Evolution of circadian rhythms in *Drosophila melanogaster* populations reared under semi-natural conditions

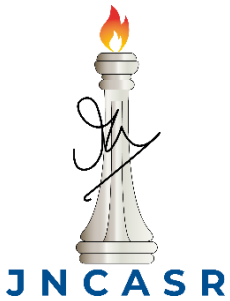
Thesis submitted

in partial fulfilment of the requirements for the degree of

Doctor of Philosophy

by

Chitrang Dani



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Dedicated to my mother, *Jayshree* and my grandmother, *Chandrika*

“*Mysteries do not lose their poetry when solved. Quite the contrary; the solution often turns out more beautiful than the puzzle and, in any case, when you have solved one mystery you uncover others, perhaps to inspire greater poetry*.”

~ Richard Dawkins ~

Contents

Declaration

The work presented in this thesis entitled "**Evolution of circadian rhythms in *Drosophila melanogaster* populations reared under semi-natural conditions**" is the result of investigations carried out by me in the Neuroscience Unit of Jawaharlal Nehru Centre for Advanced Scientific Research (JNCASR), Bengaluru, India, under the supervision of Professor Sheeba Vasu. I hereby declare that the work reported here has not been submitted elsewhere for any other degree in this or any other university.

Any part of the presented content, if an outcome of collaborative research or adopted from other studies, has been duly acknowledged within the text and in the references. Any omission which may have occurred is likely due to oversight or an error in judgment and is highly regretted.

Chitrang Dani

Bengaluru, India

October 2022

Certificate

It is certified that the work contained in the thesis titled "**Evolution of circadian rhythms in *Drosophila melanogaster* populations reared under semi-natural conditions**," is the result of investigations undertaken by **Mr. Chitrang Dani** under my supervision in the Neuroscience Unit of Jawaharlal Nehru Centre for Advanced Scientific Research (JNCASR), Jakkur, Bengaluru, India and that the results presented in the thesis have not previously formed the basis for the award of any diploma, degree or fellowship.

Prof. Sheeba Vasu
Neuroscience Unit
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List of Publications

1. Dani, C., and Sheeba, V. (2022). Drosophila populations reared under tropical semi-natural conditions evolve season-dependent differences in timing of eclosion. Front. Physiol. 13, 954731. https://doi.org/10.3389/fphys.2022.954731

2. Dani C., Kannan N.N. and Sheeba V. (2022) Environmental adaptation and evolution of circadian clocks in *Physiology of Insect Clocks* (eds.) Numata, H. and Tomioka K. *Springer* (accepted).

Glossary

Accuracy of entrainment: Inverse of day-to-day variability in phases of entrainment.

Adaptation: The process by which organisms evolve traits that confer higher fitness advantage in the organisms' habitat. Alternatively, any trait confers higher fitness to organisms in a given environment is termed an adaptation.

After-effects: Change in free-running period (FRP) as a consequence of the entraining regime.

Circadian rhythms: (Latin *circa* = about/approximately; *diēs* = day) Biological rhythms in behaviour and physiology expressed with a period of ~24 h under constant conditions (absence of external time cues/zeitgebers).

Circadian clocks: Biological time keeping mechanisms that drive circadian rhythms.

Free-running period (τ): The period of the circadian rhythms exhibited under constant conditions.

Clines: Gradual phenotypic variation across a geographical area as a consequence of variation in geophysical features such as latitudes (latitudinal clines) or altitudes (altitudinal clines).

Directional Selection: Selection for a phenotype that constitute the extremes of the phenotype distribution in the population.

Dose Response Curve (DRC): a plot of phase-shifts incurred by a circadian system in response to different doses of stimuli (either intensity or duration) at different phases of the circadian system.

Effective population size: The size of an ideal population that would undergo equal amount of genetic drift as that of a non-ideal population of size N is defined as the effective population size (N_e).

Entrainment: Entrainment refers to the process of synchronization of circadian rhythms to external time cues (zeitgeber) such that (a) the period of the entrained rhythm match that of the zeitgeber (b) the rhythms attain a stable and reproducible phase relationship with the zeitgeber (also known as phase of entrainment) and (c) upon removal of the zeitgeber, the free-running rhythm should start from the phase of entrainment established with the prior zeitgeber.

Evolutionary fitness (Darwinian): A measure of an individual's contribution to the gene pool of the next generation in a given environment.

Inbreeding: Mating among individuals with high genetic relatedness leading to increased homozygosity, isogeny and random fixation of deleterious alleles over generations.

Phase Response Curve (PRC): A PRC depicts the magnitude of response (measured as phase shifts) to a zeitgeber at different phases of the circadian cycle, and therefore is a measure of the circadian clocks' sensitivity to the zeitgeber.

Phase-control: The phenomenon wherein rhythms free-run under constant conditions post entrainment from the phase determined by the last entraining cycle.

Phase-relationship/Phase of entrainment/Phase-angle: Difference in time (either in hours or degrees or any other unit of time) between any instantaneous state (phase) of the circadian rhythm/oscillation and that of a reference phase of the environmental oscillation.

Power of a rhythm: The amplitude of a periodogram that measures robustness of τ.

T-cycle: Zeitgeber cycles of periodicity T. For instance, T-24 indicates a 24 h zeitgeber cycles with the durations of light/dark or thermophase/cryophase summing up to 24 h, T-30 a 30 h zeitgeber cycle and so on.

Temperature compensation: Temperature compensation refers to the ability of circadian clocks to maintain a stable and constant τ across different temperatures by compensating for temperature induced changes in the rate of biochemical reactions.

Zeitgeber: (German, zeit- 'time', geber- 'giver') Any forcing oscillation (with period 'T') in the environment that can entrain a biological oscillation, for instance, light/dark or temperature cycles. Zeitgeber cycles with T different from 24-h are referred to as T-cycles.

Zeitgeber Time (ZT): ZT00 refers to the time at which lights turn ON. For other zeitgebers, it is time at which the zeitgeber value starts to increase from its lowest value.

Synopsis

Under natural conditions, circadian clocks are exposed to various abiotic and biotic environmental cues such as light, temperature, humidity, sound, olfactory cues, social factors, food availability, predation etc. Compared to this, laboratory environments (where the bulk of studies on circadian rhythms are carried out), are usually composed of a simplistic regime with a single time-cue, such as a light-dark cycle (LD (12:12)) of step-up/step-down type. The fruit fly, *Drosophila melanogaster* has been an important model for chronobiology in terms of addressing fundamental questions about the nature of biological clocks to identifying components of the molecular clock. However, as stated above, most studies with this model have also been conducted in the laboratory and little is known about its timing in the wild. A feasible way to study circadian rhythms in naturalistic environments is to study them in a semi-natural enclosure with exposure to natural conditions, while mostly eliminating predation and providing complete control on food availability to the experimenter. This strategy has been used in a few recent studies to answer questions about how behaviour under semi-natural conditions differs from that in the laboratory. However, these studies were conducted with inbred strains or laboratory reared populations, which have not provided answers to questions about the evolution of clocks under such conditions.

Which clock properties would evolve to be different under semi-natural conditions compared to standard laboratory conditions? Would adaptation to semi-natural environments alter the circadian phenotype under standard laboratory conditions? It also becomes imperative to ask which time cues are important for timing behaviour and physiology when studying the evolution of clocks under semi-natural environments. Ultimately, identifying potential

selection pressures for *Drosophila* under natural conditions would prove to be useful in gaining a holistic perspective.

To address these questions, a long-term experimental evolution study was initiated comparing rhythms of four large, outbred populations of *D. melanogaster* evolving under semi-natural conditions ($NT24_{1-4}$) to their ancestral controls in the laboratory ($T24_{1-4}$). The motive underlying the establishment of these populations was to find out if circadian clocks evolve stability or flexibility of clock properties as a consequence of rearing under a complex environment with naturalistic time cues compared to standard laboratory conditions. The NT24 populations have completed 162 generations (September 2022) of rearing under semi-natural conditions. The main objectives of my work were characterizing the clock properties for activity-rest and eclosion rhythms, comparing the circadian phenotypes for these populations under standard laboratory as well as semi-natural conditions across seasons, and elucidating which time-cues are more important for phasing of rhythms under semi-natural conditions. Additionally, I surveyed several relevant life-history associated traits for both sets of populations to gain a deeper understanding of the nature of selection under semi-natural environments.

Chapter 2 describes the studies conducted to decipher the nature of selection pressures that flies might experience under semi-natural conditions. I characterized relevant life-history related traits for both sets of populations *viz.* development time, pre-adult survivorship, fecundity, heat and desiccation tolerance. I found that NT24 populations exhibit a higher offspring count under harsh semi-natural conditions, but not under moderate ones or standard laboratory conditions and also show higher heat tolerance compared to T24 populations. Along with these, some known trait correlations, e.g., correlation of development time and dry-weight at eclosion, were not consistent for the NT24 populations.

In Chapter 3, I describe the results of characterization of the eclosion rhythm for both sets of populations. Initially, I studied eclosion rhythms under standard laboratory conditions; however, there was no difference in the phasing of eclosion. Additionally, eclosion rhythms of NT24 and T24 populations also did not differ in intrinsic periodicity or power of the rhythm. On the other hand, under semi-natural conditions, NT24 populations exhibited differences in phasing of the eclosion rhythm compared to T24s. They exhibited an early phase of onset and peak of eclosion manifested under harsher semi-natural environments, but not under moderate ones. Further analysis showed that this phenotype might be responsive to changes in the temperature cycle. In order to ascertain this, I simulated specific semi-natural light and temperature regimes in the laboratory and found that NT24 populations indeed advance their phase of eclosion in response to increased magnitude of the temperature cycle.

In Chapter 4, I describe the results of investigations conducted to characterize the activity-rest rhythms of these two sets of populations. Under standard laboratory conditions, there was no difference in activity-rest behaviour. In a similar manner to that of the eclosion rhythm, when I looked at activity-rest behaviour under semi-natural conditions, I did not find any differences in activity-rest rhythms of NT24 and T24 populations under semi-natural conditions. Exploratory studies under entraining regimes in the absence of light, such as semi-natural DD conditions, step up/down and ramped temperature cycles etc., showed that NT24 populations can show slightly increased activity during daytime compared to T24 populations. These differences may vary depending on the regime used, and the underlying mechanism is not entirely understood. While there is no difference between the intrinsic periodicity and power of the rhythm, I found that the NT24 populations have evolved circadian clocks that exhibit higher precision of activity-onset under constant conditions (after ~80 generations); otherwise not differing in phasing under lab or semi-natural conditions. This is interesting because it

challenges the widespread notion of irrelevance of sustained rhythmicity under constant conditions in the context of evolution of clocks in nature.

In Chapter 5, I extend the studies on the intrinsic clock properties by investigating the state of the circadian clock in *D. melanogaster* under constant darkness and constant low temperature. Non-permissible conditions such as constant low temperature affect the persistence of rhythmicity, and this clock property is called the conditionality of rhythms. Studies to characterize conditionality of rhythms have been previously conducted on organisms such as cyanobacteria and dinoflagellates. The temperature used for my set of studies is not harmful in terms of survival and reproduction for *D. melanogaster*. I found that the rhythmicity of flies in constant darkness at lower temperature was significantly less compared to the standard temperature, which could be due to the inability of clocks to compensate for the lowered temperature, beyond a limit. From these experiments, I found that the NT24 populations exhibit higher rhythmicity than their laboratory-reared counterparts at low temperature. Interestingly, attempts to synchronize rhythms of NT24 and T24 populations under such conditions to zeitgebers showed differential responses to light and temperature.

From my results, it appears that despite being in a variable cyclic environment, the stability of the internal clock's period (precision) is under selection. This stability of the intrinsic period does not appear to evolve due to increased stability of phase relationships with zeitgebers (accuracy) under semi-natural conditions. Additionally, differences in the persistence of rhythms at low temperature also hint toward a possible difference in stability of the intrinsic period. Hence, it is interesting to speculate how enhanced stability of intrinsic period may be beneficial under naturalistic entraining conditions. Further, my results suggest that selection pressures acting on fly populations in naturalistic conditions are likely to vary across different seasons and may also be different for different rhythms such as eclosion and activity-rest. This

opens up several avenues for future research, such as characterizing the selection pressures on different rhythms of an organism due to variability in environments, whether artificially increasing fluctuations of environmental variables could result in further change of the stability of clocks, as well as if stability of internal rhythms can affect entrainment to complex natural environments.

Preface - Time, timekeepers and timekeeping

‘‘In order to see whether we had kept an exact account of the days, we charged those who went ashore to ask what day of the week it was, and they were told by the Portuguese inhabitants of the island that it was Thursday, which was a great cause of wondering to us, since with us it was only Wednesday. We could not persuade ourselves that we were mistaken; and I was more surprised than the others, since having always been in good health, I had every day, without intermission, written down the day that was current.’

- From ‘First voyage round the world by Magellan’ by Antonio Pigafetta

In our work as scientists studying biological clocks, we frequently use the term "time" without actually comprehending what it means. The topic of what time is, which has perplexed scientists, philosophers, and theologians alike for ages and is beyond the scope of this article, is in fact a tremendous effort.

Mysteriously, the idea of time being unreal had become a common one among thinkers from several disciplines. Immanuel Kant, in 1781, stated that “time is thus a purely subjective condition of human intuition, and in itself, apart from the subject, is nothing.” While acknowledging the existence of time, Kant claimed that it is subjective and therefore unreal, as in "my time" and "your time" are not always the same. Furthermore, Kant asserted that time is intuitive to humans, which, as modern day research has shown, isn't always the case. Eminent philosopher J.M.E. McTaggart (1908), well known for his writings on the philosophy of time, supported the same notion that time is unreal, that has resisted an anti-thesis for almost a century at this point. But if time is unreal, then what do we mean when we say “that place is 30 minutes away” or “we can meet after two days”? And what are we measuring using clocks? These questions lead us to subtler component of time, specifically its measurement, or

determining how long it takes between two events. The three characteristics listed below are attributed to measurements by the realist school of measurement philosophy: (i) what is measured is independent of the method used to measure it; (ii) what is being measured are attributes of things rather than the things themselves; and (iii) when measuring, numerical values are discovered rather than assigned. Thus, measurements can be described as discovered/assigned numerical values to every distinguishing attribute of an item or event. Again, by definition, we can only measure what exists and is real and time is a quality which despite evading a precise definition can be quantitatively measured.

Although a definition of time eludes us, it may be said that humans have developed atomic clocks that can measure the passage of time. Because we intuitively understand what time is, we are able to measure it using these clocks. Not just humans, life forms across almost all phyla possess biological timekeepers that function as clocks and keep time on a 24-hour basis.

Jean-Jacques d'Ortous de Mairan, a French scientist, demonstrated through experimentation that the daily leaf movements in the Mimosa plant continue even in the absence of a solar day-night cycle and suggested that organisms may possess an internal ‘clock’. de Mairan and his colleague Jean Marchant at the Royal Academy of Science even compared the phenomenon with the ability of bedridden people who never actually see daylight but could distinguish daytime from night-time. Karl von Frisch and his graduate student Beling marked individual bees and trained them to feed on sugar solution at an artificial feeder at the same time every day (once in 24 h), and on the test day did not provide the sugar solution and noted the time at which bees came to the artificial feeder. It was observed that most individuals came to the feeder within the training time, suggesting the presence of some mechanism of time measurement in bees. Additionally, while studying bird navigation, Gustav Kramer, a renowned German ornithologist found that birds can also measure the passage of time. Kramer

had shown in earlier studies that birds use the sun as a reference point to navigate successfully. In his experiment, he trained birds to eat from an artificial feeder placed at a relative position to the sun at a particular time of the day. Given that the sun's position in relation to the earth fluctuates, by ~15 degrees every hour, birds' preferences would likewise move by this amount if they were unable to measure time. However, Kramer discovered that birds prefer to keep using the same feeder that they were trained to use, showing that they are capable of correcting for the shift in the sun's position to find food sources, and in order to do so, a mechanism to assess the passing of time must exist. These studies were the first to show that animals are able to gauge the passing of time.

For organisms not believed to have consciousness, such as single-celled cyanobacteria, fungi, as well as plants, biological mechanisms serving as clocks exist. The study of rhythms in various organisms has revealed that, despite having clocks that run at somewhat different rates, animals are synchronised to the day and night cycles of their surroundings in order to find food, fend off predators, and compete for resources. Members of a species not only know what to do and when, but they may also synchronise their behaviour with those of other species to facilitate coexistence. Thus, the field has come a long way from debating about the existence of biological clocks to elucidating the nature of these clocks.

Several elegant studies have revealed how this internal clock is made up of fluctuating protein levels, is reset by outside time cues like temperature and light, and regulates a number of physiological and behavioural processes via output pathways. The molecular architecture and functional significance of this complex biological system are still being thoroughly investigated. This work is being aided by more sophisticated research techniques that combine genetics, developmental biology, evolutionary biology, endocrinology, neurobiology, molecular biology, and biophysics using a variety of model organisms.

"*Under ordinary conditions, the cycling of this innate biological clock is synchronized by the overwhelmingly greater geophysical clock on which it is modeled. But the inner clock still beats and plays a vital role. Our task is to listen for the inner beating of the biological clock in those rare situations where it can be heard independently.*"

– Arthur Winfree (Winfree, 1987)

There still exist several gaps in our understanding of critical aspects of circadian rhythms which deserve serious consideration. For instance, although the impact of circadian rhythms on a range of behavioural and physiological phenomena, such as cognition, mood, metabolism, reproduction, ageing, etc., has been widely acknowledged, the detailed description of the molecular and biochemical pathways by which the circadian system is connected to these processes is not complete. In order to fully comprehend an organism's apparent timing, various aspects of 'time' must be considered at once, some of which are internal (such as genetic elements) and others which are external (such as environmental variables). However, the disciplines of chronobiology and ecology place differing emphasis on these factors, and as a result, they have diverse perspectives on an organism's environment. Although contacts with external time are essential for the operation of internal biological clocks, the discipline of chronobiology emphasizes that rhythmicity can be sustained without them. This view contrasts with ecological perspectives that emphasize or are restricted to the manner in which an organism's environment influences how it functions. On the other hand, recent research in chronobiology has revealed differences between rhythms in the lab and in the environment, where organisms are exposed to a wide range of stimuli. A detailed description of this sub-field is in the next introductory chapter, while several questions on the evolution of clocks under natural conditions are addressed in the subsequent parts of this thesis.

Chapter 1 . Insect clocks – adaptation and evolution

Note: The contents of this chapter have been accepted to be published as:

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1.1 Introduction

Organisms face environmental challenges resulting from cyclic variations in light, temperature, humidity, etc. and thus, the need to effectively adapt to these environmental changes is hypothesized to have driven the evolution of highly conserved biological timekeeping systems across species. Circadian clocks provide extrinsic advantage to organisms by improving their ability to anticipate environmental changes and to synchronize behavioural and physiological processes with daily environmental cycles (Enright, 1980; Sharma, 2003). During evolution, the circadian timing system may have also evolved the ability to govern the timing of endogenous processes and thus confer an additional intrinsic benefit to the organism (Pittendrigh, 1993; Sharma, 2003). Insects have served as important models for studies investigating the adaptive value of circadian clocks and yet have distinct attributes from other common model systems. Various forms of the term adaptation are commonly used in two contexts - as a constant process through which organisms become suited to their surroundings or as a trait giving organisms a higher fitness in a specific environment. This chapter uses ‘adapt’ for the former and ‘adaptive/adaptation’ for the latter context. Thus, adaptive traits for an organism in terms of survival and reproductive output in a given environment are likely to

be favoured by natural selection. Individuals with such beneficial traits can be thought of as having higher evolutionary fitness and contributing proportionally more to the gene pool.

Since insects are ectothermic and have a smaller body size than other animals, they are subject to various physiological stressors with fatal or sub-lethal deleterious effects. Most insects undergo active and inactive phases throughout the day, which is thought to help them cope with physiological stress. The circadian clock regulates such daily rhythmicity in behaviour (e.g., activity, feeding, mating, and oviposition), physiological processes, and developmental events like hatching, pupariation, and eclosion. While the abiotic environment poses risks such as death or sterility due to extreme temperatures or desiccation, interactions with the biotic environment also give rise to other stressors such as energy expenditure, starvation and predation risk.

1.2 Diversity of circadian clock function in insects

Honey bees and fruit flies were among the earliest used insect models in chronobiology. Over the years, they have provided many insights into the behavioural, physiological, genetic and neuronal bases of circadian rhythms (Beer and Helfrich-Förster, 2020). Apart from the contrasting nature of their sociality, both these models also differ in aspects of clock function: in honey bee *Apis mellifera*, the circadian clock is known to play a role in time-place learning, memory and solar compass navigation, less so for the commonly studied fruit fly *D. melanogaster*. The clock in honey bees also seems to be sensitive to direct and indirect social cues such as substrate-born vibrations, volatiles and temperature cycles in the hive (Siehler and Bloch, 2020; Giannoni-Guzmán et al., 2021). Such is the diversity in clock function that these species can scarcely represent Hymenoptera and Diptera. In Hymenoptera, apart from eusocial

honey bees, there are primitively social, facultative social and solitary bees that have been shown to have diversity in clock-controlled behaviour (Shell and Rehan, 2018). Bumblebees show plasticity in rhythmic behaviour just like honey bees; however, the determinant of plasticity is not age but size (Yerushalmi et al., 2006; Eban-Rothschild et al., 2011). On the other hand, the solitary bee *Osmia bicornis* displays rhythmic locomotor behaviour and has a mature circadian system at emergence (Beer and Helfrich-förster, 2020). This has been attributed to its emergence from small nests in the spring season, where it experiences environmental changes.

In drosophilids, the variation in rhythmicity, photoperiodic response and incidence of diapause has shown that cosmopolitan species such as *D. melanogaster* may not be the best choice for studying the response to photoperiod and diapause incidence. There is a need to conduct research on typical non-model insects, perhaps better suited for addressing questions on the circadian clock's role in specific behaviours associated with seasonal environmental changes. For example, the pea aphid *Acyrthosiphon pisum* is an emerging model whose reproductive strategy varies across the year in response to photoperiodic change. Pea aphids adopt viviparous-parthenogenesis during the warmer months of spring and summer, and with the advent of shorter day length in autumn, the reproductive strategy becomes sexual, which results in the production of fertile eggs. These aphid eggs survive the harsh winter, giving rise to new parthenogenetic females (Hardie and Vaz Nunes, 2001). Recently, it was also shown that clock neurons in pea aphids neuroanatomically connect to the pars intercerebralis and the corpora allata complex; supporting the possibility of a direct link between the circadian clock and photoperiodic response to mediate hormone release (Colizzi et al., 2021). Similarly, several new perspectives have been gained by studying the role of the circadian clock and diapause induction in non-model insects such as butterflies, moths and wasps (Denlinger et al., 2017). Provision of a suitable thermoperiod of low temperature in the absence of light itself can suffice

to induce diapause in the parasitoid jewel wasp *Nasonia vitripennis* (Saunders, 2002) and some moths (Beck, 1983).

An additional factor to consider here is the frequent overbearing effect of ecology upon clock function. Antarctic midges are an excellent example of this: due to the extreme environment, *Belgica antarctica* only has a short period of time during the year with temperatures permissive for development. As a result, these midges remain active throughout the day, and despite possessing circadian clock genes, there is no cyclic pattern of expression seen in similar species living in temperate regions (Kobelkova et al., 2015). The absence of persistent rhythmicity in extreme conditions might not be as baffling as the exact opposite. *Ridgeia piscesae*, a tubeworm typically found near hydrothermal vents with extremely high temperatures, has exhibited fluctuations in density at the population level with circadian and ultradian periodicities (Cuvelier et al., 2014). Several such examples exist, reviewed in (Abhilash et al., 2017), and while an intrinsic advantage is often hypothesized for such cases, it will be interesting to see the results of future studies addressing such questions that move beyond speculation. On the other hand, studies using cosmopolitan species across environments have largely convinced us of potential environmental factors shaping rhythms and clock function (Adrion et al., 2015). This sub-field would benefit tremendously by tracing variation in genes of interest and genomic variation brought about by the environment as well as gene × environment interactions and by more studies conducted under a multitude of differing semi-natural conditions for verification of reported genetic correlations.

Another layer of variation in clock function of individuals occurs by interspecific interactions that are ecology specific, which adds to already existing complexity in circadian behaviour. This is a relatively understudied field at the interface of chronobiology and ecology in which interactions related to predation, food availability, competition, parasitism etc. have been

linked to the influence of rhythms (Kronfeld-Schor et al., 2017). For instance, in deer ticks, detachment from diurnal hosts such as hamsters has been shown to occur synchronously in the late day, which concentrates ticks in the nests of their nocturnal mouse hosts, possibly enhancing the transmission of pathogens (Mather and Spielman, 1986). Similarly, for two non-permanent ticks - *Ixodes arboricola* and *Ixodes ricinus*, detachment from their common host *Parus major* (great tit) appears to be temporally coordinated. Detachment of *I. ricinus* occurs when tits are most active during daytime while for *I. arboricola* detachment occurs during the night when the birds sleep in tree holes (Heylen and Matthysen, 2010). Temporal avoidance of competition may also be beneficial, as exemplified by the solitary bee *Proxylocopa olivieri*. This bee forages maximally at dawn and dusk, thereby avoiding a temporal overlap with other bees like *A. mellifera*, which show unimodal foraging during the day (Gottlieb et al., 2005). In cohabitating dung beetle guilds, such temporal avoidance of superior competitors has been observed (Krell-Westerwalbesloh et al., 2004). Apart from these, research on important disease vectors such as mosquitoes *Aedes aegypti* and *Anopheles gambiae* has shown over the years that several behaviours important for disease spread, such as biting, mating and flight activity are under the control of the circadian clock (Jones et al., 1967; Yee and Foster, 1992; Rund et al., 2012). Whether these behaviours also have interspecific influences might be worth investigating. These recent advances have only revealed the void in our understanding of the regulation of circadian behaviour in an ecologically realistic scenario. Hence, future research on such interspecific effects will enhance our understanding of circadian behaviour in model and non-model insects.

1.3 The impact of light and temperature cycles on circadian rhythms

Environmental light-dark cycles with changes in intensity and duration of light are believed to be the prime force of selection behind the evolution of circadian clocks (Roenneberg and Foster, 1997; Woelfle et al., 2004). It is conceivable that circadian clocks segregated daytime and night-time processes, and such temporal segregation of incompatible processes also minimized the harmful effects of the diurnal photo-oxidative environment on light-sensitive reactions (Pittendrigh, 1993). In agreement with this hypothesis, studies showed that the cells of *Chlamydomonas reinhardtii* are more sensitive to exposure of UV radiation during the evening when the UV component in solar light is lower. The timekeeping system in *C. reinhardtii* may have evolved to time the light-sensitive cell division process during the evening or in the early night and temporally segregated it from the harmful effect of UV radiation during the day (Nikaido and Johnson, 2000). In some cyanobacteria, the two incompatible and crucial metabolic processes of photosynthesis and oxygen-sensitive nitrogen fixation are temporally segregated. In others, specialized structures called heterocysts evolved to spatially segregate nitrogen fixation from photosynthesis (Stal and Krumbein, 1985; Mitsui et al., 1986).

While light-dark cycles imposed a selection pressure on early life forms to evolve an endogenous timekeeping system, changes in day length, such as extremely short and long photoperiods, may have acted as additional constraints for the evolution of circadian clocks with optimal timing to adapt to different latitudes. Hence, species belonging to divergent latitudes are likely to evolve differences in their timekeeping systems. Latitudinal clines refer to correlated phenotypic and/or genetic differences observed over a geographical area with a change in latitude. Since circadian clocks are believed to confer an adaptive advantage to their owners in natural environments, many in the field asked whether one would observe a change in circadian clock properties with latitudinal changes in natural conditions.

Several studies have surveyed circadian behaviour and clock gene variation over large geographical areas, and latitudinal clines in behaviour, physiology, gene frequencies, protein isoforms etc. have been discovered. Surveys of 57 strains of *Drosophila littoralis* (30˚N-70˚N) and 12 strains of *Drosophila subobscura* (56˚N-63˚N) revealed a latitude-dependent variation in the phase and period of the eclosion rhythm (Lankinen, 1986, 1993). Additionally, strains that naturally occurred at higher latitudes had a shorter period and advanced phase than those at lower latitudes. A study on four Japanese strains of *Drosophila auraria* (34.2˚N-42.9˚N) revealed a significant latitudinal cline in phase, lability of the period and amplitude of the Phase Response Curve (PRC) of eclosion rhythm (Pittendrigh and Takamura, 1989), revealing that *D. auraria* strains occurring at a higher latitude had a lower amplitude of phase response curve. A northern species, *Drosophila montana* is found to be widespread at high latitudes and these flies completely lack morning activity. They maintain free running periodicity better under constant light than under constant darkness and also differ in the number and location of PDF and CRY-expressing neurons compared to *Drosophila melanogaster*. High altitude species such as *Drosophila lummei*, *D. littoralis* and *Drosophila ezoana* also exhibit similar features in their activity-rest rhythms that correlate with the difference in the neurochemistry of PDF and CRY in their circadian clock network. These are likely to be the specific adaptive features of the circadian clock that evolved in *Drosophila* species in winter environments to colonize polar regions (Kauranen et al., 2012; Menegazzi et al., 2017; Beauchamp et al., 2018). In another example, *Chymomyza costata*, a related species found at latitudes above 40˚N, also becomes arrhythmic under constant darkness, however, its molecular clock remains rhythmic and uncoupled from the behavioural output (Bertolini et al., 2019). Locomotor activity pattern and clock network neurochemistry are similar in distantly related *Drosophila* species colonized at high latitudes. In contrast, phylogenetically related species living at different latitudes exhibit clock organization and coupling differences. These studies suggest that in some *Drosophila*

species, a *D. melanogaster* like ancestral fly clock network evolved with altered PDF and CRY neurochemistry to adapt and colonize in the high latitude environments (Beauchamp et al., 2018; Bertolini et al., 2019).

With respect to clines in clock gene variation and gene frequencies, the product of *timeless* gene has two allelic forms- *ls-tim* and *s-tim* varying in length due to the presence of a second start codon downstream. The presence of the *ls-tim* allele results in the formation of full length LS-TIM and truncated S-TIM while presence of *s-tim* results in the formation of the truncated S-TIM only. S-TIM is more sensitive to light whereas LS-TIM is less sensitive to light due to the weaker interaction with CRY than S-TIM (Sandrelli et al., 2007). It is reported that natural populations of *D. melanogaster* in Europe show a latitudinal cline for this polymorphism with frequency of *ls-tim* increasing from north to south of Europe (Tauber et al., 2007). The reduced light sensitivity of *ls-tim* flies prevents the enhanced TIM degradation and arrhythmicity during prolonged exposure to light under northern long summer day length. In addition to lower light sensitivity, *ls-tim* mutation induces earlier diapause in female flies during autumn. Thus it appeared that the latitudinal cline in TIM polymorphism evolved in the circadian timekeeping system to adapt to the seasonal changes in the north (Kyriacou et al., 2008). This has been substantiated by a recent study that *ls-tim*, but not *s-tim* flies can synchronize to temperature cycles under constant light and simulated northern summer conditions, and the expression of *ls-tim* in clock neurons is sufficient for this synchronization (Lamaze et al., 2022).

While light is considered the most potent time-cue for the circadian timing system in almost all organisms, temperature has also been found to entrain the circadian clocks of various organisms including *Drosophila* (Zimmerman et al., 1968; Balzer and Hardeland, 1988; Tomioka et al., 1998). Under lower temperature, *D. melanogaster* schedule a large proportion of their activity to daytime, whereas under warmer temperature they exhibit increased night-

time activity with a pronounced midday siesta (Majercak et al., 1999). Differential splicing of *per* and *tim* redistributes flies' activity pattern depending on the seasonal environmental temperature changes. Splicing of an intron (*dmpi8* intron) located in the 3' UTR of *per* mRNA is enhanced at lower temperature, accelerating the molecular clock phase and advancing the evening activity of flies under colder temperatures. This intron splicing is attenuated at higher temperatures, slowing down the pace of the clock to delay evening activity (Majercak et al., 1999). Temperature change also alters the splicing pattern of *tim* to generate four isoforms - *tim-long*, *tim-cold* and *tim-short and cold* and *tim-medium*. These *tim* isoforms act as thermal sensors regulating TIM levels at various temperatures to govern the activity pattern (Anduaga et al., 2019). *Drosophila simulans* also exhibit thermal sensitive alternate *per* splicing as an adaptation to summer in a temperate climate (Low et al., 2008).

In *D. melanogaster*, *per* encodes a continuous stretch of threonine-glycine (Thr-Gly) repeats. A latitudinal cline exists in the length of the Threonine-Glycine (TG) repeat number at the per locus in natural populations of Europe and North Africa. Two major alleles, *per* $(TG)_{17}$ and *per* $(TG)_{20}$, comprise 90% of the variation observed, with the frequency of *per* $(TG)_{20}$ decreasing, whereas that of *per* $(TG)_{17}$ increasing from north to south (Costa et al., 1992; Sawyer et al., 1997). Similarly, a cline in *per* $(TG)_{20}$ frequency is observed in Australia, though it appears less robust than in Europe (Sawyer et al., 2006). The length of TG repeats is associated with temperature compensation based on a TG monomer's structural property to confer greater thermal stability (Castiglione-Morelli et al., 1995). Assessment of the functional significance of this repeat length polymorphism showed that TG_{17} may be suitable to a thermally less variable environment, whereas TG_{20} variant may be under selection for its better temperature compensatory ability of circadian period under larger temperature fluctuations (Kyriacou et al., 2008). TG repeat length polymorphism is observed in other species such as *D. simulans,* and *D. pseudoobscura* (Costa et al., 1991; Rosato et al., 1994). The results of these cline studies

are compelling evidence that in natural environments, in addition to light, temperature changes may have contributed to the genetic variance and evolution of the circadian timing system (Figure 9.1).

In nature, animals experience varying light intensity and temperature throughout the day and seasons. Understanding the synergistic impact of such daily varying time cues on the evolution of the circadian clock and its adaptive significance is crucial. Recent studies showed that activity-rest rhythm and eclosion rhythm differ considerably under natural conditions from those observed under laboratory experiments (Vanin et al., 2012; De et al., 2013; Prabhakaran et al., 2013). Under laboratory conditions, *D. melanogaster* exhibits morning and evening peaks of activity with a siesta during the middle of the day. However, under semi-natural conditions, an additional afternoon (A) peak of activity replaces the siesta (Vanin et al., 2012). This afternoon activity peak mainly depends on temperature and requires the temperature-sensitive transient receptor potential A1 (TRPA1) ion channel (Das et al., 2015; Green et al., 2015). It is also observed that PER levels change seasonally under semi-natural conditions, whereas those of TIM remain more or less constant (Menegazzi et al., 2013). The oscillation of these proteins are decoupled in summer conditions, and how it continues to drive rhythmic behavioural output is yet to be elucidated.

1.4 Evolutionary consequences of climate change on insect clocks

As with other organisms, insects too are subject to a wide variety of environmental cues, which can dramatically affect their endogenous circadian clocks in addition to various other systems (Figure 9.3). These may modulate physiology and behaviour across generations and become differentially affected by selection pressures to produce significant shifts in the biodiversity of

insect forms. By extension, one can view climate change and urbanization as potential challenges for circadian clocks as if they were natural experiments on the adaptability and plasticity of circadian clocks on a global scale. The day length-temperature relationship, providing valuable input to circadian systems, has remained relatively consistent in which shorter day length is often associated with lower temperatures and vice-versa. However, this relationship may not hold as consistently with global warming, resulting in temperature shifts without accompanying photoperiodic change (Walker et al., 2019). Indeed, it has been observed that population peaks for certain insects have advanced in response to increasing spring temperatures, ultimately affecting the food chain (Visser et al., 1998). The rise in mean temperature over land is marked by a pattern of diurnal asymmetry, with larger tendencies of night warming than day warming (Karl et al., 1993; Alexander et al., 2006), as well as an increase in the incidence, intensity and length of warm weather and spatial changes in water availability (Tabari, 2020). Warmer temperatures at night have been shown to have a non-trivial effect on several aspects of insect life-history such as development, fecundity and survival (Zhao et al., 2014). Another aspect worthy of consideration is thermal extremes. Minor changes in maximal temperature are often overlooked but may have non-trivial effects on organismal demography and fitness (Overgaard et al., 2014; Ma et al., 2015). Moreover, the global average increase in temperature is not representative of local change as the effects of global warming are not the same everywhere (Kerr, 2007). Thus, even though general changes due to global warming can be predicted, the realized effects on local climate and their impact on the insect behaviour, life-history and rhythms in local habitats are not understood. In an overall ecological context, the importance and impact of insects is vastly underestimated and overlooked. More data and targeted studies, as well as dissemination of our understanding to the general public and policymakers, will be needed for appropriate measures to be taken to slow down the speed with which our environments are being altered.

