Sensitivity of activity/rest rhythms to temperature in populations of *Drosophila melanogaster* selected for divergent phases of adult emergence

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by

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Certificate

This is to certify that this dissertation entitled **Sensitivity of activity/rest rhythms to temperature in populations of *Drosophila melanogaster* selected for divergent phases of adult emergence** towards the partial fulfilment of the BS-MS dual degree programme at the Indian Institute of Science Education and Research, Pune represents study/work carried out by **Shephali Dansana** at Jawaharlal Nehru Centre for Advanced Scientific Research(JNCASR), Bengaluru under the supervision of Dr. Sheeba Vasu, Professor, Neuroscience Unit, JNCASR, Bengaluru during the academic year 2019-2020.

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This thesis is dedicated to

My friends and family

Declaration

I hereby declare that the matter embodied in the report entitled **Sensitivity of activity/rest rhythms to temperature in populations of *Drosophila melanogaster* selected for divergent phases of adult emergence** are the results of the work carried out by me at the Neuroscience unit, Jawaharlal Nehru Centre for Advanced Scientific Research, Bengaluru under the supervision of Dr. Sheeba Vasu and the same has not been submitted elsewhere for any other degree

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Abstract

Circadian clocks are endogenous time keeping mechanisms which help organisms to schedule their biological and physiological processes at proper times of the day. In absence of the any cyclic environmental cues, the circadian clock shows rhythms with an intrinsic period of approximately 24 hr. In the presence of cyclic environmental cues, the clock adjusts the period and phase of its oscillations such that particular phases of the overt behavior occur at specific phases of the environmental cycle. Examples of such kind of behaviors which are regulated by the circadian clocks are sleep/wake, cycle in humans, feeding rhythms, eclosion rhythm and locomotor rhythm in fruit-fly *Drosophila melanogaster.*

The phenomenon of emergence of adult insects from pupal cases termed 'eclosion' is regulated by the circadian clock such that maximum emergence occurs at dawn after lights-ON. In a continuing long-term selection study, our laboratory has derived 'early' and 'late' stocks of *Drosophila melanogaster*, exhibiting preference for emergence during morning and evening respectively. Recent results from the lab have shown that flies that have been selected for evening emergence (*late* stocks) are associated with the evolution of enhanced temperature sensitive clocks with respect to eclosion rhythm.

On the other hand, locomotor behavior in *Drosophila* is a bimodal rhythm with a morning peak around dawn and an evening peak around dusk and is thought to be regulated by the circadian clock. Previous results have also shown increased temperature sensitivity of the clock which regulates the evening bout of activity in the locomotor behavior.

By using a series of combinations of regimes where light/dark and temperature cycles were phased apart, we asked if the phase of evening peak of locomotor activity of these flies (which have been selected to emerge in the evening) more closely tracks temperature cycles rather than light/dark cycle in comparison with controls.

We found that the evening bout of activity of *late* flies tracks temperature cycles more efficiently compared to the *early* and *control* flies, although only for a limited range of light-temperature phase relationships, i.e., for the smaller phase differences between the light and the temperature cycle. However, for larger phase differences, it can be concluded that across stocks, the clock that drives activity rhythms is strongly

Influenced by light cycles rather than temperature or that the clock is responding to light and temperature equally.

Chapter-I

1.Introduction

1.1 Circadian rhythms:

Almost all organisms on the surface of earth are subjected to the daily rhythm of light-dark and also to seasonal climate changes. Trees flower and fruit at specific seasons of the year, flower petals open and closes based on the time of the day and organisms remain active only at a particular time of the day. It was thought that all these biological rhythms were in response to the cyclic temporal changes taking place in the environment. But studies done by a French astronomer De Mairan in 1729 suggested the possibility of having endogenous origins for such kind of biological rhythms (D'Ortous de Mairan, 1729). He studied the rhythmic movement of leaves of the Mimosa plant and found out that there is persistence of leaf movements in the absence of any kind of cyclical changes and he suggested that this rhythmic behavior is not just a passive response to the periodic changes to the environment, but is endogenous to the plant. Under constant conditions (in the absence of any external cues), such rhythms that persisted do not have a periodicity exactly equal to that of the earth's rotation (24 hours) and is always slightly longer or shorter, which suggests the presence of an endogenous mechanism giving rise to the behavioral rhythms (Pittendrigh, 1965).

These approximately 24-hour rhythms exhibited by the organism are called 'circadian rhythms'. The term "circadian" derives from the Latin phrase "circa diem"(circa-about + diem-day) which means "about a day" (Pittendrigh, 1965). Circadian rhythms are present in various behavioral and metabolic processes and are conserved ranging from cyanobacteria, fungi, plants to multicellular animals and are an innate response to the organism (Tarrant and Reitzel, 2013). These rhythms are generated by a biological timekeeping mechanism called the "circadian clock". Apart from the circadian rhythm, there are other rhythms which have periods other than 24 hours for example, circa tidal rhythms-rhythms that are synchronized by the tidal currents with a period of

Around 12 hr (Horacio O. de la Iglesia and Carl Hirschie Johnson, 2013) and circalunar rhythms-rhythms that are synchronized by the lunar cycle (period- 29.5 days hr)(Raible et al., 2017). Rhythms which have a period shorter than 24hr are called as ultradian rhythms, for example- respiration, heartbeat etc., while rhythms with longer periods are called as infradian rhythms (Laje et al., 2018). Amongst these, circadian rhythms are the most extensively studied and the best-understood rhythm.

1.2 Circadian clocks:

The endogenous time-keeping mechanism which generates circadian rhythms is referred to as circadian clock. The circadian clock times the internal functioning as well as the external behavior of the organism. This system helps the organism to anticipate the changes that are taking place in the environment and prepare accordingly ensuring that the organism will perform its activities at a suitable time of the day. It also provides temporal organization inside the organism which ensures coordination in the functioning of the metabolic processes.

The basic characteristic of circadian rhythms is that they persist under constant environmental conditions (in the absence of any outside environmental cues) and thus it is said to be endogenous to the organism (C. Pittendrigh,, 1960). The intrinsic period of the endogenous rhythms varies from organism to organism and is called the free-running period (FRP), which is close to but not exactly 24 hours.

The synchronizing cues present in the environment, commonly called *Zeitgeber* ('time-giver' in German) adjust the endogenous biological rhythms such that the organism synchronize or entrains its endogenous clock to the local 24hr cycles of the environment. The circadian clocks are able to adjust its period and phase of rhythms such that the period of the clock matches that of the period of the zeitgeber (C. Pittendrigh, 1960). This mechanism of synchronizing the endogenous rhythm with that of the external time cues is called "*entrainment*". As a result of entrainment, the rhythms driven by the clock and the zeitgeber attains a stable phase relationship such that particular phase of the circadian rhythms occurs only at specific phases of the

zeitgeber and thus the timing of various biological rhythms occurs at specific time of the day.

The daily cycle of light and dark is one of the most important zeitgeber for many organisms. Apart from the light-dark cycle, temperature, humidity and also various other environmental factors which have daily repetitive features play a role in synchronizing the endogenous rhythms of the organism to that of the external environment.

Another important feature of the circadian rhythms is temperature-compensation of their periods. Many of the chemical reactions taking place in our body are regulated by the increase or decrease in the temperature and this rule applies to mostly all biological processes. But the circadian clock maintains its FRP despite changes in the ambient environmental temperature within the physiological limits (Pittendrigh, 1965). However, exposure to light or temperature pulses of short durations can perturb circadian rhythms, either advancing or delaying the phase. The magnitude and direction of phase shifts is determined by the current phase of the rhythm (phase without exposure of any light and temperature pulses), intensity and duration of the zeitgeber pulse. These shifts in the phases of the circadian rhythms gives rise to a curve called as Phase Response Curve (PRC). Before a stable phase shift, the rhythm may continue to give rise to multiple shifts called as 'transients. These mechanisms are compared to the self-sustained physical oscillators, and thus, the idea of circadian clock similar to the physical oscillators, was widely believed and supported.

To summarize, a circadian clock is ubiquitous and defined as an endogenous, temperature compensated mechanism which has a fixed periodicity, and can be entrained by external cues.

1.3 Adaptive significance of circadian clock:

Darwinian fitness is defined as "the capacity of a variant type to displace the resident genotype in competition for the available resources and fitness" (Demetrius and Ziehe, 2007). According to Darwin, organisms inherited with genotypes which gives rise to greater survival and reproductive success are preferred more by the natural selection than the organisms who lacks those genotypes. These traits which help organisms fit

to their environment, enhancing their reproductive and evolutionary fitness are said to be adaptive (Gardner, 2017). Thus, any phenotype which gives the organism any kind of fitness advantage is selected by natural selection. The fact that the circadian clock times the various behaviors of the organism to particular times of the day helps the organism to perform the functionally relevant task at the proper time of the day. This function is advantageous for the organism for a variety of reasons, such as finding mates, avoiding predators and also helps the organism to utilize the resources available in the environment at a particular time (Moore-Ede, 1986). Thus, it is believed that the circadian clock confers adaptive advantage to living beings by timing their various biological functions at favorable time of the day. It also helps the organism in avoiding certain behavior at certain times of the day. These mechanisms of the circadian clock thus, appears to be naturally selected (Cloudsley Thompson,, 1960).

Circadian clocks are believed to give advantage to organisms in two ways. One by establishing an internal temporal order among various physiological cycles within the organism referred to as ‘intrinsic advantage’ and the other by facilitating entrainment of circadian rhythms with the external cycles, known as the ‘extrinsic advantage’.

1.3.1 Intrinsic advantage hypothesis:

At the organism level, Circadian rhythms are observed in the activity/rest, sleep-wake pattern, core body temperature etc., whereas at the cellular level, the rhythms are observed in various physiological cycles, for example, the blood-sugar level, transportation of various hormones and molecules from one part of the body to another (Halberg, 1960). Such physiological processes seem to follow a sequence of events and this sequence of events is controlled by the central clock (Halberg, 1960) and any disruption of such sequence could lead to disturbances in the harmony of the system which can further cause adverse fitness consequences. Thus, the circadian clock confers an intrinsic adaptive advantage to organisms by timing internal physiology and metabolism and by creating a temporal segregation between all the molecular processes taking place inside the body of the organism (Vaze and Sharma, 2013).

1.3.2. Extrinsic advantage hypothesis:

In nature, a lot of biotic and abiotic factors are present. Influence of these factors creates various favorable and unfavorable conditions for the organism (Vaze and Sharma, 2013). The organism must remain in-sync with the cyclic environment to perform various behaviors at the appropriate time of the day, for example, procurement of food when it is available, avoiding the predators etc. Thus, the circadian clock is advantageous to the organism by phasing the behavior appropriately under cyclic environmental conditions. Therefore, the synchrony of an organism with its internal and the external environment is very crucial for the survival of the organism which further indicates the importance of the circadian clock (Vaze and Sharma, 2013).

1.4. Different types of circadian rhythms:

As mentioned, these circadian clocks allow the organism to predict the cyclic changes taking place in the environment and thus increase their fitness. It provides the organism time for activities like feeding, sleeping and for reproduction (Squire, Larry R ,Zigmond and Bloom, 2008). In *Drosophila melanogaster*, it times eclosion, activity-rest behaviors, egg-laying behavior, feeding behavior etc (Tataroglu and Emery, 2014). Thus, in order to understand the mechanism of rhythm generations, some of these behavior are widely studied in *D.melanogaster*.

1.4.1. Eclosion:

Eclosion is defined as the emergence of the adult fly from the pupal case. This behavior has been very successfully used by C. Pittendrigh for the study of circadian rhythms. It has been observed that eclosion in *Drosophila* mostly occurs during dawn and the emergence decreases as the day follows. It has been shown that this behavior is under the regulation of circadian oscillators in *Drosophila pseudoobscura* (Pittendrigh, 1954) and study of circadian rhythms on this species has given very useful insight to understand the mechanism of circadian clock function. Experiments done on this behavior give information about the temperature-independent components of the timing system, which is one of the fundamental properties of the

circadian system (Pittendrigh, 1954). This behavior was also used to screen for and identify mutations in genes which are components of the molecular clock, the first of which was the *period* gene (Konopka and Benzer, 1971) followed by *timeless*- another core clock gene (Sehgal et al., 1994) However, eclosion is a population level rhythm and the act of eclosion occurs once in a lifetime. To study individual level rhythms, adult locomotor assays are a widely studied rhythm in *Drosophila melanogaster*.

1.4.2. Locomotor behavior:

This behavior is observed in adult *Drosophila* and is an individual level rhythm. *D.melanogaster* shows bimodal activity-rest behavior in LD (light: dark) 12:12 condition, such that one bout of activity occurs around lights-ON and a second bout of activity occurs during the lights-OFF. Locomotor behavior has been used to identify various cells that are part of the circadian clock and gives rise to the bouts of activity. Apart from this, the function of *per* (one of the core clock genes) has also been observed in this behavior, further supporting the role of *period* as a core clock gene (Konopka and Benzer, 1971). This behavior is measured using DAM (Drosophila Activity Monitor), the details of which are mentioned later.

