24-hour sleep/wake disorder by roughly 20% (Marqueza_2021). Phase shifting of sleep/wake rhythms in other species such as ground squirrels (Soni_2020), house sparrows (Abraham_2000), and Syrian hamsters (Hastings_1992) was also achieved by exogenous administration of melatonin.

However, these findings, even recent ones are met with some skepticism. Exogenous melatonin is able to stabilize the sleep-wake cycle, but evidence of its ability to entrain other biological rhythms is lacking, especially in humans (Foster_2021). The effects of over-the-counter melatonin supplements to treat sleep disturbance and disorders is also being investigated, and preliminary evidence is showing a high degree of misuse and potential negative health outcomes (Riha_2018, Anderson_2016, Grigg-Damberger_2017).

As showed above, when used to treat non-24 sleep/wake disorder in humans, the best study shows only 20% efficacy of melatonin administration in entraining rhythms (Marqueza_2021, Foster_2021). Evidence for exogenous melatonin entraining misaligned rhythms in cases such as jetlag, blindness, and non-24 sleep wake disorder, to name a few, is present; however, the relevance of endogenous melatonin secretion, separate from light's influence, for SCN entrainment remains to be seen. Even in melatonin's most notorious phase shifting role, re-entrainment from jetlag, it seems that the direction of travel (East vs West) and individual sensitivity to melatonin and changing photoperiods is what seems to drive this effect rather than exogenous melatonin consumption alone (Foster_2021).

#### 4.4.4 Melatonin as a seasonal zeitgeber in mammals

Despite its more supplemental role in circadian entrainment, melatonin's most prominent biological role is more likely as a seasonal timer. Given that melatonin secretion is incredibly photosensitive in mammals, it isn't surprising that changing the secretion profile of melatonin is what times the onset of seasonal behaviors (Arendt_2005, Steinlechner_1992). In many mammals, the duration and peak of melatonin secretion changes with respect to the changing night length in seasonal photoperiods

(Arendt_1998, 2005). Depending on where a species lives, its melatonin secretion profile could vary minimally or widely over the course of a year.

How drastically the light:dark ratio changes depends on where an organism lives latitudinally. At the equator, the light:dark ratio remains relatively stable with roughly 12 hours of light and 12 hours of darkness throughout the year. At the North and South Poles, the light:dark ratio changes from 0-4 hours of light (even then it can be classified as just twilight) and 20-24 hours of darkness in the winter. These photoperiods transition so rapidly that in 6 months there are 20-24 hours of light and 0-4 hours of darkness in the summer.

Organisms that live progressively further from the equator rely on seasonal entrainment just as much as they do circadian entrainment to ensure proper timing of behaviors. Animals with long breeding seasons see increasing night length, as measured by an increase in the duration of melatonin secretion, as the time to start mating so that the gestation period ends in birth around spring, where food is more readily available and environmental conditions are less harsh (Arendt_1983). On the other hand, short seasonal breeders (25-30 days gestation), such as the Syrian hamster, see increasing night length as the time to begin mating so that births occur in the spring (Demas_2004).

An animal's photic history also plays a role in how the biological clock views seasonal photoperiods. Syrian hamsters taken from an 8:16 photoperiod and placed in a 12:12 is perceived as a "long" (summer) day whereas if they were taken from a 16:8 photoperiod they would view this as a "short" (winter) day (Hastings_1989).

Neuroplastic changes are also influenced by seasonal photoperiods. In the songbird telencephalon, neuroplastic changes in the vocal control system are strongly influenced by seasonal fluctuations in testosterone. Bentley et. al. observed an increase in the volume of the song-control nucleus high vocal center during "long" days (18:6) vs "short" days (8:16). Administration of melatonin reduced the long day increase in volume in this vocal as well as decreased the volume of area X (another vocalization center in songbirds). Songbirds use vocalizations for mating and what made this study more convincing in showing direct effects of photoperiod and melatonin influence on neuroplasticity was their removal of gonadal structures. These neuroplastic changes were seen regardless of the reproductive state of the animal (Bentley_1999)

In most species, secretion of melatonin matches changes in seasonal night length (Arendt_2005). The profile is wider in the winter when night length is long, and narrower in the summer when nights are short. Infusions of melatonin that mimic long day secretion is enough to induce reproductive activity in sheep (Bittman_1984), whereas long-duration infusions of melatonin in Syrian hamsters inhibits reproductive activity while short-duration infusions increase reproduction (Arendt_2005, Goldman_1984). Melatonin supplementation needs to be given as a “block” of injections to induce these effects. This lends to the theory that melatonin is indeed a “night timer” in that its secretion is read as a block of time reflecting when it is dark (Bittman_1984, Goldman_1984, Arendt_2005).

In summary, melatonin secretion profiles typically change to match seasonal variations in night length (Garcia_2003, Waly_2015, Arendt_2005). There is a high degree of evidence that seasonal behaviors, especially breeding behaviors, are heavily influenced by melatonin and where its peak occurs. Exogenous melatonin administration is capable of inducing phase shifts in both seasonal and circadian rhythms in multiple species including humans. More research needs to be done in understanding the role seasonality plays on the biological timekeeping system and how melatonin is used as a circulating seasonal hormone.

### 4.5 Melatonin as an Antioxidant

Before melatonin signaling evolved to be a circadian and seasonal zeitgeber, melatonin was and still is a powerful antioxidant. The melatonin secretion profile closely correlates to periods of high oxidative stress in mammals, which typically occur under conditions where exposure to UV radiation and other toxic environmental conditions as well as internal energy demand, are high, (Leon_2004, Manchester_2015, Zhang_2014). Simply put melatonin secretion is tied to periods of high activity in living species.

The origin of its antioxidant properties dates back billions of years to the Great Oxygen Event (GOE). The GOE was a massive extinction event that occurred due to uncontrolled aerobic respiration that resulted in toxic levels of oxygen being released into the atmosphere (Lyons_2014). Those organisms that survived, namely cyanobacteria, did so by evolving a means of sequestering or eliminating free radicals in or from their systems. Overtime, more complex organisms evolved and in the case of eukaryotes, endocytosed cyanobacteria and retained beneficial cellular processes as they did so (Manchester_2015, Sagan_1967).

One of the more interesting evolutions from endosymbiosis of nonpurple sulfur bacteria by eukaryotes involves mitochondria, as proposed in the endosymbiotic theory by Margulis (Manchester_2015). It is thought that during this process, mitochondria assumed cellular and biological processes that conferred evolutionary advantages, one of which being using melatonin as a primary antioxidant. In support of this theory, melatonin concentration is 4x higher in mitochondria than anywhere else in living species, even the pineal gland (Reiter_2017).

### 4.5.1 Antioxidant properties of melatonin and its metabolites

Melatonin evolved alongside other antioxidants as life became increasingly dependent on the production of ATP by mitochondria for energy. Mitochondria can produce about 30 molecules of ATP per molecule of glucose oxidized inside the organelle; however, this process also produces reactive oxygen species (ROS) such as $\bullet O_2$, $H_2O_2$, and •OH, as well as reactive nitrogen species such as $\bullet NO_2$. (Alberts_2002, Zhang_2014). These byproducts in excess can be harmful to living organisms, damaging cells, DNA, and tissue, (Qi_2000, Galano_2018) and are linked to many diseases such as Alzheimer disease (Hureau_2009), cancer (Loh_2022, Harris_2020), diabetes (Nishikawa_2007), and inflammatory disorders (Zhang_2020, Lim_2021). Oxidative stress occurs whenever there is an imbalance between ROS production—on the one hand—versus the ability of antioxidants to sequester and clear them on the other. Maintaining this balance is critical: despite the fact that ROS and RNS are utilized by the body at physiologically safe levels as chemical signals, an excess of them can cause fatal harm to biological systems (Mittler_2017).

### 4.5.2 Melatonin as a direct free radical scavenger

Melatonin is a potent weapon in our biological armament used to deal with ROS and RNS. One 232.28 Da molecule of melatonin is capable of directly scavenging between 10 and 12 ROS/RNS (Tan 2007). Melatonin is canonically known to be produced by the pineal gland; however, pinealectomies fail to abolish melatonin presence outside the direct circulation of plasma (Reiter_1982, Huether_1993). Future studies identified the melatonin synthesis not only occurs in the pineal gland, there is also localized production of melatonin in the retina, gut mucosa, bone marrow, airway epithelium, and possibly other peripheral tissues (Acuna-Castroviejo 2014).

Melatonin is an *amphiphilic* hormone; the partial *hydrophilicity* enables it to freely circulate around the body and partial *lipophilicity* enables it to cross physiological barriers and membranes (Gaspar do Amaral_2018). Melatonin's *lipophilicity* is also important for its role as a potent antioxidant, as lipids are more reactive towards ROS/RNS than proteins or DNA (Cardinali_1998, Halliwell_1997). These characteristics make melatonin—outside the contribution of a signal transduction agent like a receptor—a powerful tool for maintaining the homeostatic environment needed around the production and sequestration of free radical production by biological processes.

While the direct scavenging of free radicals by melatonin's chemical structure is due to non-receptor mediated actions, there is an indirect upregulation of antioxidant enzymes through receptor-mediated mechanisms (Reiter_2017, Galano_2018). Melatonin interacts with the nuclear receptor ROR/RZR, calmodulin, and quinone reductase 2 (or MT3) (Carlberg_1995, Benítez-King_1993, Boutin_2019). However, its involvement in redox reactions is typically through its interactions with calmodulin and MT3 (Boutin_2019, Tan_2007).

Some of the most common ROS compounds are single oxygen ($^1O_2$), superoxide ($O_2^{*-}$), hydrogen peroxide ($H_2O_2$), and hydroxyl free radical ($OH^{\cdot}$). They are generated in mitochondria, during ATP production, other cellular enzymatic processes, as well as by external factors such as UV radiation and environmental toxins (Leon 2004). Mitochondria are not the main but are easily the largest source of $O_2^{*-}$ and $H_2O_2$ in the body (Zhang_2021). One of the most common reactions for producing ROS is the Fenton reaction. In mitochondria, via the Fenton reaction, $H_2O_2$ interacts with transition metals such as $Fe^{2+}$ and $Cu^{+}$ to generate $OH^{\cdot}$, one of the most toxic ROS (Thomas 2009).

Reactive nitrogen species (RNS) are free radicals that are utilized as chemical signals by the body, however, at high concentrations, they become toxic much like ROS (Moldogazieva_2018, Franson_2012). The most detrimental RNS are nitric oxide ($NO^{\cdot}$), peroxynitrite ($ONOO^{-}$), and peroxynitrate (ONOOH). The most toxic of these, $ONOO^{-}$, is degraded into other radical species that can increase the toxic effects of oxidative stress if antioxidants were unable to restore balance (van der Vliet_1994, Leon_2004, Zhang_2014).

#### 4.5.3 Melatonin metabolite antioxidant cascade

Metabolites of scavenging melatonin also act as antioxidants themselves, including cyclic-3-hydroxymelatonin (c3OHM), N-acetyl-N-formyl-5-methoxykynuramine (AFMK), and N-acetyl-5-methoxykynuramine (AMK) (Zhang 2014). These first, second, and third generation metabolites are referred to as melatonin's antioxidant cascade, enabling one molecule of melatonin to neutralize up to 10 radical products directly and indirectly. This ratio out preforms more classical free radial scavengers, which can only detoxify a single oxidizing radical per circulating molecule (Reiter 2016).

Melatonin's direct scavenging activity is conserved in most living organisms, most likely a carry-over from its original antioxidant usage in cyanobacteria millions of years ago (Manchester_2015). Adding to these multi-generation metabolites, N-acetylserotonin (NAS) and 5-methoxytryptamine (5-MT) are also lesser known but equally important members of melatonin's antioxidant cascade (Galano_2018, Reiter_2017). The first 3 (c3OHM, AFMK and AMK) are the most studied; however, NAS, 5-MT, and 4/2OHM have also been shown to have important roles as antioxidants especially when it comes to chelating metals in the body.

**4.5.3a N-acetylserotonin (NAS) and 5-methoxytryptamine**

NAS was found to protect against DNA damage from Cr(III) and UV-induced oxidative stress and inhibit iron and copper mediated lipid oxidation by sequestering metal ions from reductants. (Galano_2018, Qi_2000). It is also thought to mediate non-chemical antioxidant actions by activating antioxidant enzymes (Ding_2014, Crespo_2010). NAS is also unique in that it is both a precursor and metabolite of melatonin and is capable of mediating some of melatonin's antioxidant effects in vivo (Galano_2018, Luo_2019).

NAS has been shown to reduce light-induced degeneration of photoreceptors and act as a free radical scavenger in the retina (Ash_2014). Its most beneficial role in ROS reduction is by protecting DNA from $H_2O_2$-induced oxidative damage, UV (especially UVB (290-320nm)) exposure, and preventing lipid peroxidation (Skobowiat_2018, Janjetovic_2017). Its protection against UVB damage is thought to be mediated through NRF2 pathways (Janjetovic_2017).

The antioxidant properties of NAS are independent from those of melatonin and, depending on the species and model system, can potentially be 5 to 20 times higher than melatonin (Oxenkrug_2006). NAS has also been shown to have enhancing effects on cognition, have antidepressant and antihypertensive properties, and anti-tumor actions (Oxenkrug_2005). However, its main role seems to be as an antioxidant and a proposed method of NAS's antioxidant mechanism is through activation of MT3 receptor mediated signaling cascades (Oxenkrug_2005). Non-receptor mediated effects of NAS on RNS, via Nitrous Oxide (NO) production is thought to be through its effects on 5,6,7,8-Tetrahydrobiopterin (BH4). BH4 is an essential cofactor for nitric oxide synthase (NOS) which catalyzes NO hyperactively in its inducible form (Oxenkrug_2005, Gonçalves_2021). NAS is thought to prevent the key enzyme in BH4 production (SPR) by inhibiting its activation and blocking BH4 synthesis (Kojima_2002).

