Probing the circadian clocks of fruit fly *Drosophila melanogaster* populations selected for morning and evening adult emergence

Thesis submitted towards the partial fulfilment for the degree of

Master of Science

by
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Dedicated to
my mother …

-Flower clock proposed by Linnaeus in 1751 (From Lindauer Bilderbogen no.5)

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Declaration

I declare that the work presented in my thesis entitled, "Probing the circadian clocks of fruit fly *Drosophila melanogaster* populations selected for morning and evening adult emergence" is the result of studies carried out by me at the Chronobiology Laboratory of the Evolutionary and Organismal Biology Unit of Jawaharlal Nehru Centre for Advanced Scientific Research, Bangalore, India, under the supervision of Professor Vijay Kumar Sharma and that this work has not been submitted elsewhere for any other degree in this or any other university.

In keeping with the general practice of reporting scientific observations, due acknowledgement has been made wherever the work described has been based on the findings of other researchers. Any omission, which may have occurred is likely due to oversight or an error in judgement and is highly regretted.

Place: Bangalore Abhilash L

Date: 31.03.2015

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31 March, 2015

CERTIFICATE

This is to certify that the work described in the thesis entitiled "Probing the circadian clocks of fruit fly *Drosophila melanogaster* populations selected for morning and evening adult emergence" is the result of investigations carried out by Mr. Abhilash Lakshman in the Evolutionary and Organismal Biology Unit of the Jawaharlal Nehru Centre for Advanced Scientific Research, Jakkur, Bangalore 560064, under my supervision, and that the results presented in the thesis have not previously formed the basis for the award of any diploma, degree or fellowship.

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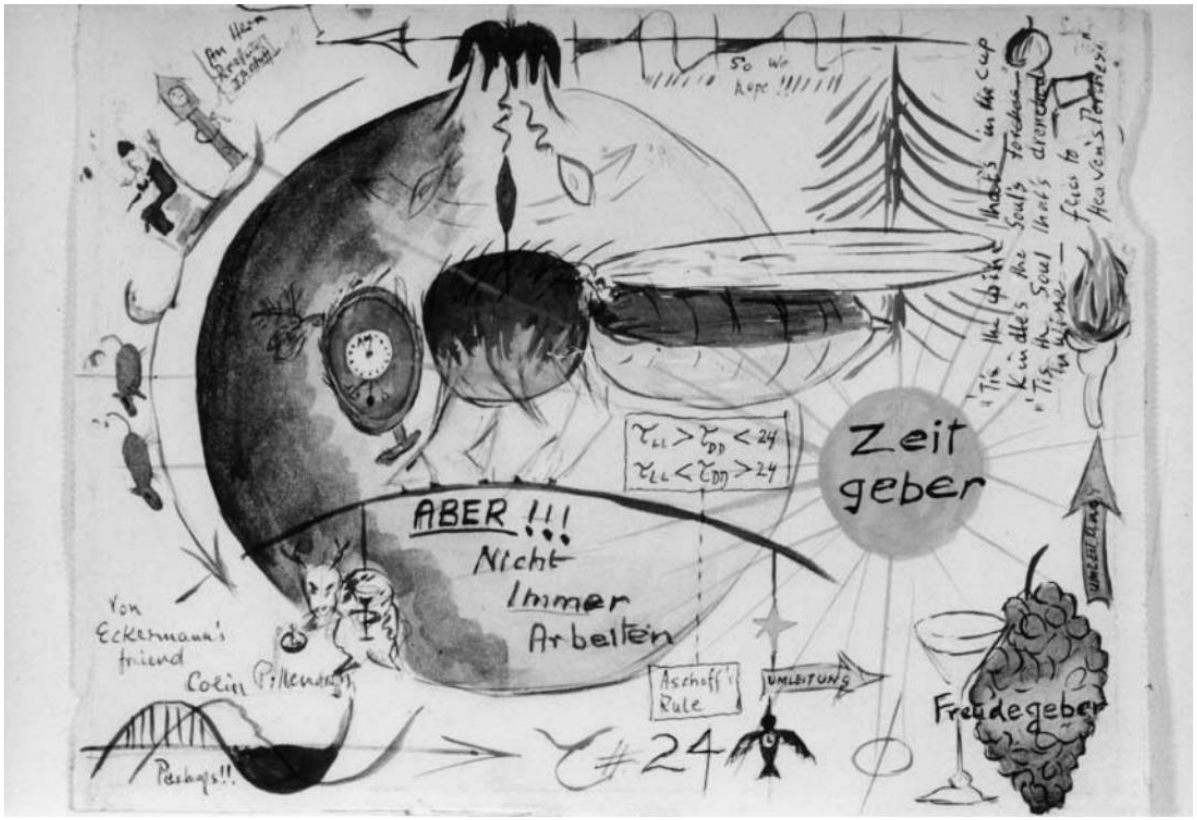

-Drawing by Pittendrigh in 1959 (From Daan, 2000)

What then is time? If no one asks of me, I know; if I wish to explain to him who asks, I know not.
-Augustine of Hippo

Symbols and abbreviations

E – Amplitude of the zeitgeber in the van der Pol oscillator model

T – Period of the zeitgeber

ε – Degree of non-linearity in a van der Pol oscillator

τ – Period of the circadian clock

φ – Phase of an entrained rhythm

ψ – Phase relationship with that of the zeitgeber

ω_0 – Frequency of the van der Pol oscillator

ω_1 – Frequency of zeitgeber entraining the van der Pol oscillator

CIRC – Circadian Integrated Response Characteristics

DD – Constant darkness

LD – Light and Dark of a light/dark cycle

LL – Constant illumination

PRC – Phase Response Curve

RH – Relative Humidity

SN – Semi-natural conditions

VRC – Velocity Response Curve

τSC – Period Setting Component

φSC – Phase of entrainment Setting Component

τ-φCCH – Period phase Coupling Component Hypothesis

Excerpt

Organisms across all taxa seem to possess networks within them that maintain time on a daily basis. These networks manifest themselves in an array of periodic physiological and behavioural programmes that we refer to as circadian rhythms. Such networks are known to maintain temporal order among various independent metabolic, physiological and behavioural programmes with their typically periodic, biotic or abiotic, environments. The network that comprises of central oscillators, the mechanisms by which these oscillators sense extrinsic periodic environments and adjust themselves and the processes by which the rhythms are affected thereon, may holistically be referred to as "circadian clocks".

In circadian biology it is widely believed that phase-relationships of rhythms with their driving oscillators are adaptive. It is therefore important to understand the mechanism by which such a phase-relationship is achieved in order to understand how circadian rhythms evolve. The phenomenon attributed, so far, for the attainment of a stable phase-relationship is called entrainment. Entrainment is defined as the process by which circadian oscillators synchronise to environmental driving oscillators.

Two significant and general mechanisms of entrainment have been proposed in the past. One is based on the idea that entrainment occurs via changes in phase progression by phase-shifts in a time dependent manner (non-parametric model) and the other is based on the idea that entrainment occurs via changes in the velocity of phase progression in a time dependent manner (parametric model). In case of the non-parametric model of entrainment, the underlying assumption is that the circadian oscillator uses the entraining stimuli only during dawn and dusk and the zeitgeber during the remaining parts of the day are superfluous. This

assumption may not always be true as has been shown experimentally in the past. This model of entrainment also falls short in attempts to explain entrainment under different day lengths. The non-parametric model also assumes that the phase-shift due to an entraining stimulus does not cause the underlying circadian period to change. Although, the parametric model is better at explaining entrainment to different day lengths and does not make an assumption of the redundancy of light during the entire day length, it assumes that the action of light affects phase progression only via velocity changes and not by phase-shifts. More recently evidence has been gathered for modes of entrainment that result in a change in phase progression via both phase-shifts and velocity changes. All these models of entrainment invoke the mechanism of entrainment occurring via changes in phase progression and a change in phase progression is only required if the periodicities of internal and external cycles do not match. Therefore, a relationship between the phases of a rhythm occurring via changes in phase progression depending on the length of the internal cycle is obvious.

To ask questions regarding the evolution of circadian clocks, selection studies were initiated in our laboratory. We imposed selection on timing of adult emergence in populations of *Drosophila melanogaster*. As a consequence of selection for morning and evening emergence, we derived two stocks viz., the *early* and *late* stocks. It was observed that these stocks evolved divergent circadian clocks underlying both adult emergence and locomotor activity rhythms as characterised by the period of these rhythms in constant darkness.

In light of the mechanisms of entrainment outlined above and data from our selection studies, we hypothesised that entrained behaviour of the locomotor activity rhythm in the *early* and

late stocks would have coevolved in response to selection on timing of adult emergence. The locomotor activity rhythm was characterised for these stocks using the overall shape of the waveform and the Centre of Mass and, to our utter surprise, no difference was seen in this rhythm among the two stocks relative to their *control* stocks. If these stocks were entraining using the mechanisms discussed above, then a correlation between period of the rhythm and its phase under entrainment are expected to be correlated. We tested for this and found that despite period differences a correlation was found only for eclosion rhythms and not for the locomotor activity rhythm. We postulate a model to explain this discrepancy and propose the existence of two components, one regulating the period of the rhythm and one its phase during entrainment and that these components are coupled and this coupling factor is a function of at least the period range in question and the kind of zeitgeber involved in entrainment. We then asked if period and phase are regulated by different components and we concluded based on our analyses that they do. The question as to how these stocks entrain was still a puzzle. A recent model of entrainment conceptualises the circadian clock as a limit cycle oscillator and uses this to explain the matching of frequencies of internal and external cycles. This model does not necessarily invoke a relationship between period and phase of the rhythm in order to entrain, thereby being a potential candidate model to explain entrainment in these stocks. We concluded from our analyses that this model also fails to explain entrainment of the locomotor activity rhythm in these stocks. Therefore, we conclude that the coupling component between the period and phase regulating components is central to our understanding of circadian entrainment.

We further wanted to analyse the genetic bases for the divergence in the emergence profiles of

our *early* and *late* stocks. Earlier studies have shown that the X-chromosome is not responsible for the underlying differences between *early* and *late* stocks and that there seems to be a major contribution of dominance and epistatic effects among alleles on the autosomes that give rise to this divergence. We used deficiency lines to screen for dominant alleles that contribute to morning and evening emergence and as of now have not found any candidate loci. Further experiments to screen other regions of the chromosome are underway.

Chapter 1

Introduction

1.1 A time keeper within?

Questions regarding certain behavioural programmes happen to be very intriguing. How do animals know when to sleep and when to forage? How do plants know when to change the orientation of their leaves towards the Sun? How do humans know the seasonal ups and downs of crop? How do flowers open and close their petals at specific times of the day? Mysteries of such nature had been floating around until when attempts were first made to study the basis for such rhythmic activity in organisms. Early philosophers asked if such rhythmic behaviours are just passive responses to cycling environmental variables as a consequence of the Earth's rotation, if they were endogenously generated and if they were innate.

The first evidence of any experimental approach adopted to answer these questions dates back to the early 18th century when the French astronomer (De Mairan, 1729; c.f Daan, 2010) showed that leaf movement rhythms persisted in the absence of any light/dark (LD) cycle suggesting that such rhythms are endogenous to organisms. Ensuing this, decades of studies by several researchers have convincingly demonstrated that such rhythms, most ubiquitously, are endogenously generated (Kleinhoonte, 1929; Bünning and Stern, 1930; c.f. Daan, 2010) and that these rhythms are not learnt but innate (Aschoff and Lohmann, 1954; c.f. Daan, 2010; Sheeba et al., 2000). Over the years it has also become clear to biologists that such physiological systems within an organism are responsible for time measurement and synchronisation of its several metabolic processes to themselves and to the daily cycles in the environment (Moore-Ede et al., 1982). These circadian (Latin: *circa*=about; *dies*=day) systems have been shown to be robust in measuring time despite fluctuations in temperature, a

phenomenon known as temperature compensation (Zimmerman et al., 1968), thereby facilitating the existence of a time keeper within us that may have adaptive value.

1.2 Do circadian clocks have any functional significance?

Darwin first recorded movements in plants and proposed that continuous or prolonged light may have deleterious effects on chlorophyll bearing leaves indicating the adaptive nature of rhythmicity in leaf movements (Darwin, 1880). Erwin Bünning later demonstrated that daily rhythms observed in plants are endogenous and have a genetic basis (Bünning, 1928; c.f. Bünning, 1936). It was he who proposed that the degree of coincidence between the zeitgeber and the endogenous oscillator might be important for the fitness of the organism (Bünning, 1932; c.f. Bünning, 1936). A couple of years after this, it was proposed that there exists qualitative differences in light sensitivity of the phases of the endogenous daily rhythm, and that this is what underlies variable effects on photoperiodic phenomena such as flowering. This work also attempted to point out the similarity in the underlying genetics of photoperiodism and daily rhythms (Bünning, 1936; c.f. Bünning, 1936). Eventually it was proposed that light during the photophase (light phase of a light/dark cycle) might enhance assimilatory processes in plants and light in the scotophase (dark phase of a light/dark cycle) might inhibit such processes. Therefore, day length might act as a trigger for photoperiodic processes (Bünning, 1950 c.f. Highkin and Hanson, 1954). The higher the degree of coincidence between the internal rhythm and the external cycle more is the precision with which organisms measure day length and therefore time functionally important events which might be what increases the fitness of the organism. Soon after this proposition, there were experiments reported which provided evidence in favour of the hypothesis put forth by

Bünning in 1950. In one particular study that was reported, the authors maintained tomato plants under different *T*-cycles. It was observed that plants maintained under conditions which deviated maximally from their intrinsic period show extremely stunted growth (Highkin and Hanson, 1954) and the same was shown by another study as well (Hillman, 1956). These results were taken as evidence in favour of the hypothesis that light in the scotophase might have deleterious effects on the organism. However, in 1959, Pittendrigh and Bruce argued that the results reported by Highkin and Hanson and Hillman can be explained by an alternative mechanism. Pittendrigh and Bruce suggest that such results should be sought in terms of the organism's clock being unable to entrain to *T*-cycles highly deviant from that of 24 h. The authors say that failure to entrain to the *T*-cycles other than that of 24 h could be either due to asynchrony among different cellular oscillations or due to severe deviation of the entraining oscillation's period from that of the natural period of the organism's rhythms which might cause significant reduction in metabolic efficiency. The latter argument illustrates the concept of circadian resonance. Since the communication of this paper it was believed that if organisms have an endogenous period resonating with that of the external time cue, then the metabolic efficiency is optimum, thereby providing a fitness advantage to organisms in terms of timing events precisely. This is what is referred to, popularly, as the circadian resonance hypothesis (Pittendrigh and Bruce, 1959).

Evidence in favour of circadian resonance

The first experimental evidence for circadian resonance came from a study on fruit flies *Drosophila melanogaster* (Pittendrigh and Minis, 1972). It was reported that in all the strains tested and across males and females, there is greater survivorship under conditions where the

internal rhythms resonate with the external environment. The authors attribute this loss in general well-being in conditions deviating from the typical 24 h *T*-cycle to internal desynchronisation, where all the cells would desynchronise and there would not be any coherence in their activity. Similar results were observed involving Blowflies (Saint Paul and Aschoff, 1978). Circadian clock mutants of *D. melanogaster* were also used to examine the circadian resonance hypothesis (Klarsfeld and Rouyer, 1998). Experiments reported in this paper show that wild type fly lines show significantly higher life span under resonating and non-resonating conditions. A particular limitation of this study was that all the fly lines were backcrossed to *Canton-S* (*CS*) flies. The *CS* flies are a highly inbred and using them for estimating fitness correlates, therefore, could yield spurious results. This is likely to be caused due to random fixation of certain alleles that may or may not have anything to do with the circadian clock. The first conclusive and very rigorous study to demonstrate the circadian resonance hypothesis came from a paper in the late 1990s. The paper reports competition experiments done under different *T*-cycles in cyanobacteria (Ouyang et al., 1998). Three strains of cyanobacteria were used to perform all the competition experiments viz., *SP22* (short period strain ~23 h), wild type strain (~25 h) and *P28* (long period strain ~30 h). It was demonstrated that lines with resonating periodicities wipe out the lines with non-resonating ones in all conditions. These results are strongly suggestive of the fact that selection favours organisms with τ closer to that of the environmental cycle, thereby providing conclusive evidence of the fact that there is some underlying fitness advantage of having a resonating circadian clock. Earlier, Pittendrigh had proposed that the fitness loss could be due to the inability to entrain to *T*-cycles deviating from that of the internal oscillator. In this paper, the authors emphasize that the PRC of cyanobacteria shows that these organisms have the ability

to entrain to ±10 h from their τ. This would mean that the strain that is losing out is not likely to be due to its inability to entrain. It was suggested with information from other experiments that the phase relationships change when clocks are entrained to different *T*-cycles and this might hamper the optimum timing of any event, thereby causing the fitness loss. This result is very interesting because it emphasised the possibility that it is the phase of a rhythm that is under selection and not the τ itself. Even though early ideas such that circadian rhythms have an adaptive advantage developed from studying movements in plants, the evidence for circadian resonance being important in determining plant fitness came much later in the 2000s. This study describes the role of circadian resonance in determining fitness of *Arabidopsis* plants (Dodd et al., 2005). The authors measured leaf chlorophyll, carbon fixation rates and biomass as correlates of fitness under conditions of monocultures and competition. The authors showed that, aerial biomass of the long period mutant was significantly lower than the short period mutant in *T*20 condition and vice versa for the *T*28 conditions. Under *T*24 the aerial biomass is significantly higher for the wild type plant as compared to the arrhythmic mutant. Similar results were obtained for carbon fixation rates and total chlorophyll in all the strains used. Under competition experiments, short period mutants were found to grow better in *T*20 and long period mutants in *T*28, as estimated from the rosette diameter and presence of chlorotic and necrotic spots on the leaves. There was no mortality observed in monoculture experiments, but high mortality was observed in competition experiments for strains that have periods significantly deviating from that of the imposed external cycle. All these evidence seem to indicate that circadian resonance is one important way by which circadian clocks enhance fitness of organisms. Another study, presumably inspired by the study on the competition experiments done on cyanobacteria, was performed in order to assay the activity

rhythms of laboratory mice in natural conditions and contrast it with the typical wheel running behaviour assayed in the lab (Daan et al., 2011). The study included competition experiments on different *mPer2* mutants to see if a dysfunctional clock renders the bearer susceptible to negative selection pressures. The *mPer2* mutants used for the experiment were, $mPer2^{++}$, $mPer2^{m+}$ and $mPer2^{mm}$. Allele frequencies of the mutant did fall initially but increased in the second year of the experiment. The authors also reported that survivorship of individuals carrying the mutation does not seem to be affected. It was in the late 1990s that researchers attempted to question the deleterious effects of light on circadian clocks. Two papers one after the other showed that light does not have deleterious effects on both fitness and the clock (Sheeba et al., 1999a; Sheeba et al., 1999b). Another paper in 2000 reported that populations of *D. melanogaster* maintained in constant illumination for over 600 generations have reduced life span, but increased fecundity than their counterparts in rhythmic conditions (Sheeba et al., 2000).

Evidence for adaptive advantage conferred by the clock other than circadian resonance

Thus far we have only discussed about the circadian clocks conferring fitness advantage to the organism via coincidence to the 24 h daily rhythm in physical parameters. This is certainly not the only possible way by which the clock confers the bearer an adaptive advantage. One can easily imagine ecological scenarios where circadian clocks are needed to anticipate changes in rhythmic environments or respond to stimuli in different ways on different days because the phase of the stimuli might be subject to unanticipated changes. Keeping all this in mind, shortly after the proposition of the concept of circadian resonance, Aschoff proposed that circadian rhythm and all other 'circa rhythms' have evolved as niches in time. He went on

to speculate that these rhythms mediate interplay between the organism and the rhythmic environment they inhabit (Aschoff, 1964). This hypothesis was only tested much later in an interesting study, which looked at the locomotor and oviposition rhythms in three *Drosophila* parasitoid wasp species (Fleury et al., 2000). The authors in this paper show that the three parasitoid wasp species have evolved differences in the phases of their locomotor and oviposition rhythms which correlated to their differential abilities to compete. The authors underline the possibility of temporal niche segregation as a means of reducing competitive disadvantage and increasing fitness.

In the 1970s there was an interesting and unconventional hypothesis as to how clocks could confer any adaptive advantage. The adaptive significance of circadian clocks was hypothesised to lie in the fact that they could adjust the daily timing of behaviour based on prior experience with a periodic environment and not on the ancestor's choice of phase of a behavioural rhythm (Enright, 1970 and 1975; c.f. Rijnsdorp et al., 1981). An interesting and extremely detailed study was carried out in order to test this hypothesis. The authors used Kestrels for this study and showed that learning the phase on prior experience is a plausible way by which circadian clocks confer fitness advantages (Rijnsdorp et al., 1981). The authors chose a particular study site and within that site observed Kestrel behaviour and isolated a zone where Kestrels do not typically feed. Then the authors started leaving mice in that zone at a particular time every day as prey for the Kestrels. It was seen that even after the authors stopped giving food, the Kestrels kept coming back to the same zone for a couple of days at the very same time at which the prey was given. The authors suggested that by synchronising the internal rhythms with that of the external cycles, the circadian clocks create a temporal

substrate on which daily experiences could act in order to fine tune timing of behavioural responses, thereby optimising the fitness of the organism. Studies performed in field also yield very interesting results and show that circadian clocks are important for fitness in ways other than just circadian resonance. In a particularly interesting study, locomotor behaviour and the chances of predation of an SCN (mammalian circadian clock) lesioned Antelope ground squirrels in their natural environment were assessed (DeCoursey et al., 1997). In the study, the authors did not find any significant difference in percentage predation for animals having an intact SCN versus animals having lesioned SCN. The authors also found a significantly higher amount of nocturnal trips by SCN lesioned animals, which is certainly a fitness disadvantage. The study was pursued further, this time in free living chipmunks with a higher sample size (DeCoursey et al., 2000). This study also showed that the central pacemaker might be essential for higher longevity and survival in the wild. The presence of the clock does nothing but tell the organism the time of the day and that is crucial information for important decisions. Apart from conferring fitness advantage to organisms for photoperiodic responses by precise measurement of day length, circadian clocks might evolve as a consequence of adaptive thermal responses and this was addressed in the pitcher plant mosquito (Bradshaw et al., 2004). The authors perform reciprocal translocation experiments to ask two questions; have mosquito populations dispersed due to evolved adaptive thermal responses? Does this dispersal involve adaptive photoperiodic responses? The authors show that species from extreme latitudes end up showing highest fitness in mid latitude conditions. This prompted the authors to conclude that these mosquito populations have evolved thermal tolerance rather than thermal specialisation. Again when assayed for correlates of fitness under benign thermal conditions, the authors find apparent reduction in fitness in the northern

and southern latitude populations. The authors attribute this to malfunctioning of the clocks under conditions of only photoperiodic changes, thereby emphasising on the complexity of clock function and its correlation to fitness components.

Molecular techniques have proven their worth in aiding the understanding of the adaptive advantage of circadian clocks as well. An elegant study in the late 1990s showed a relationship between the natural variation in a clock gene in *Drosophila* and temperature compensation (Sawyer et al., 1997). It is known that in *D. melanogaster* and *D. simulans* the *Thr-Gly* repeat in the *per* gene is polymorphic in length. The alleles that code for 14, 17, 20 and 23 dipeptide pairs make up about 99% of the variation seen in Europe. It has been observed that the *Thr-Gly 17* and *20* form a highly significant latitudinal cline. It was also seen that *Thr-Gly 17* and *20* show highly effective temperature compensation compared to the other variants. The authors therefore attribute an adaptive role to this locus, which hosts a major clock gene *period*. Another study showed that though *D. littoralis* is widely spread in Europe, there are no coding differences in the *Thr-Gly* region as one would have expected, thereby excluding the possibility that this region maintains adaptive variability in circadian clocks of this species (Lankinen and Forsman, 2006). These studies underline the difficulty in generalising any conclusions made about the adaptive significance of a trait. The obvious problems include species specific differences, species × environment interactions and the elaborate genetic basis for a complex trait under selection. *Tim*, a core clock gene was discovered to exist in two allelic forms in the laboratory. In a recent study, the authors sought to see if this polymorphism was also maintained in nature (Tauber et al., 2007). The authors also wanted to see if any of these allelic forms affect any major life history trait. Using

Tajima's D statistic the authors conclude that one of the allelic forms of *tim* is under directional selection. It is this form that also shows diapause more readily than the other form irrespective of different populations analysed, thereby suggesting that this form might have an adaptive value of considerable importance because of which it is spreading widely through Europe.

1.3 How do circadian clocks be of functional significance?

In order to be of any functional significance, circadian clocks must possess the ability to synchronise to other oscillations (Pittendrigh, 1981; Daan and Aschoff, 2001). This synchrony has been termed entrainment, a word borrowed from the oscillator theory jargon that refers to the matching of frequencies of the internal and external cycles. Initial ideas of entrainment primarily had reference to synchronisation of circadian clocks to environmental variables such as light, temperature and humidity among others that are a consequence of the rotation of the Earth, but one can, now, imagine the possibility of mutual entrainment among multiple oscillators within an organism as well (Pittendrigh and Bruce, 1959). Entrainment entails the adjustment of both period (τ) and phase (φ) of the rhythm in order to synchronise to an external cue zeitgeber (German for time-giver). It is believed that entrainment facilitates organisms to optimally time their behaviour and physiology by establishing a stable phase-relationship (ψ) with the zeitgeber and thus is considered to be adaptive (Cloudsley-Thompson, 1960; Fluery et al., 2000; O'Donell et al., 2011). Thus, entrainment becomes an indispensable phenomenon in order to understand the functional relevance of circadian clocks.

