Circadian rhythm dependent inter-organ crosstalk in *Drosophila melanogaster* haematopoiesis

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A dissertation submitted for the partial fulfillment of BS-MS dual degree in Science

Department of Biological Sciences

Indian Institute of Science Education and Research Mohali

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Dedicated to my family

Certificate of Examination

This is to certify that the dissertation titled "Circadian Rhythm dependent inter-organ crosstalk in *Drosophila melanogaster* hematopoiesis" submitted by Vaishnavi Dandavate (Reg. No. MS14081) for the partial fulfillment of B.S.-M.S. dual degree programme of the Institute, has been examined by the thesis committee duly appointed by Institute. The committee finds the work done by the candidate satisfactory and recommends that the report be accepted.

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Dated: April 26, 2019

Declaration

The work presented in this dissertation has been carried out by me under the supervision of Dr. Lolitika Mandal at the Indian Institute of Science Education and Research Mohali. This work has not been submitted in part or in full for a degree, a diploma, or a fellowship to any other university or institution. Whenever contributions of others are involved, every effort is made to indicate this clearly, with due acknowledgment of collaborative research and discussions. This thesis is a bonafide record of original work done by me and all sources listed within have been detailed in the bibliography.

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In my capacity as a supervisor of the candidate's project work, I certify that the above statements by the candidate are true to the best of my knowledge.

Dr. Lolitika Mandal

(Supervisor)

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Vaishnavi Dandavate.

“I wish it need not have happened in my time," said Frodo.
"So do I," said Gandalf, "and so do all who live to see such times. But that is not for them to decide. All we have to decide is what to do with the time that is given us.”
— J.R.R. Tolkien, The Fellowship of the Ring

Abstract

Circadian rhythm orchestrates a wide array of biological processes to period in a 24-hour rhythm, prioritizing the body functions according to the time of the day. These predictions maximize survival resulting in increased fitness of such organisms. Circadian rhythm influences several physiological processes such as the sleep-wake cycle, metabolism, immunological responses, hormonal release, body cell regeneration, ultimately regulating most of the physical, mental and behavioral changes in the body. Dysregulation of this process creates havoc and is linked to several disorders that include sleep disorders, depression, bipolar disorders, seasonal defective disorders, obesity, and diabetes. Thus, it is pertinent to understand the effects of body clocks on the regulation of various processes in the body. This study focuses on deciphering the molecular pathway that correlates circadian rhythm with hematopoiesis using *Drosophila melanogaster* as a model system. The primary organ of interest was chosen to be lymph gland, the larval hematopoietic organ in *Drosophila*. Light is a potent zeitgeber and is used as a tool to be the creator of imbalance to a perfectly regulated circadian rhythmicity. Such a turmoil in circadian rhythm in *Drosophila* larvae resulted in an elevation in the number of differentiated blood cells in lymph glands, even when the size of lymph glands remained the same. This elevation of the differentiation index is caused by the distressed levels of ecdysone signaling in the lymph gland. We have been able to chart out a link that connects the sink in ecdysone levels in animals entrained towards altered light regiment resulting in an increase in the differentiated index of lymph gland. The light is perceived and integrated into the homeostasis of *Drosophila* larvae via neurohormone Pigment-Dispersing Factor (PDF), is shown to be photoperiodic. PDF is upstream is of Prothoracicotropic hormone (PTTH), which then modulates ecdysone synthesis. Current study puts forth a linear molecular pathway linking circadian rhythm to hematopoiesis in *Drosophila melanogaster*.

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CHAPTER I

INTRODUCTION

Circadian Rhythm

The earth rotates around its axis once every 24 hours. It causes predictable changes in the light and temperature conditions throughout the day. Over an evolutionary timescale, an internal clock has been integrated within organisms ranging from bacteria to human by natural selection [1] due to its ability to anticipate future needs has provided a clear adaptative advantage. For example, the ability to predict daily environmental changes, for instance: increased energy demand or stress [2], allowing them to alter their behavior according to the conditions of the day [Figure 1]. Some species adapt to other environmental signals; like some species of crustaceans and fishes are tailored to the circatidal rhythms of 12 hours of flood and ebbing [3]. Such actions maximize survival and ultimately increase their fitness. This endogenous circuitry is called the circadian rhythm (from the Latin words 'circa diem' meaning about a day). Thus, organisms kept in constant conditions (constant dark or temperature), they follow a behavior pattern of approximate 24 hours. The average free-running period in mice and humans is 23.7 and 24.3 hours respectively [4]. The clock is adjusted according to the day-night cycle, not just the light-dark cycle. Therefore, changes in the day-night duration, attributable to the revolution of the earth due to tilting of the earth's axis, is also accommodated by such a biological clock. This mechanism accommodates the seasonal changes and acts as a season timekeeper. Although circadian rhythm is a highly self-sustained process, it has a room for integrating intracellular and environmental information into the circuitry. Environmental cues are called the '*zeitgebers*' (known as 'time givers' in German), commonly include daily light, food, or temperature cycles, to regulate various process under the control of the master clock to function according to the actual environmental conditions [5][6]. Circadian rhythms are temperature compensated, making them flexible enough to operate in a wide range of temperatures with a period of ~24 hours. Thus, the luxury of having a pliable yet a self-subsisting biological process provides significant benefits: (i) the temporal separation of chemically incompatible pathways and (ii) the limitation of potentially harmful, but necessary, chemical reactions to the times when they are required.

The first sightings of circadian rhythm observed by French astronomer Jean Jacques d'Ortous de Mairan in 1729, positioned a mimosa plant in the light-dark room which continued to open its leaves in the morning and close at night [7]. Two centuries later, the earliest mutants of the circadian clock discovered in a dipteran species, *Drosophila*

melanogaster. Ron Konopka, one of Seymour Benzer's students in his laboratory, was the first to find *period* gene mutants using the method of forward genetics [8]. In the mid-1980s two groups of Michael Young and Michael Rosbash, independently cloned the *period* gene [9][10] encoding a protein with more than 1,200 amino acids. Several early studies elucidated the localized expression of the *period* gene in a subset of lateral neurons in the brains of adult flies, which was discovered to be obligatory for the maintenance of circadian rhythms [11][12][13]. In mammalian system, the central pacemaker is positioned in the suprachiasmatic nuclei (SCN) [14], two small clusters of neurons set above the optical chiasma in the ventral hypothalamus [Figure 2], wherein the required molecular oscillations function in daily rhythmic cycles. Daily rhythms in nutrient use were first documented almost 40 years ago in cells of SCN [15][16]. SCN functions as the master clock which controls the peripheral clocks [6]. The free-running rhythm in humans slightly exceeds the 24-hour timeframe, but the master clock integrates external conditions to adjust the rhythm to approximately 24 hours. Entrainment is the process in which *zeitgebers* bring on changes in concentrations of the molecular components of clock circuitry based on the current stage of the 24-hour cycle [17], responsible for the synchrony between the clock and environmental conditions. The synchrony is primarily brought about by the photic signals received by the retina, which signals the SCN via the retinohypothalamic tract (RHT) [Figure 3] [18]. This, in turn, activates cAMP response element binding protein (CREB) in the post-synaptic neurons and leads to the expression of the *period* gene. The PERIOD protein then resets the entire clock and thus syncs the endogenous clock with the external conditions [19].

Molecular Clock

The molecular circuitry underlying circadian rhythm is a transcriptional-translational feedback loop. In *Drosophila melanogaster,* the core of the transcriptional feedback loop (TTFL) consists of positive regulators which are Circadian Locomotor Output Cycles Kaput (CLOCK a.k.a CLK) and CYCLE (CYC) proteins, and PERIOD (PER) and TIMELESS (TIM) proteins function as the negative regulators [Figure 4]. Several models were proposed to explain the functioning of *period* gene. Unfortunately, none of them could give a satisfactory explanation, until a breakthrough made by Roshash and Hall's groups demonstrating that *per* cycles in a negative autoregulatory feedback loop and the levels of period mRNA peak occurr early in the night, several hours earlier than summit in PER protein copiousness [20]. After the discovery of the *period* gene, in 1990s Michael Young’s

group found out a second clock gene *timeless* [21]. Rhythmicity similar to the ~24-hour cycle of PER seen in TIM, and these two were shown to physically interact forming a heterodimer, an association which affected the nuclear localization and PER degradation [22]. PER and TIM accumulate in the cytoplasm during the day, heterodimerize, and localize in the nucleus at night, during a specific time-window of circadian rhythm [23][24]. PER/TIM heterodimer in the nucleus promotes phosphorylation of CLK/CYC complex, thus inhibiting the activity of this complex and suppressing its affinity to DNA [25]. During daytime, PER/TIM complex undergoes phosphorylation and eventual degradation in the nucleus at late night. This degradation of PER/TIM heterodimer releases CLK/CYC, which starts a new cycle of TTFL [26]. Disruption of the heterodimer of negative regulators owed to CRYPTOCHROME (CRY). CRY is a blue-pigment photoreceptor which undergoes conformational changes upon photon absorption, allowing CRY binding to TIM [27]–[29]. This conformational change causes ubiquitylation of TIM and then proteasomal degradation, therefore resetting the endogenous clock [30]–[33]. Recent genetic analysis of circadian rhythms in flies and mammals have elucidated substantial conservation in the molecules as well as the pathways involved in the same, which opens up new avenues for using fly as a facile model in understanding the complexities of the mammalian biological clock [34]. The timing of emergence of flies from the pupae (eclosion) synced with the onset of dawn, with colder temperatures and moist conditions. Pupae entrained to 24-hour dark also eclose at subjective dawn (expected dawn), this shows that an endogenous clock regulates the eclosion timings [35]. The adult flies restrict their activities such as flight, foraging, mating to the day time, while the rest homeostasis or 'sleep' when the flies exhibit the minimal response to stimuli, is restricted to night-time. Such behavioral traits add on to their survival value [36]. Along with profound conservation with the mammalian system, flies display an array of specific physiology and behavior restricted to particular timings of the day. This along with several benefits of the model itself makes *Drosophila melanogaster* an ideal model system to work with to study circadian rhythm.

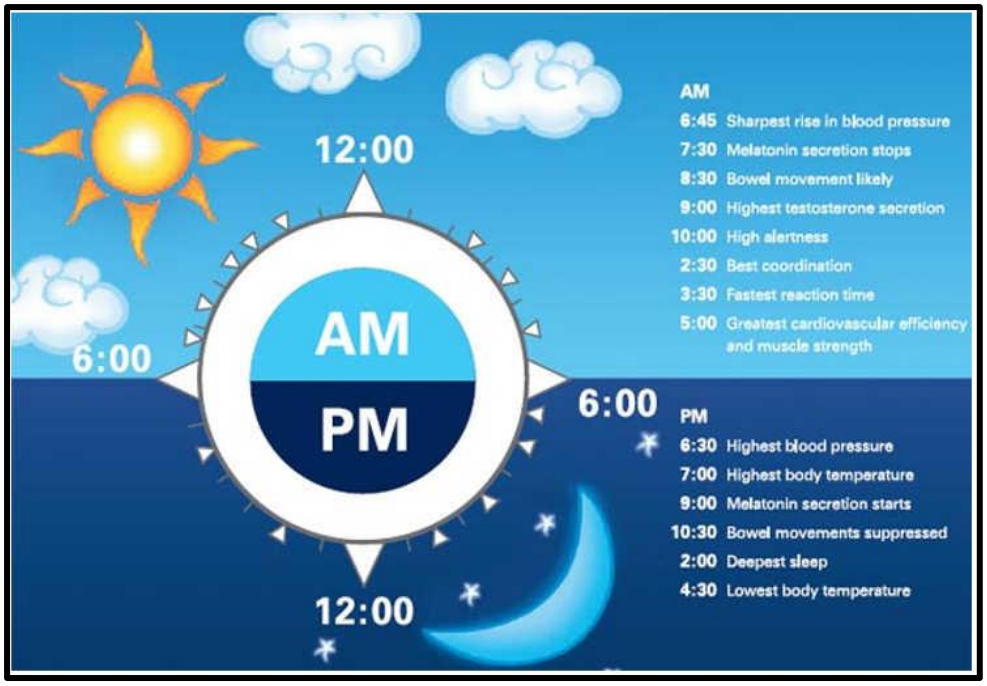


Figure 1: Daily rhythms in humans

A schematic depiction of different body function variation throughout the day, a 24-hour cycle. (https://themedcircle.com/circadian-rhythm-taking-medicines/)

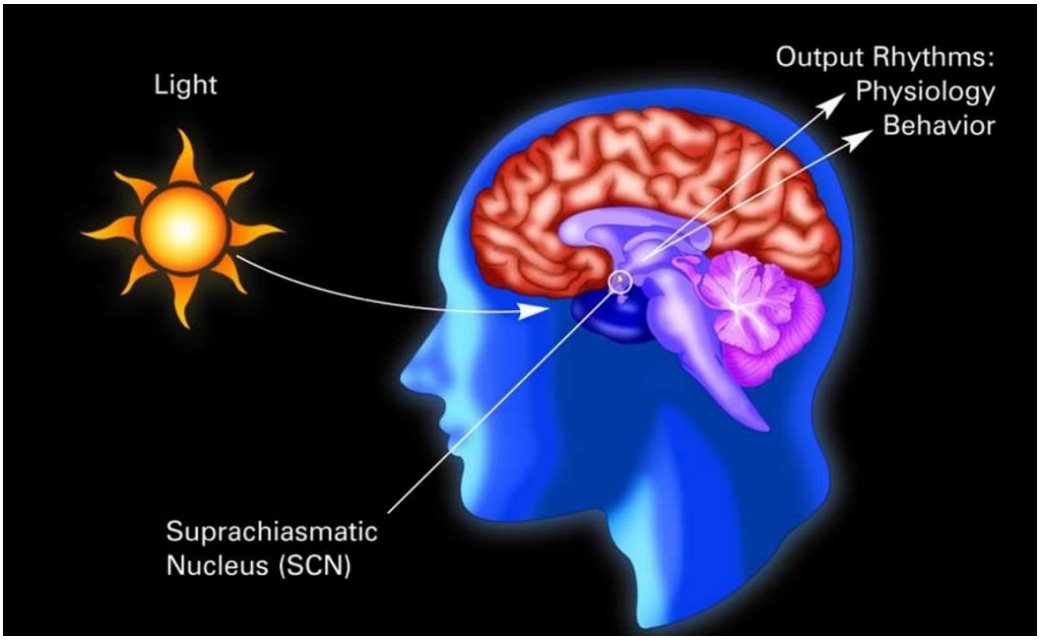


Figure 2: Suprachiasmatic nucleus in *Homo sapiens*

A person's internal body clock lies in the brain's hypothalamus and is called the suprachiasmatic nucleus (SCN). Credits: National Institute of General Medical Sciences (https://www.livescience.com/13123-circadian-rhythms-obesity-diabetes-nih.html)