1.5 Influence of circadian clocks on life-history traits

Since the advent of the field, chronobiologists have been interested in whether circadian clocks influence life-history traits to add to the fitness of organisms. As geophysical environmental cycles give opportunities or periods of risk with predictable regularity on a daily basis, internal timing systems may have mainly developed to phase activity at an appropriate time of the day (Pittendrigh, 1993). Life-history-related traits comprise size at birth, development time, age and size at maturity, number/size/sex ratio of offspring, age/size-specific reproductive investments, age/size-specific death schedules and life expectancy (Stearns, 2000). The phenotype of an organism is shaped and optimized by these features and their genetic link to constraints or trade-offs. Because life-history features are the primary components of fitness, knowing how natural selection alters an organism's fitness in response to ecological constraints is crucial (Stearns, 2000). Even though evolutionary biology and chronobiology remained as separate investigatory fields for the longest time, there was good reason to suspect the involvement of circadian clocks in the life-history of organisms (Sharma and Joshi, 2002). Despite this, the possible relationship between clocks and life-history-related traits has been scarcely investigated, especially using insect models. Barring some exceptions, most investigations at this interface too have used *Drosophila* as their organism of choice.

The circadian clock influences several aspects of the *Drosophila* life cycle. For instance, in *D. melanogaster*, a rhythmic environment such as LD (12:12) has been shown to affect several traits such as adult lifespan (Pittendrigh and Minis, 1972; Klarsfeld and Rouyer, 1998; Sheeba et al., 2000), pre-adult developmental duration (Sheeba et al., 1999a), lifetime fecundity (Sheeba et al., 2000), and larval growth rate (Sheeba et al., 2002a). The effect of the environment on insect developmental programs is considered stage-specific, and such modulation occurs by switching developmental pathways (Nijhout, 2003). Time-sensitive

stimuli are often required to form certain phenotypes, usually of the next developmental stage (Smith-gill, 1983). One such phenotype for *Drosophila* larvae at the wandering stage is pupation height in laboratory culture vials, which is thought to be a proxy for expended energy by larvae during the post-wandering stage (Chippindale et al., 1997). Light has been shown to inhibit wandering, and the higher height of pupae in darkness is speculated to be an adaptation decreasing the risk of predation, heat or desiccation (Markow, 1979; Manning and Markow, 2014). A later study (Paranjpe, 2004) verified the possible involvement of circadian clocks using various daily light durations, which were expected to give rise to pupation heights ranging from lowest in LL to highest under DD. Contrary to expectations, regimes of LD (12:12) and LD (14:14) resulted in lower pupation heights than LL, suggesting that this behaviour is influenced by complex interactions between the specific regime of development and circadian clocks.

Most studies in this context have been targeted toward verifying the adaptive value of circadian clocks concerning the Circadian Resonance hypothesis (Pittendrigh and Minis, 1972). While this has been empirically validated in cyanobacteria (Ouyang et al., 1998), studies using insect models have been limited and inconclusive. When lifespans of per^0, per^T (short period) and per^L flies were compared with wild-type flies, wild-type flies were observed to live only marginally longer under a T-24 cycle (Klarsfeld and Rouyer, 1998). In fact, there were no differences under a T-16 cycle resonating with the free-running period of per^T. The observation of clock mutant flies showing bimodal peaks of activity-rest, similar to wild-type flies under semi-natural conditions (Vanin et al., 2012), also substantiated the argument against circadian resonance being a significant influence in insects.

On the other hand, ambiguous evidence of circadian resonance conferring some advantage in flesh flies (Saunders, 1972), blowflies (von Saint Paul and Aschoff, 1978), pitcher plant

mosquitoes (Emerson et al., 2008) and ants (Lone et al., 2010) made it difficult to refute the hypothesis. Recently, a long-term study using fruit flies, spanning two years and more than 50 generations, quantified several fitness components such as fertility, mating success, pre-adult survival, reproductive output etc., for wild-type and clock mutant flies (Horn et al., 2019). This study showed that in a competition assay, wild-type flies had a clear fitness advantage over per^0 flies but this advantage also persisted in LL conditions where even wild-type flies were rendered arrhythmic. Furthermore, the resonance hypothesis was partly confirmed as per^L mutants outcompeted wild-type flies in a longer T-cycle, however, per^S mutants were unable to outcompete wild-type flies under short T-cycles. This discernibly indicated that variables other than timing also contribute to the competitive fitness advantage of wild-type flies. Contradictory evidence from jewel wasps, *Nasonia vitripennis* is clearer. When jewel wasps were subjected to light-dark T cycles ranging from 20-28 hours, no differences in longevity occurred despite differences in phase of entrainment (Floessner et al., 2019). This result is thought to be a consequence of the broad range of entrainment of jewel wasps and demonstrates how circadian resonance in higher organisms such as insects is not as pervasive.

Pre-adult development time and activity/rest rhythm had been linked in a study using D. melanogaster *per* mutants, with homozygous individuals of the short period allele of *per* (per^S) exhibiting shorter development time than wild-type flies, and individuals homozygous for the long period allele (per^L) exhibiting longer development time (Kyriacou et al., 1990). However, since inbred mutant fly lines were used here, conclusions about evolutionary fitness are unreliable. Interestingly, a later study using large, outbred populations of *D. melanogaster* under two constant conditions (LL and DD) and three symmetric light-dark cycles (T-20, T-24 and T-28) showed the influence of an entraining regime on development time (Paranjpe et al., 2005). *D. melanogaster* developed fastest under LL, followed by T-20, DD, T-24 and T-28 regimes, demonstrating the involvement of circadian clocks inappropriately time adult

emergence on maturation within a favourable 'gate' depending on phasing, periodicity and environmental conditions (Paranjpe et al., 2005).

Recently, populations of *D. melanogaster* with the per^S and per^L alleles were used to investigate the role of circadian clocks and the external cyclic environment on the speed of pre-adult development (Srivastava et al., 2018a). Although there was no difference between per^S and per^+ flies, per^L flies took longer to develop in DD and LL conditions suggesting a non-clock influence. Long and short T-cycles were also used to understand the influence of the external environment's period on the internal pacemaker and its role in determining development time. Under long T-cycles, the developmental rate of perL flies was slower compared to perS and per+; under short T-cycles, perS was faster to develop compared to per+ and perL while there was no genotype-based difference was seen under LD (12:12), establishing that the circadian clock influences pre-adult development.

Perhaps slower-running clocks drive developmental processes at slower rates, resulting in delays in development time, while faster-running clocks do the opposite to hasten development. At least over the final stages of development, the mechanistic link of clock control has been recently discovered. Recently it was shown that the *Drosophila* circadian clock imposes rhythmicity on eclosion by controlling the timing of the final steps of metamorphosis (Mark et al., 2021). However, this study shows control of the timing of eclosion to occur within a suitable gate; the mechanistic underpinnings of how the intrinsic period influences the rate of development are yet to be unearthed. A similar result to the *Drosophila* studies has been observed with two tropical ant species, night active *Camponotus compressus* and day active *Camponotus paria*, which also develop slowly under DD compared to LL and LD (Lone and Sharma, 2008). Interestingly, recent data from monarch butterflies shows that individuals reared under constant conditions (LL and DD) exhibit longer larval development times than

LD (Adams et al., 2021) with pupal development being longer in LL than DD and LD. In this context, the understanding gained from the *Drosophila* model not being entirely generalizable puts forward the requirement for more research on other insect species.

Besides the free-running period, which is a clock parameter, could other clock properties also be associated with life-history? Experimental evolution studies carried out on laboratory populations of *D. melanogaster* have shown intriguing results. Fly populations selected for the stability of phase of eclosion exhibited greater coherence in emergence time despite having no difference in mean development time (Varma et al., 2014). Additionally, there was sex-specific evolution of reduced lifespan- females of 'accurate' populations exhibited a shorter lifespan than controls. It was also observed that such sex-specific differences were attributable to the phasing of emergence. Morning emerging females had shorter lifespans than their evening emerging counterparts, however, this was compensated by higher mid-life fecundity (Varma et al., 2014). Since these populations were under selection for phase stability (eclosion occurring in a tight morning window), one can view the evening emerging flies (in a relaxed selection scenario) as ones exhibiting less phase stability. While interesting, the results observed in terms of life-history may not be generalizable. When the previously described *Early* and *Late* populations of *D. melanogaster* were assayed for changes in life-history, under LD as well as DD conditions, *Late* flies exhibited a longer duration of pre-adult development compared to the 'early' flies (Nikhil et al., 2016a). Surprisingly, the longer pre-adult duration in the *Late* flies did not result in higher body mass at pupariation or eclosion; however, *Late* females had higher fecundity and lived significantly shorter as compared to the *Early* females. Again, both these studies were carried out with *D. melanogaster* reared under crowded conditions, known to have profound effects on life-history (Mueller et al., 1993; Joshi and Mueller, 1997). Hence, more studies with a range of insect models, investigating how chronotype relates to life-history, will significantly enhance our understanding.

1.6 Does selection on life-history alter the circadian clock?

As previously stated, selection on circadian clock phenotypes can result in life-history modifications. Intuitively, the opposite - imposing selection on life-history can profoundly influence several phenotypes. Nevertheless, are the trends of change in clock properties robust enough and generalizable? For example, the correlation between speed of circadian clocks and the length of pre-adult development observed in clock mutant flies (Kyriacou et al., 1990) as well as wild-type flies (Kumar et al., 2006) discussed above lead to the hypothesis that circadian clocks track the developmental state of organisms and that there could exist genetic correlations between the two. A study selecting for faster and slower pre-adult development of melon flies *Bacterocera cucurbitae* under LD (14:10) resulted in the faster-developing lines eclosing ~3 days earlier than the controls. In contrast, at 16 generations of selection, the slower developing lines took about 5 days longer (Shimizu et al., 1997). There were changes in the activity/rest rhythm: the mean free-running period of faster-developing lines decreased by 2 h relative to the controls (24.7 h), while that of slower developing lines lengthened by 3.5 h (Shimizu et al., 1997). Thus, the authors demonstrated that mechanisms that dictate development time are also genetically correlated with circadian clock period.

In a more recent investigation, large outbreeding populations of *D. melanogaster* were used to select individuals completing faster pre-adult development. After 55 generations of selection, these faster developing (FD) populations exhibited a difference of ~29 h in development time. They also evolved a shorter free-running period by ~0.65 compared to control populations (Yadav and Sharma, 2013). However, this change can only explain ~7 h difference in developmental timing, implying that only a part of pre-adult development may be clock-controlled. Perhaps the genetic correlation between the free-running period of the clock and development time may be indirect and unlikely to be strongly mediated by the circadian clock.

The populations studied by Yadav and Sharma (Yadav and Sharma, 2013, 2014b) also yielded some interesting results from the point of view of ageing. The FD flies had a significantly shorter adult lifespan, and their activity-rest rhythms suggested that ageing of the rhythm also sets in earlier than in control flies (Yadav and Sharma, 2014a). Reduction of power of rhythmicity, activity levels, and lengthening of the free-running period under DD conditions manifested earlier in the FD populations, pointing towards aspects of physiological ageing of clocks and rhythms. Miyatake and colleagues also conducted another study on melon flies, this time selecting for early age of reproduction (10–15 days) and later age of reproduction (55–60 days) and reported correlated response to selection for mating phase and free-running period (Miyatake, 2002). The early reproducing age lines were found to mate earlier in the day than the late reproducing age lines.

Furthermore, the early reproducing age lines exhibited much shorter periodicity than the late reproducing age lines (~4 h). There is one key caveat in the interpretation of these results due to the nature of imposed selection: melon fly lines selected for reproduction at 55–60 days old would have experienced higher mortality, resulting in a smaller effective population size (N_e) than the lines selected for reproduction at 10–15 days old. It is likely to cause a larger degree of inbreeding depression in the late reproducing age lines over several generations, so the phenotypic change cannot be entirely attributable to selection but could also be an inbreeding artefact. Keeping this in mind, the results of the above study suggest that even selection on a trait manifested in a relatively late stage in the life of an organism can impact circadian clock properties.

In several insects, such as flies (Sakai and Ishida, 2001), moths (Silvegren et al., 2005), ants (McCluskey, 1967), and bees (Eban-Rothschild et al., 2011), mating activity and mating behaviour have been linked to circadian clocks. For social insects like bees and ants, regulation

of circadian rhythms has been linked to reproductive physiology. In fact, for several species of ants, it is thought that the vital role of circadian clocks is facilitating anticipation of time of mating flights, synchronizing phase within species to promote inter-colony breeding, or maintaining a stable phase difference between species to maintain reproductive isolation (McCluskey, 1967; Sharma et al., 2004). In honey bees, an interesting transition occurs in females destined to become egg-laying queens. While virgin queens rely on their circadian clocks to time their species-specific mating flight in their early life (Koeniger and Koeniger, 2000), the rhythmic output of activity-rest is lost in egg-laying honeybee queens (Harano et al., 2007). It is speculated that rhythm-independent activities such as oviposition and/or social interactions play a role here. In mosquitoes, a recent study comprising laboratory and field experiments found that constant light and higher temperatures negatively impacted mating (Wang et al., 2021). This effect of light and temperature on mosquito mating was hypothesized to occur by modulation of clock gene expression. Comparing the gene expression from heads of swarming male mosquitoes with those of resting male mosquitoes revealed a role of the circadian clock in regulating the production of several cuticular hydrocarbons, of which regulation of a hydrocarbon, 'heptacosane' was vital for attracting females (Wang et al., 2021).

The overall picture that emerges is that co-evolution of clock properties such as the phasing and periodicity of an overt behavioural rhythm is intimately linked to changes in a variety of pre-adult and adult life-history qualities, demonstrating the underlying genetic linkage between circadian timing mechanisms and life-history traits. Apart from the direct fitness effect of clock genes by circadian mechanisms, indirect pleiotropic effects on fitness may also occur. It is fair to assume that due to genetic correlations with life-history features, circadian clocks impart adaptive advantage to organisms by correctly timing rhythmic behaviours to improve their fitness in a particular environment.

1.7 Evolution of circadian rhythms - insights from laboratory selection studies

The notion that circadian clocks are innate, having a genetic basis paved the way for the idea of existent genetic variation for circadian clock-controlled behaviour. Indeed, several studies have used an experimental -evolution-based approach in insects to demonstrate various aspects of the evolution of traits such as longevity (Rose, 1984), fecundity (Rose and Charlesworth, 1981), development time (Zwaan et al., 1995), starvation tolerance (Chippindale et al., 1996) etc. Considering that it was intuitive to assume that circadian clocks evolved in response to geophysical cycles on earth, a laboratory selection approach was an attractive method to gain insights into how circadian clock properties respond to specific selection pressures.

As a concept, experimental-evolution studies are relatively straightforward. A series of replicated populations are exposed to a novel environment for many generations while, in parallel, another set is kept under the ancestral environment, serving as controls. This makes alteration of any aspect of the ancestral population's abiotic or biotic environment or its demographic condition possible. For the sake of simplicity, only one environmental variable is usually changed. However, if the experimenter introduces a novel experimental environment, it is expected to exert selection pressure, promoting evolution. Depending on the study organism and selection regime, traits may evolve due to differential selection of variants from the existing genetic variation of populations. Alternately new genetic variants may emerge (via mutation or recombination) because they are differentially favoured in the altered conditions, ultimately resulting in differential reproduction and expansion of the favoured genotypes within populations (Gibbs, 1999).

What is particularly advantageous about this approach of 'laboratory natural selection' is that having replicates at the population level allows the investigator to replicate the opportunity for

evolutionary change with every replicate population and determine if the outcome is consistent. While a laboratory selection approach does not warrant the determination of the evolutionary history of natural populations, using this approach enables the direct examination and comparison of the diversity of likely evolutionary responses. Unlike correlational studies on natural populations in which one has to make assumptions about ancestral relationships, in laboratory selection studies, there is relatively little ambiguity about the ancestry of populations (Abhilash and Sharma, 2016). Moreover, since evolution in nature occurs amidst simultaneous changes in several environmental factors that are uncontrolled and unmonitored, it is difficult to determine which aspect of the environment is causing the evolutionary change. Thus, experimental evolution using laboratory selection presents a useful strategy for isolation and analysis of the adaptive response to specific environmental factors.

As with all experimental approaches, the laboratory selection approach also suffers from some disadvantages. The requirement for replication and experimental rigour is vital, which places demands for organisms with large population sizes and short generation times. This limits the choice of model organisms used, hence biasing inference from such studies. The other shortcoming is that while laboratory selection is an excellent way to study the evolutionary response to a specific environmental factor, it is often a simplistic and unrealistic portrayal of the ecological changes naturally experienced by organisms, limiting inferential capability (Kawecki et al., 2012). The former limitation has propelled extensive use of insect model organisms in such laboratory selection studies, the most notable being *D. melanogaster*.

As previously discussed, the intrinsic advantage hypothesis proposes that circadian clocks are necessary for maintaining internal synchrony among constituent oscillators within an organism. However, it is also believed that having a biological clock in constant conditions could be unnecessary, if not harmful, because rhythmically active organisms in such environments will

be more likely to miss foraging opportunities that could be aperiodic (Poulson and White, 1969). Thus, functional circadian clocks in aperiodic environments, along with the possibility of having an intrinsic advantage, may also confer an apparent 'extrinsic disadvantage'. In such a scenario, the persistence of rhythmicity in constant environments indicates fitness benefits due to internal synchrony, possibly overriding a fitness cost due to missed foraging opportunities or predator avoidance.

Previous laboratory selection studies using *Drosophila* have shown that it is common to find that traits providing no fitness advantage to the organism under the given culture conditions become affected by random genetic drift relatively quickly within 100–200 generations (Service et al., 1988). When the specific trait has an evolutionary cost, the regression can be even faster, with mean values reverting to those of control populations in a span of ~20 generations (Teotónio and Rose, 2001). If populations are allowed to evolve in the absence of any daily time cues for sufficiently long time, one can examine whether the ability to measure time cues are retained or lost. Using a laboratory selection approach, it was found that populations of the fruit fly *D. melanogaster* reared under constant light for more than 600 generations (LL-populations) exhibited the persistence of both the population eclosion rhythm, as well as individual-level oviposition, and locomotor activity-rest under DD (constant darkness) and LD (Sheeba et al., 1999b, 2001a, 2002b) (Figure 9.2A). The persistence of circadian rhythms in DD implied that their underlying clocks had not regressed over time, whereas the behaviour in LD indicated that such clocks were capable of entrainment. Along with these observations, the group also found a significant difference between the free-running periods of eclosion, activity-rest and oviposition rhythms (Sheeba et al., 2001b). Furthermore, the ability to entrain to a wide range of LD cycles T20, T24 and T28 was also retained (Paranjpe et al., 2003).

Another group studied *D. melanogaster* stocks 'dark-flies' reared under constant darkness for ~1300 generations (Imafuku and Haramura, 2011). These were initially established in 1954 and maintained as a culture consisting of 50-200 individuals. These flies were adapted to dark conditions, reflected in higher fecundity in constant darkness compared to control lines while they did not differ under constant light (Izutsu et al., 2012). Additionally, the same group also showed a nonsense mutation in the R7 photoreceptor gene of the Dark-fly culture via genome sequencing. The targets of R7 send photic information to the clock neurons, suggesting that dark-raised flies may lose a light-input channel to the circadian clock due to being reared under DD for many generations (Saint-Charles et al., 2016). A recent study investigating the relaxed selection on the Dark-flies under normal lighting conditions found a simultaneous trade-off between vision and olfaction with the size of optic lobes increased and antennal lobes decreased at 1^{st} and 65^{th} generations compared to controls (Özer and Carle, 2020). The dark-flies have also shown differences from control flies in several other behaviours such as photokinesis, olfactory response, head bristle elongation etc. (Fuse et al., 2014).

While the former set of studies by Sheeba and colleagues used large population sizes (>1500 flies), discrete generations and multiple replicate populations, the latter ones carried out on the dark-fly culture had inbred origins (Oregon-R-S) and a relatively small population size. Hence while in Sheeba et al., results have to be interpreted with respect to selection on standing genetic variation of populations, in the case of studies on Dark-flies, evolution by mutation is the primary driver of evolutionary change. Even with a small effective population size (~90 individuals), concluding about the occurrence and fixation of a gene for arrhythmia, if beneficial for evolutionary fitness, will require approximately 3000 generations (Imafuku and Haramura, 2011) and thus warrants future investigation.

The studies mentioned above on the long term LL populations (Sheeba et al., 1999b, 2001a, 2002b) also have a major shortcoming: the lack of relevant control populations kept in a rhythmic environment such as LD (12:12) to deduce if the proportions of individuals having persisting rhythms despite being raised in LL have changed at all. Furthermore, the question of an intrinsic advantage arises only if circadian clocks were ticking under LL conditions. Previous research demonstrated that for *Drosophila* and many other organisms under LL, most behaviours and the underlying molecular clock become arrhythmic (Marrus et al., 1996). If the challenge of sustaining internal synchronization did not arise for the LL populations, why do rhythms persist in these populations under constant darkness? One explanation is that perhaps under LL, certain unknown and light-insensitive components of the circadian clock still exhibit rhythms. Another possibility is that molecular clock components may have pleiotropic functions that prevent their regression despite being in an arrhythmic state even after several hundred generations. It is also possible that not enough generations have passed to indicate any notable circadian clock regression.

To overcome these drawbacks posed by the lack of control populations, two additional sets of populations were created from the LL_{1-4} populations that were subsequently maintained under constant darkness: DD_{1-4} populations and on LD (12:12) cycle: $T24_{1-4}$ populations. After more than 330 generations under the above regimes, a study on all three sets of populations found the persistence of rhythms in behaviours such as eclosion, activity-rest, and egg-laying. The power of the activity/rest rhythm was also higher for the DD populations (Shindey et al., 2016) (Figure 9.2B). The evolution of the rhythm's robustness in DD populations may indicate the necessity for rhythm orchestration of internal physiology and metabolism. This is considered to be the selection pressure for the DD-populations, as hypothesized for organisms in aperiodic habitats (Beale et al., 2016). Notably, a follow-up to the previous study found that in comparison with the LL-populations, the DD-populations showed lower anticipation to lights-

on of the eclosion rhythm and more oviposition during the light phase (Shindey et al., 2017). Thus, despite having more robust rhythms under constant darkness, DD-populations seem to exhibit poorer entrainment to 12:12-h LD cycles than LL-populations, perhaps due to being reared in darkness for several generations.

While the persistence of rhythms in aperiodic environments is an interesting question, another aspect of clocks that fascinates chronobiologists is the control on the timing of behaviour and whether/how it evolves. Since most organisms on Earth encounter some form of daily cycling environmental cues, it is thought that circadian clocks evolved in response to selection pressures imposed by daily cycles and not a constant environment. Thus, it is reasonable to assume that selection pressures acted on the phasing of rhythmic behaviours driving the evolution of underlying circadian clock properties. Several laboratory selection studies have examined whether the phasing of rhythms changes in response to periodic selection pressures and their effects on circadian clock properties and evolutionary fitness components.

In the first study of its kind, Pittendrigh used artificial selection on populations of *D. pseudoobscura* to select for the earliest and latest eclosing flies under 12:12 h light-dark cycles (Pittendrigh, 1967). This resulted in two eclosion chronotypes: 'early' populations that eclosed earlier in the day (advanced) and 'late' populations that eclosed later in the day (delayed). It was observed that 50 generations of selection resulted in an approximately 4-hour difference in the phase of eclosion rhythm in these populations. The free-running period of eclosion rhythm of these populations also differed, with 'early' populations having a longer period than 'late' populations. However, Pittendrigh found no evidence of divergence in these populations' light-induced phase response curves (PRCs - a plot of the rhythm's shift in phase as a function of the phase of light pulse), which was unexpected according to the non-parametric model of entrainment. These differences in phasing and free-running periods of the early and late strains

were interpreted to be arising due to altered coupling between the circadian pacemaker oscillator and the driven oscillator. This interpretation gained support from a similar study conducted on the moth *Pectinophora gosypiella* (Pittendrigh and Minis, 1971); however, another study on *D. auraria* flies reported the opposite result for free-running periods of the early and late populations (Pittendrigh and Takamura, 1987). In *D. auraria* the early populations had a shorter free-running period than the late populations. These studies provided evidence that the phasing of circadian rhythms of behaviour can evolve in response to imposed selection. However, they suffered from the drawbacks of not having replication at the level of populations and confounding selection for divergent phasing with development time. Furthermore, information about the maintenance regimes was also not adequate to make reliable inferences regarding the nature of the evolutionary process that may have occurred.

To control for and correct the shortcomings mentioned above, long-term selection for 'early' and 'late' eclosing *D. melanogaster* flies was initiated by collecting individuals emerging from pupae in a 4-hour window during the morning and evening hours respectively, from control populations maintained under LD (12:12), 25°C (Kumar et al., 2007a). These *Early* and *Late* populations have shown divergence in their eclosion profiles since around 55 generations after selection. In contrast to prior research (Pittendrigh, 1967; Pittendrigh and Minis, 1971), the free-running periods of *Early* and *Late* populations became shorter and longer, respectively (Figure 9.2C, D), with about 50-minute difference in their light PRCs for eclosion rhythm (Kumar et al., 2007b) and activity-rest (Nikhil et al., 2016b) rhythms. It was also found that these two sets of populations responded to light differently in the morning and evening hours which brought about their distinct emergence patterns (Vaze et al., 2012b). The fact that circadian clocks mediate the divergence in *Early* and *Late* emergence was supported further by the discovery that the molecular clocks of these populations exhibited phase relationships corresponding to their behavioural phenotypes (Nikhil et al., 2016b). In addition, the *Late*

populations evolved high amplitude circadian clocks that exhibit higher accuracy than *Early* populations (Nikhil et al., 2015), which seems consistent with earlier latitudinal cline research (Pittendrigh and Takamura, 1989).

An especially interesting observation about these populations is that despite being reared under light cycles of LD (12:12) and constant temperature, selection for chronotype divergence has resulted in differences in responsiveness to temperature. While mean timing of eclosion for *Early* and *Control* populations occurred around the same time across different constant ambient temperatures, that was not the case for the *Late* populations. The timing of eclosion, usually occurring later in the day for the *Late* populations, advanced under the low-temperature regime, suggesting that the circadian clock of *Late* populations might have enhanced temperature sensitivity (Abhilash et al., 2019). Indeed, this was also true for the activity-rest rhythm, in which 'late' populations exhibited much faster re-entrainment to temperature cycles after a simulated jet-lag (Abhilash et al., 2020). In contrast, for the *Early* populations, it seems like part of their early emergence might be a result of masking (directly responding) to the lights-on cue (Ghosh et al., 2021).

Several studies have shown that individuals exhibiting deviant phases of activity, possessing dysfunctional circadian clocks, or exposed to exogenous cycle mismatches usually suffer fitness consequences (DeCoursey et al., 1997; Ouyang et al., 1998; Knutsson, 2003; Horn et al., 2019). Maintaining a stable phase-angle in cyclic conditions may be critical for an organism's survival and reproduction (Cloudsley-Thompson, 1960). As a result, the idea of circadian clocks evolving higher stability was intriguing, and so was the question of what other characteristics of circadian clocks may co-evolve to aid such stability.

A long-term selection study was initiated from large outbreeding Drosophila melanogaster populations by selecting individuals emerging in a narrow window of time, i.e., 1 hour (Kannan

et al., 2012c). In response to selection, after ~80 generations, the number of flies eclosing in the selection window in the selected populations increased by about 10% compared to controls (Kannan et al., 2012c). Selection for accuracy also resulted in the evolution of lower inter and intra-individual variance in eclosion and activity/rest rhythms as an associated response, revealing for the first time that circadian clocks can acquire better stability in response to selection on the timing of eclosion. These 'accurate' populations also evolved a shorter free-running period but with less inter-individual variation than the control populations, which is an interesting demonstration of the complex link between clock properties exhibited under entrained and constant conditions (Figure 9.2E). Furthermore, such stability resulting in overall robustness of the circadian system for the 'accurate' populations was prevalent not only for the eclosion rhythm (under selection directly) but also for the activity-rest rhythm (Kannan et al., 2012a).

The same set of populations has provided valuable insights into how light sensitivity of the clock may evolve. A systematic set of experiments varying the lights-on timing showed that compared to controls, populations selected for accuracy exhibited less masking to light, especially when the light was provided outside the eclosion gate; suggestive of tight gating by the circadian clock (Varma et al., 2019). The 'accurate' populations also showed increased delay phase shifts to light pulses, possibly acting via *cryptochrome*, and higher activity under orange light-dark cycles- perhaps mediated by compound eyes (Varma, 2018).

Most studies on circadian rhythms are carried out in known and controlled conditions of the laboratory. Additionally, they have mainly used light alone as the zeitgeber. In contrast, organisms in natural environments encounter multiple zeitgebers simultaneously (Helm et al., 2017). Although standard laboratory regimens have been beneficial in assessing a zeitgeber's effect under controlled settings, limited information can be gained from such studies regarding

organismal rhythms in natural situations. Thus, a lacuna exists in our understanding of how organisms entrain in the presence of multiple zeitgebers and, accordingly phase components of the respective rhythm. In some earlier studies, when fly locomotor-activity and eclosion rhythms were assayed under natural conditions for wild-type and clock mutant strains, there was a distinct contrast in activity profiles compared to the results from laboratory assays (De et al., 2012, 2013; Vanin et al., 2012). These studies showed that for most previously characterized circadian clock mutations, flies were incapable of showing rhythmic behaviour in natural-like conditions. For mutations such as per^{0}, flies could exhibit activity patterns similar to wild-type individuals probably by masking to the naturally varying time-cues (Mrosovsky, 1999). However, these studies were not designed to provide ecologically relevant insights into rhythms in naturalistic environments.

For similar insights, studies with large outbreeding *D. melanogaster* populations have also been carried out in semi-natural conditions outside the laboratory. An investigation using the *Early* and *Late* populations of *D. melanogaster* selected for divergent phasing of adult emergence showed increased divergence in the phasing of chronotypes under semi-natural conditions (Vaze et al., 2012a). The emergence waveforms also appeared to be more consolidated under semi-natural conditions than the phenotype observed in the laboratory. This was proposed to be a combined effect of multiple zeitgebers and/or twilight zones, both of which were absent in the laboratory. Similarly, populations selected for accuracy of emergence in a narrow window of time in the laboratory showed an enhanced peak and narrower gate width when assayed under semi-natural conditions (Kannan et al., 2012b). Another study compared eclosion rhythms of three closely related drosophilids – *D. melanogaster*, *D. malerkotliana* and *D. annanasae* under semi-natural conditions, which had previously shown differences in the phasing of eclosion under standard laboratory conditions (Prabhakaran et al., 2013). Surprisingly, there was no difference in the phase of eclosion even across different seasons,

which led them to conclude that these species showed a dissimilar phase of entrainment only in the presence of a light cycle. This also indicates that there is no certainty of obtaining an enhanced circadian phenotype in complex naturalistic environments compared to laboratory regimes.

1.8 Evolution of clocks in presence of naturally varying time-cues

Several questions remain in this domain, such as which clock properties would evolve to be different under semi-natural conditions compared to standard laboratory conditions. Additionally, it is interesting to ask if adaptation to semi-natural environments can alter the circadian phenotype under standard laboratory conditions. It also becomes imperative to ask which time cues are important for timing behaviour and physiology when studying the evolution of clocks under semi-natural environments. Identifying potential selection pressures for Drosophila under semi-natural conditions would prove useful in gaining a holistic perspective.

The domain of studies of clocks under natural conditions also has another angle with questions about the stability and flexibility of the circadian clock. Since these terms may appear abstract without clarification, they are being defined now: stability refers to less variability in exhibited circadian phenotypes across days. Previously, measures of precision under constant conditions and accuracy under entraining regimes have been used to infer about the stability of rhythms (Kannan et al., 2012c). On the other hand, flexibility has been used in various contexts and sometimes ambiguously. Flexibility usually has been talked about in terms of flexibility of the circadian phase of behaviour as well as reactive homeostasis mediated flexibility of circadian output from peripheral oscillators, which in turn modulates behaviour (Riede et al., 2017).

Evolving under a predictably changing environment with fluctuations presents the possibility of evolving stability or flexibility of the circadian clock. Even though it may appear that stability and flexibility are inherently opposing in definition, there is a possibility of both evolving for different phenotypes of the circadian clock in the same set of organisms. The prediction for traits to evolve stability or flexibility of the clock is indefinite as of yet.

To study how circadian clocks evolve differently in semi-natural conditions compared to standard lab conditions, in 2013, a set of four laboratory reared outbred populations of Drosophila melanogaster (T24) in the lab were used to derive one outbred population each (NT24) which have been maintained under semi-natural conditions for 162 generations (till September 2022).

1.9 Population maintenance

Drosophila melanogaster populations used in this study were initially wild-caught from South Amherst, MA, USA and reared under LL at 25˚C for over 700 generations (Joshi and Mueller, 1996; Sheeba et al., 1998). From these, four large outbreeding *D. melanogaster* populations were derived and maintained in the laboratory for 186 generations under LD 12:12 (~100 lux) at 25˚C and ~70% RH called 'T24 populations', earlier referred to as LD stocks in (Shindey et al., 2016). The $T24_{1-4}$ populations were used to derive a population each and this new set of populations - $NT24_{1-4}$ was kept under semi-natural conditions in an outdoor enclosure (De et al., 2012), located at the JNCASR campus, Jakkur, Bengaluru, India (13.06° N, 77.62° E). Up to September 2022, the T24 populations have completed 350 generations, while the NT24 populations have been under selection for 162 generations.

A pesticide-free zone was maintained (~30m radius), and light intensity, temperature, and relative humidity at the outdoor enclosure were recorded using DEnM (TriKinetics Inc., Waltham, MA, USA). Populations typically consisting of about 1500 adults (~ 1:1 sex ratio) were maintained in plexiglass cages (25 × 20 × 15 cm^3) on banana-jaggery food medium on a 21-day non-overlapping generation cycle. Before collecting eggs for the next generation, cages were provided with food plates with yeast paste for ~ 48 hours. After this, they were allowed to lay eggs on a petri-plate with fresh banana-jaggery medium for ~16 h. Eggs were collected in glass vials (9 cm height × 2.4 cm diameter) at a density of 70±10 eggs/vial in ~6 ml of food and transferred to the respective light regime.

In order to avoid the non-genetic and maternal effects caused due to the rearing regimes on the phenotype being assayed, both sets of populations are maintained together in the ancestral regime (LD 12:12, 25°C, ~70% RH) for one generation before every experiment. The progeny of these 'standardized' populations was used for all the experiments. Experiments in the laboratory were conducted in environment-controlled incubators (DR-36VL, Percival Scientific, Perry, USA).

1.10 Population ancestry

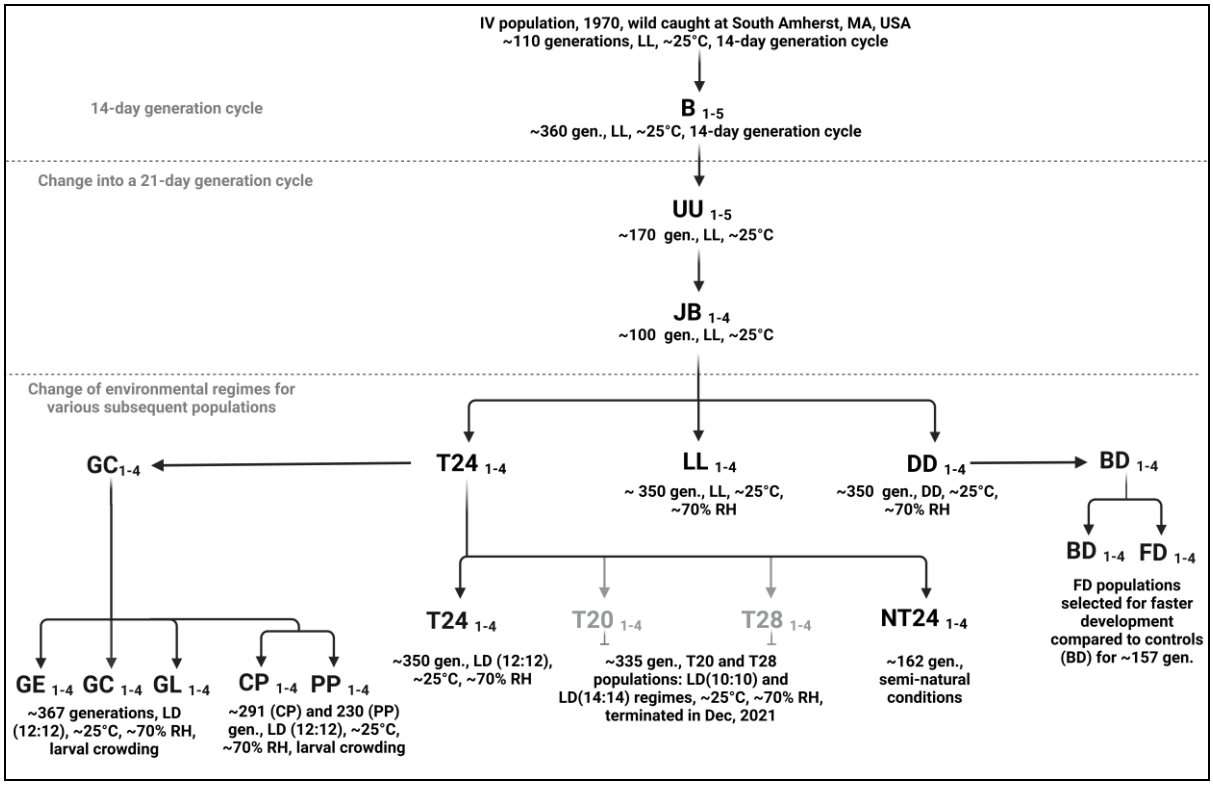

Figure 1.1 Ancestry for all *Drosophila melanogaster* populations studied for circadian rhythms in the Chronobiology and Behavioural Neurogenetics lab up to September 2022

- JB_{1-4} populations (lab of Amitabh Joshi, JNCASR) are derived from UU_{1-5} populations (lab of Laurence Mueller, UC Irvine), in which JB_4 is derived from UU_5, UU_4 was unfortunately lost during transportation.
- GC (Gate-Control) populations were derived from ~75 generations old T24 populations, subsequently maintained under larval crowding conditions to facilitate staggering of emergence, enabling selection of chronotypes.
- BD populations are synonymous in maintenance to DD populations, and served as controls for FD populations which are selected for faster development under constant darkness conditions. These populations are no longer maintained at JNCASR (shifted to SASTRA university in April 2017).