1.4 How do circadian clocks entrain to zeitgebers?

Mechanism of entrainment as we know today rests primarily on two pillars of thought. One

which proposes that entrainment occurs by discrete shifts in phase to correct for differences in period of the circadian clock (τ) and that of the zeitgeber cycle period (T) (Pittendrigh, 1981) and on the other hand we have entrainment occurring as a continuous change in τ in response to the zeitgeber in order to match the period of the zeitgeber (Aschoff, 1964; c.f. Daan, 2000). Though very elegant and holistic, these models of entrainment seem to be incomplete as will be discussed in detail later. In addition to these models, other specific mathematical models have been proposed, but they seem to be serving a more case specific understanding of entrainment rather than a more heuristic one. Recently, a more promising model which incorporates, in some sense, a fusion of the thoughts underlying both, the discrete and continuous models of entrainment has been proposed (Roenneberg et al., 2010). This model makes use of a Circadian Integrated Response Characteristic (CIRC) which shall be explained in one of the ensuing paragraphs.

Discrete model

The discrete model of entrainment is also known as the non-parametric model of entrainment and was proposed by Pittendrigh (Pittendrigh, 1981). This model invokes the concept of the zeitgeber eliciting a discrete response in the oscillator in the form of phase-shifts. This process assumes no change in the τ of the oscillator and hence is referred to as the non-parametric model. The magnitude of the phase-shift elicited by a zeitgeber is believed to be a function of the time at which the zeitgeber is provided, τ of the oscillator and strength of the zeitgeber. The relationship between magnitude of phase-shift and τ is defined by the following equation, $\Delta \varphi = T - \tau$. A plot of phase-shifts in response to zeitgeber stimulus at different times of the day is known as a phase response curve (PRC) and this was discovered to exist in

organisms as well, early on during the development of the field (Burchard, 1958; Pittendrigh, 1958; Hastings and Sweeney, 1958; Decoursey, 1960a and 1960b). A typical PRC shows that exposure to the stimulus of the zeitgeber at early subjective night causes phase delays and at late subjective night causes phase advances of overt rhythms. Since most of the phase shifts are observed during the night when the organisms does not experience any light while minimal phase shifts are observed during the day when the organisms actually experience light, it was proposed that circadian clocks use the entraining stimuli only during dawn and dusk and presence of the zeitgeber during the remaining parts of the day is redundant. This assumption may not always be true as has been shown experimentally (Hut et al., 1999; Vaze et al., 2012a). This model also fails to explain entrainment under different environmental conditions, which seems to be a considerable limitation for explaining entrainability (reviewed in Roenneberg et al., 2010).

Continuous model

The continuous model of entrainment is also known as the parametric model and was proposed by Aschoff (Aschoff, 1964; c.f. Daan, 2000). This model invokes the concept of the zeitgeber eliciting a continuous response in the oscillator in the form of gradual changes in its τ. This process requires a modification in the parameter of the clock and hence is referred to as the parametric approach. The parametric model is based on the observation that τ changes in response to a zeitgeber, is a function of the time of the day. A plot of the change in velocity ($1/\tau$) in response to a zeitgeber as a function of time of day is called the velocity response curve (VRC) (Daan and Pittendrigh, 1976). A typical VRC shows deceleration during early subjective night and acceleration during late subjective night of the individual's τ. The

parametric model of entrainment is better than the non-parametric model in explaining entrainment to different photoperiodic conditions and does not assume that light apart from dawn and dusk are redundant. However, the estimation of a VRC is made from a PRC (Daan, 1977) and therefore it suffers the same assumptions that underlie the estimation of a PRC.

Case specific models

Mathematical models have often been useful in order to understand physical and biological phenomena (Otto and Day, 2007). The same is the case for both the parametric and non-parametric models of entrainment. But, explaining entrainment using the non-parametric model is difficult in case of animals that do not see dawn or dusk as is the case with the European ground squirrel (Hut et al., 1999). This problem was overcome by a model that incorporated both the PRC and the VRC and exploited the fact that light intensity changed over the due course of time in a day (Beersma et al., 1999). Several other attempts have been made in order to explain entrainment for certain kinds of organisms or circadian oscillators of either physical or chemical nature. Pavlidis and Wever among others modelled circadian oscillators using elaborate differential equations. But, the problem with such mathematical models are that the parameters used are often less intuitive in terms of their physical meaning and biological implications (reviewed in Roenneberg, 2008). Other researchers were focussed on modelling chemical processes that give rise to circadian rhythmicity. In these models there are usually a really large number of differential equations. The parameters used in these equations have biological meaning. But, these large number of equations result in a large parameter space with very high degrees of freedom, thereby limiting the extent by which these equations are heuristic in understanding entrainment (reviewed in Leloup and Goldbeter,

2008).

Circadian Integrated Response Characteristic (CIRC)

The use of a CIRC instead of a PRC or a VRC to explain entrainment was recently proposed by Till Roenneberg (Roenneberg et al., 2010). This model relaxes some of the assumptions made under the parametric and non-parametric models of entrainment. In order to use this model one must visualise the circadian clock to be a limit cycle oscillator with a particular τ. Under entrainment this limit cycle would have to shrink or expand itself to match the periodicity of the external cycle depending on τ and T. A typical CIRC has an expansion zone during early subjective night and a compression zone during late subjective night. The only assumption of this model is that the circadian system of any organism is capable of integrating zeitgeber signals over time. The CIRC model is not really concerned about whether the matching of internal and external periodicities occurs via phase-shifts or τ changes. Only the net result of compression or expansion of the limit cycle is of significance. During entrainment, the τ of an oscillator makes an attempt to equalise itself to the T of the external cycle, and as a result of this period changes have been observed as after-effects. This periodicity of the circadian clock is considered to be the period of the oscillator under entrainment (τ_E) and is used to predict phase of entrainment, unlike in other models of entrainment put forth thus far. A diurnal animal with a τ of 23 h under the influence of an entraining stimulus of 24 h periodicity would probably change its τ to 23.6 or 23.7 h during entrainment in an attempt to equalise the T of the zeitgeber. According to Roenneberg's model this periodicity of 23.6 or 23.7 h along with the shape of the CIRC could be used to predict the phase of entrainment. One could easily imagine that τ_E is a more realistic parameter on which

the process of entrainment should depend.

The models of entrainment proposed so far, use τ or τ_E and the shape of the PRC, VRC or CIRC in order to predict phases of entrainment. Briefly put, the oscillator adjusts itself such that light falls on the part of the PRC, VRC or CIRC in order to facilitate appropriate entrainment by a change in either the phase, period or both. This adjustment is done in accordance with the oscillator's underlying τ in case of the PRC or VRC and τ_E in case of the CIRC. If a shorter than 24 h period individual has to entrain to a 24 h cycle, the individual will adjust its rhythm such that it exposes more of either the delay zone, the deceleration zone or the expansion zone of the PRC, VRC or CIRC respectively depending on which of these it uses for entrainment. As a result of this a particular phase-relationship with the zeitgeber is achieved. A longer than 24 h individual will do the exact opposite and as a result of this, the phase of entrainment for this individual will be delayed with respect to the phase of entrainment of the shorter than 24 h period individual. Based on this it was proposed that τ and phase of entrainment are correlated such that for a given T the phase of entrainment leads less or lags more relative to the zeitgeber if $\tau>T$ (Pittendrigh and Daan, 1976; Roenneberg et al., 2010). This proposition has gathered some evidence in the past. Aschoff showed this relationship to hold true for chaffinches and lizards (Aschoff, 1965). Evidence for a genetic correlation between circadian period and phase has been shown for populations selected for morning and evening emergence (Kumar et al., 2007) and for narrow gate of emergence (Kannan et al., 2012). Evidence for such a relationship also comes from data on humans (Duffy and Czeisler, 2002).

1.5 Do circadian clocks evolve under the influence of periodic selection pressures?

The above review of literature shows that we most certainly have evidence in favour of the idea that circadian clocks confer its bearers a fitness advantage over individuals with either malfunctioning clocks or with no clocks. Although few, compelling evidence in favour of the circadian resonance hypothesis exist. Results from studies in the wild do not yield very convincing or conclusive results. Light was shown to be deleterious in plants but not in animals. The fact that there are deleterious effects of constant illumination in plants has been sought after using different explanations invoking ideas of internal desynchrony, which seem to be true. Answers to adaptive questions are often very elusive, and its convoluted nature makes studies in the discipline very rare. Experimental evolution studies along with the use of genetic techniques seem to be promising ways of understanding the adaptive significance circadian clocks have in a holistic fashion. Also, these studies would help us understand how circadian clocks evolve; to determine which parameters of the clock respond to periodic selection pressures and how. The most conclusive evidence in favour of adaptive significance of circadian clocks would be to show that clocks actually evolve under periodic selection pressures. In order to address this question, attempts were made early on by Pittendrigh and other researchers. In fruit flies, *D. pseudoobscura* selection was imposed on timing of emergence, which gave rise to two strains the *early* and *late* lines. After 50 generations their emergence peak was diverged by 4 h (Pittendrigh, 1967). These flies also diverged in their τ, where the *early* lines showed a longer period and the *late* lines showed a shorter period. However, these lines did not diverge in terms of their PRC. Selection experiments in the moth, *Pectinophora gosypiella* also showed similar results (Pittendrigh and Minis, 1971). One more independent study reported effects of selection on morning and evening emergence in

two *Drosophila* populations viz., *Oregon R* and a wild caught population, *W2* (Clayton and Paietta, 1972). Although, circadian properties were not measured in these flies, it was reported that after 16 generations of selection, percentage of flies emerging in the morning and evening selection windows were significantly higher. Other selection experiments performed on the melon fly, *Bacterocera cucurbitae* populations for faster and slower rates of development showed a reduction and increase in the τ respectively (Miyatake and Shimizu, 1999). From the same group came another piece of evidence in favour of the adaptive significance of circadian clocks. It was observed that lines that were selected for later age at reproduction showed a correlated increase in τ and seemed to mate later in the day than the ones selected for early age at reproduction (Miyatake et al., 2002). Also, a genetic correlation between development time and locomotor activity rhythm was reported in *D. melanogaster* further indicating the adaptive nature of circadian clocks (Takahashi et al., 2013).

1.6 What are the GATE populations?

Experimental evolution experiments need to be designed very carefully in order to be able to interpret the results appropriately. A precise knowledge on the history of the source populations, the population sizes and population level replicates is mandatory in order to attribute the observed changes to selection pressure alone (Sharma and Joshi, 2002). One cannot rule out the effects of random genetic drift or mutations in the absence of such information. Albeit the above reviewed studies show changes in response to selection, one cannot say conclusively, that they were due to selection pressures alone because of the dearth of certain relevant information about the populations and their maintenance regimes. In order to circumvent these problems and perform a systematic analysis of how circadian clocks

evolve under periodic selection pressures, populations of *D. melanogaster* were created in our laboratory by subjecting population of flies to periodic selection pressures. Four large outbred *early (early$_{i=1..4}$), control (control$_{j=1..4}$)* and *late (late$_{k=1..4}$)* populations were initialised from four large outbred ancestral populations *(JB$_{1..4}$)*. Each of these populations caged ~1200 adult fruit flies. All three stocks are maintained in 12:12 h LD cycles under a constant temperature of about 25 °C and about 60-70% RH. All the three sets of populations that share the same subscript *(i=j=k)* have common genetic ancestry. The *early$_{i=1..4}$* lines were created by selecting individuals that emerge only between ZT21-ZT01 and the *late$_{k=1..4}$* lines were created by selecting individuals that emerge only between ZT09-ZT13. Every generation, flies emerging during the aforementioned selection window are collected from the *early* and *late* lines for three-four consecutive cycles and flies emerging throughout the day for those cycles are chosen from the *control* populations to form the breeding population for the next generation (Figure 1.1).

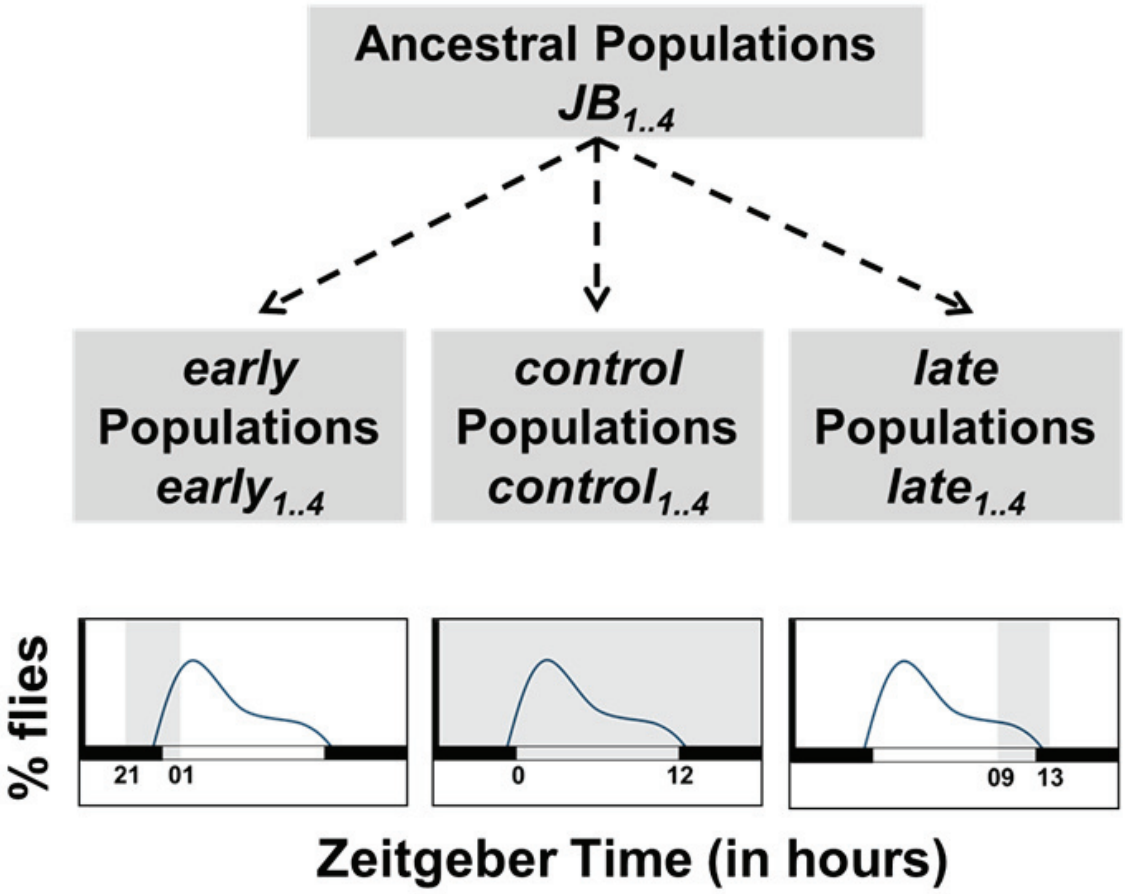

Figure 1.1: Schematic of selection protocol. Grey shaded regions in the lowermost panel indicate selection windows for the respective populations. The *early* populations are collected between ZT21-ZT01, the *control* populations are collected throughout the day and the *late* populations are collected between ZT09-ZT13.

As a direct response to selection proportion of flies emerging in the *early* and *late* stocks increased in their respective selection windows (Kumar et al., 2007). Correlated responses in these populations include change in τ of both emergence (Kumar et al., 2007) and locomotor rhythms (Figure 1.2a), photic PRCs of adult emergence rhythms (Kumar et al., 2007), phase of emergence rhythms (Figure 1.2b), shape of emergence waveforms among stocks (Figure 1.2c) and light requirement schedules for achieving entrainment to LD cycles (Vaze et al., 2012a). The *early* stocks evolved a shorter and the *late* stocks evolved a longer τ with respect to their *control* stocks. Despite this, the photic PRCs of emergence rhythms of the *early* stocks showed a greater advance than delay zone and the *late* stocks showed the exact opposite (Kumar et al., 2007). A similar trend was observed with the photic PRCs of locomotor activity rhythms (Vaze, Thesis, 2012). These are counter intuitive results and are suggestive of the fact that the non-parametric mode of entrainment may not be sufficient to explain entrainment in these populations. Also, the photic PRCs of locomotor activity rhythms did not significantly differ among the stocks (Vaze, Thesis, 2012). It was also shown that the *early* stocks need light for a longer duration in the evening and the *late* stocks need light for a longer duration in the morning in order to entrain similar to their LD12:12 profiles. This led us to conclude that the light requirement schedules for the *early* and *late* stocks have evolved to be diametrically opposite. Moreover, it was shown that under natural conditions, where there is a vast repertoire of zeitgebers and there are gradual changes in zeitgeber values over time of the day, the *early* and *late* populations show greater divergence among stocks (Vaze et al., 2012b). It was then shown that the environmental feature responsible for increased divergence between the stocks was temperature (Nikhil et al., 2014). Based on this data, the authors have suggested that the divergence in the emergence phenotypes among *early* and *late* stocks is

likely to be due to underlying differences in the B-oscillator proposed by Pittendrigh (Pittendrigh, 1981). It was also observed that there are significant period changes after entrainment of the locomotor activity rhythm to long photoperiod but the change in period was independent of the stock, which is suggestive of the fact that the parametric model of entrainment may also not be sufficient to explain entrainment of these stocks (data not shown).

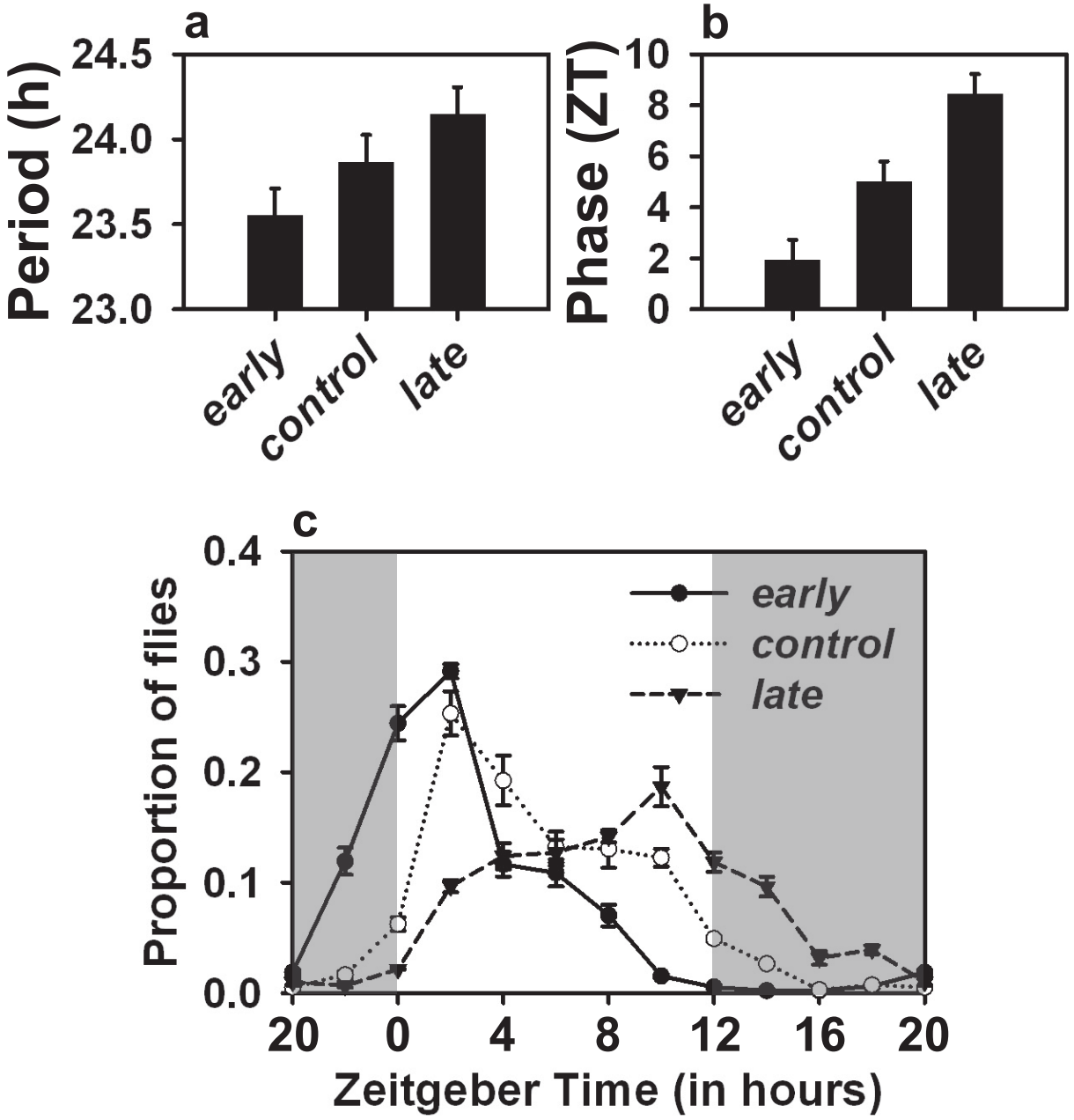

Figure 1.2: Correlated responses to selection for morning and evening emergence. (a) Period of locomotor activity rhythm; Error bars: 95% CI. (b) Phase of entrainment of emergence rhythm; Error bars: 95% CI. (c) Divergence of emergence waveforms; Error bars: SEM; Shaded regions indicate the scotophase of the LD cycle. Unshaded regions indicate the photophase of the LD cycle.

1.7 Rationale and questions put forth in this thesis

Based on the review in the previous section about our findings on the GATE populations, it is clear that the non-parametric or the parametric model of entrainment are not sufficient to explain the entrained behaviour of adult emergence rhythm in populations selected for timing of emergence under periodic LD cycles. Also, a detailed analysis of entrained behaviour of locomotor activity rhythm has not been performed in these stocks although it is known that the τ of the *early* and *late* stocks have significantly diverged. Based on arguments presented earlier it is conceivable that a systematic study of the relationship between τ and φ could lead to understanding of the mechanisms of entrainment. The goals of this thesis are, thus, to systematically characterise the entrained behaviour of locomotor activity rhythms in the GATE populations and study the relationship between τ and phase of entrainment for both emergence and locomotor activity rhythms and attempt to understand the underlying mechanisms of entrainment. Also, a further interest of this thesis is to address the genetic bases for the divergence of entrained rhythm in both adult emergence and locomotor activity between *early* and *late* stocks.

Chapter 2

Does selection for timing of emergence also lead to correlated evolution of the entrained locomotor activity rhythm?

Introduction

Entrainment of rhythms to zeitgebers could be reckoned as the phenomenon by which circadian clocks render functional advantage to its bearers. It provides means by which the circadian clock interacts with the environmental cycles in order to achieve a stable phase-relationship that may be adaptive (Pittendrigh, 1981; Cloudsley-Thompson, 1960). Based on our understanding, three general criteria for entrainment are usually defined (Moore-Ede et al., 1982): (a) *Period control:* The zeitgeber should be able to influence the rhythm such that the period of the rhythm matches that of its own, (b) *Phase control:* This refers to the fact that phase of the rhythm on the first day of constant conditions should be the same as that on the last day of periodic conditions and (c) *Stable and reproducible phase-relationship:* The phase of the rhythm with respect to the zeitgeber should be stable (little variation) and reproducible. It is generally believed that a stable phase-relationship *(ψ)* is established during entrainment, as a consequence of the circadian oscillator's attempt to match its period *(τ)* to that of the zeitgeber (*T*), a phenomenon known as period control (Pittendrigh and Daan, 1976). Due to this, τ and φ are believed to be correlated and has been shown to be true in some previous studies (Pittendrigh and Daan, 1976, Aschoff, 1965, Duffy and Czeisler, 2002, Wright et al., 2005, Kumar et al., 2007, Kannan et al., 2012). Studying the relationship between τ and φ, therefore, is central in understanding the mechanisms of entrainment.

Earlier reports have shown that mutations in the *period* gene of *D. melanogaster* affect both eclosion and locomotor activity rhythms in a similar fashion (Konopka and Benzer, 1971). This is suggestive of the fact that both these rhythms are governed by the same clock. But other reports have also suggested that in *D. pseudoobscura* there are likely to be different

circadian pacemakers that regulate eclosion and locomotor activity rhythms (Engelmann and Mack, 1978). Also, in *D. melanogaster*, mutations in several clock genes have been shown to affect only eclosion or only locomotor activity rhythms. The *ebony* mutation renders individuals arrhythmic for locomotor activity rhythm but does not seem to affect the eclosion rhythm (Newby and Jackson, 1991). The *lark* mutation on the other hand renders individuals arrhythmic for eclosion rhythms but does not seem to have any effect on adult locomotor activity rhythm (Newby and Jackson, 1993).