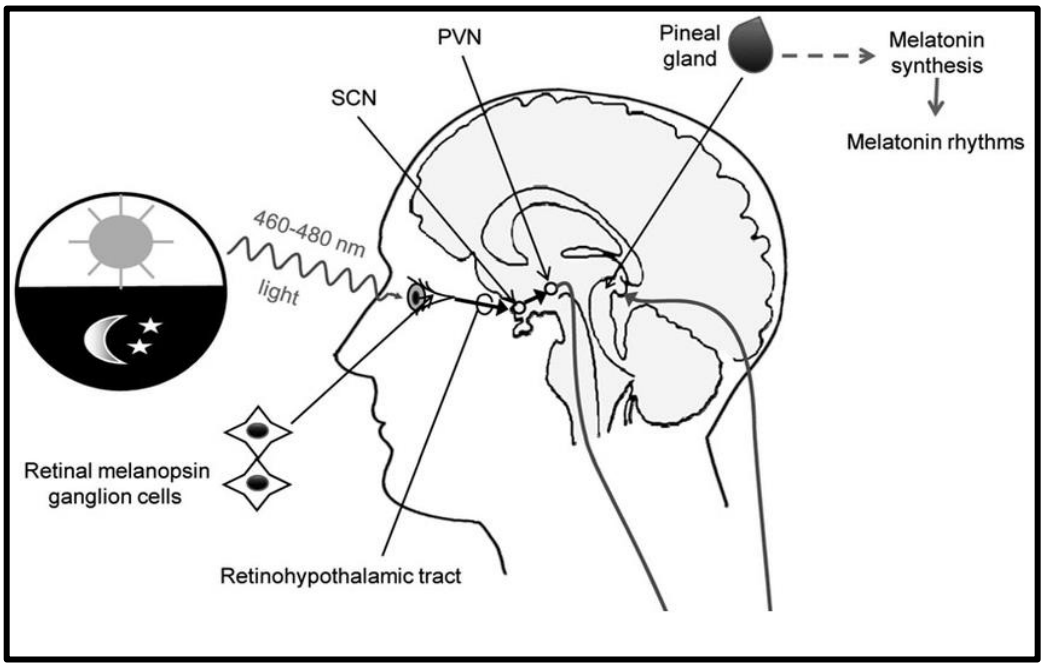


Figure 3: Retinohypothalamic Tract

A diagrammatic representation of the neural pathway connecting the retina and SCN in mammalian brain (Annals of Medicine 44(6):564-77, June 2011, DOI: 10.3109/07853890.2011.586365)

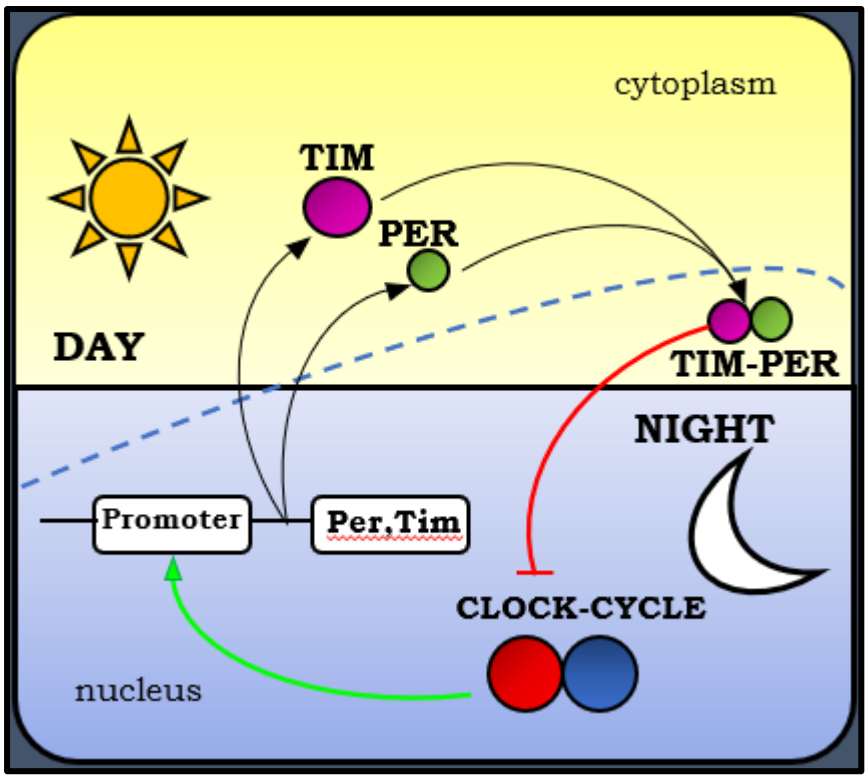


Figure 4: Molecular mechanism of Circadian rhythm

The cycling of the core clock components is presented according to the time of the day.

Drosophila melanogaster: A Genetic model

Drosophila melanogaster has been established as a stalwart model organism for more than a century now, to study an array of phenomena differing from development, neurodegenerative disorders, human genetic diseases to behavioral assays. *Drosophila melanogaster*, commonly known as the fruit-fly, belongs to the order Diptera, family Drosophilidae. At Harvard, William Ernest Castle (1867- 1962) was the first to establish Drosophila melanogaster as a genetic model on the bench. After the re-emergence of Mendelian inheritance, in 1910, the discoveries of Thomas Hunt Morgan (the father of genetics) and his colleagues, at Columbia University, elucidated several principles of heredity, using *Drosophila melanogaster* as a model system which included sex-linked inheritance, epistasis, multiple alleles, and gene mapping[37].

The genetic composition of *Drosophila melanogaster* is crisp and compact is comprising of merely four pairs of chromosomes. Its entire genome has been sequenced and mapped out, published in 2000 [38] — the information of all the genes found of the database FlyBase. After sequencing the human genome in 2000 as a part of the Human Genome Project, it has concluded that there are 60 percent of genes conserved between humans and fruit flies [39]. Further BLAST analysis of human diseases in Online Mendelian Inheritance in Man (OMIM) database with the *Drosophila* genome, revealed nearly 77 percent of human diseases having conserved or similar sequences in the flies [40]. Therefore, it is safe to say that *Drosophila melanogaster* provides an excellent model system for *in vivo* studies to explore, visualize and model any biological process involved in the development and functioning of higher order organisms.

Drosophila makes an easy and inexpensive model system to culture, with minimal space requirement. Their life cycle spans only a short duration of 10 days at optimal temperatures of 25C, leaving room for around growth of around 30 generations per year. They have incredibly high fecundity rate, can peak up to 100 eggs per day [41]. The advent of several genetic tools in the second half of the twentieth century (Gal4-UAS system regulating expression of genes spatiotemporally, site-directed mutagenesis for creating transgenes, and generation of somatic clones), sequencing of the entire genome, further increases the benefits of using *Drosophila* and making it a beguiling model for studying most of the biological processes [42][43].

Life Cycle of *Drosophila melanogaster*

Drosophila melanogaster is a holometabolous insect and thus its life cycle consists of four stages of development: egg, larvae, pupae, and adult [Figure 5]. The entire process of *Drosophila melanogaster* life cycle from fertilization to eclosion of fly takes about ten days at 25°C.

The life cycle starts with the fertilized egg which takes about twenty-four hours for hatching after traveling through 16 different stages [44]. Then the egg slides into the next chapter of its journey, which is the larval stage. The larval stage is divided into three distinct phases, namely, first instar, second instar, and third instar. The first instar takes about a day to proceed to the second instar larval stage. The second instar larvae last about only a day. The third instar is the longest stage of all larval stages spread across three consecutive days [45]. During these stages, the larvae show voracious feeding resulting in growth in the body size. At the culmination the third instar stage, larvae migrate to a dry area where they curb their movements, take on a sedentary fate and prepare themselves to undergo pupariation. The larvae evert their spiracles, secrete glue proteins, necessary for the formation of a hard, dark-colored cuticle for pupal casing which is sustained during the stage of metamorphosis. The pupal stage is the most dynamic stage where the entire metamorphosis takes place causing a complete remodeling of the body. There is a significant amount of programmed cell-death going on during this stage along with the histolysis of larval organs [46][47]. Simultaneously, over five days, newer body structures are also being created from imaginal discs that will be necessary for the survival in the adult stage. The final hurdle to cross during development is the eclosion of adult flies from the pupae. The flies have an average lifespan of about forty-five days.

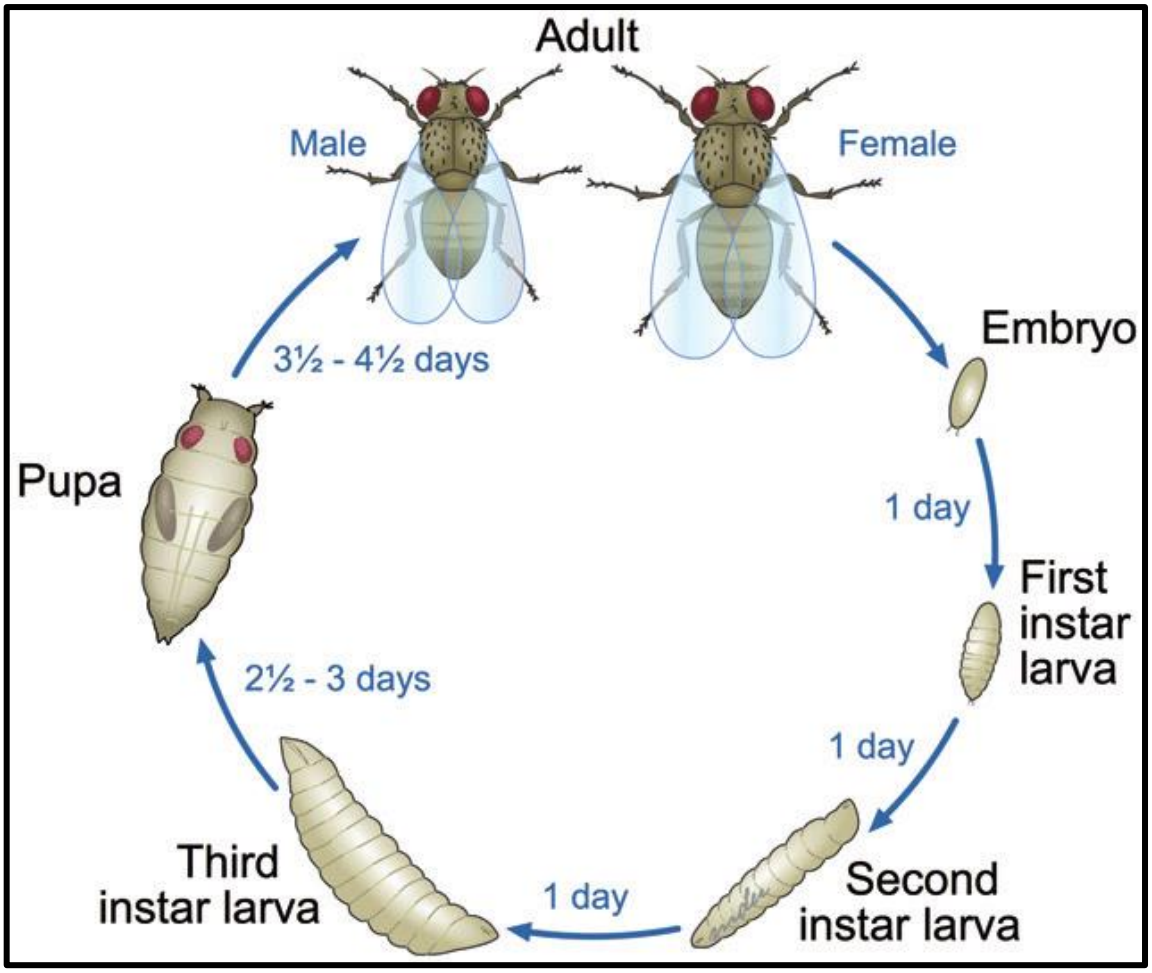


Figure 5: Life Cycle of *Drosophila melanogaster*

Schematic representation of male and female fly and different stages of the life cycle of *Drosophila melanogaster* [48].

Gal4-UAS System

The Gal4-UAS system is one of the two most famous genetic tools used in *Drosophila melanogaster* as a model system, resulting in a boost of several potentiated research in the field. It allows ectopic expression of any sequence in any tissue or cells of interest. This system of spatiotemporal regulation of expression was first discovered in 1993 by Andrea Brand and Norbert Perrimon [49].

The Gal4 protein was found in yeast *Saccharomyces cerevisiae,* and this protein binds to its target sequence in the genome known as an upstream activating sequence (UAS), which controls the expression of genes downstream to the UAS site. This approach is famously called the 'bipartite approach' where a transgenic line, commonly known as the driver line, carries a Gal4 sequence under the regulation of a specific promoter sequence. In another transgenic line, a target DNA sequence is placed downstream of UAS sequence, called the responder fly line. When these two lines are crossed, Gal4 and UAS sequences occur in the same genome in individuals of the F1 generation [Figure 6]. The spatiotemporal restrain of Gal4 expression is permitted by choice of tissue-specific or a cell-type specific promoter sequence. Activation of this Gal4 in only such selected regions allows the expression of the DNA sequence downstream of UAS site. Employing this approach, the expression pattern of the target gene can be regulated using the appropriate leverage like the promoter of the target gene in the driver line. Another perk of this method is the temperature regulation of expression. Gal4 mediated transcription of UAS element is inactivated at 16 degree Celsius, and its activity is maximum at 29 degree Celsius [50].