- CP (Control Populations) are controls for the populations selected for accuracy (PP), which have similar maintenance regime as GC populations. PP populations were selected for accuracy of emergence by selecting for flies emerging in a narrow window of time around lights-On (1 hour). These populations are no longer maintained at JNCASR (shifted to IISER-TVM in January 2019).
- T20 and T28 populations were selected for optimal reproductive output under LD (10:10) and LD (14:14) cycles respectively, for ~335 generations, with T24 populations as control. They were terminated in December 2021.
- NT24 populations were relocated to a different site within the JNCASR campus in February 2022 as the previous site of the outdoor enclosure was rendered unusable due to floods.

Chapter 2 . Evolution of life-history-related traits in Drosophila populations reared in laboratory *vs* semi-natural environments

2.1 Introduction

Environmental unpredictability shapes the structure and behaviour of all biological systems, and organisms have evolved traits and mechanisms for anticipating, recognising, and adapting to environmental changes. The evolution of traits involved in feedback mechanisms allows internal conditions to remain close to a predefined state despite a changing environment. At the same time, feedforward systems also evolve in many species enabling them to change in advance of an anticipated future condition of the environment (Bernhardt et al., 2020).

Drosophila is a widely used model in ecology, evolution, and physiology. Most drosophilids are easily reared in the lab, allowing common garden studies to be carried out with immense control over potential confounding effects such as age, reproductive status, and other environmental factors. The popularity of *Drosophila* has resulted in a plethora of laboratory-maintained stocks of various species, which supply material for many researchers lacking the time or money to acquire fresh samples from nature for each experiment. Laboratory maintenance conditions are quite different from natural conditions for Drosophila and several other model organisms. Under standard laboratory conditions, temperatures are kept constant, light cycling is either absent or a symmetric light-dark (12:12) regime, there is only one source of food provided, and the humidity levels are relatively high and mostly consistent. It is therefore, interesting to study how laboratory regimes and natural conditions may have differentially affected fly populations in the context of evolutionary change by examining life-history associated traits (Harshman and Hoffmann, 2000; Sgro and Partridge, 2000). An organism's life-history comprises events related to growth, development, reproduction and

survival. Life-history characteristics include age and size at sexual maturity, amount and timing of reproduction, survival and mortality rates (Stearns, 2000). Additionally, traits that can influence the reproductive output of organisms, such as various stress tolerance traits, are referred to as life-history associated traits. Therefore, comparing these traits across flies reared under standard laboratory and naturalistic conditions is intuitively interesting.

On a nutritious feeding medium, under typical laboratory circumstances (i.e. constant 24°-25°C and an LD (12:12) cycle), flies complete one lifecycle in 10 days. Eggs hatch within 24 h of laying under these circumstances. Larvae are the primary growth stage, with the three instars lasting around four days in total. The pupal stage lasts four to five days, following which adults emerge. Before pupa formation, larvae must reach a specific size/weight (Robertson, 1960). Moulting, which leads to the next larval instar and the conversion of third instar larvae to pupae, is correlated to pulses of the hormone ecdysone (Warren et al., 2006). As the case with several ectotherms, developmental duration in *Drosophila* is sensitive to temperature, quality of food and crowding conditions etc. In a physiologically permissible range, lowering of temperature results in slower development and vice-versa (Partridge,' Brian Barrie et al., 1994). Lower food quality and increased crowding in the culture also have a negative impact on the developmental duration of flies (Sang, 1949).

Development time has also been correlated to body size and body-weight as a consequence of resource acquisition in several studies (Zwaan et al., 1995; Prasad et al., 2001). Previous studies have found that body size may decrease as a result of laboratory adaptation (Spates and Hightower, 1970; Linnen et al., 2001; Maclean et al., 2018), suggesting that laboratory populations of flies are smaller than wild flies. Another linked correlation is that of body composition: that laboratory rearing boosts energy storage in the form of increased lipid content

(Harshman and Hoffmann, 2000). However, these results have not been consistent often showing opposing trends across species (Maclean et al., 2018).

Does the reproductive output of flies in laboratory environments differ compared to natural/semi-natural conditions? For replicate sets of populations being reared under laboratory and semi-natural conditions as discrete generations, the expected action of selection is on optimal reproductive output to maximally contribute towards the formation of the subsequent generation. In case of standard laboratory conditions, previous literature is suggestive of a positive effect on reproductive output. While older studies have reported that laboratory maintenance increases reproductive output and decreases age of reproductive maturity in *D. melanogaster* (Sgro and Partridge, 2000; Maclean et al., 2018) and *D. subobscura* (Matos et al., 2000; Simões et al., 2008), findings may not be very consistent across other species, and especially for *D. subobscura* (Maclean et al., 2018). However, this question would benefit from a systematic investigation using large replicate sets of populations with known ancestry.

The ability to survive periodic or aperiodic bouts of stress is a critical aspect for the life-history of insects under naturalistic conditions. In semi-natural conditions compared to standard laboratory regimes, heat and desiccation can be considered prevalent stressors (and starvation in natural conditions). To overcome thermal extremes and water loss, utilization of physiological mechanisms such as heat-shock proteins in drosophilids has been documented (Goto and Kimura, 1998; Hoffmann et al., 2003; Bubliy et al., 2013), along with adaptations such as waterproof cuticles (Rourke and Gibbs, 1999) . Insects can also endure heat stress by increasing water consumption (Contreras et al., 2013) and relocating to preferred temperature and humidity conditions (Heinrich, 1993; Tichy, 2003).

As to the question of how laboratory-reared *Drosophila* populations fare in stress resistance, a similar ambiguity to the one seen with fecundity exists. Some studies argue that laboratory

maintenance leads to reduced resistance to desiccation and starvation (Hoffmann et al., 2001; Simões et al., 2008), whereas others report no change or even an enhancement of stress resistance traits (Hoffmann et al., 2003; Griffiths et al., 2005). For heat tolerance, however, a negligible difference in Hsp70 expression or tolerance to heat stress between freshly collected and laboratory-maintained *Drosophila melanogaster* was found (Krebs et al., 2001).

There are some common limitations of most previous studies in this regard; firstly, the use of inbred lines or isogenic lines without sufficient diversity of lines used (Harshman and Hoffmann, 2000; Bechsgaard et al., 2013). Along with that, for the lines compared between laboratory and natural environments, the ancestry of flies was unknown. In some cases, natural collections were conducted from the same region as the laboratory ones, but not simultaneously (Maclean et al., 2018). For such cases, collection from the same site does not guarantee ancestry, especially with several years of difference in the time of collections.

While natural conditions differ from the laboratory in terms of environmental cues, food availability, predation etc., semi-natural conditions have recently been explored as a convenient alternate to conduct research in a naturalistic environment wherein to examine several of the unknowns referred to above, while retaining control over food and predation risk. This approach allows one to experiment and infer the effects of natural environmental cues on the phenotype of interest. This experimental design also does not suffer from the disadvantages stated above.

The key hypothesis for my studies is that NT24 populations (described in Chapter 1) evolving under semi-natural conditions derived from the laboratory-reared T24 populations have undergone changes in life-history associated traits. For traits to evolve by natural selection, they must confer some adaptive value, ultimately enhancing evolutionary fitness under semi-natural conditions for NT24 populations. To verify this, and characterize changes in life-

history in the NT24 populations from T24, a set of experiments assaying development time, pre-adult survivorship, fecundity and stress tolerance were carried out.

2.2 Materials and methods

2.2.1 Development time and pre-adult survivorship assay

(a) Time to pupariation: The time to pupariation for all the populations was assayed under two regimes – (i) LD (12:12), 25°C, ~70% RH and (ii) SN (July, 2019). After having provided yeast paste-supplemented media for three days, all populations were provided with media plates for one hour as a substrate for oviposition. Fresh media plates then replaced these plates for the next one hour. Eggs laid on these plates were collected, and 30 eggs were dispensed into each vial. A total of 10 such vials were used per replicate population per regime. These vials were transferred to respective regimes and monitored for the first pupariation event. After the first puparium was observed, vials were checked every two hours to count the number of puparia formed thereafter, and the assay was terminated when no pupariation event was seen for 24 consecutive hours.

(b) Egg-to-adult development time assay: After two days of the end of pupariation, flies started to emerge. The earliest emergence was monitored, following which the assay vials were subjected to two hourly checks to count the number of flies that emerged thereafter. The assay was terminated when no emergence event was observed for 24 h.

(c) Dry weight: Emerged adults were used for dry weight measurements after all emergence had ended in both T24 and NT24 assay vials (these vials were kept in parallel with the development time vials). Five flies of both males and females were taken in each micro centrifuge tube of 5 replicates for the T24 and NT24 populations in LD (12:12) and SN (July

2019) regimes. They were frozen at -20ºC and then desiccated for 36 hours at 70˚C in a convection oven after which their dry weight was measured.

2.2.2 Fecundity assay

Mid-life fecundity at 10 days post eclosion or 21st day of generation cycle of populations was assayed. One mating pair/food vial was used for the assay with 40 replicates/population. Food was changed every three days. On the 8th day post eclosion, flies were flipped into a new food vial with a yeast drop for ~48h according to maintenance protocol. On the 10th day post eclosion, flies were transferred to fresh food vials for the assay. After ~16 h, flies were removed from the vials and egg-counting for each vial was carried out in a blinded manner. After egg-counting, vials were maintained in the corresponding regime till the progeny of the flies assayed eclosed, and the number of offspring were recorded.

An extra set of flies preserved before the assay was used for determining the pre-fecundity assay dry weight of female flies. Flies used for the assay, which were removed before egg counting, were preserved and used for determining the post-fecundity assay dry weight of female flies. Five flies were taken in each micro centrifuge tube of 5 such replicates. Flies were frozen at -20ºC and then desiccated for 36 hours at 70˚C before weighing.

2.2.3 Heat tolerance assay

From each population of T24 and NT24, an egg collection was done and after 10 days of collection when emergence occurred, they were sexed. Four- day old flies (200 females and 200 males) from both regimes with five flies in each vial for 10 replicates were taken, who were exposed to both the regimes for 3 days, in both LD and SN conditions.

The sexed flies were transferred from food vials to empty vials and given a heat shock of 37°C for 2 hours in the incubator. The temperature of 37°C was already manually set up an hour

before the introduction of vials in the incubator. After the heat shock, the flies were transferred to fresh food vials and checked on the next day for recovery.

2.2.4 Desiccation tolerance assay

For this assay, males and females aged 7 days post eclosion were sexed and 5 male flies or 5 female flies were transferred to an empty vial. Every population had 10 such replicate vials for male and female flies each. The assay was conducted in an incubator with light, 25ºC, and humidity maintained at 35-40% RH using $CaCl_2$ as a desiccant. Hourly checks were carried out to check for deaths until the death of all flies.

2.2.5 Statistical analysis

All data was analysed using a randomized block design, mixed model ANOVA approach with 'Block' as the random factor. The details of these analyses are specified, along with the results of each assay. Tukey's honest significant difference (HSD) tests were used to perform all post-hoc multiple comparisons for statistically significant effects of interest from ANOVA results, contained in Appendix 1. All statistical tests were carried out using STATISTICA v7.0 (StatSoft, Tulsa, OK, USA). The results were deemed significant at $\alpha = 0.05$.

2.3 Results

2.3.1 Selection under semi-natural conditions does not alter development time and pre-adult survivorship

As an initial step to understanding whether rearing under semi-natural conditions had led to differences in life-history, I carried out assays in standard laboratory conditions (LD) and semi-natural conditions (SN) for estimating the development time of NT24 and T24 populations. I observed a significant effect of 'regime' on mean pupariation time (Three-way ANOVA with *Selection*, *Block*, *Regime* with main effect of *Regime*: $F_{1,3} = 121.32$, $p =$

0.0016) and mean emergence time (Three-way ANOVA with *Selection*, *Block*, *Regime* with main effect of *Regime*: $F_{1,3} = 223.18$, $p = 0.00065$) which can be seen in Fig. 2.1 A and 2.1 B respectively, which shows that flies take longer to complete development in SN compared to LD. However, there is no significant difference between the NT24 and T24 populations in terms of both pupariation and emergence time. I also measured the pre-adult survivorship for the same set of flies (Fig. 2.1 C) and did not find that to be significantly different either (Three-way ANOVA with *Selection*, *Block*, *Regime*; main effect of *Selection* and *Regime*, and *Selection* × *Regime* interaction being statistically non-significant, $p > 0.05$).

2.3.2 Selection under semi-natural conditions resulted in reduced dry-weight at eclosion

I compared dry-weights of NT24 and T24 populations developing under both LD and SN regimes (Fig. 2.1 D). Four-way ANOVA: significant main effects for *Selection* ($F_{1,3} = 17.07$, $p = 0.0257$), *Regime* ($F_{1,3} = 674.8$, $p = 0.00013$), *Sex* ($F_{1,3} = 249.4$, $p = 0.00055$), and a significant interaction of *Selection* × *Regime* × *Sex* ($F_{1,3} = 22.14$ $p = 0.0182$). This indicates that while flies developing in SN regimes had significantly lower dry-weight compared to LD, across both regimes, NT24 flies had significantly lower dry-weight compared to T24. Additionally, this effect of lowered dry-weight was starker in the case of NT24 females. In a separate assay, I also verified if there was a difference in sex ratio between NT24 and T24 populations in LD and SN regimes. This was carried out at the normal maintenance density of egg collection (~70 eggs/vial). The M/F ratio was ~1 with no difference between NT24 and T24 populations, Three-way ANOVA with *Selection*, *Block*, and *Regime*, $p > 0.05$ for all main effects and interactions (Appendix 1 – Table 2.24, 2.25).

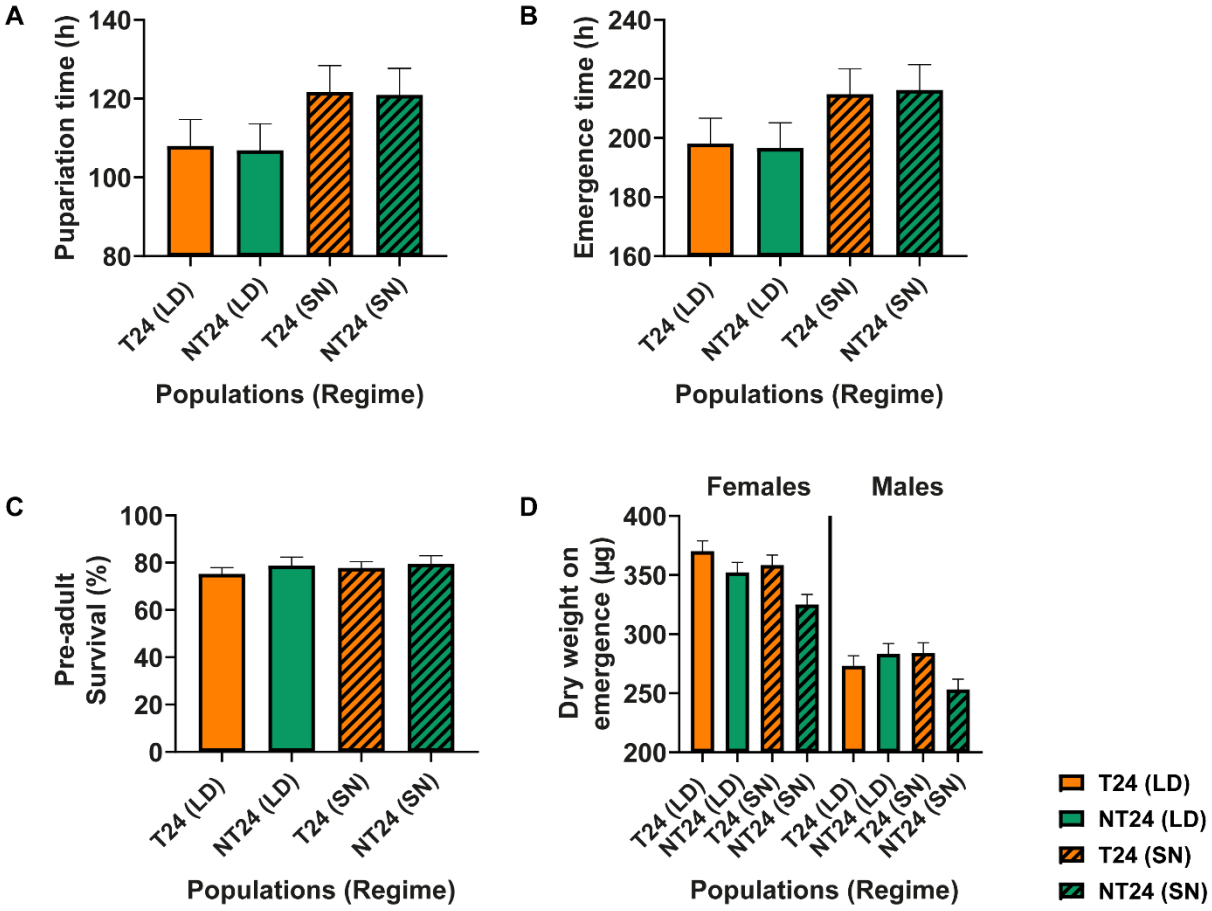

Fig. 2.1 Development time, pre-adult survivorship and dry-weight of NT24 and T24 populations in standard laboratory and semi-natural regimes A) pupariation time B) adult emergence time C) pre-adult survival D) dry-weight at eclosion in standard lab (LD) and semi-natural conditions (SN). Error bars are 95% CI (Tukey's HSD).

2.3.3 NT24 flies exhibit unchanged development time and lower dry-weight even in the absence of environmental cues impacting development time

Since light and temperature influence development time (reviewed in Prasad and Joshi, 2003), I wanted to know the extent of environmental influence on the effects seen above for development time and dry-weight. To examine that, I assayed the development time and

measured dry-weights for NT24 and T24 flies under constant darkness at 25°C (DD). I found that just as in LD and SN regimes, there was no significant difference between the NT24 and T24 populations in terms of both pupariation time (Two-way ANOVA with *Selection* and *Block*, $p > 0.05$) and emergence time Two-way ANOVA with *Selection* and *Block*, $p > 0.05$) under DD (Fig. 2.2 A and B) as well as for pre-adult survivorship (Fig. 2.2 C, Two-way ANOVA with *Selection* and *Block*, $p > 0.05$). However, consistent with previous results in LD and SN regimes, the dry-weight of NT24 flies was significantly lower compared to T24 in DD 25°C too (Fig. 2.2 D). Three-way ANOVA with *Selection*, *Block*, and *Sex*: significant main effects for *Selection* ($F_{1,3} = 44$, $p = 0.007$) and *Sex* ($F_{1,3} = 802.7$, $p = 9.7 \times 10^{-5}$).

2.3.4 Under harsh semi-natural conditions, NT24 populations exhibit higher fecundity

Since our maintenance of flies under semi-natural conditions or standard laboratory conditions as discrete generations required collection of eggs at a fixed age- ten days post eclosion; I reasoned that populations under these conditions are ultimately selected for optimal reproductive output around mid-life. Hence I compared the mid-life fecundity in the form of offspring/female from the NT24 and T24 populations. I found that under standard laboratory conditions, NT24 females did not differ from T24 females in terms of either egg output or viability (Fig. 2.3 A, B), Two-way ANOVA, $p > 0.05$. Since fecundity has been shown to vary widely with temperature and flies show phenotypic plasticity for the same (Flatt, 2020), I carried out fecundity assays under semi-natural conditions in three different seasons: April 2019 (summer), July 2019 (monsoon) and December-January 2019-20 (winter). I found that NT24 populations had significantly higher offspring/female in summer (Two-way ANOVA, *Selection*: $F_{1,3} = 145.4$, $p = 0.00123$) and winter (Two-way ANOVA, *Selection*: $F_{1,3} = 15.4$, $p = 0.0295$) compared to T24, but not in monsoon (Two-way ANOVA, *Selection*: $p > 0.05$), (Fig. 2.3 C).

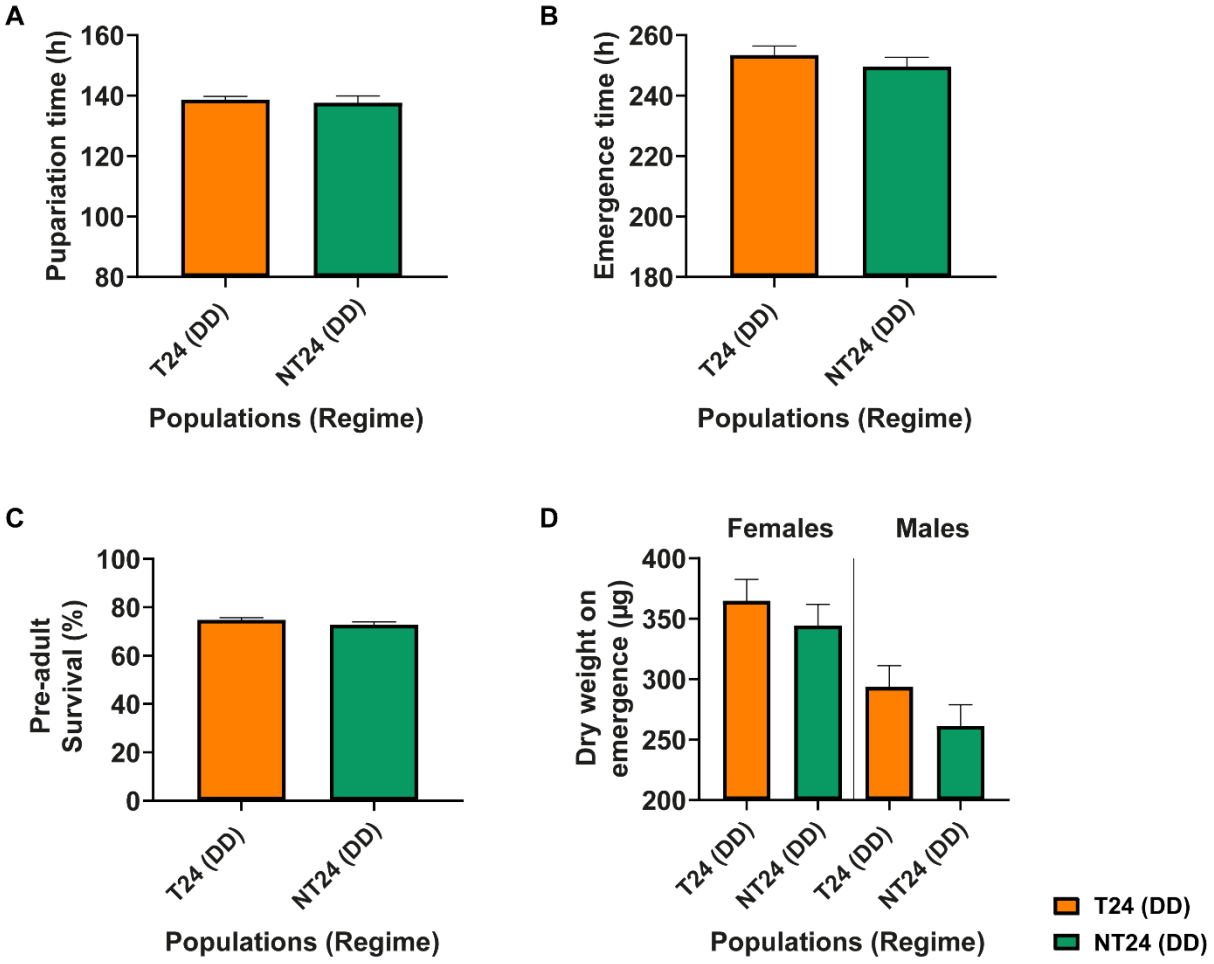

Fig. 2.2 Development time, pre-adult survivorship and dry-weight of NT24 and T24 populations under constant darkness, 25°C A) pupariation time B) adult emergence time C) pre-adult survivorship D) dry-weight at eclosion under constant darkness (DD) at 25°C. Error bars are 95% CI (Tukey's HSD).

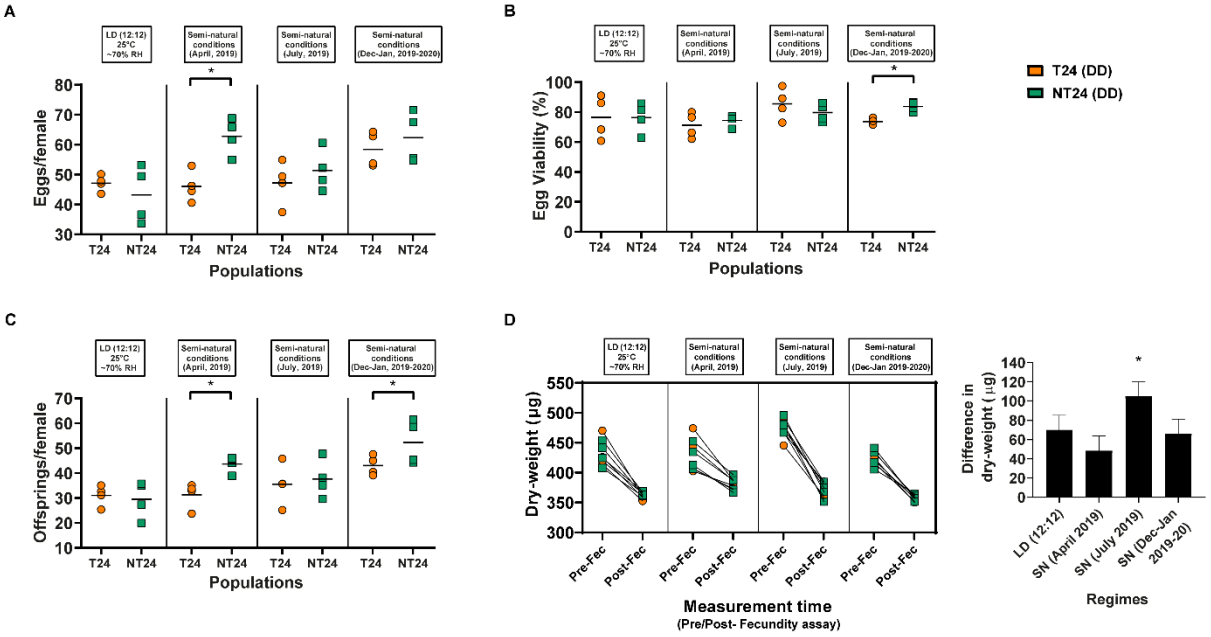

Fig. 2.3 Fecundity of NT24 and T24 populations under standard laboratory and semi-natural environments. A) Egg output/female B) Egg viability C) Offspring/female D) Dry-weight and difference in dry-weight for NT24 and T24 populations under LD (12:12) at 25°C and ~70% RH; semi-natural conditions in April 2019 (summer); semi-natural conditions in June 2019 (monsoon); and semi-natural conditions in December-January 2019-2020 (winter). Dashed lines indicate means, error bars indicate 95% CI, and asterisks indicate significant differences ($\alpha = 0.05$).

2.3.5 NT24 populations may be exhibiting higher offspring output compared to T24 by different reproductive strategies in summer and winter conditions

To assay fecundity, I quantified the eggs laid per female and their viable offspring. Since there was a difference in offspring/female for only summer and winter seasons (analyses of individual assays and combined analysis with 'Regime' as a factor), I wanted to identify if the difference was in egg output itself or viability of the eggs laid or both. Comparisons between the NT24 and T24 populations revealed that in April 2019 (summer), NT24 flies had higher

egg output but no difference in the viability of eggs resulting in a higher offspring count (Fig. 2.3 A, C), Two-way ANOVA, *Selection*: $F_{1,3} = 42.4$, $p = 0.0074$. While in December-January 2019-20 (winter), higher viability of eggs but no difference in egg output lead to higher offspring/female (Fig. 2.3 B, C), Two-way ANOVA, *Selection*: $F_{1,3} = 17.24$, $p = 0.0254$. I additionally also measured the dry-weight of flies before egg-laying (10 days post eclosion) and after the fecundity assay, referred to as Pre-Fecundity assay dry-weight and Post-Fecundity assay dry-weight, respectively for LD (12:12), SN (April 2019, July 2019, December-January 2019-20) regimes (Fig. 2.3 D). While there was an expected decrease in weight between the Pre-Fecundity assay and Post-Fecundity assay for all regimes, the decrease in dry-weight in July 2019 was significantly higher than other regimes, Three-way ANOVA with *Selection*, *Block*, *Regime*: Main effect of *Regime*; $F = 11.94$, $p = 0.00172$.

2.3.6 In response to desiccation stress, females survive for a longer duration than males, however there are no differences between NT24 and T24 populations

In tropical conditions, water loss due to desiccation can be a major stressor for flies (Chown and Nicolson, 2005). Our environmental records showed that relative humidity regularly dropped to very low levels (20-30%) for a few hours in the summers. I wanted to check if rearing under semi-natural conditions would have imposed a selection pressure for higher tolerance to desiccation stress. Hence I carried out a desiccation tolerance assay for the NT24 and T24 populations in which I quantified the time till the death of flies due to desiccation stress. I found that apart from the expected difference between males and females, there was no difference between the NT24 and T24 populations for tolerance to desiccation stress. Three-way ANOVA with *Selection*, *Block*, *Sex* showed a significant main effect for *Sex*: $F_{1,3} = 1315.12$, $p = 4.6 \times 10^{-5}$, whereas for main effect of *Selection* and *Selection* × *Sex* interaction, $p > 0.05$.

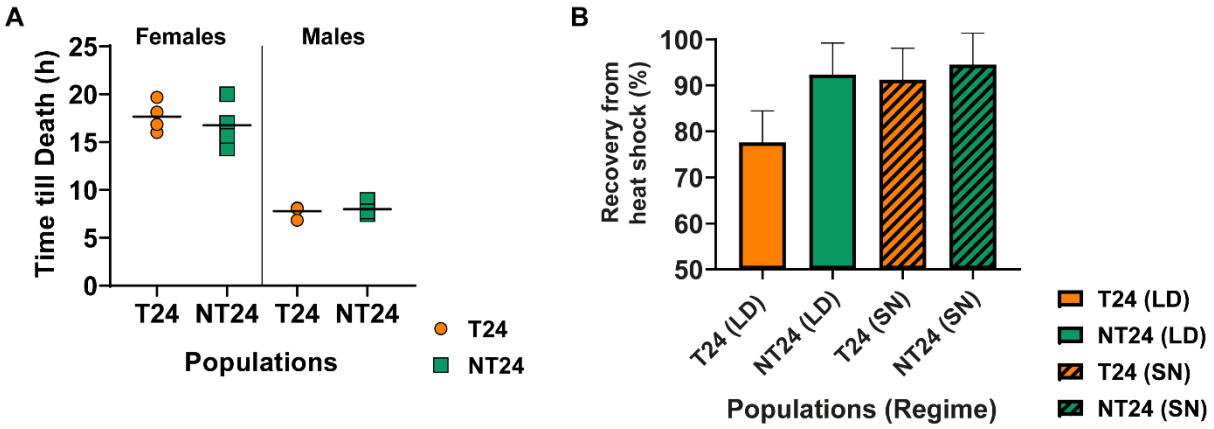

Fig. 2.4 Testing the tolerance of NT24 and T24 flies to heat and desiccation stress. A) Percentage of flies recovered from heat shock at 37°C for 2h. Flies were either maintained in standard lab conditions or semi-natural conditions for 5 days prior to the assay B) Time to death of flies under desiccation stress of 40% RH. Dashed lines indicate means, error bars indicate 95% CI.

2.3.7 In response to heat stress, NT24 populations exhibit higher tolerance than T24 populations

It has been noted that thermal tolerance limits predict the geographical distribution of *Drosophila* in the wild (Hoffmann et al., 2013). Since temperature is constant and optimal in the laboratory, *Drosophila* populations do not experience any heat stress. In contrast, natural conditions, especially tropical summer, may impose high levels of heat stress on flies. It has also been observed that acclimation to temperature stress, via a process referred to as hardening, can influence response to temperature stress (Mathur and Schmidt, 2017).

I wanted to verify if this is the case as it would imply a selection pressure for tolerance to heat stress in semi-natural conditions for the NT24 populations. I carried out an assay

exposing NT24 and T24 flies to heat stress of 37°C for 2 hours and checked for recovery from the heat shock in the form of activity after a considerable rest period. I found that for the set maintained in standard lab conditions, NT24 flies exhibited higher recovery compared to T24 flies. However, for the set that was exposed to semi-natural conditions prior to the assay, I saw no difference in recovery between the NT24 and T24 flies. Four-way ANOVA with *Selection, Block, Sex, Regime of Exposure* showed significant main effects for *Sex*: $F_{1,3}$ = 42.5, p = 0.0073, and *Regime of Exposure*: $F_{1,3}$ = 85.45, p = 0.0027, and significant interaction effect- *Selection* × *Regime of Exposure*: $F_{1,3}$ = 34.31, p = 0.0099. This shows that while NT24 populations are more heat tolerant than T24 populations, the regime of exposure prior to heat shock tolerance assay can impact the assay outcome.

2.4 Discussion

NT24 populations exhibited differences in fecundity compared to T24 populations in a season-dependent manner. I also found that NT24 populations showed higher heat tolerance but not higher desiccation tolerance compared to controls. Surprisingly, the development time of NT24 and T24 populations is not different for both regimes despite the clear differences in dry-weight. Under semi-natural conditions, development is delayed for both sets of populations, and occurs synchronously, possibly due to variation in temperature due to temperature cycling.

Speculating what advantages might be entailed for flies by having lower dry weight as an evolutionary response to semi-natural conditions is interesting. One of the changes might be a difference in the critical body mass under these conditions. Alternatively, the NT24 flies may not differ from T24 flies in their wet weights, indicating different adaptations to semi-natural conditions.

A point to note here would be that based on previous studies (Zwaan et al., 1995), it would seem counter-intuitive for flies having lower dry-weight to lay more eggs. However, I found

that the dry-weight of NT24 flies before the fecundity assay was not different from that of T24 flies, indicating that in ~9 days post eclosion, they might be compensating for the difference in dry-weight by increased feeding and/or by less energy expenditure. However, our results clearly show that the strong correlation between development time and dry-weight at eclosion seen very frequently in laboratory conditions may not always manifest in naturalistic conditions.

Our results suggest that in semi-natural conditions, NT24 populations may have experienced selection for high egg output and viability, varying degrees based on the environment, resulting in a higher offspring count compared to the control T24 populations. A number of aspects differ between the environments of winter and summer, some of which, e.g. temperature and humidity (Winkler et al., 2020; Maurya et al., 2021), have been demonstrated to affect fecundity in drosophilids. Moreover, variation in the substrate for egg-laying, known to be a contributing factor (Yang et al., 2008) due to environmental effects, may also play a role here. Additionally, I see that the difference in fecundity between NT24 and T24 populations under semi-natural conditions appears to vary based on the harshness of the season. This suggests that the selection on fecundity is likely to be variable across the year.

Chapter 3 . Evolution of seasonal differences in eclosion timing of flies reared under semi-natural conditions

Note: The contents of this chapter have been published as:

Dani, C., and Sheeba, V. (2022). Drosophila populations reared under tropical semi-natural conditions evolve season-dependent differences in timing of eclosion. Front. Physiol. 13, 954731. https://doi.org/10.3389/fphys.2022.954731

3.1 Introduction

Circadian clocks drive rhythms in behaviour, physiology and metabolism in several species and are thought to have evolved multiple times independently during the course of evolution (Dunlap et al., 2004). They are characterized by properties such as free-running period (innate periodicity exhibited by individuals under constant conditions) and phase-angle of entrainment (timing of behavioural or physiological events with respect to the external cycles). These circadian clock properties exhibit variation across species (Pittendrigh and Daan, 1976) and may even represent adaptations to local environments within a species (Daan, 1981). The range of entrainment, phase-angle (ψ) and entrained amplitudes have been shown to vary systematically not only with respect to the zeitgeber but also with intrinsic clock properties (Aschoff, 1960; Winfree, 2001; Schmal et al., 2020).