In the light of these observations, it is reasonable to expect divergence of locomotor activity rhythm in the *early* and *late* stocks relative to their *control* stocks. Selection on timing of emergence led to evolved differences in the τ of locomotor activity rhythm (Figure 1.2a). Albeit this selection pressure revealed that the underlying circadian clocks of the *early* and *late* lines have diverged, it is not clear if the circadian clocks underlying any adult behavioural rhythm under entrainment have also changed. Adult emergence is a once in a lifetime phenomenon for an individual fly whereas locomotor activity is a regular and daily affair. It would, therefore, be interesting to test if the entrained behaviour of locomotor activity rhythm has also diverged in our stocks.

In order to characterise the locomotor activity rhythm of the *early*, *control* and *late* stocks under entrainment we use two measures viz., the shape of the waveform and φ of the rhythm. These features of the locomotor activity rhythm were estimated under their native light conditions and low light intensity conditions. Low light intensity was used to nullify the potential effects of masking on the φ of the rhythm (Moore-Ede et al., 1982). Further we

wanted to ask if the underlying differences in circadian clock periods also lead to differences in the way these rhythms entrain. In order to answer that we analysed systematically the $\tau - \varphi$ relationship of adult emergence and locomotor activity rhythms under native lighting conditions and low light intensities, among and within populations. It has also been reported previously that such $\tau - \varphi$ relationships are obscured under abrupt LD cycles and that such associations become more apparent under gradually cycling light conditions (Sharma and Chandrasekharan, 1998). In order to make sure that our conclusions are not biased by such an artefact, we also assayed the phase of entrainment under semi-natural conditions of the *control* stocks (both sexes) and the same individuals' circadian period under constant darkness to estimate the $\tau - \varphi$ relationship.

Materials and Methods

Population maintenance and laboratory selection protocol

Four replicates of *early* ($early_{i=1...4}$), *control* ($control_{j=1...4}$) and *late* ($late_{k=1...4}$) populations were derived from four common ancestral, large, outbred populations ($JB_{1...4}$). The *early*, *control* and *late* populations have been maintained as independent populations for more than 200 generations. The $early_i$, $control_j$ and $late_k$ populations that share the same subscript $(i = j = k)$ indicate common ancestry. This would mean that populations of *early, controls* and *late* having different subscripts have independent genetic substructure. Given that there will be ancestral genetic similarity between *early*, *control* and *late* sharing the same subscript, the replicate populations have been used as 'blocks' in the statistical analyses. All the three sets of populations are maintained on a 21 day discrete generation cycle, in cubicles maintained under LD12:12 (~70-80 lux) conditions at about 25 °C and about 60-70% RH. The time at which

lights come ON is labelled as ZT00 (Zeitgeber Time 00) and lights go OFF at ZT12. Eggs from all the populations are collected at an egg density of about 300 eggs in ~6-8 mL of food in long vials. Flies emerging over the 9^{th}-13^{th} day after egg collection are collected and transferred into Plexiglas cages. All the populations are maintained on Banana-Jaggery food medium. The populations are given fresh food supplies in a petri-plate every alternate day.

Flies emerging between ZT21 to ZT01 on days 9^{th} to 13^{th} are collected to form the breeding population for the next generation for the *early* populations. Flies emerging during ZT09 to ZT13 on days 9^{th} to 13^{th} are collected to form the breeding population for the next generation for the *late* populations. Flies emerging throughout the day, on days 9^{th} to 13^{th} post egg collection are collected to form the next generation of *control* populations. On the 18^{th} day after the previous egg collection all the three sets of populations are provided with live yeast paste. This yeast plate remains for three days and on the 21^{st} day after the previous egg collection, eggs from all these populations are collected in the similar fashion in order to initiate the next generation. Before the assays fly populations were subjected to one generation of common rearing (standardisation). This common rearing condition for one generation provides considerable buffer against possible maternal and non-genetic inheritance effects. The standardisation protocol therefore, also enables us to appropriately assess the effects of selection alone.

Locomotor rhythm assay

The locomotor activity rhythm was assayed for all four blocks of all the three stocks. Sixty-four flies were used per sex per population for blocks 1 and 2 and thirty two flies were used

per sex per population for blocks 3 and 4 for this assay. The locomotor activity rhythm of both males and females were first monitored under conditions of LD12:12 (~1 lux and ~70 lux) at ~25 °C and ~70% RH or semi-natural conditions (SN) for about five to seven days. Data from these seven days were used to estimate the phase of the rhythm under entrained condition. On the 8th day, flies were transferred into new locomotor tubes with fresh food and were recorded under constant darkness (DD) with the same temperature and humidity. The flies were kept in this condition for about five to seven days. Data from these days were used to estimate the free-running period of each fly.

Adult emergence rhythm assay

The adult emergence or eclosion assay was performed on all four blocks of all the three stocks. The adult emergence profiles were drawn separately for males and females under conditions of LD12:12 (~70 lux) at ~25 °C and ~70% RH. Approximately 300 eggs were collected from standardised populations and dispensed into each glass vial with Banana-Jaggery food medium. Eight to nine vials per population per block were used as replicates for the ~1 lux experiment and six vials per population per block were used as replicates for the ~70 lux experiment. Post egg collection the racks with all the vials were subject to the experimental condition. From initiation of emergence flies emerging from each vial in every 2 h interval were sexed and recorded for 3-4 days consecutively. Only vials which had at least 15 flies of each sex over the duration of one cycle, and vials that showed robust rhythms over at least three cycles were included in the analyses.

Estimating φ of the rhythms

In order to estimate the phase of entrainment *(φ)* for the locomotor activity rhythm of individuals, freshly eclosed male and female flies of age ~3-5 days were recorded using Drosophila Activity Monitors (Trikinetics, Waltham, MA, USA), for 5-7 days under LD12:12 cycles at two light intensities viz., ~1 lux and ~70 lux. Activity counts were summed into bins of 60 min each, across all the days of recording. Proportion of activity at each time point is computed by normalising the activity counts at each time point by the total amount of activity in each cycle and then averaging these normalised values over cycles. Centre of Mass (CoM) is used as the phase marker of the rhythms for all our analyses. This measure of phase is used because it is not subjective like other phase markers are, and also incorporates differences in the overall shape of the rhythm and other phase markers. Each ZT point is converted to its corresponding degrees using the equation, φ_i = (ZT*360)/24. Each of these φ_i is then used to compute the sine and cosine components of each of these time points. The proportion of activity is then multiplied with their corresponding sine and cosine components of their respective time points. Sum of the product of proportion activity and corresponding sine components *(y)* and of proportion activity and corresponding cosine components *(x)* are computed respectively. The φ is then computed according to the following equation, $\varphi = atan(y/x)$. This φ is the computed CoM for each individual (Zar, 1999).

In order to estimate the phase of entrainment *(φ)* for locomotor activity rhythm of populations, first, proportion of activity at each time point is computed by normalising the activity counts at each time point by the total amount of activity in each cycle and then averaging these normalised values over cycles and over individuals. Then this proportion of activity over time

points for a population is used in order to compute the CoM for the population using the same procedure mentioned above. Since the locomotor activity rhythm is bimodal, a transformation referred to as angle doubling is performed. In this transformation the angles corresponding to a given ZT is doubled and any angle greater than 360 degrees is coverted to a scale of 0-360 degrees by subtracting 360 from those values. This procedure converts a single day with two peaks of activity to a format such that the two activity peaks coincide in time (Zar, 1999).

Phase of entrainment *(φ)* for eclosion rhythms of populations was computed similar to that of locomotor activity rhythm using percentage emergence instead of percentage activity.

Estimating τ of locomotor activity rhythm

In order to estimate τ of free-running locomotor activity rhythm in these individuals and populations for which φ was computed, flies were transferred to activity tubes with fresh food and transferred into DD. Under DD, flies were allowed to free-run for at least 10 days. Data collected was then analysed using CLOCKLAB (Actimetrics, IL, USA). Chi-square periodogram analysis was used to scan for significant periodicity in the data for each individual. Population τ was estimated by averaging individual τ values from the respective population.

Statistical procedures

In order to compare the waveform of entrained locomotor activity rhythms among populations, a three factor mixed model analysis of variance (ANOVA) was performed on proportion of activity (*arcsine* square root transformed) on the data using STATISTICA v5.0 (StatSoft Inc.,

Tulsa, OK). Stock (S) and time point (T) were treated as fixed factors and the blocks (B) were treated as a random factor. Any significant effect of S × T interaction would mean that the entrained waveforms of locomotor activity rhythms have evolved. A Tukey's Honestly Significant Difference (HSD) test was performed for multiple comparisons where required (Zar, 1999).

In order to compare the τ among populations, block mean values of τ were taken for analyses. A two factor mixed model ANOVA was performed with stock (S) as a fixed factor and block (B) as a random factor using STATISTICA v5.0 (StatSoft Inc., Tulsa, OK). A Tukey's Honestly Significant Difference (HSD) test was performed for multiple comparisons where required (Zar, 1999).

In order to compare the φ among populations, block mean values of φ were taken for analyses. A three factor mixed model ANOVA was performed with stock (S) and light regime (L) as fixed factors and block (B) as a random factor using STATISTICA v5.0 (StatSoft Inc., Tulsa, OK). A Tukey's Honestly Significant Difference (HSD) test was performed for multiple comparisons where required (Zar, 1999).

In order to study the degree of association between τ and φ correlation analyses were performed. A circular linear correlation was performed in this case because one variable in our study is a linear random variable (τ) and the other variable in our study is a circular random variable (φ) (Mardia, 1976). These correlation analyses were carried out using the CircStats toolbox written for MATLAB (Berens, 2009).

All statistical results were considered significant at $\alpha=0.05$.

Results

Entrained locomotor waveform

The overall shape of entrained waveforms of locomotor activity rhythm was analysed to assess whether the GATE stocks have evolved differences in the way they entrain their locomotor activity rhythms to LD cycles. ANOVA showed no differences in the overall waveforms of the *early*, *control* and *late* stocks as revealed by the fact that there is no significant interaction between stock and time point ($F_{46,138}=0.652$; $p>0.05$). This suggests that proportion of activity at any given time point across the day is not dependent on the stocks. However, there seems to be a significant effect of the interaction of stock, time point and light intensity ($F_{46,138}=2.035$; $p<0.05$). This is likely to be due to the highly significant effect of the interaction between time point and light intensity ($F_{23,69}=16.653$; $p<0.05$). Overall it can be concluded that the entrained shape of the locomotor activity rhythm waveform does not significantly differ among the stocks under any light intensity (Figure 2.1, Table 2.1). Given that, no statistically significant interaction between stock and time point was found, no further post-hoc comparisons were necessary.

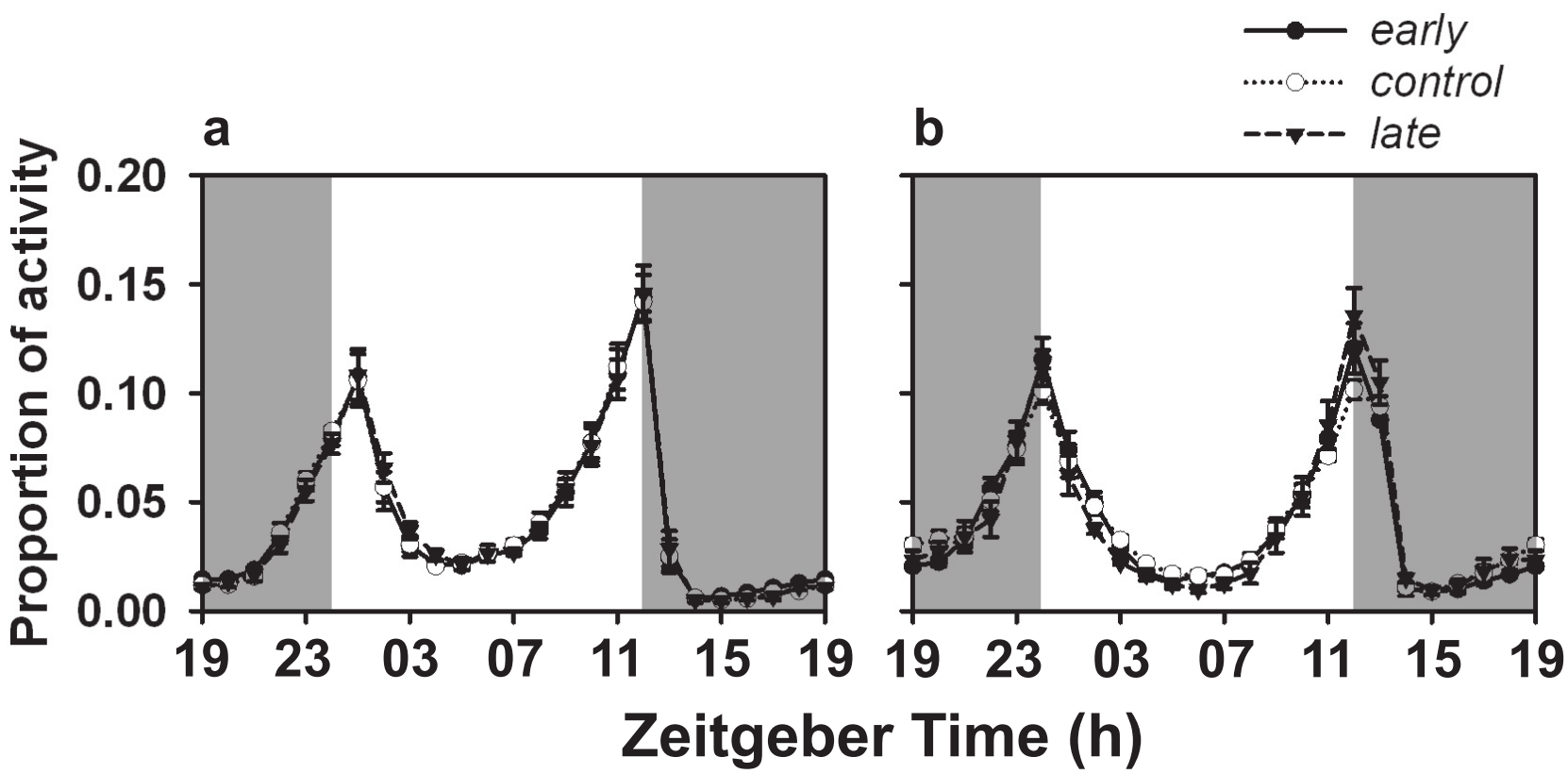

Figure 2.1: Entrained locomotor activity rhythm waveform of the GATE stocks under LD12:12 cycles of (a) ~1 lux and (b) ~70 lux. Proportion of activity is plotted across time of the day (Zeitgeber Time). All error bars are SEM. Grey shaded regions indicate the scotophase of the LD cycle and the unshaded regions indicate the photophase of the LD cycle.

	df Effect	MS Effect	df Error	MS Error	*F*	*p-level*
Stock (S)	2.00	0.00	6.00	0.00	2.10	0.20
Time Point (T)	*23.00*	*0.15*	*69.00*	*0.00*	*160.58*	*0.00*
Light Intensity (L)	1.00	0.00	3.00	0.00	6.26	0.09
Block (B)	3.00	0.00	0.00	0.00	--	--
S × T	46.00	0.00	138.00	0.00	0.65	0.95
S × L	2.00	0.00	6.00	0.00	1.99	0.22
T × L	*23.00*	*0.02*	*69.00*	*0.00*	*16.65*	*0.00*
S × B	6.00	0.00	0.00	0.00	--	--
T × B	69.00	0.00	0.00	0.00	--	--
L × B	3.00	0.00	0.00	0.00	--	--
S × T × L	*46.00*	*0.00*	*138.00*	*0.00*	*2.04*	*0.00*
S × T × B	138.00	0.00	0.00	0.00	--	--
S × L × B	6.00	0.00	0.00	0.00	--	--
T × L × B	69.00	0.00	0.00	0.00	--	--
S × T × L × B	138.00	0.00	0.00	0.00	--	--

Table 2.1: Results of analysis of variance (ANOVA) performed on mean proportion of activity across a day. Stock (S), Time Point (T) and Light Intensity (L) are used as fixed factors and Block (B) is used as a random factor. Italicised effects are significant at alpha=0.05.

Free-running period of locomotor activity rhythm

The mean τ significantly differed between the *early*, *control* and *late* stocks. The *early* stocks evolved a shorter period (~23.5 h) and the *late* stocks evolved a longer period (~24.1 h) relative to the *control* stocks (~23.8 h). A Tukey's HSD post-hoc test following a mixed model ANOVA revealed that the mean circadian period of the *early* and *late* stocks significantly differed from each other but neither of them significantly differed from that of the *control* stocks (Figure 2.2a, Table 2.2).

Phase of locomotor activity rhythm

The mean phase of the *early*, *control* and *late* stocks did not significantly differ from each other under either their native light intensity or low light intensity. This result is surprising as the underlying circadian period of these individuals differ significantly. The *early*, *control* and *late* stocks had their CoMs occurring at ZT~23.29, ~23.16 and ~23.27 respectively under their native light intensity (~70 lux) and at ZT~22.9, ~22.88 and ~23.18 respectively under low light intensity (~1 lux) (Figure 2.2b, Table 2.3a). ANOVA revealed no statistically significant effect of either stock, light intensity or their interaction hence a post-hoc comparison was not necessary.

Phase of eclosion rhythms

The mean phase of the *early*, *control* and *late* stocks significantly differed from each other under both, their native and low, light intensities. The *early*, *control* and *late* stocks had their CoMs occurring at ZT~1.93, ~5 and ~8.43 respectively under their native light intensity (~70 lux) and at ZT ~2.12, ~3.92 and ~6.27 respectively under low light

intensity (~1 lux). Mixed model ANOVA revealed a statistically significant effect of stock ($F_{2,6}$=313.08, p<<0.05) and stock × light intensity interaction ($F_{2,6}$=8.88, p<0.05). A Tukey's HSD post-hoc test revealed that *early*, *control* and *late* stocks were significantly different from each other (Figure 2.2c, Table 2.3b). A post-hoc comparison of the interaction effect revealed that only the *late* stocks differed in their mean phases between the two light intensity conditions, whereas neither the *early* nor the *control* stocks differed in their phases between the two light intensities.

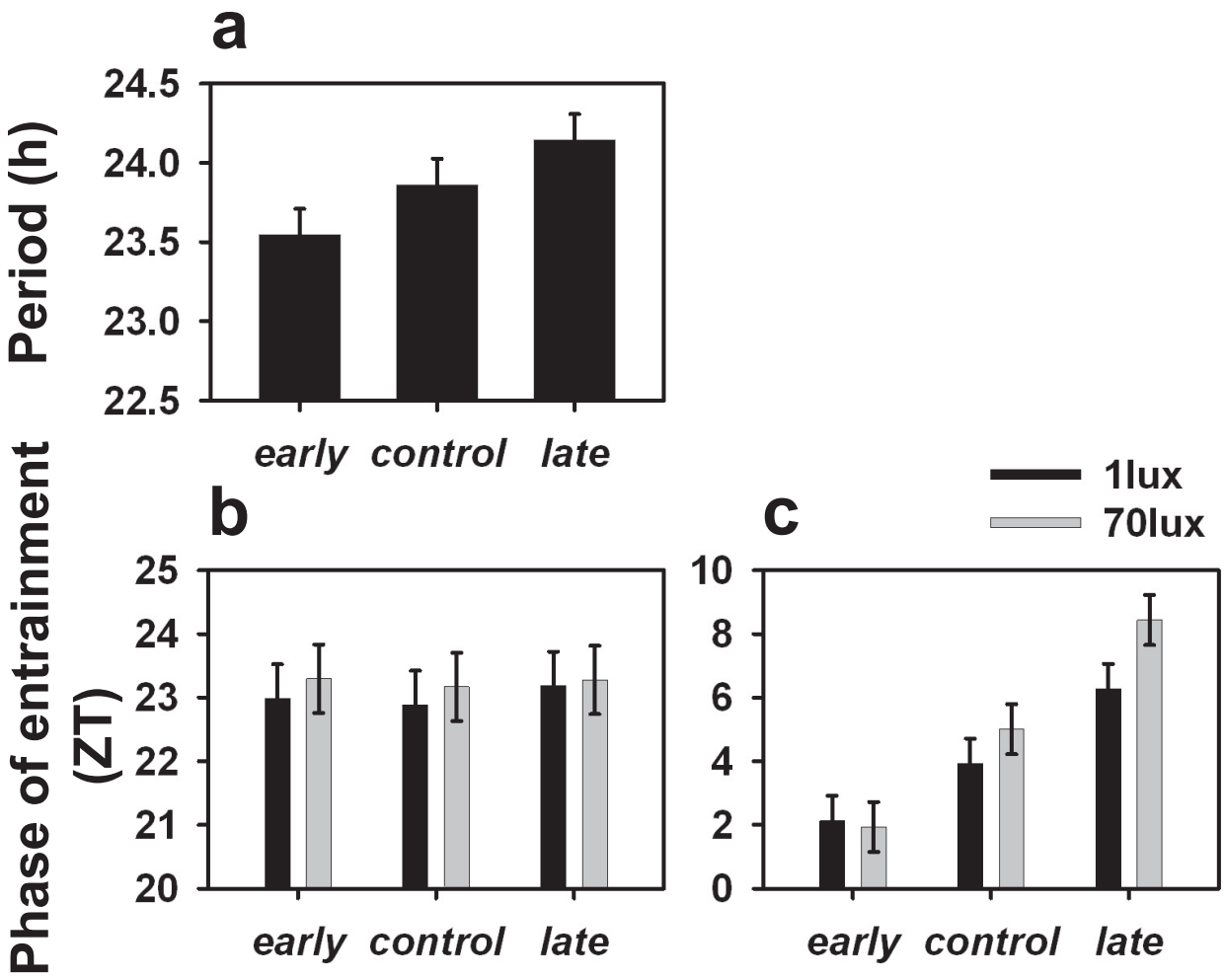

Figure 2.2: (a) Mean circadian period of locomotor activity rhythm of the GATE stocks. (b) Mean phase (expressed in Zeitgeber Time) of locomotor activity rhythm under ~1 lux and ~70 lux of the GATE stocks. (c) Mean phase (expressed in Zeitgeber Time) of eclosion rhythm under ~1 lux and ~70 lux of the GATE stocks. All error bars are 95% CI computed after a Tukey's HSD test, therefore all non overlapping error bars indicate significant differences between the means.

	df Effect	MS Effect	df Error	MS Error	*F*	*p-level*
Stock (S)	*2.00*	*0.36*	*6.00*	*0.02*	*16.12*	*0.00*
Block (B)	3.00	0.12	0.00	0.00	--	--
S × B	6.00	0.02	0.00	0.00	--	--

Table 2.2: Results of analysis of variance (ANOVA) performed on mean circadian period of locomotor activity rhythm. Stock (S) is used as a fixed factor and Block (B) is used as a random factor. Italicised effects are significant at alpha=0.05.

a

	df Effect	MS Effect	df Error	MS Error	*F*	*p-level*
Stock (S)	2.00	0.08	6.00	0.08	1.03	0.41
Light Intensity (L)	1.00	0.32	3.00	0.93	0.34	0.60
Block (B)	3.00	0.54	0.00	0.00	--	--
S × L	2.00	0.03	6.00	0.15	0.19	0.83
S × T	6.00	0.08	0.00	0.00	--	--
S × B	3.00	0.93	0.00	0.00	--	--
S × L × B	6.00	0.15	0.00	0.00	--	--

b

	df Effect	MS Effect	df Error	MS Error	*F*	*p-level*
Stock (S)	*2.00*	*56.78*	*6.00*	*0.18*	*313.09*	*0.00*
Light Intensity (L)	1.00	6.20	3.00	0.71	8.72	0.06
Block (B)	3.00	0.27	0.00	0.00	--	--
S × L	*2.00*	*2.78*	*6.00*	*0.31*	*8.88*	*0.02*
S × T	6.00	0.18	0.00	0.00	--	--
S × B	3.00	0.71	0.00	0.00	--	--
S × L × B	6.00	0.31	0.00	0.00	--	--

Table 2.3: Results of analysis of variance (ANOVA) performed on mean CoM. (a) ANOVA on CoM of locomotor activity rhythm. (b) ANOVA on CoM of eclosion rhythm. Stock (S) and Light Intensity (L) are used as fixed factors and Block (B) is used as a random factor. Italicised effects are significant at alpha=0.05.

Correlation between τ and φ among populations in the lab

Circular-linear correlation analyses revealed that mean circadian period of locomotor activity rhythm of all the twelve populations for both sexes are significantly correlated with the mean phase of entrainment of emergence rhythms under ~1 lux light intensity (r=+0.65, p<0.05) (Figure 2.3a, Table 2.4). The analyses also revealed the absence of any such correlation between the mean circadian period of locomotor activity rhythm and the mean phase of entrainment of locomotor activity rhythm under ~1 lux light intensity (r=+0.26, p>0.05; Figure 2.3b, Table 2.4). When such associations were looked at under ~70 lux light intensity, similar results were obtained. Circadian period of locomotor activity rhythm and phase of entrainment of emergence rhythm were significantly correlated (r=+0.88, p<0.05; Figure 2.3c, Table 2.4) but that of locomotor activity rhythms were not (r=+0.23, p>0.05; Figure 2.3d, Table 2.4).