Both these behaviors are used as circadian read-outs to determine the period of the internal circadian clock, the effects of various types of light inputs on the circadian clock, to understand the molecular mechanism of the clock and various other aspects of the circadian clock and circadian rhythms. Since the intrinsic period of the organism does not exactly match the period of the environmental cycle, the zeitgeber contributes to the entrainment of the rhythms by either delaying or advancing the phase of the circadian clock. This further indicates the importance of external factors in the behavior of the organism (Volpato and Trajano, 2005).

1.5 Circadian rhythms under different zeitgebers

1.5.1. Circadian rhythms under light as a zeitgeber:

As mentioned, biological rhythms are entrained by the environmental cues such that even a single light or a temperature pulse can easily entrain the circadian clock and the clock can gradually align itself to a new cycle. Almost all the behavior including eclosion and locomotor behavior are also entrained by the environmental cue. Out of all the environmental cues, the light-dark cycle is considered as the major entraining zeitgebers. Under LD 12:12 condition, two bouts of activity are observed- the morning bout of activity and the evening bout of activity which occur during dawn and dusk respectively (Rosato and Kyriacou, 2006).

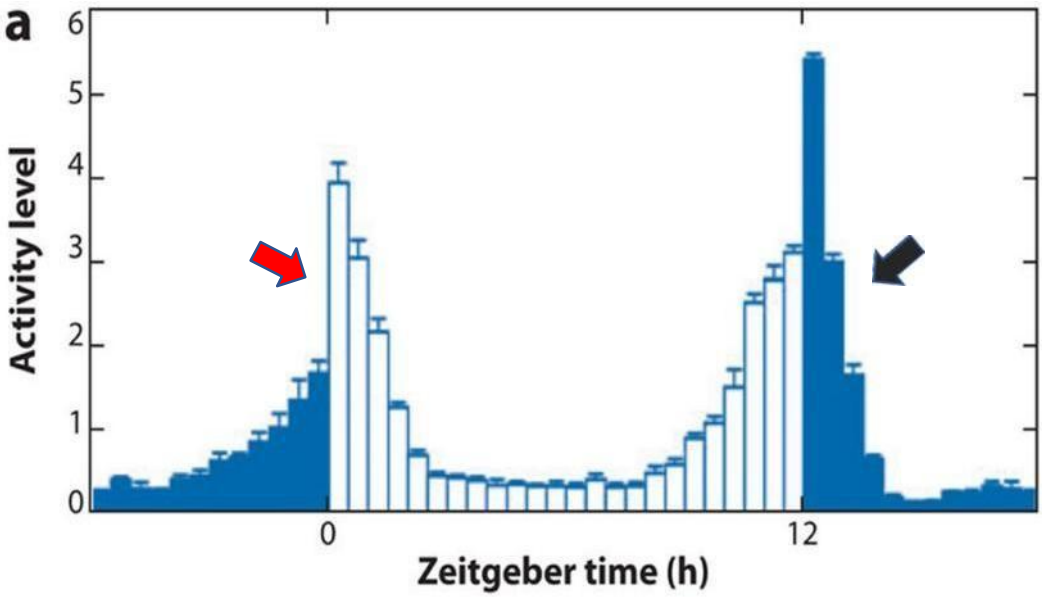


Figure 1: Representative profile of *Drosophila* circadian locomotor behavior. Zeitgeber Time 00 represents the time of lights-ON under a LD12:12 regime. ZT12 represents 12 hours after the lights-ON. Blue shaded bars represent the dark phase, non-shaded bars represent light phase. The red arrow depicts the morning bout of activity and the black arrow depicts the evening bout of activity (Ravi Allada, 2010).

But with variation in the photoperiod (light phase duration) flies show a change in phase of the activity bouts. With longer photoperiod (increase in day length) there is an increase in phase between the morning and the evening bout of activity and vice versa (Rieger et al., 2007). Also, the behavior of the flies under rectangular LD cycle (lab like environmental condition) shows direct response to the change in lights (the activity bouts were very close to lights-ON and lights-OFF). With longer photoperiod the M-peak follows the lights-ON unlike the evening peak, which did not track the lights-OFF (Rieger et al., 2012). The behavior of the flies changes also with respect to the change in intensity of light. Results suggest that flies tend to be more active in dim light in comparison to high light intensity (Rieger et al., 2007) (Rieger et al., 2012).

1.5.2. Circadian rhythms under temperature as a zeitgeber:

Apart from the light-dark cycle, temperature also acts as a very powerful zeitgeber in natural as well as in laboratory conditions in organisms starting from fungi to vertebrates (Rensing and Ruoff, 2002). Too high temperature during the day may be dangerous for the organism and too low temperature during the night can lead to immobility. Thus, optimum temperature conditions are required for the proper functioning of all the behavior of the organism. It has been observed that in the case of low temperature (18^{o} C), the two bouts of activity come closer to each other and in case of higher temperature (29^{o} C) the bouts of activity are far from each other. Both of them are in comparison to the phases at 25^{o} C (Majercak et al., 1999) (Miyasako et al., 2007)

Both temperature cycles and constant ambient temperatures have different effects on the behavior of the organism. Previous experiments on *Drosophila melanogaster* have shown that temperature cycles (TCs) alone are powerful enough as a zeitgeber to shift the phases of the bouts of activity in activity-rest behavior (Yoshii et al., 2009). It has also been shown that there is a difference in the phasing of the activity-rest pattern of the fly in case of rectangular TC and in case of nature like TC. The phase relationship between the morning and the evening bout of activity in case of locomotor behavior is also affected by the overall mean temperature of the environment (Yoshii et al., 2009).

1.6. Neuronal and molecular basis of the circadian clock in Drosophila:

1.6.1. Neuronal basis of the circadian clock:

As mentioned earlier, rhythms of organisms in constant conditions (no external cues) free run with their endogenous period. Although the molecular clock is present in many cells and tissues, but the free-running rhythm of locomotor activity is generated by a circadian pacemaker or central oscillator which is present in the brain. It also controls various other behavioural circadian rhythms and other peripheral oscillators. It is localized to specific neural structures, which includes the optic lobe of the cockroaches (Lubinski and Page, 2016), and the hypothalamus of various birds and mammals (Takahashi and Menaker, 1982). All the neuronal subgroups in the fly brain which have been identified as the circadian pacemaker neurons express core clock genes- *period* (PER), *timeless* (TIM) and the neurotransmitter Pigment Dispersing Factor (PDF). The group of neurons are divided into two groups- LN (lateral neurons) and DN (dorsal neurons). The lateral neurons consist of ventral lateral neuron (LNv) and dorsal lateral neurons (LNd) (Kaneko and Hall et al, 2000). LNv consist of four group of neurons called small ventral lateral neurons (s-LNv) which express both PER and PDF (Helfrich-Forster et al, 1997). The DN neurons are further subdivided into DN1, DN2 and DN3. The DN1 and DN2 neurons also express PER (Kaneko and Hall et al, 2000). Altogether this network of ~150 neurons forms the pacemaker of the *Drosophila* circadian system.

1.6.2. Molecular basis of the circadian clock:

The molecular mechanism which gives rise to various circadian rhythms are due to interactions between the various core clock genes and proteins. Clock genes that have been identified till now are *period (per)*(Konopka and Benzer, 1971), *timeless (tim)* (Sehgal et al., 1994), *clock (clk)* (Allada et al., 1998), *cycle(cyc)* (Rutila et al., 1998), *doubletime (dbt), cryptochrome(cry)*(Stanewsky et al., 1998), and many others. It is believed that the function of the circadian clock is regulated by a rhythmic transcription-translation feedback loop. The feedback loop is regulated by the PER/TIM dimer and

the CLK/CYC dimer (Hardin et al., 1990). The heterodimer CLK/CYC binds to specific promoters and drives the expression of *per* and *tim* from the mid-day till early in night (Darlington et al., 1998). The per and tim transcripts level peaks during the early night time but the protein levels of PER and TIM peaks at late night (Hardin et al., 1990). The PER/ TIM complex transports inside the nucleus where the PER represses the transcription of CLK/CYC. However, in the presence of light, CRY protein degrades TIM and thus the PER also gets unstable and is also degraded, leading to the functioning of CLK/CYC transcription (Busza et al., 2004). Such translation-transcription feedback loop give rise to mRNA oscillations of these core clock genes giving rise to overt rhythmic behaviors. Disturbance or delay in the accumulation of the PER and TIM might lead to disturbance in generating a stable 24-hour period rhythm (Tataroglu and Emery, 2015)

1.7 Organization of the circadian clock:

1.7.1 Morning and Evening oscillator model:

It is known that under a 12:12 light-dark cycle, the activity/rest rhythms of *D.melanogaster* shows a bimodal profile with a morning bout of activity occurring during dawn and an evening bout of activity occurring close to dusk (Helfrich-Förster, 2001). There is also presence of anticipatory activity before the light-ON and before lights-OFF. The dual oscillator model has been originally developed for nocturnal rodents and it assumes two separate circadian oscillator (M and E oscillator) that gives rise to activity and responds differently to light (Pittendrigh and Daan, 1976). It has been found out that in *D.melanogaster* , different subsets of clock neurons are responsible for the morning and evening bout of activity(Stoleru et al., 2004). the M-cells give rise to the morning peak and the cells called the E-cells regulate the evening peak (Grima et al., 2004; Stoleru et al., 2004) . The PDF (pigment dispersing factor) expressing cells-the sLNvs are thought to constitute the M-cells and it also determines the phase and amplitude of the morning activity under a LD 12:12 regime. On the other hand, the PDF negative and CRY positive, 3 LNds and the 5th sLNv together constitute the E-cells (Grima et al., 2004). All the cells have different functions with respect to the

presence and absence of zeitgebers. Together, activity/rest rhythms in *D. melanogaster is* regulated by a neuronal network which is composed of distinct sets of neurons and it regulates the overt rhythm of the behavior under different environmental conditions.

1.7.2 A and B oscillator model:

According to this model, there are two oscillators-the A-oscillator and the B-oscillator. The A-oscillator is self-sustaining and can be entrained by the light regime of the environment but is temperature independent. It is also coupled with and drives the B-oscillator. The rhythmic behavior of the B-oscillator in the system is directly reflected in the fly's overt rhythm, which means the phases of the overt rhythms of the fly matches with the phase of the B-oscillator. The B-oscillator is temperature-sensitive in the way that it can be entrained by the external temperature cycles and the period of the B -oscillator is also temperature-dependent. It was also predicted that there is also partial feedback from the B-oscillator to the A-oscillator. This model was predicted based on the experiment performed by Pittendrigh and Bruce in 1959 on the adult eclosion rhythm in *Drosophila pseudoobscura* using a set of conflicting zeitgeber experiments (in the presence of two zeitgebers). They were intended to find out the relative strengths of the light-dark cycle and the temperature cycle in the entrainment of the oscillators which controls the adult eclosion phenomena in *Drosophila pseudoobcura* (Pittendrigh and Bruce et al, 1959).

1.8 Rational for the present study:

The circadian clock regulates various types of rhythmic behavior taking place in our day to day life. Sleep-wake pattern is one such behavior regulated by the clock. In a given population, this pattern differs from individual to individual such that some individuals are more active during the morning hours-get up early and go to bed early whereas, the others are more active in the late afternoon or at night (Roenneberg et al., 2003). This individual difference in the timing of sleep/wake schedule is described in terms of 'chronotypes'. Individuals who have earlier sleep wake schedules are called as the 'morning type' or the 'early-types' and are usually labeled

as ‘larks’. Their alertness and cognitive performance peaks early in the morning. On the other hand, individuals with a later sleep wake schedules are called as ‘evening type’ or ‘late-type’. This evening type chronotypes are usually called as ‘owls’ and evening is the peak performance time for individuals of this chronotypes (Baehr, 2000).

Although the terms-larks and owls are used metaphorically to represent the human chronotypes, it can also represent two extreme distribution in any diurnal behavior. For example, fruit flies show diurnal rhythm in the adult emergence and is known to be under the regulation of the circadian clock. Under the laboratory LD 12:12 condition, most of the emergence occurs during the lights-ON followed by a decrease in the rate of emergence as the day progresses. Previously there have been studies associating the period of the clock to the morning- evening chronotype, which suggests the involvement of circadian clock in the variation of the sleep-wake schedules (Baehr, 2000) (Roenneberg et al., 2003) (Duffy et al., 2001). To understand the mechanism of how the clock regulates the different chronotypes, in a continuing long-term selection study, our laboratory has derived ‘*early’* and ‘*late*’ stocks of *Drosophila melanogaster,* exhibiting preference for emergence during morning and evening, respectively (Kumar et al., 2007).