Eclosion is a critical event for the fruit fly *Drosophila melanogaster*, and as it occurs only once in the lifetime of an individual insect, rhythmic eclosion requires synchronized emergence of several adults from pupae. Thus, the eclosion rhythm is inherently a population-level rhythm, its waveform reflecting average population behaviour and inter-individual variation in the population. The eclosion rhythm was one of the earliest rhythms to undergo systematic investigation (Pittendrigh, 1954b, 1967; Pittendrigh et al., 1958; Chandrashekaran, 1967;

Skopik and Pittendrigh, 1967; Zimmerman et al., 1968) and recent efforts have elucidated its anatomical and physiological basis (Krüger et al., 2015; Selcho et al., 2017) and circadian control of the developmental process (Mark et al., 2021). Further, the act of eclosion is considered a fixed action pattern (Kim et al., 2006), hence compared to other behavioural rhythms such as activity, feeding etc., and is unperturbed by other behavioural outputs, interspecific interactions, or motivational state. However, it is disrupted in core clock mutants such as those of *period* and *timeless* under constant as well as cyclic conditions (Sehgal et al., 1994; Qiu and Hardin, 1996; De et al., 2012; Ruf et al., 2021). As a result, it appears to be a more reliable indicator of core clock output when compared to other rhythms.

Recent evidence also indicates the necessity of a functional molecular clock for appropriately timing eclosion under semi-natural conditions (Ruf et al., 2021). According to evolutionary theory, heritable variation is the substrate upon which selection acts and allows for the adaptive evolution of the trait. Thus, the maintenance of genetic variation in clock properties in populations may facilitate adaptation to new selection pressures or environments. With this, I asked if subjecting large outbreeding laboratory populations to rearing under semi-natural conditions could result in changes in the circadian clock i.e. if adaptation to semi-natural environments altered the circadian phenotype under standard laboratory conditions. If so, what clock properties would evolve to be different? I also hypothesized that specific time cues or aspects of time cues could be more important in terms of the phasing of the eclosion rhythm under semi-natural conditions.

To study how circadian clocks evolve differently in semi-natural conditions compared to standard lab conditions, outbred populations of *Drosophila melanogaster* were reared under semi-natural conditions for 159 generations (NT24) along with control populations (T24) in the laboratory (as of July 2022). Here I show that (i) under laboratory regimes- light-dark cycles

and constant conditions, NT24 and T24 populations show similar patterns of phasing and intrinsic free-running period, respectively (ii) under semi-natural conditions, NT24 populations exhibit an advanced phase of eclosion compared to T24 controls in a season-dependent manner (iii) NT24 populations do not track the timing of a particular environmental variable across all seasons (iv) Difference in the phasing of eclosion for NT24 populations compared to T24 appears to be in response to the magnitude of temperature cycle variables.

3.2 Materials and methods

3.2.1 Adult Eclosion Rhythm assay

For the eclosion assay, ~250 eggs/vial (9 cm height × 2.4 cm diameter) were collected in 10 vials/population, each containing 10 ml of banana-jaggery (BJ) medium, and transferred into the respective assay regime. Upon initiation of emergence, the number of flies emerging every 2-h was recorded for four days. This assay was carried out in four different conditions: a) Standard laboratory conditions – LD (12:12), 25°C, ~70%RH, b) Semi-natural conditions (SN) multiple times at different times of the year – seasonally varying light, temperature, and humidity, c) Constant darkness (DD) – 25°C, ~70%RH d) Simulated semi-natural conditions with varying light and temperature cycles.

3.2.2 Quantification of rhythm parameters

I quantified three phase markers to assess aspects of the rhythm that change under each of the assay settings of entraining regimes (LD/SN): (i) Phase of Onset of eclosion- the time point when the percentage of emerging flies surpassed 5% of total emergence (ii) Phase of Offset- the time point when the percentage of emerging flies surpassed 95% of total emergence of the cumulative distribution of eclosion over one cycle/vial (iii) Phase of Peak as the time point when the most flies emerged from 1 cycle/vial. If two successive time points had the same

maximum number of eclosing flies, the average time between the two time points was calculated to determine the phase of Peak.

3.2.3 Analysis of environmental data

To obtain phase markers for environmental variables under semi-natural conditions, for light, the average and maxima for each day were extracted (since the minima of light intensity was 0 lux). For temperature and humidity, average, maxima, minima and amplitude for each day were extracted from environmental recording. Average values of environmental variables across assay days for each month were used to check correlations between environmental variables (Pearson's correlation coefficient) and for a Principal Component Analysis (PCA).

3.2.4 Data analysis and statistics

Values for eclosion rhythm phase markers for LD and SN regimes were computed using custom MATLAB scripts. For analysis of DD data, I estimated the free-running period and power of rhythm using autocorrelation in RhythmicAlly (Abhilash and Sheeba, 2019a), based on R (v3.6.3). All data were statistically tested using a randomized block design, mixed model ANOVA approach with *Block* i.e. population as the random factor. The normality of distribution was ascertained using the Shapiro-Wilk test. The details of these analyses are mentioned with the results of each assay, and statistical tables are contained in Appendix 2. Tukey's honest significant difference (HSD) tests were used to perform all post-hoc multiple comparisons. All statistical tests were carried out using STATISTICA v7.0 (StatSoft, Tulsa, OK, USA), as well as the results were deemed significant at $\alpha = 0.05$.

3.3 Results

3.3.1 Adaptation to semi-natural environments has not changed features of rhythmic eclosion under standard laboratory light-dark cycles

As an initial step to understand whether rearing under semi-natural conditions had led to phenotypic differences in circadian behaviour in their ancestral regime, I assayed the eclosion rhythm of NT24 and T24 populations under standard laboratory conditions (LD). I examined 3 phase markers – Onset, Peak, and Offset of the rhythm and found no difference between NT24 and T24 populations (Fig. 3.1A). Two-way ANOVA with *Selection* and *Block*, phase of Onset: $F_{1,3} = 0.643$, $p = 0.481$; phase of Peak: $F_{1,3} = 0.18$, $p = 0.697$; phase of Offset: $F_{1,3} = 1.08$, $p = 0.374$.

3.3.2 Selection under semi-natural conditions has not changed free-running period and power of the eclosion rhythm under constant conditions

To characterize intrinsic clock properties, I assayed the eclosion rhythm under constant darkness (DD) (Fig. 3.1B). I found that NT24 populations did not differ from T24 in free-running period (Fig. 3.1C) or power of the rhythm (Fig. 3.1D). Two-way ANOVA with *Selection* and *Block*, for free-running period: $F_{1,3} = 0.75$, $p = 0.451$; power of rhythm: $F_{1,3} = 0.998$, $p = 0.391$.

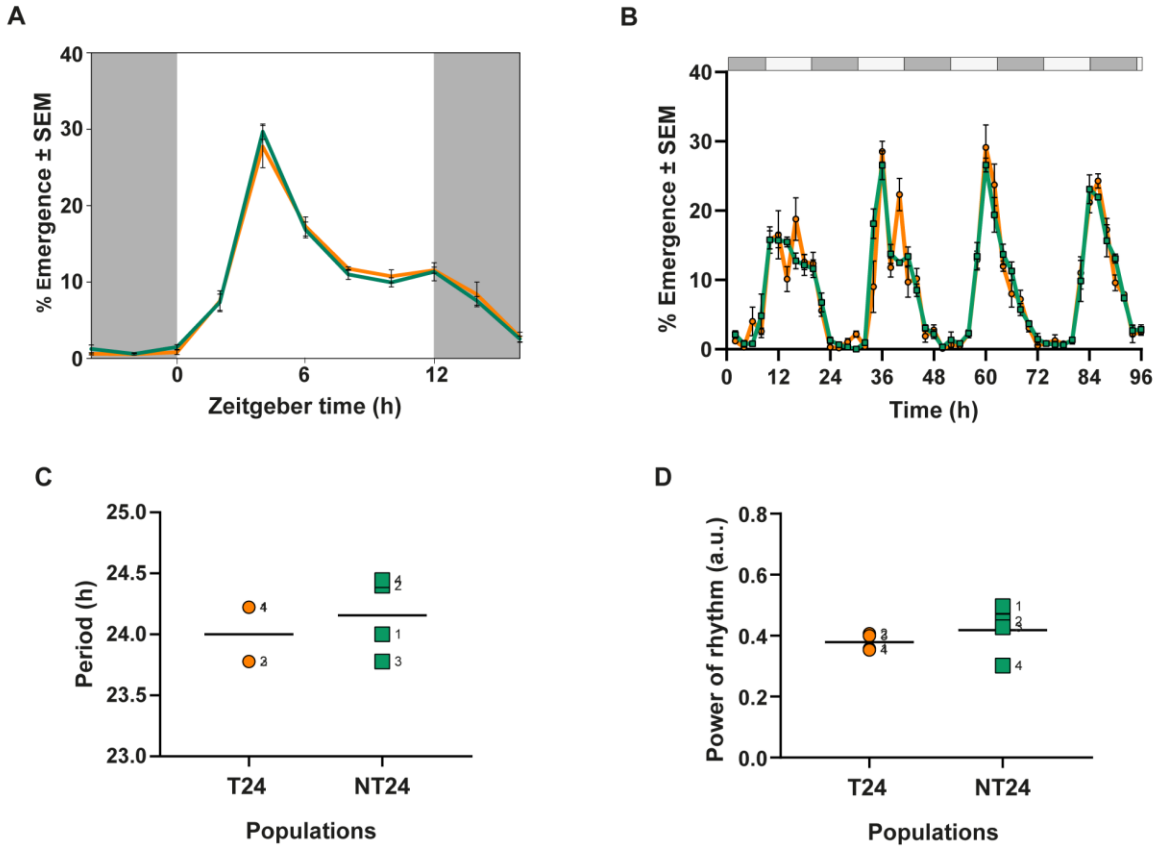

Fig. 3.1 Eclosion rhythm under standard laboratory conditions and constant conditions
A) Eclosion profiles for NT24 (green) and T24 (orange) plotted as daily average percentage of total individuals, averaged across 10 replicate vials under LD (12:12), 25°C, ~70% RH

B) Time series of daily percentage emergence for NT24 and T24 populations under constant darkness (DD), Error bars = SEM

C) Free-running period (autocorrelation) under DD

D) Power of rhythm (autocorrelation) under DD. For C and D, individual points represent independent replicate populations, and dashed line represents mean.

3.3.3 Under semi-natural conditions NT24 populations have a season-dependent advanced phase of eclosion due to earlier Onsets and Peaks

I assayed the eclosion rhythm of both sets of populations in our outdoor semi-natural enclosure during various times of the year from November 2017 to January 2019 (Figure 3.2). As explained in the methods section, the populations were subjected to one generation of common rearing under LD12:12 or ancestral regime before all of the assays. Even at a tropical latitude with small changes in photoperiod, various aspects of light, temperature and humidity changed across the assays conducted (see Fig.3.2). I asked whether these environmental changes may have impacted the eclosion phenotype of NT24 populations since they were reared under semi-natural conditions for at least 80 generations. I find that NT24 populations show an earlier phase of Onset (Fig. 3.3A) and Peak (Fig. 3.3B) in the months of November 2017, February 2018, April 2018, and January 2019 compared to T24 populations, Three-way ANOVA, phase of Onset- *Selection*: $F_{1,3} = 136.276$, $p = 0.0014$*; Month*: $F_{6,18} = 82.701$, $p < 10^{-6}$; *Selection* × *Month*: $F_{6,18} = 3.813$, $p = 0.013$; phase of Peak- *Selection*: $F_{1,3} = 818.14$, $p = 9.4\times10^{-5}$*; Month*: $F_{6,18} = 58.97$, $p < 10^{-6}$. Interestingly, there was a strong but statistically insignificant trend in advance of the phase of Offset across seasons between the two sets of populations (Fig. 3.3C), Three-way ANOVA, phase of Offset- *Selection*: $F_{1,3} = 27.42$, $p = 0.014$, n.s. via Tukey's HSD*; Month*: $F_{6,18} = 21.99$, $p < 10^{-6}$.

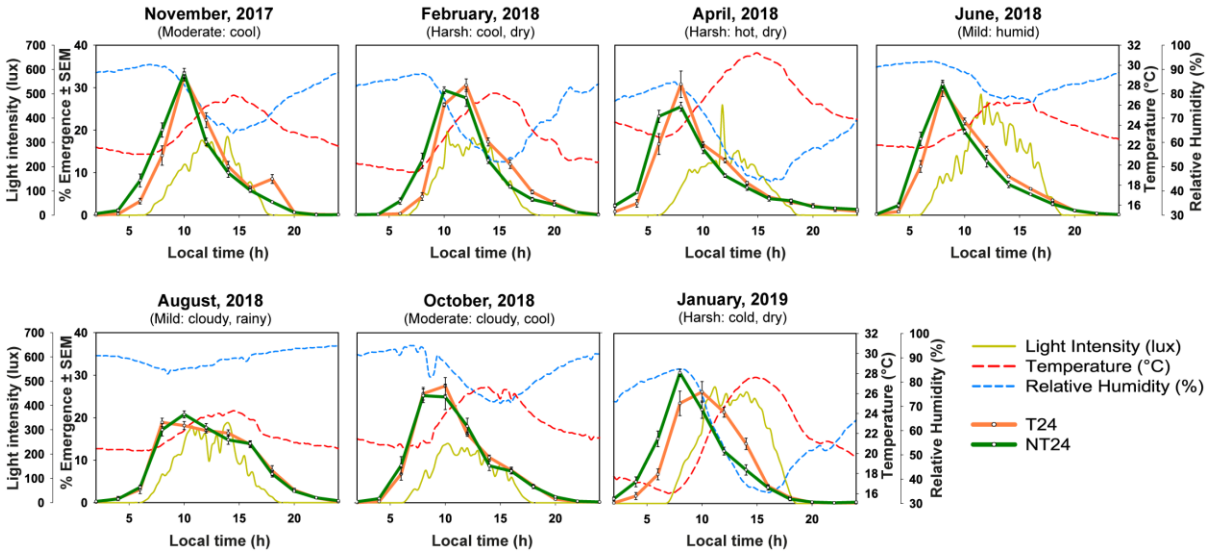

Fig. 3.2 Eclosion rhythm under changing semi-natural conditions (Nov-2017 to Jan-2019)

Eclosion profiles for NT24 (green) and T24 (orange) plotted as daily numbers of emerging flies, averaged across 10 replicate vials, for assays conducted across various months of the year with weather description based on existing knowledge of fly preferences. Assays were conducted in 7 different months from 2017-2019 under semi-natural conditions and average profiles for environmental variables of light (yellow-solid curve), temperature (red-dashed curve) and humidity (blue-dashed curve).

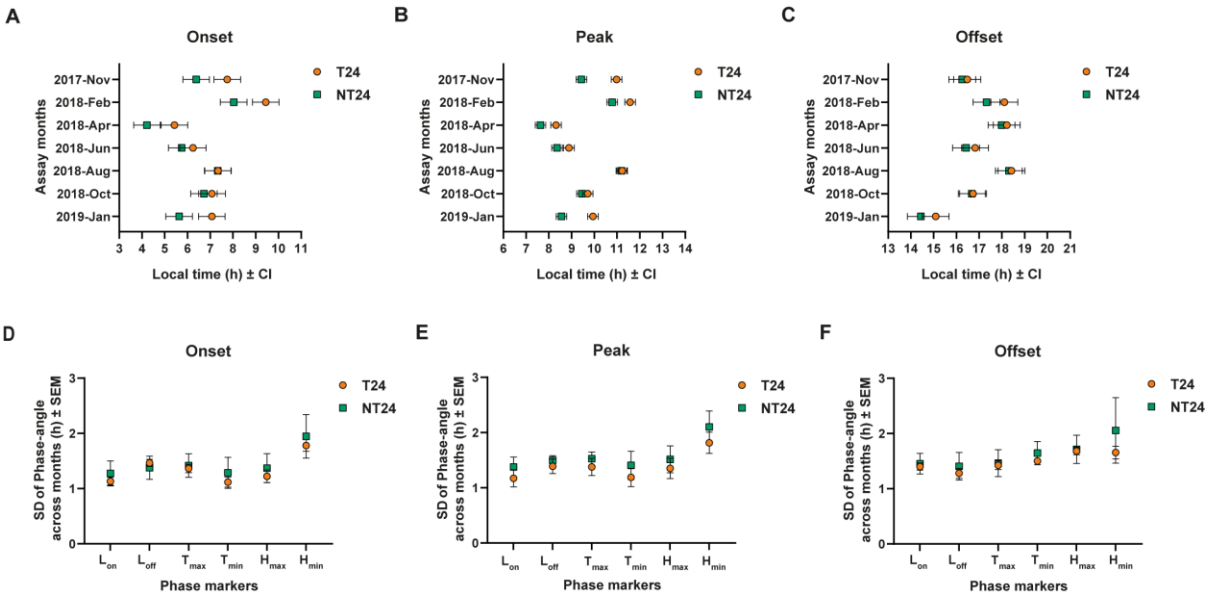

Fig. 3.3 Phasing of the eclosion rhythm under semi-natural conditions and phase-angle variation

A) Average phase of Onset B) Average phase of Peak C) Average phase of Offset, error bars are 95% CI via Tukey's HSD. Variation in phase angle across months for phase markers of environmental variables viz. Lights On (L_{ON}), Lights Off (L_{OFF}), Maximum temperature (T_{MAX}), Minimum temperature (T_{MIN}), Maximum humidity (H_{MAX}) and Minimum humidity (H_{MIN}) for D) average phase of Onset E) average phase of Peak F) average phase of Offset, error bars are SEM.

3.3.4 The advancement of phase of eclosion of NT24 populations is not due to tracking of a specific environmental variable across seasons

Earlier studies have proposed the role of light intensity and photoperiods in mediating seasonal variation in phase-angle observed across several organisms (Daan and Aschoff, 1975). In our regime, various aspects of light, temperature and humidity change across seasons with comparatively little photoperiodic variation. I wanted to check if NT24 populations track a

specific environmental phase marker across seasons and use it to advance the phase of eclosion observed in Figs. 3.2, 3.3 A, B. It was hypothesized that this would be reflected in across-season variation in the phase-angle of eclosion such that the variation in NT24 for such an environmental phase marker would be lower than that for T24 populations. I specifically asked if variation in the phase-angle with different environmental phase markers was different between the NT24 and T24 populations. To do this, I compared the standard deviation of phase-angle across months for Onset, Peak, and Offset. I found no significant difference between the NT24 and T24 populations (Fig. 3.3 D, E, F), suggesting that NT24 populations do not track a specific phase marker of light, temperature, and humidity. Three-way ANOVA, for SD of phase of Onset- *Selection*: $F_{1,3} = 0.307$, $p = 0.618$; *Selection* × *Phase marker*: $F_{5,15} = 1.864$, $p = 0.161$; *Phase marker*: $F_{5,15} = 129.43$, $p < 10^{-6}$; for SD of phase of Peak- *Selection*: $F_{1,3} = 0.774$, $p = 0.444$; *Selection* × *Phase marker*: $F_{5,15} = 0.556$, $p = 0.732$; *Phase marker*: $F_{5,15} = 158.255$, $p < 10^{-6}$; for SD of phase of Offset- *Selection*: $F_{1,3} = 1.162$, $p = 0.36$; *Selection* × *Phase marker*: $F_{5,15} = 1.22$, $p = 0.347$; *Phase marker*: $F_{5,15} = 4.232$, $p = 0.013$.

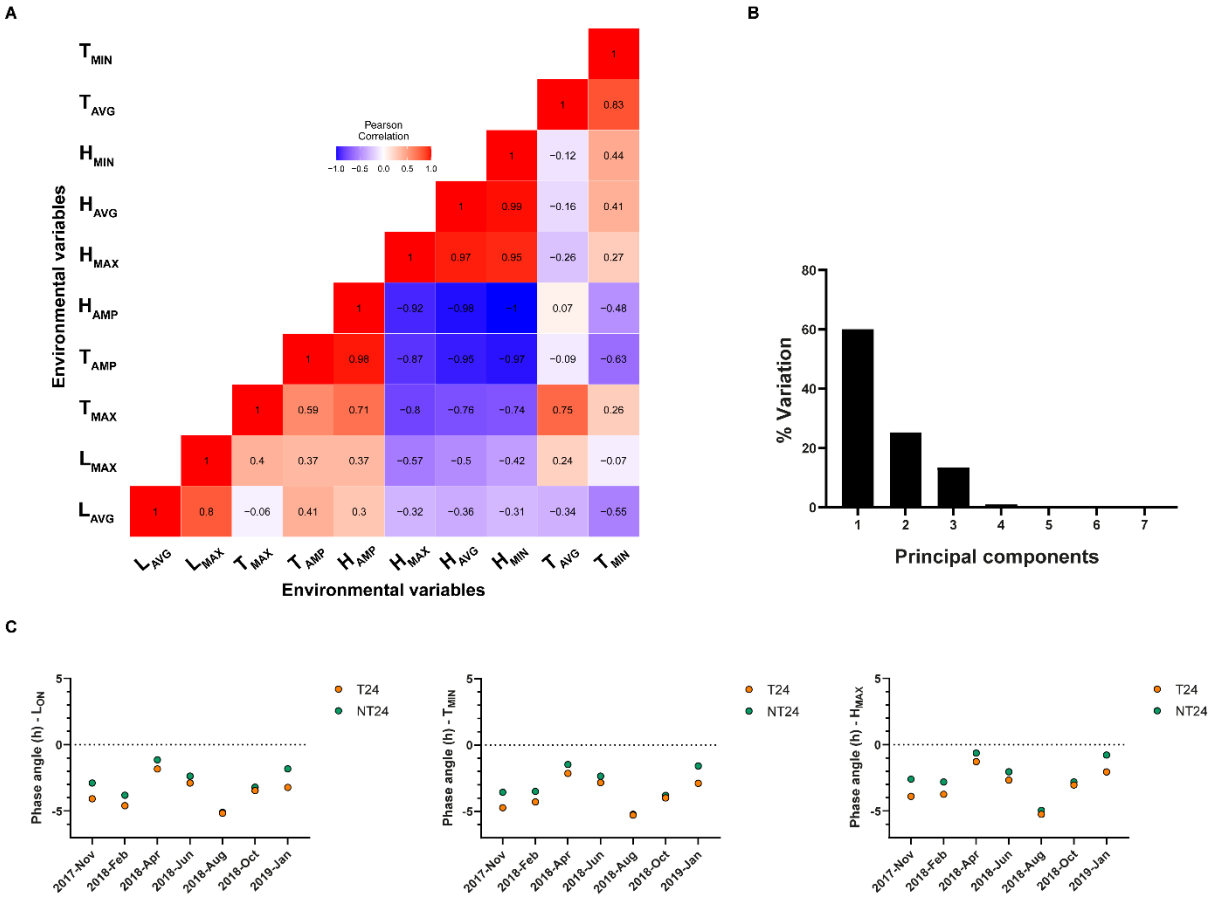

Fig. 3.4 Analysis of environmental data and phase-angle of Peak with early daytime environmental phase markers

A) Heatmap correlation matrix of environmental variables using Pearson's correlation coefficient B) Histogram showing percentage of variation explained by various principal components derived from environmental variables: PC1 (60.06%), PC2 (25.3%), PC3 (13.4%), PC4 (1%), PC5 (0.2%), PC6 and PC7 (0%). C) Phase-angle for assay months with Peak of eclosion and environmental phase markers of early daytime: Lights-On (L_{ON}), Temperature-Minima (T_{MIN}), Humidity-Maxima (H_{MAX})

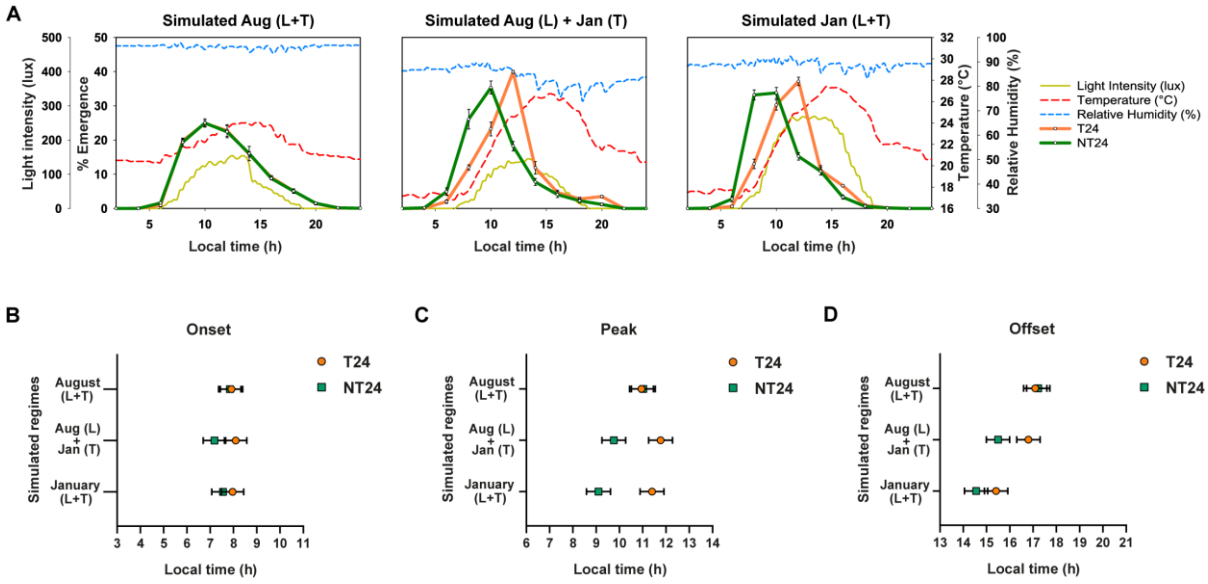

Fig.3.5 Phasing of the eclosion rhythm under simulated natural light and temperature cycles

A) Eclosion profiles for NT24 (green) and T24 (orange) populations, under three simulated semi-natural conditions in laboratory incubators with average profiles for environmental variables of light (yellow-solid curve), temperature (red-dashed curve) and humidity (blue-dashed curve). Header indicates similarity of simulated regime to that observed in our outdoor enclosure during specific months (as Fig.2) B) Average phase of Onset C) Average phase of Peak D) Average phase of Offset, Error bars are 95% CI via Tukey's HSD.

3.3.5 NT24 populations advance their phase of eclosion compared to T24 in response to increase in magnitude of Temperature cycle variables

Since the intensity of the zeitgeber can alter the phasing of rhythms (Johnson et al., 2003), advance in phases of onset and peak may be altered based on the magnitude of light, temperature and humidity cycles. Analysis of environmental data revealed a high positive or negative correlation among several environmental variables (Fig. 3.4 A). I then carried out a

Principal Component Analysis to better understand the contribution of various environmental variables to the total environmental variation observed across the year (Fig. 3.4 B). I found that temperature cycle variables (T_{MAX}, T_{MIN}, T_{AVG}, T_{AMP}) were major constituents of the primary principal component (Appendix 2 - 1). To test the hypothesis that NT24 populations respond differently to temperature cycle shifts in different seasons under semi-natural conditions, I conducted experiments simulating previously observed semi-natural light and temperature regimes in the laboratory. Accordingly, I simulated three regimes (Fig. 3.5 A): 1) light and temperature cycles of August 2018; 2) light cycle of August 2018, temperature cycle of January 2019; 3) light and temperature cycles of January 2019. I found a significant difference in phase of onset between the two sets of populations (Fig. 3.5 B) via Three-way ANOVA, *Selection*; $F_{1,3} = 12.162$, $p = 0.04$, however not in the subsequent post-hoc test - Tukey's HSD (n.s. for *Selection*), *Regime*; $F_{2,6} = 1.363$, $p = 0.325$, *Selection* × *Regime*; $F_{2,6} = 3.137$, $p = 0.117$. For the phase of Peak, as expected, there was no difference in phasing between the NT24 and T24 populations for the August regime; however, with the change in temperature cycle, NT24 populations significantly advanced their phase (Fig. 3.5 C). The January light-temperature regime, however, was not significantly different from the regime with only January temperature cycle, Average phase of Peak: Three-way ANOVA, *Selection*; $F_{1,3} = 86.1$, $p = 0.003$, *Regime*; $F_{2,6} = 10.1$, $p = 0.012$, *Selection* × *Regime*; $F_{2,6} = 27.9$, $p = 0.0009$. I also saw a minor but significant advance in phasing of Offset, although it was not consistent with introduction of January light-temperature regime: Three-way ANOVA, *Selection*; $F_{1,3} = 13.59$, $p = 0.035$, *Regime*; $F_{2,6} = 70.717$, $p = 6.7\times10^{-5}$, *Selection* × *Regime*; $F_{2,6} = 9.422$, $p = 0.014$, Tukey's HSD (significant for *Selection* × *Regime*). Thus the difference in phasing observed in certain semi-natural regimes can be attributed primarily to the increase in magnitude of the temperature cycle.

3.4 Discussion

While studies on circadian rhythms under naturalistic regimes have been carried out previously, most have not been explicitly designed to provide evolutionary insights. Our study describes an experimental system tailored explicitly for testing such hypotheses (Abhilash and Sharma, 2016). I found that rearing *D. melanogaster* populations under semi-natural conditions resulted in phenotypic change compared to the ancestral controls. Interestingly, adaptation to semi-natural conditions did not alter the circadian phenotype measured under standard laboratory conditions (Fig. 3.1 A) and that the two sets of populations did not differ in their intrinsic free-running period or power of the rhythm (Fig. 3.1 C, D). This is intriguing as it suggests that a genetic trade-off (Matos et al., 2000) in terms of circadian rhythm phenotypes of phasing and periodicity may not be required for populations adapting to a novel semi-natural environment coming from many generations of laboratory rearing and maintenance.

However, in outdoor experiments, under semi-natural conditions, differences between NT24 and T24 populations were revealed specifically under certain seasons. After conducting experiments in different seasons across the span of 15 months, I found that NT24 populations have a season-dependent advanced phase of eclosion due to advances in the phases of Onset and Peak. These differences were more pronounced under conditions considered harsh for *Drosophila* (Hoffmann, 2010). Though the magnitude of the difference in phasing may appear small (~1.5 h), it is likely to be biologically significant. This is because a) I observed consistency in phasing across most replicates (n=10 vials per population) for each population despite being large and outbred and b) the advance in phasing occurs at a timing considered ecologically significant for the eclosion rhythm (Pittendrigh, 1954b; Cloudsley-Thompson, 1960). Moreover, I also observed an increase in the magnitude of phase difference even under simulated natural light and temperature cycles (~2 h), consistent with what is seen under natural

conditions. One reason for this could be an absence of ultradian fluctuations in simulated conditions in the laboratory compared to the natural environment, which may have enhanced the magnitude of phase advance for NT24 populations, an interesting possibility for future testing.

The motivation to compare variation in phase-angle across seasons was to reveal the relative importance of phasing of an environmental cue compared to the absolute magnitude of the cue for entrainment of an oscillation across seasons. Since there was no difference in across-season variation of phase-angle (Fig. 3.3 D, E), it appears that NT24 populations do not track the timing of a specific environmental variable across seasons for advancing the phase of eclosion. However, the phasing of eclosion rhythm in NT24 flies appears to be sensitive to the magnitude of variables of the temperature cycle. I verified this by checking whether a change in magnitude of the temperature cycle results in the expected differences in phase-angle between the two sets of populations. Indeed, the simulated temperature cycle with greater contrast between T_{MIN} and T_{MAX} resulted in an advance in phasing for NT24 populations that remained unchanged with the addition of a similar change in the light cycle (Fig. 3.5 D). This suggests that altered sensory integration as input to the circadian clock may occur for flies under naturalistic regimes due to differences in the magnitude of temperature cycle variables experienced. Previously, similar results have been reported and elucidated for the integration of light input to the clock in various organisms (Lall et al., 2010; Vinayak et al., 2013; Piechura et al., 2017; Woelders et al., 2018). The basis of such altered sensory integration for temperature inputs is currently unknown and requires further investigation.

The differential responsiveness of populations reared under semi-natural conditions to temperature, hints that they may be undergoing selection for temperature-directed phasing of eclosion rhythm. Thus, the role of temperature in the presence of other time cues in regulating

eclosion rhythm may be more critical than previously thought. Apart from light and temperature, humidity has been implicated in influencing the timing of rhythms (Clayton and Paietta, 1972), however, without much supporting evidence. Eclosion was thought to be mainly limited to the early part of the day as an adaptation to limit water loss and allow optimal wing unfolding (Clayton and Paietta, 1972; Pittendrigh, 1993). Recently, however, humidity cycles (70:30 RH) were found to be insufficient to entrain the eclosion rhythm in *D. melanogaster* lines, and the effect of drastically low humidity (2% RH) on successful eclosion and wing extension was minimal (Ruf et al., 2021). In this context, it seems surprising that NT24 populations, in adapting to semi-natural conditions, have evolved to advance the phase of eclosion to the early morning hours very close to when humidity levels peak (Fig. 3.4 C). Even though low humidity does not affect successful eclosion and wing extension, one still cannot rule out the possibility of the phase of eclosion determining evolutionary fitness later in life via effects on lifespan or fecundity, and this remains to be tested.

As a natural physical consequence, temperature and humidity cycles are highly correlated and anti-phasic in occurrence (Fig. 3.4 A). Advancing the phase of eclosion not only makes NT24 populations emerge close to H_{MAX}, but also close to T_{MIN}. Since NT24 populations advance the eclosion phase by responding to changes in the magnitude of temperature cues, it would be interesting to test the limits of the range of temperature cycles for which such a response is possible. It is also interesting to speculate if and how the circadian clock could differentiate sudden and potentially harmful changes in temperature from the necessary temperature changes required for phasing eclosion rhythm output. Our knowledge of temperature entrainment in *Drosophila* is still limited (George and Stanewsky, 2021), despite recent advances in the characterization of peripheral clocks in temperature entrainment and elucidation of the differential role of ion channels at different temperatures. Future studies on the NT24 and T24

populations will hopefully unravel the mechanistic bases of this response of the oscillation to the magnitude of temperature and the contribution of central vs peripheral clocks in the same.

Chapter 4 . Evolution of differences in activity-rest rhythms in populations reared under semi-natural conditions

4.1 Introduction

Locomotor activity-rest rhythms are the most popular behavioural rhythms assayed in *Drosophila*. The overt activity-rest rhythms are thought to be a readout of the central pacemaker. To reliably convey information about time, clocks require consistency in maintaining a stable periodicity under constant conditions and a consistent phase-angle under entrained conditions. These properties, namely precision and accuracy of circadian clocks, need to be characterized for a deeper understanding of the system when overt rhythms are used to derive conclusions about the central pacemaker. Precision of the clock is defined as inverse of the standard deviation of free-running period measured across days, indicating its day-to-day internal stability (Daan and Beersma, 2002), while accuracy is defined as inverse of the standard deviation of the phase relationship with a zeitgeber measured across days thereby reflecting the stability of entrainment (Beersma et al., 1999; Daan and Beersma, 2002). These two characteristics are proposed to have a strong association with the absolute value of the free-running period with the prediction that precision would be higher for clocks with period values close to 24 hours (Pittendrigh and Daan, 1976).

Besides these two clock properties, the free-running period has also been correlated with the entrained phase, such that rhythms with longer periods are expected to have a delayed phase compared to those with shorter periods. Also, for rhythms of a given period, phase relationships will get advanced with an increase in the length of the zeitgeber cycle (Pittendrigh and Daan, 1976; Aschoff and Pohl, 1978). In the same manner, the free-running period has also been observed to have a definitive relationship with activity/rest durations, amplitude, and power or robustness of the rhythm under constant as well as entrained conditions (Pittendrigh and Daan,

1976). Although these speculations have been tested and found to be true in a few empirical studies, there have been instances where either no clear pattern is observed or unexpected relationships are observed.

Since most organisms live in a rhythmic environment, the prevalent notion is that the circadian system is likely to be selected for functioning best in the presence of time cues (Roenneberg and Merrow, 2002). Despite no consistent correlation between precision and accuracy (Srivastava et al., 2019), the question of whether an imprecise internal pacemaker under constant conditions will have impaired functions under rhythmic conditions has not been entirely resolved. Two of the most important functions of clocks i.e., conservation of phase-angle and estimation of day/night length for regulation of seasonal changes, are performed under rhythmic conditions in many organisms. The other important function of the pacemaker is to track the passage of time, especially in the absence of external cues. The free- running period of the rhythm under constant conditions is also subject to changes such as lability with age, in response to environmental conditions like temperature or constant light (Aschoff, 1960, 1981; Barrett and Page, 1989), in terms of after-effects (Aschoff, 1960, 1981) as well as developmental plasticity (Srivastava et al., 2018).

Lability of the free-running period can be defined as the variation observed in the period due to fluctuations in external or internal physiological variables (Pittendrigh and Daan, 1976; Aschoff, 1979). Some studies have examined this aspect of the pacemaker as an important clock property, although its relationship with mean internal period value has not been clearly understood (Pittendrigh and Daan, 1976; Aschoff, 1979). Overt activity/rest rhythms depend on locomotor behaviour as well as circadian clocks, which, like most physiological functions, deteriorate with age. In addition to the internal physiological state, external environmental variables such as light also affect the clock period. For instance, wild-type and *per* mutant flies

exhibit a lengthening of the period under constant light with low intensity (Konopka et al., 1989), eventually driving wild-type flies into a state where splitting of rhythms occurs, resulting in arrhythmicity (Sheeba et al., 1999b). The period and amplitude of circadian rhythms are affected by illumination and is a function of its intensity (Aschoff, 1960, 1981).