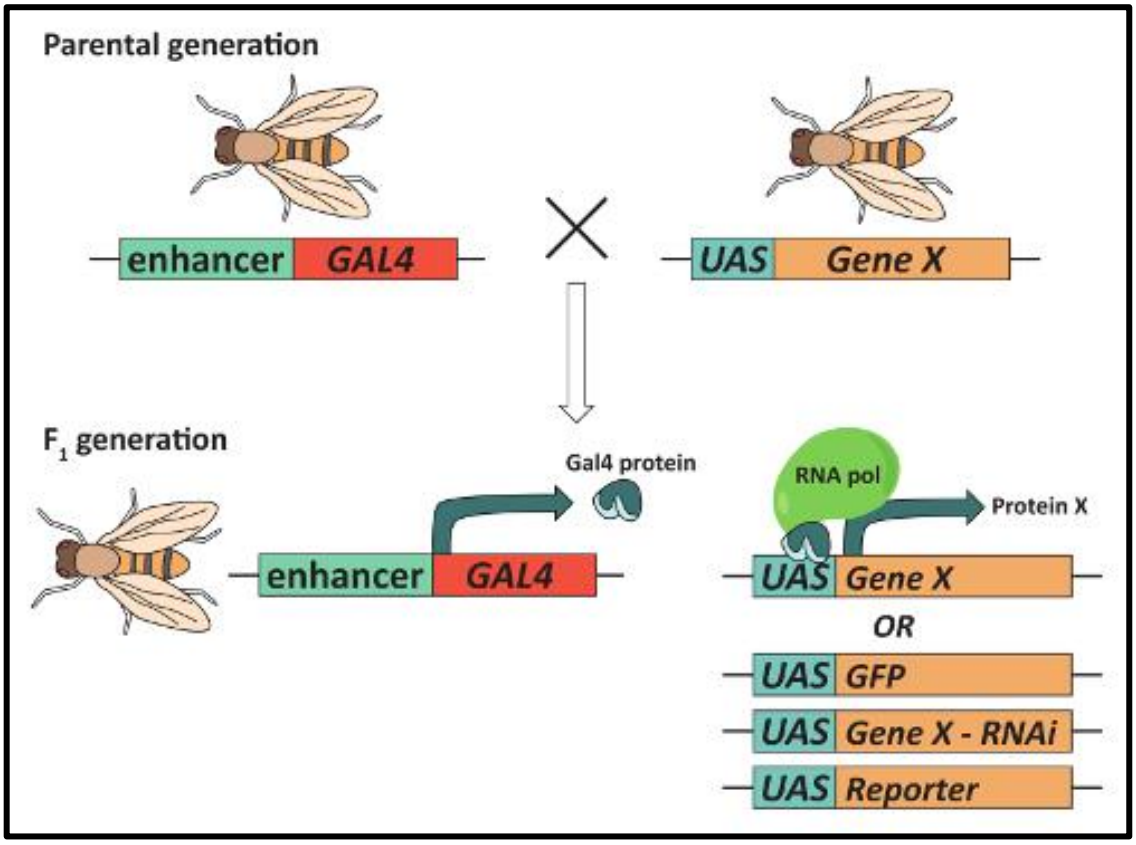


Figure 6: The Gal4-UAS system

Schematic representation of the Gal4-UAS system which is used for targeted gene expression in *Drosophila melanogaster* [51].

Hematopoiesis in *Drosophila melanogaster*

Hematopoiesis is a dynamic process of formation of distinct blood cellular components from pluripotent precursors or hematopoietic stem cells (HSCs) [52]. Vertebrate hematopoietic development occurs in two stages, spaced spatiotemporally. Phase one termed as 'primitive hematopoiesis,' located in the extraembryonic sac during embryogenesis which is erythroid (giving rise to erythrocytes and macrophages) in nature [53]. This is a transient phase and is present only for the production of red blood cells to facilitate oxygen consumption as a developing embryo requires a huge reservoir of energy [54]. These cells lack pluripotency and stemness. Phase two is known as 'definitive hematopoiesis' beginning at the later stages of development. They include multipotent cells, giving rise to all cell types of adult phase. In vertebrates, this process befalls in the mesodermal aorta-gonad-mesonephros (AGM) of the embryo proper. These cells initially home in the fetal liver, but subsequently, as the organism develops, they begin populating the bone marrow, location of HSCs for adults, and an entire load of hematopoiesis now shifts to bone marrow [55].

Recent developments have illustrated significant conservation between *Drosophila* and vertebrate signaling pathways and transcription factors [Table 1] in charge of proliferation, differentiation, and determination processes involved in hematopoiesis [56]. Thus, using *Drosophila melanogaster* as a model system to understand the underlying mechanism of hematopoiesis is a pragmatic decision. The process of hematopoiesis in *Drosophila* is a biphasic process (occurs in two waves) of development [Figure 8], spread across space and time, to populate the developmental stages with mature blood cells or hemocytes [57].

The first phase of hematopoiesis is the **'primitive hematopoiesis.'** The characterization of hemocytes begins as early as stage 5 of embryogenesis. Eventually, they hemocytes migrate throughout the embryo. These circulating hemocytes are called plasmatocytes, and approximately 95% hemocytes in the embryo populated by these cells. The other 5% occupied by crystal cells, but their role in embryonic stage is still unknown. The head mesoderm produces approximately 700 plasmatocytes and 36 crystal cells. The hemocytes from primitive phase are also present in the larval stages, and a few persist until the adult stage of *Drosophila* [58]. These cells originate from the head mesoderm, explicitly from the procephalic mesoderm. They are first distinguished by the expression of Serpent (Srp), a GATA family zinc finger transcription factor. The expression of Srp is the key for taking

on the fate of hemocytes [59], [60]. The differentiated hemocytes migrate from their place of origin and populate the entire embryo by following some attraction cues [58].

The second wave of hematopoiesis is called **'definitive hematopoiesis.'** This process occurs first in a specialized organ in the larval stages called the lymph gland or the blood forming organ of the larvae [61] and later in the specialized domains in the abdomen of the adult fly. During embryogenesis, a small population of cells from the cardiogenic mesoderm called the lymph gland arises in the lateral mesoderm, which is responsible for the manufacturing of hemocytes. Similar to the precursors of hemocytes located in the head mesoderm, the cells of lymph gland also express Srp.

Conserved Transcription Factors	
Drosophila	*Mammals*
Serpent	*GATA*
U-Shaped	*Friend of GAT (FOG)*
Lozenge	*Runx*
Cut	*Cux*
Dorsal/Cactus	*NFkB/IkB*
Collier	*Early B Factor*
Notch/ Serrate	*Notch/ Jagged*
Hop/ STAT 92E	*JAK/ STAT*
Toll	*Toll-like Receptor*

Table 1: Conservation of Transcription factors during hematopoiesis between *Drosophila* and mammals

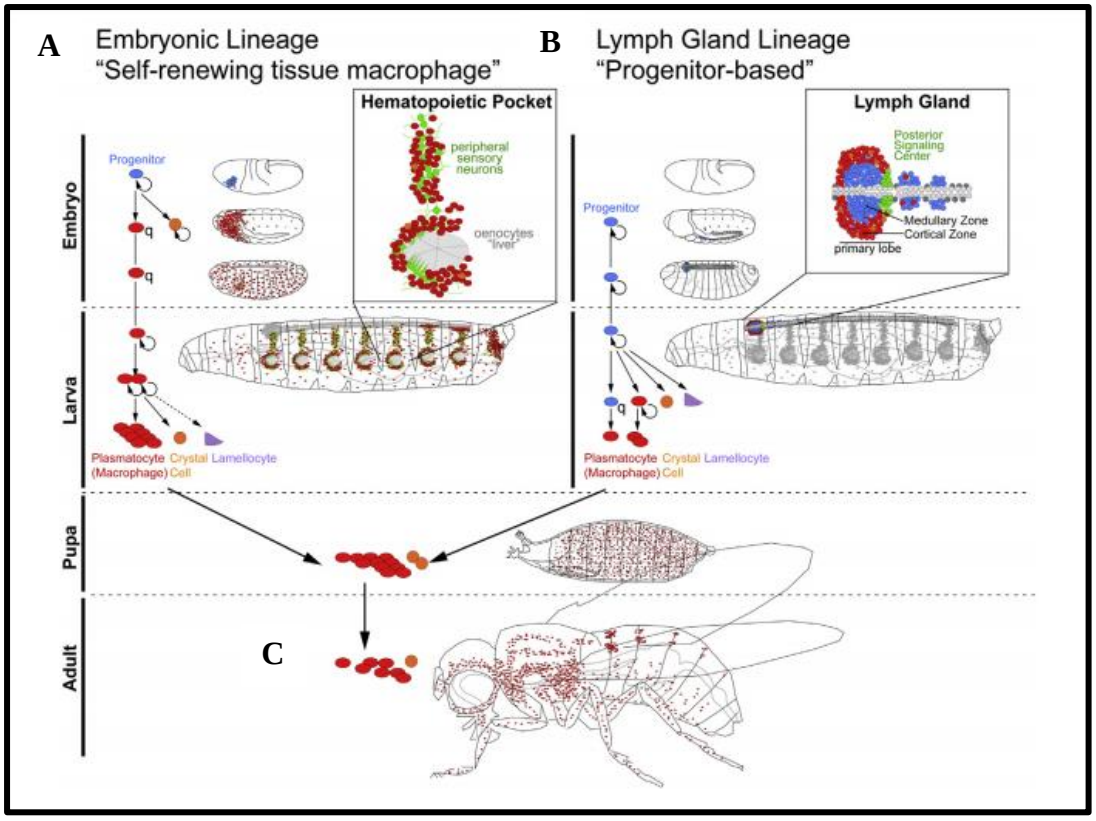


Figure 8: The two waves of hematopoiesis in *Drosophila melanogaster*

A) Represents the primitive phase of hematopoiesis and its cell-lineage which occurs in the head mesoderm

B) Characterizes definitive stage, the genesis of larval hematopoietic organ- the lymph gland

C) Depicts an adult fly having blood contribution from both waves of hematopoiesis. [62]

Lymph Gland: Organ for definitive hematopoiesis

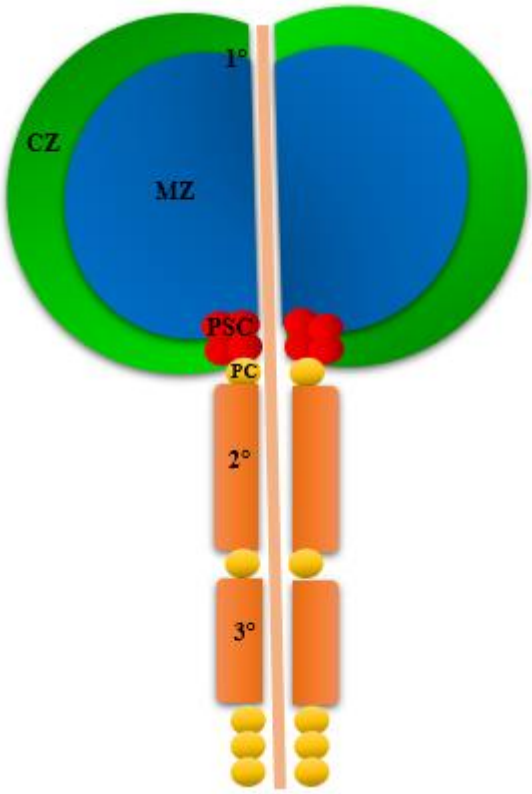
During embryogenesis, dorsal mesoderm originates from lateral mesoderm, further giving rise to cardiogenic mesoderm and visceral mesoderm. The cardiogenic mesoderm differentiates into three different cell types i) vascular cells (cardioblasts): giving rise to blood vessels, ii) pericardial cells: nephrocyte functions, iii) lymph gland: giving rise to hemocytes.

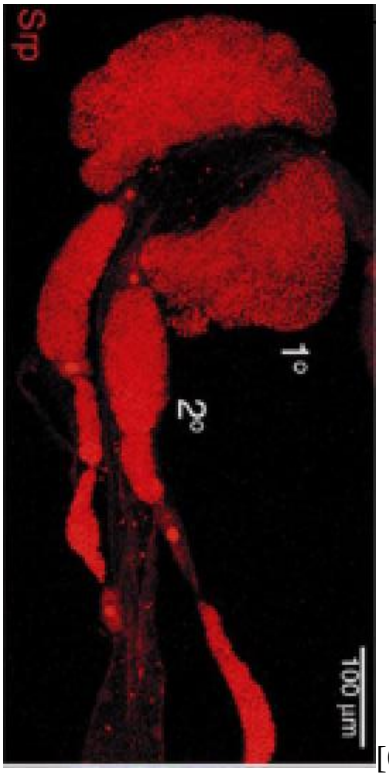
Similar to the precursors of hemocytes from the head mesoderm, the cells of lymph gland also express Srp [59]. On the contrary to the prohemocytes of head mesoderm, cells of lymph gland multiply for the first half of larval development, before they undergo differentiation. These proliferating cells aggregate to form lobes, bilaterally flanking the dorsal vessel. They are called the primary lobes carrying approximately 20 cells in each lobe. Increasing their numbers up to 200 cells, two to three clusters of cells bilaterally form secondary and tertiary lobes, in the second instar larvae [63]. These hemocytes show the first indications of differentiation in the second instar stages [59]. The differentiation regime remains fairly restrained to the primary lobes, and it continues till the third instar larval stage. Secondary lobes undergo differentiation during the inception of metamorphosis or sometimes during some immune challenge. The hemocytes of lymph gland are released into circulation after the beginning of metamorphosis and populate the adult fly [64]. The lymph gland divided into a distinct zone, and each zone has a unique characterization. These regions are Posterior signaling center (PSC), Medullary zone (MZ), and Cortical zone (CZ) [Figure 9] [65].