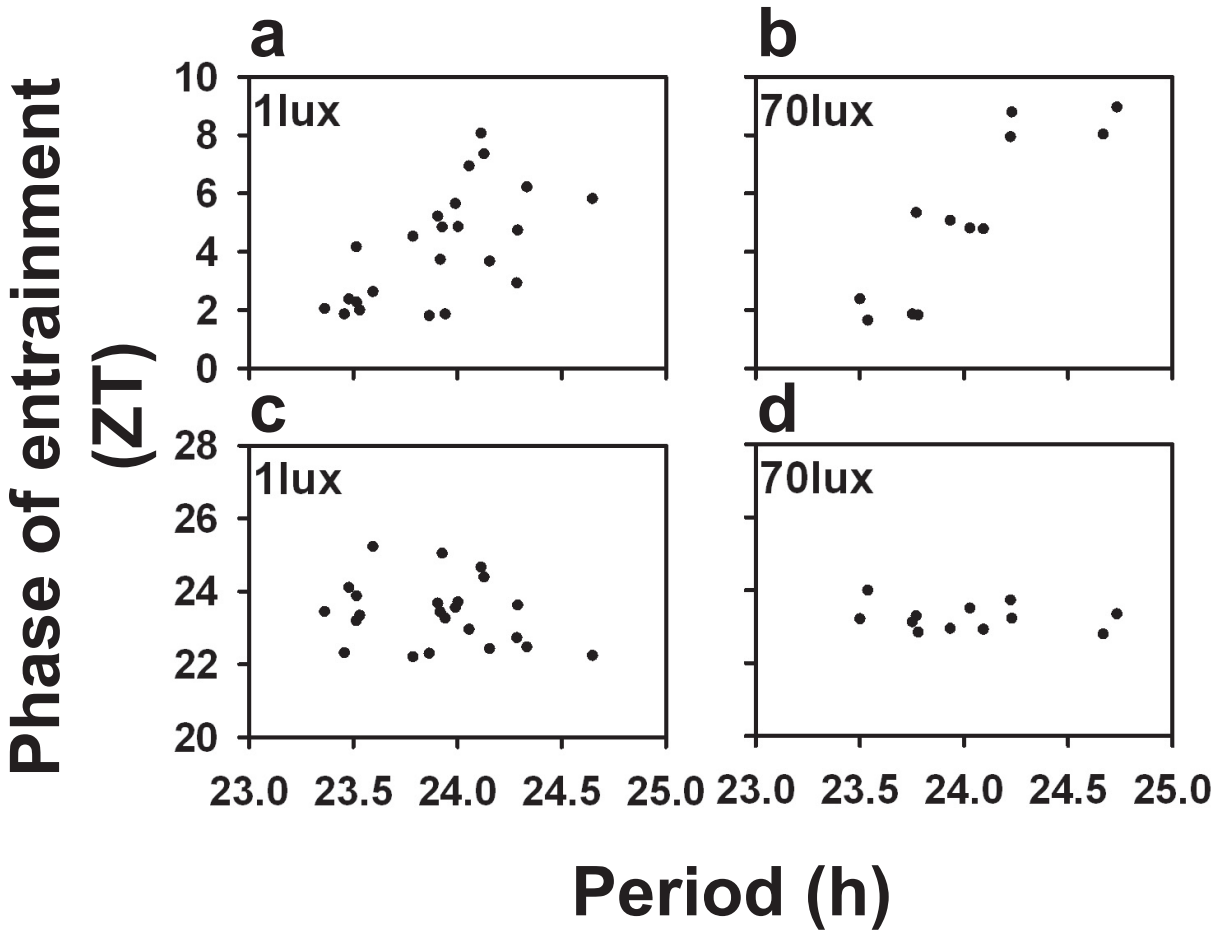

Figure 2.3: Circular-linear correlation between circadian period of locomotor activity rhythm and phase of entrainment across populations. (a) and (b) With phase of emergence rhythm under ~1 lux and ~70 lux respectively. (c) (d) With phase of locomotor activity rhythm under ~1 lux and ~70 lux respectively. Each dot in (a) and (c) represents mean circadian period and mean phase of entrainment for each block of each stock for each sex and in (b) and (d) represents the same for each block of each stock. (a) and (b) report significant correlations whereas (c) and (d) report non-significant correlations.

Correlation between τ and φ within populations in the lab

Circular-linear correlation analyses were performed to see if within population circadian period and phase of entrainment of locomotor activity rhythms are associated. Under a light intensity of ~1 lux, there was no such correlation within the *early* (pooled over all four blocks) (r=+0.16, p>0.05; Figure 2.4a, Table 2.4) and *late (pooled over all four blocks)* (r=+0.06, p>0.05;Figure 2.4c, Table 2.4) stocks. However, a significant correlation was observed within the *control* (pooled over all four blocks) stocks (r=+0.27, p<0.05; Figure 2.4b, Table 2.4-). When data from all these stocks were pooled, no such correlation between circadian period and phase of entrainment was observed (r=+0.05, p>0.05; Figure 2.4d, Table 2.4). Similar analyses on data collected under entrainment to an LD cycle with ~70 lux light intensity revealed no statistically significant correlation whatsoever (Figure 2.5a-d, Table 2.4). Interestingly, across a range of period values, the phase of entrainment was similar. This can be observed across all stocks and light intensities and is suggestive of the fact that phase of entrainment would not covary with circadian period.

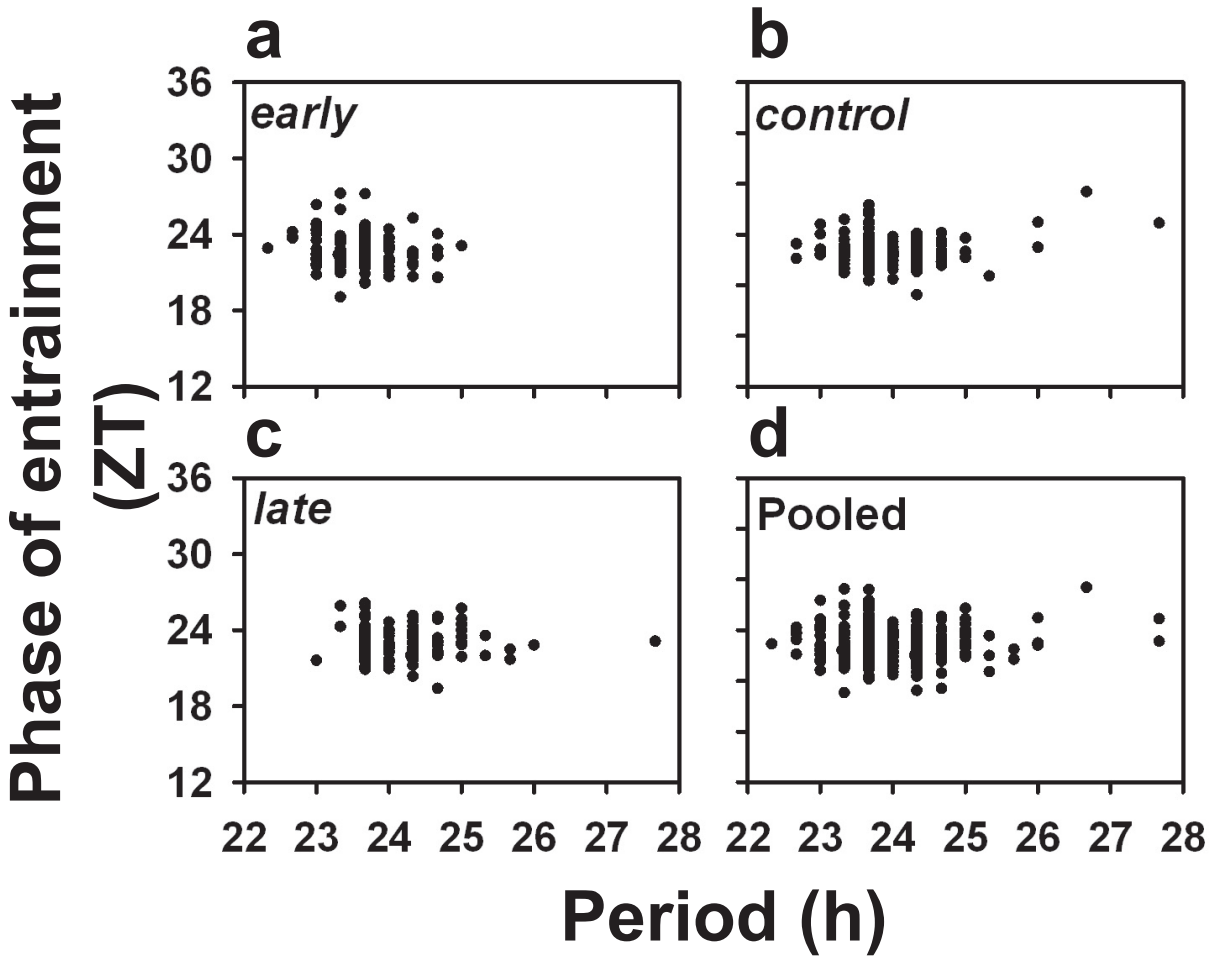

Figure 2.4: Circular-linear correlation between circadian period of locomotor activity rhythm and phase of entrainment within populations. (a) *early* (all 4 blocks) stocks. (b) *control* (all 4 blocks) stocks. (c) *late* (all 4 blocks) stocks. (d) All stocks and all blocks pooled. Each dot in (a), (b), (c) and (d) represents each individual fly's circadian period and its corresponding phase of entrainment under ~1 lux light intensity. All plots, except (b), report non-significant correlations.

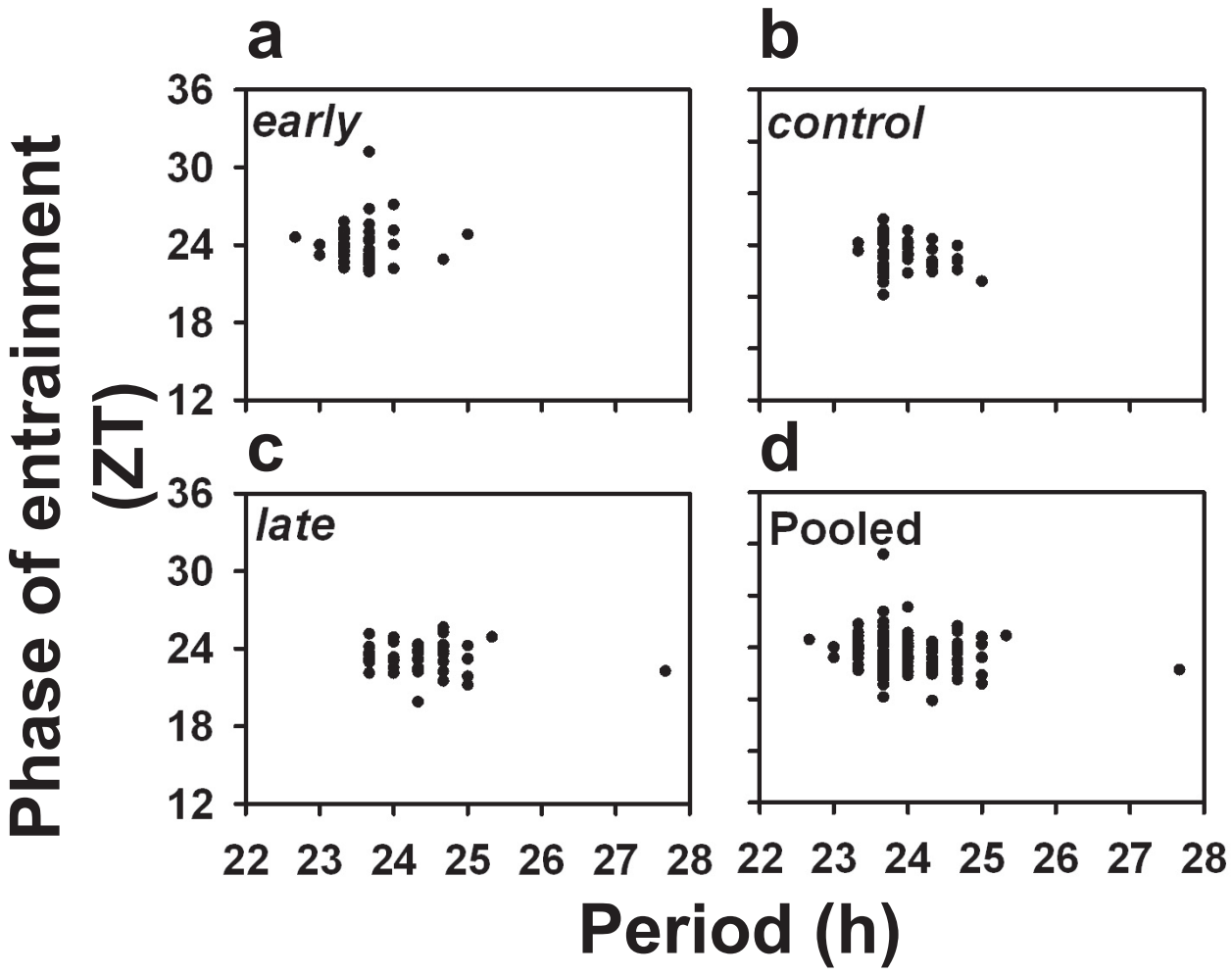

Figure 2.5: Circular-linear correlation between circadian period of locomotor activity rhythm and phase of entrainment within populations. (a) *early* (all 4 blocks) stocks. (b) *control* (all 4 blocks) stocks. (c) *late* (all 4 blocks) stocks. (d) All stocks and all blocks pooled. Each dot in (a), (b), (c) and (d) represents each individual fly's circadian period and its corresponding phase of entrainment under ~70 lux light intensity. All plots report non-significant correlations.

Correlation between τ and φ within populations in the nature

It has been earlier reported that circadian period and phase of entrainment may not show any association under abrupt LD cycles, but under a gradually changing zeitgeber cycle such associations become apparent (Sharma and Chandrasekharan, 1998). We hypothesised that we see no correlation between circadian period and phase of entrainment, precisely due to this reason. Hence, individuals from the *control* stocks (pooled over all four blocks) were assayed for their phase of entrainment and then their circadian period was estimated. Using this data set when a circular-linear correlation was performed, no such association between these two variables was observed (r=+0.27, p>0.05; Figure 2.6, Table 2.4).

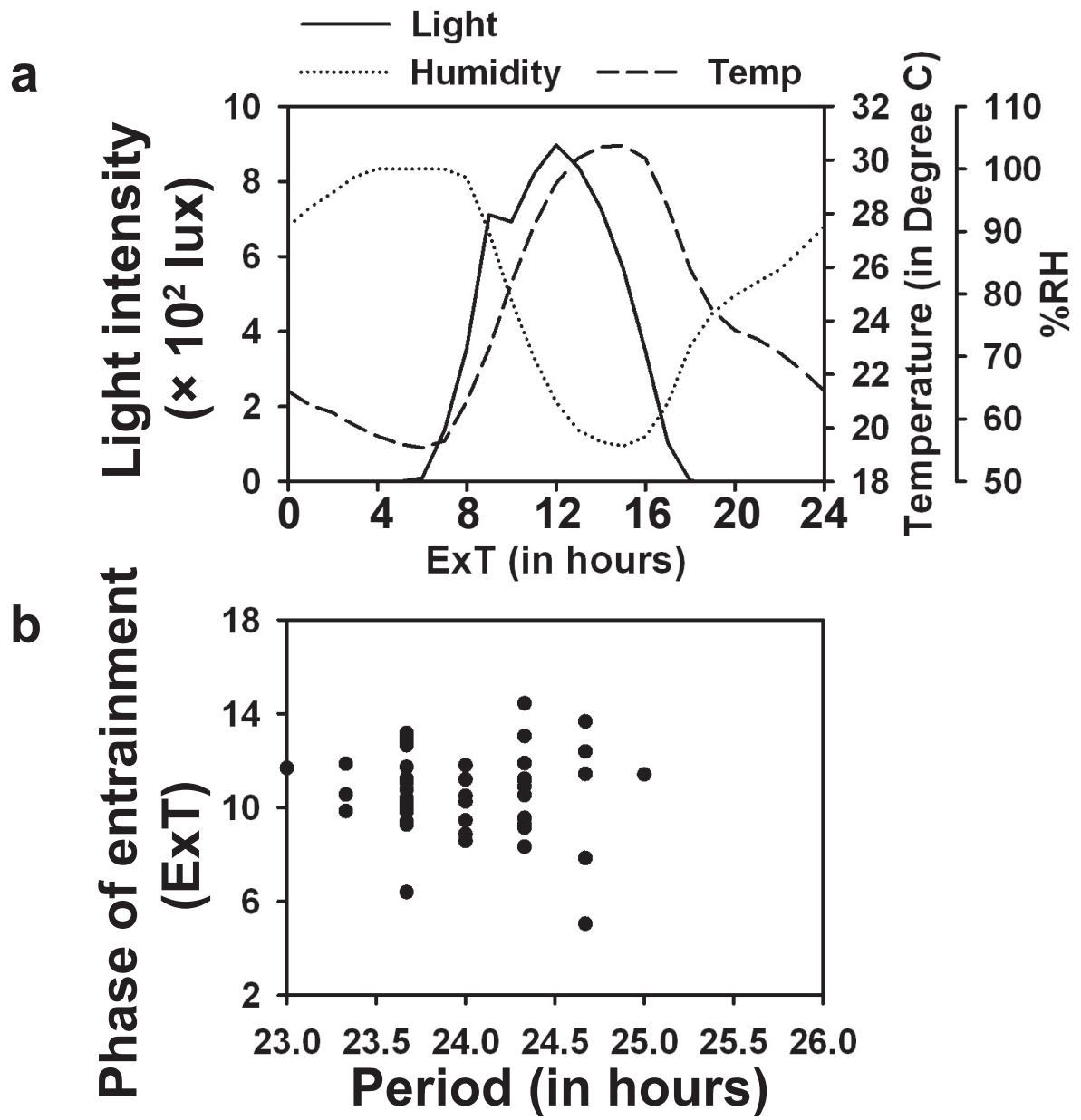

Figure 2.6: Circular-linear correlation between circadian period of locomotor activity rhythm and phase of entrainment in nature. (a) Light, temperature and humidity profiles under natural conditions, averaged over all the days when locomotor activity was recorded. (b) Correlation with phase of locomotor activity rhythm of *control* (all 4 blocks and both sexes) stocks. Each dot in (b) represents each individual fly's circadian period and its corresponding phase of entrainment under semi-natural conditions. (b) reports a non-significant correlation.

Rhythm	Light Intensity (in lux)	Nature of comparison	Correlation Coefficient *(r)*	*p-value*
Eclosion	***1***	***Across***	*0.66*	*0.01*
Eclosion	***70***	***Across***	*0.88*	*0.01*
Locomotor	**1**	**Across**	0.27	0.44
Locomotor	**70**	**Across**	0.23	0.72
Locomotor	**1**	**Within (*early*)**	0.16	0.16
Locomotor	**70**	**Within (*early*)**	0.10	0.82
Locomotor	***1***	***Within (control)***	*0.27*	*0.00*
Locomotor	**70**	**Within (*control*)**	0.23	0.34
Locomotor	**1**	**Within (*late*)**	0.07	0.74
Locomotor	**70**	**Within (*late*)**	0.13	0.69
Locomotor	**1**	**Within (pooled)**	0.05	0.55
Locomotor	**70**	**Within (pooled)**	0.14	0.28
Locomotor	**Semi Natural**	**Within (*control*)**	0.28	0.18

Table 2.4: Results of circular-linear correlations performed on circadian period of locomotor activity rhythm and phase of entrainment. Nature of comparisons are made either across populations or within them. Italicised comparisons report significant correlations at alpha=0.05.

Discussion

Earlier studies have reported and stressed on the fact that the evolved emergence waveforms in GATE stocks stem from underlying differences in the circadian clocks of the *early*, *control* and *late* stocks (Kumar et al., 2007, Vaze et al., 2012a). The circadian period of locomotor activity and that of eclosion rhythms have been shown to be highly significantly correlated (Kumar et al., 2007). Therefore, all further comparisons will be made with the circadian period of locomotor activity rhythms. We, in this study, wanted to first characterise if such underlying differences in the circadian clocks of the *early*, *control* and *late* stocks also resulted in the evolution of locomotor activity waveforms. Analyses revealed that difference in activity levels across time of the day was not dependent on the stocks (Figure 2.1; Table 2.1). Also, the phase of entrainment was not significantly different among the stocks (Figure, 2.2b; Table 2.3a). Both these results suggest that the underlying difference in the circadian clock periods of the *early*, *control* and *late* stocks do not translate into any observable difference in either the shape of the locomotor activity waveform or the phase of entrainment for these populations. This advocated a possibility of there being a distinct component that regulates the phasing of the locomotor activity rhythm. These results pose a challenge towards explaining entrainment of this rhythm in these populations using the non-parametric mode of entrainment.

Albeit, the entrainment of locomotor activity rhythm to LD12:12 cycles of different light intensities are not in accordance with the classical models of entrainment, the entrainment of eclosion rhythms, however, are. As expected, based on the classical model of entrainment, *early* stocks entrain with an advanced phase relative to the *control* stocks and the *control* stocks in turn entrain with an advanced phase relative to the *late* stocks (Figure 2.2c; Table

2.3b). Such differences in phases corroborate nicely with the underlying differences of the period of their circadian clocks (Figure 2.2a). Although, the results do seem to comply with the non-parametric model of entrainment, previous studies have shown that the non-parametric model of entrainment may not be sufficient to explain entrainment of the eclosion rhythms of these stocks (Vaze et al., 2012).

We further wanted to analyse if there is any correlation between circadian period and phase of entrainment. It was observed that under both the light intensities used (~l lux and ~70 lux) there is a statistically significant correlation across populations between period and phase of entrainment of emergence rhythm but not for locomotor activity rhythm (Figure 2.3; Table 2.4). We then looked for associations within populations and found that except in one condition and one stock, all other stocks revealed no correlation whatsoever (Figure 2.4, 2.5; Table 2.4). It was reported earlier that, in mice, the presence of abrupt LD cycles abolishes such associations between τ and φ (Sharma and Chandrasekharan, 1998) and in order to test if this was indeed the case in our stocks, we looked at the *control* stocks. There was no correlation between period and phase of entrainment under semi-natural conditions within the *control* stocks across both sexes (Figure 2.6; Table 2.4).

It is certainly intriguing as to why there is no change in φ as a consequence of change in τ. It is now very well established that phase-relationship with the zeitgeber is of immense significance in terms of being adaptive (reviewed in Vaze and Sharma, 2013). Studies done previously have reported that phasing activity to certain times of the day is adaptive in terms of finding mates, avoiding predation and finding food (Cloudsley-Thompson, 1960). It has

also been reported that *Drosophila* parasitoid species segregate their phases of activity in order to reduce among strain competition (Fleury et al., 2000). It had been discussed that phase of entrainment in period mutants of cyanobacteria are maladaptive, thereby reducing fitness under competition in a non-resonating condition (Ouyang et al., 1998). In another study it was shown that the malarial parasites' in-host survival and between-host transmission potential was reduced due to desynchrony in phases of the parasite and the host (O'Donnell et al., 2011). All such literature surveys indicate towards the fact that the primary role of possessing circadian clocks is to time behaviour and physiology appropriately. If this were the case, then it does seem intuitive that relatively small differences in circadian period might not translate into corresponding differences in phases of entrainment as predicted by either the parametric or non-parametric models of entrainment.

The question now is how circadian oscillators entrain. What is the mechanism by which differences in circadian period are restricted from translating into corresponding, covarying differences in phases of entrainment? The non-parametric model posits that a difference in τ and T is corrected for by appropriate phasing of the rhythm such that required regions of the PRC are exposed to light (Pittendrigh and Daan, 1976). Had this been the case, a difference in the phases of locomotor activity rhythms of the *early*, *control* and *late* stocks would be expected given the difference in their τ (Figure 2.2a). The parametric model considers entrainment by changes in the velocity of phase progression of the oscillator in a phase dependent manner (Swade, 1969). It has been shown that in wild type and *per* mutants of *Drosophila*, with increasing light intensity above 0.1 lux, the circadian period of locomotor activity rhythms increases (Konopka et al., 1989; Matsumoto et al., 1994). If this were the

case, then it is justified to expect that long photoperiods induce circadian period lengthening and this is what we find. Following entrainment to a long photoperiod (LD18:06), the circadian period significantly lengthens compared to that post entrainment to a regular photoperiod (LD12:12) in the GATE stocks (data not shown). This is in contrast to what has been shown previously in *Drosophila* (Tomioka et al., 1997). One reason for such a discrepancy could be due to the fact that in Tomioka's study the photoperiod condition was provided during development and not in adult stages. Although, there seems to be evidence in favour of parametric effects of light on the circadian period of the GATE stocks, these effects do not differ among the *early* and *control* and *late* stocks i.e. the interaction effect between stock and photoperiod on circadian period is not significant. Given this information, one can imagine two possible hypotheses to explain such discrepancy.

τ-φ Coupling Component Hypothesis (CCH)

From the association studies between circadian period of locomotor activity and the phase of locomotor activity rhythm and that of eclosion rhythm it is clear that period and phase are regulated by different components. Had they been outputs of the same oscillator, one would observe covariance between these variables. But, in our experiments we observe that for a range of period values the phase of entrainment assumed by the individuals is similar, thereby indicating a role for a secondary component. The fact that period and phase could be outputs of different oscillators was well recognised by Pittendrigh (Pittendrigh, 1981). Pittendrigh found that the phase-relationship of the eclosion peak with that of the LD cycle was not temperature compensated in *D. pseudoobscura*. Based on this he proposed that there must be a second oscillator viz., a “slave” or peripheral oscillator that directly regulates the overt

rhythm's timing and is temperature dependent (Pittendrigh, 1981). Pittendrigh, however, firmly believed that the phase of this "slave" oscillator is driven strongly by that of the "master" oscillator or the circadian pacemaker.