Additionally, light can have a tonic / continuous effect of increasing the speed of the clock at certain phases and reducing it at certain other phases, which can be depicted using Velocity Response Curves (Daan and Pittendrigh, 1976). In addition to the effects of environment on the pacemaker's state during exposure, after-effects of these conditions are also known to affect the state of the pacemaker. The steady-state free-run can show an effect of preceding regimes, such as the length of the entraining cycle or the length of the photoperiod (Aschoff, 1979).

Another crucial aspect of the free-running period of circadian clocks is its temperature-compensated nature, which facilitates the conservation of the phase-angle of entrainment (Pittendrigh, 1993) against daily temperature fluctuations, especially in poikilotherms (Zimmerman et al., 1968; Menaker and Wisner, 1983; Chiba et al., 1993). Temperature compensation has been demonstrated in heterotherms (Menaker, 1959; Lee et al., 1990), and obligate homeotherms (Grahn et al., 1994) as well.

In the context of NT24 populations, rearing under semi-natural conditions may have led to the selection of either increased or decreased stability of the intrinsic clock, depending on what might ultimately contribute positively towards evolutionary fitness. As discussed in Chapter 1, the waveform of activity is different under semi-natural conditions compared to standard laboratory environments. This raises important questions about the prevalence as well as the relevance of conventional phase markers such as morning and evening peaks, which are examined in further experiments. I studied activity-rest rhythms of NT24 and T24 populations under (i) Standard laboratory conditions (LD (12:12), 25°C, ~70% RH) (ii) Semi-natural

conditions (SN) (iii) Semi-natural-DD conditions (SN-DD) and (iv) Constant darkness conditions (DD, 25°C, ~70% RH).

4.2 Materials and methods

4.2.1 Activity-rest recording

Activity of flies was recorded using the Trikinetics Drosophila Activity Monitors (DAM) system (Trikinetics, Waltham, MA, USA). Individual flies were loaded into glass tubes of 5 mm diameter with food at one end and a cotton plug at the other. The tubes were placed in the channels of Drosophila Activity Monitors such that an infrared beam passed through the middle of each tube. When these DAM monitors are connected to a computer, the movement of the flies in the tube is recorded by beam breaks in the middle of the tubes every minute, each beam break recorded as one activity count. 3-5 days old virgin male flies were used for the locomotor activity-rest assays. For all activity-rest experiments, n = 32 / population, of which > 85% were alive and rhythmic in all assays.

4.2.2 Data analysis and statistics

Analysis of entrained rhythms: Locomotor activity-rest behaviour of flies was recorded for seven days (laboratory) and ten days (semi-natural conditions), and the activity counts were binned into 15-minute intervals. The proportions of such patterns of activity were averaged across the four populations for each set. They were used to create activity profiles after the exclusion of arrhythmic and dead individuals for further analysis. For accuracy estimation, the inverse of the standard deviation of phase-angle was calculated for each individual fly.

Analysis of free-running rhythms: Locomotor activity-rest behaviour of flies was recorded in DD for seven days, and the activity counts were binned into 15-minute intervals. These data were used to calculate the free-running period and power by Chi-square periodogram as well

as to mark phases of Onset and Offset of activity for precision analysis using *RhythmicAlly* (Abhilash and Sheeba, 2019b) in R (v3.6.3). Precision was estimated as the inverse of the standard deviation of daily periodicity for each fly.

Data for both sets of populations were statistically analysed using a randomized block design, mixed model ANOVA approach with *Block* as the random factor. Normality of distributions was ascertained using the Shapiro-Wilk test. The details of these analyses are mentioned with the results of each assay, and statistical tables are contained in Appendix 3. Tukey's honest significant difference (HSD) tests were used to perform post-hoc multiple comparisons wherever necessary. All statistical tests were carried out using STATISTICA v7.0 (StatSoft, Tulsa, OK, USA), and the results were deemed significant at $\alpha = 0.05$.

4.3 Results

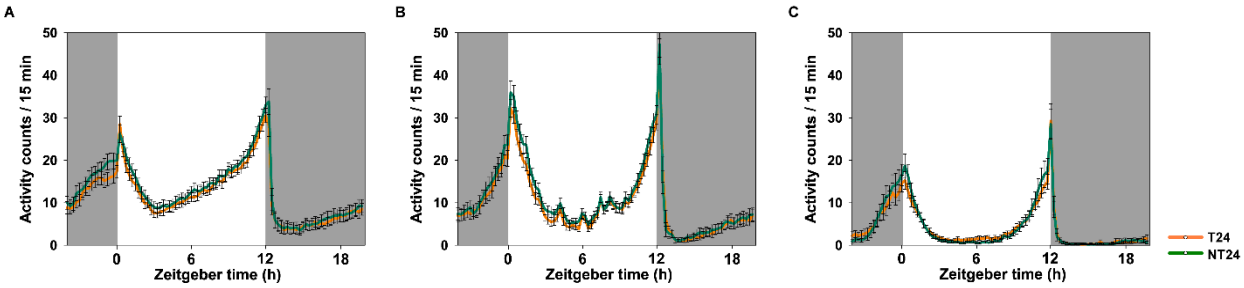

Fig. 4.1 Activity-rest rhythms of NT24 and T24 populations under standard laboratory conditions Assays under standard conditions of LD (12:12), 25°C, ~70% RH, were carried out under three different light intensities A) 0.1 lux B) 100 lux C) 1000 lux. White and grey shading indicates light and dark respectively, error bars indicate SEM.

4.3.1 Under standard laboratory conditions, activity-rest rhythms of NT24 and T24 populations do not differ

In order to ascertain whether rearing NT24 populations under semi-natural conditions altered the circadian behaviour under their ancestral regime of standard laboratory conditions, I assayed activity-rest rhythms for both sets of populations under LD (12:12), 25°C, ~70% RH. These assays were carried out at three different light intensities – 0.1 lux (Fig 4.1 A), 100 lux (Fig 4.1 B), and 1000 lux (Fig 4.1 C) to rule out light intensity-dependent artefacts. NT24 populations did not differ from T24 at any of these regimes (Two-way ANOVA, *Selection*, *Block*, $p > 0.05$), thus confirming that evolution under semi-natural conditions has not altered circadian behaviour under the ancestral regime.

4.3.2 Activity-rest rhythms of NT24 and T24 populations do not differ under SN conditions

I carried out a series of locomotor rhythm assays on NT24 and T24 populations under semi-natural conditions across the year in November 2017, February 2018, April-May 2018, June 2018, August 2018, and October 2018 (Fig. 4.2 A). Despite variation across months in activity-rest profiles for all populations in general, there was no difference in activity-rest profiles between NT24 and T24 populations (Three-way ANOVA, *Selection*, *Block*, *Timepoint*, $p > 0.05$).

4.3.3 Activity-rest rhythms under SN conditions exhibit a pronounced and consistent E- peak that correlates with Lights-off timing

It was interesting to note that under semi-natural conditions, the evening peak was much more pronounced in amplitude and consistency in phasing across individuals than the morning peak, which in comparison was diminished in amplitude and more variable (Fig. 4.2 A). To examine phase variation in activity-rest behaviour across different months, I carried out a correlational

analysis of the phases of morning and evening activity peaks with environmental phase markers of Lights-On (L_{ON}), Lights-Off (L_{OFF}), Maximum Temperature (T_{MAX}), Minimum temperature (T_{MIN}), Maximum humidity (H_{MAX}) and Minimum humidity (H_{MIN}) across months (Fig. 4.2 B). Interestingly, I found that the evening peak phase was strongly correlated with L_{OFF} (Pearson's coefficient, r = 0.994) across months (Fig. 4.2 B, black asterisk); other correlations are reported in Appendix 3.

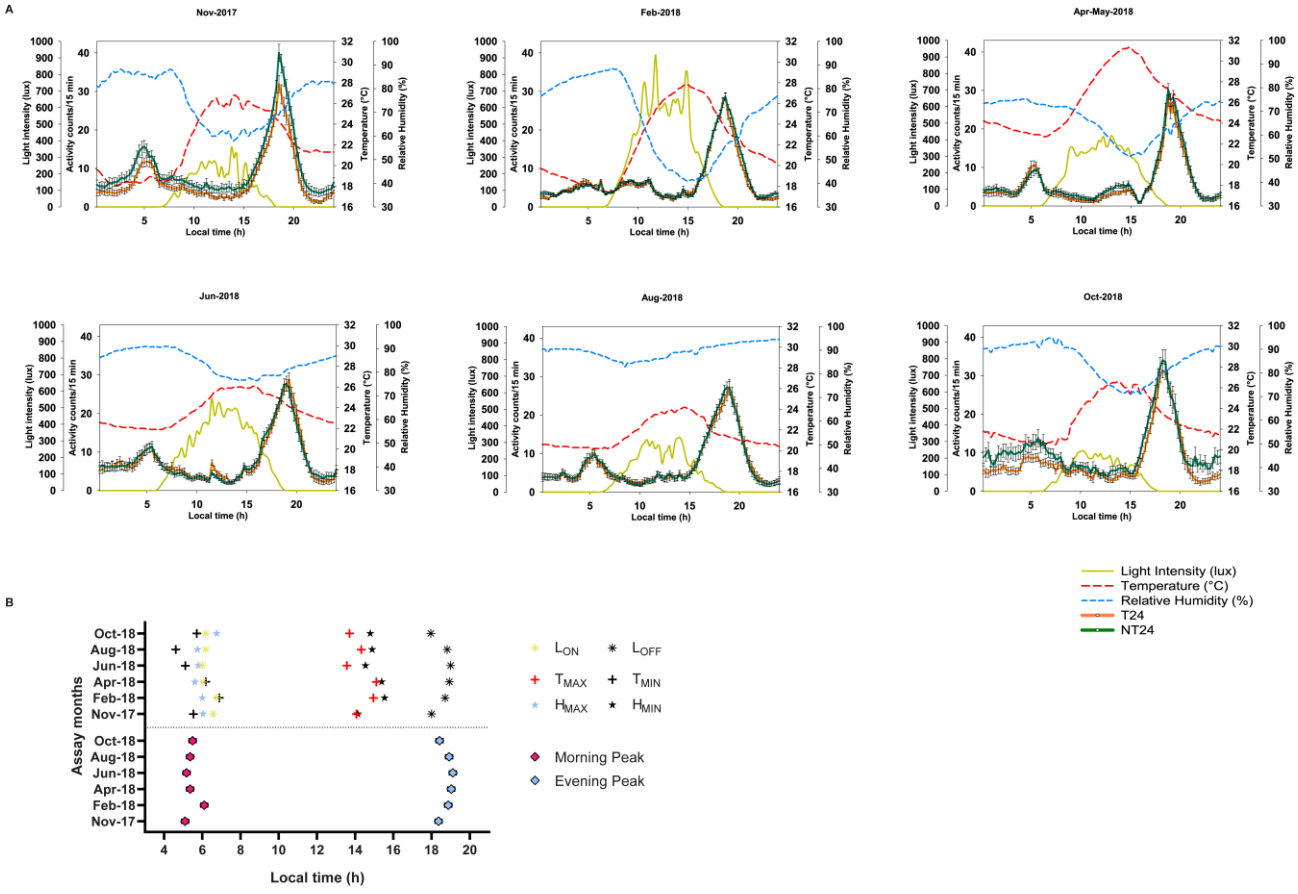

Fig. 4.2 Activity-rest rhythms of NT24 and T24 populations under semi-natural conditions (SN): A) Activity-rest profiles of assays carried out under 6 different months across the year, viz. November, February, April, June, August and October B) Phasing across months of morning and evening peaks as well as environmental variables viz. Lights-On (L_{ON}), Lights-

Off (L_{OFF}), Maximum Temperature (T_{MAX}), Minimum temperature (T_{MIN}), Maximum humidity (H_{MAX}) and Minimum humidity (H_{MIN}).

4.3.4 Under SN-DD conditions, NT24 populations exhibit higher activity levels during daytime without affecting phasing

Along with assays under semi-natural conditions, a parallel set of both populations were assayed such that they only experienced the temperature and humidity cycles (SN-DD) and light information was blocked within the same enclosure (from November 2017 – October 2018 (Fig. 4.3 A)). The hypothesis was that NT24 and T24 populations would experience zeitgebers such as temperature and humidity like the set of assays under the previously described semi-natural conditions, hence could be compared for differential responses to those in the absence of light. Interestingly, there was a timepoint-dependent difference in activity levels in three of the six months tested, without a clear difference in the phasing of the waveform. Three-way ANOVA, *Selection*, *Block*, *Timepoint* showed NT24 populations to have significant *Selection* × *Timepoint* interaction for November 2017 ($F_{23,69} = 3.494$, $p = 0.00003$), April-May 2018 ($F_{23,69} = 15.846$, $p < 10^{-6}$) and August 2018 ($F_{23,69} = 5.195$, $p < 10^{-6}$), while there was no difference for February, June and October 2018 ($p > 0.05$). The higher activity levels in the three months with significant differences between NT24 and T24 populations occurred at timepoints during the mid-day, but not consistently, and without affecting the waveform of activity. There were also significant *Selection* × *Block* interactions; thus I proceeded to systematically test for differences due to temperature cycles by subjecting the two sets of populations to temperature cycles in a laboratory incubator.

4.3.5 NT24 and T24 populations do not differ in phasing of activity-rest rhythms under ambient step-up/step-down temperature cycles and ramped temperature cycles

To test if NT24 and T24 populations differ in their response to temperature cycles, I attempted to mimic the temperature cycles of our enclosure (November, 2017) in the ambient range (27-20°C) within the laboratory in two ways - (i) step-up/step-down and (ii) ramped. On comparing both sets of data (Three-way ANOVA, *Selection*, *Block*, *Timepoint*), I found that NT24 populations showed a significant *Selection* × *Timepoint* interaction under step-up/step-down ($F_{23,69}$ = 4.129, p = 0.000003, Fig. 4.3 B), as well as ramped temperature cycle regime ($F_{23,69}$ = 3.44, p = 0.000038, Fig. 4.3 C). However, there were significant *Selection* × *Block* interactions for both assays and the increased activity for NT24 populations did not occur at any time windows that could be attributed to any functional significance (as in Fig. 4.3 A). Hence, this result was not examined any further. I also observed a rapid increase and dip in activity in response to small increases in temperature, which may be attributed to startle activity responses to the small temperature step-ups implemented in the incubator.

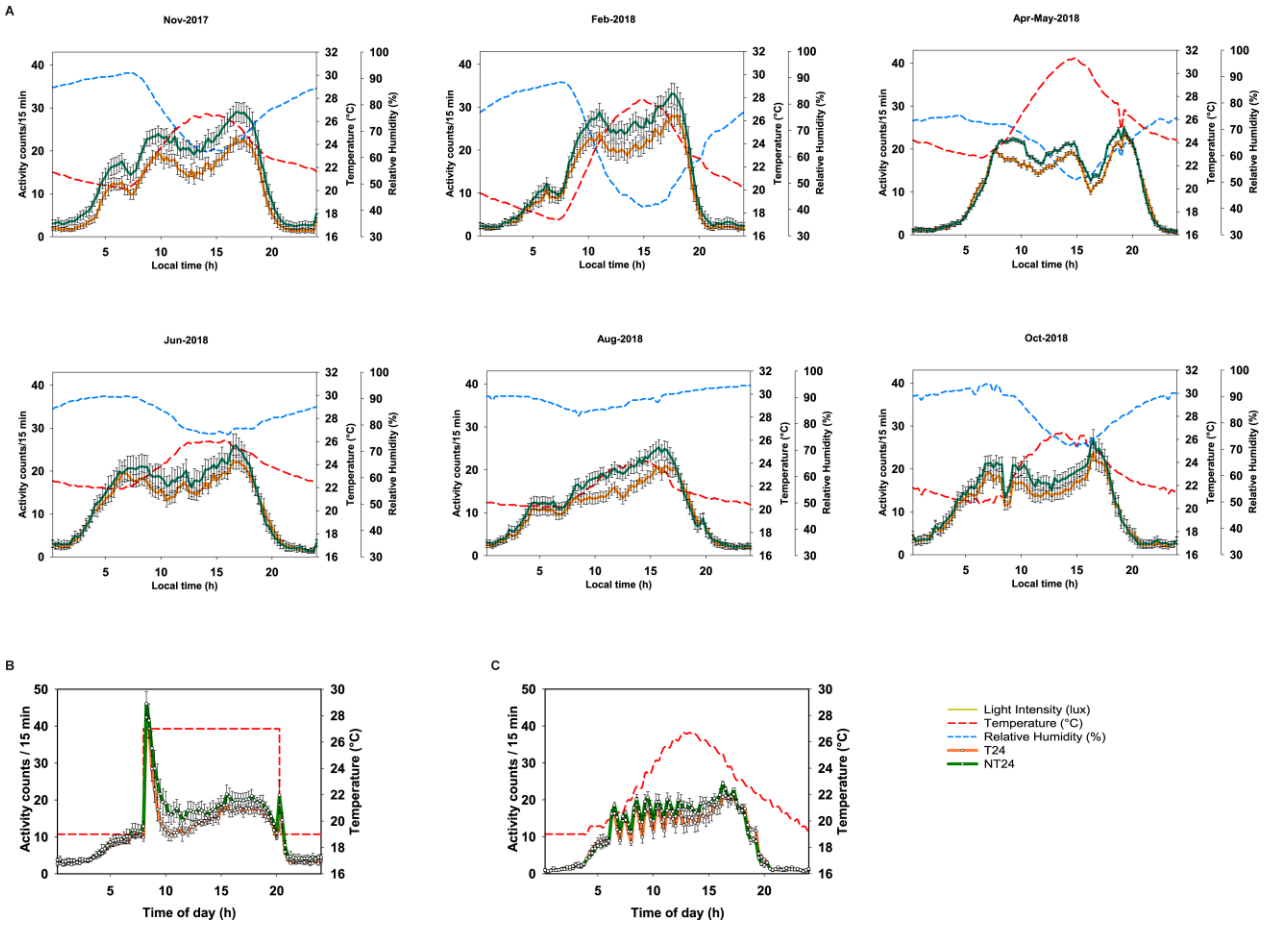

Fig. 4.3 Activity-rest rhythms of NT24 and T24 populations under natural cycles of temperature and humidity (SN-DD). A) Activity-rest profiles of NT24 and T24 populations under A) assays carried out under SN-DD conditions in 6 different months across the year viz. November, February, April, June, August and October B) Step-up / step-down temperature cycles (27-20°C) and C) Ramped temperature cycles (27-20°C). Error bars = SEM.

4.3.6 NT24 and T24 populations do not show a difference in period and power of rhythm under constant darkness conditions

I carried out activity-rest assays under constant conditions (DD – 25°C) to find out if NT24 and T24 populations differed in their free-running rhythms. Both populations exhibited a period

close to 24 hours (Fig. 4.4 A) and a similar power of the rhythm (Fig. 4.4 B), Two-way ANOVA, $p > 0.05$.

4.3.7 NT24 and T24 populations exhibit a difference in precision of Onset of activity under constant darkness

To examine the stability of the free-running period, I estimated the precision of the intrinsic clock using the phase of Offset, which has been an oft-used phase-marker for *Drosophila* activity-rest rhythms in previous studies (Srivastava et al., 2019). I found no difference in phase of Offset between the NT24 and T24 populations (Two-way ANOVA, $p > 0.05$, Fig. 4.4 C). Upon examining the usually more variable phase of Onset, I found that NT24 populations exhibited significantly higher precision than T24 populations (Fig. 4.4 D). When tested across generations, it is apparent that increased precision evolved over time (Fig. 4.4 D). Three-way ANOVA with *Selection*, *Block*, *Generation*, shows significant *Selection* × *Generation* interaction ($F_{10,30} = 6.423$, $p = 0.00003$). When visualized as the difference between the precision of the Onset of NT24 and T24 populations, a linear trend of increase in precision values can be observed (Fig. 4.4 E).

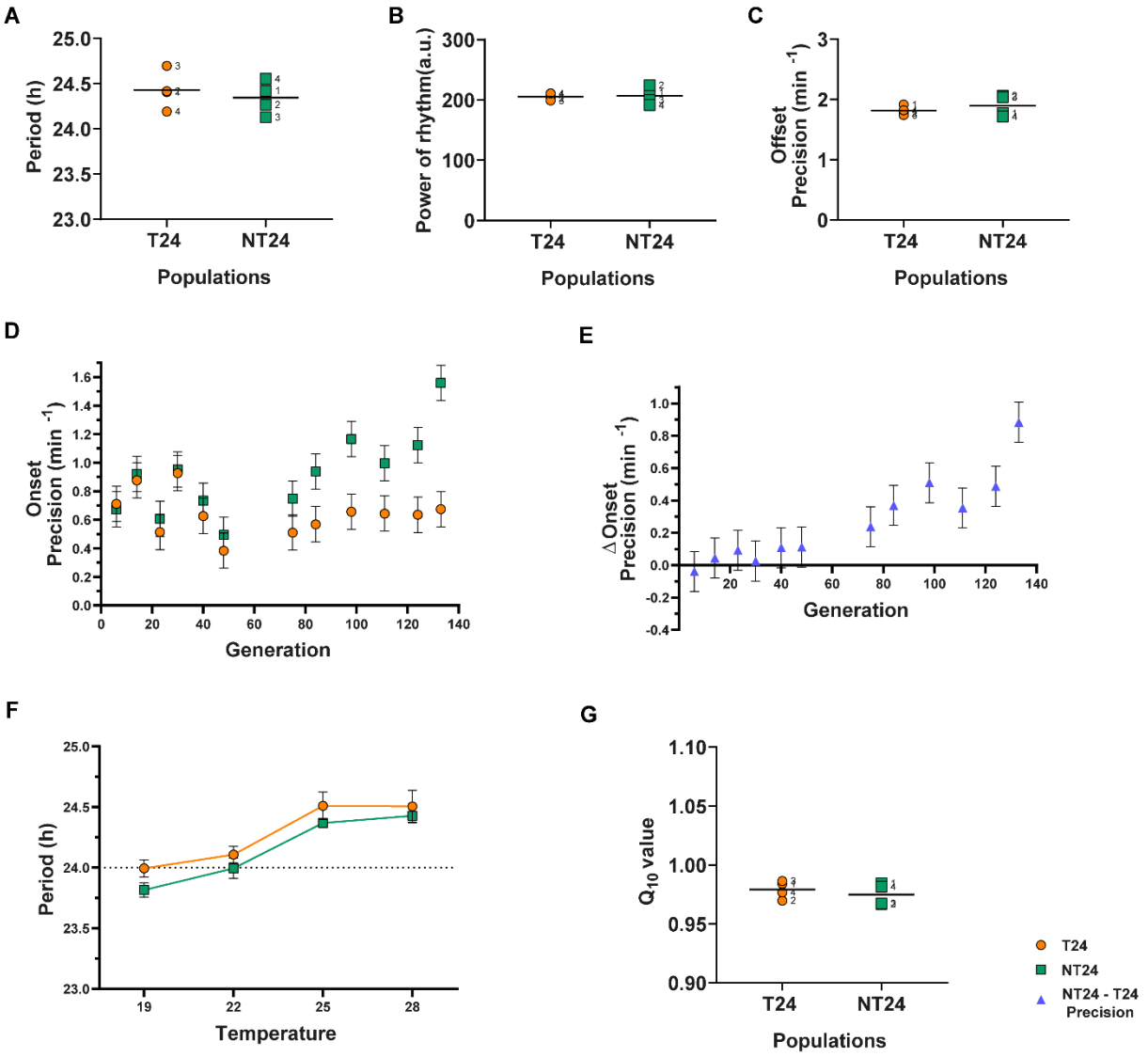

Fig. 4.4 Activity-rest behaviour under constant darkness conditions. Comparison of A) Free-running period B) Power of the rhythm C) Precision of activity Offset (Gen. 133) D) Precision of activity Onset (across generations) of NT24 and T24 populations under DD – 25°C. E) Difference in precision (across generations) of Onset between NT24 and T24 populations F) Free-running period at a range of temperatures (19-28°C) G) Comparison of Q_{10} values Dashed lines indicate means, error bars indicate 95% CI (Tukey's HSD).

4.3.8 NT24 and T24 populations do not differ in temperature compensation of free-running period in the ambient temperature range

Since temperature compensation is implicated in maintaining a stable period, I asked whether the two sets of populations have diverged in terms of temperature compensation of intrinsic period in the ambient temperature range of 19-28°C. On examining the period values across the range of temperatures, I found that both sets of populations maintain periodicities close to 24 h (Fig. 4.4 F). Comparing their Q_{10} values, I found no difference in temperature compensation between NT24 and T24 populations (Two-way ANOVA, *Selection*, *Block*, $p > 0.05$).

4.3.9 Evolution of increased precision in NT24 populations is not a consequence of correlated evolution of accuracy

Under semi-natural conditions that NT24 populations experience, precision of the free-running period is thought to not be directly expressed. Thus, one possibility driving the evolution of increased precision is its correlated evolution with accuracy (Pittendrigh and Daan, 1976). While this claim has been disputed in a recent study (Srivastava et al., 2019), I attempted to quantify accuracy to find out if correlated evolution may have occurred and thereby also test whether precision and accuracy are correlated in NT24 populations. Since multiple environmental cues are present under semi-natural conditions, ascertaining the evolution of accuracy required that accuracy measurements be made with respect to all phase markers across seasons. On comparing the accuracy of Onset of activity of NT24 and T24 populations with respect to Lights-On (L_{ON}), Lights-Off (L_{OFF}), Maximum Temperature (T_{MAX}), Minimum temperature (T_{MIN}), Maximum humidity (H_{MAX}) and Minimum humidity (H_{MIN}), I found no significant difference between the two sets of populations (Three-way ANOVA, *Selection*, *Block*, *Month*) for *Selection* and *Selection* × *Month*, $p > 0.05$ (Fig. 4.5), except for H_{MIN}, that

showed a trend - significant *Selection* × *Month* interaction ($F_{5,15} = 3.38$, $p = 0.03$; Tukey's HSD = n.s.). Interestingly, there was a significant effect of *Month* for each environmental phase marker due to higher accuracy in April 2018 (L_{ON}, L_{OFF}, T_{MAX}, H_{MIN}) as well as seasonal variation across the year.

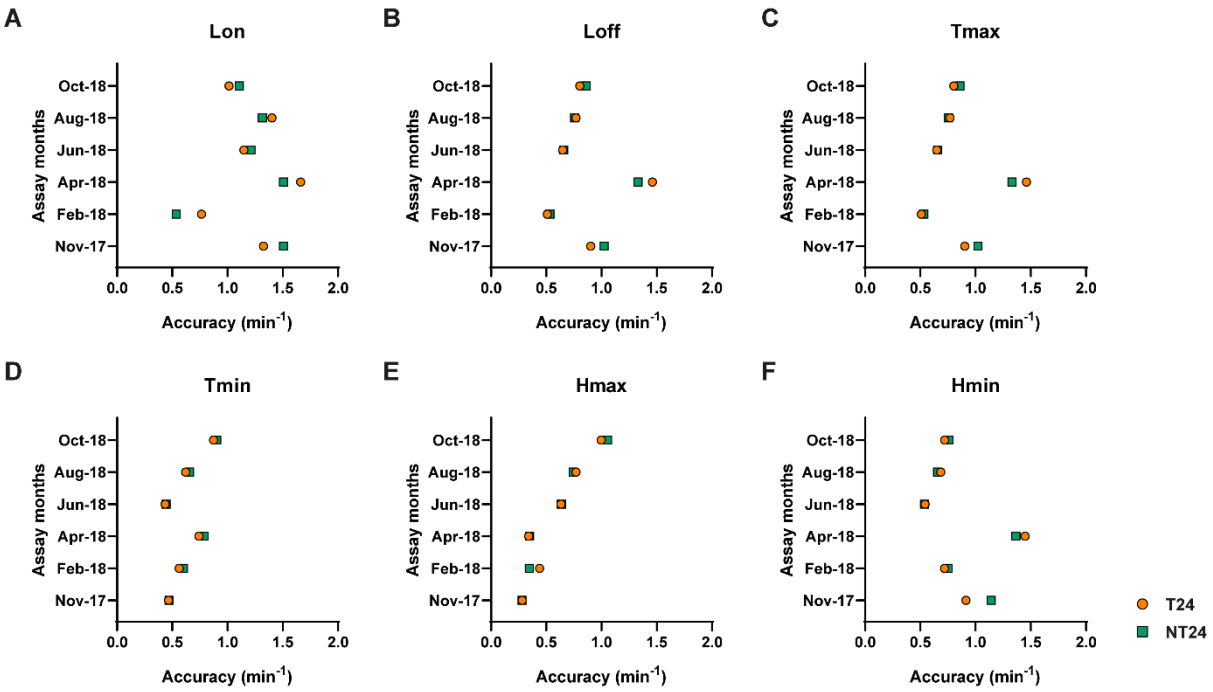

Fig. 4.5 Accuracy of NT24 and T24 populations under semi-natural conditions. Accuracy of the phase of Onset with A) Lights-On (L_{ON}) B) Lights-Off (L_{OFF}) C) Maximum Temperature (T_{MAX}) D) Minimum temperature (T_{MIN}) E) Maximum humidity (H_{MAX}), and F) Minimum humidity (H_{MIN}) under semi-natural conditions. Error bars (not visible) = SEM.

4.4 Discussion

After a systematic investigation of activity-rest behaviour under standard laboratory and semi-natural conditions, I found that, similar to eclosion rhythms, the activity-rest rhythms of NT24 populations have not diverged from T24 populations when assayed under the standard laboratory regime (Fig. 4.1 A, B, C). However, in contrast to the eclosion assays, activity rhythm assays conducted across the year under semi-natural conditions did not reveal any differences between the two sets of populations (Fig. 4.3 A). There were, overall, no differences in the waveform of activity-rest behaviour under semi-natural – DD conditions except at certain timepoints during the mid-day, where NT24 populations exhibited higher activity in certain seasons (Fig. 4.3 A), as well as under temperature cycles (Fig. 4.3 B). Elevated activity levels during the mid-day may reflect differences at the metabolic level for NT24 populations, which may be temperature-dependent. Overall, however, the lack of a clear difference in the phasing of activity-rest rhythms is in contrast to the results seen with the eclosion rhythm described in Chapter 3.

Surprisingly, NT24 populations exhibited higher precision of Onset of activity under constant conditions (Fig. 4.4 D), despite no changes in the free-running period (Fig. 4.4 A). While a combined analysis showed differences to be significant statistically after ~80 generations of selection, analyses of individual assays from generation 40 also showed a significant increase in precision for NT24 populations. This result is especially interesting as the evolution of increased precision in NT24 populations compared to T24 is not due to correlated evolution of accuracy under semi-natural conditions (Fig. 4.5).

The evolution of precision of Onset of activity in NT24 populations shows that despite being in a variable cyclic environment, the stability of the intrinsic period is under selection. This challenges the notion that phase-angle and accuracy under entrained conditions are the primary

determinants of adaptive advantage to the organism (Roenneberg and Merrow, 2016). However, the mechanism by which increased precision may be adaptive is currently unknown. One possibility by which precision might play a role in entrainment is by affecting the stability of period via parametric entrainment. In my examination of the stability of periods across a range of ambient temperatures, I did not find a difference in the temperature compensation of the clock (Fig. 4.4 F, G). It is therefore unclear whether or how such a dynamic change would be brought about on a daily basis in NT24 populations without affecting the phase-angle, particularly as the molecular correlates of these clock properties are currently unknown and require further investigation.

Chapter 5 . Differences in conditionality of circadian rhythms in fly populations reared in laboratory *vs* semi-natural environments

5.1 Introduction

A clock property which is discussed relatively less frequently in literature is the conditionality of circadian rhythms. Conditionality refers to the loss of rhythmicity due to exposure to a single aperiodic environmental variable or multiple aperiodic factors that were ineffective individually (Njus et al., 1977). Such a loss of rhythmicity is reversible, and in the recovered rhythm (following some treatment), the original phase is not retained. The new phase depends only on the time of return to normal conditions. Common aperiodic variables that have been used in this regard are low temperature (Njus et al., 1977), anoxic conditions (Pittendrigh, 1954a), bright light (Bruce et al., 1960) etc.

It is obvious that this property pertains to poikilothermic organisms and hence has been mainly investigated in plants (Bünning, 1964), dinoflagellates (Hastings and Sweeney, 1957; Njus et al., 1977), fungi (Francis and Sargent, 1979) and cyanobacteria (Murayama et al., 2017). In insects such as *Drosophila*, there are a few reports of conditionality, such as susceptibility of the eclosion rhythm to low temperature in the case of *Drosophila pseudoobscura* at ~10.5°C (Zimmerman et al., 1968) and maintenance of rhythmicity in activity-rest behaviour of cold resistant *Drosophila melanogaster* lines at ~12°C (Maguire et al., 2014). In the study on *D. pseudoobscura*, the low temperature was hypothesized to have nullified the rhythm by affecting the synchronisation of pupae to eclose periodically. In the latter study on *D. melanogaster* it was thought that cold-adaptation results in the persistence of circadian rhythms despite the lowered temperature. It is unclear whether the persistence of activity rhythms at the low

temperatures tested was due to activity output being unaffected by low temperature or the core clock oscillation being more robust in the cold-resistant lines. In *Drosophila* which underwent anoxia on nitrogen exposure, similar conditionality of rhythms has been observed (Pittendrigh, 1954a).

Research on dinoflagellates and cyanobacteria has provided key insights in the general context of what we know regarding the conditionality of rhythms to low temperature. The core oscillation persisting at normal temperatures is abolished below a certain critical temperature (Njus et al., 1977; Murayama et al., 2017). Prior to this, some changes in the amplitude of the oscillation can be seen; however, persistence of the oscillation is usually unaffected (Njus et al., 1977). In cyanobacteria, the current understanding is that low temperature causes a transient change in the state of the oscillation from self-sustained to damped, a phenomenon known as Hopf bifurcation (Murayama et al., 2017).

I found no difference in the temperature compensation of NT24 and T24 populations in the above range (Chapter 4), however, at 19°C more individuals with complex rhythms were observed. Hence, I wanted to investigate the persistence of rhythms at lower temperatures for T24 and NT24 populations with the expectation that NT24 populations to have increased persistence than T24 populations. The reasons for this expectation are: (i) under standard 25°C, NT24 populations show an increased precision of activity-rest rhythm, a phenotypic output of the intrinsic clock (ii) NT24 populations have experienced more temperature fluctuations and have been exposed to seasonally occurring lower temperature conditions than T24 populations which are reared in a constant temperature environment.

5.2 Materials and methods

5.2.1 Fly populations

Apart from NT24 and T24 populations described previously, other fly populations used in the study are: *Drosophila melanogaster* (originally wild-caught population from around Bengaluru, independent in origin from NT24 and T24 populations), *Drosophila malerkotliana*, *Zaprionus indianus*, *Drosophila nasuta*, *Drosophila ananassae* (see Fig. 5.2 A for taxonomic details). These drosophilids have since been maintained as outbred cage populations under standard laboratory conditions (LD (12:12), 25°C, ~70%RH). The number of generations of laboratory maintenance for *Drosophila malerkotliana* and *Drosophila melanogaster* are 367, *Drosophila ananassae* – 298, *Zaprionus indianus* – 285 and *Drosophila nasuta* – 286 generations. The founding size of the *D. ananassae* population was ~300 and for all other populations was ~70 individuals.

5.2.2 Activity-rest behaviour at constant low temperature

3-5-day old virgin male flies cultured under the standardized maintenance regime (LD (12:12), 25°C, ~70%RH) were used for experiments in constant conditions. Locomotor activity assay setups are as described in Chapter 4. The typical length of each assay was 12 days at DD – 14°C. The experiment was replicated 6 times, and to rule out the possibility of driving the circadian oscillator to a state of singularity, the time of shifting to DD -14°C varied from ZT8 – ZT14 as well as transition to low temperature was carried out gradually over 60 min (as opposed to a cold shock) from 25°C to 14°C.

5.2.3 Activity-rest behaviour under cyclic conditions at low temperature

Flies were exposed to synchronizing conditions after 12 days of exposure to DD-14°C. There were four such zeitgeber conditions used: (i) LD (12:12) 100 lux, TC (18-14°C) in phase (ii)

TC (18-14°C) (iii) LD (12:12) 1 lux at 14°C (iv) LD (12:12) 100 lux at 14°C. All zeitgeber regimes used were step-up/step-down. To avoid biasing of inferences due to transients occurring at the beginning of synchronizing regimes, data from last 3 days of the regime (manually verified to be stable in phasing) was used to create activity profiles and further analysis. All individual activity-rest experiments were carried out by loading n = 32 flies / population.