In this selection study, the ‘*early’* stocks of Drosophila have been selected to emerge during a 4-hr window in the morning, whereas the ‘*late’* stocks have been selected to emerge during a 4-hr window in the evening. These *early* and *late* populations are derived from the *control* population. As a response to selection, the *early* flies exhibit a preference of emerging in the morning and the *late* flies in the evening. The *early* flies have shorter free-running period (~23.4 hr) while the *late* population has a longer free-running period (~24.5 hr) compared to the free running period of the *control* population (~23.8 hr). The emergence waveform of the early stocks was advanced and the late stocks were delayed relative to the *control* which suggests differential entrainment of the underlying circadian clocks of the flies of the *early* and *late* stocks which is observed in the free running periods of the stocks.

All the three stocks are maintained in LD 12:12 (~70lux) and at ~25^{o}C (details of the selection protocol can be found in the methods and materials). Recent experiments done on these population has demonstrated that the flies which have been selected

for evening emergence (the *late* stocks) is associated with co-evolution of enhanced temperature sensitivity in their clock circuits for the adult emergence rhythms (Abhilash et al., 2019a). On the other hand, previous results on the same population have shown increased temperature sensitivity of the clock which regulates the activity/rest behavior. This increased sensitivity was predominantly observed in the evening bout of activity in comparison to the morning bout of activity. (Abhilash et al., 2019b). Considering the above results, we asked if the phase of evening peak of locomotor activity of these populations of flies more closely tracks temperature cycle rather than light/dark cycle in comparison with *control* populations. We intend to observe the phases of these populations in the presence of both the temperature cycle and the light- dark cycle (conflicting zeitgeber conditions).

Chapter-2

2.Methods and materials

2.1. Selection protocol employed to derive early and late populations:

All the experiments are done using 4 large, outbreeding, genetically independent sets of population, *early* (1-4), *control* (1-4) and *late* (1-4) where the *early* populations have been artificially selected for high morning emergence and the *late* populations has been selected for high evening emergence. These populations henceforward will be referred to as 'stocks'. Figure-2 represents a schematic of the selection protocol followed to derive *early* and *late* populations from *control* populations. The *early* and *late* stocks were initiated such that block 1 of *early* and *late* (*early*1 and *late*1) were initiated from block 1 of *control* (*control 1)* and similarly for the other three blocks, and thus all the *early* and *late* stocks share a common ancestry with their *control* stocks. All the 12 populations (4 populations each of *early, control* and *late*) were maintained on a 21 day discrete generation cycle. They were maintained in plexiglass cages of dimension 25 × 20 × 15 cm with ~1500-2000 individuals per cage (sex ratio ~1:1) and were provided with banana-jaggery medium as food.

Every generation of the populations were provided with food and yeast (to increase fecundity) for two and a half days before egg collection. ~300 eggs were collected and put into culture vials (16 vials for *control*, 24 for *early* and 48 for *late*) containing the banana jaggery food. They are maintained in a light, temperature and humidity-controlled cubicle under LD 12:12 (light for 12 hours and darkness for 12 hours) at 25 ± 0.5 ^{o}C temperature and 65 ± 5% relative humidity. On the 9th -13th day after the egg collection, the flies emerging in a four-hour window-from 3 hours before lights-ON to 1 hour after lights-ON or from ZT21-ZT01(where ZT is the zeitgeber time and ZT00 refers to the time of lights-ON) are collected to form the next generation of *early* flies. Whereas the flies that are collected from ZT09-ZT13 form the next generation of the *late* population. The *control* population is formed from the flies that are collected throughout the day, without any selection imposed on the timing of emergence.

To minimize the effects of non-genetic inheritance due to different selection regimes, all the populations were passed through one generation of standardization (Bonduriansky and Day, 2009). These populations are referred to as standardized populations. No selection on timing of emergence was applied during the collection of these flies. The population size is kept constant at ~1500-2000 flies for all the population. All the experiments were done on the progenies of the standardized populations. Thus, our populations are maintained on a 21-day discrete generation cycle and the flies used in all the experiments have undergone over 300 generations of selections.

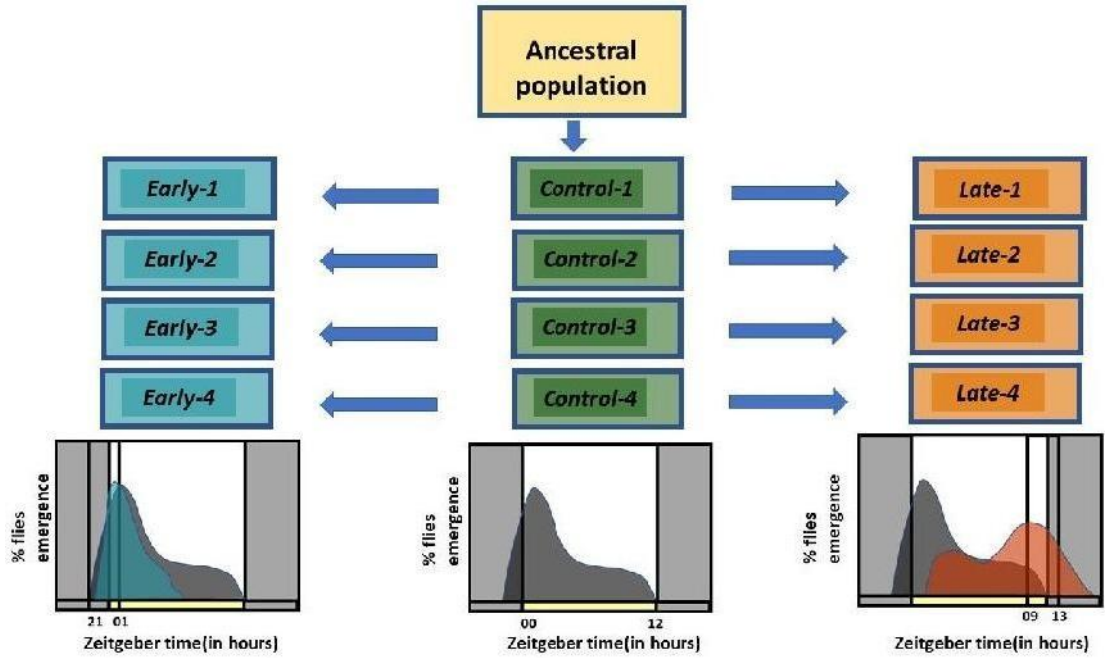


Figure 2: Schematic of laboratory selection protocol employed to derive the *early* and *late* populations. Zeitgeber Time 00 or ZT00 represents the time of lights-ON under a LD12:12 regimes. ZT21-01 represents the morning window during which flies for *early* populations are collected and ZT09-13 represents the evening window during which flies for *late* populations are collected. The emergence profile in the black depicts the *control* emergence profile, blue depicts the emergence profile of *early,* and red depicts the emergence profile of *late* populations.

2.2 Behavioral experiments:

Approximately 300 eggs from each of the populations were collected and were kept in 5-10 vials and were maintained under the standard regime. From those flies, 3-5-day old virgin males (n = 32 per population) were collected and were transferred into 5mm locomotor tubes. One side of the tube was closed with food while the other side was closed with cotton plugs. The locomotor tubes were then loaded into *Drosophila* Activity Monitor (DAM) and the data was recorded using the system. The monitors were kept inside metal boxes inside which LD 12:12 regimes were maintained using timers. The light intensity used during the experiments was ~70 lux. The boxes were kept inside incubators which were programmed to set temperature cycles TC 12:12 (thermophase: 28 ^{o}C; cryophase: 21 ^{o}C) which were variously phased with reference to the LD 12:12 cycle. Details of the different experimental regimes that were used are provided in the table below:

Phase difference		
between light	**Light conditions**	**Temperature conditions**
and temperature		
4 Hour	**Lights ON- ZT00**	**Thermophase ON 28 ^{o}C- ZT04**
	Lights OFF- ZT12	**Cryophase ON 21 ^{o}C- ZT16**
8 Hour	**Lights ON- ZT00**	**Thermophase ON 28 ^{o}C- ZT08**
	Lights OFF- ZT12	**Cryophase ON 21 ^{o}C- ZT20**
12 Hour	**Lights ON- ZT00**	**Thermophase ON 28 ^{o}C- ZT12**
	Lights OFF- ZT12	**Cryophase ON 21 ^{o}C- ZT00**
16 Hour	**Lights ON- ZT00**	**Thermophase ON 28 ^{o}C- ZT16**
	Lights OFF- ZT12	**Cryophase ON 21 ^{o}C- ZT04**

20 Hour	**Lights ON- ZT00**	**Thermophase ON 28 ºC- ZT20**
	Lights OFF- ZT12	**Cryophase ON 21 ºC- ZT08**
24 Hour	**Lights ON- ZT00**	**Thermophase ON 28 ºC- ZT00**
	Lights OFF- ZT12	**Cryophase ON 21 ºC- ZT12**

Table-1: Table depicts the various light regimes that are used in this study. Column 1 denotes the phase difference between the light and the temperature. Column 2 gives information about the time of onset and offset of time (in terms of zeitgeber, ZT00-lights-ON). Column 3 is information about the onset and offset of temperature.

For the first 4-5 days, all the flies of all the populations were subjected to only LD 12:12 cycle with constant 21 ºC after which the temperature cycle was introduced. The temperature cycle was adjusted such that the temperature started to rise (28 ºC) four hours after the lights-ON (in case of regime-I). Similarly, the temperature started to fall (21 ºC) four hours after the lights-OFF. Similar set up was done for all the regimes keeping the phase difference between the LD and TC into consideration. The flies were kept in LDTC condition for ~12 days. After this, the flies were subjected to DD (constant darkness) at constant 21 ºC for 5-6 days in which the flies were maintained in free-running conditions for the estimation of period of the circadian clock of the flies.

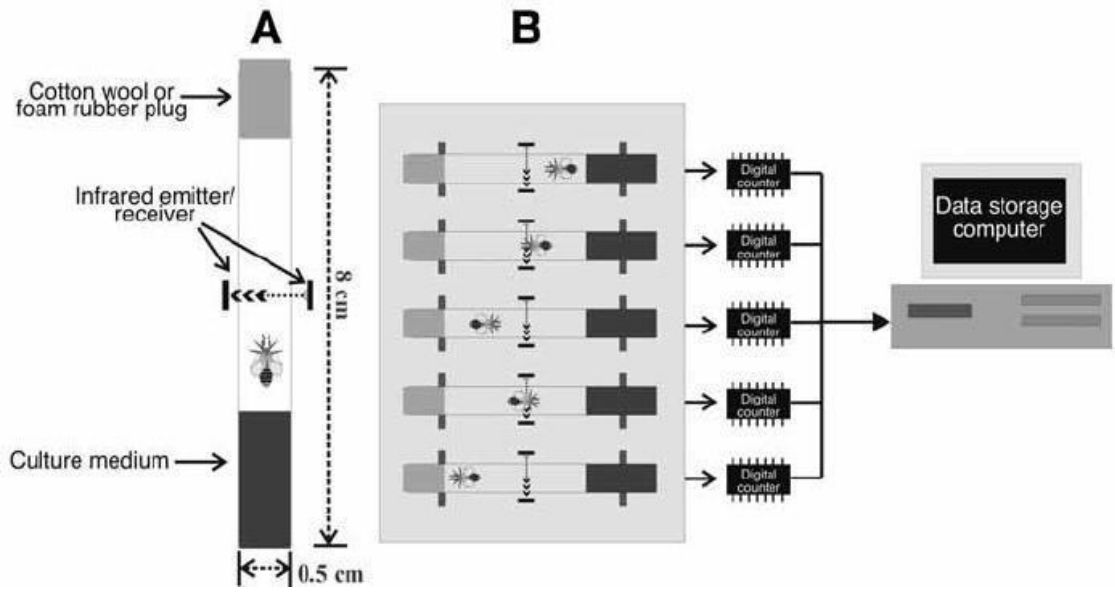


Figure 3: Schematic of the *Drosophila* Activity Monitor system (DAM). A) Schematic of the locomotor tube where one side of the tube is filled with the food medium and the other side of plugged using a cotton plug. B) The tubes are then loaded into DAM monitors through which infrared light is passed. The monitors are connected to the computer system and data is recorded (Mauro A. Zordan, 2007).

2.3. Data analysis:

2.3.1 Activity profiles:

Activity data from the recordings were used to plot the activity profiles for all the population. Raw DAM data was scanned and were saved in 20-mins bins and were analyzed using Rhythmically (Abhilash and Sheeba, 2019). Individual profiles were downloaded and were analyzed using 20-mins bins. The activity profiles were plotted using the activity counts of all the flies for each block averaged across days. The profiles were made separately for the 1st 5-days (21 ^{o}C LD) and for the next 12 days (LDTC) which will give us insights about the difference in activity in the absence and presence of temperature cycle.