PSC is the master regulator of lymph gland, commonly called a hematopoietic niche. This zone modulates the stemness by sending out highly integrative signals based on the current requirements of the animal. It is placed the posterior tip of the primary lobes. By the close of the third instar stage, there are on an average of about 50 cells in the niche. The cells of PSC express a protein specific to them named Antennapedia (Antp). This homeobox protein begins its expression right after lymph gland genesis. It helps in the specification of the niche. Several other markers give a unique identification to the cells of PSC, for example, reporter lines *unpaired-gal4, dorothy-gal4* [65], transcription factors Collier (Col) [66] and Notch ligand Serrate (Ser) [59]. The medullary zone is the homing zone for prohemocytes. Cells of this zone exhibit stem-like properties; thus, these cells are commonly called progenitor cells of lymph gland. They express specific cell adhesion

markers like DE cadherin/ Shotgun, or this zone is marked by using reporter lines *domeless-gal4* and *TepIV*. The cortical zone encloses differentiating hemocytes, and this zone characterized by Hemolectin (Hml) [67], Peroxidasin (Pxn) [68] and Lozenge (Lz).

There is tight regulated signaling crosstalk between the three zones of lymph gland, and the absence of these signals can disrupt the maintenance and differentiation of the progenitor cells.





[65]

CZ: Cortical zone

MZ: Medullary zone

PSC: Posterior signalling centre (Niche)

PC: Pericardial cells

1°: Primary Lobes

2°: Secondary Lobes

3°: Tertiary Lobes

Figure 9: Lymph gland: hematopoietic organ of *Drosophila melanogaster*

Representation of the hematopoietic organ of the fly with its well-characterized primary lobes divided into three distinct zones.

Objectives

Hematopoietic stem cells (HSCs) are used as a model to understand tissue stem cells and their role in aging and oncogenesis. Furthermore, following the process of hematopoiesis is crucial to elucidate signaling pathways underlying blood disorders and cancers. Just like most of the operations in the body, hematopoiesis has not been able to evade the clutches of the biological rhythms. The snowballing pool of evidence presented in the field talk about the connection between the two. The influence ranges from circulation and trafficking of hematopoietic stem cells (HSCs) to their differentiation and proliferation (reviewed in [69]). There has been documentation stating the existence of clock genes in bone marrow [70], [71] and in HSCs [72], [73], but their function has not been elucidated. As new reports continuingly indicate a growing association between circadian rhythm and hematopoiesis, it calls upon for establishing the molecular signaling that links these two processes. Thus, this project, therefore, focuses on answering the following questions:

1. Does circadian rhythm regulate the process of hematopoiesis in *Drosophila melanogaster*?
2. How does the hematopoietic organ respond to alteration of the circadian rhythm?
3. What are the molecular players involved in the crosstalk between circadian rhythm and hematopoiesis?

CHAPTER II

MATERIALS AND METHODS

2.1 Fly Rearing and Stocks used

Flies were reared at 25°C on standard agar-corn-yeast media. Fruit plates made of a mix of fruit juice-sugar-agar medium were used for egg collection and synchronization of the larvae. The newly hatched eggs were transferred onto food plates and kept at in a unique experimental setup described below.

The fly stocks used for this thesis were procured from various stock centers, while some were gifted fly lines. They are described in detail as follows-

1. Oregon R: Wild-type laboratory stock of Drosophila.
2. W^{1118}: This line has been procured from BDSC (Bloomington Drosophila Stock Center, Indiana University), BL3605.
3. W^{1118}*; hhF4f-GFP; +*: This transgenic fly line, a gift from Utpal Banerjee, UCLA.
4. *DomeEBFP; +; +*: This stock was a gift from Utpal Banerjee, UCLA.
5. W^{1118}*; Hml-Gal4; +*: This line has been procured from BDSC, BL30140.
6. W^{1118}*; +; UAS-GFP*: This line has been obtained from BDSC.
7. W^{1118}*; Timeless-Gal4; +*: This line has been acquired from BDSC, BL7126
8. W^{1118}*; +; UAS-nls GFP*: This line has been purchased from BDSC, BL4776.
9. W^{1118}*; +; Clock-Gal4*: This line has been procured from BDSC, BL 36316.
10. W^{1118}*; UAS-Tim RNAi; +*: This line has been procured from BDSC, BL 4089.
11. W^{1118}*; +; UAS-Per RNAi*: This line has been procured from BDSC, BL31285, 31659.

2.2 Experimental setup

The setup was made using cardboard boxes with 2 LED lights attached on to the roof of the box. The intensity of the light combined was 2000 lux. Two boxes were used: one for control conditions and the other one for experimental conditions. Control condition regiment proceeded as follows: 12 hours of light and 12 hours of dark conditions. The experimental condition regiment included 24 hours of continuous light. The entrainment started at 0 hours after egg hatching (AEH). These conditions were continued till 96h AEH and samples were processed for dissections or RNA isolation

12h Light-12h Dark

LD (control)

24h Light

LL (experiment)

Synchronization and Exposure to Light — **0h**

24h

48h

72h

Dissection and observation — **96h**

Experimental setup and experimental time-line.

Fruit Plates

10g agar and 8g sugar were added in a conical flask. 100ml dH_2O was added. The solution was heated for 2 min intended for homogenization. Fruit juice was added for color and fragrance. The solution was heated again for 1 min. Juice was added until the solution was similar to running water. The solution was poured into plates and kept aside until the solution solidified. Plates were stored at 4°C.

10X PBS

For a volume of 500ml, 40g NaCl, 1g KCl, 7.2g Na_2HPO, and 1.2g K_2HPO_4 was weighed. 490 ml dH_2O was added. pH was adjusted to 7.2. The volume was scaled to 500ml.

2.3 Immunofluorescence

The larvae were dissected in 1X PBS (Phosphate-Buffered Saline) and transferred to 1X PBS containing cavity blocks. The tissues were fixed using 4% PFA (Paraformaldehyde) for 55 min, at room temperature, on a shaker with 60 rpm (rotation per min). The tissues were washed with 1X PBS for 5 min, at room temperature, on a shaker with 60 rpm. Next, the tissues were permeabilized using 0.3% PBT. Four washes were given, 15 min each, at room temperature, on a shaker with 60 rpm. Samples were blocked with 5% BSA (Bovine Serum Albumin) in 1X PBS, for 1h, room temperature, on a shaker with 60 rpm. The samples were cleaned off of extra tissue and transferred to Nunc microwell plates with a 10µl primary antibody solution.

**(Antibody was diluted in 5% BSA (depends on the antibody type). The concentration of dilution varies according to the antibody used).

The nunc microwell plates were transferred to 4°C for 16h. Next day, samples were moved back into cavity blocks. Four washes were given using 0.3% PBT, 15 min each, at room temperature, on a shaker with 60 rpm. A second block was provided using 5% BSA for 1h, at room temperature, on a shaker with 60 rpm. The cavity blocks were transferred to 4°C for 16h. Four washes were given using 1X PBS, 15 min each, at room temperature, on a shaker with 60 rpm. Tissues were stained using DAPI for 1h, at room temperature, on a shaker with 60 rpm. Two washes with 1X PBS were given, 10 min each, at room temperature, on a shaker with 60 rpm. Tissues were mounted in VECTASHIELD Mounting Medium.

2.4 Antibodies used

PDF (1:100, mouse), Antp (1:10, mouse), Hnt (1:5, mouse) were obtained from Developmental Studies Hybridoma Bank, Lowa 7E8A10. P1 (1:100, mouse) was gifted to the lab by Prof. Istavan Ando.

2.5 Imaging

The mounted samples were imaged in a confocal microscope (Leica SP8), courtesy of IISER Mohali confocal facility. The images were further processed and analyzed using ImageJ (NIH) software.

2.6 RNA Isolation

Lymph glands were dissected (or the third instar larvae were selected) and collected in 500µl TRIzol, in Eppendorf tubes kept on ice. Using autoclaved pestles, the samples were crushed and kept them overnight in -80°C. The samples were thawed on ice. Next, 100µl chloroform was added in the samples then shaken vigorously for 15 seconds at room temperature. Kept the samples upright at room temperature for the next 3 min. Samples were centrifuged at 4°C, 12000g for 15 min. Collected the supernatant in separate autoclaved DEPC (Diethyl pyrocarbonate) treated tubes. Added equal volume of isopropanol. The samples were kept overnight at -80°C. Samples were centrifuged at 4°C, 12000g for 15 min. Discarded the supernatant without disturbing the pellet. Later the pellet was washed with 75% chilled ethanol and centrifuged samples at 4°C, 10000g for 10 min. Discarded the supernatant and centrifuged samples at 4°C, 7500g for 5 min. Lastly, the pellet was dissolved in 20µl autoclaved miliQ water. The RNA was quantitated using a NanoDrop (Thermofisher).

2.7 DNase Treatment

The isolated RNA was incubated at room temperature for 5 minutes. 2µl of RDD buffer and 0.5 µl of DNase was added and the samples and kept in the heating block at 25°C for 30 min. Next, 100 µl RNase free water, 350 µl RLT mix, and 250 µl 100% ethanol were sequentially added, and the entire solution was added to a column. Samples were centrifuged at 8000 rcf for 15 sec, and flow-through was discarded. 500 µl RPE was added, and samples were centrifuged again at 8000 rcf for 1 min, flowthrough discarded. Dry spin for 2 min. The column was placed in a new 1.5ml tube. 10 µl of RNase free water was

added and incubated for 2min. Centrifuged at 10,000 rcf for 1 min and dry spun for 2 min. A further collection was done by repeating the last two steps.

2.8 cDNA synthesis

Verso cDNA synthesis kit

Components	**1X**
RNA + water	10µl
cDNA mix	7µl
RNA primer mix	2µl
Verso Enzyme Mix	1µl

Reaction Time:

	Temperature	**Time**	**Number of cycles**
cDNA synthesis	42°C	30 min	1
Inactivation	95°C	2 min	1

2.9 Polymerase Chain Reaction

Components	**1X**
10X PCR Buffer	2
5mM dNTPs	0.8
Forward Primer	0.4
Reverse Primer	0.4
Template DNA	1
Taq DNA polymerase	0.125
Water	15.275

Reaction Time:

	Temperature	Time	Number of cycles
Denature template	94°C	0:30 min	40
Anneal template	56°C	0:30 min	
Extension	72°C	1 min	
	72°C	5 min	1
Forever	4°C	∞	-

2.10 Real-Time Polymerase Chain Reaction

Components	1X
Sybr Green	10µl
Forward Primer	1µl
Reverse Primer	1µl
Template DNA	1µl
Water	7µl

Reaction Time:

Temperature	Time	Number of cycles
94°C	0:10 min	40
56°C	0:45 min	
95°C	0:30 min	1
65°C	0:05 min	1
4°C	∞	-

2.11 Statistical Analysis

All the processing of the data was done in Microsoft Word and the statistical test used was TTEST with two-tailed distribution with two-sample unequal variance.

CHAPTER III

RESULTS AND DISCUSSION

3.1 Circadian rhythm is disrupted by entrainment to a 24-hour light regiment

Circadian rhythm is a self-sustained endogenous clock which regulates various bodily functions. This rhythm is aligned with the external conditions via *Zeitgebers*, such as light, temperature, food availability and social behavior. The predictable changes that occur throughout the day help this biological clock in maintaining a precise 24-hour molecular timekeeping mechanism. Light is a crucial input that regulates this 24-hour rhythmicity in animals and plants. Light activates the intracellular blue-pigment photoreceptor CRYPTOCHROME (CRY) by bringing about conformational changes in this protein. This change in the conformation of CRY enables it to bind to TIMELESS (TIM), and this particular association initiates proteasomal degradation of TIM via JETLAG, containing E3 ubiquitin ligase [74]–[76]. This light is responsible for the rapid deterioration of TIM, which is essential for the resetting of the circadian pacemaker, in turn regulating the behavioral rhythms [29], [77].

Therefore, when the light-dark cycles are disrupted, there are severe defects in photoreception and animals lose their rhythmicity [78]. *Drosophila melanogaster* is no exception to this rule; these flies in constant light become arrhythmic [79]. Our study demonstrated this onset of arrhythmicity in the flies by using constant light conditions, i.e. 24-hour light regiment (experimental conditions/ LL) opposed to control states of 12-hour light: 12-hour dark regiment (control conditions/ LD). Using synchronized larval population reared at 25 degree Celsius, 96-hour larvae post egg hatching were dissected, and levels of *timeless* gene expression in the central clock were measured in the brains by using the recombinant line W^{1118}*; timeless-Gal4, UAS-nls GFP; +*. Pigment dispersing factor (PDF), an ortholog of the crustacean "pigment-dispersing hormone" (PDH) family [80], explicitly expressed in the circadian neurons, thus co-expressing with clock genes: *timeless (tim)* [Figure 10] [81] and *period (per)* [82]. It is a well-established marker for circadian neurons, therefore, utilized in this study for the same purpose. Our results establish that the rhythmic oscillations of *timeless* gene that are usually seen in control conditions are lost in animals with 24-hour light entrainment (LL), thus advocating circadian disruption under constant light conditions. Timeless levels are at the highest peak during the dark phase, which is evident in the 12-hour light: 12-hour dark regiment (LD). As the larvae become arrhythmic under entrainment to constant light conditions, the single peak of control conditions shows a shift in phase and dampening of amplitude [Graph 1].