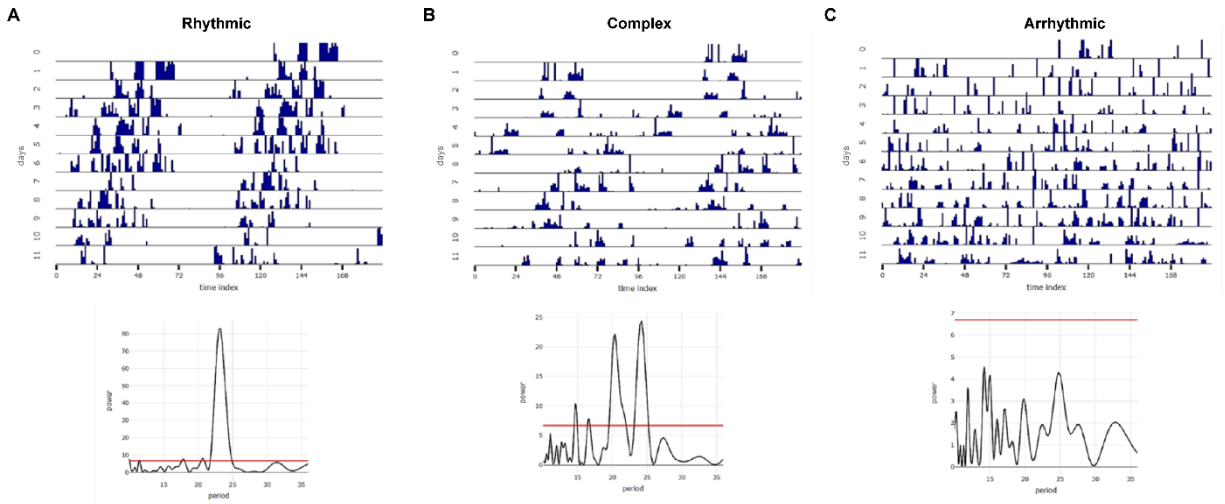

Fig. 5.1 Classification of individuals exhibiting rhythmic, complex and arrhythmic behaviour Representative actograms (above) and periodograms (below) of individuals exhibiting A) rhythmic B) complex and C) arrhythmic behaviour based on Lomb-Scargle periodogram analysis.

5.2.4 Data analysis and statistics

12 days of activity-rest data was used for the DD – low temperature regime, while ten days of data was used for the LL regime. Periodogram analysis was conducted using the Lomb-Scargle periodogram. Free-running periods in the range of 16-32 hours were considered to be circadian. Categorization of individuals into ‘rhythmic’, ‘complex’ or ‘arrhythmic’ categories was carried

out objectively using a pre-defined criterion based on the periodogram analysis (Fig. 5.1). For this categorization, the range of periodicities used was 10-36 h. To eliminate bias resulting from the bimodality of activity-rest bout patterns, peaks in the non-circadian range, exactly 0.5τ of the circadian range, were ignored. Individuals with no significant peak in the circadian range were considered 'arrhythmic'. Individuals showing more than one peak in the periodogram, with at least one in the circadian range and another in the circadian/non-circadian range and having greater than 50% amplitude of the highest peak, were considered as showing 'complex' rhythms. Individuals with a single significant peak in the circadian range or with multiple peaks where the subsidiary peaks have less than 50% amplitude of the highest peak were categorized as 'rhythmic'. Statistical analysis was done using randomized block design ANOVA and Tukey's HSD post-hoc tests were carried out when required (Appendix 4).

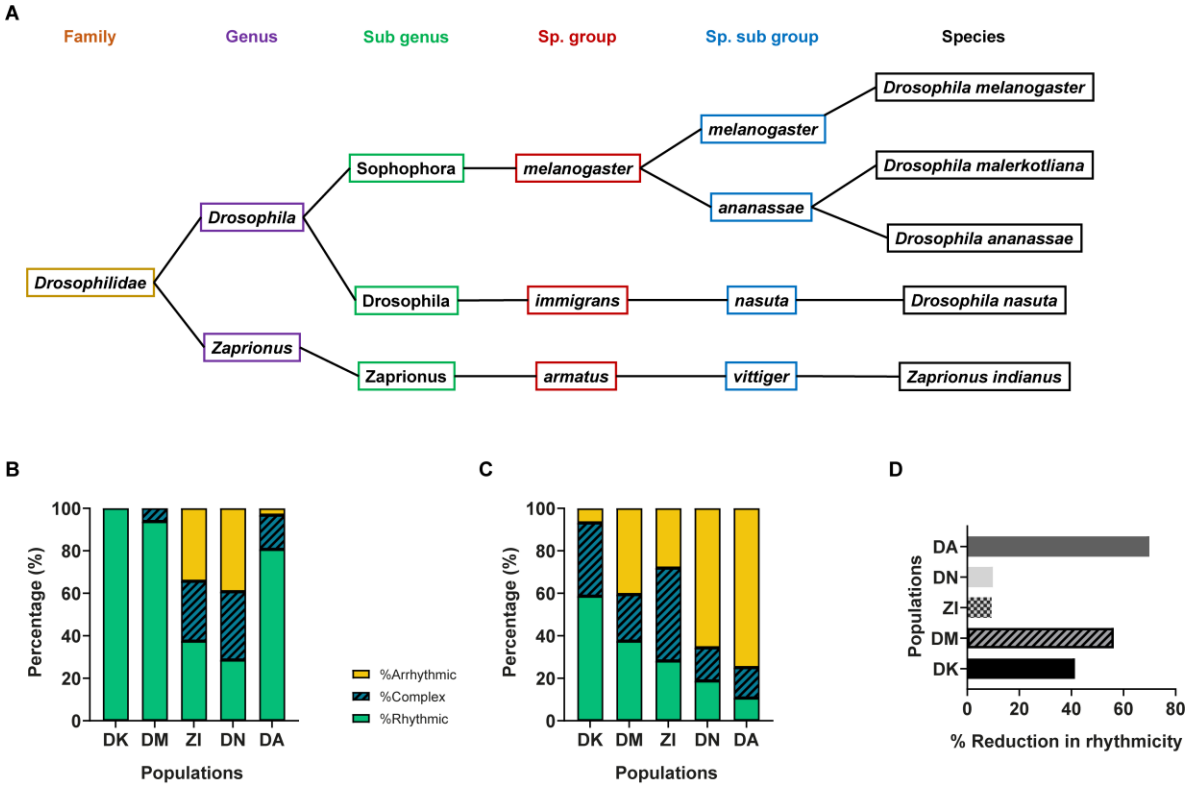

Fig. 5.2 Activity-rest rhythms of drosophilid species under constant darkness – low temperature (14°C) A) Taxonomic details of species used in the study B) Activity-rest behaviour of different species under constant darkness (DD) at 25°C C) Activity-rest behaviour of different species under constant darkness (DD) at 14°C D) Difference in percentage of rhythmic individuals from 25°C to 14°C.

5.3 Results

5.3.1 Constant darkness and constant low temperature lead to a breakdown of activity-rest rhythms

Assaying activity-rest rhythms of flies under DD – 14°C showed that rhythmicity is negatively impacted across species compared to rhythmicity exhibited under DD – 25°C (Figure 5.2 B, C). *D. malerkotliana* and *D. melanogaster* exhibit higher persistence of rhythmicity at low temperature than *D. ananassae,* as expected from previous knowledge of robustness of their activity-rest rhythms (Prabhakaran and Sheeba, 2012, 2013). This establishes the constant darkness – low temperature paradigm as one that tests the limits of persistence of rhythms in fruit-flies.

5.3.2 NT24 populations show higher persistence of rhythmicity at low temperature compared to T24 populations

Since the NT24 and T24 populations are *D. melanogaster* but of different ancestry (temperate, see Chapter 1) from the *D. melanogaster* population assayed above (tropical), we wanted to see how they compared in terms of levels of rhythmicity. We found that NT24 populations consistently exhibited a higher percentage of flies with persistent circadian rhythms than T24 populations (Fig. 5.3 A, B), Two-way ANOVA, *Selection*, $F_{1,3} = 27.69$, $p = 0.0134$. There was no difference in the percentage of flies exhibiting complex rhythms and arrhythmic behaviour across both sets of populations (Fig. 5.3 C, D), Two-way ANOVA, *Selection*, $p > 0.05$.

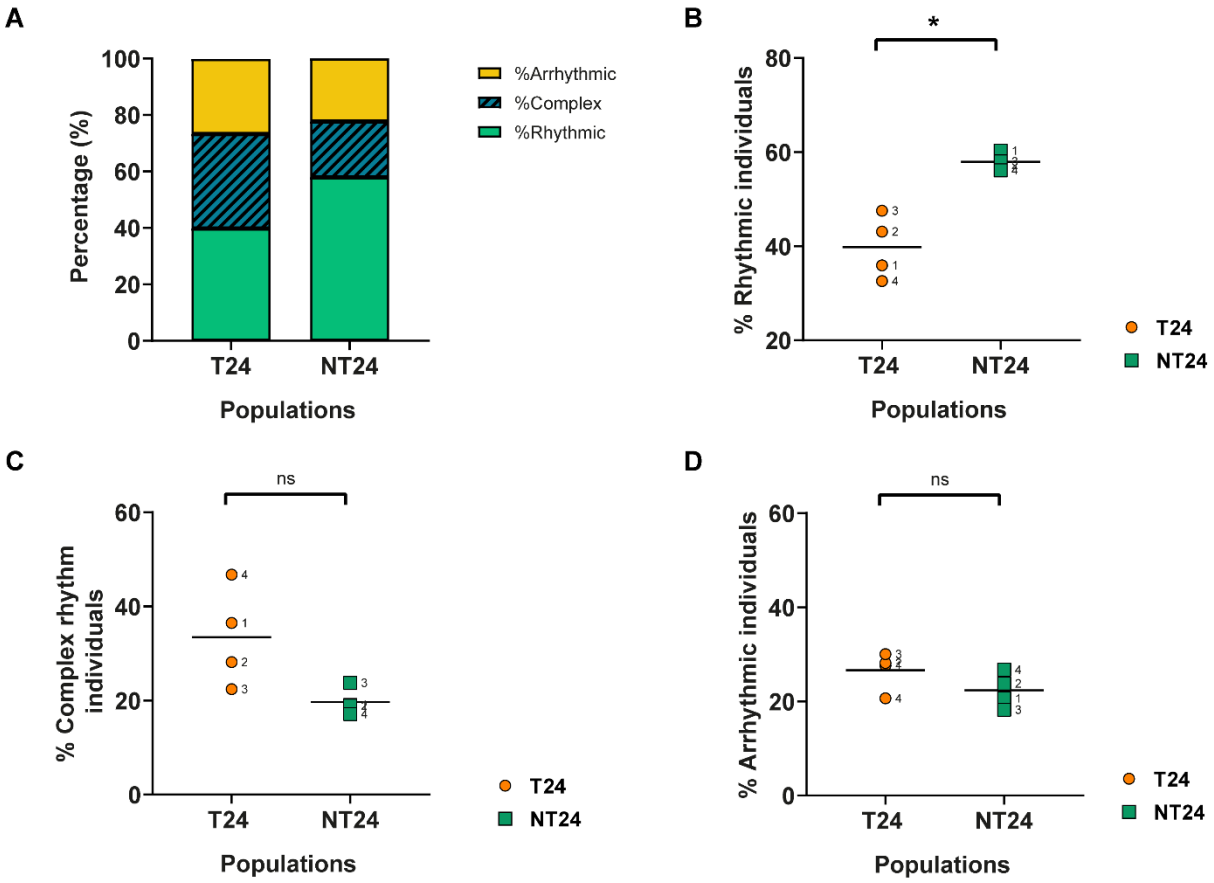

Fig. 5.3 NT24 and T24 populations show differences in persistence of rhythmicity under constant darkness – low temperature (14°C). A) Activity-rest behaviour under constant darkness (DD) at 14°C (n > 181 / population, data pooled across 6 experiments). Further shown is the comparison of B) percentage of rhythmic individuals, C) percentage of individuals showing complex rhythms D) percentage of arrhythmic individuals of NT24 and T24 populations under DD - 14°C. Dashed lines indicate means, and asterisks indicate significant differences ($\alpha = 0.05$).

5.3.3 NT24 and T24 populations respond differentially to light and temperature zeitgebers at low temperature

To probe whether the state of the circadian clock at low temperature permits synchronization to zeitgebers, I carried out a series of experiments using light and temperature cycles. While

all four regimes used (described above in methods) synchronized activity-rest behaviour of NT24 and T24 populations, there were; however, some differences observed. While the LD (12:12, 100 lux) + TC (18-14˚C) regime did synchronize the clock, there was no difference in the activity profile of NT24 and T24 populations (Fig. 5.4 A, Three-way ANOVA, *Selection* × *Timepoint*, $p > 0.05$). Under the DD-TC (18-14˚C) regime, however, T24 populations exhibited a higher startle response to the onset of warm temperature (Fig. 5.4 B) (Three-way ANOVA, *Selection* × *Timepoint*, $F_{23,69} = 34.68$, $p < 10^{-13}$). When tested under LD regimes of differing light intensities (and thereby zeitgeber strengths) at 14˚C, there was no significant difference in activity-rest behaviour under low light intensity (1 lux) LD (12:12) conditions (Fig. 5.4 C, Three-way ANOVA, *Selection* × *Timepoint*, $F_{23,69} = 2.02$, $p = 0.0132$, Tukey's HSD = n.s.). In contrast, the activity profile of NT24 populations was drastically different when zeitgeber strength was increased (100 lux) (Three-way ANOVA, *Selection* × *Timepoint*, $F_{23,69} = 9.46$, $p = 1.37 \times 10^{-13}$). NT24 populations also appeared to show higher anticipation to the onset of light under LD (100 lux) at 14˚C (Fig. 5.4 D).

5.3.4 Phase control is impacted at low temperature despite synchronization to light and temperature zeitgebers

Despite almost all flies synchronizing to the above four zeitgeber regimes, I found that a substantial proportion did not exhibit phase control across regimes (except the DD-TC regime). Contrary to the differences observed in activity profiles of NT24 and T24 populations under DD-TC and LD (1 lux) regimes, there was no difference in phase control among the populations for these regimes (Two-way ANOVA, *Selection*, $p > 0.05$). However, NT24 populations exhibited significantly higher phase control than T24 populations under LD-TC (Two-way ANOVA, *Selection*, $F_{1,3} = 14.25$, $p = 0.0326$) and low light intensity (1 lux) LD (12:12) (Two-way ANOVA, *Selection*, $F_{1,3} = 20.16$, $p = 0.0206$).

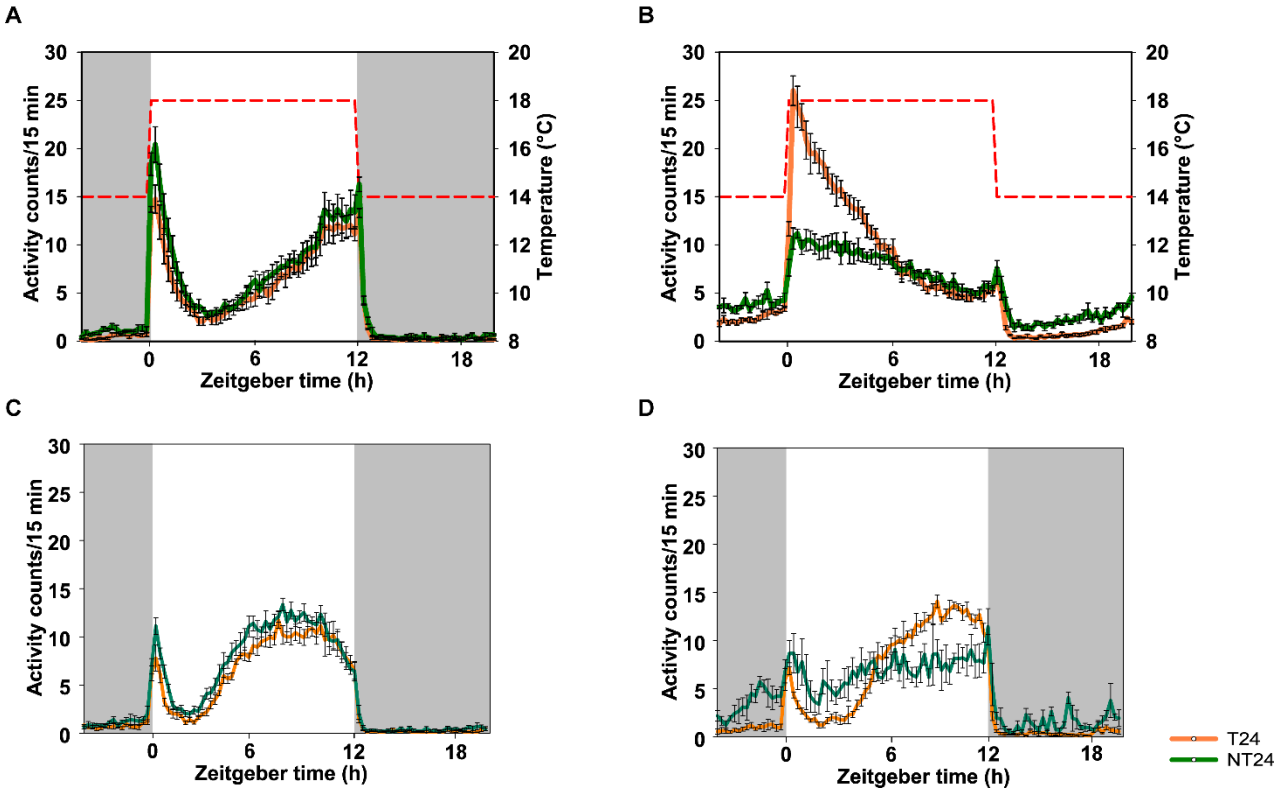

Fig. 5.4 Synchronization of NT24 and T24 populations to light and temperature zeitgebers Activity-rest profiles of NT24 and T24 populations under A) Light and temperature cycling: LD (12:12) – 100 lux, TC (18-14°C) B) Temperature cycling: TC (18-14°C) C) Light cycling (low intensity): LD (12:12) – 1 lux at 14°C D) Light cycling (high intensity): LD (12:12) – 100 lux at 14°C. The light cycle is indicated by white-grey shading while the dashed red line indicates the temperature cycle. Error bars indicate SEM, n > 30 / population.

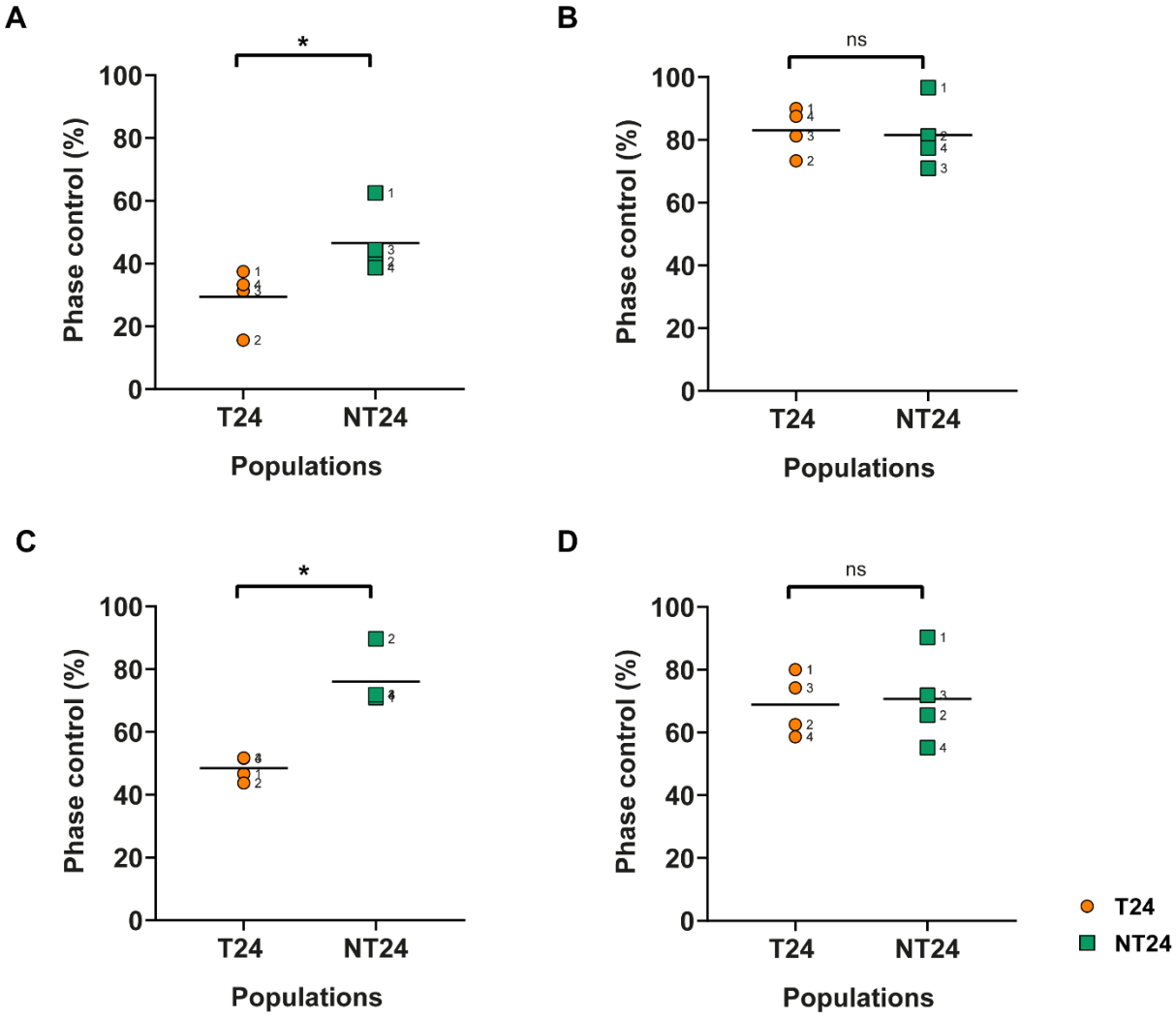

Fig. 5.5 Phase control of NT24 and T24 populations synchronized to light and temperature zeitgeber cycles Phase control using the phase of onset of activity on day 1 of DD - 14°C after 8 days of entraining regime of A) Light and temperature cycling: LD (12:12) – 100 lux, TC (18-14°C) B) Temperature cycling: TC (18-14°C) C) Light cycling (low intensity): LD (12:12) – 1 lux at 14°C D) Light cycling (high intensity): LD (12:12) – 100 lux at 14°C. Dashed lines indicate means, and asterisks indicate significant differences ($\alpha = 0.05$).

5.3.5 NT24 and T24 populations exhibit similar persistence of rhythms under constant light

As constant light renders *D. melanogaster* arrhythmic via degradation of TIM (Marrus et al., 1996), I used low intensity (0.1 lux) constant light at 25°C to test persistence of rhythmicity in

NT24 and T24 populations and determined the proportions of individuals exhibiting rhythmic, complex and arrhythmic behaviour (Fig. 5.6 A). On comparing the percentage of individuals showing free-running rhythms (Fig. 5.6 B), complex rhythms (Fig. 5.6 C) and arrhythmicity (Fig. 5,6 D), I found no significant difference between the NT24 and T24 populations (Two-way ANOVA, $p > 0.05$).

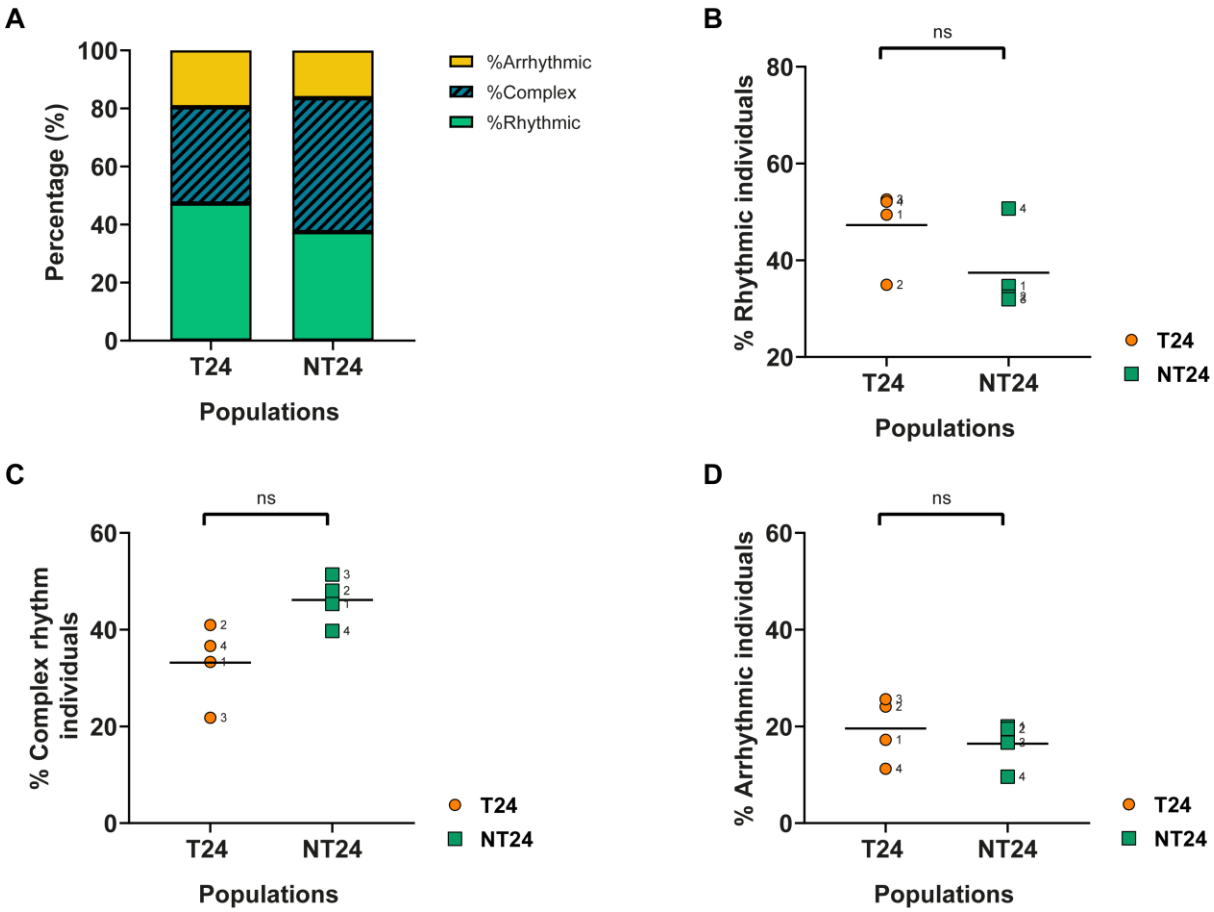

Fig. 5.6 NT24 and T24 populations show no difference in persistence of rhythmicity under constant light (25°C) A) Activity-rest behaviour under 0.1 lux constant light (LL) at 25°C (data from 3 experiments). Further shown is the comparison of B) percentage of rhythmic individuals, C) percentage of individuals showing complex rhythms D) percentage of arrhythmic individuals of NT24 and T24 populations under 0.1 lux LL at 25°C. Dashed lines indicate means, and asterisks indicate significant differences ($\alpha = 0.05$).

5.4 Discussion

When assayed at constant darkness - low temperature (14˚C), activity-rest rhythms in flies are severely affected, and phenomena such as splitting and complex activity-rest rhythms are observed under constant darkness, conventionally thought to be characteristics of constant light conditions. My results show that this conditionality of circadian rhythms in activity-rest is pervasive across various drosophilids (Fig. 5.2). Moreover, in the study system of NT24 and T24 populations, I found higher persistence of rhythmicity in NT24 populations compared to T24 (Fig. 5.3). On testing for synchronization and entrainment to light and temperature cycle regimes, NT24 and T24 populations showed very distinct differences. While there was a higher startle response to the warm phase of the temperature cycle by T24 populations under TC (18-14˚C) (Fig. 5.4 B), on increasing light intensity under only light cycles, NT24 populations exhibited increased anticipation as well as an overall altered activity profile (Fig. 5.4 D).

Assessing phase control is the cleanest way to infer about entrainment to the four zeitgeber regimes used. My results show that entrainment does not seem to occur for a large proportion of flies subjected to a DD – low temperature regime (except for temperature cycles) (Fig. 5.5). In case of temperature cycles, it is still debatable if the effectiveness of temperature as a solo zeitgeber leads to high phase control rather than the increase in absolute/average temperature in the cycling regime. It is rather peculiar that the two regimes to show differences between NT24 and T24 populations in phase control (Light + Temperature cycle and Light cycle – low intensity) are the ones where differences in activity profiles are not seen. This phenotype variability requires further examination of the phenotypes of entraining and non-entraining flies separately with a larger cohort. Overall, NT24 and T24 populations exhibit clear differences in the sensitivity of their rhythms to light and temperature cues under low temperature. When

tested for differences in circadian photosensitivity using constant light at ambient temperature, there was no difference between the two sets of populations (Fig. 5.6 B, C, D).

NT24 populations exhibit increased persistence of rhythms under physiologically non-harmful low temperatures than T24, despite not being reared in a constant environment. This result further supports the hypothesis of selection on the stability of intrinsic period under semi-natural conditions, for which I also described increased precision of intrinsic period as initial evidence in Chapter 4.

Chapter 6 . Conclusions and future directions of investigation with NT24 and T24 populations

This study began with questions asking which clock properties would evolve by selection under semi-natural conditions, whether the circadian phenotype under ancestral conditions would be altered, what would dictate timing under semi-natural conditions and the general nature of selection under such regimes. My results have provided some answers to all of these questions. Additionally, several new questions have arisen – a few along the lines of the evolution of clocks under semi-natural conditions and others on our fundamental understanding of clocks and clock function.

6.1 Selection on life-history

To begin with, what does selection under semi-natural conditions act upon in terms of phenotypes reaping life-history dividends? From the results of experiments testing reproductive output and recovery from heat stress of NT24 and T24 populations, it appears that under semi-natural conditions, there is selective pressure for increased fecundity and heat tolerance. This selective pressure on fecundity could be variable across the year, as evidenced by the differences in egg output and viability. An interesting question is whether there is a direct link between heat tolerance and the observed differences in fecundity. This can be easily tested by conducting a targeted experiment on both sets of populations to elucidate if prior heat stress results in differences in egg output or viability. Although it was a reasonable expectation, I did not find differences in desiccation tolerance of NT24 and T24 populations. There could be several reasons for this – selection on desiccation tolerance may be weak or inconsistent through the year and thus may not result in the continual evolution of the trait. A case of highly inconsistent selection can be verified by looking at the effect of the season of collection on the

desiccation tolerance of NT24 populations. Similarly, I did not obtain differences in development time under laboratory or semi-natural conditions (June 2019). However, there is a possibility that changes in development time across seasons might be different for NT24 and T24 populations which can be verified by testing development time across an array of semi-natural regimes. Despite unaltered development time of NT24 flies, there could be several ways by which differences in dry-weight at eclosion may arise: different critical weights; alterations in the duration of certain developmental stages e.g. wandering stage post-attainment of critical weight; or differential efficiency of feeding or food assimilation. Curiously, there are no differences in dry-weight ~10 days post eclosion that are suggestive of differences in feeding volumes as young adults. All of these possibilities remain to be tested.

6.2 Circadian clock evolution

In the context of circadian clock evolution in NT24 and T24 populations, two overt rhythms were tested – eclosion and activity-rest. The eclosion rhythm is a population-level rhythm that is also tied to development; in contrast, the locomotor activity-rest rhythm is a rhythmic output at the level of individuals linked to several other rhythms in adult *Drosophila*. In the case of eclosion rhythm, I found that NT24 populations exhibit temperature-directed phase advance of eclosion under semi-natural conditions as well as natural light and temperature cycles simulated in the lab, depending on the magnitude of temperature cues. While the direct adaptive value of such advance in phase (~2 hours) is unknown, according to previous literature, it can be speculated to be protective against harsh weather or it could also occur due to the evolution of another correlated trait. On the other hand, no differences were observed in phasing for the activity-rest rhythm under semi-natural conditions. This is not contradictory and is especially interesting because eclosion and activity-rest are considered to be under the control of different peripheral oscillators. Thus, NT24 populations may have evolved differences in temperature

sensitivity at the level of the peripheral oscillator for eclosion, which might not be the case for activity-rest behaviour. While the peripheral oscillator for eclosion is known to be in the prothoracic gland, the exact location of the peripheral oscillator for activity-rest is still not elucidated.

Of all results, the most surprising and yet consistent one has been the difference in stability of intrinsic period observed between the NT24 and T24 populations. This phenotype was investigated in detail across generations and it was found that NT24 populations have evolved to have increased precision of Onset of activity compared to T24 populations. Despite the inter-individual variation and noise in activity-rest assays, increased precision of Onset first appeared after ~40 generations of selection under semi-natural conditions but became apparent only after ~80 generations. The Offset of activity, which generally is less variable for Drosophila, did not show a difference between the two sets of populations. The question of how higher precision in Onset of activity could be selected is still open as I did not find it to be a case of correlated evolution with accuracy under semi-natural conditions. Interestingly enough, accuracy under semi-natural conditions showed a strong seasonal effect across all populations with the hot season bringing about significant increase in accuracy for almost all environmental phase markers. This is an exciting premise for further investigations using *D. melanogaster* and comparisons across various species.

A possible explanation for the evolution of increased precision is that parametric entrainment-changes in the clock's speed to match the external entraining cycle might be operative under semi-natural conditions, and increased stability of the free-running period might be aiding that. This can be verified by looking for: (i) after-effects of SN regimes under DD and (ii) dose-response curves with light and temperature. The molecular or neuronal bases for clock precision are unknown at this stage; however, it would be interesting to study isogenized lines

derived from NT24 and T24 populations and compare protein sequences of known kinases and phosphatases affecting the general stability of molecular oscillations for changes. Another paradigm of thought is that of clock properties, such as precision being network-level properties, requiring investigation into synchrony between different neuronal oscillators comprising the circadian clock.

Even though it has not been studied extensively in the past, the low temperature – constant darkness paradigm is an unambiguous and promising method of investigating the state of the circadian clock. In line with the hypothesis of NT24 populations having evolved increased stability of intrinsic period, under low temperature – constant darkness, NT24 populations exhibit higher persistence of rhythmicity than T24 populations. While differential cold tolerance could also contribute to this, the circadian clock of NT24 populations appears to be differentially responsive to light and temperature cues in that state. I also found that while the light and temperature regimes used to probe into the state of the oscillator were effective at synchronization, a substantial proportion of individuals did not entrain to the zeitgebers, as evidenced by the analysis of phase control. It would be interesting to check using further experiments and analysis whether there is a discernible pattern among individuals that have persistent rhythms and those which entrain to the time cues.

An essential technical diversion at this point would be to discuss the use of phase markers for analysis and inference. Most *Drosophila* circadian biologists who study activity-rest rhythms use the phase of Offset as a standard phase marker and often do not compare other phase markers. The reason cited is usually about the phase of Offset being more ‘reliable’, implying lesser daily variation. However, as observed in my data on precision differences between NT24 and T24 populations, several aspects of activity-rest patterns may be overlooked due to this, and thus exploratory studies might benefit from letting go of this view. The other error one

may make by using the phase of Offset for phase control in DD conditions (especially when using first-day values) is that there is a noticeable change in the phase of Offset due to the tonic effect of absence of light. In such a case, using the phase of Onset is relatively 'reliable'. In the same way, other phase markers, such as acrophase and centre of gravity (CoG) of the oscillation, may also prove to be more reliable depending on the context.

6.3 Future perspectives

In the case of overall differences in circadian phenotypes of NT24 and T24 populations, probing the outputs of eclosion and activity-rest has provided valuable insights into how multiple circadian outputs may evolve differentially under naturalistic conditions. The next step in future investigations would be to similarly characterize differences in the circadian outputs of feeding and oviposition. Elucidating the nature of these four rhythmic outputs for NT24 and T24 populations would provide a more holistic overview of circadian clock control and aid immensely in attributing genetic changes to specific phenotypes. For the latter, it would be helpful to carry out sequencing of pooled genomes of NT24 and T24 populations to gain information on loci at which these evolutionary differences may manifest. Additionally, pilot experiments testing the effect of standardization under laboratory conditions on activity-rest behaviour showed that non-standardized NT24 populations might show differences compared to both T24 and standardized NT24 populations under semi-natural conditions. This is an exciting prospect for future studies as an effect of rearing and/or exposure to laboratory vs natural zeitgeber cycles directing differences in circadian behaviour suggests a role for epigenetic or direct environmental effects that need further characterization.

Revisiting the phenotypes observed to be different compared to their ancestral controls, NT24 populations (i) exhibit lower dry-weight at eclosion, which equalizes at ten days post-eclosion, (ii) have differences in fecundity despite same dry-weight (10 days post-eclosion) and (iii)

exhibit increase in mid-day activity levels under temperature cycles. These suggest that NT24 populations have an overall or age-dependent altered metabolism compared to T24 populations. These differences could be present at the level of altered metabolic rates, metabolic oscillations, or even altered metabolic pathways. An altered metabolism might lead to increased feeding or assimilation in adult life, efficiency for allocating resources towards reproductive output, and also temperature-directed change in activity levels without changes in the circadian phase. Though currently unexplored, this is a promising avenue for future research with the NT24 and T24 populations, which may aid in explaining some of the observed phenotypic differences.