**4.5.3b Cyclic 3-hydroxymelatonin**

Cyclic 3-hydroxymelatonin (c3OHM) has been shown to act through hydrogen atom and single electron transfer and radical adduct formation (Tan_2002). Hydrogen and single electron transfer antioxidant reactions exist both to prevent damage from oxidative stress as well as help repair damage to other antioxidants. This versatility allows c3OHM to effectively scavenge OH and 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid ($ABTS^+$) and peroxy-radicals (Tan_2003).

Protection against HOO-induced damage is most effective in aqueous solutions but scavenges OH at diffusion limited rates. ROS damage to DNA and OH lesions from Fenton reagents are inhibited by c3OHM in vitro. C3OHM has also been shown to have chelating effects on Cu(II) (Galano_2015, 2017, 2018).

#### 4.5.3c $N^1$-acetyl-$N^2$-formyl-5-methoxykynuramine (AFMK)

One of the most notable metabolites of melatonin would have to be $N^1$-acetyl-$N^2$-formyl-5-methoxykynuramine (AFMK). This metabolite has antioxidant actions against radiation, DNA-damage by ROS and RNS, and $H_2O_2$-induced oxidative damage in cells (Manda_2007, Galano_2018). AFMK is also a highly potent scavenger of OH, the most toxic and reactive of all oxidative species under conditions of OS (Tan_2001).

Like its precursor melatonin, AFMK is also a chelator of metals involved in Fenton reactions such as Cu(II) and Fe(II)+ by acting to sequester the metal ions from their reductants (much like NAS) (Tan_2001, Galano_2015). It should be noted that most of these experiments are in vitro under physiological relevant environments and concentrations, but convincing evidence for this chelating antioxidant effect has yet to be done in vivo.

#### 4.5.3d N1-acetyl-5-methoxykynuramine (AMK)

$N^1$-acetyl-5-methoxykynuramine (AMK) is a metabolite of AFMK and regulates antioxidant enzymes by up or down regulating their activity and is more active in scavenging ROS and RNS compared to AFMK. (Hurdeland_2017). A mechanism of action was shown in a study Kelly et. al., which showed AMK inhibiting cyclooxygenase (COX), specifically COX-2, via activation of enzymatic proteins rather than affecting gene expression (Kelly_1984).

Most of AMKs effects are like those of AFMK, with a notable exception of its reduction of HOCL concentrations in neutrophils with anti-inflammatory properties (Hurdeland_2017, Manchester_2015). Also different from AFMK is AMK's potent scavenging of singlet oxygen (Schaefer_2009). AMK is over 150-fold better at scavenging free radicals and is 1.6 times more potent than melatonin itself as an antioxidant (Schaefer_2009). In addition to scavenging singlet oxygen, AMK also scavenges hydroxyl, peroxyl, and carbonate radicals. (Hardeland_2017)

**4.5.4 Chelating effects of melatonin on metal production of ROS and RNS**

In addition to binding and sequestering ROS/RNS, melatonin and its metabolites also bind and sequester heavy metals. Melatonin has been shown capable of binding metals such as aluminum, cadmium, iron, zinc, copper, and lead (Limson_2007). Besides directly scavenging the hydroxyl product from the Fenton reaction, melatonin chelates Fe(III) and Fe(II) metals needed for this reaction to take place (Gulcin_2003).

One could argue that melatonin does a better job at binding heavy metals compared to the more commonly thought of metallothionein. Metallothionein is a protein capable of binding metals in the brain; however, metallothionein is also damaged by the free radical produced from binding the metal (Reiter 2016, Asaad 2018). Melatonin on the other hand, is capable of binding the same metals while also neutralizing the free radicals they produce. Melatonin's metabolites (c3OHM, AFMK, and AMK) also are capable of binding metals in the body without damage from free radical production (Reiter 2016).

Little research has been done to further the understanding of melatonin's role in chelating metals in a biological system. A recent study by Wang et. al. showed in an external system, melatonin has pro-oxidant actions when added to a redox system with low concentrations of copper and quercetin (Que) or (-)-epigallocatechin-3-gallate (EGCG) (Wang_2019). In this condition, melatonin enhances ROS production as it reduced cupric to cuprous ions; however, the ROS production was reversed with the addition of DNA which bound copper and prevented its reduction by melatonin. They also evaluated melatonin's role in vivo in rodents.

In the presence of high levels of copper, Que and EGCG melatonin's role reversed and became that of an antioxidant and reduced ROS production. When DNA was added to this high concentration system, melatonin prevented DNA damage by reducing ROS production. More importantly when melatonin was added to rodents in vivo, melatonin retained its antioxidant effect of protecting DNA damage by reducing ROS production through its chelation of copper (Wang_2019).

Melatonin's indirect role is typically through melatonin receptor 3 (MT3) mediation of quinone reductase 2 (QR2) by donating an electron to the enzymes co-factor, flavin adenine dinucleotide (FAD) (Tan 2007). QR2 is both a detoxifying and antioxidant enzyme with activity dependent on availability of substrates. Melatonin, whose availability is dependent on the light/dark cycle, on the other hand, provides seasonal and circadian control over the behavior of QR2 indicating a seasonal or circadian component to antioxidant actions of melatonin and chelation of metal.

# Chapter 5

## Seasonal melatonin secretion in *Drosophila ananassae*

**5.1 Background**

Research on the use of melatonin as a chronobiotic and antioxidant has increased exponentially over the past 20 years (Meng_2021). Studies have examined its therapeutic potential in treating sleep and circadian-related disorders (Arendt_2008, Sakellaropoulou_2022), mood disorders (De Crescenzo_2017), its use as adjunct intervention alongside chemotherapy to treat cancer (Cutando_2012, Farhood_2018), and in neurodegenerative diseases such as Parkinson and Alzheimer disease (Pérez-Lloret_2021, Hossain_2021). What makes melatonin an attractive therapeutic for these diseases are their shared comorbidity of sleep/circadian disruptions, mitochondrial dysfunction, and higher free-radical production of reactive oxygen and nitrogen species (Misrani_2021, Phillipson_2017, McClung_2013, Hardeland_2009).

Resolving the evolutionary progression along which melatonin gained chronobiotic and seasonal properties—in addition to its primordial antioxidant functions—is essential for understanding: (1) the divergent mechanisms by which melatonin assumed control over distinct processes related to timekeeping in living organisms; (2) the pathways connected to melatonin's antioxidant versus chronobiotic actions; and (3) melatonin's implementation as a cytoprotective agent across different species. *Drosophila* have been one of the founding models for investigating and understanding circadian biology. *Drosophila* also can help us discriminate where in the evolutionary tree the divergence between antioxidant and chronobiotic likely occurred.

*Drosophila* are also an ideal model to start exploring this divergence in that they are a more cost effective and time-efficient model species than other higher order species such as rodents and avian species. *Drosophila melanogaster* also have a plethora of genetic and molecular tools available for researchers to explore genetic and cellular processes and neural mechanisms underlying various physiological behaviors. Unfortunately, our lab uses *Drosophila ananassae* to study circadian phase shifting and although behaviorally they are better for these types of experiments, this species do lack the molecular and genetic tools available for *Drosophila melanogaster*. These studies would need to be replicated in *Drosophila melanogaster* in order to take advantage of those tools to pick apart melatonin's mechanistic role as an antioxidant and chronobiotic in flies.

However, *Drosophila ananassae* do contribute to the overall exploration of if melatonin has rhythmic cycling in *Drosophila* and inferences about what the rhythm contributes to within this species. Since *Drosophila* have conserved circadian mechanisms and behaviors to humans with regards to the biological timekeeping system, our findings have a fair degree of translatability from invertebrate to vertebrate species, giving us a future means of furthering our understanding of exactly what the function of endogenous melatonin is, how its rhythmic secretion times various seasonal and circadian behaviors, and more information about its importance as an antioxidant (Bosler_2015, Binkley_1993).

The *Drosophila* molecular clock mechanism provided the backbone for today's modern understanding of mammalian circadian genetics and molecular-cellular biology. Although there are a myriad of papers concerning *Drosophila* circadian timekeeping, research on the *Drosophila* melatonin system is surprisingly lacking. To date, there are only 2 studies that quantify melatonin secretion over 24 hours in flies (Finocchiaro_1998, Hintermann_1996). Both studies show a rise of melatonin during the evening hours and into the night and fall in melatonin levels at the end of the night and into the afternoon hours but did not characterize the system further. This gap in knowledge motivated us to explore how melatonin is seasonally and endogenously regulated in Drosophila.

Using *Drosophila ananassae*, we measured the diurnal profile of melatonin secretion, the effects of seasonality on this secretion profile, and the potential suppression of melatonin secretion by light exposure. Establishing the presence versus absence of these canonical timekeeping functions of melatonin in *Drosophila* will establish if melatonin's chronobiotic function is potentially conserved in the genus. It will also provide a preliminary understanding of how the melatonin 24-hour secretion profile is tied to its role as an antioxidant. Lastly, this study will contribute to how melatonin evolved its chronobiotic functions and where it the antioxidant functions diverged into other chronobiotic roles such as sleep timing (Hardeland_2006), seasonal reproduction (Abrieux_2020), and temperature tolerance (Li_2018).

## 5.2 Methods

*D. ananassae* were derived from an isofemale line maintained at the Drosophila Species Stock Center (DSSC) at Cornell University's College of Agriculture and Life Science (stock # 14,024–0371.16). Stocks were reared at 25 ºC in DigiTherm® incubators (Tritech Research, Inc., Los Angeles, CA) and entrained to a 12:12 light-dark (LD) cycle (broad-spectrum light source: 4 watt cold-cathode fluorescent light tube with step-up inverter, freely mounted with no fixture, illuminance at rack level = 887.7 lux; Tritech model DT2-LB-F12IN/ CIRC-L-INV; lights-on at 07.00 h, MST). The stocks were transferred daily to generate a steady supply of offspring. Flies were reared on a mixture fresh food (corn-flour–nutritional yeast–agar medium—0.8% agar, 3.5% sucrose, 1.7% glucose, 6% fine-grained masa and 1% yeast) which was also used as the food source during experiments.

Virgin male and female flies (n = 300 total; sex-balanced for each circadian hour surveyed) were selected as late-stage, "pharate-adult" pupae, moved onto fresh food, and housed in groups of 25-50 per tube segregated by sex. Eclosed adult animals were maintained in one of four seasonal photoperiods for 7 days within climate-controlled vivariums. On the 7$^{th}$ day, at bi-hourly intervals, animals were harvested in either light or dark conditions (depending on the phase of the prevailing photoperiod) and placed in foil wrapped tubes (n = 300). Tubes were immediately frozen at -80 °C, then transferred on ice to the CORE facility on the University of Arizona North Campus located in the BIO5 Institute. Sample extraction, preparation, and quantification of melatonin by mass spectroscopy were conducted on site by project collaborators (Dr. Justin Snider and colleagues).

### Harvesting Schedule

Harvesting of 7-day old male and female flies occurred at bi-hourly intervals at either 12:00, 14:00, or 16:00 local time. The photoperiods that were programmed in each vivarium were time-staggered to ensure that harvesting for each timepoint (bi-hourly from ZT0-to-ZT24; 12 timepoints in all) occurred at the same 3 chronological (local) times each day. Animals were placed in foil-wrapped, light-protected tubes whether they were harvested during the day (photophase) or night (scotophase). Male and female flies were combined in the same tube for 1-2 minutes pre-freeze as the researcher walked them to the freezer.

The control photoperiod experienced at the equator mostly year-round (12:12) was programmed for harvesting according to the following schedule. Lights-on was programmed at 7:00 in the vivarium and lights-off was programmed for 19:00 for harvests at ZT 3, 5, 7. Lights-on was programmed at 1:00 in the vivarium and lights-off was programmed for 13:00 for harvests at ZT 9, 11, 13. Lights-on was programmed at 19:00 in the vivarium and lights-off was programmed for 7:00 for harvests at ZT 15, 17, and 19. Finally, lights-on was programmed at 1:00 in the vivarium and lights-off was programmed for 13:00 for harvests at ZT 21, 23, and 1.

The high winter photoperiod experienced at 30° latitude (8:16) was programmed for harvesting according to the following schedule. Lights-on was programmed at 7:00 in the vivarium and lights-off was programmed for 15:00 for harvests at ZT 3, 5, 7. Lights-on was programmed at 1:00 in the vivarium and lights-off was programmed for 9:00 for harvests at ZT 9, 11, 13. Lights-on was programmed at 19:00 in the vivarium and lights-off was programmed for 3:00 for harvests at ZT 15, 17, and 19. Finally, lights-on was programmed at 13:00 in the vivarium and lights-off was programmed for 21:00 for harvests at ZT 21, 23, and 1.

The high summer photoperiod experienced at 30° latitude (16:8) was programmed for harvesting according to the following schedule. Lights-on was programmed at 7:00 in the vivarium and lights-off was programmed for 23:00 for harvests at ZT 3, 5, 7. Lights-on was programmed at 1:00 in the vivarium and lights-off was programmed for 17:00 for harvests at ZT 9, 11, 13. Lights-on was programmed at 19:00 in the vivarium and lights-off was programmed for 11:00 for harvests at ZT 15, 17, and 19. Finally, lights-on was programmed at 13:00 in the vivarium and lights-off was programmed for 5:00 for harvests at ZT 21, 23, and 1.