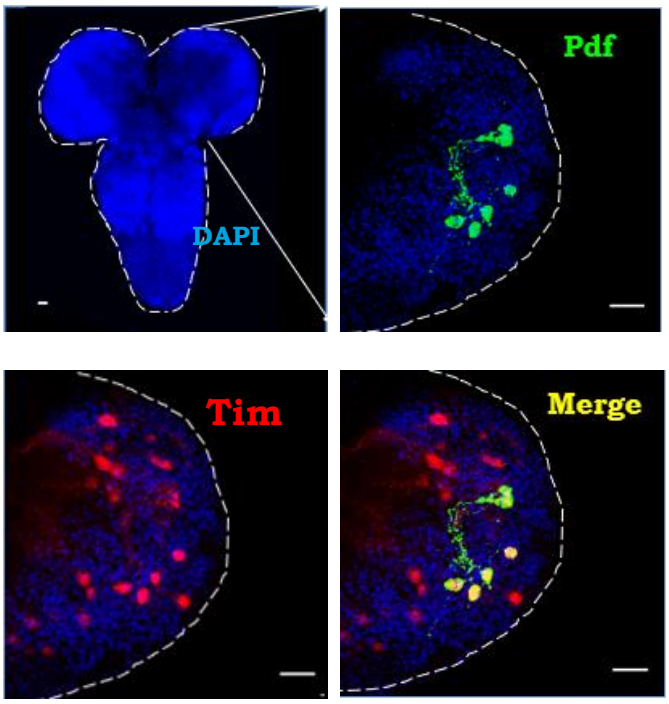
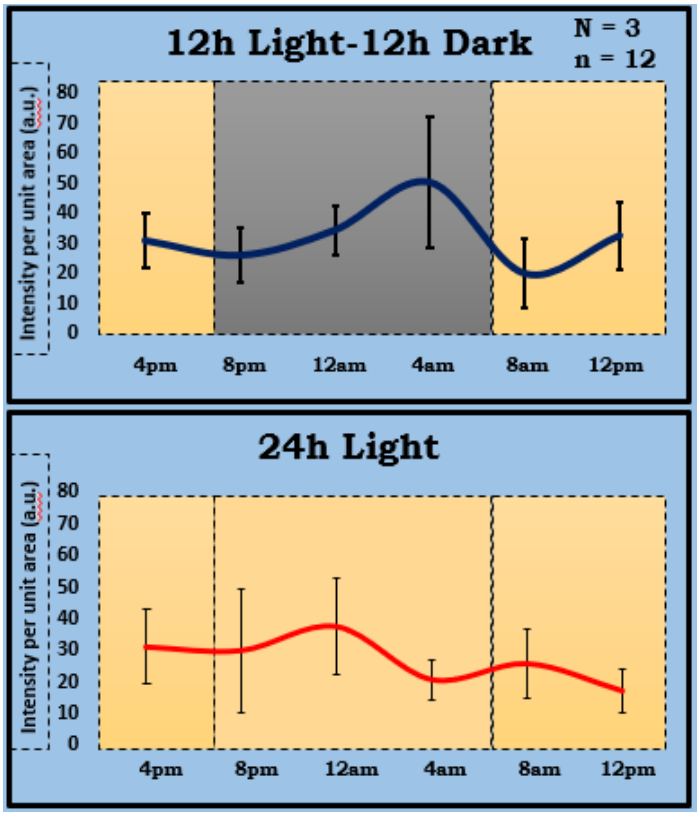


Figure 10: Expression of Timeless neurons

Confocal slices of 3rd instar larval brain with expression of TIM in neurons. The intensity of TIM expression is measured only in neurons which are positive for Pigment dispersing factor (Pdf).



Graph 1: Rhythmicity of *timeless* gene over a period of 24-hours

Graphical representation of the intensity of expression of Timeless-Gal4, UAS-nlsGFP in clock neurons. The two graphs show the comparison in the oscillations of TIM protein in 12-hour light: 12-hour dark vs 24-hour light regiment.

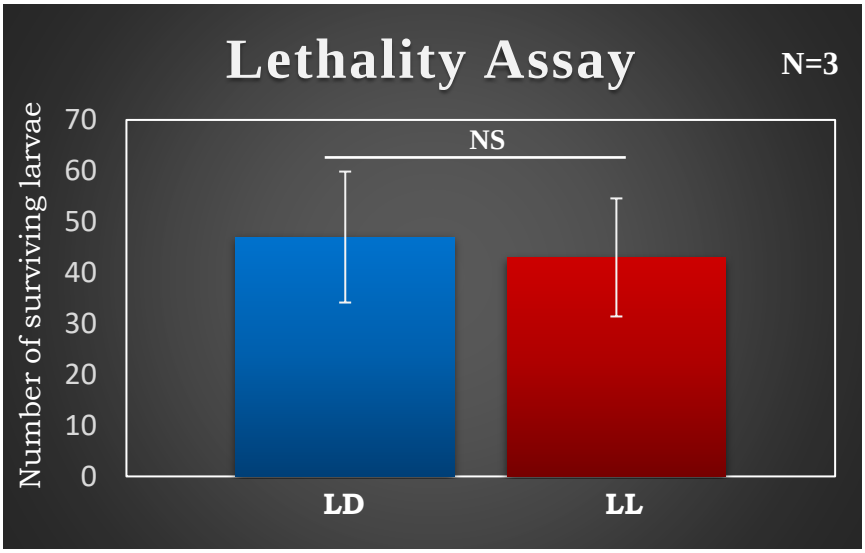
3.2 Lethality is unaffected in experiment vs control conditions

Several studies have indicated that constant light conditions can cause severe damage to the animal by elevation of light-induced stress [83]–[85]. By collecting the surviving larvae (larvae metamorphized into the pupal state) and counting the number of larvae those did and others that did not survive, this study showed that the numbers were not polarized between the control and the experimental conditions. There was no statistical difference in the larvae that survived in 12-hour light: 12-hour dark entrained batch vs. the 24-hour light-entrained batch [Graph 2]. From this result, we concluded it to be fit to use these conditions to conduct further experiments.

3.3 Entrainment to constant light regime results in an elevation in the numbers of differentiated cells in the lymph gland

The lymph gland is divided into three zones which home distinct sets of cells; each zone is homing a different cell population [64]. These zones are the Posterior signaling center (PSC) the hematopoietic niche, Medullary zone (MZ) with closely filled cells located proximally to the dorsal vessel, and Cortical zone (CZ) with larger and loosely packed cells. Lymph gland homes a population of multipotent stem-like progenitors, maintained by combined signaling pathways and cues from diverse cellular entities within the organ. Hedgehog (Hh) is secreted by the PSC which mediates progenitor maintenance in the MZ [85]. Apart from Hh, the differentiating cells of CZ are also involved in determining the fate of the progenitors of MZ [64]. These two independent pathways regulate prohemocyte maintenance. Apart from intra-organ signaling pathways, several other signals from the rest of the body can cause alternations in the size of the hematopoietic niche [86]–[89], which in turn leads to changes in the topographical landscape of the lymph gland.

Our study exhibited that the landscape of the primary lobes in lymph gland changed under constant light regiment. The lymph glands of the larvae exposed to a 24-hour light regiment displayed a **38.6%** increase [Graph 3] in the differentiation index compared to the lymph glands of larvae entrained to 12-hour light: 12-hour dark regiment [Figure 11]. This difference was an ectopic differentiation. Differentiation index is the ratio of the area of the cortical zone to the area of the entire primary lobe. The genetic line used here was *DomeEBFP; Hml-Gal4, UAS-GFP;* +. As stated earlier, *domeless*-Gal4 marks MZ and Hemolectin (Hml) marks the CZ; this particular line marked both these zones simultaneously. DAPI was used to label the nuclei of the cells in lymph glands [86]



Graph 2: Lethality assay of *Drosophila* larvae in control vs experimental conditions

The graph represents the number of surviving larvae when entrained to 24-hour light regiment compared to the 12-hour light: 12-hour dark regiment. The blue bar represents the number of larvae going into the pupal stage when kept in to 12-hour light: 12-hour dark light regiment (control conditions/ LD) and red bar represents the number of larvae going into pupal stage when kept in 24-hour light regiment (experimental conditions/ LL). TTEST score, p value: 0.906

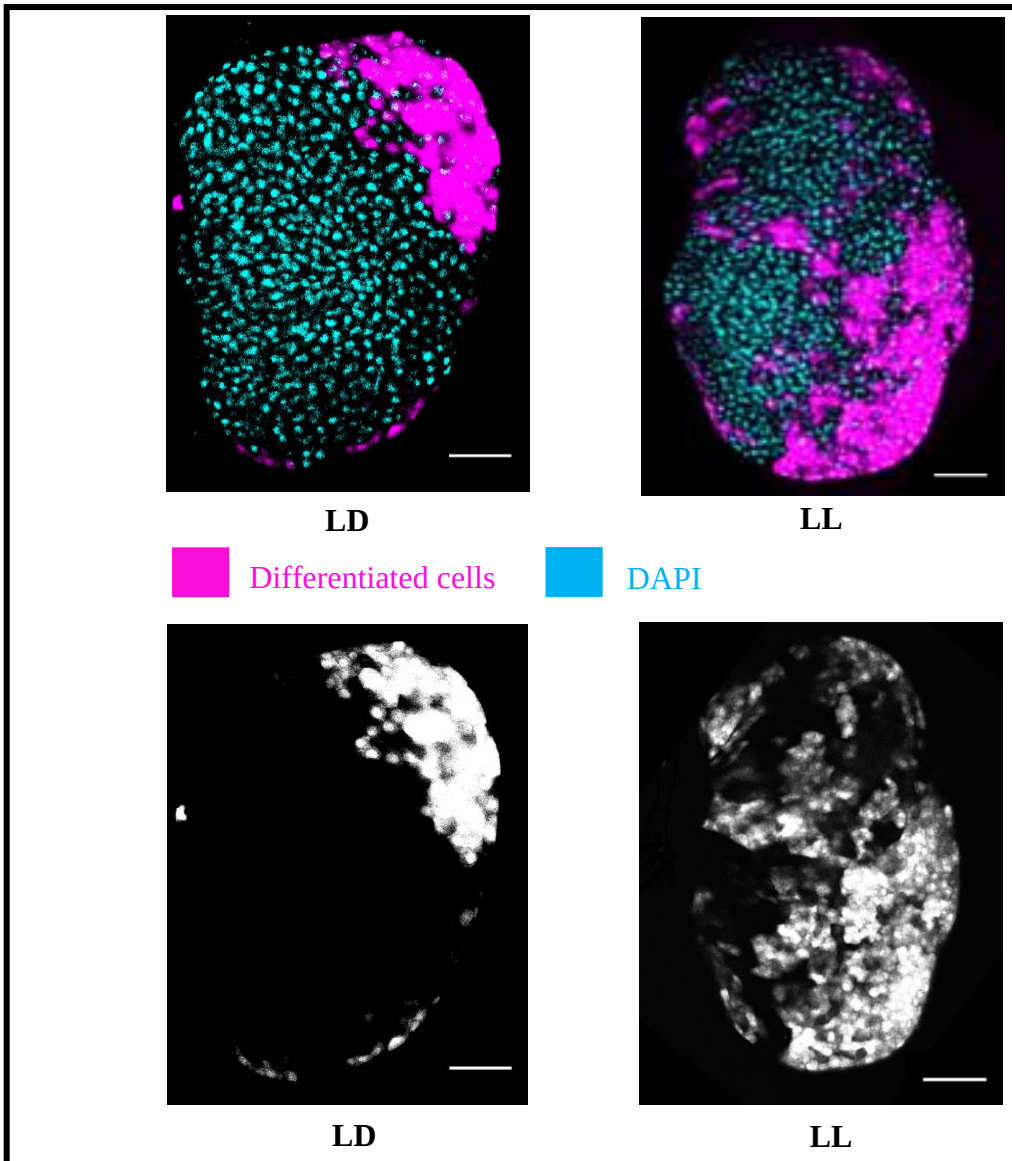
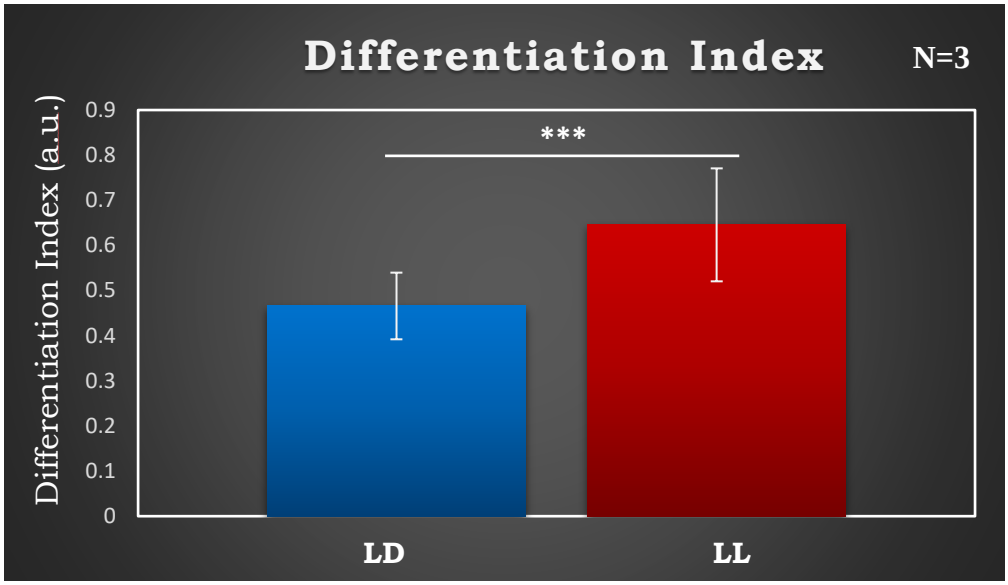


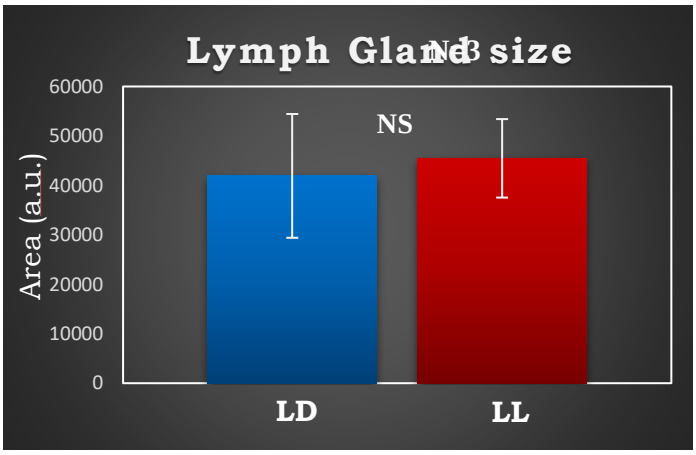
Figure 11: Differentiation index of lymph glands in control vs experiment conditions

Confocal slices of the elevation in the differentiation index of lymph glands in control vs experiment conditions. The line used is *DomeEBFP; Hml-Gal4, UAS-GFP*; +. The magenta marks the differentiated cells (Hml), while cyan is a color indicated for DAPI staining in the lymph gland. Scale bar: 40µm



Graph 3: Differentiation Index comparison

A graphical representation of the comparison of in the elevation in differentiation index of lymph glands entrained to LL vs LD, with 38.6% increase. The TTEST was conducted with p-value: 1.71E-05.