Multiple phenotypic differences evolved in NT24 populations compared to T24 appear to arise due to variable selection across the year. An exciting way to think about the evolution of NT24 populations under semi-natural conditions is to consider the summation of all selective pressures experienced across one year as an annual selection cycle. This enables the conceptualization of the evolutionary history of NT24 populations as a series of annual selection cycles. According to our current maintenance regime, there are ~17 generations in one such annual selection cycle; thereby, the rearing of NT24 populations under semi-natural conditions for 162 generations comprises nine complete and one ongoing annual selection cycle. The phenotypic differences in NT24 populations from their ancestral controls can thus arise due to selection pressure directly on the trait for a part of the year or due to phenotypic plasticity. If there is selection pressure directly acting on the trait in question, one would expect the mean trait value to change after a sufficient number of selection cycles. However, in the case of selection on phenotypic plasticity, seasonal variation in mean trait value is expected to be present even after a large number of selection cycles. It would be interesting to test whether phenotypic changes in NT24 populations have come about due to changes in the mean trait value of various traits or phenotypic plasticity for specific traits.

In conclusion, my results are based on experimentation conducted under semi-natural conditions in a typical tropical savannah climate, devoid of extreme weather conditions. Although these results are expected to hold true for insects under tropical natural conditions, reproducibility in similar studies with different organisms in which these hypotheses are applicable while further examining the mechanisms underlying these results will ultimately determine how generalizable the conclusions of my thesis are.

Appendix 1

Table 2.1 Univariate Tests of Significance for % Survival (LD, SN) - Over-parameterized model Type III decomposition

	Effect	SS	Degr. of	MS	Den.Syn.	Den.Syn.	F	p
Intercept	Fixed	96980.34	1	96980.34	3	547.2477	177.2147	0.000916
SEL	Fixed	27.56	1	27.56	3	48.1736	0.5721	0.504384
BLOCK	Random	1641.74	3	547.25	4.632449	64.8403	8.4399	0.024774
REGIME	Fixed	10.56	1	10.56	3	19.8032	0.5334	0.518057
SEL*BLOCK	Random	144.52	3	48.17	3	3.1366	15.3587	0.025182
SEL*REGIME	Fixed	3.67	1	3.67	3	3.1366	1.1712	0.358379
BLOCK*REGIME	Random	59.41	3	19.8	3	3.1366	6.3137	0.08222
SEL*BLOCK*REGIME	Random	9.41	3	3.14	0	0		
Error			0					

Table 2.2 Univariate Tests of Significance for Pupariation time (LD, SN) - Over-parameterized model Type III decomposition

	Effect	SS	Degr. of	MS	Den.Syn.	Den.Syn.	F	p
Intercept	Fixed	209214.8	1	209214.8	3	99.25632	2107.823	0.000023
SEL	Fixed	3.2	1	3.2	3	2.69393	1.188	0.35547
BLOCK	Random	297.8	3	99.3	3.4631	7.56451	13.121	0.022292
Regime	**Fixed**	**768.8**	**1**	**768.8**	**3**	**6.33729**	**121.318**	**0.001603**
SEL*BLOCK	Random	8.1	3	2.7	3	1.46672	1.837	0.314984
SEL*Regime	Fixed	0.2	1	0.2	3	1.46672	0.118	0.753515
BLOCK*Regime	Random	19	3	6.3	3	1.46672	4.321	0.13024
SEL*BLOCK*Regime	Random	4.4	3	1.5	0	0		
Error			0					

Table 2.3 Univariate Tests of Significance for Emergence time (LD, SN) - Over-parameterized model Type III decomposition

	Effect	SS	Degr. of	MS	Den.Syn.	Den.Syn.	F	p
Intercept	Fixed	681881.4	1	681881.4	3	75.52596	9028.438	0.000003
SEL	Fixed	0	1	0	3	8.29642	0.001	0.971652
BLOCK	Random	226.6	3	75.5	3.01908	10.79614	6.996	0.071507
REGIME	**Fixed**	**1324.2**	**1**	**1324.2**	**3**	**5.93309**	**223.181**	**0.000651**
SEL*BLOCK	Random	24.9	3	8.3	3	3.43336	2.416	0.243833
SEL*REGIME	Fixed	8.8	1	8.8	3	3.43336	2.554	0.208319
BLOCK*REGIME	Random	17.8	3	5.9	3	3.43336	1.728	0.332138
SEL*BLOCK*REGIME	Random	10.3	3	3.4	0	0		
Error			0					

Table 2.4 Univariate Tests of Significance for Dry-weight (LD, SN) - Over-parameterized model Type III decomposition

	Effect (F/R)	SS	Degr. of Freedom	MS	Den.Syn. Error df	Den.Syn. Error MS	F	*p*
Intercept	Fixed	3124667	1	3124667	3.000000	978.8474	3192.190	0.000012
SEL	**Fixed**	**2587**	**1**	**2587**	**3.000000**	**151.5756**	**17.069**	**0.025727**
BLOCK	Random	2937	3	979	0.000000			
REGIME	**Fixed**	**1698**	**1**	**1698**	**3.000000**	**2.5156**	**674.802**	**0.000125**
SEX	**Fixed**	**48589**	**1**	**48589**	**3.000000**	**194.8267**	**249.395**	**0.000552**
SEL*BLOCK	Random	455	3	152	4.412703	298.2956	0.508	0.695800

SEL*REGIME	Fixed	1587	1	1587	3.000000	231.4333	6.856	0.079110
BLOCK*REGIME	Random	8	3	3	4.845095	325.9704	0.008	0.998938
SEL*SEX	Fixed	480	1	480	3.000000	81.8926	5.867	0.093977
BLOCK*SEX	Random	584	3	195	4.931121	176.4296	1.104	0.430010
REGIME*SEX	Fixed	197	1	197	3.000000	109.5674	1.801	0.272110
SEL*BLOCK*REGIME	Random	694	3	231	3.000000	15.0304	15.398	0.025094
SEL*BLOCK*SEX	Random	246	3	82	3.000000	15.0304	5.448	0.098707
SEL*REGIME*SEX	**Fixed**	**333**	**1**	**333**	**3.000000**	**15.0304**	**22.143**	**0.018162**
BLOCK*REGIME*SEX	Random	329	3	110	3.000000	15.0304	7.290	0.068495
SEL*BLOCK*REGIME*SEX	Random	45	3	15	0.000000	0.0000		
Error			0					

Table 2.5 Univariate Tests of Significance for Pupariation time (DD) - Over-parameterized model Type III decomposition

	Effect	SS	Degr. of	MS	Den.Syn.	Den.Syn.	F	p
Intercept	Fixed	152655.8	1	152655.8	3	18.40792	8292.94	0.000003
SEL	Fixed	2.1	1	2.1	3	7.94458	0.264	0.64254
BLOCK	Random	55.2	3	18.4	3	7.94458	2.317	0.254037
SEL*BLOCK	Random	23.8	3	7.9	0	0		
Error			0					

Table 2.6 Univariate Tests of Significance for Emergence time (DD) - Over-parameterized model Type III decomposition

	Effect	SS	Degr. of	MS	Den.Syn.	Den.Syn.	F	p
Intercept	Fixed	506168.9	1	506168.9	3.000000	65.90458	7680.329	0.000003
SEL	Fixed	28.5	1	28.5	3.000000	7.96125	3.580	0.154835
BLOCK	Random	197.7	3	65.9	3.000000	7.96125	8.278	0.058088
SEL*BLOCK	Random	23.9	3	8.0	0.000000	0.00000		
Error			0					

Table 2.7 Univariate Tests of Significance for % Survival (DD) - Over-parameterized model Type III decomposition

	Effect	SS	Degr. of	MS	Den.Syn.	Den.Syn.	F	p
Intercept	Fixed	43610.89	1	43610.89	3	2.481481	17574.54	0.000001
SEL	Fixed	8	1	8	3	6.037037	1.33	0.333094
BLOCK	Random	7.44	3	2.48	3	6.037037	0.41	0.757789
SEL*BLOCK	Random	18.11	3	6.04	0	0		
Error			0					

Table 2.8 Univariate Tests of Significance for Dry-weight (DD) - Over-parameterized model Type III decomposition

	Effect	SS	Degr. of	MS	Den.Syn.	Den.Syn.	F	p
Intercept	Fixed	1597612	1	1597612	3	7.73741	206478.9	0
SEL	**Fixed**	**2813**	**1**	**2813**	**3**	**63.86778**	**44**	**0.006972**
BLOCK	Random	23	3	8	1.456101	55.69	0.1	0.92677
SEX	**Fixed**	**23808**	**1**	**23808**	**3**	**29.65444**	**802.9**	**0.000097**

SEL*BLOCK	Random	192	3	64	3	37.83222	1.7	0.338821
SEL*SEX	Fixed	140	1	140	3	37.83222	3.7	0.150066
BLOCK*SEX	Random	89	3	30	3	37.83222	0.8	0.576954
SEL*BLOCK*SEX	Random	113	3	38	0	0		
Error			0					

Table 2.9 Univariate Tests of Significance for Egg output (LD (12:12), 25°C, ~70% RH) - Over-parameterized model Type III decomposition

	Effect	SS	Degr. of	MS	Den.Syn.	Den.Syn.	F	p
Intercept	Fixed	16323.99	1	16323.99	3	55.39945	294.6597	0.000431
SEL	Fixed	30.13	1	30.13	3	42.26383	0.7129	0.460504
BLOCK	Random	166.2	3	55.4	3	42.26383	1.3108	0.414634
SEL*BLOCK	Random	126.79	3	42.26	0	0		
Error			0					

Table 2.10 Univariate Tests of Significance for % Viability (LD (12:12), 25°C, ~70% RH) - Over-parameterized model Type III decomposition

	Effect	SS	Degr. of	MS	Den.Syn.	Den.Syn.	F	p
Intercept	Fixed	46737.93	1	46737.93	3	19.5559	2389.965	0.000019
SEL	Fixed	0.15	1	0.15	3	283.6621	0.001	0.982988
BLOCK	Random	58.67	3	19.56	3	283.6621	0.069	0.972739
SEL*BLOCK	Random	850.99	3	283.66	0	0		
Error			0					

Table 2.11 Univariate Tests of Significance for Offspring / Female (LD (12:12), 25°C, ~70% RH) - Over-parameterized model Type III decomposition

LD12:12	Effect	SS	Degr. of	MS	Den.Syn.	Den.Syn.	F	p
Intercept	Fixed	7001.882	1	7001.882	3.000000	53.95195	129.7800	0.001451
SEL	Fixed	17.331	1	17.331	3.000000	61.44216	0.2821	0.632152
BLOCK	Random	161.856	3	53.952	3.000000	61.44216	0.8781	0.541294
SEL*BLOCK	Random	184.326	3	61.442	0.000000	0.00000		
Error			0					

Table 2.12 Univariate Tests of Significance for Egg output (March 2019) - Over-parameterized model Type III decomposition

	Effect (F/R)	SS	Degr. of Freedom	MS	Den.Syn. Error df	Den.Syn. Error MS	F	p
Intercept	Fixed	23712.98	1	23712.98	3.000000	49.53510	478.7105	0.000209
SEL	**Fixed**	**562.80**	**1**	**562.80**	**3.000000**	**13.28396**	**42.3670**	**0.007366**
BLOCK	Random	148.61	3	49.54	3.000000	13.28396	3.7289	0.154178
SEL*BLOCK	Random	39.85	3	13.28	0.000000	0.00000		
Error			0					

Table 2.13 Univariate Tests of Significance for % Viability (March 2019) - Over-parameterized model Type III decomposition

	Effect (F/R)	SS	Degr. of Freedom	MS	Den.Syn. Error df	Den.Syn. Error MS	F	p
Intercept	Fixed	3867.045	1	3867.045	3.000000	74.06184	52.21373	0.005466
SEL	Fixed	190.171	1	190.171	3.000000	39.89933	4.76627	0.117000
BLOCK	Random	222.186	3	74.062	3.000000	39.89933	1.85622	0.312057

SEL*BLOCK	Random	119.698	3	39.899	0.000000	0.00000		
Error			0					

Table 2.14 Univariate Tests of Significance for Offspring / Female (March 2019) - Over-parameterized model Type III decomposition

Mar-Apr	Effect	SS	Degr. of	MS	Den.Syn.	Den.Syn.	F	p
Intercept	Fixed	11251.88	1	11251.88	3	35.25341	319.1712	0.000382
SEL	**Fixed**	**304.74**	**1**	**304.74**	**3**	**2.09549**	**145.4245**	**0.001227**
BLOCK	Random	105.76	3	35.25	3	2.09549	16.8234	0.022178
SEL*BLOCK	Random	6.29	3	2.10	0	0.00000		
Error			0					

Table 2.15 Univariate Tests of Significance for Egg output (July 2019) - Over-parameterized model Type III decomposition

	Effect (F/R)	SS	Degr. of Freedom	MS	Den.Syn. Error df	Den.Syn. Error MS	F	p
Intercept	Fixed	19458.71	1	19458.71	3.000000	93.53552	208.0355	0.000722
SEL	Fixed	34.86	1	34.86	3.000000	6.79354	5.1315	0.108395
BLOCK	Random	280.61	3	93.54	3.000000	6.79354	13.7683	0.029297
SEL*BLOCK	Random	20.38	3	6.79	0.000000	0.00000		
Error			0					

Table 2.16 Univariate Tests of Significance for % Viability (July 2019) - Over-parameterized model Type III decomposition

	Effect (F/R)	SS	Degr. of Freedom	MS	Den.Syn. Error df	Den.Syn. Error MS	F	p
Intercept	Fixed	54584.36	1	54584.36	3	116.6791	467.8162	0.000216
SEL	Fixed	68.83	1	68.83	3	27.3063	2.5205	0.210573
BLOCK	Random	350.04	3	116.68	3	27.3063	4.2730	0.131937
SEL*BLOCK	Random	81.92	3	27.31	0	0.0000		
Error			0					

Table 2.17 Univariate Tests of Significance for Offspring / Female (July 2019) - Over-parameterized model Type III decomposition

July SN	Effect	SS	Degr. of	MS	Den.Syn.	Den.Syn.	F	p
Intercept	Fixed	10732.96	1	10732.96	3	125.7032	85.38332	0.002682
SEL	Fixed	8.66	1	8.66	3	2.1184	4.08948	0.136360
BLOCK	Random	377.11	3	125.70	3	2.1184	59.33843	0.003604
SEL*BLOCK	Random	6.36	3	2.12	0	0.0000		
Error			0					

Table 2.18 Univariate Tests of Significance for Egg output (December 2019) - Over-parameterized model Type III decomposition

	Effect - (F/R)	SS	Degr. of - Freedom	MS	Den.Syn. - Error df	Den.Syn. - Error MS	F	p
Intercept	Fixed	29173.2	1	29173.2	3	103.0646	283.0575	0.000457
SEL	Fixed	29.84	1	29.84	3	3.742	7.9738	0.06653
BLOCK	Random	309.19	3	103.06	3	3.742	27.5428	0.011015
SEL*BLOCK	Random	11.23	3	3.74	0	0		
Error			0					

Table 2.19 Univariate Tests of Significance for % Viability (December 2019) - Over-parameterized model Type III decomposition

	Effect - (F/R)	SS	Degr. of - Freedom	MS	Den.Syn. - Error df	Den.Syn. - Error MS	F	p
Intercept	Fixed	49048.61	1	49048.61	3	2.31353	21200.76	0.000001
SEL	**Fixed**	**214.86**	**1**	**214.86**	**3**	**12.46361**	**17.24**	**0.025393**
BLOCK	Random	6.94	3	2.31	3	12.46361	0.19	0.899921
SEL*BLOCK	Random	37.39	3	12.46	0	0		
Error			0					

Table 2.20 Univariate Tests of Significance for Offspring / Female (December 2019) - Over-parameterized model Type III decomposition

	Effect	SS	Degr. of	MS	Den.Syn.	Den.Syn.	F	p
Intercept	Fixed	18501.67	1	18501.67	3	80.22049	230.6351	0.000620
SEL	**Fixed**	**157.75**	**1**	**157.75**	**3**	**10.24966**	**15.3911**	**0.029464**
BLOCK	Random	240.66	3	80.22	3	10.24966	7.8266	0.062490
SEL*BLOCK	Random	30.75	3	10.25	0	0.00000		
Error			0					

Table 2.21 Univariate Tests of Significance for Diff. dry-weight - Over-parameterized model Type III decomposition

	Effect	SS	Degr. of	MS	Den.Syn.	Den.Syn.	F	p
Intercept	Fixed	167155.3	1	167155.3	3	542.1355	308.3275	0.000403
SEL	Fixed	1.6	1	1.6	3	589.1199	0.0026	0.962279
BLOCK	Random	1626.4	3	542.1	3.981618	738.2483	0.7344	0.583814
REGIME	**Fixed**	**13404.4**	**3**	**4468.1**	**9**	**374.2544**	**11.9387**	**0.001723**
SEL*BLOCK	Random	1767.4	3	589.1	9	225.1261	2.6168	0.115163
SEL*REGIME	Fixed	439	3	146.3	9	225.1261	0.6499	0.602602
BLOCK*REGIME	Random	3368.3	9	374.3	9	225.1261	1.6624	0.230334
SEL*BLOCK*REGIME	Random	2026.1	9	225.1	0	0		
Error			0					

Table 2.22 Univariate Tests of Significance for % Survival (Heat tolerance) - Over-parameterized model Type III decomposition

	Effect	SS	Degr. of	MS	Den.Syn.	Den.Syn.	F	p
Intercept	Fixed	233392.6	1	233392.6	3	21.02918	11098.51	0.000002
SEL	Fixed	762.1	1	762.1	3	88.91742	8.57	0.061123
BLOCK	Random	63.1	3	21	2.407402	92.58982	0.23	0.87215
SEX	**Fixed**	**395.3**	**1**	**395.3**	**3**	**9.30183**	**42.5**	**0.007332**
REGIME of exposure	**Fixed**	**866.9**	**1**	**866.9**	**3**	**10.14463**	**85.45**	**0.002678**
SEL*BLOCK	Random	266.8	3	88.9	0			
SEL*SEX	Fixed	4.6	1	4.6	3	9.01007	0.51	0.525971
BLOCK*SEX	Random	27.9	3	9.3	0.045649	6.16152	1.51	0.912612
SEL*REGIME of exposure	**Fixed**	**329.8**	**1**	**329.8**	**3**	**9.61253**	**34.31**	**0.009923**
BLOCK*REGIME of exposure	Random	30.4	3	10.1	0.054766	6.76399	1.5	0.900623
SEX*REGIME of exposure	Fixed	50.4	1	50.4	3	33.28711	1.51	0.306336
SEL*BLOCK*SEX	Random	27	3	9	3	36.13565	0.25	0.858052
SEL*BLOCK*REGIME of exposure	Random	28.8	3	9.6	3	36.13565	0.27	0.847219
SEL*SEX*REGIME of exposure	Fixed	3.9	1	3.9	3	36.13565	0.11	0.76526
BLOCK*SEX*REGIME of exposure	Random	99.9	3	33.3	3	36.13565	0.92	0.526114
SEL*BLOCK*SEX*REGIME of exposure	Random	108.4	3	36.1	0	0		
Error			0					

Table 2.23 Univariate Tests of Significance for Time to death (Desiccation tolerance) - Over-parameterized model Type III decomposition

	Effect	SS	Degr. of	MS	Den.Syn.	Den.Syn.	F	p
Intercept	Fixed	2518.158	1	2518.158	3	1.386772	1815.841	0.000028
SEL	Fixed	0.509	1	0.509	3	3.90926	0.13	0.742028
BLOCK	Random	4.16	3	1.387	0.018985	0.431031	3.217	0.948016
SEX	**Fixed**	**347.589**	**1**	**347.589**	**3**	**0.264302**	**1315.124**	**0.000046**
SEL*BLOCK	Random	11.728	3	3.909	3	3.742531	1.045	0.486129
SEL*SEX	Fixed	1.235	1	1.235	3	3.742531	0.33	0.605928
BLOCK*SEX	Random	0.793	3	0.264	3	3.742531	0.071	0.971816
SEL*BLOCK*SEX	Random	11.228	3	3.743	0	0		
Error			0					

Table 2.24 Sex ratios of NT24 and T24 populations in LD and SN (July 2021) regimes

Population	Regime	Replicate (Block)	M/F ratio
T24	**LD**	1	0.9592
		2	0.9436
		3	1.0472
		4	1.1114
	SN	1	1.1468
		2	0.9768
		3	1.082
		4	1.0394

NT24	**LD**	1	1.0026
		2	0.999
		3	0.9756
		4	1.0246
	SN	1	1.1632
		2	1.1442
		3	1.015
		4	0.993

Table 2.24 Univariate Tests of Significance for M/F Ratio - Over-parameterized model Type III decomposition

	Effect	SS	Degr. of	MS	Den.Syn.	Den.Syn.	F	p
Intercept	Fixed	17.2715	1	17.2715	3	0.001951	8852.809	0.000003
SEL	Fixed	0.00001	1	0.00001	3	0.007512	0.001	0.977104
BLOCK	Random	0.00585	3	0.00195	5.295918	0.015579	0.125	0.941357
REGIME	Fixed	0.01545	1	0.01545	3	0.008959	1.725	0.280514
SEL*BLOCK	Random	0.02253	3	0.00751	3	0.000892	8.423	0.056783
SEL*REGIME	Fixed	0.00106	1	0.00106	3	0.000892	1.184	0.356071
BLOCK*REGIME	Random	0.02688	3	0.00896	3	0.000892	10.046	0.044965
SEL*BLOCK*REGIME	Random	0.00268	3	0.00089	0	0		
Error			0					

Appendix 2

Table 3.1 Loading values for environmental variables (vertical) in the Principal Component Analysis (various principal components (horizontal), represented as PC1 for component 1, PC2 for component 2 etc.)

	PC1	PC2	PC3	PC4	PC5	PC6	PC7
L_{AVG}	-0.25335	0.16699	0.259808	0.097753	-0.00067	-0.00179	0.0000559
L_{MAX}	-7.13336	0.171101	-0.03733	-0.04071	0.0000732	0.0005	-0.0000116
T_{AVG}	1.412908	0.117591	-0.02804	-0.05857	-0.00029	-0.00652	0.000865
T_{AMP}	1.826569	0.244255	0.010026	-0.05341	0.004201	0.009802	0.00082
T_{MAX}	1.311052	0.11417	-0.04151	-0.03088	0.002916	-0.00298	0.00229
T_{MIN}	1.482119	0.114616	-0.01968	-0.07859	-0.00573	-0.0048	-0.00197
H_{AVG}	-0.00699	-0.42088	-0.01762	0.027096	-0.01354	0.003492	0.00114
H_{AMP}	1.272233	0.302663	-0.11748	0.118378	-0.0015	0.002867	-0.00171
H_{MAX}	-0.31829	-0.37627	-0.07876	0.119222	0.008745	-0.00284	0.000124
H_{MIN}	0.407113	-0.43423	0.070579	-0.10028	0.005804	0.002275	-0.00161

Table 3.2 Univariate Tests of Significance for Onset (LD) - Over-parameterized model Type III decomposition

	Effect	SS	Df	MS	Den.Syn. error df	Den.Syn.	F	p
Intercept	Fixed	756.569	1	756.569	3	1.073584	704.7133	0.000117
SEL	Fixed	0.2059	1	0.2059	3	0.320292	0.6428	0.481342
BLOCK	Random	3.2208	3	1.0736	3	0.320292	3.3519	0.173522
SEL*BLOCK	Random	0.9609	3	0.3203	0	0		
Error			0					

Table 3.3 Univariate Tests of Significance for Peak (LD) - Over-parameterized model Type III decomposition

	Effect	SS	Df	MS	Den.Syn. error df	Den.Syn.	F	p
Intercept	Fixed	1367.572	1	1367.572	3	0.034713	39396.66	$<10^{-6}$
SEL	Fixed	0.06	1	0.06	3	0.326061	0.18	0.697351
BLOCK	Random	0.104	3	0.035	3	0.326061	0.11	0.950821
SEL*BLOCK	Random	0.978	3	0.326	0	0		
Error			0					

Table 3.4 Univariate Tests of Significance for Offset (LD) - Over-parameterized model Type III decomposition

	Effect	SS	Df	MS	Den.Syn. error df.	Den.Syn.	F	p
Intercept	Fixed	3772.185	1	3772.185	3	0.146809	25694.54	0.000001
SEL	Fixed	0.009	1	0.009	3	0.00832	1.08	0.374474
BLOCK	Random	0.44	3	0.147	3	0.00832	17.65	0.020744
SEL*BLOCK	Random	0.025	3	0.008	0	0		
Error			0					

Table 3.5 Univariate Tests of Significance for Period - Over-parameterized model Type III decomposition

	Effect	SS	Df	MS	Den.Syn. error df	Den.Syn.	F	p
Intercept	Fixed	4637.915	1	4637.915	3.000000	0.104362	44440.59	$<10^{-6}$
SEL	Fixed	0.048	1	0.048	3.000000	0.064856	0.75	0.451221
BLOCK	Random	0.313	3	0.104	3.000000	0.064856	1.61	0.352734
SEL*BLOCK	Random	0.195	3	0.065	0.000000	0.000000		
Error			0					

Table 3.6 Univariate Tests of Significance for Power - Over-parameterized model Type III decomposition

	Effect	SS	Df	MS	Den.Syn. error df	Den.Syn.	F	p
Intercept	Fixed	1.270749	1	1.270749	3.000000	0.004571	277.9745	0.000470
SEL	Fixed	0.003037	1	0.003037	3.000000	0.003042	0.9982	0.391373
BLOCK	Random	0.013714	3	0.004571	3.000000	0.003042	1.5026	0.373020
SEL*BLOCK	Random	0.009127	3	0.003042	0.000000	0.000000		
Error			0					

Table 3.7 Univariate Tests of Significance for Onset (SN) across months - Over-parameterized model Type III decomposition

	Effect	SS	Df	MS	Den.Syn. error df	Den.Syn.	F	p
Intercept	Fixed	2546.627	1	2546.627	3	0.762781	3338.61	0.000011
SEL	**Fixed**	**11.265**	**1**	**11.265**	**3**	**0.082664**	**136.276**	**0.00135**
BLOCK	Random	2.288	3	0.763	0.31581	0.040943	18.63	0.482092
MONTH	**Fixed**	**70.939**	**6**	**11.823**	**18**	**0.142961**	**82.701**	**$<10^{-6}$**
SEL*BLOCK	Random	0.248	3	0.083	18	0.184682	0.448	0.722015
SEL*MONTH	**Fixed**	**4.225**	**6**	**0.704**	**18**	**0.184682**	**3.813**	**0.012534**
BLOCK*MONTH	Random	2.573	18	0.143	18	0.184682	0.774	0.70367
SEL*BLOCK*MONTH	Random	3.324	18	0.185	0	0		
Error			0					

Table 3.8 Univariate Tests of Significance for Peak (SN) across months - Over-parameterized model Type III decomposition

	Effect	SS	Df	MS	Den.Syn. error df	Den.Syn.	F	p
Intercept	Fixed	5287.972	1	5287.972	3	0.05648	93624.84	$<10^{-6}$
SEL	**Fixed**	**7.786**	**1**	**7.786**	**3**	**0.009516**	**818.14**	**0.000094**
BLOCK	Random	0.169	3	0.056	0	-	-	-
MONTH	**Fixed**	**72.577**	**6**	**12.096**	**18**	**0.205138**	**58.97**	**$<10^{-6}$**
SEL*BLOCK	Random	0.029	3	0.01	18	0.310943	0.03	0.99254
SEL*MONTH	Fixed	3.707	6	0.618	18	0.310943	1.99	0.121042
BLOCK*MONTH	Random	3.692	18	0.205	18	0.310943	0.66	0.807055
SEL*BLOCK*MONTH	Random	5.597	18	0.311	0	0		
Error			0					

Table 3.9 Univariate Tests of Significance for Offset (SN) across months - Over-parameterized model Type III decomposition

	Effect	SS	Df	MS	Den.Syn. error df	Den.Syn.	F	p
Intercept	Fixed	16085.36	1	16085.36	3	0.309098	52039.66	$<10^{-6}$
SEL	**Fixed**	**1.72**	**1**	**1.72**	**3**	**0.062764**	**27.42**	**0.013557**
BLOCK	Random	0.93	3	0.31	12.59546	0.495563	0.62	0.612576
MONTH	**Fixed**	**73.64**	**6**	**12.27**	**18**	**0.558207**	**21.99**	**$<10^{-6}$**
SEL*BLOCK	Random	0.19	3	0.06	18	0.125408	0.5	0.686667
SEL*MONTH	Fixed	0.9	6	0.15	18	0.125408	1.2	0.35185
BLOCK*MONTH	Random	10.05	18	0.56	18	0.125408	4.45	0.001366
SEL*BLOCK*MONTH	Random	2.26	18	0.13	0	0		
Error			0					

Table 3.10 Univariate Tests of Significance for SD of Onset across env. phase markers - Over-parameterized model Type III decomposition

	Effect	SS	Df	MS	Den.Syn. error df	Den.Syn.	F	p
Intercept	Fixed	93.44122	1	93.44122	3	0.184392	506.7536	0.000192
SEL	Fixed	0.11538	1	0.11538	3	0.375655	0.3071	0.618075
BLOCK	Random	0.55318	3	0.18439	2.88515	0.368429	0.5005	0.708886
PHASE MARKER	**Fixed**	**2.42861**	**5**	**0.48572**	**15**	**0.003753**	**129.431**	**$<10^{-6}$**
SEL*BLOCK	Random	1.12697	3	0.37566	15	0.010978	34.2174	0.000001
SEL*PHASE MARKER	Fixed	0.10232	5	0.02046	15	0.010978	1.864	0.160769
BLOCK*PHASE MARKER	Random	0.05629	15	0.00375	15	0.010978	0.3418	0.977198
SEL*BLOCK*PHASE MARKER	Random	0.16468	15	0.01098	0	0		
Error			0					

Table 3.11 Univariate Tests of Significance for SD of Peak across env. phase markers - Over-parameterized model Type III decomposition

	Effect	SS	Df	MS	Den.Syn. error df	Den.Syn.	F	p
Intercept	Fixed	104.5884	1	104.5884	3.00000	0.284457	367.6775	0.000310
SEL	Fixed	0.4277	1	0.4277	3.00000	0.552325	0.7744	0.443656
BLOCK	Random	0.8534	3	0.2845	2.88472	0.541645	0.5252	0.696001
PHASE MARKER	**Fixed**	**2.4680**	**5**	**0.4936**	**15.00000**	**0.003119**	**158.2546**	**$<10^{-6}$**
SEL*BLOCK	Random	1.6570	3	0.5523	15.00000	0.013799	40.0264	$<10^{-6}$
SEL*PHASE MARKER	Fixed	0.0384	5	0.0077	15.00000	0.013799	0.5563	0.731687

BLOCK*PHASE MARKER	Random	0.0468	15	0.0031	15.00000	0.013799	0.2260	0.996714
SEL*BLOCK*PHASE MARKER	Random	0.2070	15	0.0138	0.00000	0.000000		
Error			0					

Table 3.12 Univariate Tests of Significance for SD of Offset across env. phase markers - Over-parameterized model Type III decomposition

	Effect	SS	Df	MS	Den.Syn. error df	Den.Syn.	F	p
Intercept	Fixed	116.4518	1	116.4518	3	0.081005	1437.595	0.00004
SEL	Fixed	0.2096	1	0.2096	3	0.180277	1.162	0.359924
BLOCK	Random	0.243	3	0.081	4.21675	0.217576	0.372	0.778105
PHASE MARKER	**Fixed**	**1.474**	**5**	**0.2948**	**15**	**0.069651**	**4.232**	**0.013383**
SEL*BLOCK	Random	0.5408	3	0.1803	15	0.032352	5.572	0.008997
SEL*PHASE MARKER	Fixed	0.1973	5	0.0395	15	0.032352	1.22	0.347377
BLOCK*PHASE MARKER	Random	1.0448	15	0.0697	15	0.032352	2.153	0.074456
SEL*BLOCK*PHASE MARKER	Random	0.4853	15	0.0324	0	0		
Error			0					

Table 3.13 Univariate Tests of Significance for Onset (Simulated SN) - Over-parameterized model Type III decomposition

	Effect	SS	Df	MS	Den.Syn. error df	Den.Syn.	F	p
Intercept	Fixed	1444.214	1	1444.214	3	0.221341	6524.83	0.000004
SEL	**Fixed**	**1.286**	**1**	**1.286**	**3**	**0.105773**	**12.162**	**0.03984**
BLOCK	Random	0.664	3	0.221	0.838948	0.077154	2.869	0.440646
REGIME	Fixed	0.232	2	0.116	6	0.085158	1.363	0.325062
SEL*BLOCK	Random	0.317	3	0.106	6	0.113777	0.93	0.481998
SEL*REGIME	Fixed	0.714	2	0.357	6	0.113777	3.137	0.116803
BLOCK*REGIME	Random	0.511	6	0.085	6	0.113777	0.748	0.63302
SEL*BLOCK*REGIME	Random	0.683	6	0.114	0	0		
Error			0					

Table 3.14 Univariate Tests of Significance for Peak (Simulated SN) - Over-parameterized model Type III decomposition

	Effect	SS	Df	MS	Den.Syn. error df	Den.Syn.	F	p
Intercept	Fixed	2731.205	1	2731.205	3	0.020542	132957.1	$<10^{-6}$
SEL	**Fixed**	**11.952**	**1**	**11.952**	**3**	**0.138825**	**86.1**	**0.002649**
BLOCK	Random	0.062	3	0.021	1.574244	0.131563	0.2	0.916232
REGIME	**Fixed**	**2.295**	**2**	**1.148**	**6**	**0.113415**	**10.1**	**0.011957**
SEL*BLOCK	Random	0.416	3	0.139	6	0.120676	1.2	0.40247
SEL*REGIME	**Fixed**	**6.737**	**2**	**3.369**	**6**	**0.120676**	**27.9**	**0.000914**
BLOCK*REGIME	Random	0.68	6	0.113	6	0.120676	0.9	0.529063
SEL*BLOCK*REGIME	Random	0.724	6	0.121	0	0		
Error			0					

Table 3.15 Univariate Tests of Significance for Offset (Simulated SN) - Over-parameterized model Type III decomposition

	Effect	SS	Df	MS	Den.Syn. error df	Den.Syn.	F	p
Intercept	Fixed	6216.36	1	6216.36	3	0.75933	8186.637	0.000003
SEL	Fixed	2.766	1	2.766	3	0.203513	13.59	0.034602
BLOCK	Random	2.278	3	0.759	2.632634	0.223636	3.395	0.190334
REGIME	**Fixed**	**19.016**	**2**	**9.508**	**6**	**0.134452**	**70.717**	**0.000067**
SEL*BLOCK	Random	0.611	3	0.204	6	0.11433	1.78	0.250771
SEL*REGIME	**Fixed**	**2.154**	**2**	**1.077**	**6**	**0.11433**	**9.422**	**0.014088**
BLOCK*REGIME	Random	0.807	6	0.134	6	0.11433	1.176	0.424501
SEL*BLOCK*REGIME	Random	0.686	6	0.114	0	0		
Error			0					

Appendix 3

Table 4.1 Univariate Tests of Significance for Activity levels in LD(12:12) 0.1 lux - Over-parameterized model Type III decomposition

	Effect	SS	Degr. of	MS	Den.Syn.	Den.Syn.	F	p
Intercept	Fixed	29902.99	1	29902.99	3	46.3592	645.0276	0.000134
SEL	Fixed	52.09	1	52.09	3	139.4	0.3737	0.584218
TIMEPOINT	**Fixed**	**8376.55**	**23**	**364.2**	**69**	**1.6118**	**225.9541**	**0**
BLOCK	Random	139.08	3	46.36	2.96891	138.677	0.3343	0.80396
SEL*TIMEPOINT	Fixed	50.71	23	2.2	69	2.3348	0.9442	0.543691
SEL*BLOCK	Random	418.2	3	139.4	69	2.3348	59.7043	0
TIMEPOINT*BLOCK	Random	111.22	69	1.61	69	2.3348	0.6903	0.936895
SEL*TIMEPOINT*BLOCK	Random	161.1	69	2.33	0	0		
Error			0					

Table 4.2 Univariate Tests of Significance for Activity levels in LD(12:12) 100 lux - Over-parameterized model Type III decomposition

	Effect	SS	Degr. of	MS	Den.Syn.	Den.Syn.	F	p
Intercept	Fixed	18128.58	1	18128.58	3	73.30507	247.3032	0.000559
SEL	Fixed	51.93	1	51.93	3	27.24117	1.9064	0.261247
TIMEPOINT	**Fixed**	**10148.75**	**23**	**441.25**	**69**	**3.02618**	**145.8109**	**0**
BLOCK	Random	219.92	3	73.31	2.51527	24.97066	2.9356	0.226361
SEL*TIMEPOINT	Fixed	85.66	23	3.72	69	5.29669	0.7031	0.826387
SEL*BLOCK	Random	81.72	3	27.24	69	5.29669	5.1431	0.002874
TIMEPOINT*BLOCK	Random	208.81	69	3.03	69	5.29669	0.5713	0.989325
SEL*TIMEPOINT*BLOCK	Random	365.47	69	5.3	0	0		
Error			0					