The final photoperiod, arctic high summer, (20:4) was programmed for harvesting according to the following schedule. Lights-on was programmed at 7:00 in the vivarium and lights-off was programmed for 3:00 for harvests at ZT 3, 5, 7. Lights-on was programmed at 1:00 in the vivarium and lights-off was programmed for 21:00 for harvests at ZT 9, 11, 13. Lights-on was programmed at 19:00 in the vivarium and lights-off was programmed for 15:00 for harvests at ZT 15, 17, and 19. Finally, lights-on was programmed at 13:00 in the vivarium and lights-off was programmed for 9:00 for harvests at ZT 21, 23, and 1.

**Mass Spectrometry**

Frozen flies were added to flatbottom bead-beater tubes containing 200 µL of pure ethanol, 20-30 zirconia beads, and 25 µL of D4 melatonin, an internal standard used widely for quantification of melatonin via mass spectrometry. One ml of ethyl acetate was added to each tube. Samples were vortexed to ensure mixing, and a fast-prep bead-beater was used at 6.5 strokes/sec for 40 seconds to homogenize the flies within solution. Tubes were then centrifuged at 4 °C at 14,000 RPM for 10 minutes with high brake. Supernatant was transferred from sample tubes to 10-mm x 75-mm glass tubes. Extracts were then desiccated in a nitrogen dryer and resuspended in 100 ml of pure ethanol.

After vortexing and centrifugation at 3000 RPM for 15 minutes, the extracts were transferred to 2-ml acls vials with inserts and refrigerated before being placed in a Shimadzu Nexera XR UHPLC tandem Sciex 5500 Qtrap MS. For melatonin quantification, each extract was processed by LC/MS analysis on the Shimadzu Nexera XR UHPLC tandem Sciex 5500 Qtrap MS under conditions similar to those described in Zhao et.al. (Zhao_2016).  A two-ANOVA with a post-hoc TUKEY HSD was applied to the data with significance set to a p-value of .05.

## 5.3 Results

Roughly 14,000 virgin male and female *Drosophila ananassae* were used to generate melatonin secretion profiles across 12 timepoints in each of 4 seasonal photoperiods (8:16, 12:12, 16:8 and 20:4). Data are still preliminary but future projects will seek to extend them. The analysis conservatively assumes that there is no interaction between seasonality and time-of-day. In other words, the effect of seasonality on melatonin secretion -- if there is one -- will be observed at the same circadian phases in each photoperiod.

The analysis of melatonin secretion during each of the 4 examined seasonal photoperiods indicates potential effects of both seasonality and time-of-day on the melatonin secretion profile (Figure 1; $p < 0.05$, two-way ANOVA). Seasonality accounted for 44.8% of the total variance in the data ($F_{3,33} = 15.27$), with less than a 0.0001% chance of our findings being due to chance and season having no effect ($p < 0.0001$). Time-of-day accounted for 22.9% of the variance ($F_{11,33} = 2.13$), with a 4.6% chance that our findings were due to chance ($p = 0.0459$).

To investigate how these effects were affecting seasonal melatonin secretion, we used a Tukey Honest Significant Difference (HSD) post-hoc analysis for a multiple comparisons test between the seasonal melatonin profiles. Results showed a significant difference between the 8:16 (winter) photoperiod secretion profile compared to the other 3 photoperiods (12:12 “equatorial,” $p < 0.0001$; 16:8 “summer,” $p < 0.0001$; and 20:4 “extreme summer,” $p = 0.0005$). All other photoperiods were not significantly different from each other. This difference is most clearly seen in a shift in the first peak of melatonin secretion in the winter photoperiod. The winter peak in melatonin secretion occurs at CT9 whereas in the other 3 photoperiods peak melatonin secretion is achieved at CT13 (Figure 1).

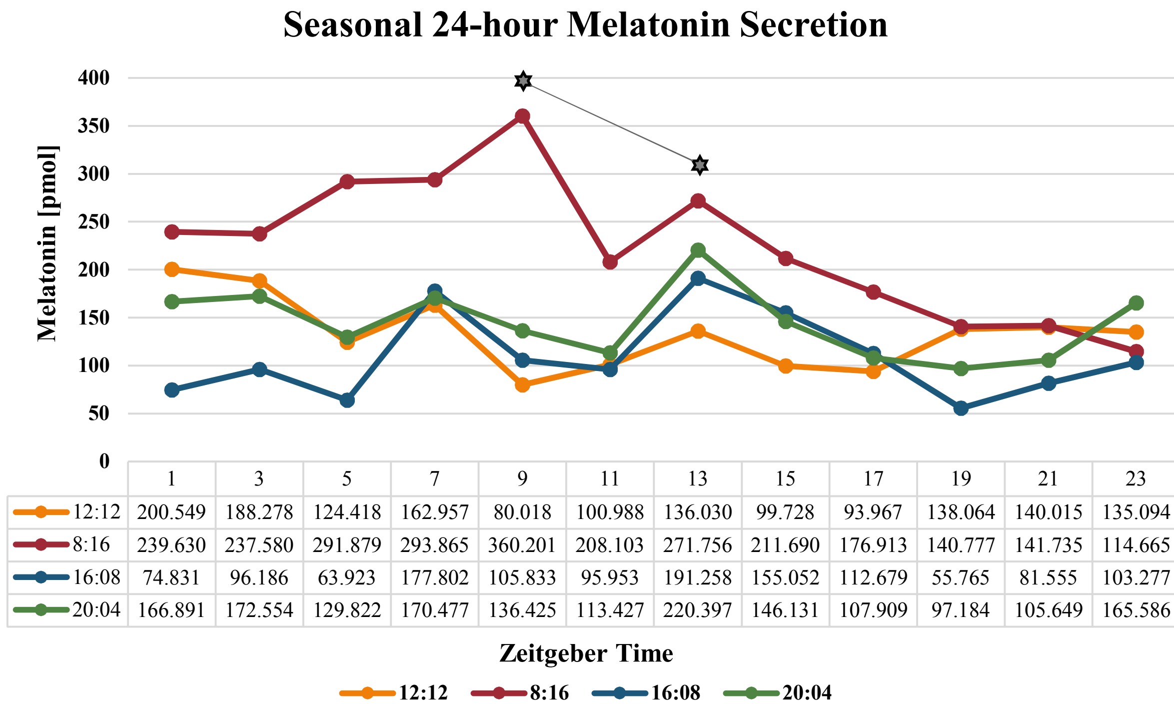
However, it cannot be concluded whether this shift is significant and will hold with repeated experiments. As stated before, although each time point is generated from 300 flies, they are singular timepoints (N=1), and further replication studies are needed to determine if the secretion profiles can be reproduced and show statistically significant differences.

Irrespective of photoperiod, bi-modal peaks of melatonin secretion were evident and localized to phase positions within the photophase (daytime) and/or scotophase (night), including a peak at CT13 in all photoperiods and a peak that occurred several hours after subjective dawn at CT7 in control, summer, and high-summer photoperiods but appeared shifted to CT9 under the winter photoperiod (Figure 1). Interestingly, both peaks in the high summer photoperiod occurred during the photophase at CT9 and CT13 (Figure 1; seen more clearly in Figure 2B).

This finding is somewhat surprising given that melatonin levels in mammals are understood to be low during daylight hours. However, this might be due, in part, to an experimental design flaw since we used whole fly homogenates. In *Drosophila*, melatonin synthesis may not be localized to a specific brain structure or region, but also synthesized in non-neuronal tissues and circulated widely through the hemolymph (Chapter 4.1). As stated, very little research has been done on endogenous melatonin in *Drosophila*, including where it is synthesized and if there is an area of synthesis that is synonymous to mammalian pineal melatonin synthesis and secretion.

There has been research investigating the expression of the *Drosophila* homolog of the rate-limiting enzyme (AANAT1) in mammalian melatonin synthesis (AANAT). Hintermann et. al. reported that mRNA for AANAT1 is present in the gut, brain, and ventral cord and possibly oenocytes in *Drosophila* (Hintermann_1996). In a more recent paper, Davla et. al. found that AANAT1 is also present in *Drosophila* astrocytes and that loss of astrocytic AANAT1, which synthesizes melatonin, causes an increase in daytime sleep recovery following sleep deprivation (Davla_2020). Accordingly, astrocytes may be the proper source for isolating melatonin samples in order to see the canonical nighttime peaking and daytime depletion of melatonin that is seen in mammals as opposed to using whole bodies for sampling. Alternatively, melatonin in *Drosophila,* and possibly insects, may have a divergent role.

The second preliminary finding is the phase control of melatonin secretion being anchored to lights-on (dawn) across all seasonal photoperiods (Figure 2B). This finding is opposite to the phase anchoring of the *D. ananassae* seasonal phase response curve to light to lights-off (dusk) seen in our previously published work (Dollish_2022). This reciprocal phase control by the biological clock for activity and melatonin rhythms bodes well for preliminary research given these opposing themes of activity and melatonin rhythms are also seen in humans (Cajochen_2003). Replications of these photoperiods would need to be done in order to assess a significant shift in the first winter melatonin peak and phase-lock to lights-on.




| | 1 | 3 | 5 | 7 | 9 | 11 | 13 | 15 | 17 | 19 | 21 | 23 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 12:12 | 200.549 | 188.278 | 124.418 | 162.957 | 80.018 | 100.988 | 136.030 | 99.728 | 93.967 | 138.064 | 140.015 | 135.094 |
| 8:16 | 239.630 | 237.580 | 291.879 | 293.865 | 360.201 | 208.103 | 271.756 | 211.690 | 176.913 | 140.777 | 141.735 | 114.665 |
| 16:08 | 74.831 | 96.186 | 63.923 | 177.802 | 105.833 | 95.953 | 191.258 | 155.052 | 112.679 | 55.765 | 81.555 | 103.277 |
| 20:04 | 166.891 | 172.554 | 129.822 | 170.477 | 136.425 | 113.427 | 220.397 | 146.131 | 107.909 | 97.184 | 105.649 | 165.586 |

**Figure 1**: Melatonin secretion over 4 seasonal photoperiods (12:12 (control), 8:16 (winter), 16:8 (summer) and 20:4 (high summer)) shows an increase in melatonin secretion overall for the winter photoperiod (red) relative to melatonin secretion in summer (blue), high summer (green) and control (orange) photoperiods for CT1 through CT17. There is a shift in the peak of melatonin secretion in the winter photoperiod compared to the other 3 photoperiods (grey stars). Data below show the concentration of melatonin (picomoles) measured by mass spec at each circadian time point (n= 150 females and 150 males per time point (N = 48)).

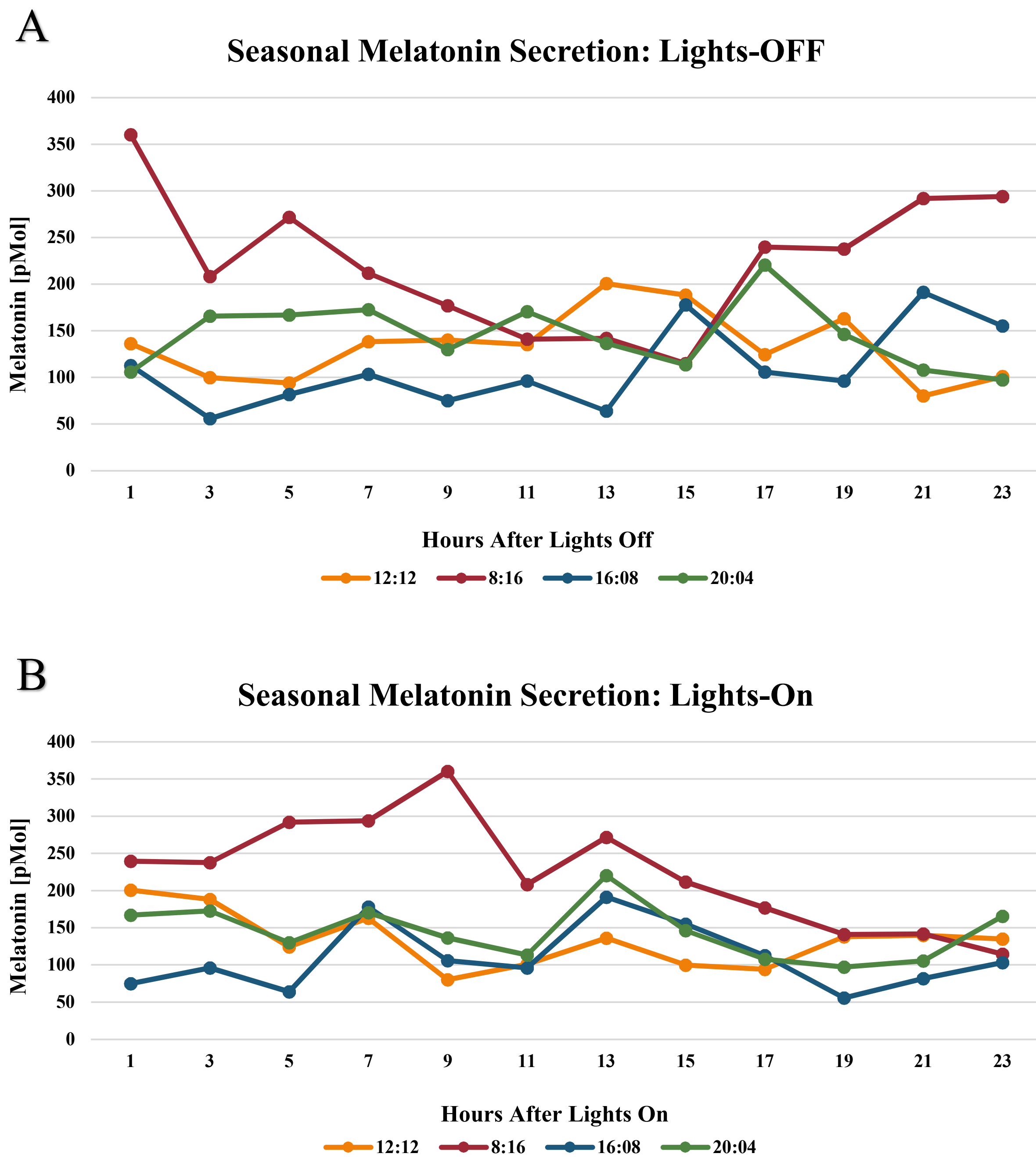



**Figure 2:** Seasonal photoperiods were phase aligned to lights-off (A) and lights-on (B). X-axis shows hours after lights-on when melatonin was collected. Y-axis shows melatonin concentration in picomoles (pMol). Control 12:12 (orange) , summer 16:8 (blue), high summer 20:4 (green) and winter 16:8 (red). Melatonin shows 2-peaks that are phase locked to the timing of lights on (subjective sunrise) (B).