Graph 4: Lymph Gland size comparison

Graphical representation of the size of the primary lobes of lymph gland. The sizes of the primary lobes do not vary dramatically between LD vs LL conditioned larvae. TTEST, p-value: 0.375294711

The size of the primary lobes of lymph glands fairly remains constant between the two differently conditioned larvae [Graph 4]. This observation advocates a rise in the number of differentiated cells in lymph glands of larvae entrained to 24-hour light regiment compared to the ones entrained to 12-hour light: 12-hour dark regiment.

3.4 Knockdown of circadian genes expression in the master neurons causes an increase in the differentiation index in the lymph gland

To show a direct connection between circadian rhythm and hematopoiesis, a knock-down of transcripts of two clock genes (*timeless* and *period*) in the circadian pacemaker neurons [Figure 12] was orchestrated for this study. The samples were exposed to control conditioned environment of 12-hour light: 12-hour dark regiment, with the temperature maintained at 25 degree Celsius. RNAi lines were employed under the Gal4- UAS genetic tool. The following crosses were used-

- W^{1118}*; Clock-Gal4; +* x W^{1118}*; +; UAS-Per RNAi*
- W^{1118}*; Clock-Gal4; +* x W^{1118}*; +; UAS-Tim RNAi*
- W^{1118}*; Clock-Gal4; +* x w^{1118}*; +; +*

To mark the differentiated cell in the lymph gland, P1 antibody was used. P1 antigen also called the Nimrod, is a potent marker for the differentiated cells of the cortical zone in lymph gland [87]. Nimrod belongs to a family of ten related proteins the majority of which are expressed by hemocytes [88]. The lymph glands were labeled using anti-P1 to mark the differentiated cells of lymph glands from the crosses mentioned above. Our study discovered that disruption of clock genes in the central pacemaker of the fly lead to an increase in the differentiation index of the lymph gland under control conditions [Figure 13] and the increase was statistically significant [Graph 5]. This result is a robust experimental indication that circadian rhythm and hematopoiesis are linked in *Drosophila melanogaster*.

3.5 Brain sizes remain unaffected in larvae exposed to constant light

Reports display that environmental conditions bring about changes in the development of larval brains in *Drosophila melanogaster* [89]. As a consequence, there are anatomical changes in the larval brains. Our study has revealed that the brain sizes are unaffected in the larvae exposed to a continuous light regiment. The larval brain sizes are comparable between the control vs. the experimental conditions [Graph 6].

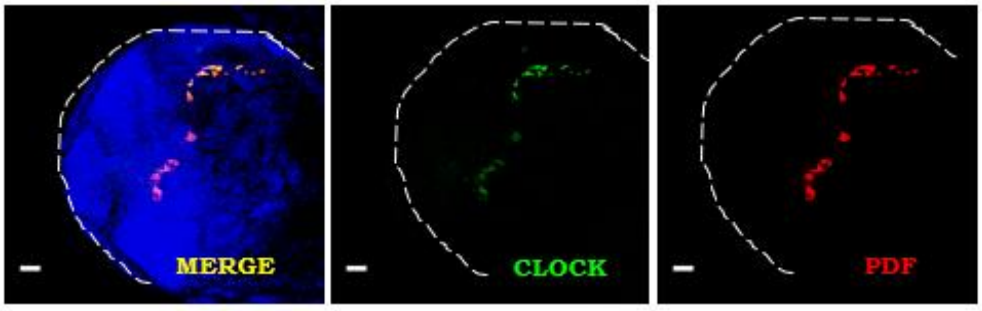
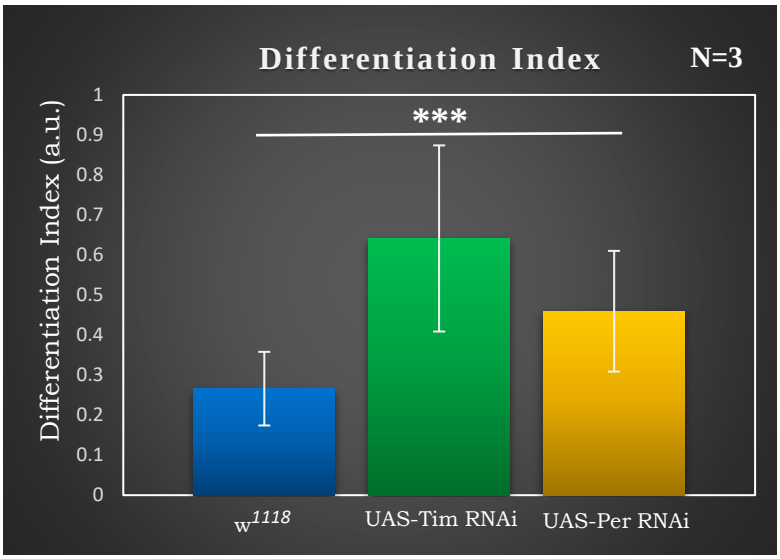


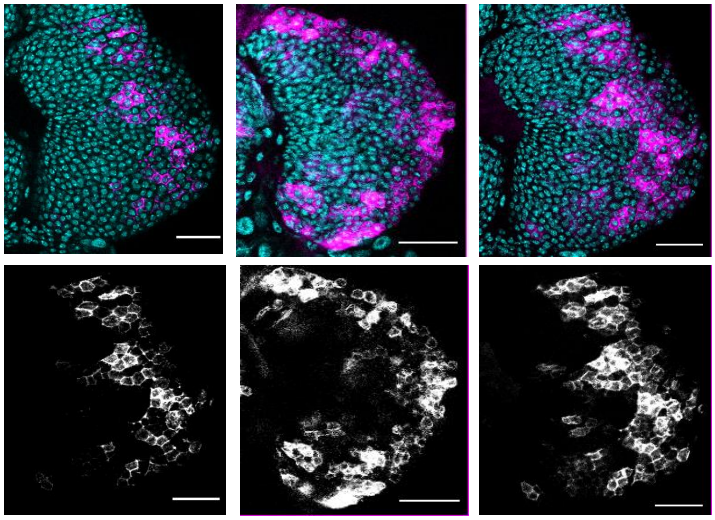
Figure 12: CLOCK-Gal4 is explicitly expressed in circadian pacemaker neurons

Confocal images of the co-expression of PDF and CLOCK in the master circadian pacemaker neurons. The green colour represents *Clock-Gal4, UAS-nls GFP* while red colour represents the Pigment Dispersing factor (PDF), marking the circadian neurons. Scale bar: 20µm



Graph 5: Graphical representation of an elevation in the differentiation index after knocking down circadian rhythm genes *timeless* and *period*

Graphical representation of an increase in differentiation index on knocking down circadian rhythm genes timeless and period using *Clock-Gal4* as the driver line. There was a 140% and 73% increment on using *UAS-Tim RNAi and UAS-Per RNAi* lines respectively. The TTEST for the values in the graph was performed with a p-value of 2.03164E-06.

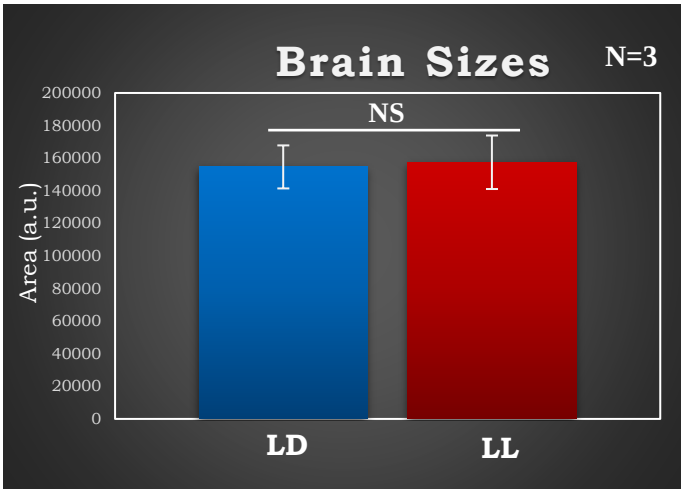


Clock-Gal4, w^{1118} | Clock-Gal4, UAS-Tim RNAi | Clock-Gal4, UAS-PerRNAi

Differentiated cells DAPI

Figure 13: Increase in differentiation index by knocking down the expression of *timeless* and *period* genes

Representation of elevation in the differentiation index of lymph glands after knocking down the expression of *timeless* and *period* genes under the driver line *Clock-Gal4.* Scale bar 40µm.



Graph 6: Larval brain size

Graphical representation of the larval brain size at 96-hour after egg hatching (AEH). There is no significant change in the brain sizes between the animals exposed to LL vs LD conditions. TTEST, p-value: 0.652666291

3.6 Disrupted circadian rhythm shows no effect on the number of cells in the Posterior signaling center (Niche)

The posterior signaling center works as the conductor in maintaining homeostasis in progenitor population and differentiated cell population of the lymph gland. PSC also integrates information from the rest of the organs and alters this homeostasis according to the need of the animal. Our study exhibited that under the altered light conditions, there is no significant difference in the number of cells of the niche as compared to the control conditions [Figure 4] [Graph 7].

3.7 Crystal cell number is unaffected by continuous light regime

The cortical zone of hematopoietic organ, the lymph gland, houses differentiating blood cells. There are three types of hemocytes named plasmatocytes, crystal cells, and lamellocytes. They are found in *Drosophila* circulating system, plasmatocytes being the major constituent (approximately 90%) [90]. Maximum of remaining hemocytes are crystal cells, with only a few to no lamellocytes under normal growth conditions. The primary function of crystal cells is the process of melanization. Notch is responsible for promoting crystal cell differentiation. Notch targets *klumpfuss* (ERG/WT1 family) and *pebbled/hindsight* (RREB1 homolog), which bring about the differentiation of crystal cells [91]. Thus, *hnt* is a useful marker for crystal cells in lymph gland.

Our study indicated that the number of crystal cells does not significantly change in constant light conditions compared to control conditions [Figure 15]. Their numbers remained comparable to wild type even when the number of differentiating cells increased in a constant light regiment [Graph 8]. Although there was no difference between the averages in two the conditions, the number of crystal cells in lymph glands of constant conditions showed a considerable variation, and there was a variance between the two lobes of the same lymph gland.

3.8 Absence of clock genes in lymph glands

In mammals, the central circadian pacemaker in the suprachiasmatic nuclei (SCN) synchronizes the circadian pacemakers in different tissues [92]. But in insects, several

peripheral clocks are autonomous, entrained by external light which penetrates through the translucid cuticle, or temperature cycles [93].

But this is not the case of lymph glands in *Drosophila melanogaster*. There was an absence of any transcripts of different clock genes (*timeless, period* and *cryptochrome* used for this purpose) in the transcript pool isolated from the lymph glands of larvae dissected at 96-hour AEH [Figure 16]. This was the hint that there was some external signaling at work, and not the presence of a peripheral clock, which brings about an elevation in the differentiation of lymph gland in constant light exposure.

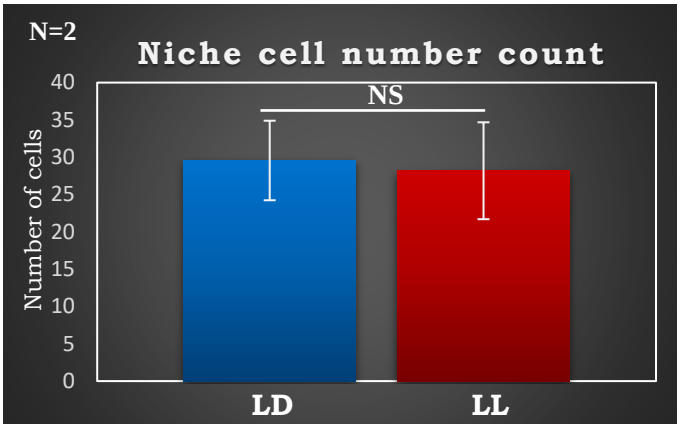
3.9 Stages of metamorphosis are unsynchronized in animals exposed to 24-hour light conditions

The endocrine system is the primary mode of communication between the central clock and the peripheral clocks [94]. The PG is an endocrine gland in *Drosophila melanogaster* and some other insects, that is responsible for the production of the steroid hormone 20-hydroxyecdysone (20E), which is the principal molting hormone in insects [95], [96]. Several reports repeatedly prove that the prothoracic gland (PG) clock is under the control of the central clock of the brain [97][98]. The central clock and clock in PG, together as a team restrict the emergence time window within a day, which is also species-specific [99] [100].

This study confirms the findings in the literature by displaying a disruption in the restricted time-window of pupation and eclosion timings in animals exposed to constant light (LL). Reports indicate that pupation occurs 120 AEH and eclosion occurs during early dawn [101]. This time-window is lost in larvae entrained to constant light [Graph 9].

3.10 Ecdysone signaling gets perturbed in 24-hour light conditions

A daily rhythmic pattern is observed for hormones in several insects [102], [103]. The first demonstration of the circadian regulation of an invertebrate hormone, ecdysone, was shown in *Rhodnius prolixus* in 1991[104]. Ecdysone is the primary steroid molting hormone, and its titer peaks in the hemolymph are responsible for developmental transitions in *Drosophila* and other insects [98], [105]. Ecdysone (E) is synthesized in the prothoracic gland, and when released into the hemolymph, E gets converted into its active form 20-



Graph 7: Niche cell number count

Graphical representation of the number of cells in the niche in larvae entrained to LL vs LD conditions.