I refer to the "master" oscillator as the τ setting component (τSC) and the slave oscillator as the φ setting component (φSC). The CCH (Figure 2.7) can be formalised as follows:

a) There is one τSC that acts as a pacemaker and sets the free-running period. Evidence for this comes from the fact that disruption of the *period* gene affects the free-running period of eclosion and locomotor activity rhythms in *D. melanogaster* in a similar fashion (Konopka and Benzer, 1971). Another evidence comes from a strong positive correlation between the circadian period of eclosion rhythm and that of locomotor activity rhythm (Kumar et al., 2007).

b) There is a φSC for each overt rhythm which is directly responsible for phasing the rhythm and there is a coupling component between the τSC and the φSC for each behaviour. Evidence for this come from results reported here. For the same difference in period values among *early*, *control* and *late* stocks, phases of eclosion rhythm differ whereas that of locomotor activity rhythm do not under their native as well as low light intensities (Figure 2.2). Also, for the same range of period values there exist a significant correlation with phase of emergence across populations under different light intensities and there is an absence of such association in case of locomotor activity rhythm across and within populations under different light intensities and semi-natural conditions (Figures 2.3, 2.4, 2.5 and 2.6).

c) The expression of the coupling component between the τSC and the φ SC is driven by at least two features viz., the nature of the zeitgeber and the range of period values generated by

the τSC. Based on the aforementioned data set (Figures 2.2, 2.3, 2.4, 2.5 and 2.6) one can conclude that either the coupling component between the τSC and the φ SC of eclosion rhythm is stronger than that of the locomotor activity rhythm or the coupling component is just turned ON or OFF in the presence or absence of a particular zeitgeber. It is seen that the *early*, *control* and *late* stocks significantly differ in their phases of entrainment of locomotor activity rhythm under temperature cycles as predicted based on their circadian period (Nikhil KL, unpublished data). Moreover it is observed that the extent by which *early* or *late* stocks differ from their *control* stocks are not affected by whether the rhythm under study is eclosion or locomotor activity (data not shown). If the coupling component between the τSC and φSC of the eclosion rhythm was indeed stronger than that of the locomotor activity rhythm, then for the same difference in period, phase divergence between the *early* and *late* stocks would be greater in case of eclosion rhythm than that in locomotor activity rhythm. Given that this is not the case, we can safely conclude that the strength of this coupling component does not differ between the two rhythms, but it just gets either turned ON or OFF based on the zeitgeber in question. If indeed light was keeping the coupling component between the τSC and φSC of locomotor activity rhythm OFF, then how can one explain the difference in phases of entrainment of the short and long period mutants in *D. melanogaster* as compared with their wild type controls (Hamblen-Coyle et al., 1992)? This can be explained by a second condition that may activate the coupling component. The circadian system attempts to maintain a stable, adaptive phase-relationship with the zeitgeber. In order to do so, it must be buffered against perturbations in period values, but only to a certain extent. One can imagine a range around the mean period value of any stock on either side. Within this range the differences in period values are unlikely to get translated into corresponding, covarying phases of entrainment.

Within this range, all period values will attempt to maintain a similar phase of entrainment. Outside this range there exists a different range which has its own corresponding phase of entrainment. The mean phases of entrainment corresponding to such ranges are correlated with the mean circadian period of the concerned range. This would explain the correlation between period and phase of locomotor activity rhythm in the *period* mutants of *D. melanogaster*. Experimental evidence favours this notion as it has been shown that there is no correlation within short or long *period* mutant stocks of *D. melanogaster* but there is a significant correlation across stocks (Manishi et al., unpublished data). From data reported here it is likely that although period values of *early*, *control* and *late* stocks differ significantly, they still belong within the range and do not therefore get translated into corresponding, covarying phases of entrainment. A careful observation of Figure 2.4b will reveal the presence of a few individuals with really long period values and these individuals also seem to have an overall delayed phase of entrainment as compared with the remaining bulk of the individuals. It is likely that those individuals contribute towards the significant correlation between period and phase of locomotor activity rhythm within the *control* stocks (Figure 2.4b; Table 2.4). These arguments therefore provide evidence in favour of the conjecture that the expression of the coupling component is driven by the nature of the zeitgeber and the range of period values generated by the τSC. Therefore, the CCH provides a plausible mechanism by which differences in period can be restricted from translating into covarying phases of entrainment.

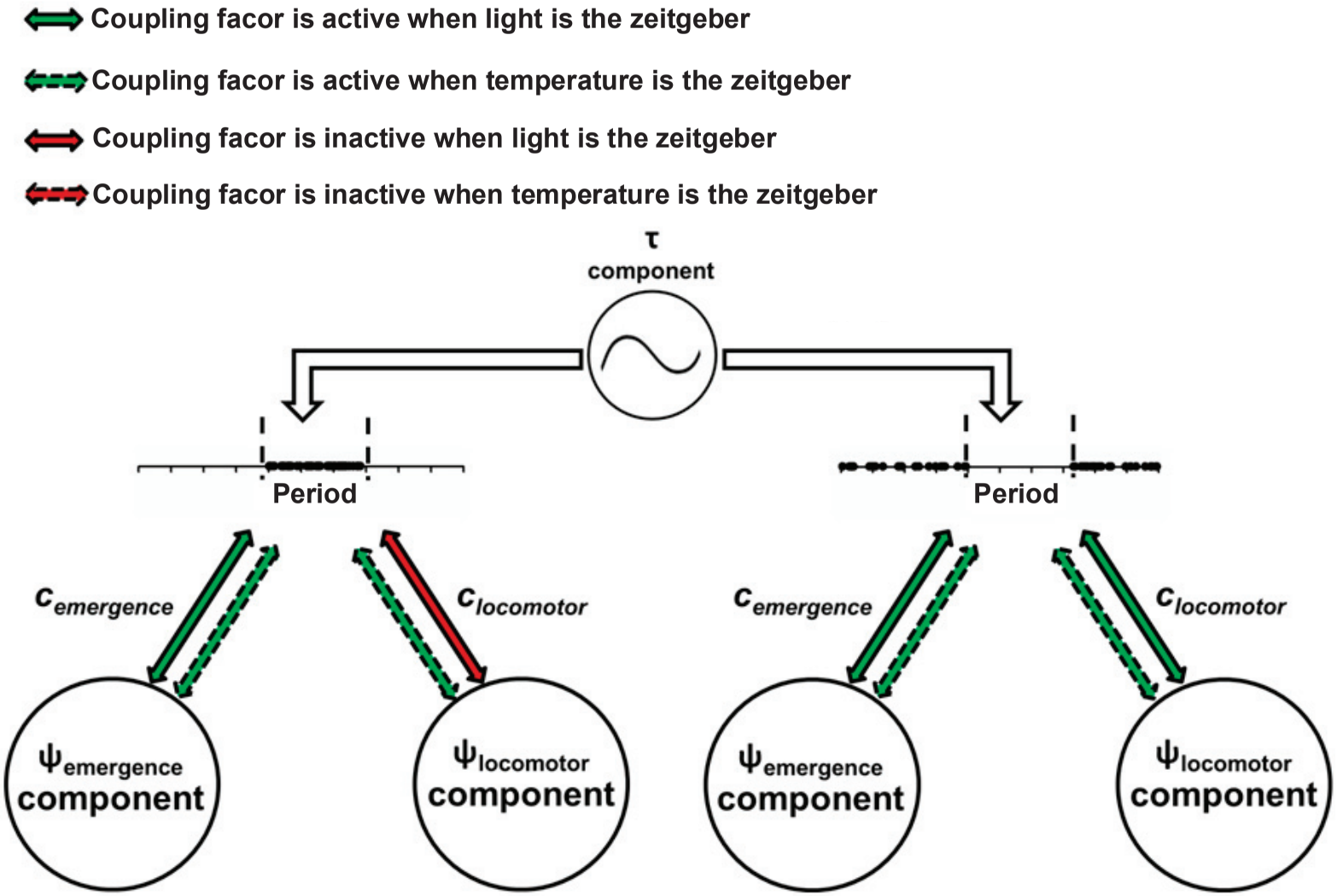

Figure 2.7: A schematic for the Coupling Component Hypothesis (CCH) to aid understand the functional organisation of the circadian system in order to maintain adaptive phase relationships. The black dashed lines indicate the threshold of period values beyond which the coupling factor ($c_{emergence/locomotor}$) dynamics change (See text).

CIRC

τ-φ correlations invariably emerge when one attempts to explain entrainment using traditional mechanisms. The circadian integrated response characteristic was proposed recently and this allowed for the possibility of entrainment of oscillators with different period values without a difference in the underlying phase of entrainment (Roenneberg et al., 2010). This model assumes that the circadian system is capable of integrating zeitgeber signals over time. This is a reasonable assumption to make and has gained experimental evidence from a study that showed the ability of mammalian SCN sections to integrate light signals over time (Dkhissi-Benyahya et al., 2000). Also the nature of the model is such that it does not invoke the use of either the pacemaker or the peripheral oscillators in order to explain the mechanism of entrainment. This model could be used to treat each rhythm as a system attempting to entrain by integrating zeitgeber signals over time in order to expand or compress its limit cycle based on its circadian period. In keeping with this model, one could imagine how *early*, *control* and *late* stocks maintain similar phase-relationships with the zeitgeber despite having significant differences in their period values. These stocks could have possibly evolved asymmetry in their expansion to compression zones, thereby still attaining the necessary light input in order to entrain without shifting their phases. Therefore, the use of the CIRC provides the second plausible hypothesis which could explain entrainment patterns of locomotor activity rhythm in the GATE stocks.

The following chapters will deal with the attempt towards finding evidence in favour of each of these hypotheses. The next chapter will discuss the possibility of τ and φ being outputs of different components and whether or not we can find evidence in favour of distinct genetic

components that regulate only circadian period or only phase of entrainment. The chapter after that will discuss the general use of the CIRC for entrainment to LD cycles and will explore the hypothesis of differential evolution of the asymmetry in the expansion to compression zone between the GATE stocks.

Chapter 3

Are circadian period and phase of entrainment regulated by different components?

Introduction

Periodicity and phase of entrainment are among the defining features of circadian rhythms and the relationship between them has been believed to be of ecological and physiological relevance (Cloudsley-Thompson, 1960; reviewed in Vaze and Sharma, 2013). It was predicted from oscillation theory that oscillators with higher frequencies (shorter periodicities) will entrain with an advanced phase, relative to those with low frequencies (longer periodicities) (c.f. Aschoff, 1965). In an early study Aschoff (1965) showed that this was also true for the activity/rest rhythm of lizards and chaffinches. Pittendrigh also proposed that rhythms with shorter periodicities will phase lead the rhythms with longer periodicities with respect to a phase of reference of the zeitgeber (Pittendrigh and Daan, 1976). Evidence for these as discussed in previous chapters have been documented in the *period* mutants in *Drosophila* (Hamblen-Coyle et al., 1992), for the GATE stocks (Kumar et al., 2007), for *Drosophila* populations under selection for a narrow window of adult emergence (Kannan et al., 2012) and in humans (Duffy and Czeisler, 2002; Wright et al., 2005). However, the fact that such relationships are not ubiquitous has been exemplified in the previous chapter. We proposed that there is a possibility of two distinct components governing the features of circadian rhythms viz., first is the oscillator that governs the periodicity under constant conditions and the other is a phasing component. We also went on to suggest that the relationship of phase of entrainment with the circadian period is dictated by a zeitgeber dependent coupling components as delineated in the previous chapter under the τ-φ Coupling Component Hypothesis (CCH). Before dealing with the coupling component it would be important to gain evidence suggesting that such a dual component model is indeed plausible. In order to do so we adopted two approaches. First, we used a van der Pol oscillator model to simulate the

process of entrainment and study the relationship between circadian period and phase of entrainment. The van der Pol oscillator is modelled using a second order differential equation which resembles a simple harmonic oscillator but with the added component of a non-linear damping term ($\varepsilon(1-x^2)$). The non-linear damping term determines how quickly the oscillator will dampen its rhythm under constant conditions. This model has earlier been used to understand entrainment of circadian rhythms but asked different questions from those asked here (Pavlidis, 1973). Therefore, we know the parameter space for the oscillator that best models entrainment in reference to circadian rhythms. Given that this is a single oscillator model, we hypothesised that the absence of τ-φ relationship for all parameter combinations explored could be taken as evidence for the fact that if τ and φ were outputs of the same oscillator they cannot be uncorrelated. This must hence mean that τ and φ are indeed regulated by different components. Second, we use *Drosophila* deficiency lines from the DrosDel deficiency kit (Ryder et al., 2007) to screen for genetic regions that may solely regulate circadian period or phase of entrainment.

Materials and Methods

Simulating the τ-φ relationship using a van der Pol oscillator

In order to model the τ- φ relationship where both τ and φ are outputs of the same oscillator, we use a forced van der Pol oscillator. The equation has the following form:

$$\boldsymbol{\frac{d^2x}{dt^2} + \varepsilon(1-x^2)\frac{dx}{dt} + \omega_0^2 x = E\cos\omega_1 t} \quad \textbf{.....(i)}$$

where, x is the output of the oscillator or the state variable, $\boldsymbol{\varepsilon}$ is the degree of non-linearity of the oscillator, ω_0 is the free-running frequency of the circadian oscillator, E is the zeitgeber amplitude and ω_1 is the frequency of the zeitgeber.

Let,

x=y(1).....(ii)

and

dx/dt=y(2).....(iii)

Substituting ***(iii)*** in ***(i)*** we get,

$$dy(2)/dt = E\cos\omega_1 t - \varepsilon(1-y(1)^2)y(2) - \omega_0^2 y(1) \quad \text{.....(iv)}$$

From ***(ii)*** and ***(iii)***, we get,

$$dy(2)/dt = y(2) \quad \text{.....(v)}$$

Equations ***(iv)*** and ***(v)*** have been used to numerically solve for ***x***. First we sample 300 τ values randomly from a uniform distribution of τ between 19 and 29 h. These τ values are then converted to frequencies using the relation, $\omega_0 = 2*pi/\tau$. Then, using the ordinary differential equation (ODE) solver inbuilt for MATLAB (ode45) we solve this differential equation for 2400 time steps starting from 0.1 in intervals of 0.1 for all these values of τ (Appendices A1 and A2). Given that this equation has been solved as an initial value problem, we discard the first 10 cycles of the solution in order to allow the system to stabilise (each cycle is of 24 h consisting of 240 time steps each). We then average the output of this oscillator i.e., the value of ***x*** over 30 cycles for all period values. This is done in order to account for error due to approximations during integration by the ODE solver. The CoM of these averaged profiles is then computed for each value of τ as described in Chapter 2. Analytical studies done earlier showed that quality of entrainment is a function of ε and E_L (Pavlidis, 1973). Pavlidis showed that stable entrainment can occur if, $0.2<\varepsilon<0.65$ and $2<E_L/E<3$. Therefore, the parameter values of ε used in our simulations are 0.35, 0.45, 0.55 and 0.65. Pavlidis derived a

relationship to compute E_L which has been outlined below.

$$E_L = 1.414(\omega_0^2 - \omega_1^2) + \varepsilon\omega_1/1.414$$

The range of ω_0 in our simulations is very small. Therefore, the mean of all the ω_0 in every simulation is computed and is used to calculate E_L. From this E_L the minimum and maximum required E are computed. Then, 1000 E values are randomly chosen between the minimum and maximum values from a uniform distribution of E values. The minimum and maximum E values among the 1000 generated E values are used for every ε. This allowed us to study the relationship of τ and φ when both these are outputs of the same oscillator.

Our hypothesis was that there is a secondary component that regulates the φ such that it does not change for a range of τ values. To do this, we chose three ranges of period values viz., 19-22.5, 22.5-25 and 25-29 h. Corresponding phases to each period values within each of these ranges of period values were averaged and their standard deviation was computed. For each period range now, we randomly draw phase values for each period value with a mean phase of that range and half the standard deviation of the phases in that range of period values. This manipulation to the single oscillator, thus, simulates the condition where a secondary component determines the phases of entrainment within a range of period values.

Deficiency screening

In order to find out if there were distinct genetic components that regulate period alone or phase alone a preliminary deficiency screening was performed. We used deficiency lines from the DrosDel Deficiency Kit (Ryder et al., 2007). These are deficiency lines maintained over balancers and all the deficiencies are created on a w^{1118} genetic background. The advantage of

using such lines is that all deficiencies on the same chromosome have the same balancers, thereby reducing the components that could lead to variation in any trait. Virgin males and females were collected from each of these lines and six males and six females (aged to 3-4 days) for each deficiency line were used for preliminary screening. All these flies were loaded into DAM systems and their activity was recorded under LD12:12 (~70 lux) at 25 °C and ~70% RH for six days and then were transferred to constant darkness to estimate their free-running periodicities. They were recorded in DD for another six days. All these experiments were done in locomotor activity tubes with standard corn medium. CoM for each individual and for each line was computed as described in Chapter 2. Free-running period was also calculated using methods described in Chapter 2. Only lines that had at least four flies till the end of the experiment were used in any of our analyses. All comparisons of period or phase for each line have been made with w^{1118} lines.

Statistical procedures

All τ-φ circular-linear correlations from simulation data were carried out as described in Chapter 2. Multiple one factor ANOVAs were carried out to test for the effect of deficiency line on mean period and mean phase. A Tukey's Honestly Significant Difference test was performed for post-hoc where required (from Zar, 1999). Levene's test (Levene, 1960) was performed in order to test if among strain variance in period was different from among strain variance in phase of entrainment. Different Levene's tests were performed for lines deficient for different regions of the 2nd and 3rd chromosomes respectively. All the analyses were carried out using STATISTICA v5.0 (StatSoft Inc., Tulsa, OK). All results were considered significant at alpha=0.05.

Results

τ-φ correlation

Circular-linear correlation analyses were performed to see if across a range of period values within a set parameter space whether or not there can be a scenario where τ-ψ are uncorrelated if they are both outputs of the same circadian oscillator. Results suggested that for either of the ε value tested and for either of the E values sampled, there was a strong positive correlation of phase of entrainment with period (Figures 3.1, 3.2; Table 3.1). It was observed that with increasing period values phase lag with respect to the zeitgeber increased as predicted earlier (Pittendrigh and Daan, 1976).

When a secondary component is present that is supposed to restrict such covariance of period and phase within a small range of period values, we see yet again that a very strong association between period and phase still exists across the whole range of period values (Figures 3.1, 3.2; Table 3.1). The presence of this secondary component, however, is successfully able to restrict the covariation among period and phase within small period ranges (Table 3.2). There is no significant correlation between period and phase for all combinations of ε and E for all small period ranges, except one. This one positive correlation explains only ~32% of the variation and is also likely to be an artefact of large number of sampled period values within that range.

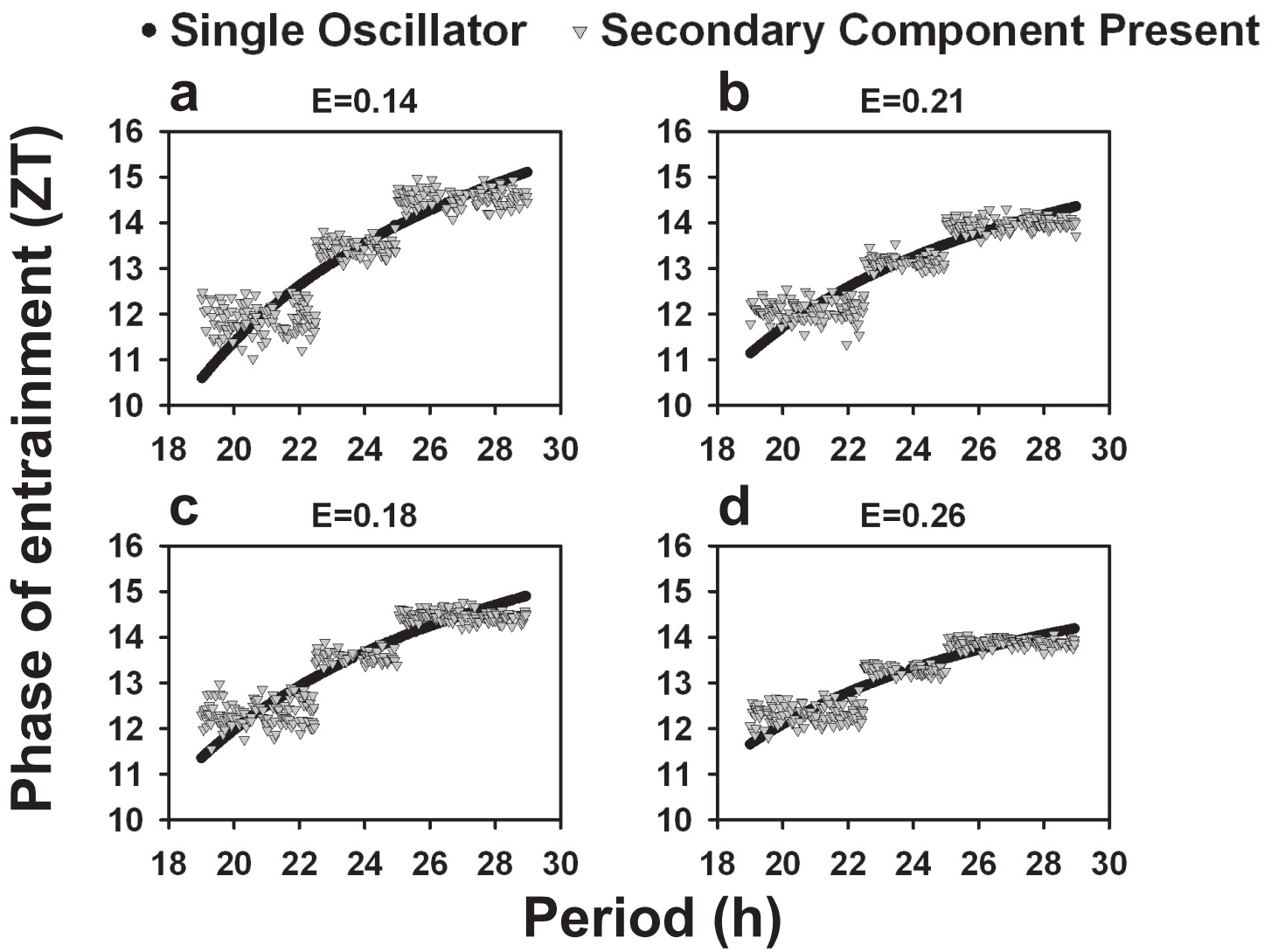

Figure 3.1: Circular-linear correlation between sampled circadian period and simulated phase of entrainment across period ranges. (a) and (b) Correlation at ε=0.35. (c) and (d) Correlation at ε=0.45. All correlations reported here are significant at alpha=0.05. Note that the scales are different.

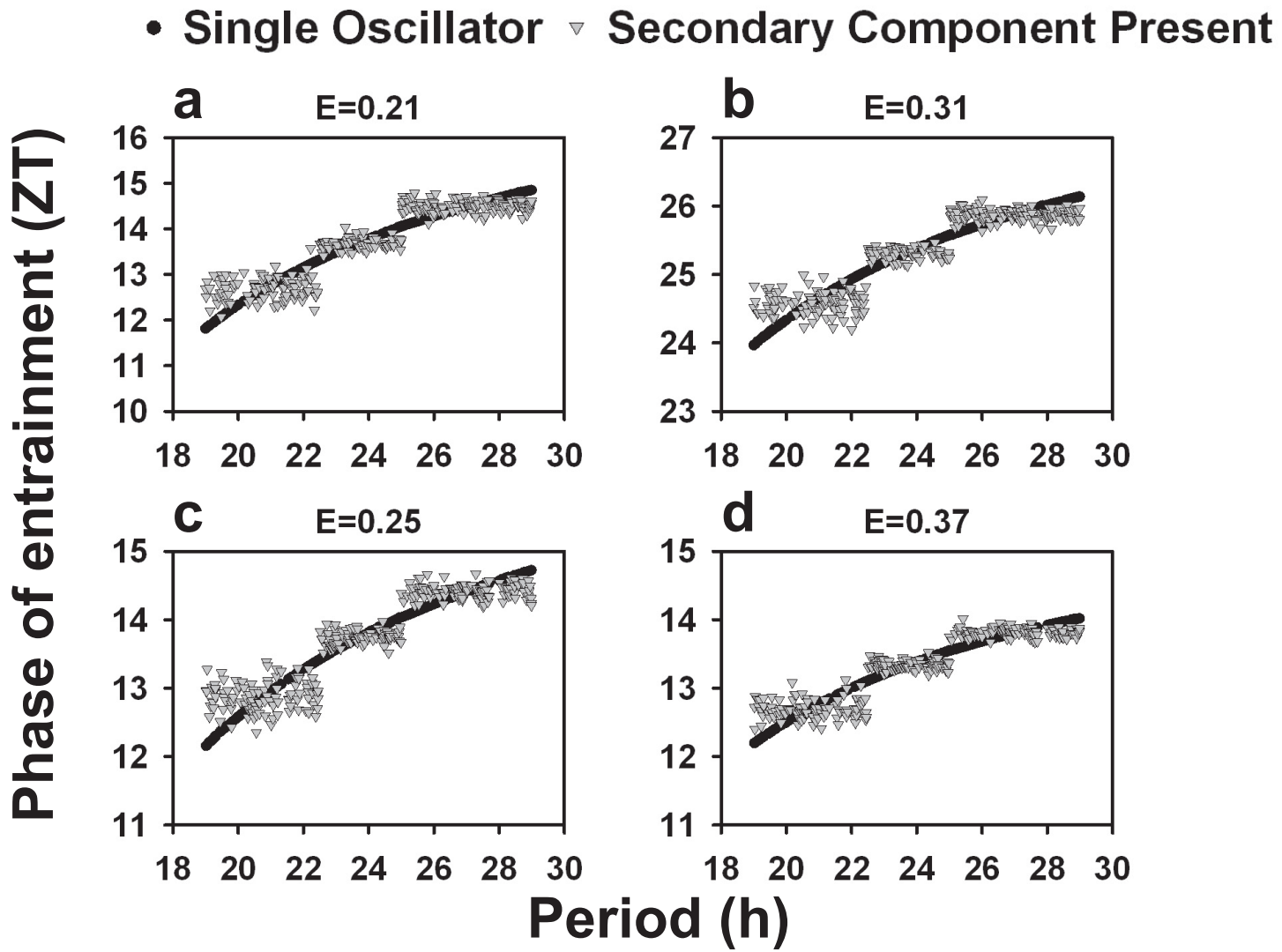

Figure 3.2: Circular-linear correlation between sampled circadian period and simulated phase of entrainment across period ranges. (a) and (b) Correlation at ε=0.55. (c) and (d) Correlation at ε=0.65. All correlations reported here are significant at alpha=0.05. Note that the scales are different.