**5.4 Discussion**

Preliminary results show promise for melatonin as a seasonal timekeeper in *Drosophila ananassae*. The winter photoperiod especially seems to be important compared to summer and equatorial photoperiods given it was different compared to the other 3. This may be due to winter being an especially taxing season for animals as far as environmental conditions and resource availability. *Drosophila* reproduction is postponed in winter months and considering melatonin's role as a reproductive timer, the increase in winter melatonin levels might drive the delay of reproductive behaviors (Mitrovski_2001).

Stress and cold tolerance, seen in sustained winter activity in *Drosophila suzukii*, have also been reported along with underdeveloped ovaries and a decrease in their survival to pathogenic fungi (Toxopeus_2016). Expression of stress genes is also transcriptionally altered during the winter to increase stress resilience (Toxopeus_2016). Studies in *D. suzukii* have shown that preoviposition times are longer compared to flies in summer or 12:12 conditions, indicating reproductive diapause (Wallingford_2016). The integration of seasonal temperature and light inputs might be the role of dorsal neurons (DNs). DNs integrate both light and temperature information (specifically low temperatures) in light induced transcription of *timeless* (Chen_2006). This might allow DNs to time seasonal reproduction and eclosion by regulating *timeless* transcription and production.

Bi-modal peaks were observed at very similar phase positions across all seasonal photoperiods tested irrespective of the acute presence of light during daytime (Figure 1). Light is typically suppressive of melatonin in mammals; however, it was shown to be suppressive mostly during periods of darkness or subjective darkness and also has the opposite effect in insects (Arendt_1998). Interestingly, humans also exhibit a bi-modal peak in melatonin secretion that do occur during daylight hours (Wehr_1995, Quian_2022).  Potential reasons for these daylight peaks might be due to (1) melatonin's role as an antioxidant and (2) suppression of melatonin expression taking place in the brain but not the periphery.

One reason for the daytime peak(s) in melatonin might be tied to the diurnal nature of the animal as well as high levels of UV exposure from sunlight. *Drosophila* are diurnal meaning their active period occurs during the day. High periods of activity result in increased free radical production as mitochondria produce ATP for the body to use as fuel. Reactive oxidative and nitrogen species (ROS and RNS) rise in concentration, putting the animal in a state of oxidative stress unless counter-balanced by antioxidant sequestration and elimination of ROS and RNS. Another reason for a daytime increase in body-wide melatonin is also tied to exposure to solar UV radiation. UV radiation is damaging to DNA and various cellular processes as well as increasing free radical production. For small animals, such as *Drosophila*, UV penetration may be high and may have more damaging consequences. Accordingly, this may result in a need for increased antioxidant availability and activity during the day when light intensity is greatest. Given that melatonin is a powerful antioxidant capable of directly and indirectly sequestering up to 10 ROS per one molecule of melatonin, it makes sense for melatonin levels to rise during periods of oxidative stress (see Chapter 4.3).

The *Drosophila* biological clock, although conserved in its molecular and genetic functions, differs from mammals in that clock nuclei are spread throughout the brain and not localized like they are in the SCN. If you look at each lateral and dorsal nuclei in isolation from the others, the increase and decrease in activity would not translate into a representation of the animal's endogenous rhythm or entrainment to the external 24-hour daily period. However, where looked at as a network, the rhythmic activity of these neurons do indeed provide a robust circadian output that can translate into a representation of the 24-hour clock (Taghert_2016).

It may be that the melatonin system in *Drosophila* has an analogous anatomical differentiation from mammals, in that the center of melatonin production in the circadian system and the pineal gland might diffuse into different nuclei in the *Drosophila* brain much like how the lateral and dorsal neurons diffuse from the prototypical mammalian central pacemaker. *Drosophila* might not have a melatonin production center but rather a melatonin production network that differentially secretes melatonin at different phases of the photoperiod. Secretion of melatonin in areas that secrete melatonin as a timer of night length might also overlap with areas that secrete melatonin at different rhythmic amounts for antioxidant functions. Global peaks might mask a more nuanced system of secretion like that of the lateral and dorsal neuron biological timekeeping network.

Our last preliminary finding concerned the phase control of the seasonal melatonin rhythm: it was phase-locked to the timing of lights-on (sunrise). This is interesting as a previous study by our lab found that the *D. ananassae* seasonal phase response curve to light was reciprocally anchored to the timing of lights-off (dusk) (Dollish_2022). Melatonin and light work in opposing synchrony to entrain and synchronize circadian and seasonal rhythms. Having light-induced activity rhythms differentially anchored to melatonin secretion provides an interesting overlap between the *Drosophila* and mammalian systems. It also highlights the dualistic functions that light and melatonin have in entraining and synchronizing internal rhythms and the regulatory feedback they have on each other.

# Appendix A

**Abstract**

Photic history, including the relative duration of day versus night in a 24-hour cycle, is known to influence subsequent circadian responses to light in mammals. Whether such modulation is present in *Drosophila* is currently unknown. To date, all photic phase-response curves (PRCs) generated from *Drosophila* have done so with animals housed under seasonally agnostic equatorial photoperiods with alternating 12-hour segments of light and darkness. However, the genus contains thousands of species, some of which populate high and low-latitude habitats (20-50° north or south of the Equator) where seasonal variations in the light-dark schedule are pronounced. Here, we address this disconnect by constructing the first high-resolution *Drosophila* seasonal atlas for light-induced circadian phase-resetting. Testing the light responses of over 4,000 *Drosophila* at 120 timepoints across 5 seasonally-relevant rectangular photoperiods (i.e., LD 8:16, 10:14, 12:12, 14:10, and 16:8; 24 hourly intervals surveyed in each), we determined that many aspects of the fly circadian PRC waveform are conserved with increasing daylength. Surprisingly though, irrespective of LD schedule, the start of the PRCs always remained anchored to the timing of subjective sunset, creating a differential overlap of the advance zone with the morning hours after subjective sunrise that was maximized under summer photoperiods and minimized under winter photoperiods. These data suggest that there may be differences in flies versus mammals as to how the photoperiod modulates the waveform and amplitude of the circadian PRC to light. On the other hand, they support the possibility that the lights-off transition determines the phase-positioning of photic PRCs across seasons and across species. More work is necessary to test this claim and whether it might factor into the timing of seasonal light responses in humans.

**Key words:** light, circadian rhythms, phase shift, photoperiod, photic history, season

## 1. INTRODUCTION

The brain's circadian pacemaker integrates information about ambient light and darkness to calibrate endogenous rhythms of physiology along a schedule that matches the 24-hour schedule set by the Earth's rotation.[1,2] This biological adherence enables animals to occupy a temporal niche phase-locked in alignment with the day (diurnal), night (nocturnal), or the temporal poles stationed between them at dawn and dusk (crepuscular), thereby organizing behaviors related to sleep, feeding, and reproduction to times of the solar cycle optimal for survival.[3] The pacemaker not only evolved to anticipate daily recurring episodes of consolidated light exposure followed by darkness but also to annual variations in the length of the day *relative to the night*.[4] Owing to the Earth's 23.45° axial tilt and orbit around the Sun, the ratio of day versus night changes systematically across the year, with the degree of change dependent on latitudinal (angular) distancing north or south from the equator. When the boundaries separating the day from the night are moved by shortening or extending the duration of the daily light period, the pacemaker will continue to direct 24-h rhythms while repositioning the onset and offset of many physiological indices to the new transitions marking sunrise and sunset.[4-7] The most conspicuous of these indices in mammals is the onset and offset of nightly melatonin secretion, which imprints a biological representation of daylength animals use to make forward-looking seasonal adaptations (e.g., gonadal regression, hibernation) to predictable environmental challenges introduced at times of year when the number of daylight/night hours becomes significantly skewed (e.g., severe decreases in temperature and food availability in winter when the night is extended at the expense of the day).[7-9]

While much is known about the independent mechanisms that subserve the pacemaker's operation as a circadian clock versus photoperiodic seasonal timer[10-23], comparably less is understood about the intersection of these two areas. Per most current data, the pacemaker's phase responses to acute light exposure change systematically across the subjective night in the service of photoentrainment to an equinoctial 12:12 light-dark (LD) cycle.[24] Here, light administration in the first half of the evening will produce phase *delays* in behavioral-physiological rhythms, whereas administration in the latter half will produce phase *advances* (light stimulation during the subjective day tends to provoke little phase movement either way).[24,25] It is assumed that these responses stem from the pacemaker's differing interpretations of light exposure in the several hours subsequent to dusk as opposed to the several hours preceding dawn. In the former, any incidence of light telegraphs an extension of the sunset requiring delays in activity offset that will maximize a diurnal animal's contact with daylight; in the latter, light signals an earlier sunrise requiring advances in activity onset that will enable a diurnal animal to awake in alignment with the start of the morning (reciprocal relationships hold for nocturnal animals). When graphing these responses across hourly intervals, it is customary to plot the magnitude of delays with negative numbers and advances with positive numbers.[26] The resulting visual that emerges—termed the circadian phase response curve (PRC)—takes on the appearance of a sigmoidal-like wave that zeroes out near the scheduled transitions of the light-dark cycle.[24-26]

The circadian PRC to light has been predominantly studied—and modeled—in laboratory animals entrained to seasonally agnostic LD schedules comprised of light and darkness available in equal measure for 12 hours.[27,28] In the context of these wider characterizations, targeted work has also described how previous entrainment to short versus long days (i.e., winter-like versus summer-like rectangular photoperiods generated by electric lighting) can alter the PRC's waveform characteristics and response amplitude. Hamsters or birds housed under long 16-hour nights, for example, demonstrate subsequent circadian responses to light exposure that occur over a broader range of the 24-h cycle and/or are as much as two-fold greater compared to animals housed under short 8-hour nights[29-31]. While seasonal influences are apparent in the shape of the hamster and avian PRC to light and have been hinted at in humans[32-33], no work has described analogous seasonal changes in *Drosophila*, which have otherwise provided fundamental insights into circadian timekeeping[34].

Under short days, female *Drosophila* enter a state of reproductive dormancy referred to as ovarian diapause[35]. Here, in a light and temperature-dependent fashion, they secrete less juvenile hormone and restrict uptake of hemolymph yolk protein by oocytes[36]. The suite of physiological responses exhibited by female *Drosophila* under simulated winter conditions is analogous to the suite of photoperiodic responses made by seasonal breeders such as Syrian and Siberian hamsters when those animals are situated in electric LD cycles providing less than 10 hours of light[37,38]. Given that flies and hamsters are both photoperiodic species, we wondered if there were other parallels related to seasonal changes in the circadian PRC to light. To our knowledge, a comprehensive PRC atlas detailing how patterns of circadian photoresponsivity change as a function of daylength across several seasonal photoperiods has never been constructed for any lab model. Such an atlas has the potential to open a discussion about which principles of seasonal change are universal in the photic PRCs of animals and which are more likely to be species centric. Though humans are endemically exposed to artificial photoperiods that differ markedly from natural photoperiods, many live 20-50° N/S from the Equator (NASA Socioeconomic Data and Applications Center, **Supplementary Figure 1**), placing them within a geographic distribution where summer and winter photoperiods range from LD 16:8 to 8:16, respectively. There is an inferential gap when generalizing results from flies and rodents → humans; Nevertheless, these data may contribute (at least in small part) to existing considerations for optimizing the timing of human bright light therapy.

In the current study, we used *Drosophila* to construct high-resolution PRCs for 5 photoperiods exhibiting incremental changes in the ratio of light and darkness (LD 8:16, 10:14, 12:12, 14:10, and 16:8).

This range provided an accurate representation of winter and summer LD schedules across high and low latitudes, allowing us to infer how circadian photoresponsivity might change across the seasons in geographical areas removed from the Equator. We outline the results below and discuss their relevance with respect to the fly's PRC waveform and its localization relative to the timing of sunset and sunrise.

## 2. METHODS

*D. Ananassae* (melanogaster species subgroup) were derived from an isofemale line maintained at the *Drosophila Species Stock Center* (DSSC) at Cornell University's College of Agriculture and Life Science (stock # 14024-0371.16). Stocks were reared at 25°C in DigiTherm® incubators (Tritech Research, Inc., Los Angeles, CA) and entrained to a 12:12 LD cycle (broad-spectrum light source: 4 watt cold cathode fluorescent light tube with step-up inverter, freely mounted with no fixture, illuminance at rack level = 887.7 lux; Tritech model DT2LBF12IN/CIRC-L-INV; lights-on at 07.00 h, MST). The stocks were transferred daily to generate a steady supply of offspring. For phase-shifting experiments, female flies were selected as late-stage, "pharate-adult" pupae, moved onto fresh food, and housed in groups of 5 to 6. A few days post-eclosion, animals were singly housed in Pyrex glass chambers (5 mm outside diameter, 65 mm long) containing a solid aqueous plug of food medium (4.7% sucrose, 1.9% agar; MoorAgar, Inc., Rocklin, CA) on one end and a cotton fitting on the other, and loaded into Trikinetics DAM2 Drosophila Activity Monitors (TriKinetics, Inc., Waltham, MA). The monitors were situated within climate-controlled vivariums identical to the ones used in colony management (25°C, 887.7 lux) but with the photoperiod maintained, extended, or retracted to one of five possible seasonal schedules: **LD 8:16** (lights-on at 0.700; lights-off at 15.00), **LD 10:14** (lights-on at 07.00; lights-off at 17.00), **LD 12:12** (lights-on at 07.00; lights-off at 19.00), **LD 14:10** (lights-on at 07.00; lights-off at 21.00), or **LD 16:8** (lights-on at 07.00; lights-off at 23.00). Throughout the experiment, the flies' motion was independently tracked by cross-sectioned infrared beams, which transmitted movement information over modem/USB to a computer acquisition software (DAMSystem-3) every 30 seconds. A preliminary set of 160 flies was screened to confirm seasonal activity profiles during 5 days of housing under each photoperiod (32 animals per group; eduction plots and population actograms provided in **Figure 1A-B**).