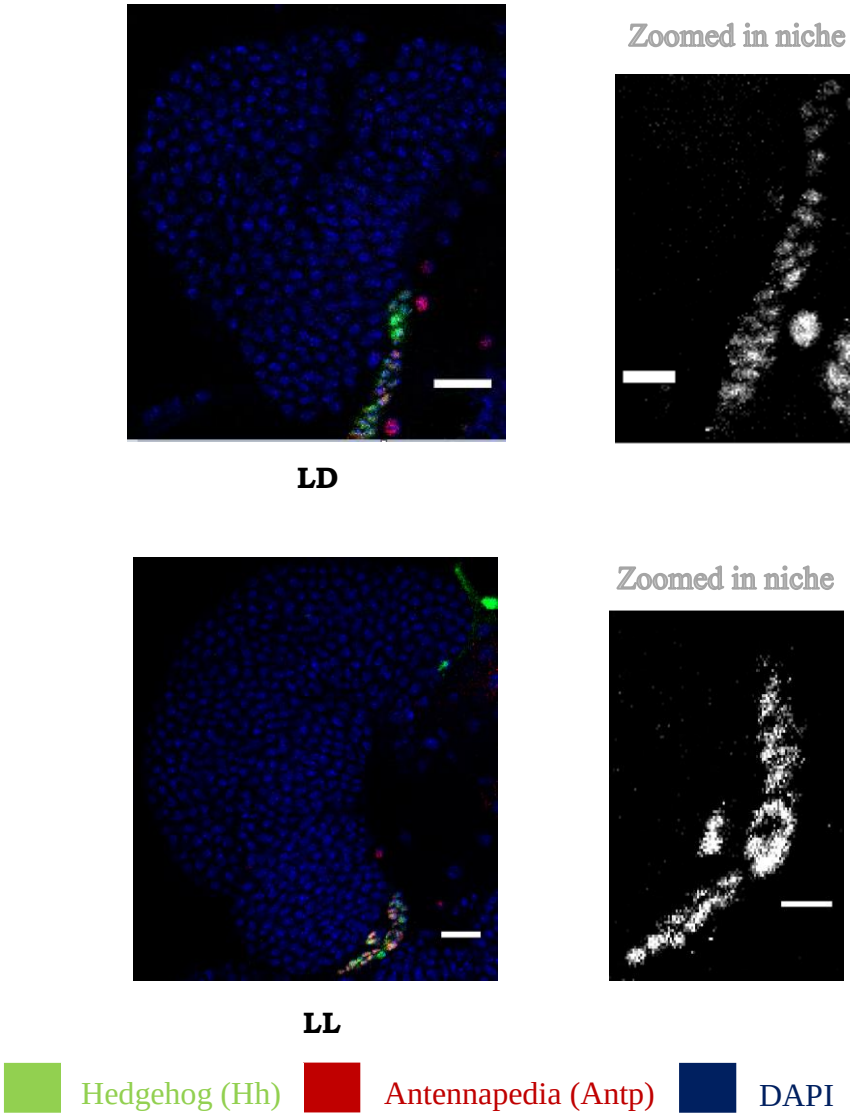
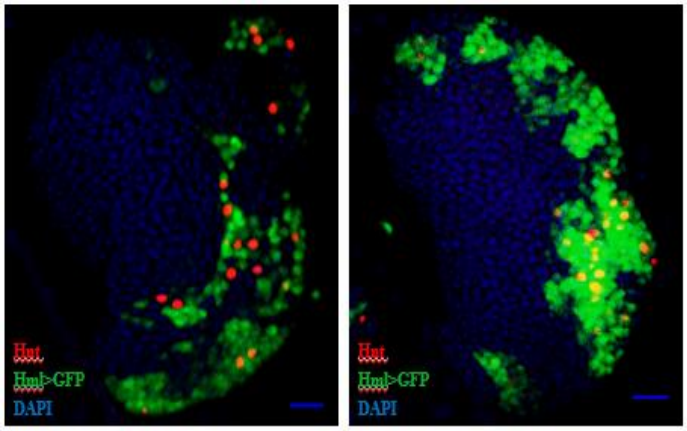


Figure 14: Niche of Lymph gland

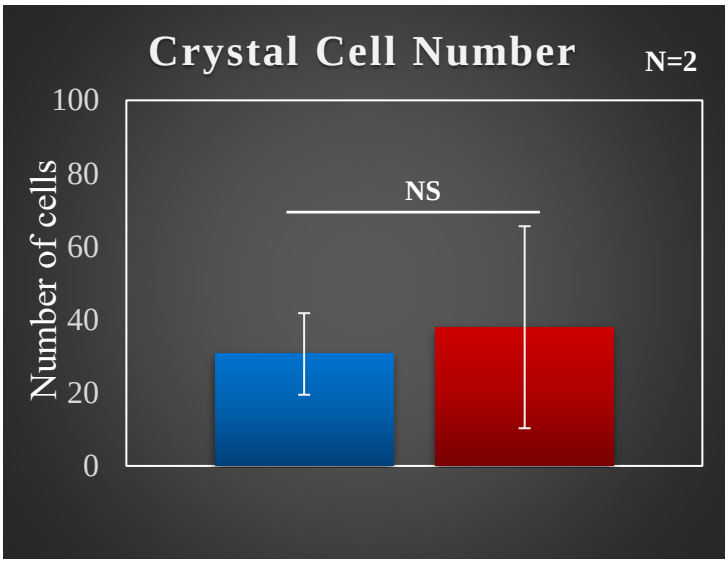
Confocal slices of niche of the lymph gland of third instar larvae exposed to LL vs LD conditions. Hh and Antp are co-expressed in the niche cells. Scale bar: 20µm (entire lobe), 10µm (zoomed in niche).



LD LL

Figure 15: Crystal cells in Lymph Gland

Representation of crystal cell number in LL vs LD conditions. Scale bar: 20µm.



Graph 8: Crystal cell number

Graphical representation of total number of crystal cells in lymph glands of larvae entrained to constant light (LL) vs control light (LD) conditions. P-value: 0.94

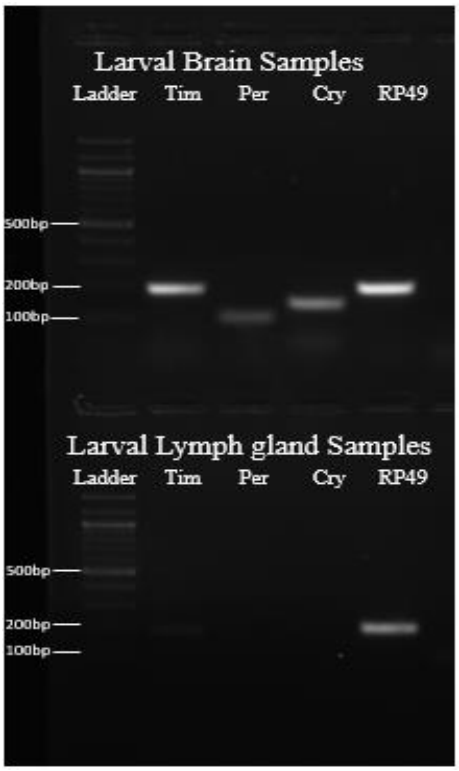
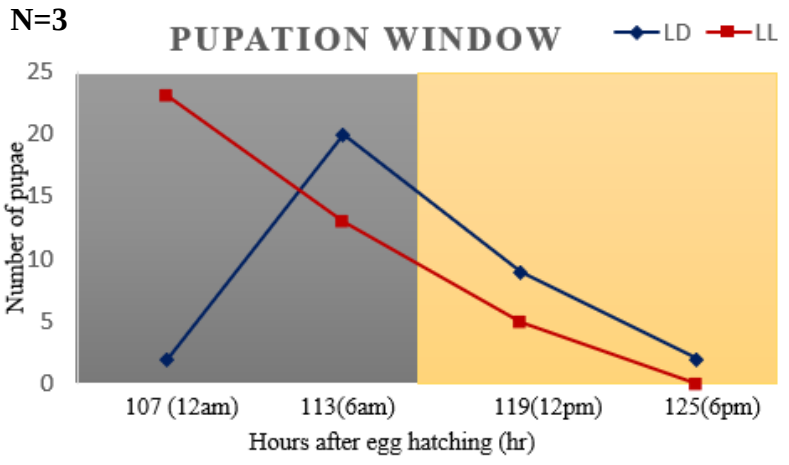
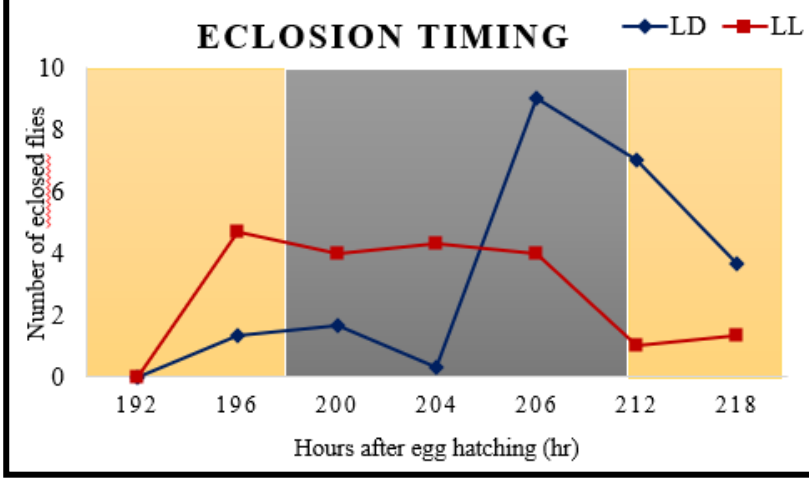


Figure 16: Absence of clock genes expression in lymph glands of *Drosophila melanogaster*

Gel image of different clock genes *timeless*, *period* and *cryptochrome* in brain and lymph gland samples. The constitutive gene RP49 is the positive control used for the experiment.

Timeless: 211 bps; Period: 101 bps; Cryptochrome: 141 bps; RP49: bps.





Graph 9: Pupation time window and Eclosion timings

Graphical representation of pupation timings and eclosion timing of flies entrained to control (LD) and experimental (LL) light conditions. The time windows for both the phases of metamorphosis is abrogated and flies become arrhythmic in constant light conditions.

hydroxyecdysone (20E). 20E binds to the nuclear receptor and initiates gene expression which is responsible for the processes of metamorphosis [103]. Ecdysone and 20E are usually used interchangeably for convenience. Ecdysteroid synthesis is arrhythmic in flies entrained to LL conditions [106]. According to Asburner *et al.* (1974), a hierarchical mode exists for the downstream expression pattern by ecdysone. The model states that ecdysone, bound to its specific receptor protein, performs two key roles: the repression of premature late gene expression and the direct activation of the early genes. Ecdysone induces activation of transcription factors, and these subsequently activate secondary response genes [Figure 17] [107]. The late response genes are thought to function as effector genes responsible for the larval and imaginal tissues toward their destined fate by activating diverse developmental pathways during metamorphosis. Kenneth Burtis extended the above model stating that ecdysone activation of early regulatory genes and the overlapping ecdysone titer spurts, directs unique patterns of late gene expression that define the morphological and functional properties of each target tissue at each stage in its development [108] [109]. The three early response genes were characterized as *Broad complex* (Br-C), *E74* and *E75.*

Our data showed a dip in the transcript levels by relative quantitative reverse transcriptase–polymerase chain reaction (RT-PCR), of the early ecdysone response genes *Br-C* and *E74* in the lysate of the larvae exposed to constant light conditions compared to the ones entrained to 12-hour light: 12-hour dark conditions [Graph 10]. There was a 25% and 15% decrease in expression of *E74* and *Br-C* respectively. The same trend was observed in the progenitor cells isolated from the lymph gland using Fluorescence assisted cell sorting (FACS) (data not shown). This result indicates a change in the ecdysone levels as the ecdysone downstream genes show deviation than control conditions.

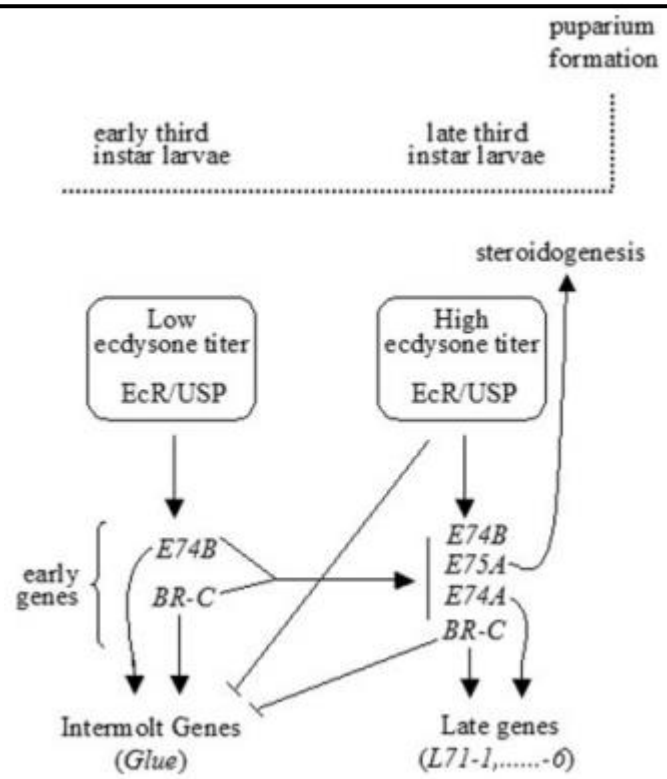
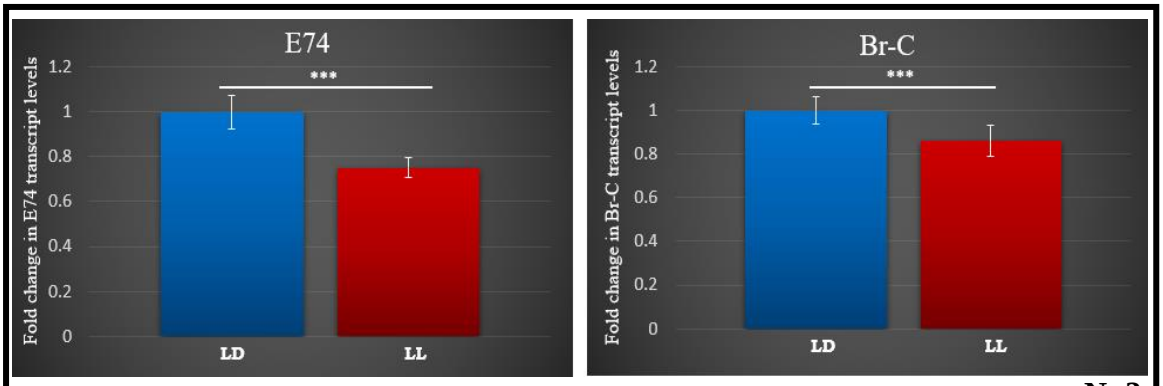


Figure 17: Ecdysone Response Genes

The figure adapted from L.R. Basso Jr. M. de C. Neves N. Monesi and M.L. Paco´-Larson 2007, shows multiple ecdysone-triggered regulatory hierarchies direct the onset of Drosophila metamorphosis. This figure summarizes regulatory interactions discussed in the aforementioned text.
[110]



N=3

Graph 10: Expression levels of early ecdysone response genes

Graphical representation of early ecdysone response genes *E74* and *Br-C.* TTEST, p-value: 0.000315172 and 0.000285068 for *E74* and *Br-C* respectively.