2.3.2 Centre of Mass calculation:

The Centre of Mass (CoM) was used as an objective phase marker to quantify the changes observed in the waveform of the activity-rest profiles. We intended to calculate the CoM separately for the morning bout of activity and the evening bout of activity and thus divided the whole 24 hour day into two halves- 6 hour before the transition from dark to light and 6 hours after the transition (12 hours) was considered as the morning half and the rest 12 hours were considered as the evening half. CoM was calculated for both bouts of activity in the presence and the in the absence of temperature cycles for all the regimes. The CoM values are available in degrees, so for better comparisons, the values were converted into ZT. All the ZT values for each block were calculated separately for the morning and the evening bout of activity. All the ZT values were calculated separately for each block and then averaged to get the block means. Same was repeated for all the 3 populations.

2.3.3 SSD calculation:

We were also interested in comparing the waveform across the population, in the absence of the temperature cycle and the presence of the temperature cycle. We calculated the SSD (sum of squared difference) for all the populations and then compared between the waveform of the populations. For this, we used the 20-min bin activity profiles of individual flies and computed the difference between the activity levels at each time point between the two conditions. The difference was further squared and the sum of this squared difference was calculated for the entire cycle. We did these calculations separately for the morning bout of activity and the evening bout of activity to understand the potential differences between the SSDs of the two activity bouts.

2.3.4 Phase control calculation:

This measurement gives information about the phase of the activity on the first day in constant darkness and constant temperature with respect to the phase on the last of LDTC conditions. For this calculation, the daily onset and offset of the activity rhythm under constant darkness were estimated for all individual flies, using Rhythmically. The phase of the first day in DD was predicted using the marked onset and offset phases. The phase of the first day in DD was then subtracted from the phase of the last day in LDTC. The difference was then statistically analyzed to determine whether the overall difference is significantly more than zero. We concluded that phase control was not maintained if the phase on the first day in DD is not in the same phase as that of the last day in LDTC or we can say it is not a phase control.

2.3.5 Free-running period calculation:

Free-running period (FRP) for all the flies kept under constant darkness at 21^{o}C were analyzed using Rhythmically. Periods of all the individuals were marked manually and was averaged within stocks first and then across blocks.

2.4 Statistics:

The activity counts at every phase was calculated separately for each block which was used as data points for doing analysis using two-way Analysis of Variance (ANOVA) and to test for statistically significant differences among populations. All the further comparisons were done using Tukey HSD with a 95% CI.

To compare the waveform of the locomotor activity rhythms among populations, CoM was calculated separately for the morning bout of activity and the evening bout of activity for the LDTC condition. These were analyzed using two factors (selection and block) two-way randomized block design ANOVA using block means. Out of which the selection was used as a fixed factor and block was used as a random factor. A Tukey honestly significant difference (HSD) test was done on all the results after the statistical analysis in order to generate to error bar of 95% confidence interval.

Similarly, for the SSD calculation, the SSD calculation was done separately for the morning and the evening bout of activity. Two-factor (selection and block), two-way randomized block design ANOVA was done followed by a Tukey HSD test on all the results. All the error bars on the graph are of 95% CI.

For phase control statistical analysis Wilcoxon signed rank test was done on each block for all the population. The significant level was taken as $\alpha = 0.05$.

For the free-running period calculation, similar statistical analysis was done taking the selection and block as two factors and taking the selection factor fixed and the block as the random factor for the two-way ANOVA. A Tukey HSD test was done on the results taking 95% CI for constructing the error bars in the graph.

Chapter-3

3.Results and Observations

To observe the behavior of the flies and the phases of the evening and the morning bout of activity in the presence of both the light-dark cycle and the temperature cycle, we made average activity profiles for all the stocks in the LD 12:12, constant temperature condition and in the LD 12:12, temperature cycle(LDTC) condition (Figure-4). It is to be noted that the regime LD 12:12 and constant temperature is referred to as 'before' (before the temperature cycle was introduced) and is denoted by the blue curve. Whereas the regime LD 12:12 and TC 21 ^{o}C -28 ^{o}C is referred to as 'after' (after the temperature cycle was introduced). It is denoted by the green curve. The yellow shaded region of the graph is the 'lights-ON', non- shaded region- 'lights- OFF', maroon shaded region denotes the 'thermophase' and non-shaded region is the cryophase (Fig-4).

3.1. Activity profiles:

Visual inspection of the average activity-rest profiles of *early*, *control* and the *late* stocks shows that in both the regime, the flies displayed a characteristic bimodal profile, showing an evening peak of activity which coincides with the end of the lights-ON and a morning peak at the transition from lights-OFF to lights-ON.

A 4-hr phase difference (4-hr PD) between LD and TC was introduced via a 4-hr delay of TC relative to LD (leaving LD unchanged) (Fig-4A). Comparing the 'before' and 'after' curves of the 4-hr PD we observed that both the profiles for the morning bout of activity are similar for all the population (Fig-4A: i, ii, iii). On the other hand, the evening bout of activity for the *late* stocks in the 'after' curve seemed to have shifted to the right direction (Fig-4A: iii) in comparison to the 'before' curve. The same held true for *late* 'after' curve when compared to the 'after' curves of *early* and *controls* (Fig-4A: I, ii.).

For the 8-hr PD regime, an 8-hr phase difference has been introduced via an 8-hr delay of TC relative to LD cycle (Fig-4B). Comparisons similar to the 4-hr PD was made between the stocks and it was again observed that the 'after' curve of the evening bout of activity in the *late* stocks seems to have shifted to right (Fig-4B: iii) in comparison to the 'before' curve of the *late* stocks. The same was observed for *late* 'after' curve in comparison to those of *early* and *controls* (Fig-4B: i, ii,). However, both the curves in the morning bout of activity have similar profiles.

For the 12-hr PD, 16-hr PD, 20-hr PD and the 24-hr PD (Fig-4:C, D, F) both the profiles of 'before' and the 'after' curves for the morning and the evening bouts of activity was found to be similar in the *early*, *control* and *late* stocks by manual inspection(Fig-4:C(i, ii, iii), D(i, ii, iii), F(i, ii, iii)).

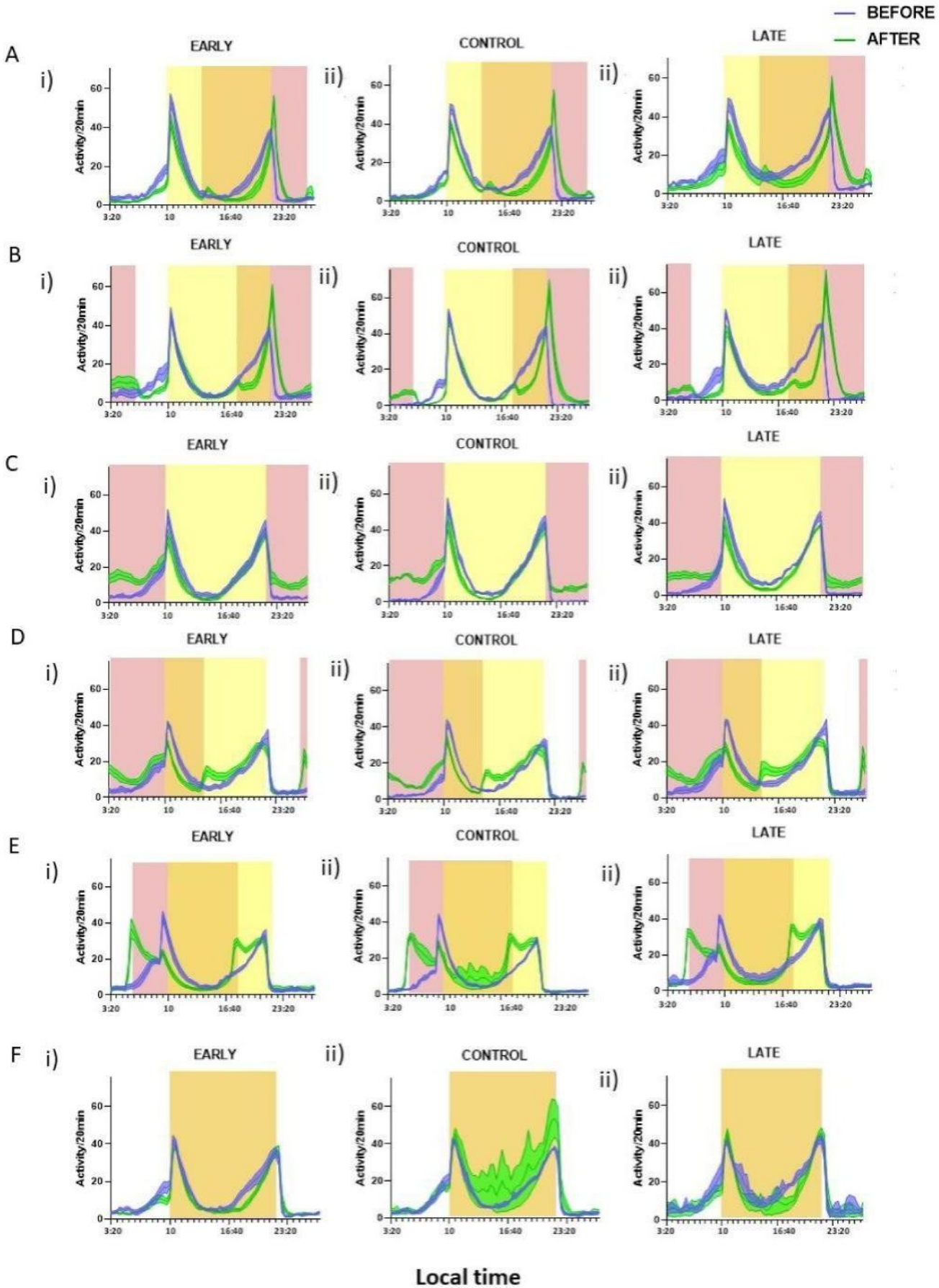

BEFORE
AFTER
A
B
C
D
E
F
i)
ii)
EARLY
CONTROL
LATE
Activity/20min
0
20
40
60
3:20
10
16:40
23:20
Local time

Figure-4: Locomotor activity profiles of *early*, *control* and *late* stocks under LD 12:12 at 21 °C (blue-curve) and LD 12:12 TC 21 °C-28 °C (green curve) of A) 4hr PD, B) 8hr PD, C)12hr PD, D)16hr PD, E)20hr PD, F)24hr PD. Yellow shaded regions in panel depicts the light phase of the LD cycle and the non-shaded part depicts the dark phase. The maroon shaded part depicts the thermophase, non-shaded part- cryophase. Error bars in this panel are standard error of the mean (SEM). Profiles are averaged over cycles and then averaged over flies.

3.2. Centre of Mass:

For the CoM, we found out that under the LDTC 12:12, 21 ^{o}C-28 ^{o}C the phase of CoM was not significantly different between stocks in both morning (fig-5) and evening (fig-5) bout of activity, in any of the regime used. We also observed that till the 16-hr PD regime, the *early, control* and *late* stocks seems to be advancing their phases, but for the 20-hr PD and 24-hr PD, the CoM phases starts delaying compared to the previous regimes (Fig-5). Even though, the shift in the evening bout of activity in the *late* stocks was observed in the average activity profiles, significant differences between the stocks was not observed in the CoM calculation. Thus, we quantified differences between the stocks using the SSD (sum of squared difference) calculation for the morning bout of activity and the evening bout of activity (Fig-6: A, B).