An Aschoff Type II paradigm was used to quantify the effects of light exposure on phase resetting of the flies' locomotor activity rhythms. This procedure is ideal for pooling data from a large population of subjects because they are at about the same phase of their cycles when the stimulus is delivered, and offers an accurate assessment of the natural field shape of the PRC vis-à-vis photoentrainment by avoiding the amplitude inflation that develops after long-term housing in DD[26,39]. This approach to pacemaker analysis might be especially relevant for applying data across animal and human models[40], and has been previously employed to evaluate how photoperiod length modulates light-induced circadian resetting in house sparrows (*Passer domesticus*)[31] as well as Siberian hamsters (*Phodopus sungorus*)[41]. For the various PRC experiments, animals were entrained to the LD schedule to which they were introduced for 3 days. After lights-off on the last day of the entrainment period, separate cohorts received a single 15-min pulse at one of the hourly intervals spanning the next 24-h cycle (i.e., 24 independent groups tested per seasonal schedule; 120 groups tested in total, with ~32 flies per group; see **Supplementary Tables 1-5**). This was accomplished by software-controlled activation of the house lamp (887.7 lx, white fluorescent light; Tritech Research, DeviceCom3™). Post-pulse, animals were left to free-run in constant darkness (DD) for 4-5 days.

Actogram plots reflecting the daily activity profile for each fly in a given treatment group were created by binning raw 30-sec time series data of individual *D. ananassae*. Phase shifts of behavior were calculated for each fly (one fly = one 5×65 mm tube) by determining the horizontal distance between regression lines fitted through software-called activity onsets 2 days prior and 2-4 days after light administration (ClockLab Analysis Version 6, Actimetrics, Wilmette, IL). Initial experiments assessing the flies' activity profile within each photoperiod showed that the activity onsets of *D. ananassae* were phase-locked to the timing of lights-on in each seasonal schedule (i.e., 07.00 h, MST). Post-pulse in DD, transients are observed for a day, but the flies' behavioral rhythms invariably reset by the second DD cycle[42] (see also **Supplementary Figure 2** for a few individual actograms from this study demonstrating phase stability of the activity rhythms 2-4 days after light pulsing in the delay and advance zones). To correct for phase movements that might simply accompany transitions from LD to DD in each seasonal condition, a control group was transferred into DD without any light treatment. Final phase-shift values were calculated by subtracting out the average phase movement exhibited by the no-light-exposure group under each seasonal condition (mean ± SEM, n = 32 flies per control group; **LD 8:16**, 2.37 ± 0.09 h advance relative to lights-on; **LD 10:14**, 0.75 ± 0.10 h advance relative to lights-on; **LD 12:12**, 1.04 ± 0.11 h delay relative to lights-on; **LD 14:10**, 2.85 ± 0.09 h delay relative to lights-on; and **LD 16:8**, 4.98 ± 0.14 h delay relative to lights-on; these control data are summarized in **Figure 1C**; see **Supplementary Figure 2**, first row of panels, for example actograms taken from individual flies).

For analyses, one-sample *t*-tests were used to determine whether the net phase shift produced by light exposure at a given hour for a given seasonal schedule was greater than zero. Where appropriate, one-way or two-way ANOVAs with Tukey's post hoc correction were used to assess seasonal variations in phase-shift magnitude across select intervals encompassing the delay and advance zones. Significance was set at $p = 0.05$. In total, 4,094 animals were independently evaluated to measure the effects of seasonality on light-induced circadian resetting (control and light-treatment groups combined; represented in **Figure 1C** and **Figures 2-4**, respectively). Please note that the phase-shift values for the hourly intervals between ZT13 to ZT23 in the 12:12 LD condition are reproduced from Kaladchibachi et al., 2018[43], which reports on a battery of experiments largely contemporaneous to the ones discussed in the current manuscript.

Please also note that the general outline for our assessment is consistent with the standard (long-held) practices of the *Drosophila* PRC and light resetting literature[44-62]. In these studies, generally[60-62]:

1. Aschoff II paradigms (often referred to by the moniker "anchored" PRC) are used to assess light-induced resetting of locomotor activity rhythms.
2. Due to the appearance of transients, the first DD cycle post-pulse is not taken into account when measuring the size of a phase shift.
3. The second day is a point of emphasis in phase-shift quantification, either as part of a regression or as a standalone value (i.e., the position of the phase reference in the second DD cycle determines the absolute size of the phase shift).
4. A no pulse control group is used as a correction factor in phase resetting.
5. Animals are left to free-run in DD for 3-5 days after light treatment.

## 3. RESULTS

Our preliminary screen suggested that *D. ananassae* maintained robust 24-h patterns of locomotion under each photoperiod tested. In all cases, 5-day activity profiles were marked with morning and evening peaks that were coincident with the timing of lights-on and lights-off in the LD schedule (**Figure 1A-B**). While morning peaks of activity gave way to similar declining rates of movement leading into midday, evening peaks scaled with the number of hours available in the photophase; the longer the daylength, the more that evening bouts of activity encroached into the photophase, encompassing a span of about 6 hours when animals were housed in a LD 16:8 (**Figure 1A-B**). Morning anticipatory activity was not a general feature observed in the photoperiods that were tested, though such patterns emerged after a few days of housing under a high-winter 8:16 LD schedule (**Figure 1B**). It is worth noting that this anticipation can be variable between flies and across days for the same fly. While anticipation activity is clock influenced, it occurs independently of the morning activity peak coincident with lights-on, which is controlled by the fly circadian pacemaker network and accurately reflects the pacemaker's phase positioning[63].

Subsequent experiments in separate groups of control animals (i.e., those released into constant darkness without a light pulse after 3 days of entrainment to one of the five photoperiods) confirmed that activity onsets were increasingly phase-delayed with respect to the timing of lights-on (dawn) in the previous photoperiod; the longer the daylength in the previous schedule, the larger the delay repositioning that was observed when the LD cycle was eliminated (**Figure 1C**; $F_{4,155} = 726.3$, $p < 0.0001$). This photoperiod alteration of the phase relationship between activity onset and the time of lights-on (dawn) is consistent with observations made by Binkley and Mosher in house sparrows[31] and with the photoperiodic aftereffects noted by Pittendrigh in the *Drosophila* eclosion rhythm upon release into DD from a range of seasonally relevant photoperiods[64]. The latter data, collected from flies after 5 days of photoperiod exposure[64], suggest that the locomotor activity rhythm of *D. ananassae* in the present study was likely seasonally entrained after just 3 days (however, see further discussion of this point below). The irradiance spectrum for the broadspectrum light source used to generate the photoperiods and construct the PRCs described below is provided in **Figure 1D**.

Raw data from the PRC experiments are reported in five scatterplots visualizing phase shifts in the locomotor activity rhythm for individual animals exposed to a 15-min pulse at one of the hourly intervals of the 24-h day (**Figure 2**; descriptive statistics are available in **Supplementary Tables 1-5**). These data were averaged at each timepoint to generate high-resolution PRCs that illustrated how the circadian pacemaker's sensitivity changed as a function of seasonal photohistory (**Figure 3**). In theory, seasonal differences in the PRC might manifest as differences in the fraction of the circadian cycle that light-induced resetting of any kind occurs. If this fraction remains constant, there might still be changes in the amplitude of the delay or advance zone (i.e., scaling of the maximal shifts achievable by saturating light exposure), retractions or expansions in the widths of these regions (i.e., changes in the proportion of the PRC where light exposure will elicit a delay versus an advance shift), or changes in the positioning of the delay and advance zones with respect to the timing of lights-on (dawn) and lights-off (dusk) in the LD schedule.

In the midst of all these possibilities, we found very targeted effects of seasonality on the circadian PRC to light. Primarily, this involved phase-positioning of the entire curve relative to the transition borders of the LD cycle. Irrespective of season, photic PRCs were always anchored to the timing of lights-off (subjective sunset) in the LD schedule, producing a staggered realignment of the advance zone relative to the timing of lights-on (subjective sunrise) (**Figure 3A-B**). The absolute widths of the delay and advance zones were conserved—comprising approximately 5 and 10 hours of the circadian cycle, respectively, along with a 9-hour deadzone (**Figure 3B-D**)—though *minor* photoresponsivity differences were distinguishable across hourly intervals within each region. A two-way ANOVA for the phase-shift data within the first 5 hours of the scotophase, the interval marking the delay zone, indicated a photoperiod/seasonal effect ($F_{4,1007} = 9.471$, $p < 0.0001$), circadian phase effect ($F_{5,1007} = 344.3$, $p < 0.0001$), and an interaction between season and circadian phase ($F_{20,1007} = 19.69$, $p < 0.0001$) such that photoresponsivity peaked later with summer-like photoperiods possessing ≥ 14 hours of daylight (**Figure 3C**). A two-way ANOVA for the phase-shift data within the 10-hour block of the circadian cycle aligned with the delay-to-advance transition (marking the advance zone) also indicated a photoperiod/seasonal effect ($F_{4,1576} = 9.448$, $p < 0.0001$), circadian phase effect ($F_{9,1576} = 170.5$, $p < 0.0001$), and an interaction between season and circadian phase ($F_{36,1576} = 7.759$, $p < 0.0001$) such that photoresponsivity was greater within the first two hours of the advance zone under longer photoperiods with short nights (**Figure 3D**). The net effect of these changes was to create a sharper inflection in the waveform of the PRC as it transitioned from the most photoresponsive area of the delay zone to the most photoresponsive area of the advance zone (**Figure 3B**).

In one final analysis, we replotted phase-shift data relative to the timing of lights-on (subjective sunrise) in each photoperiod to quantify the number of hours within the first 8-hour segment of the morning/afternoon that remain circadian responsive by virtue of the morning's overlap with the PRC advance zone (**Figure 4**). Overlap of the advance zone with the morning was minimized under short, winter-like photoperiods (e.g., LD 8:16 and 10:14) and accentuated under long, summer-like photoperiods (e.g., LD 14:10 and 16:8). At the seasonal poles, we found that the pacemaker's photoresponsivity was effectively extinguished by sunrise during high winter but robust throughout much of the first-half of the day in high summer.

## 4. DISCUSSION

Here, we report development of the first seasonal atlas for light-induced phase resetting of circadian rhythms. After probing 120 timepoints across 5 seasonally relevant photoperiod schedules, we determined that many aspects of the *Drosophila* circadian photic PRC are conserved with increasing daylength, including the widths and amplitudes of the delay and advance zones and the proportion of the curve comprised by each region. The single largest impact of seasonality on the PRC was to change the extent to which circadian photoresponsivity in the advance zone extended into the morning hours after sunrise. By virtue of the PRCs' anchor to sunset, this intersection was maximized under summer photoperiods and limited under winter photoperiods with ≥ 14 hours of darkness.

It is worth noting that there has been only one previous *systematic* attempt to understand how the pacemaker's light-induced phase-shifting responses are affected by photoperiod (though some work has established photoperiod-dependent changes in other outputs such as the shortening/lengthening interval of photic induction of c-fos expression and *Per1* induction)[14,20,65,66]. In studies that were never formally published, but disseminated as part of a larger book chapter, Elliott and Pittendrigh found that the amplitude of the hamster PRC to light was significantly larger when animals were housed under a LD cycle with short days (LD 10:14) versus one with long days (LD 18:6)[67]. Though it was never directly articulated by the authors, examination of their plots suggests that both PRCs were pegged to the timing of lights-off (subjective sunset) in each photoperiod, with a resulting: 1. several-hour overhang of the advance zone with the morning/afternoon hours of the photophase in the LD 18:6 schedule and 2. absence of circadian photosensitivity in the morning hours of the winter-like LD 10:14 schedule (see Figure 5 of reference 67). These data, collected across two photoperiods probing 9 timepoints in each, are limited relative to the current seasonal atlas but are consistent with some of the findings we have made. While seasonal differences are bound to exist in the pacemaker's responses to dim versus bright light, or broadspectrum versus narrowband emission, or light delivered along other physical exposure dimensions, the current study establishes that the PRCs reflecting maximal responses across the seasons do not generally change in their waveform characteristics or their phase-positioning relative to sunset in *Drosophila*. All the aforementioned considered, one of these tenets, the invariable phase-alignment of the PRC to sunset, has the potential to be species generalizable. With further attempts to compare seasonal PRCs across model systems, this "sunset anchor" might eventually prove relevant for optimizing bright light therapy in humans[68-70].