3.11 Levels of Prothoracicotropic hormone are downregulated in continuous light-entrained larvae

Now, that it had been established that disruption of circadian rhythm affects the expression of ecdysone response genes, more in-depth investigations were necessary to find out the root of this aberration. Ecdysone is produced in the prothoracic gland (PG), but the cue for its synthesis is given through the neuropeptide Prothoracicotropic Hormone (PTTH) [111]. Temporal signals from the central clock is transmitted via the neuropeptide, sNPF, to non-clock neurons that yield PTTH. PTTH is released in daily rhythms from the brain during larval development and is under the circadian regulation [97]. In LL conditions, PTTH release in brain ceases [106]. The fruit flies' brain and the ring gland are proximal in the fly larva, and it has been observed that PTTH neurons project onto the PG [112]. PTTH, in turn, triggers a cascade of reactions culminating in the synthesis of ecdysone [113], [114]. Ablation of PTTH neurons revealed that PTTH was not required for molting or metamorphosis, but that removal of the source of PTTH resulted in delayed development, accompanied by a low 20E titer. The clock in the PG is responsive to both, zeitgeber from the outside environment (light) and internal cues (PTTH rhythmicity). But in vivo, PTTH signal is the dominant entraining force once rhythmicity is set in and PG clock ignores the light. The eclosion of larger adults with more cells suggested that the final body size of this insect, and perhaps all insects, is a resultant of the growth regulator (20E, insulin) concentration and developmental timing cues provided by PTTH, the latter by modulating the duration of the larval feeding period [115]–[117].

Our study revealed that the expression of *ptth* gene gets significantly reduced in the larvae entrained to constant light conditions [Graph 11]. This could be the explanation of the reduction in the expression of ecdysone as to what is the real cause for the phenotype observed in animals exposed to constant light. The decrease in the source of PTTH leads to the lower synthesis of ecdysone, thus lower 20E titer levels and depressed expression of ecdysone response genes. But this hypothesis needs further experiments for validation.

3.12 Pigment dispersing factor is elevated in 24-hour light entrainment

The central pacemaker clock in *Drosophila melanogaster* resides in 150 clock neurons [118]. Among these neurons, eight pars of neurons express Pigment-dispersing factor (PDF) which modulates adult emergence [99], [119], and acts as an intercellular signal that

is necessary as a coordinator of phase and amplitude of circadian protein oscillations among the different pacemaker neurons [120]. PDF behaves as a neuromodulator in the dorsal brain that is involved in the rhythmic control of behavior [121], [122]. Loss of rhythmicity has been observed in *pdf* mutants kept in constant darkness (DD) after 2-3 days. PDF-containing neurons maintain rhythmicity by signaling the PER-containing neurons in the dorsal brain [123]. *pdf* RNA abundance shows an absence of diurnal rhythm [124], the genes *clk* and *cyc*, control *pdf* expression. In the absence of CLK or CYC product, *pdf* RNA and protein are reduced or eliminated within the clock neurons [124], [125].

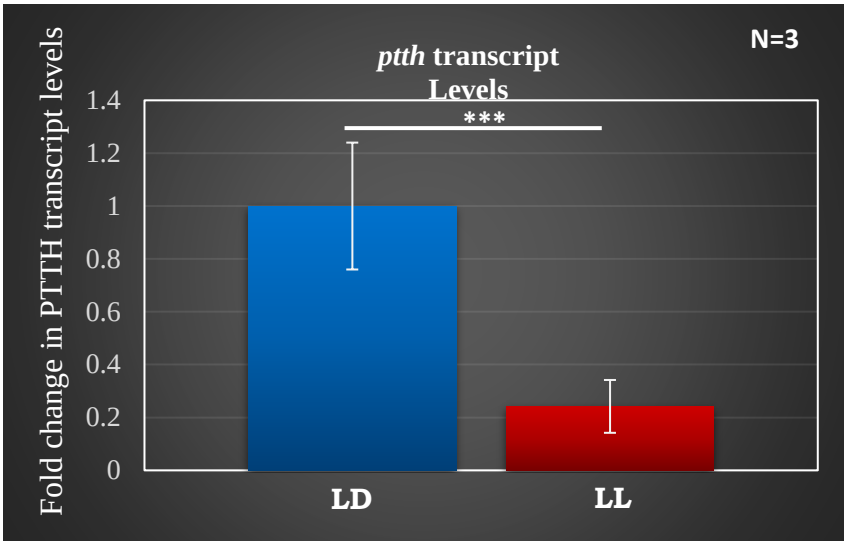
In the *Drosophila* larval brain, the PDF-expressing clock neurons known as 'lateral neurons' (LNs) project to the dorsal region of the brain and terminate in close proximity, especially in the pharate adult brains in particular, to the dendritic arbours of neurons that produce the prothoracicotropic hormone (PTTH), which controls ecdysone production by the PG [101]. Reports have shown that *Drosophila* larvae with ablated PDF neurons display an increase in PTTH periodicity and higher PTTH transcript levels [101]. Moreover, PDF presents photoperiodism and is observed to be elevated in continuous light conditions [126].

Our study demonstrates this relationship between light, PDF, and PTTH. Transcript levels of PDF were elevated in constant light (LL) compared to control conditions (LD) [Graph 12]. Immunostaining data of PDF antibody staining reaches the same conclusion. The PDF antibody intensity surges in animals entrained to a continuous light regiment (data not shown).

3.13 Proposed Model

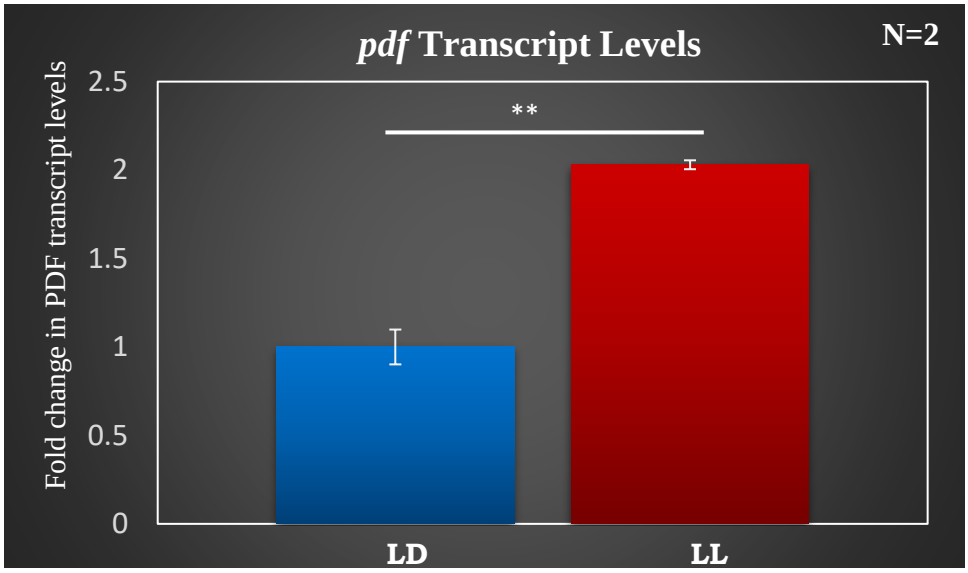
Our findings elucidate the peptidergic pathway that connects the brain clock to the peripheral PG clock to regulate the timing of ecdysone synthesis, which in turn governs the homeostasis in the zonation in lymph gland. Below is a working model of all results obtained so far [Figure 18].

But as so several processes in biology are never a straight-forward connection, there are many more layers to be added to this current hypothesis. Pooling up the already known facts from the literature, the revised version of the theory looks the following [Figure 19].



Graph 11: PTTH transcript levels

Graphical representation of a depression in *ptth* gene transcript in constant light conditions compared to control light conditions with TTEST, p-value: 9.94127E-06.



Graph 12: PDF transcript levels

Graphical representation of an elevation in *pdf* gene transcript in constant light conditions compared to control light conditions with TTEST, p-value: 0.00154

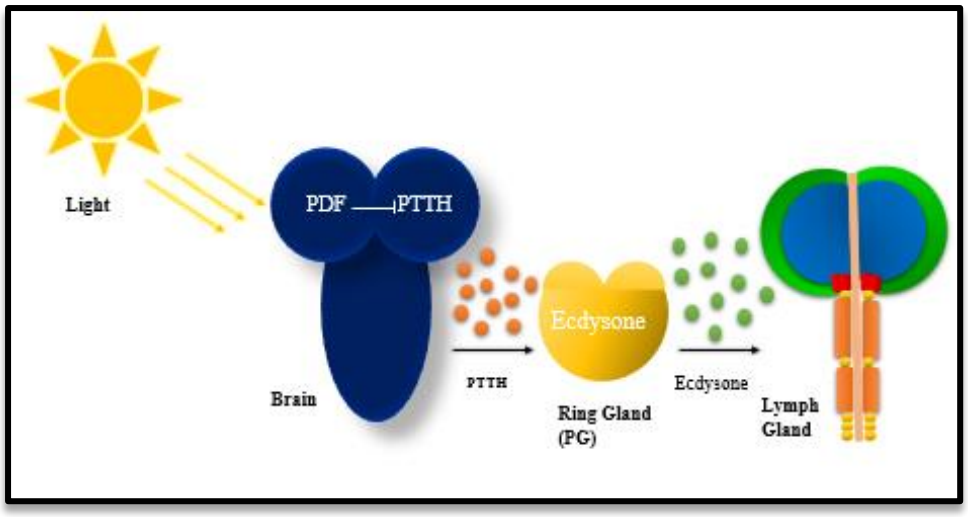


Figure 18: The working model

A schematic representation of the conclusion drawn up from this study. In the presence of light, levels of PDF increase. PDF suppresses PTTH expression thus causing a decrease in the ecdysone levels. The lowered ecdysone levels cause an elevation of the differentiation of the lymph gland.

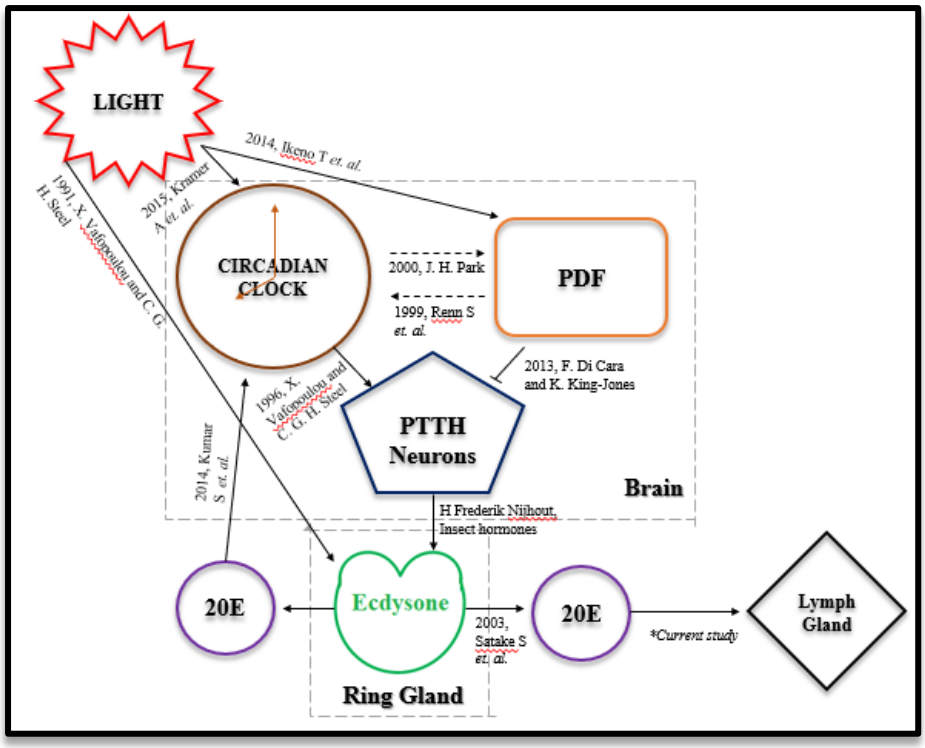


Figure 19: Peptidergic network regulating hematopoiesis in *Drosophila melanogaster*

Schematic representation of the pooled-up data from the literature along with the current study.

CHAPTER IV

FUTURE DIRECTIONS AND SIGNIFICANCE

As stated earlier, several reports are linking the process of hematopoiesis with the biological clock [69], [70], [127]. But the clear molecular link between these processes eludes us. This study is novel in its way of elucidating the molecular pathway interlinking these two; complex, yet quite interesting body functions. It helps us to take a step further in a better understanding of physiology and try to design treatments to several blood-related diseases and disorders.

Our study tried to tackle another area related to how the disrupted circadian rhythm wreaks havoc upon the process of hematopoiesis and the molecules that play assist in this damage. Due to the advent of technology and modernization, it has become impossible to 'darken oneself' in the literal meaning of the term, escape from the constant bombardment of light that is laid upon us. This has become a rising global health issue, not only for humans but also for animals and birds exposed to these conditions. As one cannot evade the clutches of bright lights, we have to should understand the costs that are associated with light pollution. Once we know the wirings of the diseases that can be caused under such circumstances, we could work towards finding out precautionary measurements and cures for them.

Literature indicates the occurrence of developmental defects upon disruption of ecdysone-controlled differentiation and proliferation in multiple tissues, thus aiding as models to study cancer development [128], [129]. Recently, the human ortholog of ecdysone gene hEcd has been identified, as a binding partner of p53 for maintaining stability and function of p53 [130]. Thus, understanding the role of ecdysone is imperative if we need to follow in greater details the onset of cancers.

CHAPTER V

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