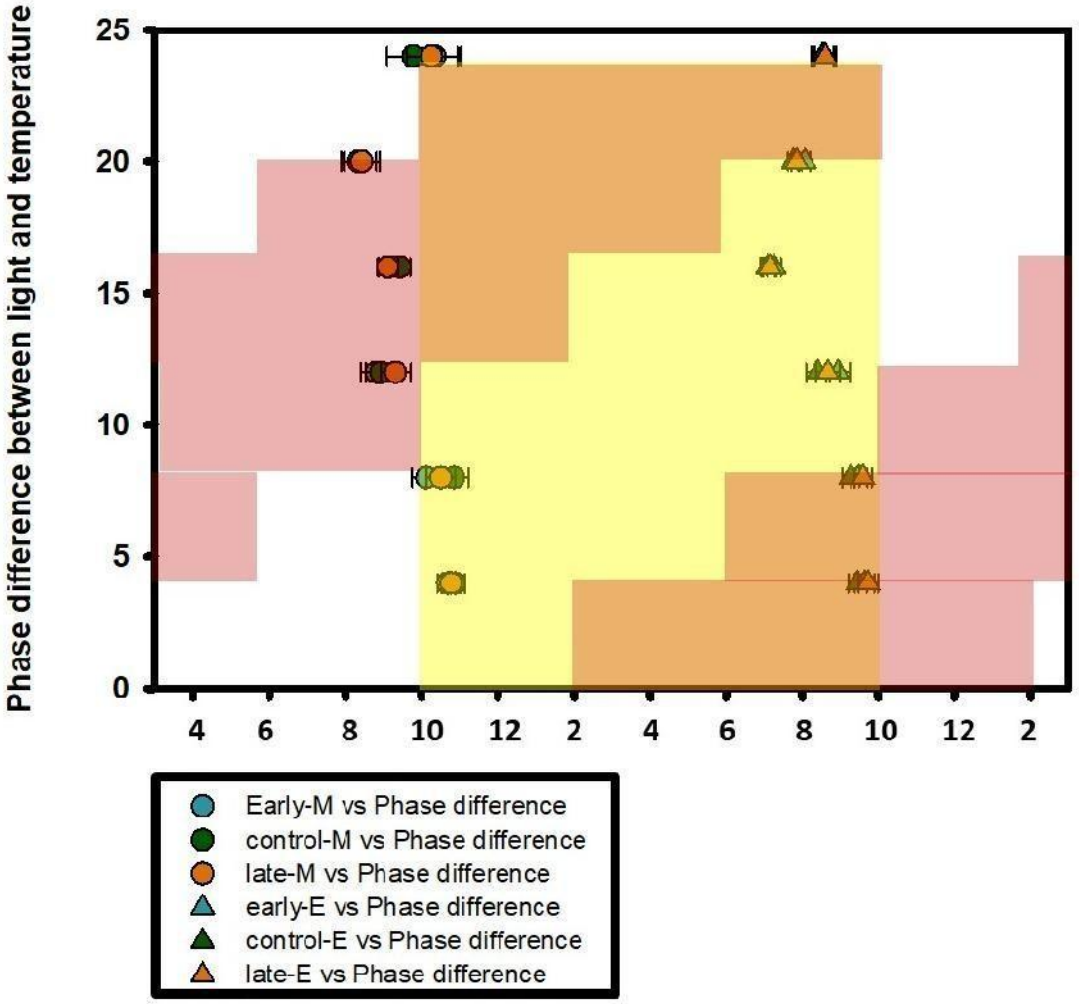


Figure-5: Phases of CoM of *early*, *control* and *late* stocks under LD 12:12 at 21 °C (blue-curve) and LD 12:12 TC 21 °C-28 ºC for the morning and evening bout of activity of 4hr PD, 8hr PD, 12hr PD, 16hr PD, 20hr PD, 24hr PD. Yellow shaded regions in panel depicts the light phase of the LD cycle and the non-shaded part depicts the dark phase. The maroon shaded part depicts the thermophase, non-shaded part- cryophase. Analyzed using two factors (selection and block) two-way randomized block design ANOVA, Error bars in this panel are 95% CI from a Tukey HSD test at α=0.05. Thus, means with overlapping error bars are not statistically significantly different from each other.

3.3 Sum of squared difference:

We found out that there are no significant differences in the SSD between the *early*, *control* and *late* stocks in the morning bout of activity in any of the regime used (fig- 6A: i, ii, iii, iv, v, vi). Whereas, we found significant differences between the SSD of the *early* and the *late* stocks of the evening bout of activity in the 4-hr phase difference regime (Fig-6B: i).Significant differences in the SSD was also observed between the *early* and the *late* stocks in the evening bout of activity for the 8-hr phase difference regime (Fig-6B: ii). No difference was observed in the morning bout of activity for this regime (Fig-6A: ii).Further, no significant differences in SSD was observed either in the morning bout of activity (Fig-6A: iii, iv, v, vi) or in the evening bout (Fig-6B: iii, iv, v, vi) of activity in the 12-hr, 16hr, 20-hr and 24-hr phase difference regimes.

All the results from the SSD calculations were consistent with the observations that were made from the average activity profiles of all the regimes. It also suggests the possibility that the evening bout of activity of the late stocks is more sensitive to temperature cycle rather than the light dark cycle, at least when the phase relationships between light and temperature cycle are smaller (4 and 8 hours). But, this preference for temperature cycle more than the light dark cycle is only present in the 4-hr PD and 8-hr PD and not in the 12-hr, 16-hr, 20-hr and 24-hr phase difference regimes.

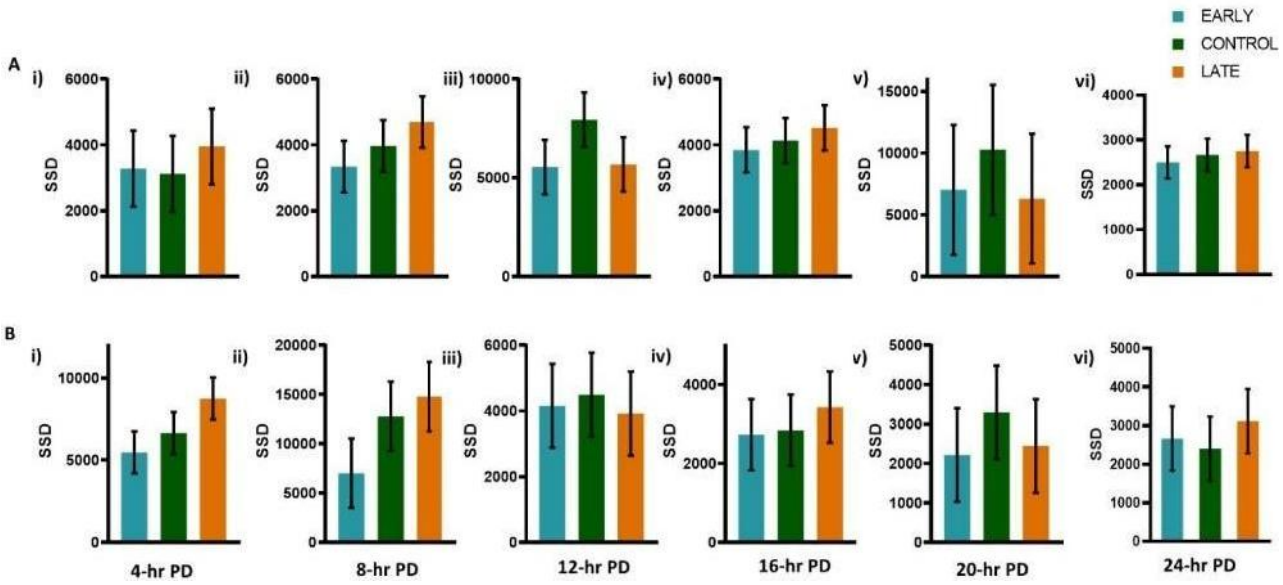
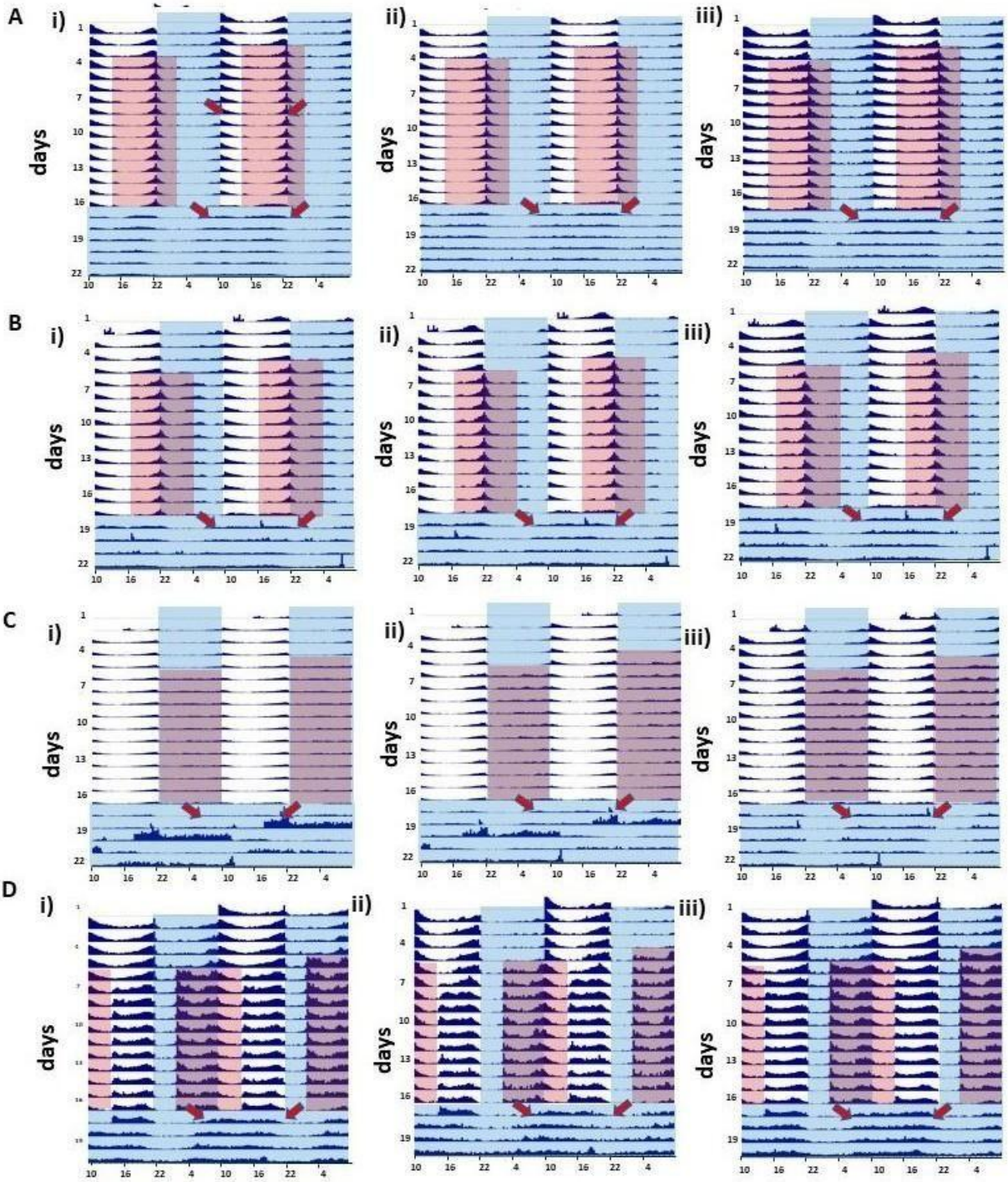


Figure 6-SSD (sum of squared difference) of *early*, *control* and *late* stocks in LD 12:12 TC 21 °C - 28 °C for i)4hr PD, ii)8hr PD iii)12hr PD, iv)16hr PD, v)20hr PD, vi)24hr PD. A) SSDs for the all the regimes for morning bout of activity, B) SSDs for all the regimes for the evening bout of activity. A significant difference is observed between the early and late in the evening bout of activity for 4hr PD (B-i) and in the 8hr PD (B-ii). These were analyzed using two factors (selection and block) two-way randomized block design ANOVA. All error bars are 95% CI following a Tukey's HSD test at α = 0.05. Thus, means with non-overlapping error bars are significantly different from each other and vice- versa

3.4. Phase control calculation from actograms:

Apart from the CoM and the SSD calculation, phases of the onset of the activity and the offset of the activity were marked under constant darkness-DD at 21 ^{o}C. This was marked using the actograms-which is a graphical representation of the activity-rest behavior of an animal with respect to the environmental cues (Fig-7). All the depicted actograms are the representative average actograms of all the individuals of a particular stock (Fig-7). The dark blue band in the actograms represents period of activity and the rest depicts the period of rest. The phases that have been marked with

the arrows are marked to calculate the phase control to the last day of the entrained condition. The red arrows depict the onset and offset of activity respectively.