The present study has a few limitations. The first involves the three-day entrainment window used for each seasonal lighting condition and a consideration of whether that time enabled the flies to adjust to the various photoperiods. In hamsters, photoperiodic responses associated with reproduction require several weeks of seasonal re-entrainment by the pacemaker[37,38]. In *Drosophila*, indices related to diapause can develop in about 14 days[35]. Pittendrigh[64], along with recent work by Vaze and Helfrich-Förster[71], noted seasonal changes in the phase relationship between *Drosophila* eclosion/activity peaks and dawn/dusk that emerge upon release into DD after 5-6 days exposure to the prevailing LD cycle. We observed a very similar phenomenon in the current dataset after 3 days exposure to the photoschedule (**Figure 1C**). In so far as this phase relationship reflects a measure of seasonal entrainment, it suggests that the circadian responses quantified in the present study were grounded in animals that had stably entrained their locomotor activity rhythm to each lighting condition prior to the probe pulse. There may be differences in the duration of housing necessary to trigger photoperiodic responses linked to reproduction compared to those that enable entrainment of behavioral activity[72,73]. Since we did not directly test this dissociation, the possibility remains that the photoperiod was not *completely* encoded by the pacemaker (i.e., as such, the data may relate more to the influence of acute photic history on the PRC and less to direct seasonal changes). Future experiments will be necessary to scrutinize this issue further, along with efforts to better simulate seasonal progression by incrementally adjusting the timing of both dawn and dusk rather than conserving the time of lights-on and migrating only the time of lights-off to generate the experimental photoperiod.

Another limitation of the present study concerns the possibility that other aspects of daylight and twilight might add important nuances to how *Drosophila* photic PRCs are influenced by seasonality. For instance, seasonal variations in the spectral composition of sunlight—and patterns of spectral change over the daytime—mark the photoperiod at all latitudes.[74] Introduction of more natural "dynamic" lighting conditions to phase-shifting experiments, though rarely (if ever) practiced in the laboratory, might thus refine *Drosophila* PRC waveforms and have special significance for identifying changes to photic PRCs constructed using narrowband and intermittent photic stimuli.[75,76] A final future consideration involves the use of graded twilight simulations. Though virtually all phase-shifting studies in *Drosophila* to date have been conducted in the laboratory under square-wave LD cycles carrying abrupt (switch-like) transitions between lights-on and lights-off, inclusion of natural twilight progressions—over the course of 60-90 minutes—might signal (or aid in the communication of) other seasonal factors to the pacemaker that impact the shape of the PRC manifesting under different photoperiods.[77-80] All of these considerations underscore the need for characterization of the circadian pacemaker outside the typical contexts (e.g., equatorial LD schedules) in which it has been historically studied.

In summary, we have established a seasonal atlas for circadian phase-shifting in *Drosophila* and, therein, identified the advance zone as the major site of the PRC undergoing photoperiod-dependent realignments in positioning with respect to the LD cycle. These data have the potential to enhance the visibility of seasonal PRC research in *Drosophila*. They also lay the groundwork for future experiments seeking to identify other seasonal factors that might contribute to circadian light responses in laboratory models.

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**Conflict of Interest.** The authors declare no competing interests.

**Author Contributions.** F. Fernandez developed the study concept, oversaw its experimental design, drafted the paper, and procured funding for all aspects of the work. Behavioral testing and data collection were performed by H. Dollish, S. Kaladchibachi, and D. Negelspach. All the authors contributed to discussing and interpreting the findings and approved the final version of the manuscript for submission.

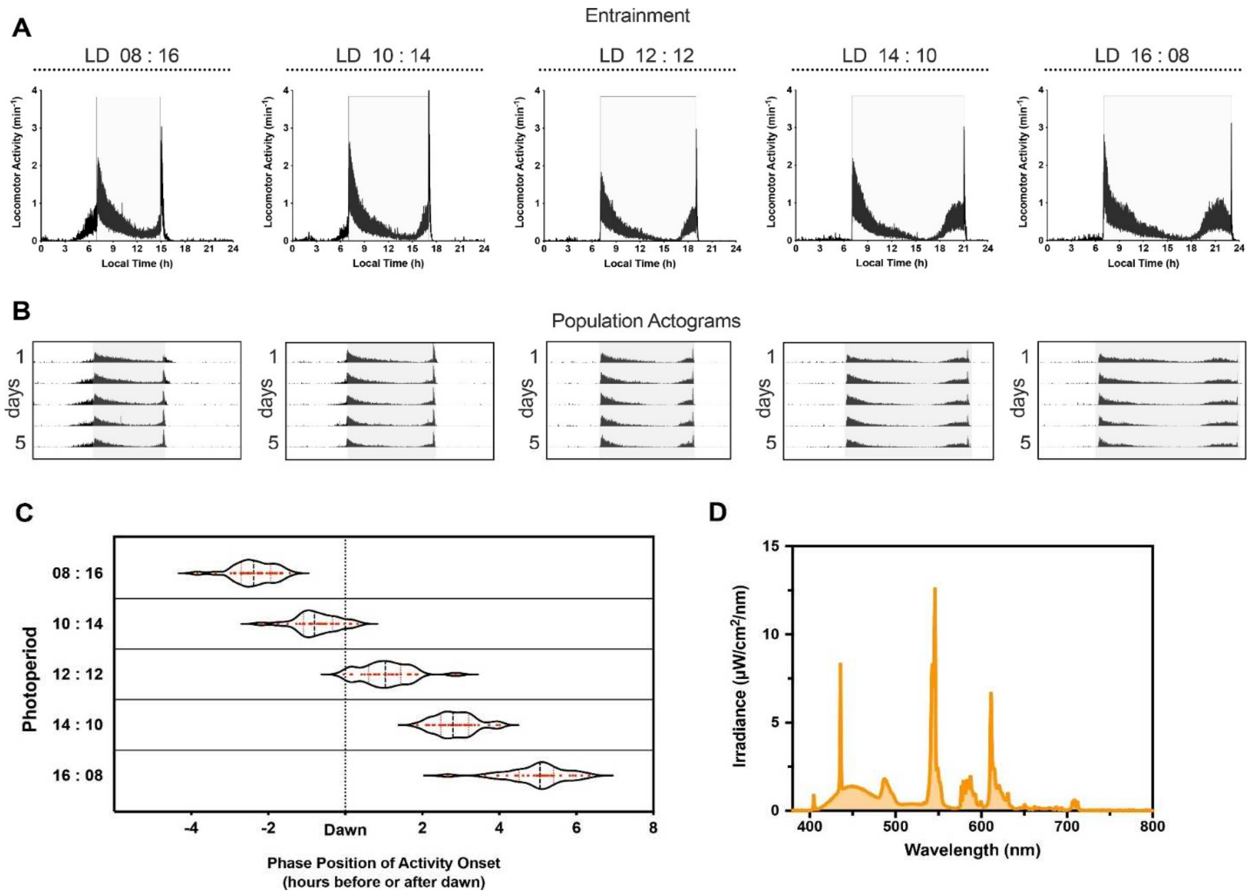


**Figure 1**. Entrainment to seasonal photoperiods. (**A**) Eduction plots showing the 24-h activity profiles ($min^{-1}$) for flies maintained for 5 days under the various photoperiods. The LD cycles are labeled overhead, left to right, with the light phase in each bracketed in light gray. Activity profiles were created by binning raw 60-sec time series data of individual flies, averaging these data across animals, and then averaging across each corresponding 60-sec epoch of each day under study. (**B**) Population actograms for flies housed under each photoperiod are provided, situated below each of the activity profiles to which they correspond. Black bars indicate 30-sec epochs where the animals registered breaks in the infrared-tracking beams, smoothed over each two minutes of recording. Data are vertically aligned, such that one 24-h day of movement is shown per line, with successive days appearing one below the other. The portion of the LD schedule where the lights are on is highlighted in grey (local time of lights-on in each photoperiod = LD 08:16, 07.00-15.00; LD 10:14, 07.00-17.00; LD 12:12, 07.00-19.00; LD 14:10, 07.00-21.00; and LD 16:08, 07.00-23.00). (**C**) Control data are reported for flies housed under each photoperiod for 3 days and then released into DD. Phase movements associated with removal of the LD cycle were calculated by determining the horizontal distance between regression lines fitted through software-called activity onsets 2 days prior and 2-4 days after the light schedule transition. Violin plots show the migration of each animal's locomotor activity onset. Data are plotted with respect to the timing of lights-on in the previous schedule (Dawn). The overall distribution of the data points (red) is reflected by changes in width that occur along each plot. The median value and quartiles of the samples are marked with a thick fragmented line and thinner red-dotted lines, respectively. (**D**) Irradiance spectrum for the broadspectrum light source used in the study. Illustrated within are the dominant wavelengths produced by the emission.

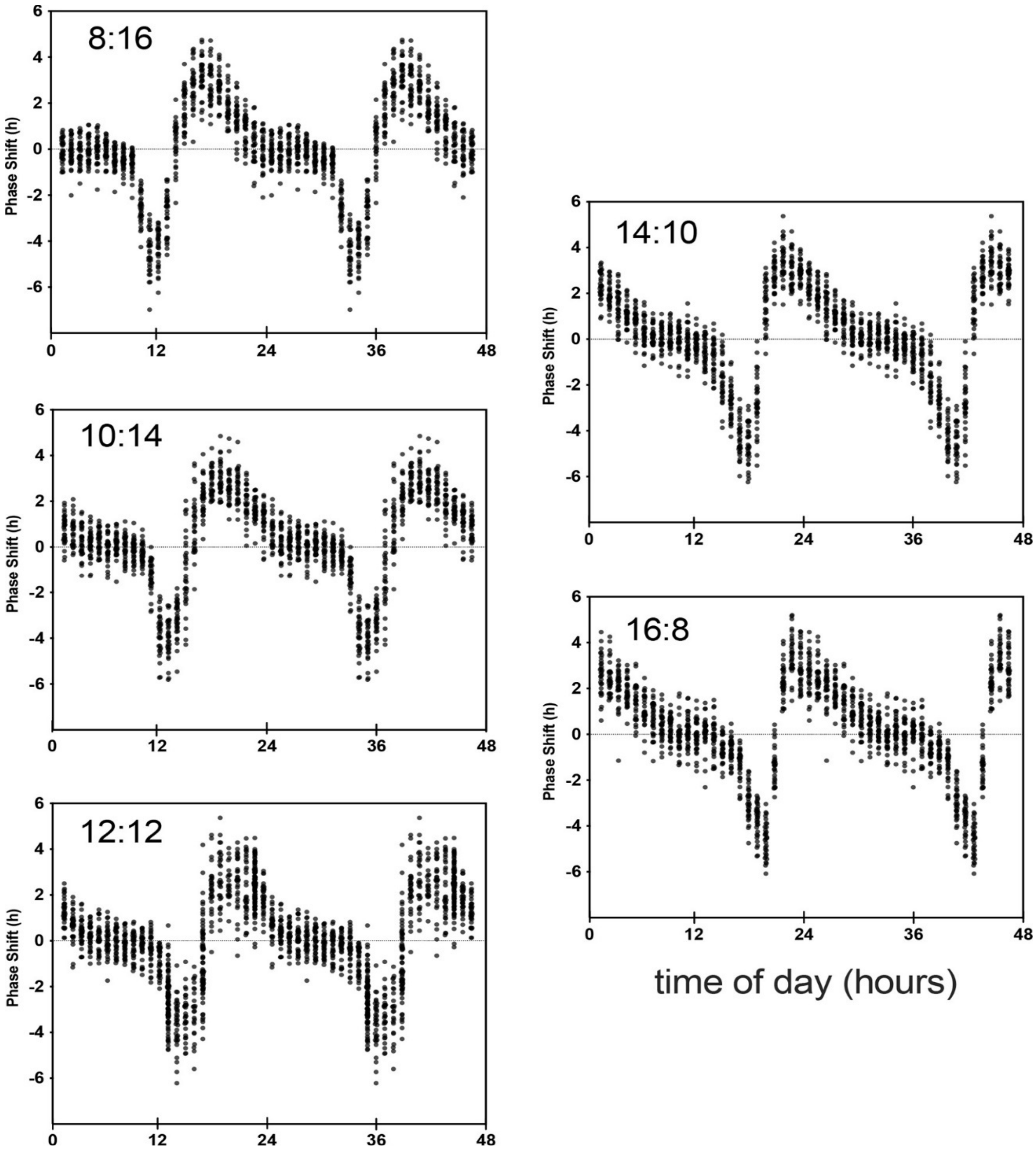


**Figure 2**. Phase-shifting responses across the seasons: Raw data. Phase shifts of locomotor activity (h) are quantified for individual flies (n = 3,934) stimulated with a 15-min pulse (white fluorescent light, 887.7 lx) at one of the hourly intervals associated with the 24-h cycle starting after the last day of entrainment to each LD schedule (post-pulse, animals were maintained in DD). Data collected from animals housed under each photoperiod are independently graphed and labeled from top-to-bottom. The phase shift observed in each fly's activity rhythm (1 black circle = 1 animal) is shown in scatterplot with respect to the timing of the pulse. X-axes start at ZT0, which reflects the timing of lights-on (dawn) in the previous LD schedule. Data are double-plotted for ease of visualization.