		Output of Single Oscillator		Secondary Component Present	
ε	**E**	**r**	***p***	**r**	***p***
0.35	***0.14***	*0.99*	*<<0.05*	*0.91*	*<<0.05*
	0.21	*0.99*	*<<0.05*	*0.92*	*<<0.05*
0.45	***0.18***	*0.99*	*<<0.05*	*0.92*	*<<0.05*
	0.26	*0.99*	*<<0.05*	*0.92*	*<<0.05*
0.55	***0.21***	*0.99*	*<<0.05*	*0.92*	*<<0.05*
	0.31	*0.99*	*<<0.05*	*0.91*	*<<0.05*
0.65	***0.25***	*0.99*	*<<0.05*	*0.92*	*<<0.05*
	0.37	*0.99*	*<<0.05*	*0.92*	*<<0.05*

Table 3.1: Results of circular-linear correlations performed on sampled circadian period and simulated phases of entrainment across period ranges. Italicised comparisons report significant correlations at alpha=0.05.

		Short period range		Intermediate period range		Long period range	
ε	**E**	**r**	***p***	**r**	***p***	**r**	***p***
0.35	**0.14**	0.05	>0.05	0.03	>0.05	0.03	>0.05
	0.21	0.14	>0.05	0.08	>0.05	0.18	>0.05
0.45	**0.18**	0.11	>0.05	0.10	>0.05	0.11	>0.05
	0.26	0.13	>0.05	0.28	>0.05	0.12	>0.05
0.55	**0.21**	0.14	>0.05	0.26	>0.05	0.15	>0.05
	0.31	0.05	>0.05	0.06	>0.05	0.14	>0.05
0.65	**0.25**	0.11	>0.05	*0.33*	*<0.05*	0.10	>0.05
	0.37	0.10	>0.05	0.06	>0.05	0.15	>0.05

Table 3.2: Results of circular-linear correlations performed on sampled circadian period and simulated phases of entrainment within period ranges. Italicised comparisons report significant correlations at alpha=0.05.

Mean period of deficiency lines

There was no significant effect of deficiency lines on the mean circadian period ($F_{17,70}$=0.84, p>0.05). Therefore, no post-hoc analysis was necessary (Figures 3.3a and b; Table 3.3a).

Mean phase of entrainment of deficiency lines

Mean phases of entrainment also did not significantly differ among deficiency lines ($F_{17,105}$=0.89, p>0.05). Therefore, there was no need for a post-hoc analysis (Figures 3.3c and d; Table 3.3b). However, the mean variation in phases between the lines was high.

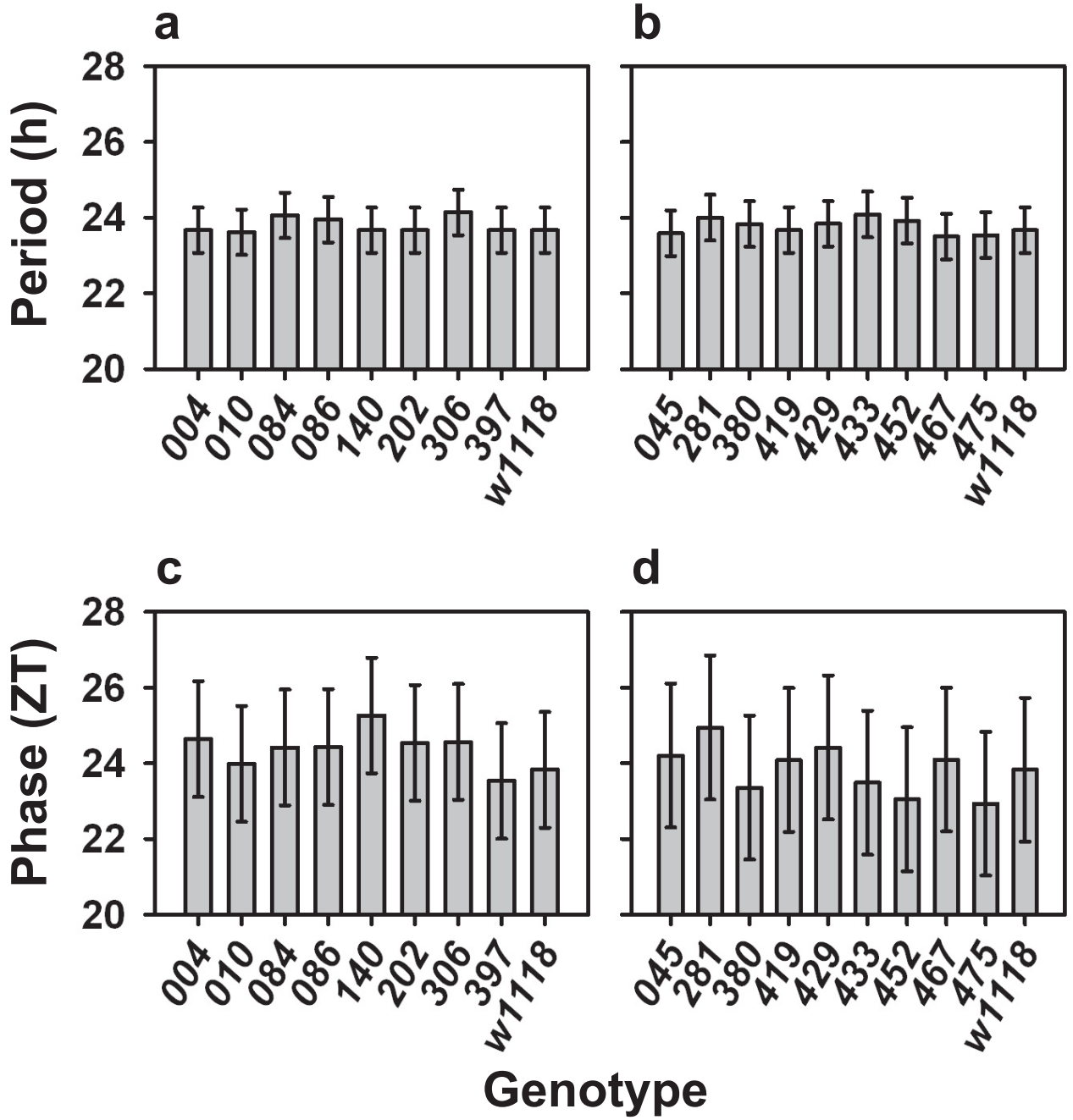

Figure 3.3: Deficiency screening for circadian period (in DD) and phase of entrainment of locomotor activity rhythm (~70 lux, LD 12:12). Mean period of genotypes with deficiencies in (a) The 2nd chromosome and (b) The 3rd chromosome. Mean phase of lines with deficiencies in (c) The 2nd chromosome and (d) The 3rd chromosome. Error bars are 3xSD of mean across genotypes. Non overlapping error bars, thus, indicate outliers.

a

	df Effect	MS Effect	df Error	MS Error	*F*	*p-level*
Deficiency Line	17.00	0.20	70.00	0.24	0.84	0.65

b

	df Effect	MS Effect	df Error	MS Error	*F*	*p-level*
Deficiency Line	17.00	2.59	105.00	2.90	0.89	0.58

Table 3.3: Results of analysis of variance (ANOVA) performed to test the effect of Deficiency Line. (a) ANOVA on mean circadian period of locomotor activity rhythm. (b) ANOVA on mean CoM of locomotor activity rhythm. Deficiency Line is used as a fixed factor. Italicised effects are significant at alpha=0.05.

Among lines variance in period and phase of entrainment

While analysing the mean data for phases of entrainment, it was observed that, though no significant effect of deficiency lines on phase existed, there were some lines that showed differences in mean phase by an order of about 1 h, suggesting contribution of genetic material to overall variance in the phenotype. The activity profiles of these lines have been shown in Figures 3.4a and 3.4b. In order to quantify whether among line variance was significantly higher in case of phase of entrainment than in circadian period, Levene's test for homogeneity of variances was performed. This test was performed separately for lines that have deficiency in the 2nd chromosome and that have deficiency in the 3rd chromosome. This was done in order to avoid the possibility of balancers on each of these chromosomes having a contribution to overall phenotypic variance. It was seen that there was no significant difference in variances between period and phase for lines having deficiency in the 2nd chromosome although there was a highly significant difference in variances between the period and phase of lines having deficiency in the 3rd chromosome (Table 3.4). The among line variance is higher for phases by 10 fold as compared with that of circadian period in the lines with a deficiency in the 3rd chromosome and by a slightly smaller margin in case of lines having a deficiency in the 2nd chromosome (Figure 3.5).

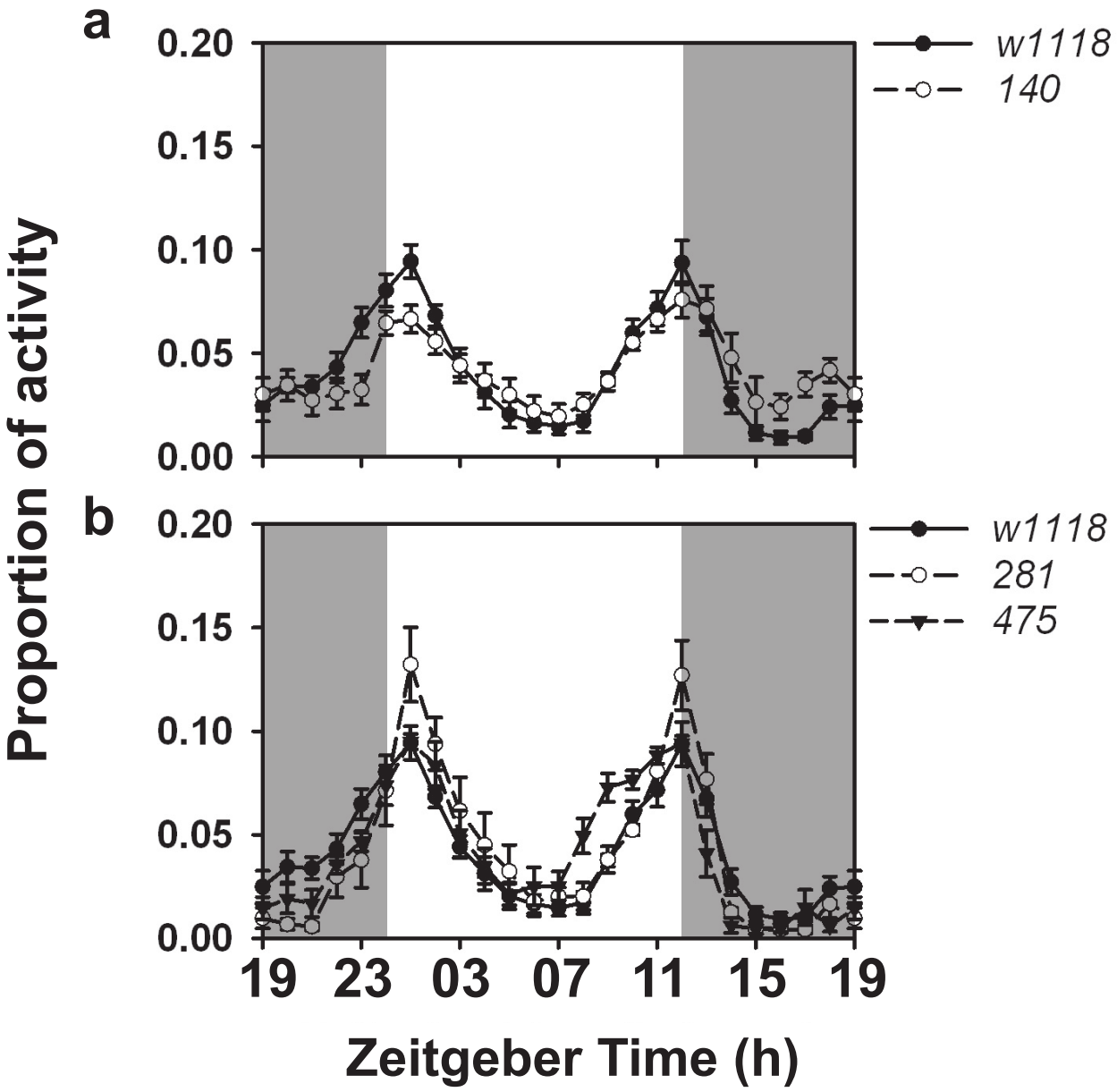

Figure 3.4: Normalised mean activity profiles of deficiency lines exhibiting, albeit non-significant, highly deviant phases of entrainment. (a) Lines with deficiency in the 2nd chromosome. (b) Lines with deficiency in the 3rd chromosome. Error bars are SEM. Grey shaded regions indicate scotophase of the LD cycle and the unshaded regions indicate the photophase.

a

	MS Effect	MS Error	*F*	*p-level*
Group	0.20	0.05	3.84	0.07

b

	MS Effect	MS Error	*F*	*p-level*
Group	*0.57*	*0.06*	*9.42*	*0.01*

Table 3.4: Results of Levene's test to detect differences in inter-strain variance between circadian period and phase of entrainment. (a) Test for deficiency of the 2nd chromosome. (b) Test for deficiency of the 3rd chromosome. Italicised effects are significant at alpha=0.05.

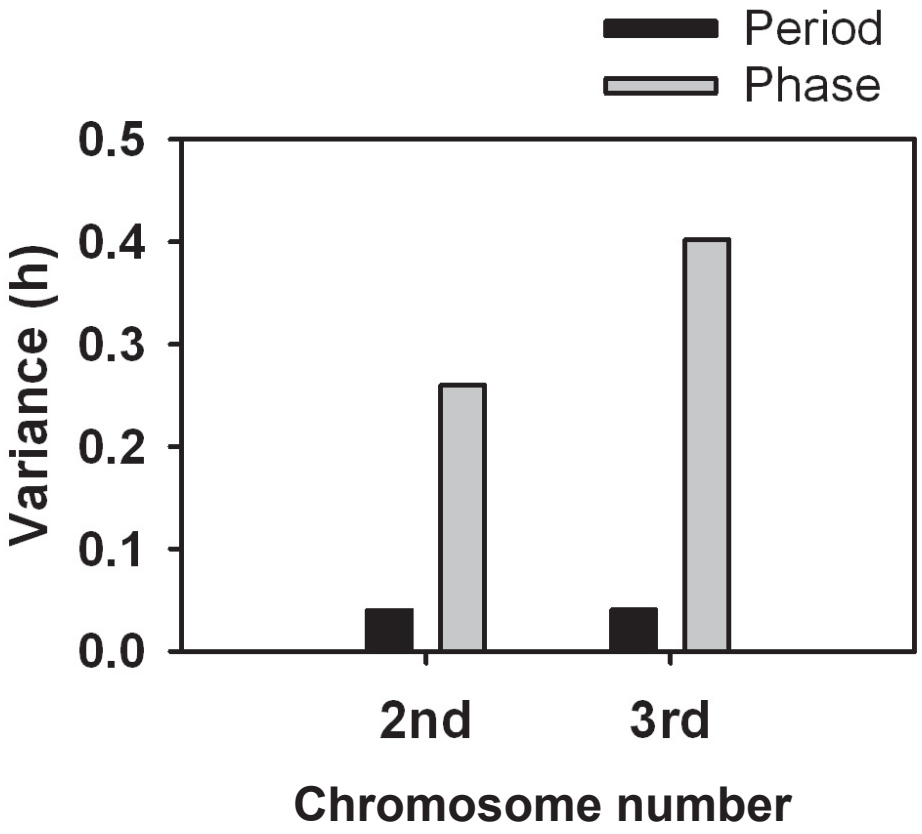

Figure 3.5: Variance in period and phase across deficiency lines. Variance in phase of entrainment is significantly higher among deficiency lines of the 3rd chromosome as compared with variance in circadian period.

Discussion

The τ-φ relationship that we simulated distinctly shows that they cannot be uncorrelated if both were outputs of the same oscillator. The linear correlations performed across the entire period range exhibit correlation coefficients over 0.9 in all cases (Table 3.1). This implies that a straight line can be used to model this relationship with >90% of the variation being explainable. If τ and φ could vary independent of each other, then the slope of the line modelling a φ versus τ scatter would have a slope of 0 or infinity. It is clear from our data that this is not the case (Figures 3.1, 3.2). Though suggestive, this forms a mathematical foundation for us to believe that there is no possibility of τ and φ being uncorrelated if they are outputs of the same oscillator. The possibility that different oscillators could be responsible for regulating circadian period and timing of an overt rhythm was recognised early on in the field (Pittendrigh, 1981). But, how this knowledge builds our understanding of maintenance of adaptive phase-relationships and entrainment mechanisms has not been particularly clear.

However, if a secondary component (i.e. the φSC) was present in the system and there was some crosstalk between the φSC and the τSC, then one could imagine the possibility of there being maintenance of adaptive phase-relationships within a range of period values. The coupling component could then be involved in determining the role of τSC in regulating the functioning of the φSC. If this were indeed the case, then across a large range of period values τ and φ could still covary significantly but within small ranges of period values there may not be any covariance of these two (Figures 3.1, 3.2; Table 3.2; Refer to Figure 2.7). This could be taken as evidence in favour of the fact that there could be two distinct components that regulate τ and φ respectively.

The use of deficiency lines did not yield very clear results on whether or not we could identify distinct genetic components that regulate period and phase. It was seen that for neither period nor phase of entrainment there was a significant effect of deficiency lines (Table 3.3). This implies that the mean period and phase values do not differ from each other or with respect to their background controls (Figure 3.3). However, the phenotypic variation in period was lower by ~10 fold as compared with that in phase of entrainment (Figure 3.5). This information allows us to carefully speculate that there is a greater genetic contribution to variation in phase values as compared with that in period values. Phases of entrainment seem to be more labile and are subject to change more readily than period values due to genetic changes. This is, although not conclusive, preliminary evidence towards the fact that period and phase may be regulated by different genetic components.

One deficiency line tested (DGRC Id: 150140) has the region of chromosome missing between 23C4 and 23F6 on the cytological map. This is the region of the chromosome that houses a core clock gene *timeless*. The *tim* null mutants are arrhythmic for both eclosion and locomotor activity rhythms (Sehgal et al., 1994). The deficiency line housing *tim,* as compared with its wild type controls, shows a very small change in mean period (~0 h, Figure 3.3a) and a large change in phase of entrainment (~1.5 h, Figures 3.3c, 3.4a). Similar features were observed for two other deficiency lines. Genotypes *281* (DGRC Id: 150281) with deficiency between 75B1 and 75C6 on the cytological map and *475* (DGRC Id: 150475) with a deficiency between 99A5 and 99C1 on the cytological map showed a difference in period of about 0.3 h and 0.1 h (Figure 3.3b) but showed a difference in phase of about 1.12 h and 0.89 h (Figures 3.3d and 3.4b) respectively as compared with their controls. These are again suggestive of the

fact that haploid copies of certain genes could potentially affect only phase and not period of at least locomotor activity rhythm.

All these deficiency lines are heterozygous with balancers on the other homolog. Therefore any sets of genes that only have a dominant effect on the period or phase could be detected via the preliminary screen reported here. One reason for not detecting differences in period or phases among lines could be due to the fact that the deficiencies in the regions tested house genes that may have a recessive or epistatic effect on either of these traits. Also, all the tested regions cover only a very small region of the entire genome of *D. melanogaster*. Hence, no major conclusions can be drawn from these small number of lines tested. A larger scale screening with large sample sizes from each deficiency line needs to be performed before any conclusions can be made on the different genetic components regulating period and phase of circadian rhythms.

Other indirect evidence also exist which suggest that period and phase are regulated by different components. It was shown in an earlier study that males have a significantly shorter period of locomotor activity rhythm than females and they also have an advanced phase of morning peak of activity relative to that of females in the three strains of *Drosophila* (Helfrich-Förster, 2000). Some of our experiments reveal that males from large, outbred populations of *Drosophila* have significantly advanced phases of emergence relative to that of females. But in these populations the period does not differ among males and females (data not shown). This implies that there must be some genetic bases for distinct regulation of period and phase.

Although, not substantial and conclusive, there is compelling evidence towards the possibility of there being two distinct components that regulate circadian period and phase of entrainment viz., the τSC and the φSC.

Chapter 4

Do populations of *Drosophila melanogaster* use the Circadian Integrated Response Characteristic to entrain?

Introduction

The means of entrainment, as argued earlier, is the key to rendering the circadian clock's functionality to any organism. Most organisms live in rhythmic conditions and therefore the functionality of a circadian clock lay more in its ability to tell external time than maintain a precise internal time. This allows the organism to maintain an adaptive phase-relationship with the zeitgeber (Cloudsley-Thompson, 1960; Pittendrigh, 1981). One can imagine that the organism associates cycling of the zeitgeber to something relevant in its ecology that is crucial for its survival and perpetuation. The organism, thus, tries to recognise external time as accurately as possible.

Entrainment is the mechanism by which circadian clocks attempt to equalise its period to that of the zeitgeber. How it manages to do so has been a mystery for a very long time. Two major theories of entrainment form the backbone of what we understand about the mechanisms of entrainment even today. On the one hand we have the PRC or non-parametric or phasic theory (Pittendrigh, 1981) and on the other we have the VRC or parametric or tonic theory (Aschoff, 1964; c.f. Daan, 2000; Swade, 1969). The PRC theory of entrainment suggests that an organism with a given inherent clock period adjusts its phase such that the zeitgeber stimulus falls on that region of the PRC that elicits a shift in the oscillator's phase by a magnitude equal to the difference between its own period and that of the zeitgeber. It is believed that zeitgeber stimuli during dawn and dusk are sufficient for entrainment of the circadian clock (Pittendrigh and Daan, 1976). The PRC theory assumes that zeitgeber during other times of the day are redundant (for a review on PRC theory of entrainment see, Johnson, 1999). There are evidences that this need not necessarily be true. It has been shown in the recent past that

eclosion waveforms of populations of *Drosophila* under skeleton photoperiods (light only during dawn and dusk) are different from the ones under full photoperiods of equal light and dark durations (Vaze et al., 2012a). There are some animals that live in burrows and therefore do not see light during dawn or dusk such as the European ground squirrel. Explaining entrainment of circadian clocks in such organisms is problematic using the non-parametric model (Hut et al., 1999). Contributions of light to dark and dark to light transitions to phase-shifts have also been studied using the step PRC approach (Kramm, 1974; Subbaraj and Chandrashekaran, 1981; Albers, 1986; Aschoff, 1994; Comas et al., 2008). Such studies conclude that the non-parametric mechanism of entrainment is not sufficient to explain entrainment. It was also observed that dark to light transitions during subjective dawn caused greatest phase delays and light to dark transitions during subjective dusk produced largest phase advances (Comas et al., 2008). This led the authors to conclude that the circadian clock does not shift its phase by non-parametric responses alone. The VRC theory of entrainment suggests that the change in velocity of phase progression in response to zeitgeber stimuli is a function of time of the day (Swade, 1969). This model rests heavily on the notion that zeitgebers have a continuous effect on circadian clocks. This model predicts steady state phase-relationship of circadian rhythms under long photoperiods reasonably well (Comas et al., 2006, 2007, Taylor et al., 2010), which is a significant limitation of the PRC theory (Remi et al., 2010). Despite these advantages, ultimately, the VRC can be estimated from the PRC (Daan, 1977) which makes both the models suffer from the same kind of assumptions that, in case of a PRC the steady state shift in phase is not due to transient changes in velocity of the clock and vice versa for a VRC.