A
i)
ii)
iii)
B
i)
ii)
iii)
C
i)
ii)
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D
i)
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days

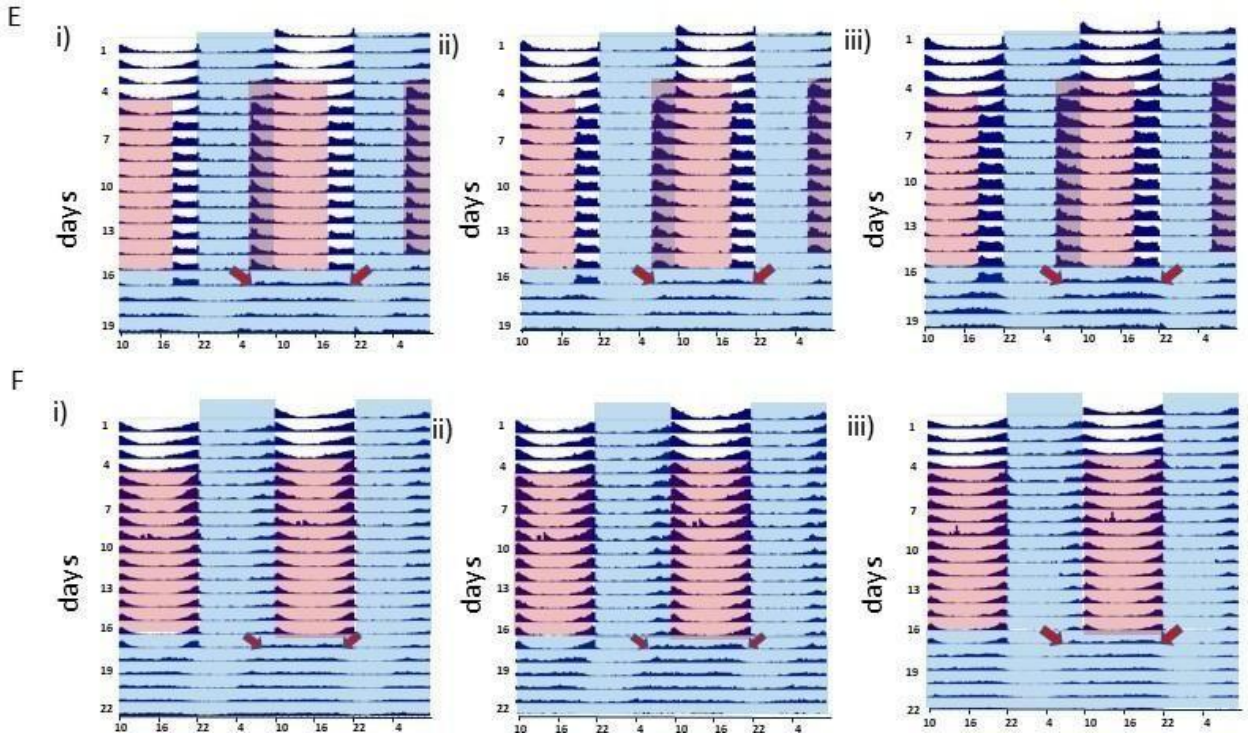

Figure-7: Representative actograms of flies experiencing LD 12:12 at 21 ºC (1st 4 days) and LD 12:12 TC 21 ºC-28 ºC (12-13 days after LD). Blue shaded region depicts the dark phase of LD and the non- shaded region depicts the light phase. Red shaded region indicates the thermophase (28 °C) and no shaded region indicates cryophase (21 °C). After LDTC, flies were kept in DD 21 ºC. The dark blue band represents period of activity and the rest depicts the period of rest. The red arrows show the morning and evening bout of activity in the LDTC and it denotes onset and offset of activity in DD. Also shown are phase-relationship between the LD and the TC in the LDTC region- A) 4hr PD, B)8hr PD, C)12hr PD, D) 16hr PD, E) 20hr PD, F) 24hr PD.

To observe if the flies have entrained to the light-dark cycle or the temperature cycle, the flies were introduced in DD after the LDTC regime. If the flies would have entrained to the light dark cycle in the LDTC regime, the phase of the onset and offset (phase markers) of the 1st day in DD would be similar to the phase of the onset and offset of

the LD cycle in the last day of LDTC regime. If the phase of the last day of LDTC was same with the phase of the 1st day in DD, then it was said to be phase control.

Since our aim was to compare the phases of the onset and offset of the activity of the flies in DD and the onset and offset of activity in LDTC, Wilcoxon signed-rank test was done which would be useful in detecting differences from the phases in the presence of the conflicting zeitgeber and in DD. Table summarizing if the 1st day in DD is phase control to the last day of the LDTC is given.

ONSET												
PHASE DIFFERENCE	GE1	GE2	GE3	GE4	GC1	GC2	GC3	GC4	GL1	GL2	GL3	GL4
4hr-PD	yes	yes	yes	yes	no	no	no	no	yes	yes	no	no
8hr-PD	yes	yes	no	no	no	no	no	no	no	no	no	no
16hr-PD	no	no	no	yes	no	no	yes	yes	no	no	yes	yes
20hr-PD	no	no	no	no	no	no	no	no	no	no	no	no
24hr-PD	no	yes	yes	Yes	no	yes	yes	yes	yes	yes	yes	yes

Table-2: Depicted here is summary of results of whether the 'onset' of first day in DD is entrained to the last day of in LD 12:12 TC 21 ºC- 28 °C. First column- list of regimes, third row-list of the stocks, 'yes' denotes- entrained to LDTC, 'no' denotes-not entrained to LDTC (Based on the criteria given in results and discussion).

OFFSET												
PHASE DIFFERENCE	GE1	GE2	GE3	GE4	GC1	GC2	GC3	GC4	GL1	GL2	GL3	GL4
4hr-PD	yes	yes	no	no	yes	yes	no	Yes	yes	yes	yes	yes
8hr-PD	yes	no	yes	No	yes	no	yes	No	yes	yes	yes	yes
16hr-PD	yes	no	no	no	no	yes	no	no	yes	yes	yes	yes
20hr-PD	no	yes	no	No	no	no	no	No	no	yes	yes	no
24hr-PD	no	no	no	No	no	no	yes	Yes	no	no	no	yes

Table-3: Depicted here is summary of results of whether the 'offset' of first day in DD is entrained to the last day of in LD 12:12 TC 21 ºC- 28 °C. first column- list of regimes, second row-list of the stocks, 'yes' denotes- entrained to LDTC, 'no' denotes-not entrained to LDTC (Based on the criteria given in results and discussion).

The activity-rest behavior in DD can be classified as free-run if both the onset and offset in the first day of DD is not a phase control with the last day of LDTC. It can be

considered as not entrained in case any one phase marker is a phase control. It can only be considered as entrained if both the onset and the offset is phase control. Since, very few of the stocks were completely entrained to the light-dark cycle in all the regimes, the results from this observation was inconclusive. The DD data for 12-hr PD could not be calculated because all the flies were showing unusual free running behavior because of which the onset and offset could not be marked.

In the actograms (Fig-7), an anticipatory behavior was observed to the lights-ON and lights-OFF for the *early, control* and *late* stocks in the 4-hr and 8-hr PD but no such anticipatory behavior was observed in response to the thermophase, thus appearing to primarily follow the light-dark cycle (Fig-7 A,B). It was also observed that before the temperature cycle was introduced, most of the activity in the morning and the evening bout of activity) would occur in the light-region, But in the presence of the thermophase, some activity is also taking place after the transition from light to dark in the evening bout of activity. But after the end of the thermophase there is decrease in the activity. Similar behavior is observed in the 8-hr phase difference regime (Fig-7 B).

For the 12-hr phase difference regime, the behavior is similar to that of fly which are exposed to only the LD cycle (Fig-7 C). However, both the morning and the evening bout of activity in this regime were not very sharp compared to the activity bouts of the 4-hr and 8-hr phase difference. Same observation was made with the activity peaks of the 16-hr, 20-hr and 24-hr PD regime (Fig-7 D, E, and F). This observation may be due to the high activity just after the onset of the temperature regime, during the thermophase and also after the end of the thermophase. It appears that the activity bout due to the temperature and the morning bout of activity together are giving rise to a long, but not sharp activity bout as observed in the 16-hr PD and 20-hr PD. The night time activity in these regimes is also very high due to the presence of thermophase in the dark region of the light-dark cycle. Similar thing is observed in the evening bout of activity, the end of thermophase is giving rise to a bout of activity, which is getting merged with the evening activity bout. It appears that, night time activity is happening because of the presence of temperature.

3.5. Free-running period:

We then examined the FRP of these stocks under DD at 21 ºC after entrainment to the LDTC condition. We found that there was no statistically significant difference between the *early*, *control* and *late* stocks in any of the regime (fig-8)

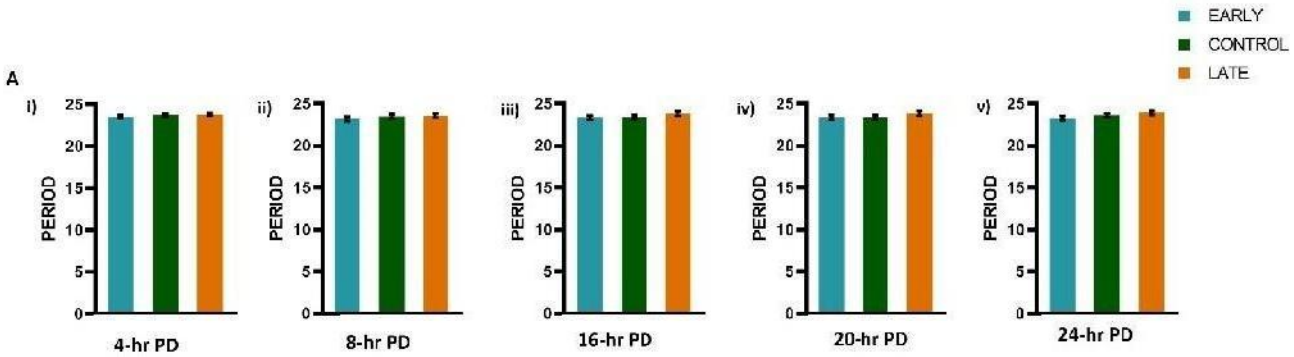

Figure 8-Free-running period of early, control and late stocks post entrainment to LD 12:12 TC 21 °C - 28 °C for i)4hr PD, II)8hr PD iii)16hr PD, iv)20hr PD, v)24hr PD. These were analyzed using two factors (selection and block) two-way randomized block design ANOVA. All error bars are 95% CI following a Tukey's HSD test at α = 0.05. Thus, means with non-overlapping error bars are significantly different from each other.

We can observe that the early, *control* and *late* stocks have no significant difference in their FRP in any of the regimes used (Fig-5A-i, ii, iii, iv, v).

Apart from the above observations, a startle activity was also observed during the transition from the thermophase to cryophase and also in the transition from the cryophase to thermophase in the 'after' curve in all the stocks in the average activity-rest profiles (Fig-4). Sudden change in light or temperature can often cause direct effects on the activity level of the organism that "mask" the endogenous rhythm of the circadian system.

Chapter-4

4.Discussion

Circadian research in *Drosophila melanogaster* has been mostly focused on behaviors in response to either light or temperature. However, the clock network has evolved to show behaviors within different environmental condition containing different zeitgebers. It has been previously observed that the *late* stocks which have been selected for the evening emergence has co-evolved enhanced temperature sensitivity of the circadian clock circuit with respect to the adult emergence rhythms (Abhilash et al., 2019a). On the other hand, it has also been observed that this population is also associated with increased temperature sensitivity of the clock especially in the evening bout of activity in activity-rest rhythm (Abhilash et al., 2019b). In this study we were interested in examining the activity-rest behavior of the *early*, *control* and the *late* stocks under a variety of conflicting zeitgeber regimes to observe their behavior in the presence of both temperature and light, the working hypothesis being in *late* flies' evening activity bout is more temperature sensitive compared to that of *early* and *controls*

It was observed from the average activity profiles (Fig-3: A, B) of the evening bout of activity for the 4-hr phase difference regime and the 8-hr phase difference regime have significant differences with respect to the presence or absence of temperature. The SSD analysis supports the observation (Fig 4B: i, ii). On the other hand, in other phase relationship regimes- the 12-hr PD, 16-hr PD, 20-hr PD and the 24-hr PD, all three stocks have similar activity profiles for morning as well as evening bout of activity (Fig-3: C, D, E, F).

It has been previously shown that different groups of cells responsible for the control of the circadian clock in the fly brain regulate different kinds of rhythmic processes all over the body, for example- the locomotor activity and the eclosion behavior are regulated by different clock cells in the body (Blanchardon et al., 2001; Castillon et al., 2003).

As mentioned before, it was proposed that the eclosion behavior was regulated by the A-B oscillator model in which the A-oscillator or the master clock is light sensitive and temperature compensated oscillator, whereas the B-oscillator is temperature sensitive and is not affected by light (Pittendrigh, 1960). On the other hand, the fly's locomotor behavior is thought to be regulated by the M and E oscillator model, where the M oscillator regulates the morning bout of activity and the E oscillator is responsible for the evening bout of activity.

With respect to previous results (Abhilash et al., 2019b, 2019a), since the late stocks have evolved temperature sensitive components with respect to the eclosion rhythm and the evening bout of activity (regulated by the E-oscillator) in the activity-rest behavior is also temperature sensitive, we hypothesized that there might be some overlap between the temperature sensitive components of the B-oscillator and the temperature sensitive components of the E-oscillator. It was also suggested that the evening bout of activity of the *late* stocks should track temperature instead of light in the presence of a conflicting zeitgeber regime.

We observed in the activity profiles for the 4-hr PD and 8-hr PD that the evening bout of activity (in the presence of temperature cycle) in the *late* stocks seems to shift its phase towards the thermophase (Fig-3A: iii, B: iii). Whereby, for the 12-hr PD regime (the anti-phasic regime) and after, there was no difference in the activity profiles of the 'before' and 'after' (in the absence and presence of temperature cycle) (Fig-3C). In the first two regimes the evening bout of activity appears to follow the temperature, it could be because of less phase difference between the light-dark cycle and the temperature cycle. And in those phase differences the temperature sensitive components of the *late* stocks could be dominating over the light sensitive components. And for the anti-phasic phase difference (12-hr PD), the light sensitive components might be more dominant than the temperature sensitive components of the clock. The intensity of the light is also a very important component for the behavior of the organism. It might be possible that the light intensity used in our studies (70 lux) is too high for the amplitude of the temperature cycle that is being used. Therefore, the temperature cycle might not be able to overhaul the light cycle and the observed behavior of the *late* stocks is with respect to only the light-dark cycle since they are not able to track the temperature cycle but only the light-dark cycle.