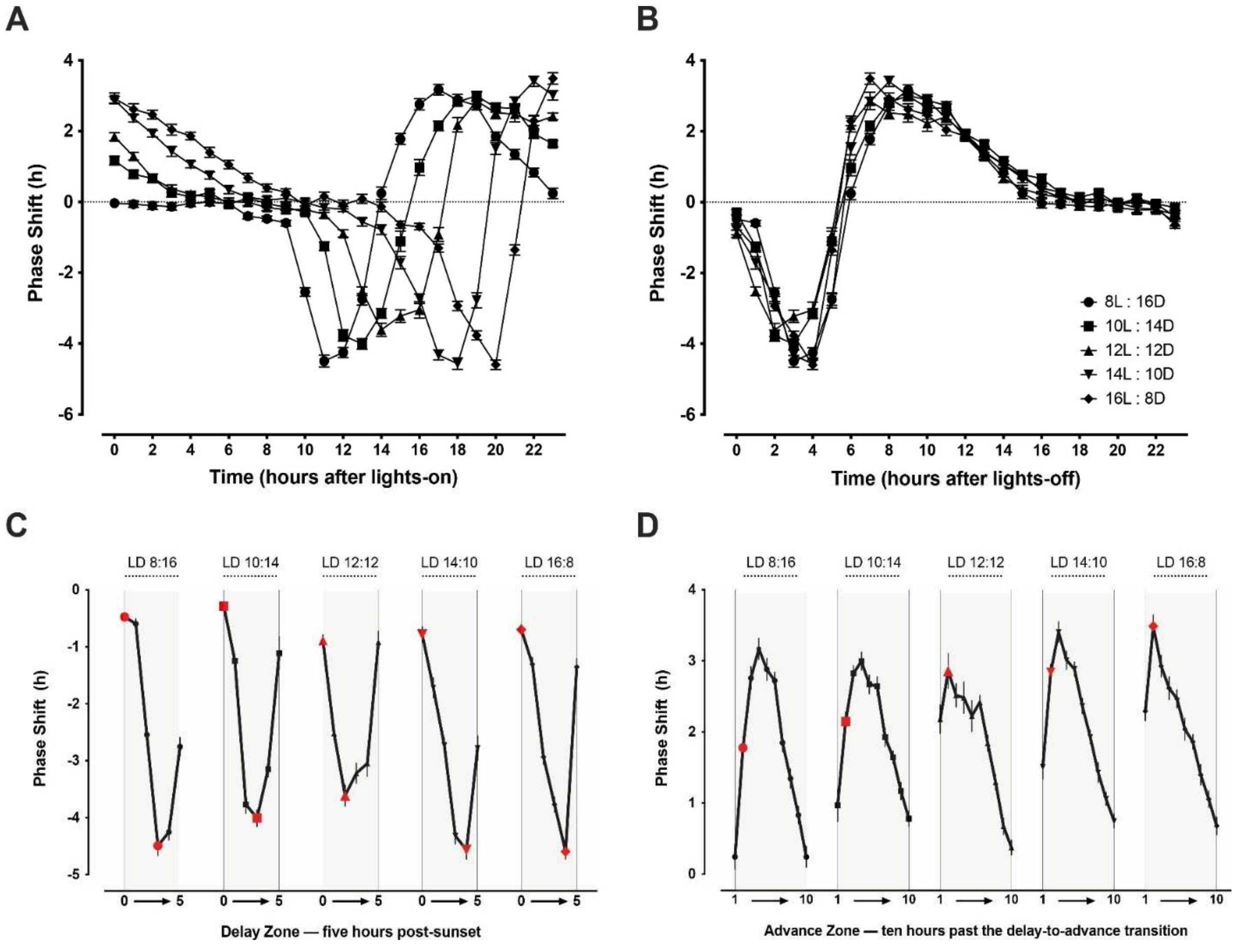


**Figure 3**. Phase-shifting responses across the seasons: Averaged data. (**A-B**) The PRCs generated after entrainment to each photoperiod are co-plotted with respect to the timing of either lights-on (subjective sunrise, **A**) or lights-off (subjective sunset, **B**) in the previous LD schedule. Independent of photoperiod, PRCs are anchored to the timing of lights-off. (**C-D**) The parts of the PRC corresponding to the delay (**C**) and advance (**D**) zones are isolated for each photoperiod (labeled left-to-right) to visualize similarities in the trajectories of circadian photoresponsivity occurring along each curve. Red points highlight hourly stretches where these trajectories were slightly, but statistically different. Inflections of photoresponsivity (moving from the amplitude of the delay → advance zone) were sharper for longer photoperiods with shorter nights.

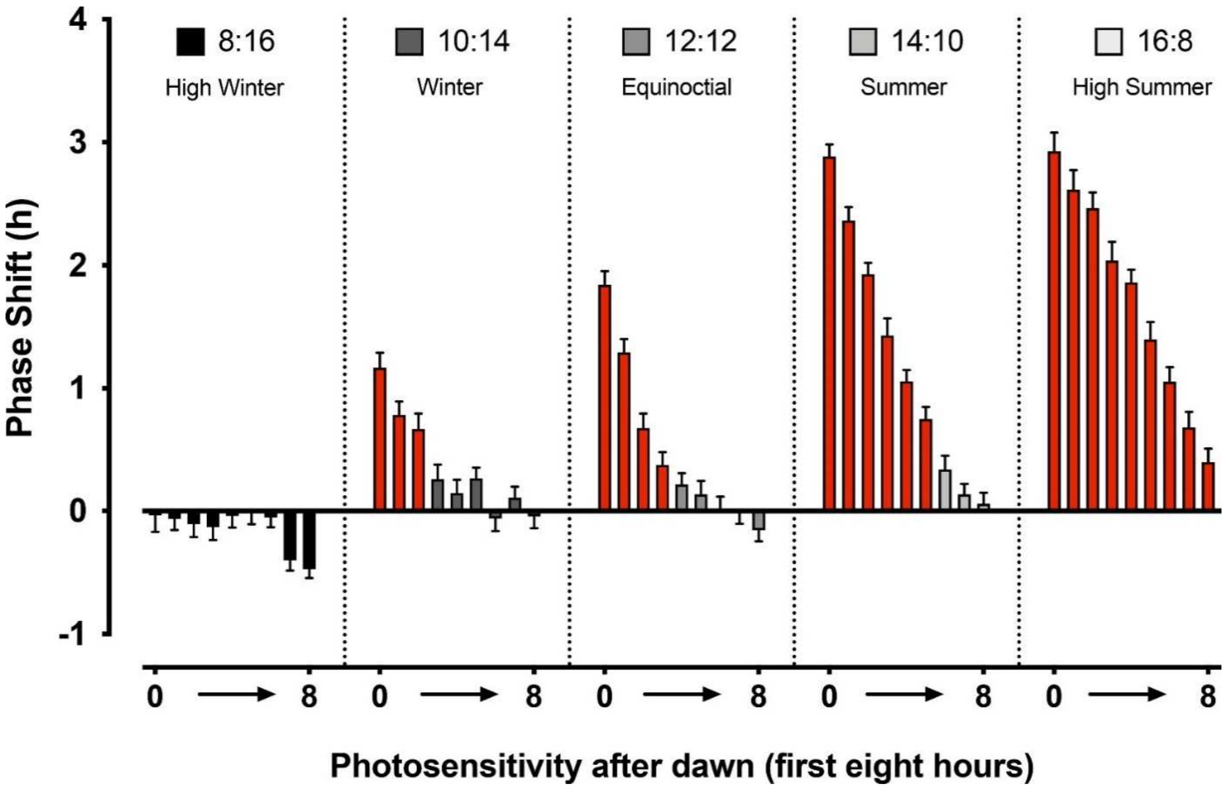


**Figure 4**. Phase-shifting responses across the seasons: Focus on the morning hours. The average phase-shift achieved with a 15-min pulse was replotted for each seasonal photoperiod (labeled left-to-right) and referenced to the hourly intervals of the circadian cycle coinciding with the first 8 hours of the morning/afternoon after lights-on (dawn). Red bars indicate times where light exposure produced a net phase-advance greater than zero (one-sample *t*-test, $p < 0.05$). Overlap of circadian photoresponsivity with the morning/afternoon hours was maximized in summer photoperiods and minimized (or effectively absent) in winter photoperiods.

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# Appendix B

**Supplemental Materials for:** The Drosophila Circadian Phase Response Curve to Light: Conservation across Seasonally Relevant Photoperiods and Anchorage to Sunset

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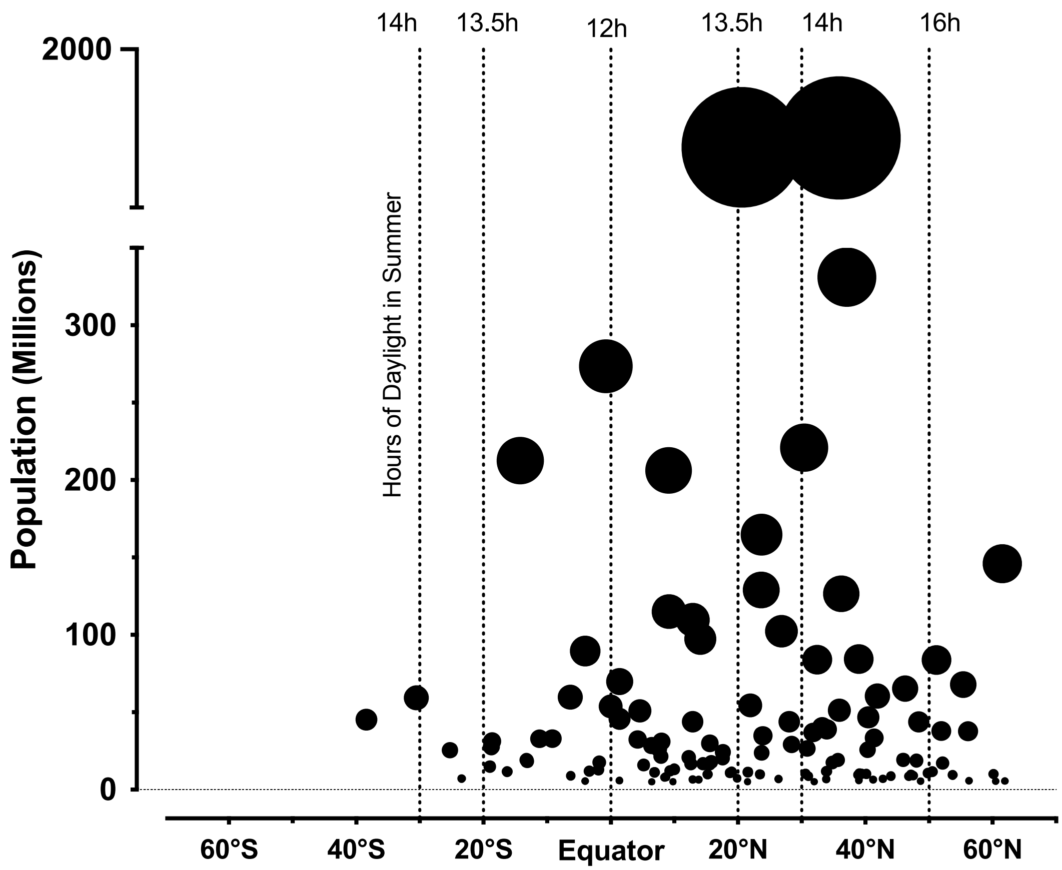
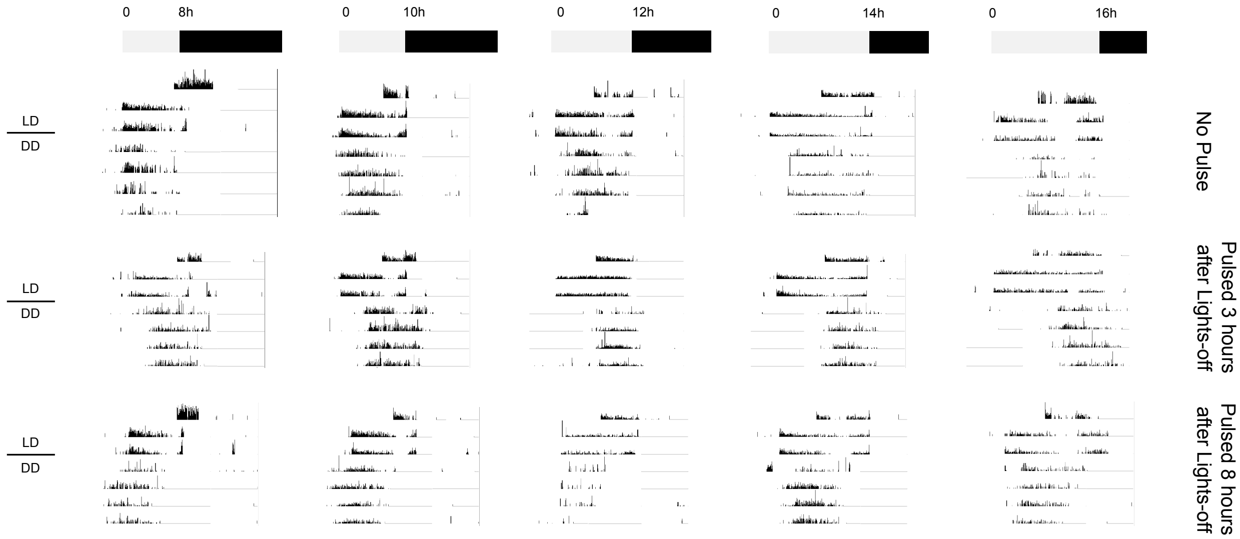
**Supplementary Figure 1.** Population vs. Latitude: Countries in the world are plotted as individual points




with respect to their central latitude (°N/S) and population (in the millions). The size of each point is scaled to population level (referenced from United Nations Population Division). Dotted lines intersecting each latitude mark the seasonal swings in daylight associated with countries located at each deviation from the Earth's Equator. The number of daylight hours during summer (and the inverse in winter) increases moving away from this midline. Most of the human population lives between 20-50° N, where up to 8-hour swings in the photoperiod occur every 6 months.




**Supplementary Figure 2.** Representative *Drosophila* actograms: Representative actograms taken from flies receiving either no light pulse before entry into constant darkness (DD control) (upper panels), or 15-min light pulse 3 hours (delay zone; middle panels) or 8 hours (advance zone; bottom panels) after the final transition of the previous light-dark (LD) cycle. Grey and black bars seated at the top of the figure show the timing of the previous LD schedule (ZT0, lights-on at 07.00). Post pulse, animals were left to free-run in DD for 4 days. Within each actogram, black bars indicate raw 30-s epochs where the animals registered breaks in the activity-tracking beams, smoothed over each 3-minute interval of recording. Data are vertically aligned, such that one 24-h day of movement is shown per line, with successive days appearing one below the other. To help visualize shifts in locomotor rhythms produced by the light pulse or by removing the light regime, example actograms are organized in a temporally aligned column, with a dotted line marking the hours just before dawn. The phase relationship between activity onset and the timing of dawn in the previous LD schedule is increasingly delayed as a function of daylength when the animals enter DD without any further light exposure. Relative to this phase-positioning, the magnitude of delays and advances elicited by a 15-min pulse is similar.