More recently another generalistic model of entrainment was proposed that attempted to reconcile the two models of entrainment (Roenneberg et al., 2010). This model, as discussed earlier, uses the CIRC to explain entrainment of circadian programmes. The CIRC theory of entrainment requires the conceptualisation of circadian clocks as limit cycle oscillators. The fact that this conceptualisation may be meaningful has gathered evidence from a large body of theoretical work (Winfree, 1970, 1971; Peterson, 1980, 1981; Pavlidis, 1981; Taylor et al., 1982; Jewett et al., 1991; Johnson and Kondo, 1992). The CIRC proposes that the change in radius of the limit cycle representing the circadian clock in response to zeitgeber stimuli is a function of time of the day. During early subjective night or dusk the circadian limit cycle expands and during late subjective night or dawn it compresses to achieve entrainment with the zeitgeber. So far, all the other models of entrainment have used the circadian period to predict whether the clock needs to phase delay or advance more or it needs to accelerate or decelerate more in order to entrain. The CIRC model proposes that the circadian clock during entrainment changes its period in an attempt to equalise it to that of the zeitgeber. This period can be estimated by letting the clock free-run immediately post the entrainment protocol and is referred to as τ_E (τ during entrainment). It is τ_E and not τ that is central to predicting phases of entrainment in this model. Therefore, circadian limit cycles with τ_E shorter than T would adjust its rhythm such that a greater portion of the expansion zone of the CIRC is exposed to zeitgeber. The only assumption this model makes is that circadian timing systems are capable of integrating zeitgeber signals over time. Thus, the area under the curve of the CIRC region exposed to the zeitgeber is equal to the difference between τ_E and T. That circadian timing systems are capable of integrating light signals over time has found empirical evidence as well, thereby making this assumption a plausible one (Dkhissi-Benyahya et al., 2000). The

CIRC model is based on only two parameters, viz., the asymmetry factor (a) which is a measure of the asymmetry between the expansion and compression zones, and the shape factor (s) which is a measure of the extent of the dead zone.

So far, all models of entrainment reveal a fundamental relationship between the circadian period and the phase of entrainment. Depending on the circadian period, rhythms would be adjusted such that they expose the advance or delay, acceleration or deceleration, or expansion or compression zones of the PRC, VRC, or CIRC respectively. The CIRC theory, however, has an alternative possibility of entrainment. Depending on τ_E, the CIRC could alter the asymmetry between its expansion and compression zones such that the area under the curve could be different for different τ_E values. This mechanism would allow different periodicities to assume the same phase-relationship with the zeitgeber, and is possible only because circadian timing systems are assumed to have the ability of integrating zeitgeber signals over time. The *early* and *late* stocks, as shown in Chapter 2, have circadian periodicities coming from different distributions. Despite this they do not show any difference in their phases of entrainment. This provides evidence that the classical models discussed above are not sufficient to explain entrainment of circadian clocks in these populations. We hypothesised that these populations use CIRC to entrain, and therefore have evolved different asymmetry factors between the expansion and compression zones.

In order to test this we further hypothesised that relative to an LD12:12 protocol, subjecting the clock to a longer photoperiod (LD18:06) would expose different amounts of expansion and compression zones if the CIRC was asymmetric. This would lead to a difference between the

phase under LD12:12 and phase under LD18:06 of a magnitude greater than a scenario where the CIRCs are symmetric. Therefore, we studied the phase of entrainment of eclosion and locomotor activity rhythms of the GATE stocks under LD12:12 and LD18:06. To further specifically examine if asymmetries for locomotor activity rhythms were different among the *early* and *late* stocks, we modelled the CIRC and attempted to find the best fitting parameter values and the CIRC defined by it.

Materials and methods

Eclosion rhythm assay

The adult emergence or eclosion assay was performed on all four blocks of all the three stocks. These experiments were done under conditions of LD12:12 (~70 lux) and LD18:06 (~70 lux) at ~25 °C and ~70% RH. Approximately 300 eggs were collected and dispensed into each glass vial with Banana-Jaggery food medium. Six vials per population per block per photoperiod were used as replicates for the experiment. Post egg collection the rack with all the vials were subject to the experimental condition. When emergence was about to begin, the assay was initiated. Flies emerging from each vial in every 2 h interval were recorded for 3-4 days consecutively. Only vials which had at least 20 flies of each sex over the duration of one cycle and vials that showed robust rhythms over at least three cycles were included in the analyses.

Locomotor activity rhythm assay

The locomotor activity rhythm was assayed for all four blocks of all the three stocks. Freshly eclosed virgin flies were aged to 3-5 days and were then loaded into locomotor tubes and their

activity was recorded using the Drosophila Activity Monitoring System (DAM system, Trikentics, USA). Thirty two flies were used per block per photoperiod for this assay. The locomotor activity rhythm was first monitored under conditions of DD for about 7 days. Post this condition flies were transferred to fresh locomotor activity tubes with fresh food, were subjected to LD12:12 and LD18:06 (~70 lux) and were recorded for another 6-7 days. Following this, flies were subjected to DD for about 5 days. All these recordings were done at ~25 °C and ~70% RH.

Estimating phase of the rhythm

Centre of mass (CoM) is used as a measure of phase of the rhythm and the reference timing scale used is ZT. Computation of this phase marker is done as explained in Chapter 2.

Estimating the τ_E

The activity of flies post the LD protocol that was recorded in DD was used to compute the period. This was done based on ~5 days of data because allowing them to free run for a longer duration would eventually lead to a case where $\tau_E = \tau$. This would then defeat the purpose of computing τ_E. The free-running period is calculated using the Lomb-Scargle periodogram.

Experimental assessment of asymmetry factor

The CoM for each block of each stock for each assayed rhythm is computed under both photoperiods. The absolute difference between the phases (CoM) of the waveforms shown in Figures 4.1a-c and 4.2a-c are computed for each block of each stock for both rhythms. We believe this difference is a proxy to the measure of asymmetry between the stocks or rhythms.

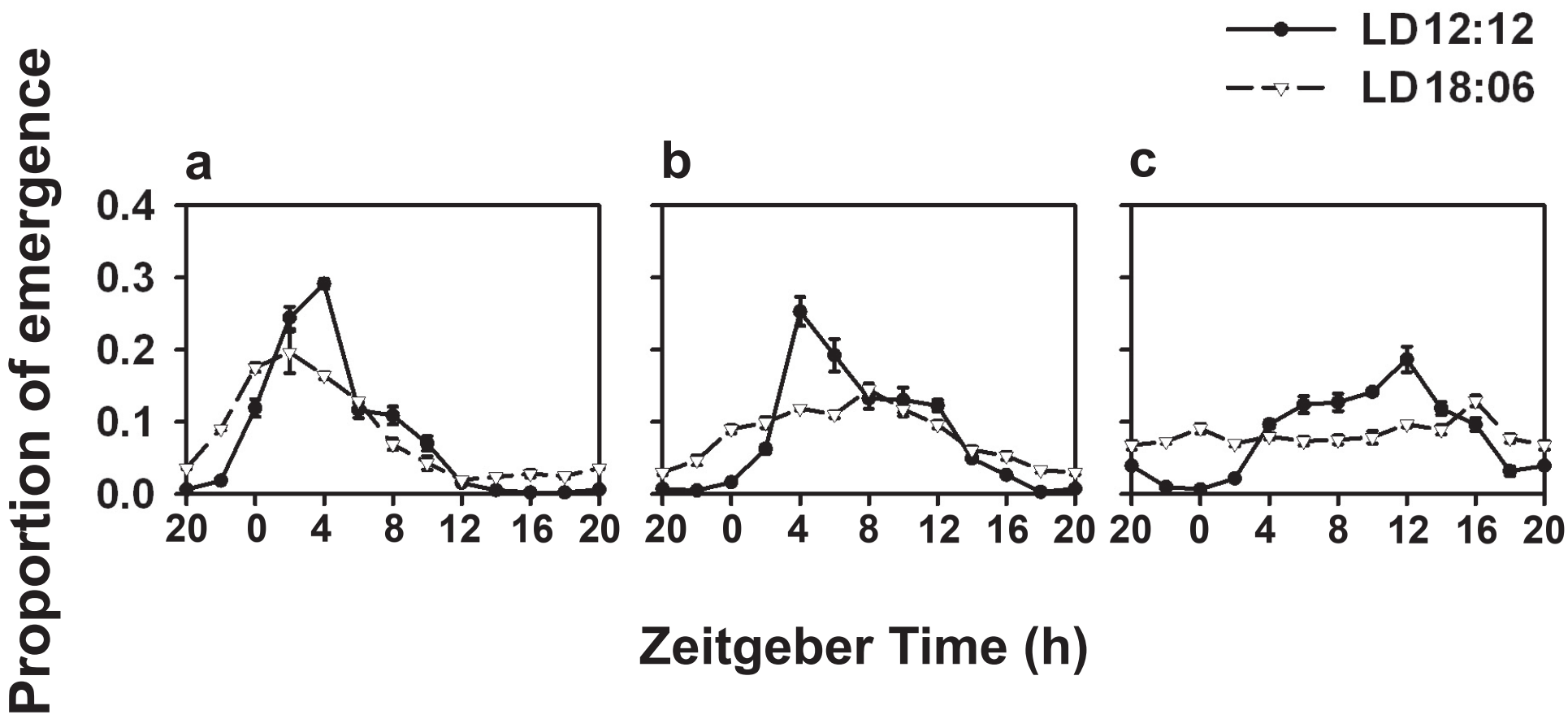

Figure 4.1: Adult emergence profile of GATE stocks under different photoperiods under T24 at ~70 lux. Profiles of (a) *early* stocks, (b) *control* stocks and (c) *late* stocks. All error bars are SEM. Lights-ON is at Zeitgeber Time 00 for both LD12:12 and LD18:06. Lights-OFF is at Zeitgeber Time 12 and 18 for LD12:12 and LD18:06 respectively.

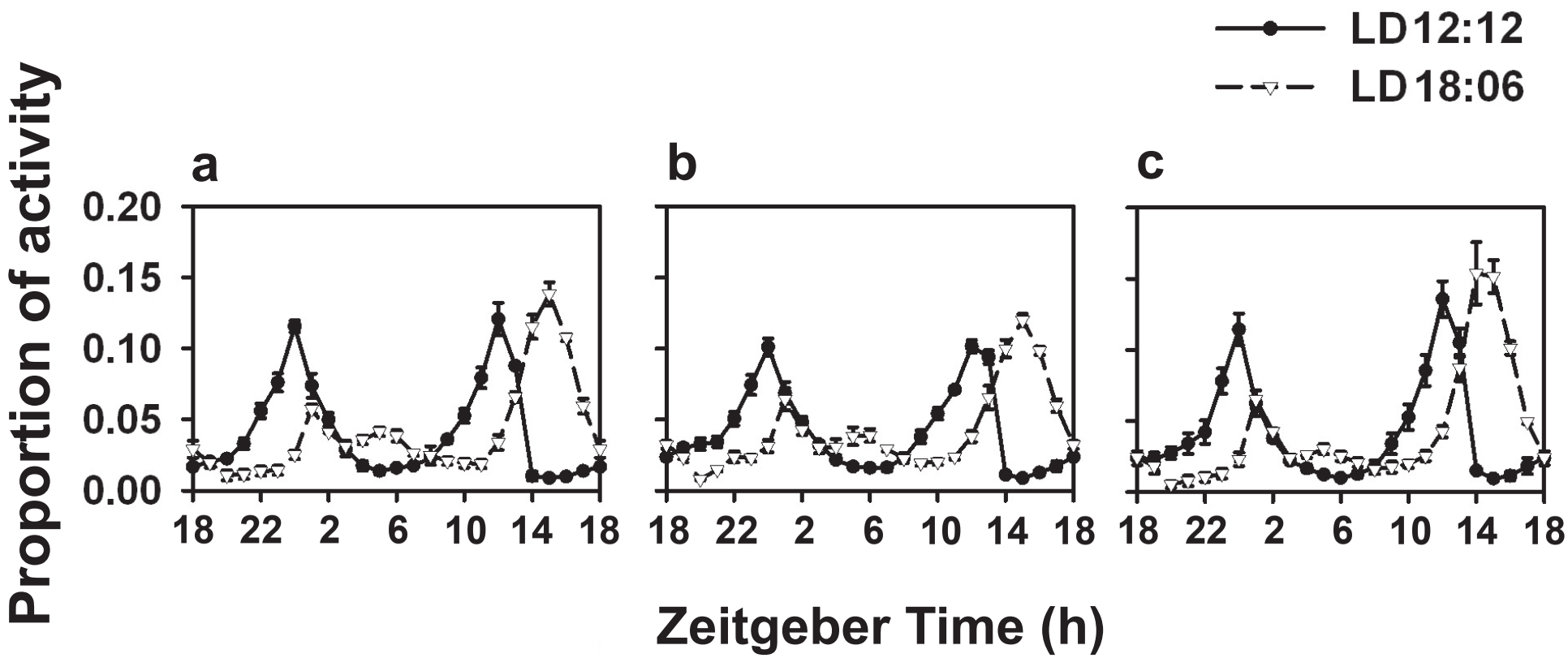

Figure 4.2: Locomotor activity profile of GATE stocks under different photoperiods under T24 at ~70 lux. Profiles of (a) *early* stocks, (b) *control* stocks and (c) *late* stocks. All error bars are SEM. Lights-ON is at Zeitgeber Time 00 for both LD12:12 and LD18:06. Lights-OFF is at Zeitgeber Time 12 and 18 for LD12:12 and LD18:06 respectively.

Modelling the best fitting CIRC

The basic CIRC is modelled using a sine curve and its first harmonic with certain conditions as specified below.

$$c = \sin\varphi + s(\sin 2\varphi),$$

where c is the CIRC response, φ is time of the day in radians (0-2π) and s is the shape factor.

Condition 1a: ***For $c_{0\text{-}\pi}$, if c<0, then c=0***

Condition 1b: ***For $c_{0\text{-}\pi}$, if a<1, then c=ca***

Condition 2a: ***For $c_{\pi\text{-}2\pi}$, if c>0, then c=0***

Condition 2b: ***For $c_{\pi\text{-}2\pi}$, if a>1, then c=c/a***

where a is the asymmetry factor.

Using this equation and its ensuing conditions we generated CIRC responses as a function of time of the day for all combinations of a and s, and normalised it such that the maximum or minimum CIRC response is 1. We varied s from 0.1 to 0.5. It is clear from the model that there is good correspondence between the extent of the dead zone of the CIRC and the PRC. We know that the PRC of the GATE stocks show a small dead zone (Vaze Thesis, 2012) and therefore chose s values appropriately. We varied a from 0.1 to 2, with a=1 meaning complete symmetry between the expansion and compression zones. We then generated zeitgeber arrays of LD12:12 and of LD18:06 with light values corresponding to 1 and dark values corresponding to 0. These arrays were then shifted in phase by 6 min. This led to 240 LD12:12 and 240 LD18:06 scenarios of different phase-relationships with all the CIRCs. We had experimental values of τ_E for both LD12:12 and LD18:06 for all four blocks. In both these cases T=24. So, for different photoperiods we obtained different $\tau_E - T$ values. We then computed the area under the curve for each CIRC under each photoperiod for all the phases of

entrainment. Given the number of combinations and loops it would have been tedious to integrate under the curve algorithmically. Thus, we computed the area under the curve as the sum of all the products of corresponding values of the zeitgeber and the CIRC responses. The area under the curve for each phase-relationship of the CIRCs with the zeitgeber was then subtracted from $\tau_E - T$. This difference was squared and the minimum was then used to find the corresponding phase-relationship that yielded this minimum difference between ∫CIRC and $\tau_E - T$. We, therefore, obtain eight modelled phase-relationships, one for each block and each photoperiod for each combination of *a* and *s*. We then subtracted these predicted phase-relationships for each block and each photoperiod for all the combinations of *a* and *s* from the experimental phase-relationship for that block. We squared these differences and added them across all the four blocks and two photoperiods. Thus, we get one Sum of Squared Difference (SSD) for each combination of *a* and *s*. The combination of *a* and *s* that yield the minimum SSD across the four blocks and two photoperiods is then thought to be a representative CIRC for that stock and that behaviour. We wrote a programme on MATLAB (Appendix B) to perform this simulation and the programme was run separately for each stock and each rhythm.

Statistical procedures

In order to estimate the difference between phases under LD12:12 and LD18:06 for each rhythm and each stock a three way mixed model ANOVA was performed on absolute difference in phases of entrainment between the two aforementioned photoperiods using STATISTICA v5.0 (StatSoft Inc., Tulsa, OK). Stock (S) and rhythm type (R) were treated as fixed factors and the blocks (B) were treated as a random factor. A Tukey's Honestly

Significant Difference (HSD) Test was performed for multiple comparisons where required (from Zar, 1999).

All statistical results were considered significant at α=0.05.

Results

The asymmetry between expansion and compression zones are different for the eclosion rhythm but not for the locomotor activity rhythm

The ANOVA on phase of entrainment revealed a significant effect of stock (S) ($F_{2,6}$=54.56, p<<0.05) and an interaction of stock by rhythm type (S×R) ($F_{2,6}$=29.42, p<<0.05) (Table 4.1). A Tukey's HSD post-hoc comparison revealed that the relative degrees of asymmetry was significantly different among all, *early*, *control* and *late,* stocks for the eclosion rhythm (Figure 4.3). However, this was not the case for the locomotor activity rhythm (Figure 4.3). Although overall high, none of the stocks differed among each other in their relative degrees of asymmetries for this rhythm.

Furthermore, it was observed that in the *control* stocks there is no difference in the degree of asymmetry between eclosion and locomotor activity rhythms. In the *early* stocks the eclosion rhythm shows a significantly lower degree of asymmetry as compared with locomotor activity rhythm and vice versa in case of the *late* stocks (Figure 4.3).

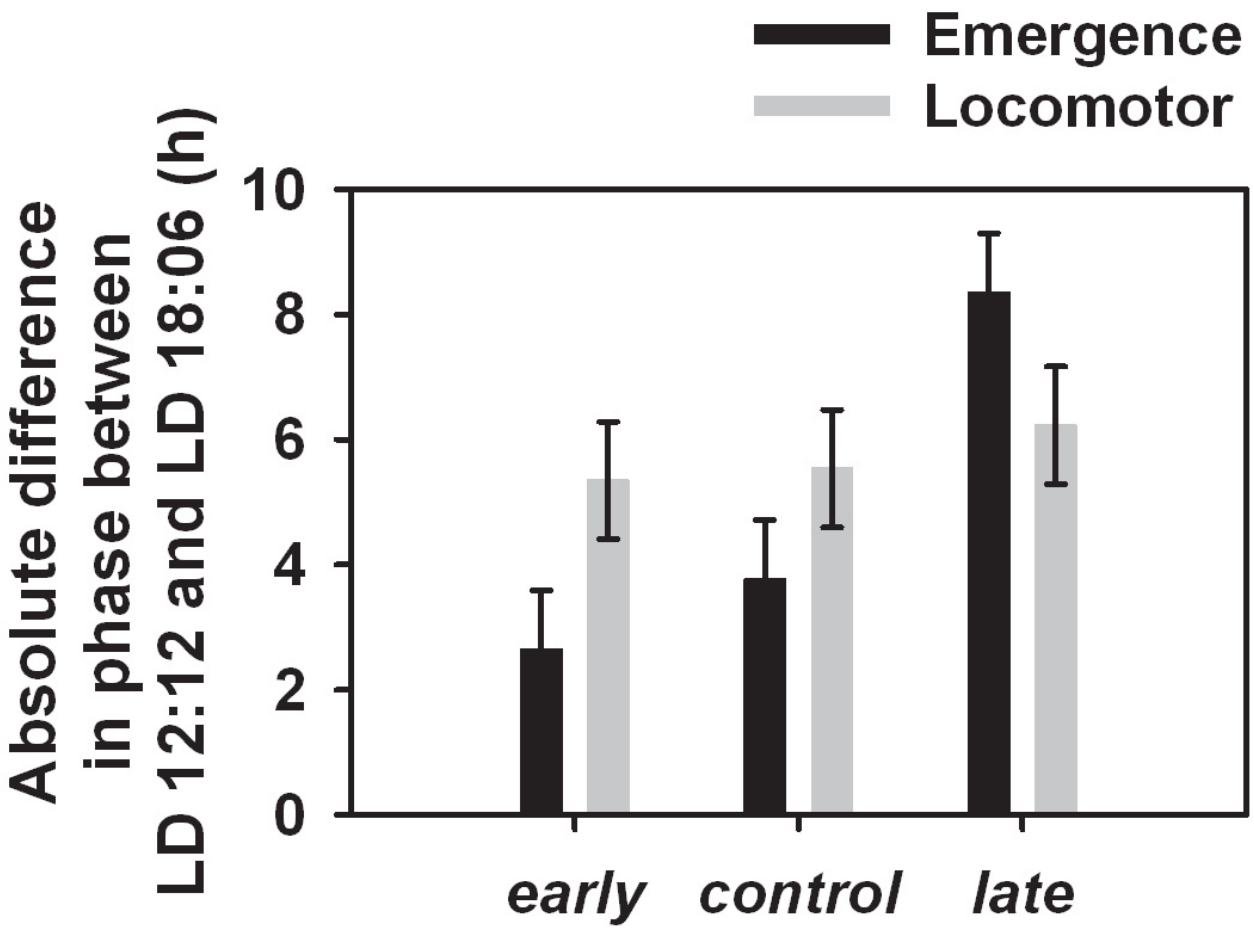

Figure 4.3: Experimental assessment of relative degrees of CIRC asymmetries of emergence and locomotor activity rhythms in the GATE stocks. Error bars are 95% CI. Therefore all non overlapping error bars indicate significantly different means at alpha=0.05.

	df Effect	MS Effect	df Error	MS Error	*F*	*p-level*
Stock (S)	*2.00*	*24.28*	*6.00*	*0.45*	*54.56*	*0.00*
Rhythm (R)	1.00	3.62	3.00	0.75	4.80	0.12
Block (B)	3.00	0.11	0.00	0.00	--	--
S × R	*2.00*	*13.09*	*6.00*	*0.44*	*29.42*	*0.00*
S × B	6.00	0.45	0.00	0.00	--	--
R × B	3.00	0.75	0.00	0.00	--	--
S × R × B	6.00	0.44	0.00	0.00	--	--

Table 4.1: Results of analysis of variance (ANOVA) performed on difference in phases between LD12:12 and LD18:06 across all three stocks and both, eclosion and locomotor activity, rhythms. Stock (S) and Rhythm type (R) are used as fixed factors and Block (B) is used as a random factor. Italicised effects are significant at alpha=0.05.

Prediction of the CIRC for the eclosion and locomotor activity rhythms of the GATE stocks

The results from modelling matched closely to those obtained empirically. A general view of the plot of SSD as a function of *a* and *s* reveals that the asymmetry factors change only for eclosion rhythm across stocks and not for locomotor activity rhythm (Figure 4.4). For eclosion rhythm, the *control* stocks exhibit a degree of asymmetry such that there is a larger expansion zone than compression zone (a=0.5) (Figure 4.4a, Table 4.2). The *early* stocks show relatively low asymmetry (a=1.13) (Figure 4.4b, Table 4.2), whereas the *late* stocks show a large compression zone (a=2) (Figure 4.4c, Table 4.2). For locomotor activity rhythm, all three stocks show relatively large expansion zones (a=0.1) (Figures 4.4d-f, Table 4.2). There seems to be very good agreement between empirical and theoretical phases of entrainment for both the rhythms studied (Figures 4.5a-c, 4.6a-c). A measure of the SSD reveals that the differences are in the order of about 3.55 radians on an average across 8 data points (~13 h) for eclosion rhythm and about 0.433 radians on an average across 8 data points (~1.7h) for locomotor activity rhythm (Figures 4.5d, 4.6d, Table 4.2). The predicted CIRCs for all stocks and both rhythms from the modelling results are plotted as a function of internal time (Figures 4.5e, 4.6e).