Similar other experiments have shown that flies exposed to the conflicting zeitgeber conditions showed an advance phase shift in the evening bout of activity in comparison to flies not exposed to any such conditions. However, In this case, the phase difference between the light and the temperature was 6-hr where the temperature was 6-hr advanced than the light-dark cycle. (Harper et al., 2016). This study further supports the idea of the evening bout of activity preferring the temperature cycle more than the light dark cycle, although, for some particular phase differences.

It has been observed that in a conflicting zeitgeber condition, light does indeed dominate temperature in case of maximal phase difference between LD and TC and the evening bout of activity in the flies are predominantly entrained to the temperature cue compared to the morning bout of activity (Harper et al., 2016).

It has also been shown that, 8 to 10-hr of misalignments between the LD and TC showed a change in the evening bout of activity with respect to the temperature but, the preference was changed to light signal for larger misalignments (Yoshii et al., 2010), which supports our observations. However, other observations also show, temperature being a very important parameter for the morning activity in natural conditions (Vanin et al., 2012).

It has been found out that with delays of 5-7 hr of LD with respect to TC, the evening activity peaks broke down and gave rise to a plateau (P) behavior (Harper et al., 2016). This behavior was associated with a reduction of amplitude of the molecular rhythms demonstrating that sensory conflict alters the state of the circadian oscillator which supports our observations in the activity profiles of the flies. Together all these results show the importance of a particular zeitgeber in different contexts. It appears that at this particular light intensity and amplitude of temperature cycle that we used, the *Drosophila* circadian system is preferring temperature for behavioral rhythms only for a limited range of light-temperature phase relationships, i.e., for the smaller misalignments of 4-hr PD and the 8-hr PD, however for larger misalignments, the clock seems to have more preference towards light to exhibit the locomotor behavior.

CoM calculations:

For the CoM calculation, we observed that there was no significant difference between the stocks in either the morning bout of activity or the evening bout of activity(Fig-4) in any of the phase regime used although we could observe a significant phase shift of the evening bout of activity in a 4-5 hour window after the light-to-dark transition in the presence of temperature(data not provided).Significant differences was also observed between the stocks in the SSD calculation in the 4-hr PD and the 8-hr PD for the evening bout of activity (Fig-5B: i, ii).

As mentioned before, for the calculation of CoM, the 24-hr day was divided into two equal parts, first 12-hr part had the morning bout of activity and the later12-hr part had the evening bout of activity. The reason, we could observe significant differences between the stocks in the SSD and not in the CoM calculation might be because, for a 12-hr window, all the activities got averaged at a single point, which includes night time as well as the sudden activity during temperature transition. And thus, the activity other than the desired 4-5 hr (where the difference between stocks was visible) window would also have been averaged in the total CoM, giving us no significant difference.

The other observation that was made using the CoM calculation, that till 16-hr PD regime the stocks seems to be advancing their phases (Fig-4), whereas the stocks for both morning and evening bout of activity for the 20-hr PD and the 24-hr PD are delaying their phases. This behavior could be explained using the individual's circadian clock's phase response curve (PRC). A phase response curve is a plot, which gives information about phase-shifts done by the individual's clock when a stimulus of certain strengths is provided at different times of the internal time of the organism. An individual's clock exhibits either delay or advance in its phase with response to the stimulus. In case of a phase delay, the peak levels of the rhythms reach later than they would, if the rhythms had not been shifted, whereas, in case of phase advance, the peak levels are reached earlier than they would have, had it not been phase shifted. Exposure of light to organism's early part of the night causes a phase delay and exposure of light in the later part of the night causes a phase advance while light during the day time produces no phase shifts.

According to the PRC (constructed using light as the stimulus), the 20-hr PD and the 24-hr PD would have been created a very huge phase delay, which would have been very difficult for the organism to adjust and thus, it tried to phase advance its phase. And thus, it showed advance phase with respect to phases in other regimes and with respect to lights-OFF.

This would have been the response, for a light PRC but in a conflicting zeitgeber condition (our case); the response of the organism might change. It is also possible that, the observed response of the organism is based only on light, and not the temperature, for which it can be said that light has more effect on the behavior of the organism than temperature does for the 20-hr PD and 24-hr PD. But, since very little is known about the PRC for light and temperature both, the observed behavior cannot be further explained.

From actograms:

It was observed that in the LDTC regime, small amount of activity was observed during the dark phase, in the presence of temperature cycle, but similar behavior was not observed in the LD conditions (with constant temperature). The conflicting regime (and thus, the periodic presence of temperature) appears to have some effects on the flies experiencing the 4-hr and 8-hr PD. But, since this behavior was not observed for the 12-hr, 16-hr, 20-hr and 24-hr PD regimes, it can be suggested that the light-dark cycle is dominant over temperature cycle in these regimes, such that the behavior due to temperature is getting suppressed.

For phase control:

According to theoretical considerations, it is believed that the two distinct group of neurons (two oscillators) which give rise to the morning bout of activity and the evening bout of activity respectively can function autonomously, but studies also indicates that they can be functionally coupled(Stoleru et al., 2004). Based on our observations and results of the phases of the onset and offset of the activity bout in constant darkness condition, it can be suggested that the underlying circadian clock is giving weightage

to both the zeitgebers, giving rise to an average phase with respect to the phases of both LD and TC. It might be possible that, the oscillators are giving rise to an activity pattern in response to both light dark cycle and the temperature cycle and therefore, the onset and offset phases might not be able to synchronize either to light- dark cycle or to temperature cycle.

From the above observations, the preference of the *late* stocks towards the light-dark cycle or the temperature cycle is unclear. Since the flies of the *late* stocks are preferring temperature in some phase differences and light-dark cycle in others, we cannot comment about the overlap of the B-oscillator (in adult emergence rhythm) and E-oscillator (in locomotor rhythm) as hypothesized above.

Other similar studies:

Apart from experiments done on *Drosophila melanogaster*, there are several other organisms on which different kinds of behavior are observed under conflicting zeitgeber conditions. There are studies done on zebrafish, using the conflicting zeitgeber cycles and it has been found out that zebrafish entrain their circadian rhythms to both light and thermocycle. They display most of their activity during the daytime regardless of the phase of the temperature cycle (López-Olmeda et al., 2006) They also entrain to temperature cycles of 24-hr in the presence of constant dim light. Under constant dim LL condition, their free-running behavior was observed with an average period of 23.3-hr. All these observations provided the insights of the influence of daily light and temperature related changes on the locomotor activity rhythms of the fish. It also revealed the ability of thermocycle to entrain the circadian activity rhythms in zebrafish (López-Olmeda et al., 2006).

Studies have also been done on the eclosion and activity-rest behavior of *Osmania bicornis (*a bee species). It has been shown that this bee species synchronizes its daily rhythms of emergence to the temperature cycles rather than the light-dark cycles and the locomotion rhythm is triggered by the light-dark cycles (Beer et al., 2019).

Experiments were also done on rabbit using light and food access as the two zeitgeber for the conflicting zeitgeber regimes. The results of the experiment indicated that rabbit has two circadian oscillator systems, out of which one oscillator is entrained by the

light-dark cycle whereas the other is entrained by the feeding-fasting cycle. However, both the oscillators show common control over multiple other behaviors (Jilge and Stahle, 1993).

Conclusion and Future directions

From all the above observations and calculations, it appears that the evening bout of activity of flies that have been selected for evening emergence *(late* stocks) is preferring temperature for behavioral rhythms only for a limited range of light-temperature phase relationships, i.e., for the smaller phase differences of 4-hr PD and the 8-hr PD, however for larger phase differences, it can be concluded that either the clock seems to have more preference towards light than towards temperature or the clock is responding to light and temperature equally to exhibit the locomotor behavior.

To understand the clear preference of the flies in the conflicting zeitgeber condition in the future we intend to do another behavioral experiment with a different regime- The flies will be kept in LDTC in-phase followed by LDTC out of phase with the same light and temperature cycle. This will be done for the 4-hr phase difference and the 8-hr phase difference regimes. Since, only a phase difference will be introduced and all other conditions will remain same, this might give us clear information about the preference of the flies toward the temperature cycle or the light-dark cycle. We could also do behavioral experiments with less light and intensity than 70lux and the same temperature cycle, which will give us insights to comment about the behavior of flies in response to the temperature cycle

In order to understand the underlying molecular mechanism, we intend to examine the states molecular clocks in different components of the circadian circuit in the fly brain under specific conflicting zeitgeber regimes.

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Appendix

	Effect (F/R)	SS	Degr. of Freedom	MS	Den.Syn. Error df	Den.Syn. Error MS	F	p
Intercept	Fixed	7.261724	1	7.261724	3	0.027982	259.5186	0.00052
sel	Fixed	0.070964	2	0.035482	6	0.060488	0.5866	0.585214
block	Random	0.083945	3	0.027982	6	0.060488	0.4626	0.718707
sel*block	Random	0.362926	6	0.060488	0	0		
Error			0					

Table 1. Results of ANOVA performed on CoM of the morning bout of activity for early, control and late stocks for 4-hr phase difference regime.

	Effect (F/R)	SS	Degr. of Freedom	MS	Den.Syn. Error df	Den.Syn. Error MS	F	p
Intercept	Fixed	1617.643	1	1617.643	3	0.050755	31871.54	0
sel	Fixed	0.163	2	0.081	6	0.051899	1.57	0.283449
block	Random	0.152	3	0.051	6	0.051899	0.98	0.463063
sel*block	Random	0.311	6	0.052	0	0		
Error			0					

Table 2. Results of ANOVA performed on CoM of the evening bout of activity for early, control and late stocks for 4-hr phase difference regime.

	Effect (F/R)	SS	Degr. of Freedom	MS	Den.Syn. Error df	Den.Syn. Error MS	F	p
Intercept	Fixed	2.950116	1	2.950116	3	0.03503	84.21777	0.002736
sel	Fixed	1.049125	2	0.524562	6	0.12408	4.2276	0.071513
block	Random	0.105089	3	0.03503	6	0.12408	0.28231	0.836672
sel*block	Random	0.744483	6	0.12408	0	0		
Error			0					

Table 3. Results of ANOVA performed on CoM of the morning bout of activity for early, control and late stocks for 8-hr phase difference regime.

	Effect (F/R)	SS	Degr. of Freedom	MS	Den.Syn. Error df	Den.Syn. Error MS	F	p
Intercept	Fixed	1574.923	1	1574.923	3	0.052425	30041.2	0
sel	Fixed	0.227	2	0.113	6	0.041691	2.72	0.144127
block	Random	0.157	3	0.052	6	0.041691	1.26	0.369747
sel*block	Random	0.25	6	0.042	0	0		
Error			0					

Table 4. Results of ANOVA performed on CoM of the evening bout of activity for early, control and late stocks for 8-hr phase difference regime.

	Effect (F/R)	SS	Degr. of Freedom	MS	Den.Syn. Error df	Den.Syn. Error MS	F	p
Intercept	Fixed	11.55289	1	11.55289	3	0.410207	28.16353	0.013063
sel	Fixed	0.57273	2	0.28637	6	0.137032	2.08978	0.20477
block	Random	1.23062	3	0.41021	6	0.137032	2.99351	0.117383
sel*block	Random	0.82219	6	0.13703	0	0		
Error			0					

Table 5. Results of ANOVA performed on CoM of the morning bout of activity for early, control and late stocks for 12-hr phase difference regime.

	Effect (F/R)	SS	Degr. of Freedom	MS	Den.Syn. Error df	Den.Syn. Error MS	F	p
Intercept	Fixed	1368.326	1	1368.326	3	0.183227	7467.936	0.000003
sel	Fixed	0.571	2	0.285	6	0.084003	3.398	0.103084
block	Random	0.55	3	0.183	6	0.084003	2.181	0.191237
sel*block	Random	0.504	6	0.084	0	0		
Error			0					

Table 6. Results of ANOVA performed on CoM of the evening bout of activity for early, control and late stocks for 12-hr phase difference regime.

	Effect (F/R)	SS	Degr. of Freedom	MS	Den.Syn. Error df	Den.Syn. Error MS	F	p
Intercept	Fixed	7.191277	1	7.191277	3	0.375349	19.15889	0.022069
sel	Fixed	0.247951	2	0.123976	6	0.060038	2.06495	0.207797
block	Random	1.126048	3	0.375349	6	0.060038	6.25186	0.028161
sel*block	Random	0.360228	6	0.060038	0	0		
Error			0					

Table 7. Results of ANOVA performed on CoM of the morning bout of activity for *early, control* and *late* stocks for 16-hr phase difference regime.