**Supplementary Table 1**. Seasonal PRCs to light: Summary of phase-shifting data in a high winter-like photoschedule. Response after a 15-min broadspectrum pulse.

| Zeitgeber Time | Photoperiod (Composition of Light-Dark Cycle) | Δ Phase Shift, h (*n*) |
|---|---|---|
| Lights-on 0 | 8 hours of light • 16 hours of darkness | -0.04 ± 0.13 (32) |
| 1 | 8 hours of light • 16 hours of darkness | -0.06 ± 0.09 (32) |
| 2 | 8 hours of light • 16 hours of darkness | -0.11 ± 0.11 (32) |
| 3 | 8 hours of light • 16 hours of darkness | -0.13 ± 0.11 (31) |
| 4 | 8 hours of light • 16 hours of darkness | -0.04 ± 0.10 (32) |
| 5 | 8 hours of light • 16 hours of darkness | -0.00 ± 0.10 (32) |
| 6 | 8 hours of light • 16 hours of darkness | -0.05 ± 0.08 (32) |
| 7 | 8 hours of light • 16 hours of darkness | -0.40 ± 0.08 (32) |
| 8 Lights-off | 8 hours of light • 16 hours of darkness | -0.47 ± 0.07 (32) |
| 9 | 8 hours of light • 16 hours of darkness | -0.60 ± 0.09 (32) |
| 10 | 8 hours of light • 16 hours of darkness | -2.54 ± 0.12 (32) |
| 11 | 8 hours of light • 16 hours of darkness | -4.49 ± 0.17 (31) |
| 12 | 8 hours of light • 16 hours of darkness | -4.25 ± 0.14 (32) |
| 13 | 8 hours of light • 16 hours of darkness | -2.75 ± 0.16 (32) |
| 14 | 8 hours of light • 16 hours of darkness | 0.24 ± 0.18 (30) |
| 15 | 8 hours of light • 16 hours of darkness | 1.78 ± 0.16 (31) |
| 16 | 8 hours of light • 16 hours of darkness | 2.76 ± 0.16 (32) |
| 17 | 8 hours of light • 16 hours of darkness | 3.17 ± 0.15 (32) |
| 18 | 8 hours of light • 16 hours of darkness | 2.88 ± 0.15 (32) |
| 19 | 8 hours of light • 16 hours of darkness | 2.72 ± 0.12 (31) |
| 20 | 8 hours of light • 16 hours of darkness | 1.85 ± 0.12 (32) |
| 21 | 8 hours of light • 16 hours of darkness | 1.34 ± 0.14 (32) |
| 22 | 8 hours of light • 16 hours of darkness | 0.83 ± 0.12 (32) |
| 23 | 8 hours of light • 16 hours of darkness | 0.24 ± 0.15 (31) |

**Supplementary Table 2**. Seasonal PRCs to light: Summary of phase-shifting data in a winter-like photoschedule. Response after a 15-min broadspectrum pulse.

| Zeitgeber Time | Photoperiod (Composition of Light-Dark Cycle) | Δ Phase Shift, h (*n*) |
|---|---|---|
| Lights-on 0 | 10 hours of light • 14 hours of darkness | 1.17 ± 0.12 (32) |
| 1 | 10 hours of light • 14 hours of darkness | 0.78 ± 0.11 (32) |
| 2 | 10 hours of light • 14 hours of darkness | 0.67 ± 0.13 (26) |
| 3 | 10 hours of light • 14 hours of darkness | 0.26 ± 0.12 (32) |
| 4 | 10 hours of light • 14 hours of darkness | 0.15 ± 0.11 (32) |
| 5 | 10 hours of light • 14 hours of darkness | 0.26 ± 0.09 (32) |
| 6 | 10 hours of light • 14 hours of darkness | -0.06 ± 0.10 (32) |
| 7 | 10 hours of light • 14 hours of darkness | 0.11 ± 0.09 (32) |
| 8 | 10 hours of light • 14 hours of darkness | -0.04 ± 0.10 (32) |
| 9 | 10 hours of light • 14 hours of darkness | -0.15 ± 0.09 (32) |
| 10 Lights-off | 10 hours of light • 14 hours of darkness | -0.29 ± 0.08 (32) |
| 11 | 10 hours of light • 14 hours of darkness | -1.25 ± 0.12 (32) |
| 12 | 10 hours of light • 14 hours of darkness | -3.77 ± 0.16 (32) |
| 13 | 10 hours of light • 14 hours of darkness | -4.01 ± 0.15 (32) |
| 14 | 10 hours of light • 14 hours of darkness | -3.15 ± 0.14 (32) |
| 15 | 10 hours of light • 14 hours of darkness | -1.11 ± 0.29 (32) |
| 16 | 10 hours of light • 14 hours of darkness | 0.97 ± 0.22 (31) |
| 17 | 10 hours of light • 14 hours of darkness | 2.14 ± 0.14 (31) |
| 18 | 10 hours of light • 14 hours of darkness | 2.82 ± 0.11 (31) |
| 19 | 10 hours of light • 14 hours of darkness | 2.99 ± 0.13 (31) |
| 20 | 10 hours of light • 14 hours of darkness | 2.67 ± 0.13 (30) |
| 21 | 10 hours of light • 14 hours of darkness | 2.64 ± 0.13 (29) |
| 22 | 10 hours of light • 14 hours of darkness | 1.93 ± 0.13 (32) |
| 23 | 10 hours of light • 14 hours of darkness | 1.64 ± 0.09 (32) |

**Supplementary Table 3**. Seasonal PRCs to light: Summary of phase-shifting data within an equinoctial photoschedule. Response after a 15-min broadspectrum pulse.

| Zeitgeber Time | Photoperiod (Composition of Light-Dark Cycle) | Δ Phase Shift, h (*n*) |
|---|---|---|
| Lights-on 0 | 12 hours of light • 12 hours of darkness | 1.84 ± 0.11 (32) |
| 1 | 12 hours of light • 12 hours of darkness | 1.29 ± 0.11 (32) |
| 2 | 12 hours of light • 12 hours of darkness | 0.67 ± 0.12 (32) |
| 3 | 12 hours of light • 12 hours of darkness | 0.37 ± 0.10 (32) |
| 4 | 12 hours of light • 12 hours of darkness | 0.22 ± 0.09 (32) |
| 5 | 12 hours of light • 12 hours of darkness | 0.14 ± 0.11 (32) |
| 6 | 12 hours of light • 12 hours of darkness | 0.01 ± 0.11 (32) |
| 7 | 12 hours of light • 12 hours of darkness | -0.01 ± 0.10 (32) |
| 8 | 12 hours of light • 12 hours of darkness | -0.16 ± 0.09 (32) |
| 9 | 12 hours of light • 12 hours of darkness | -0.22 ± 0.10 (32) |
| 10 | 12 hours of light • 12 hours of darkness | -0.22 ± 0.10 (32) |
| 11 | 12 hours of light • 12 hours of darkness | -0.34 ± 0.12 (32) |
| 12 Lights-off | 12 hours of light • 12 hours of darkness | -0.89 ± 0.10 (32) |
| 13 | 12 hours of light • 12 hours of darkness | -2.52 ± 0.12 (85) |
| 14 | 12 hours of light • 12 hours of darkness | -3.61 ± 0.19 (33) |
| 15 | 12 hours of light • 12 hours of darkness | -3.22 ± 0.18 (30) |
| 16 | 12 hours of light • 12 hours of darkness | -3.05 ± 0.23 (25) |
| 17 | 12 hours of light • 12 hours of darkness | -0.92 ± 0.19 (70) |
| 18 | 12 hours of light • 12 hours of darkness | 2.18 ± 0.20 (30) |
| 19 | 12 hours of light • 12 hours of darkness | 2.86 ± 0.24 (30) |
| 20 | 12 hours of light • 12 hours of darkness | 2.52 ± 0.16 (22) |
| 21 | 12 hours of light • 12 hours of darkness | 2.48 ± 0.22 (30) |
| 22 | 12 hours of light • 12 hours of darkness | 2.22 ± 0.22 (33) |
| 23 | 12 hours of light • 12 hours of darkness | 2.42 ± 0.10 (92) |

**Supplementary Table 4**. Seasonal PRCs to light: Summary of phase-shifting data in a summer-like photoschedule. Response after a 15-min broadspectrum pulse.

| Zeitgeber Time | Photoperiod (Composition of Light-Dark Cycle) | Δ Phase Shift, h (*n*) |
|---|---|---|
| Lights-on 0 | 14 hours of light • 10 hours of darkness | 2.88 ± 0.10 (32) |
| 1 | 14 hours of light • 10 hours of darkness | 2.36 ± 0.11 (32) |
| 2 | 14 hours of light • 10 hours of darkness | 1.93 ± 0.09 (32) |
| 3 | 14 hours of light • 10 hours of darkness | 1.43 ± 0.14 (32) |
| 4 | 14 hours of light • 10 hours of darkness | 1.06 ± 0.09 (32) |
| 5 | 14 hours of light • 10 hours of darkness | 0.75 ± 0.10 (32) |
| 6 | 14 hours of light • 10 hours of darkness | 0.34 ± 0.11 (32) |
| 7 | 14 hours of light • 10 hours of darkness | 0.14 ± 0.08 (32) |
| 8 | 14 hours of light • 10 hours of darkness | 0.06 ± 0.09 (31) |
| 9 | 14 hours of light • 10 hours of darkness | 0.10 ± 0.09 (32) |
| 10 | 14 hours of light • 10 hours of darkness | -0.02 ± 0.10 (32) |
| 11 | 14 hours of light • 10 hours of darkness | -0.17 ± 0.12 (32) |
| 12 | 14 hours of light • 10 hours of darkness | -0.20 ± 0.08 (32) |
| 13 | 14 hours of light • 10 hours of darkness | -0.56 ± 0.11 (32) |
| 14 Lights-off | 14 hours of light • 10 hours of darkness | -0.78 ± 0.14 (31) |
| 15 | 14 hours of light • 10 hours of darkness | -1.72 ± 0.17 (32) |
| 16 | 14 hours of light • 10 hours of darkness | -2.73 ± 0.14 (32) |
| 17 | 14 hours of light • 10 hours of darkness | -4.32 ± 0.14 (31) |
| 18 | 14 hours of light • 10 hours of darkness | -4.57 ± 0.16 (31) |
| 19 | 14 hours of light • 10 hours of darkness | -2.78 ± 0.22 (32) |
| 20 | 14 hours of light • 10 hours of darkness | 1.51 ± 0.17 (32) |
| 21 | 14 hours of light • 10 hours of darkness | 2.84 ± 0.11 (32) |
| 22 | 14 hours of light • 10 hours of darkness | 3.40 ± 0.14 (31) |
| 23 | 14 hours of light • 10 hours of darkness | 3.01 ± 0.13 (32) |

**Supplementary Table 5**. Seasonal PRCs to light: Summary of phase-shifting data in a high summer-like photoschedule. Response after a 15-min broadspectrum pulse.

| Zeitgeber Time | Photoperiod (Composition of Light-Dark Cycle) | Δ Phase Shift, h (*n*) |
|---|---|---|
| Lights-on 0 | 16 hours of light • 8 hours of darkness | 2.93 ± 0.15 (32) |
| 1 | 16 hours of light • 8 hours of darkness | 2.62 ± 0.16 (31) |
| 2 | 16 hours of light • 8 hours of darkness | 2.47 ± 0.13 (32) |
| 3 | 16 hours of light • 8 hours of darkness | 2.04 ± 0.15 (31) |
| 4 | 16 hours of light • 8 hours of darkness | 1.86 ± 0.10 (32) |
| 5 | 16 hours of light • 8 hours of darkness | 1.40 ± 0.14 (30) |
| 6 | 16 hours of light • 8 hours of darkness | 1.06 ± 0.12 (32) |
| 7 | 16 hours of light • 8 hours of darkness | 0.68 ± 0.13 (32) |
| 8 | 16 hours of light • 8 hours of darkness | 0.40 ± 0.11 (32) |
| 9 | 16 hours of light • 8 hours of darkness | 0.28 ± 0.09 (32) |
| 10 | 16 hours of light • 8 hours of darkness | -0.10 ± 0.13 (32) |
| 11 | 16 hours of light • 8 hours of darkness | 0.16 ± 0.12 (32) |
| 12 | 16 hours of light • 8 hours of darkness | -0.08 ± 0.13 (32) |
| 13 | 16 hours of light • 8 hours of darkness | 0.09 ± 0.13 (32) |
| 14 | 16 hours of light • 8 hours of darkness | -0.14 ± 0.14 (32) |
| 15 | 16 hours of light • 8 hours of darkness | -0.64 ± 0.10 (31) |
| 16 Lights-off | 16 hours of light • 8 hours of darkness | -0.70 ± 0.09 (32) |
| 17 | 16 hours of light • 8 hours of darkness | -1.31 ± 0.12 (32) |
| 18 | 16 hours of light • 8 hours of darkness | -2.93 ± 0.13 (32) |
| 19 | 16 hours of light • 8 hours of darkness | -3.77 ± 0.13 (32) |
| 20 | 16 hours of light • 8 hours of darkness | -4.60 ± 0.13 (32) |
| 21 | 16 hours of light • 8 hours of darkness | -1.35 ± 0.15 (30) |
| 22 | 16 hours of light • 8 hours of darkness | 2.29 ± 0.14 (32) |
| 23 | 16 hours of light • 8 hours of darkness | 3.49 ± 0.16 (32) |

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