Table 4.3 Univariate Tests of Significance for Activity levels in LD(12:12) 1000 lux - Over-parameterized model Type III decomposition

	Effect	SS	Degr. of	MS	Den.Syn.	Den.Syn.	F	p
Intercept	Fixed	2846.606	1	2846.606	3	11.01763	258.3682	0.000524
SEL	Fixed	0.202	1	0.202	3	2.23312	0.0902	0.783478
TIMEPOINT	**Fixed**	**3961.502**	**23**	**172.239**	**69**	**2.01612**	**85.4312**	**0**
BLOCK	Random	33.053	3	11.018	4.15999	2.70265	4.0766	0.099881
SEL*TIMEPOINT	Fixed	58.552	23	2.546	69	1.54659	1.646	0.058454
SEL*BLOCK	Random	6.699	3	2.233	69	1.54659	1.4439	0.237549
TIMEPOINT*BLOCK	Random	139.112	69	2.016	69	1.54659	1.3036	0.136636
SEL*TIMEPOINT*BLOCK	Random	106.715	69	1.547	0	0		
Error			0					

Table 4.4 Univariate Tests of Significance for Activity levels under SN (Nov-2017) - Over-parameterized model Type III decomposition

	Effect	SS	Degr. of	MS	Den.Syn.	Den.Syn.	F	p
Intercept	Fixed	13757.79	1	13757.79	3	35.34297	389.2651	0.000285
SEL	Fixed	369.53	1	369.53	3	51.78299	7.1361	0.075603
TIMEPOINT	**Fixed**	**9030.7**	**23**	**392.64**	**69**	**2.83586**	**138.4549**	**0**
BLOCK	Random	106.03	3	35.34	3.12969	52.89516	0.6682	0.623975
SEL*TIMEPOINT	**Fixed**	**75.01**	**23**	**3.26**	**69**	**1.72369**	**1.892**	**0.022324**
SEL*BLOCK	Random	155.35	3	51.78	69	1.72369	30.042	0
TIMEPOINT*BLOCK	Random	195.67	69	2.84	69	1.72369	1.6452	0.020186
SEL*TIMEPOINT*BLOCK	Random	118.93	69	1.72	0	0		
Error			0					

Table 4.5 Univariate Tests of Significance for Activity levels under SN (Feb-2018) - Over-parameterized model Type III decomposition

	Effect	SS	Degr. of	MS	Den.Syn.	Den.Syn.	F	p
Intercept	Fixed	9266.512	1	9266.512	3	41.05143	225.7293	0.00064
SEL	Fixed	10.945	1	10.945	3	48.46369	0.2258	0.667076
TIMEPOINT	**Fixed**	**6862.884**	**23**	**298.386**	**69**	**2.26194**	**131.9159**	**0**
BLOCK	Random	123.154	3	41.051	2.76585	46.54374	0.882	0.545487
SEL*TIMEPOINT	Fixed	41.018	23	1.783	69	4.1819	0.4265	0.987619
SEL*BLOCK	Random	145.391	3	48.464	69	4.1819	11.5889	0.000003
TIMEPOINT*BLOCK	Random	156.074	69	2.262	69	4.1819	0.5409	0.994192
SEL*TIMEPOINT*BLOCK	Random	288.551	69	4.182	0	0		
Error			0					

Table 4.6 Univariate Tests of Significance for Activity levels under SN (Apr-May-2018) - Over-parameterized model Type III decomposition

	Effect	SS	Degr. of	MS	Den.Syn.	Den.Syn.	F	p
Intercept	Fixed	6346.334	1	6346.334	3	18.31784	346.4564	0.000338
SEL	Fixed	25.682	1	25.682	3	6.73379	3.8139	0.145859
TIMEPOINT	Fixed	6480.486	23	281.76	69	1.90437	147.9544	0
BLOCK	Random	54.954	3	18.318	3.47794	7.26946	2.5198	0.213981
SEL*TIMEPOINT	Fixed	51.974	23	2.26	69	1.36871	1.651	0.057365
SEL*BLOCK	Random	20.201	3	6.734	69	1.36871	4.9198	0.003723
TIMEPOINT*BLOCK	Random	131.402	69	1.904	69	1.36871	1.3914	0.086324
SEL*TIMEPOINT*BLOCK	Random	94.441	69	1.369	0	0		
Error			0					

Table 4.7 Univariate Tests of Significance for Activity levels under SN (Jun-2018) - Over-parameterized model Type III decomposition

	Effect	SS	Degr. of	MS	Den.Syn.	Den.Syn.	F	p
Intercept	Fixed	11829.64	1	11829.64	3	36.18576	326.9143	0.000369
SEL	Fixed	0.56	1	0.56	3	23.98021	0.0234	0.888038
TIMEPOINT	**Fixed**	**7568.97**	**23**	**329.09**	**69**	**4.05983**	**81.0589**	**0**
BLOCK	Random	108.56	3	36.19	3.51045	25.96061	1.3939	0.379325
SEL*TIMEPOINT	Fixed	51.28	23	2.23	69	2.07944	1.0723	0.396738
SEL*BLOCK	Random	71.94	3	23.98	69	2.07944	11.5321	0.000003
TIMEPOINT*BLOCK	Random	280.13	69	4.06	69	2.07944	1.9524	0.003038
SEL*TIMEPOINT*BLOCK	Random	143.48	69	2.08	0	0		
Error			0					

Table 4.8 Univariate Tests of Significance for Activity levels under SN (Aug-2018) - Over-parameterized model Type III decomposition

	Effect	SS	Degr. of	MS	Den.Syn.	Den.Syn.	F	p
Intercept	Fixed	9852.822	1	9852.822	3	38.29774	257.269	0.000527
SEL	Fixed	0.509	1	0.509	3	0.98144	0.5183	0.523605
TIMEPOINT	**Fixed**	**8069.556**	**23**	**350.85**	**69**	**2.85173**	**123.0308**	**0**
BLOCK	Random	114.893	3	38.298	3.51129	1.36097	28.14	0.006191
SEL*TIMEPOINT	Fixed	23.8	23	1.035	69	2.4722	0.4186	0.989026
SEL*BLOCK	Random	2.944	3	0.981	69	2.4722	0.397	0.75557
TIMEPOINT*BLOCK	Random	196.769	69	2.852	69	2.4722	1.1535	0.277337
SEL*TIMEPOINT*BLOCK	Random	170.582	69	2.472	0	0		
Error			0					

Table 4.9 Univariate Tests of Significance for Activity levels under SN (Oct-2018) - Over-parameterized model Type III decomposition

	Effect	SS	Degr. of	MS	Den.Syn.	Den.Syn.	F	p
Intercept	Fixed	16028.21	1	16028.21	3	184.588	86.83234	0.002617
SEL	Fixed	267.96	1	267.96	3	290.2213	0.9233	0.407495
TIMEPOINT	**Fixed**	**8745.56**	**23**	**380.24**	**69**	**5.6377**	**67.44598**	**0**
BLOCK	Random	553.76	3	184.59	3.0151	290.9551	0.63442	0.641001
SEL*TIMEPOINT	Fixed	168.8	23	7.34	69	4.904	1.49655	0.101847
SEL*BLOCK	Random	870.66	3	290.22	69	4.904	59.18106	0
TIMEPOINT*BLOCK	Random	389	69	5.64	69	4.904	1.14963	0.282037
SEL*TIMEPOINT*BLOCK	Random	338.37	69	4.9	0	0		
Error			0					

Table 4.10 Pearson's correlation coefficients with each environmental phase marker reported for M and E peaks

	L_{ON}	L_{OFF}	T_{MAX}	T_{MIN}	H_{MAX}	H_{MIN}
M-Peak	0.563803	0.112411	0.534916	0.72928	0.183061	0.785461
E-Peak	-0.43174	**0.994038**	0.33005	-0.04019	-0.76285	0.499909

Table 4.11 Univariate Tests of Significance for Activity levels under SN-DD (Nov-2017) - Over-parameterized model Type III decomposition

	Effect	SS	Degr. of	MS	Den.Syn.	Den.Syn.	F	p
Intercept	Fixed	32459.69	1	32459.69	3	99.13945	327.4145	0.000368
SEL	**Fixed**	**791.87**	**1**	**791.87**	**3**	**39.4678**	**20.0637**	**0.020746**
TIMEPOINT	**Fixed**	**12721.95**	**23**	**553.13**	**69**	**3.96893**	**139.3647**	**0**

BLOCK	Random	297.42	3	99.14	3.27495	41.24861	2.4035	0.233191
SEL*TIMEPOINT	**Fixed**	**175.84**	**23**	**7.65**	**69**	**2.18811**	**3.4941**	**0.000031**
SEL*BLOCK	Random	118.4	3	39.47	69	2.18811	18.0374	0
TIMEPOINT*BLOCK	Random	273.86	69	3.97	69	2.18811	1.8139	0.007215
SEL*TIMEPOINT*BLOCK	Random	150.98	69	2.19	0	0		
Error			0					

Table 4.12 Univariate Tests of Significance for Activity levels under SN-DD (Feb-2018) - Over-parameterized model Type III decomposition

	Effect	SS	Degr. of	MS	Den.Syn.	Den.Syn.	F	p
Intercept	Fixed	38254.53	1	38254.53	3	80.2331	476.7921	0.00021
SEL	Fixed	325.63	1	325.63	3	131.3822	2.4785	0.213483
TIMEPOINT	**Fixed**	**19143.63**	**23**	**832.33**	**69**	**3.3006**	**252.1747**	**0**
BLOCK	Random	240.7	3	80.23	2.82216	127.4387	0.6296	0.645886
SEL*TIMEPOINT	Fixed	209.61	23	9.11	69	7.2442	1.2581	0.229817
SEL*BLOCK	Random	394.15	3	131.38	69	7.2442	18.1362	0
TIMEPOINT*BLOCK	Random	227.74	69	3.3	69	7.2442	0.4556	0.999343
SEL*TIMEPOINT*BLOCK	Random	499.85	69	7.24	0	0		
Error			0					

Table 4.13 Univariate Tests of Significance for Activity levels under SN-DD (Apr-May-2018) - Over-parameterized model Type III decomposition

	Effect	SS	Degr. of	MS	Den.Syn.	Den.Syn.	F	p
Intercept	Fixed	27746.14	1	27746.14	3	78.43921	353.7279	0.000328
SEL	**Fixed**	**88.61**	**1**	**88.61**	**3**	**5.2386**	**16.9139**	**0.026038**
TIMEPOINT	**Fixed**	**11333.01**	**23**	**492.74**	**69**	**4.43987**	**110.9807**	**0**
BLOCK	Random	235.32	3	78.44	4.774	6.75472	11.6125	0.012262
SEL*TIMEPOINT	**Fixed**	**182.75**	**23**	**7.95**	**69**	**2.92375**	**2.7176**	**0.000738**
SEL*BLOCK	Random	15.72	3	5.24	69	2.92375	1.7917	0.156837
TIMEPOINT*BLOCK	Random	306.35	69	4.44	69	2.92375	1.5186	0.042481
SEL*TIMEPOINT*BLOCK	Random	201.74	69	2.92	0	0		
Error			0					

Table 4.14 Univariate Tests of Significance for Activity levels under SN-DD (Jun-2018) - Over-parameterized model Type III decomposition

	Effect	SS	Degr. of	MS	Den.Syn.	Den.Syn.	F	p
Intercept	Fixed	32256.16	1	32256.16	3	234.4236	137.5977	0.001331
SEL	Fixed	95.54	1	95.54	3	133.3235	0.7166	0.459443
TIMEPOINT	**Fixed**	**9874.03**	**23**	**429.31**	**69**	**6.9776**	**61.5266**	**0**
BLOCK	Random	703.27	3	234.42	3.13147	136.2244	1.7209	0.32807
SEL*TIMEPOINT	Fixed	103.41	23	4.5	69	4.0767	1.1029	0.365012
SEL*BLOCK	Random	399.97	3	133.32	69	4.0767	32.704	0
TIMEPOINT*BLOCK	Random	481.45	69	6.98	69	4.0767	1.7116	0.013522
SEL*TIMEPOINT*BLOCK	Random	281.29	69	4.08	0	0		
Error			0					

Table 4.15 Univariate Tests of Significance for Activity levels under SN-DD (Aug-2018) - Over-parameterized model Type III decomposition

	Effect	SS	Degr. of	MS	Den.Syn.	Den.Syn.	F	p
Intercept	Fixed	24853.34	1	24853.34	3	67.09825	370.4022	0.000306
SEL	Fixed	187.43	1	187.43	3	36.32329	5.16	0.107769
TIMEPOINT	**Fixed**	**8312.56**	**23**	**361.42**	**69**	**3.45804**	**104.5147**	**0**
BLOCK	Random	201.29	3	67.1	3.27562	37.96474	1.7674	0.314951
SEL*TIMEPOINT	**Fixed**	**217.05**	**23**	**9.44**	**69**	**1.81658**	**5.1948**	**0**
SEL*BLOCK	Random	108.97	3	36.32	69	1.81658	19.9954	0
TIMEPOINT*BLOCK	Random	238.6	69	3.46	69	1.81658	1.9036	0.004126
SEL*TIMEPOINT*BLOCK	Random	125.34	69	1.82	0	0		
Error			0					

Table 4.16 Univariate Tests of Significance for Activity levels under SN-DD (Oct-2018) - Over-parameterized model Type III decomposition

	Effect	SS	Degr. of	MS	Den.Syn.	Den.Syn.	F	p
Intercept	Fixed	28535.89	1	28535.89	3	99.8613	285.7553	0.000451
SEL	Fixed	122	1	122	3	141.8846	0.8598	0.422178
TIMEPOINT	**Fixed**	**8568.96**	**23**	**372.56**	**69**	**2.6912**	**138.4376**	**0**
BLOCK	Random	299.58	3	99.86	2.92051	139.9965	0.7133	0.607349
SEL*TIMEPOINT	Fixed	82.56	23	3.59	69	4.5793	0.7839	0.738586
SEL*BLOCK	Random	425.65	3	141.88	69	4.5793	30.9837	0
TIMEPOINT*BLOCK	Random	185.69	69	2.69	69	4.5793	0.5877	0.985631
SEL*TIMEPOINT*BLOCK	Random	315.97	69	4.58	0	0		
Error			0					

Table 4.17 Univariate Tests of Significance for Activity levels under DD-TC (27-20°C) Step-up/Step-down cycles - Over-parameterized model Type III decomposition

	Effect	SS	Degr. of	MS	Den.Syn.	Den.Syn.	F	p
Intercept	Fixed	24711.68	1	24711.68	3	246.7628	100.1435	0.002124
SEL	Fixed	251.95	1	251.95	3	45.2643	5.5662	0.099465
TIMEPOINT	**Fixed**	**7817.88**	**23**	**339.91**	**69**	**7.0102**	**48.4873**	**0**
BLOCK	Random	740.29	3	246.76	3.67421	50.1216	4.9233	0.087589
SEL*TIMEPOINT	**Fixed**	**204.44**	**23**	**8.89**	**69**	**2.1529**	**4.1286**	**0.000003**
SEL*BLOCK	Random	135.79	3	45.26	69	2.1529	21.0246	0
TIMEPOINT*BLOCK	Random	483.71	69	7.01	69	2.1529	3.2562	0.000001
SEL*TIMEPOINT*BLOCK	Random	148.55	69	2.15	0	0		
Error			0					

Table 4.18 Univariate Tests of Significance for Activity levels under DD-TC (27-20°C) ramped cycles - Over-parameterized model Type III decomposition

	Effect (F/R)	SS	Degr. of Freedom	MS	Den.Syn. Error df	Den.Syn. Error MS	F	p
Intercept	Fixed	16582.69	1	16582.69	3.00000	85.18230	194.6729	0.000797
SEL	Fixed	71.96	1	71.96	3.00000	24.66150	2.9181	0.186127
TIMEPOINT	**Fixed**	**8087.18**	**23**	**351.62**	**69.00000**	**5.03445**	**69.8421**	**0.000000**
BLOCK	Random	255.55	3	85.18	3.78029	27.71249	3.0738	0.159960
SEL*TIMEPOINT	**Fixed**	**171.18**	**23**	**7.44**	**69.00000**	**1.98346**	**3.7523**	**0.000011**
SEL*BLOCK	Random	73.98	3	24.66	69.00000	1.98346	12.4335	0.000001
TIMEPOINT*BLOCK	Random	347.38	69	5.03	69.00000	1.98346	2.5382	0.000076
SEL*TIMEPOINT*BLOCK	Random	136.86	69	1.98	0.00000	0.00000		
Error			0					

Table 4.19 Univariate Tests of Significance for Period - Over-parameterized model Type III decomposition

	Effect	SS	Degr. of	MS	Den.Syn.	Den.Syn.	F	p
Intercept	Fixed	4757.437	1	4757.437	3	0.002473	1923473	0
SEL	Fixed	0.014	1	0.014	3	0.074487	0	0.689281
BLOCK	Random	0.007	3	0.002	3	0.074487	0	0.990314
SEL*BLOCK	Random	0.223	3	0.074	0	0		
Error			0					

Table 4.20 Univariate Tests of Significance for Power - Over-parameterized model Type III decomposition

	Effect	SS	Degr. of	MS	Den.Syn.	Den.Syn.	F	p
Intercept	Fixed	339735.3	1	339735.3	3	95.0231	3575.29	0.00001
SEL	Fixed	6.4	1	6.4	3	141.5403	0.046	0.844666
BLOCK	Random	285.1	3	95	3	141.5403	0.671	0.624372
SEL*BLOCK	Random	424.6	3	141.5	0	0		
Error			0					

Table 4.21 Univariate Tests of Significance for Precision of Offset - Over-parameterized model Type III decomposition

	Effect	SS	Degr. of	MS	Den.Syn.	Den.Syn.	F	p
Intercept	Fixed	27.58216	1	27.58216	3	0.00888	3106.211	0.000013
SEL	Fixed	0.01417	1	0.01417	3	0.028627	0.495	0.532439
BLOCK	Random	0.02664	3	0.00888	3	0.028627	0.31	0.818975
SEL*BLOCK	Random	0.08588	3	0.02863	0	0		
Error			0					

Table 4.22 Univariate Tests of Significance for Precision of Onset across generations - Over-parameterized model Type III decomposition

	Effect	SS	Degr. of	MS	Den.Syn.	Den.Syn.	F	p
Intercept	Fixed	49.00401	1	49.00401	3.00000	0.005768	8495.845	0.000003
SEL	**Fixed**	**0.96222**	**1**	**0.96222**	**3.00000**	**0.016453**	**58.483**	**0.004643**
GEN	**Fixed**	**2.08766**	**10**	**0.20877**	**30.00000**	**0.010515**	**19.855**	**0.000000**
BLOCK	Random	0.01730	3	0.00577	2.41368	0.015409	0.374	0.781825
SEL*GEN	**Fixed**	**0.74241**	**10**	**0.07424**	**30.00000**	**0.011559**	**6.423**	**0.000033**
SEL*BLOCK	Random	0.04936	3	0.01645	30.00000	0.011559	1.423	0.255333
GEN*BLOCK	Random	0.31544	30	0.01051	30.00000	0.011559	0.910	0.601426
SEL*GEN*BLOCK	Random	0.34676	30	0.01156	0.00000	0.000000		
Error			0					

Table 4.23 Univariate Tests of Significance for Q_{10} value - Over-parameterized model Type III decomposition

	Effect	SS	Degr. of	MS	Den.Syn.	Den.Syn.	F	p
Intercept	Fixed	7.637488	1	7.637488	3	0.000089	85344.04	0
SEL	Fixed	0.000036	1	0.000036	3	0.000054	0.67	0.473193
BLOCK	Random	0.000268	3	0.000089	3	0.000054	1.65	0.345171
SEL*BLOCK	Random	0.000163	3	0.000054	0	0		
Error			0					

Table 4.24 Univariate Tests of Significance for Accuracy of Onset (L_{ON}) - Over-parameterized model Type III decomposition

	Effect	SS	Degr. of	MS	Den.Syn.	Den.Syn.	F	p
Intercept	Fixed	70.10289	1	70.10289	3.00000	0.089437	783.8226	0.000100
SEL	Fixed	0.00609	1	0.00609	3.00000	0.076580	0.0795	0.796355
BLOCK	Random	0.26831	3	0.08944	4.13549	0.096047	0.9312	0.501397
MONTH	**Fixed**	**4.33497**	**5**	**0.86699**	**15.00000**	**0.054169**	**16.0054**	**0.000015**
SEL*BLOCK	Random	0.22974	3	0.07658	15.00000	0.034701	2.2068	0.129589
SEL*MONTH	Fixed	0.25316	5	0.05063	15.00000	0.034701	1.4591	0.260657
BLOCK*MONTH	Random	0.81253	15	0.05417	15.00000	0.034701	1.5610	0.199131
SEL*BLOCK*MONTH	Random	0.52052	15	0.03470	0.00000	0.000000		
Error			0					

Table 4.25 Univariate Tests of Significance for Accuracy of Onset (L_{OFF}) - Over-parameterized model Type III decomposition

	Effect	SS	Degr. of	MS	Den.Syn.	Den.Syn.	F	p
Intercept	Fixed	67.89020	1	67.89020	3.00000	0.056943	1192.246	0.000053
SEL	Fixed	0.00923	1	0.00923	3.00000	0.027879	0.331	0.605279
BLOCK	Random	0.17083	3	0.05694	3.35583	0.033736	1.688	0.325491
MONTH	**Fixed**	**5.25989**	**5**	**1.05198**	**15.00000**	**0.027258**	**38.593**	**0.000000**
SEL*BLOCK	Random	0.08364	3	0.02788	15.00000	0.021401	1.303	0.310093
SEL*MONTH	Fixed	0.12398	5	0.02480	15.00000	0.021401	1.159	0.373784
BLOCK*MONTH	Random	0.40888	15	0.02726	15.00000	0.021401	1.274	0.322698
SEL*BLOCK*MONTH	Random	0.32102	15	0.02140	0.00000	0.000000		
Error			0					

Table 4.26 Univariate Tests of Significance for Accuracy of Onset (T_{MAX}) - Over-parameterized model Type III decomposition

	Effect	SS	Degr. of	MS	Den.Syn.	Den.Syn.	F	p
Intercept	Fixed	35.00054	1	35.00054	3.00000	0.006862	5100.731	0.000006
SEL	Fixed	0.00136	1	0.00136	3.00000	0.007769	0.176	0.703409
BLOCK	Random	0.02059	3	0.00686	0.38302	0.003287	2.087	0.640790
MONTH	**Fixed**	**3.72667**	**5**	**0.74533**	**15.00000**	**0.005223**	**142.696**	**0.000000**
SEL*BLOCK	Random	0.02331	3	0.00777	15.00000	0.009705	0.801	0.512714
SEL*MONTH	Fixed	0.07029	5	0.01406	15.00000	0.009705	1.449	0.263975
BLOCK*MONTH	Random	0.07835	15	0.00522	15.00000	0.009705	0.538	0.879146
SEL*BLOCK*MONTH	Random	0.14557	15	0.00970	0.00000	0.000000		
Error			0					

Table 4.27 Univariate Tests of Significance for Accuracy of Onset (T_{MIN}) - Over-parameterized model Type III decomposition

	Effect	SS	Degr. of	MS	Den.Syn.	Den.Syn.	F	p
Intercept	Fixed	19.05102	1	19.05102	3.00000	0.000262	72800.51	0.000000
SEL	Fixed	0.00913	1	0.00913	3.00000	0.003493	2.61	0.204432
BLOCK	Random	0.00079	3	0.00026	5.63905	0.006116	0.04	0.986999
MONTH	**Fixed**	**1.19705**	**5**	**0.23941**	**15.00000**	**0.005497**	**43.55**	**0.000000**
SEL*BLOCK	Random	0.01048	3	0.00349	15.00000	0.002875	1.22	0.338433
SEL*MONTH	Fixed	0.00289	5	0.00058	15.00000	0.002875	0.20	0.957114
BLOCK*MONTH	Random	0.08246	15	0.00550	15.00000	0.002875	1.91	0.110406
SEL*BLOCK*MONTH	Random	0.04312	15	0.00287	0.00000	0.000000		
Error			0					

Table 4.28 Univariate Tests of Significance for Accuracy of Onset (H_{MAX}) - Over-parameterized model Type III decomposition

	Effect	SS	Degr. of	MS	Den.Syn.	Den.Syn.	F	p
Intercept	Fixed	15.71014	1	15.71014	3.00000	0.003652	4301.787	0.000008
SEL	Fixed	0.00100	1	0.00100	3.00000	0.004762	0.209	0.678462
BLOCK	Random	0.01096	3	0.00365	0.50633	0.002755	1.326	0.652049
MONTH	**Fixed**	**3.30482**	**5**	**0.66096**	**15.00000**	**0.006395**	**103.359**	**0.000000**
SEL*BLOCK	Random	0.01428	3	0.00476	15.00000	0.008402	0.567	0.645328
SEL*MONTH	Fixed	0.02441	5	0.00488	15.00000	0.008402	0.581	0.714158
BLOCK*MONTH	Random	0.09592	15	0.00639	15.00000	0.008402	0.761	0.698118
SEL*BLOCK*MONTH	Random	0.12602	15	0.00840	0.00000	0.000000		
Error			0					

Table 4.29 Univariate Tests of Significance for Accuracy of Onset (H_{MIN}) - Over-parameterized model Type III decomposition

	Effect	SS	Degr. of	MS	Den.Syn.	Den.Syn.	F	p
Intercept	Fixed	34.93672	1	34.93672	3.00000	0.009873	3538.566	0.000010
SEL	Fixed	0.01117	1	0.01117	3.00000	0.006826	1.636	0.290824
BLOCK	Random	0.02962	3	0.00987	3.44495	0.009105	1.084	0.462712
MONTH	**Fixed**	**3.96683**	**5**	**0.79337**	**15.00000**	**0.009058**	**87.588**	**0.000000**
SEL*BLOCK	Random	0.02048	3	0.00683	15.00000	0.006779	1.007	0.416931
SEL*MONTH	**Fixed**	**0.11455**	**5**	**0.02291**	**15.00000**	**0.006779**	**3.379**	**0.030436**
BLOCK*MONTH	Random	0.13587	15	0.00906	15.00000	0.006779	1.336	0.290809
SEL*BLOCK*MONTH	Random	0.10168	15	0.00678	0.00000	0.000000		
Error			0					

Appendix 4

Table 5.1 Univariate Tests of Significance for % rhythmic individuals under DD at 14°C - Over-parameterized model Type III decomposition

	Effect	SS	Degr. of	MS	Den.Syn.	Den.Syn.	F	p
Intercept	Fixed	19104.20	1	19104.20	3.000000	25.16372	759.1963	0.000105
SEL	**Fixed**	**659.37**	**1**	**659.37**	**3.000000**	**23.81068**	**27.6922**	**0.013371**
BLOCK	Random	75.49	3	25.16	3.000000	23.81068	1.0568	0.482414
SEL*BLOCK	Random	71.43	3	23.81	0.000000	0.00000		
Error			0					

Table 5.2 Univariate Tests of Significance for % individuals showing complex rhythms under DD at 14°C - Over-parameterized model Type III decomposition

	Effect	SS	Degr. of	MS	Den.Syn.	Den.Syn.	F	p
Intercept	Fixed	5649.539	1	5649.539	3.000000	34.24555	164.9715	0.001018
SEL	Fixed	377.775	1	377.775	3.000000	85.66213	4.4101	0.126564
BLOCK	Random	102.737	3	34.246	3.000000	85.66213	0.3998	0.764370
SEL*BLOCK	Random	256.986	3	85.662	0.000000	0.00000		
Error			0					

Table 5.3 Univariate Tests of Significance for % arrhythmic individuals under DD at 14°C - Over-parameterized model Type III decomposition

%AR	Effect	SS	Degr. of	MS	Den.Syn.	Den.Syn.	F	p
Intercept	Fixed	4797.782	1	4797.782	3.000000	2.06452	2323.923	0.000020
SEL	Fixed	36.560	1	36.560	3.000000	28.60013	1.278	0.340435
BLOCK	Random	6.194	3	2.065	3.000000	28.60013	0.072	0.970950
SEL*BLOCK	Random	85.800	3	28.600	0.000000	0.00000		
Error			0					

Table 5.4 Univariate Tests of Significance for activity profile under LD (12:12) 100 lux + TC(18-14°C) - Over-parameterized model Type III decomposition

	Effect	SS	Degr. of	MS	Den.Syn.	Den.Syn.	F	p
Intercept	Fixed	3018.749	1	3018.749	3.00000	37.29055	80.95213	0.002898
SEL	Fixed	40.236	1	40.236	3.00000	4.09475	9.82625	0.051877
TIMEPOINT	**Fixed**	**3524.006**	**23**	**153.218**	**69.00000**	**2.48141**	**61.74611**	**0.000000**
BLOCK	Random	111.872	3	37.291	5.31506	5.50173	6.77797	0.029315
SEL*TIMEPOINT	Fixed	34.114	23	1.483	69.00000	1.07443	1.38047	0.153334
SEL*BLOCK	Random	12.284	3	4.095	69.00000	1.07443	3.81108	0.013749
TP*BLOCK	Random	171.218	69	2.481	69.00000	1.07443	2.30951	0.000318
SEL*TIMEPOINT*BLOCK	Random	74.136	69	1.074	0.00000	0.00000		
Error			0					

Table 5.5 Univariate Tests of Significance for activity profile under TC(18-14°C) - Over-parameterized model Type III decomposition

	Effect	SS	Degr. of	MS	Den.Syn.	Den.Syn.	F	p
Intercept	Fixed	6192.258	1	6192.258	3.00000	12.33579	501.9751	0.000195
SEL	Fixed	17.295	1	17.295	3.00000	4.84106	3.5725	0.155139
TP	**Fixed**	**3692.665**	**23**	**160.551**	**69.00000**	**1.83818**	**87.3421**	**0.000000**
BLOCK	Random	37.007	3	12.336	4.26939	5.79736	2.1278	0.232040
SEL*TP	**Fixed**	**703.397**	**23**	**30.582**	**69.00000**	**0.88187**	**34.6789**	**0.000000**
SEL*BLOCK	Random	14.523	3	4.841	69.00000	0.88187	5.4895	0.001928
TP*BLOCK	Random	126.835	69	1.838	69.00000	0.88187	2.0844	0.001322
SEL*TP*BLOCK	Random	60.849	69	0.882	0.00000	0.00000		
Error			0					

Table 5.6 Univariate Tests of Significance for activity profile under LD (12:12) 1 lux at 14°C - Over-parameterized model Type III decomposition

	Effect	SS	Degr. of	MS	Den.Syn.	Den.Syn.	F	p
Intercept	Fixed	3159.984	1	3159.984	3.00000	7.439512	424.7569	0.000250
SEL	Fixed	41.610	1	41.610	3.00000	9.944799	4.1841	0.133338
TP	**Fixed**	**3508.172**	**23**	**152.529**	**69.00000**	**0.865867**	**176.1578**	**0.000000**
BLOCK	Random	22.319	3	7.440	2.74953	9.525635	0.7810	0.583140
SEL*TP	**Fixed**	**59.739**	**23**	**2.597**	**69.00000**	**1.285031**	**2.0212**	**0.013243**
SEL*BLOCK	Random	29.834	3	9.945	69.00000	1.285031	7.7390	0.000158
TP*BLOCK	Random	59.745	69	0.866	69.00000	1.285031	0.6738	0.948300
SEL*TP*BLOCK	Random	88.667	69	1.285	0.00000	0.000000		
Error			0					

Table 5.7 Univariate Tests of Significance for activity profile under LD (12:12) 100 lux at 14°C - Over-parameterized model Type III decomposition

	Effect	SS	Degr. of	MS	Den.Syn.	Den.Syn.	F	p
Intercept	Fixed	3563.624	1	3563.624	3.00000	35.90095	99.26266	0.002152
SEL	Fixed	3.752	1	3.752	3.00000	36.35003	0.10322	0.769078
TP	**Fixed**	**2318.369**	**23**	**100.799**	**69.00000**	**2.40494**	**41.91325**	**0.000000**
BLOCK	Random	107.703	3	35.901	3.03557	36.57123	0.98167	0.504981
SEL*TP	**Fixed**	**475.093**	**23**	**20.656**	**69.00000**	**2.18374**	**9.45908**	**0.000000**
SEL*BLOCK	Random	109.050	3	36.350	69.00000	2.18374	16.64574	0.000000
TP*BLOCK	Random	165.941	69	2.405	69.00000	2.18374	1.10129	0.344875
SEL*TP*BLOCK	Random	150.678	69	2.184	0.00000	0.00000		
Error			0					

Table 5.8 Univariate Tests of Significance for Phase Control after LD (12:12) 100 lux + TC(18-14°C) - Over-parameterized model Type III decomposition

	Effect - (F/R)	SS	Degr. of - Freedom	MS	Den.Syn. - Error df	Den.Syn. - Error MS	F	p
Intercept	Fixed	22776.99	1	22776.99	3	156.2996	145.7264	0.001223
SEL	Fixed	341.7	1	341.7	3	23.9767	14.2516	0.032552
BLOCK	Random	468.9	3	156.3	3	23.9767	6.5188	0.078975
SEL*BLOCK	Random	71.93	3	23.98	0	0		
Error			0					

Table 5.9 Univariate Tests of Significance for Phase Control after TC(18-14°C) - Over-parameterized model Type III decomposition

	Effect	SS	Degr. of	MS	Den.Syn.	Den.Syn.	F	p
Intercept	Fixed	54184.2	1	54184.2	3	123.4489	438.9199	0.000238
SEL	Fixed	4.18	1	4.18	3	51.0186	0.0818	0.793435
BLOCK	Random	370.35	3	123.45	3	51.0186	2.4197	0.243508
SEL*BLOCK	Random	153.06	3	51.02	0	0		
Error			0					

Table 5.10 Univariate Tests of Significance for Phase Control after LD (12:12) 1 lux at 14°C - Over-parameterized model Type III decomposition

	Effect - (F/R)	SS	Degr. of - Freedom	MS	Den.Syn. - Error df	Den.Syn. - Error MS	F	p
Intercept	Fixed	31016.27	1	31016.27	3	21.37839	1450.823	0.00004
SEL	Fixed	1529.58	1	1529.58	3	75.88078	20.158	0.020617
BLOCK	Random	64.14	3	21.38	3	75.88078	0.282	0.837083
SEL*BLOCK	Random	227.64	3	75.88	0	0		
Error			0					

Table 5.11 Univariate Tests of Significance for Phase Control after LD (12:12) 100 lux at 14°C - Over-parameterized model Type III decomposition

	Effect - (F/R)	SS	Degr. of - Freedom	MS	Den.Syn. - Error df	Den.Syn. - Error MS	F	p
Intercept	Fixed	38948.61	1	38948.61	3	297.6442	130.8563	0.001434
SEL	Fixed	7.17	1	7.17	3	19.7647	0.3627	0.589483
BLOCK	Random	892.93	3	297.64	3	19.7647	15.0594	0.02588
SEL*BLOCK	Random	59.29	3	19.76	0	0		
Error			0					

Table 5.12 Univariate Tests of Significance for % rhythmic individuals under LL (0.1 lux) at 25°C - Over-parameterized model Type III decomposition

	Effect	SS	Degr. of	MS	Den.Syn.	Den.Syn.	F	p
Intercept	Fixed	14348.64	1	14348.64	3	104.4946	137.3146	0.001335
SEL	Fixed	192.85	1	192.85	3	44.2404	4.3591	0.12804
BLOCK	Random	313.48	3	104.49	3	44.2404	2.362	0.249337
SEL*BLOCK	Random	132.72	3	44.24	0	0		
Error			0					

Table 5.13 Univariate Tests of Significance for % individuals showing complex rhythms under LL (0.1 lux) at 25°C - Over-parameterized model Type III decomposition

	Effect	SS	Degr. of	MS	Den.Syn.	Den.Syn.	F	p
Intercept	Fixed	12577.93	1	12577.93	3	23.48899	535.482	0.000177
SEL	Fixed	335.26	1	335.26	3	68.19766	4.9159	0.113339
BLOCK	Random	70.47	3	23.49	3	68.19766	0.3444	0.797648
SEL*BLOCK	Random	204.59	3	68.2	0	0		
Error			0					

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Table 5.14 Univariate Tests of Significance for % arrhythmic individuals under LL (0.1 lux) at 25°C - Over-parameterized model Type III decomposition

	Effect	SS	Degr. of	MS	Den.Syn.	Den.Syn.	F	p
Intercept	Fixed	2591.374	1	2591.374	3	54.67643	47.39473	0.006278
SEL	Fixed	19.563	1	19.563	3	12.19114	1.60469	0.29465
BLOCK	Random	164.029	3	54.676	3	12.19114	4.48493	0.124675
SEL*BLOCK	Random	36.573	3	12.191	0	0		
Error			0					

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