	Effect (F/R)	SS	Degr. of Freedom	MS	Den.Syn. Error df	Den.Syn. Error MS	F	p
Intercept	Fixed	1008.603	1	1008.603	3	0.244463	4125.786	0.000008
sel	Fixed	0.06	2	0.03	6	0.028074	1.069	0.400823
block	Random	0.733	3	0.244	6	0.028074	8.708	0.013215
sel*block	Random	0.168	6	0.028	0	0		
Error			0					

Table 8. Results of ANOVA performed on CoM of the evening bout of activity for *early, control* and *late* stocks for 16-hr phase difference regime.

	Effect (F/R)	SS	Degr. of Freedom	MS	Den.Syn. Error df	Den.Syn. Error MS	F	p
Intercept	Fixed	31.80613	1	31.80613	3	0.178537	178.1483	0.000909
sel	Fixed	0.01635	2	0.00817	6	0.183779	0.0445	0.956811
block	Random	0.53561	3	0.17854	6	0.183779	0.9715	0.465552
sel*block	Random	1.10268	6	0.18378	0	0		
Error			0					

Table 9. Results of ANOVA performed on CoM of the morning bout of activity for *early, control* and *late* stocks for 20-hr phase difference regime.

	Effect (F/R)	SS	Degr. of Freedom	MS	Den.Syn. Error df	Den.Syn. Error MS	F	p
Intercept	Fixed	1175.307	1	1175.307	3	0.044157	26616.83	0.000001
sel	Fixed	0.193	2	0.097	6	0.014928	6.46	0.031844
block	Random	0.132	3	0.044	6	0.014928	2.96	0.119744
sel*block	Random	0.09	6	0.015	0	0		
Error			0					

Table 10. Results of ANOVA performed on CoM of the evening bout of activity for *early, control* and *late* stocks for 20-hr phase difference regime.

	Effect (F/R)	SS	Degr. of Freedom	MS	Den.Syn. Error df	Den.Syn. Error MS	F	p
Intercept	Fixed	0.208304	1	0.208304	3	0.729005	0.285738	0.630043
sel	Fixed	0.769711	2	0.384855	6	0.383259	1.004166	0.42056
block	Random	2.187014	3	0.729005	6	0.383259	1.902121	0.230376
sel*block	Random	2.299553	6	0.383259	0	0		
Error			0					

Table 11. Results of ANOVA performed on CoM of the morning bout of activity for *early, control* and *late* stocks for 24-hr phase difference regime.

	Effect (F/R)	SS	Degr. of Freedom	MS	Den.Syn. Error df	Den.Syn. Error MS	F	p
Intercept	Fixed	1340.808	1	1340.808	3	0.030789	43547.96	0
sel	Fixed	0.015	2	0.007	6	0.06934	0.11	0.900631
block	Random	0.092	3	0.031	6	0.06934	0.44	0.730274
sel*block	Random	0.416	6	0.069	0	0		
Error			0					

Table 12. Results of ANOVA performed on CoM of the evening bout of activity for *early, control* and *late* stocks for 24-hr phase difference regime.

	Effect (F/R)	SS	Degr. of Freedom	MS	Den.Syn. Error df	Den.Syn. Error MS	F	p
Intercept	Fixed	142633050	1	142633050	3	992849	143.6603	0.001249
sel	Fixed	1561955	2	780978	6	1129270	0.6916	0.536695
block	Random	2978548	3	992849	6	1129270	0.8792	0.502772
sel*block	Random	6775621	6	1129270	0	0		
Error			0					

Table 13. Results of ANOVA performed on SSD of the morning bout of activity for *early, control* and *late* stocks for 4-hr phase difference regime.

	Effect (F/R)	SS	Degr. of Freedom	MS	Den.Syn. Error df	Den.Syn. Error MS	F	p
Intercept	Fixed	580953548	1	580953548	3	3007795	193.1493	0.000806
sel	Fixed	22102259	2	11051130	6	1390035	7.9503	0.020563
block	Random	9023386	3	3007795	6	1390035	2.1638	0.193406
sel*block	Random	8340211	6	1390035	0	0		
Error			0					

Table 14. Results of ANOVA performed on SSD of the evening bout of activity for *early, control* and *late* stocks for 4-hr phase difference regime.

	Effect (F/R)	SS	Degr. of Freedom	MS	Den.Syn. Error df	Den.Syn. Error MS	F	p
Intercept	Fixed	192100773	1	192100773	3	5811715	33.05406	0.010453
sel	Fixed	3682554	2	1841277	6	521131	3.53323	0.096823
block	Random	17435145	3	5811715	6	521131	11.15211	0.007239
sel*block	Random	3126787	6	521131	0	0		
Error			0					

Table 15. Results of ANOVA performed on SSD of the morning bout of activity for *early, control* and *late* stocks for 8-hr phase difference regime.

	Effect (F/R)	SS	Degr. of Freedom	MS	Den.Syn. Error df	Den.Syn. Error MS	F	p
Intercept	Fixed	1.59E+09	1	1.59E+09	3	2773848	574.3976	0.000159
sel	Fixed	1.30E+08	2	6.50E+07	6	10502179	6.1877	0.034813
block	Random	8.32E+06	3	2.77E+06	6	10502179	0.2641	0.849071
sel*block	Random	6.30E+07	6	1.05E+07	0	0		
Error			0					

Table 16. Results of ANOVA performed on SSD of the evening bout of activity for *early, control* and *late* stocks for 8-hr phase difference regime.

	Effect (F/R)	SS	Degr. of Freedom	MS	Den.Syn. Error df	Den.Syn. Error MS	F	p
Intercept	Fixed	489587987	1	489587987	3	6230404	78.58046	0.003027
sel	Fixed	14575622	2	7287811	6	1613128	4.51781	0.063546
block	Random	18691212	3	6230404	6	1613128	3.86231	0.074858
sel*block	Random	9678768	6	1613128	0	0		
Error			0					

Table 17. Results of ANOVA performed on SSD of the morning bout of activity for *early, control* and *late* stocks for 12-hr phase difference regime.

	Effect (F/R)	SS	Degr. of Freedom	MS	Den.Syn. Error df	Den.Syn. Error MS	F	p
Intercept	Fixed	210278839	1	210278839	3	3834310	54.84137	0.005093
sel	Fixed	664806	2	332403	6	1382253	0.24048	0.79348
block	Random	11502931	3	3834310	6	1382253	2.77396	0.132975
sel*block	Random	8293520	6	1382253	0	0		
Error			0					

Table 18. Results of ANOVA performed on SSD of the evening bout of activity for *early, control* and *late* stocks for 12-hr phase difference regime.

	Effect (F/R)	SS	Degr. of Freedom	MS	Den.Syn. Error df	Den.Syn. Error MS	F	p
Intercept	Fixed	208237353	1	208237353	3	2258768	92.19068	0.002397
sel	Fixed	916984	2	458492	6	399006	1.14909	0.378013
block	Random	6776304	3	2258768	6	399006	5.66099	0.034886
sel*block	Random	2394035	6	399006	0	0		
Error			0					

Table 19. Results of ANOVA performed on SSD of the morning bout of activity for *early, control* and *late* stocks for 16-hr phase difference regime.

	Effect (F/R)	SS	Degr. of Freedom	MS	Den.Syn. Error df	Den.Syn. Error MS	F	p
Intercept	Fixed	107754223	1	107754223	3	1584383	68.01022	0.003733
sel	Fixed	1123073	2	561537	6	695380	0.80752	0.489143
block	Random	4753148	3	1584383	6	695380	2.27844	0.179651
sel*block	Random	4172281	6	695380	0	0		
Error			0					

Table 20. Results of ANOVA performed on SSD of the evening bout of activity for *early, control* and *late* stocks for 16-hr phase difference regime.

	Effect (F/R)	SS	Degr. of Freedom	MS	Den.Syn. Error df	Den.Syn. Error MS	F	p
Intercept	Fixed	743170545	1	743170545	3	1733781	42.86414	0.007244
sel	Fixed	35698812	2	17849406	6	2346465	0.76063	0.507669
block	Random	52013450	3	17337817	6	2346465	0.73883	0.56633
sel*block	Random	140798792	6	23466465	0	0		
Error			0					

Table 21. Results of ANOVA performed on SSD of the morning bout of activity for *early, control* and *late* stocks for 20-hr phase difference regime.

	Effect (F/R)	SS	Degr. of Freedom	MS	Den.Syn. Error df	Den.Syn. Error MS	F	p
Intercept	Fixed	84315037	1	84315037	3	551423	152.9044	0.001139
sel	Fixed	2582564	2	1291282	6	1194953	1.0806	0.397363
block	Random	1654269	3	551423	6	1194953	0.4615	0.719412
sel*block	Random	7169720	6	1194953	0	0		
Error			0					

Table 22. Results of ANOVA performed on SSD of the evening bout of activity for *early, control* and *late* stocks for 20-hr phase difference regime.

	Effect (F/R)	SS	Degr. of Freedom	MS	Den.Syn. Error df	Den.Syn. Error MS	F	p
Intercept	Fixed	83583084	1	83583084	3	4001468	20.8881	0.019652
Selection	Fixed	132086	2	66043	6	110724	0.59646	0.580413
Block	Random	12004404	3	4001468	6	110724	36.13903	0.000309
Sel*block	Random	664346	6	110724	0	0		
Error			0					

Table 23. Results of ANOVA performed on SSD of the morning bout of activity for *early, control* and *late* stocks for 24-hr phase difference regime.

	Effect (F/R)	SS	Degr. of Freedom	MS	Den.Syn. Error df	Den.Syn. Error MS	F	p
Intercept	Fixed	89089999	1	89089999	3	4277133	20.82937	0.019727
Selection	Fixed	1053999	2	526999	6	589286	0.8943	0.457167
Block	Random	12831398	3	4277133	6	589286	7.25816	0.020181
Sel*block	Random	3535714	6	589286	0	0		
Error			0					

Table 24. Results of ANOVA performed on SSD of the evening bout of activity for *early, control* and *late* stocks for 24-hr phase difference regime.

	Effect (F/R)	SS	Degr. of Freedom	MS	Den.Syn. Error df	Den.Syn. Error MS	F	p
Intercept	Fixed	6735.335	1	6735.335	3	0.026124	257826	0
SEL	Fixed	0.153	2	0.077	6	0.029172	2.6	0.151356
BLOCK	Random	0.078	3	0.026	6	0.029172	0.9	0.49595
SEL*BLOCK	Random	0.175	6	0.029	0	0		
Error			0					

Table 25. Results of ANOVA performed on free running period of the for *early*, *control* and *late* stocks for 4-hr phase difference regime.

	Effect (F/R)	SS	Degr. of Freedom	MS	Den.Syn. Error df	Den.Syn. Error MS	F	p
Intercept	Fixed	6578.492	1	6578.492	3	0.027073	242992.8	0
SEL	Fixed	0.347	2	0.174	6	0.051031	3.4	0.102838
BLOCK	Random	0.081	3	0.027	6	0.051031	0.5	0.677775
SEL*BLOCK	Random	0.306	6	0.051	0	0		
Error			0					

Table 26. Results of ANOVA performed on free running period of the for *early*, *control* and *late* stocks for 8-hr phase difference regime.

	Effect (F/R)	SS	Degr. of Freedom	MS	Den.Syn. Error df	Den.Syn. Error MS	F	p
Intercept	Fixed	6633.687	1	6633.687	3	0.111619	59431.57	0
SEL	Fixed	0.562	2	0.281	6	0.058872	4.78	0.057437
BLOCK	Random	0.335	3	0.112	6	0.058872	1.9	0.231355
SEL*BLOCK	Random	0.353	6	0.059	0	0		
Error			0					

Table 27. Results of ANOVA performed on free running period of the for *early*, *control* and *late* stocks for 16-hr phase difference regime.

	Effect (F/R)	SS	Degr. of Freedom	MS	Den.Syn. Error df	Den.Syn. Error MS	F	p
Intercept	Fixed	6645.168	1	6645.168	3	0.049122	135278.3	0
SEL	Fixed	0.62	2	0.31	6	0.077629	4	0.078912
BLOCK	Random	0.147	3	0.049	6	0.077629	0.6	0.62041
SEL*BLOCK	Random	0.466	6	0.078	0	0		
Error			0					

Table 28. Results of ANOVA performed on free running period of the for *early*, *control* and *late* stocks for 20-hr phase difference regime.

	Effect (F/R)	SS	Degr. of Freedom	MS	Den.Syn. Error df	Den.Syn. Error MS	F	p
Intercept	Fixed	6685.595	1	6685.595	3	0.083042	80509.05	0
SEL	Fixed	0.964	2	0.482	6	0.053783	8.96	0.015778
BLOCK	Random	0.249	3	0.083	6	0.053783	1.54	0.297304
SEL*BLOCK	Random	0.323	6	0.054	0	0		
Error			0					

Table 29. Results of ANOVA performed on free running period of the for *early*, *control* and *late* stocks for 24-hr phase difference regime.