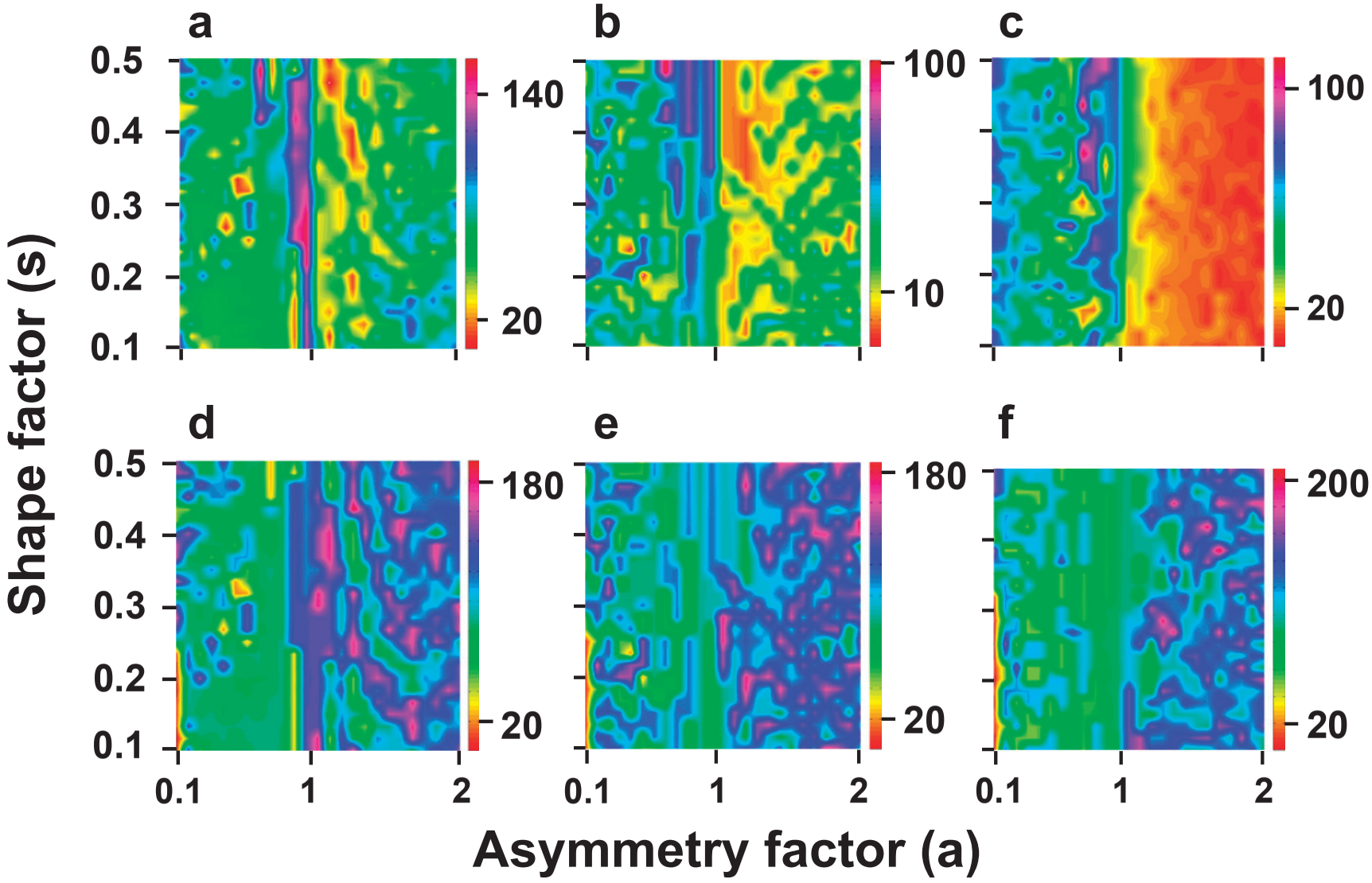

Figure 4.4: Sum of squared differences between experimental and predicted phases of entrainment as a function of shape and asymmetry factors. (a), (b) and (c) are for emergence rhythm. (d), (e) and (f) are for locomotor acitivty rhythm. (a) and (d) represent *early* stocks. (b) and (e) represent *control* stocks. (c) and (f) represent *late* stocks. Regions that are darkest red indicate best fit.

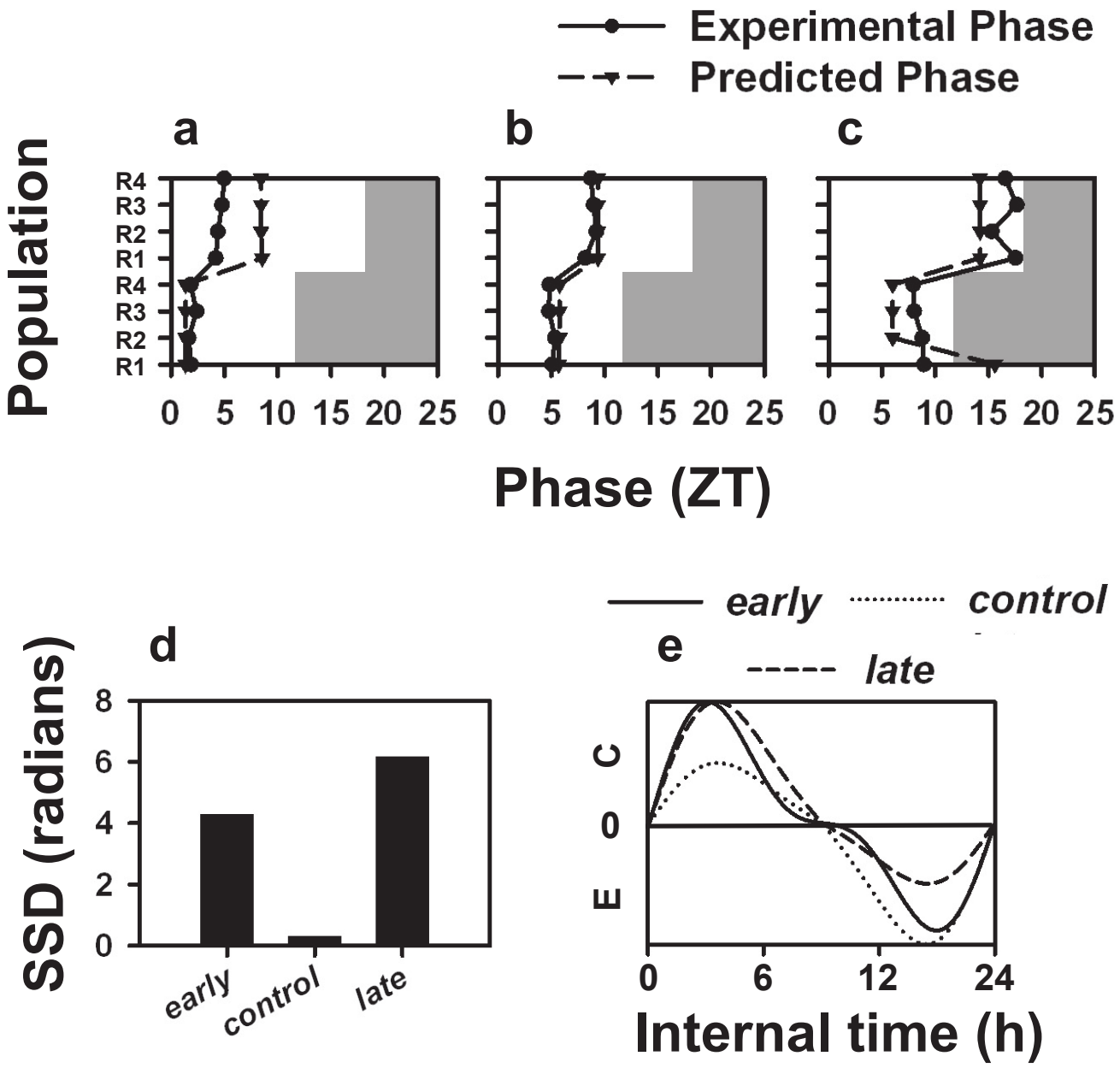

Figure 4.5: Results of best fitting CIRCs for the emergence rhythm. Comparison of experimental and predicted phases from the best fitting CIRC for (a) *early*, (b) *control* and (c) *late* stocks. (d) Sum of squared differences (SSD) between experimental and predicted phases for all stocks. (e) Predicted, best fitting CIRCs for all the stocks. (e) C and E refer to the compression and expansion zones respectively. Gray shaded regions in (a), (b) and (c) mark the scotophase of the LD cycle and the unshaded regions mark the photophase.

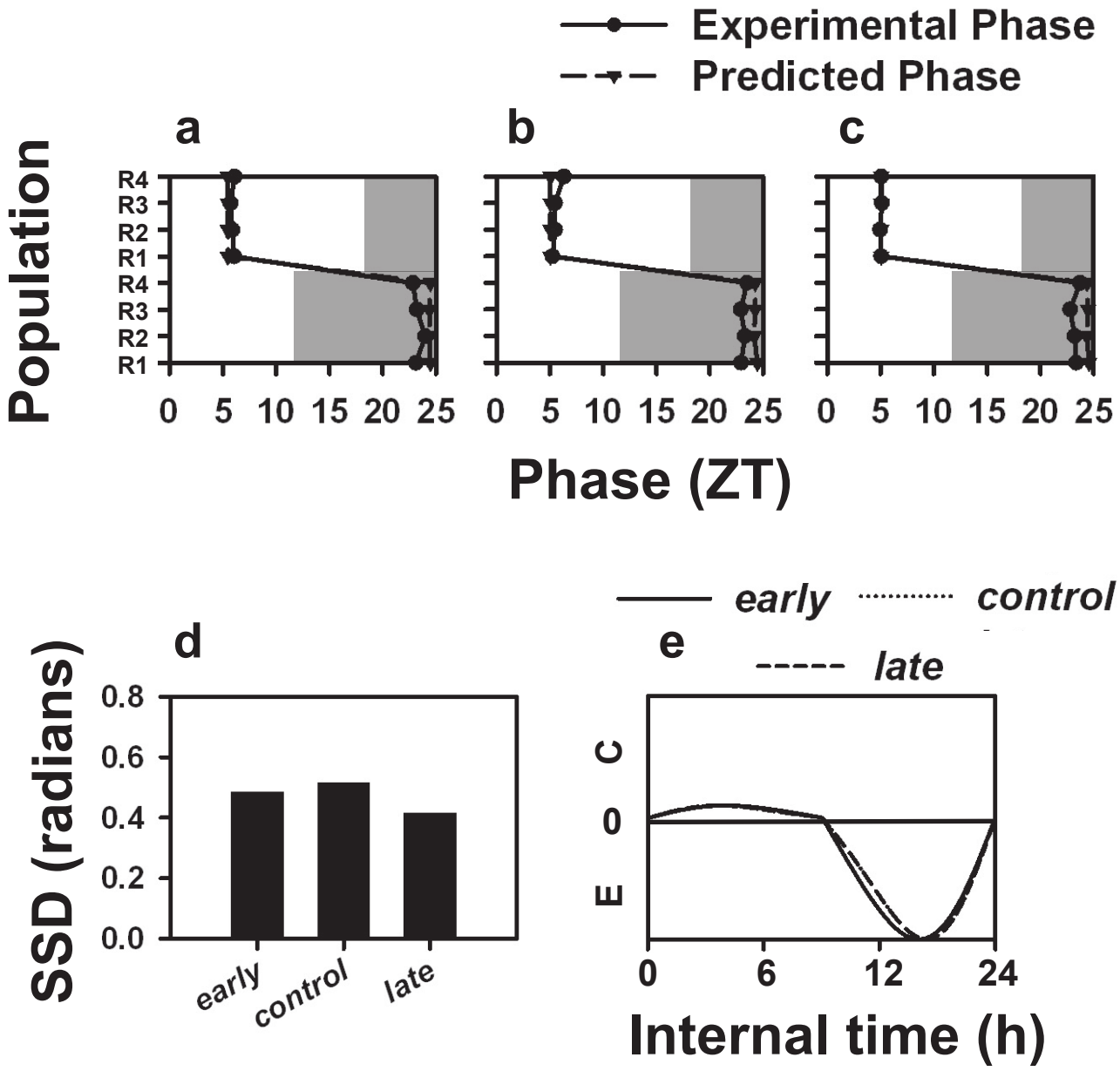

Figure 4.6: Results of best fitting CIRCs for the locomotor rhythm. Comparison of experimental and predicted phases from the best fitting CIRC for (a) *early*, (b) *control* and (c) *late* stocks. (d) Sum of squared differences (SSD) between experimental and predicted phases for all stocks. (e) Predicted, best fitting CIRCs for all the stocks. (e) C and E refer to the compression and expansion zones respectively. Gray shaded regions in (a), (b) and (c) mark the scotophase of the LD cycle and the unshaded regions mark the photophase.

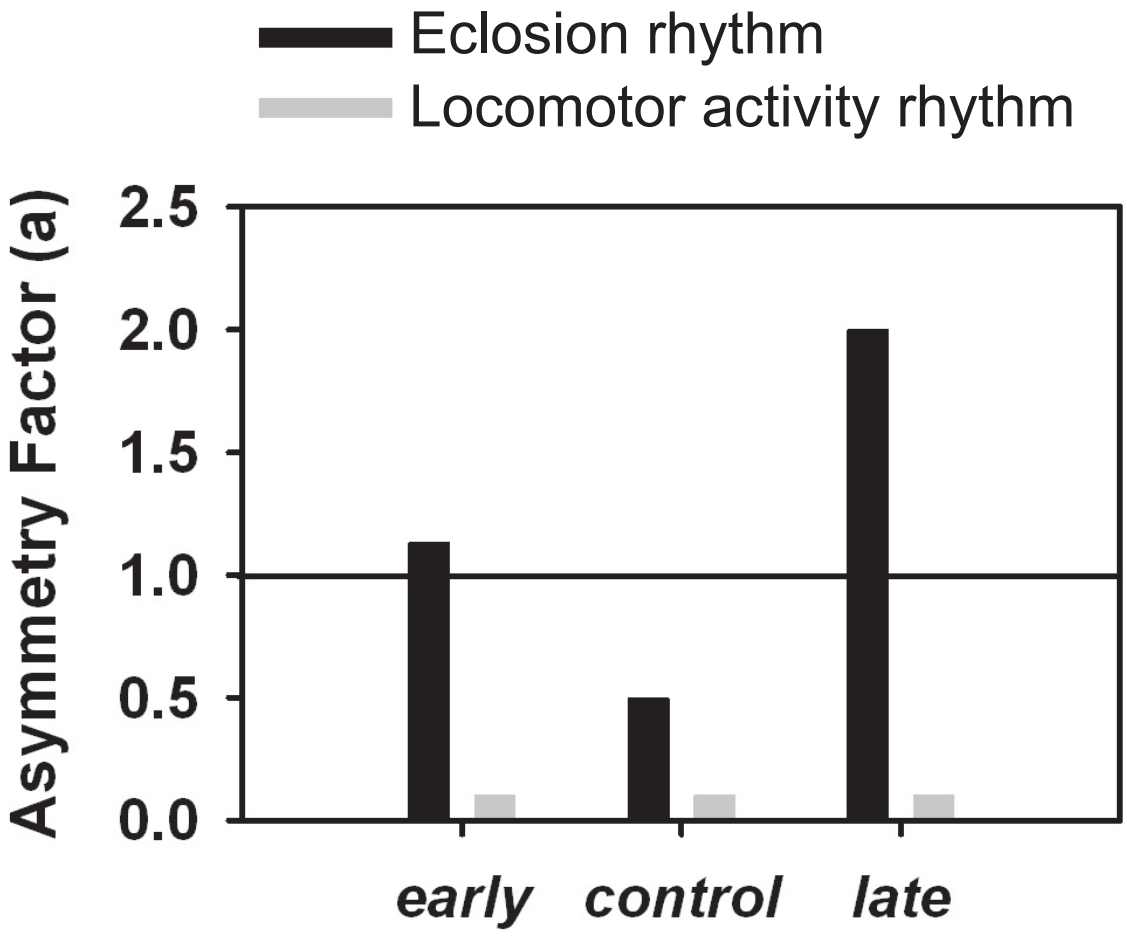

Figure 4.7: Estimate of asymmetry factors of the CIRCs of eclosion and locomotor activity rhythms for all the three stocks respectively. The black line parallel to the X-axis at Y-axis=1 refers to an asymmetry factor of 1 which indicates CIRC with completely symmetric compression and expansion zones. Any deviation of asymmetry factor below 1 refers to a larger expansion zone and any deviation above 1 refers to a larger compression zone.

		a	*s*	SSD
Eclosion Rhythm	***early***	1.13	0.47	4.29
	control	0.50	0.20	0.31
	late	2.00	0.22	6.16
Locomotor activity Rhythm	***early***	0.10	0.10	0.48
	control	0.10	0.20	0.52
	late	0.10	0.20	0.42

Table 4.2: Results of the optimisation process for estimating parameters of the CIRC for all three stocks and both kinds of rhythms. *a* refers to the asymmetry factor, *s* refers to the shape factor and SSD is the sum of squared differences between experimental phases and those predicted by CIRCs with the corresponding *a* and *s*.

Discussion

Emergence rhythm CIRC

We found, experimentally, that the asymmetry factors are significantly different among stocks (Figure 4.3). The *control* stocks show high asymmetry with a large expansion zone. This is understandable given that the τ_E of these stocks is lower than 24 h. The circadian clocks of these stocks need to expand their limit cycle every day in order to entrain. In case of the *early* stocks, there is selection pressure for an advanced phase of emergence. Albeit, the τ_E of these stocks is further lower than that of the *control* stocks, it could be speculated that this would expose a larger expansion zone of the CIRC than required in order to entrain. In order to compensate for this, the circadian clocks of these stocks have evolved reduced asymmetry thereby increasing the compression zone available to light. This would allow efficient entrainment in these stocks. The *late* stocks, however, are selected for a greatly delayed phase of emergence. These stocks have a τ_E greater than 24 h, which means that they would have to compress their limit cycles every day in order to entrain. One could speculate, again, that the delayed phase of emergence in these stocks would expose the compression zone, but the magnitude of compression required is likely to be greater than what has been exposed to light. This drives the *late* stocks to evolve an asymmetry factor of 2 that reduces the expansion zone and increases the compression zone, thereby facilitating entrainment.

Locomotor activity rhythm CIRC

We found, experimentally, that the asymmetry factors, although high, are essentially the same among stocks (Figure 4.3). This result is surprising as this theory also fails to explain entrainment of locomotor activity rhythms in the GATE stocks.

The idea of the CIRC theory is certainly appealing in understanding how entrainment occurs. An earlier report suggested that *early* and *late* stocks differ in their photosensitivity and using this hypothesis explained the entrainment profiles of these stocks to different skeleton photoperiods (Vaze et al., 2012a). At least with respect to eclosion rhythms the CIRC theory can explain entrainment without invoking any difference in photosensitivities of the circadian clocks of the *early* and *late* stocks for which we, as of now, have no evidence. In this paper it was shown that *control* stocks need more light in the evening to entrain with a profile similar to that in an LD12:12 conditions. However, why this happens is not discussed in the paper. Using the CIRC theory these data can be reconciled. The *early* and *control* stocks have shorter than 24 h period values that indicate that they need more light in the expansion zone i.e. in the evening for them to be able to entrain and this is what is seen. On the other hand the *late* stocks have longer than 24 h period values that indicate that they need more light in the compression zone i.e. in the morning for them to be able to entrain and this is again what we see experimentally as well.

Despite all this, a solution to the puzzle of mechanism of entrainment still remains elusive. If phase-relationships are adaptive, maintaining them irrespective of period fluctuations within a range seems to be among the primary goals of a circadian clock. Again, one has to revert back to the τ-φCCH (Figure 2.7) in order to understand how this role of the circadian clock may be fulfilled.

Chapter 5

Preliminary studies to identify the genetic bases of the divergent entrained eclosion rhythm of *early*, *control* and *late* stocks

Introduction

Quantitative traits are measurable traits that form a continuous distribution of trait values and have no naturally occurring categorical discontinuities (Falconer and Mackay, 1996). Quantitative traits, therefore by definition, are characterised by a considerable amount of variation. This variation is mediated in part by variation at the genetic level, in part by variation due to different environmental conditions and in part by an interaction between the two. It is also well established that such variations and departure from Mendelian ratios of traits are a consequence of small contributions of multiple alleles segregating simultaneously at multiple loci (Falconer and Mackay, 1996; Lynch and Walsh, 1998). The nature of such small contributions of alleles segregating at multiple loci simultaneously depend on allelic interactions and three major classes of allelic interactions are thought to give rise to quantitative trait variation; a) *Additive interactions:* Alleles within the same locus and across multiple loci contribute additively to the final phenotype, b) *Dominance interactions:* Alleles within a locus interact and produce effects such as dominance that contributes to the deviation in the final phenotype and c) *Epistatic interactions:* Non-additive effects of interactions among alleles at two or more loci on the phenotype (Falconer and Mackay, 1996).

Many circadian clock features are also quantitative traits and variations in these clock features such as those introduced in the previous paragraph exist for these traits as well. It has been shown that there is such continuous variation in circadian period of locomotor activity in mice (Hofstetter et al., 2003), in precision of circadian clocks in mice (Sharma and Chandrashekaran, 1999), in circadian period and phase of entrainment in *Neurospora* (Kim et al., 2007) and in phase of entrainment of *Drosophila* (Kumar et al., 2007). It has been

suggested that, indeed, the variation in such circadian clock features are contributed to by alleles at multiple loci segregating simultaneously (Mayeda et al., 1996; Shimomura et al., 2001; Hofstetter et al., 2007; Kim et al., 2007).

Several earlier studies have indicated an association between the variation in circadian clock genes and the potential adaptive significance of such variation. In *D. melanogaster* and *D. simulans*, the *Thr-Gly* repeat in the *per* gene is polymorphic in length. Different alleles code for different dipeptide pairs but the alleles that code for 14, 17, 20 and 23 dipeptide pairs make up to about 99% of the variation seen in Europe. Among them, the *Thr-Gly 17* and *20* form a significant latitudinal cline (Costa et al., 1992) and have been shown to have the ability to maintain periodicity with highest stability in response to fluctuating temperatures (Sawyer et al., 1997). The authors therefore attribute an adaptive role to this locus. Latitudinal clines in circadian period and phases of entrainment have been observed for *D. littoralis* and *D. subobscura* (Lankinen, 1986,1993), in circadian period and PRC of *D. auraria* (Pittendrigh and Takamura, 1989), and in circadian period of leaf movement rhythm of *Arabidopsis thaliana* (Michael et al., 2003). Although indirect, a strong association of variation in a trait with geographical location is reasonable evidence towards the adaptive value of the trait (reviewed in the context of rhythms in Hut et al., 2013).

In light of the fact that variation exists for circadian clock properties and that they may be adaptive, one may ask the genetic bases for such variation and the contributions of additive, dominance, epistatic or maternal effects to such variation. The answers to these questions may lead us to finding loci that maintain such adaptive variation. Studies have been done in the

past to answer such questions in the pitcher plant mosquito, *Wyeomyia smithii* (Mathias et al., 2006) and in the bean beetle, *Callosobruchus chinensis* (Harano and Miyatake, 2010).

We have an elaborate body of work that has enabled us to understand the molecular framework of circadian rhythms (Sehgal, 2004, Ko and Takahashi, 2006, Hardin, 2011) and we have evidence for adaptive variations in known clock genes that was discussed in the previous passages. Despite this, we know very little about the genetic bases for evolutionary changes in circadian clocks. Periodic selection pressures on phases of emergence in fruit flies and moth have revealed that underlying circadian clocks evolve (Pittendrigh, 1967; Pittendrigh and Minis, 1971; Kumar et al., 2007). Such an experimental evolution system, therefore, seems to be ideal to investigate the genetic players maintaining such variation in circadian clock features. Questions that one could ask include what kinds of allelic interactions give rise to evolutionary change in clock properties, and are there genetic players that are different from the ones that regulate the core clock that initially change in response to selection.

As a first step towards answering these questions, preliminary genetic studies were carried out on the GATE stocks (Vaze et al., 2013). The goal of this study was to identify the genetic bases of morning and evening emergence, and to identify the relative contributions of different allelic interactions to the phenotype. The authors found that morning and evening emergence are traits regulated primarily by autosomal genes and that the genetic architecture underlying morning and evening emergence is complex and primarily include dominance and epistatic allelic interactions. In order to further characterise the underlying autosomal regulators of morning and evening emergence, we used lines deficient for regions on the 2nd and 3rd chromosomes from the DrosDel Deficiency Kit (Ryder et al., 2007). Using deficiency lines to

map QTLs have proven to be an efficient method in *D. melanogaster* (Pasyukova et al., 2000).

Materials and methods

Deficiency lines and maintenance protocol

All the deficiency lines were ordered from the DrosDel Kit (Ryder et al., 2007). All these lines are generated on a w^{1118} genetic background and are maintained as heterozygotes over balancers on the respective chromosome. Each of these deficiency lines contains a P-element mediated deletion on a specific region of the chromosome. We obtained lines that covered different regions on the 2nd and 3rd chromosomes. About 39 lines were used to screen for dominant effects of alleles on proportion emergence in the morning and evening windows (Table 5.1). These lines are maintained in standard cornmeal medium. Flies are transferred to fresh food vials and are allowed to mate and lay eggs. On the 7th day the older flies are discarded and the current culture vial is maintained at ~25 °C and ~60-70% RH under LD12:12 cycles (~70 lux).

Deficiency Line ID	Chromosome	Deleted Segment	Deficiency Line ID	Chromosome	Deleted Segment
399	2	34A4-34B6	433 **	3	70A3-70C10
200 **	2	22A6-22D3	230 **	3	66D12-67B3
306	2	35B10-35D4	073 *	3	79C2-80A4
296	2	27E4-28B1	429 *	3	68A6-68E1
086	2	28F1-29A3	431 **	3	69A5-69D3
397	2	33B8-34A3	419 *	3	61A5-61B1
364	2	28C4-28D3	045 *	3	89E11-90C1
315	2	37B1-37C5	423 *	3	63A6-63B7
002 *	2	21D1-21E2	426	3	66A22-66C5
140 *	2	23C4-23F6	428 *	3	67E2-68A7
010	2	26B1-26D7	467 **	3	94B5-94E7
004 *	2	24F4-25A7	380	3	64B9-64C13
084 **	2	28E1-28E9	471	3	97E2-98A7
016 *	2	29E1-29F5	525	3	85D19-85F8
202 **	2	31E1-32A4	442 **	3	78D5-79A2
007	2	25F2-26B2	281	3	75B1-75C6
			280 **	3	73A1-73B5
			072 *	3	70A3-71E1
			143 **	3	82E8-83A1
			253 *	3	96A7-96C3
			475 **	3	99A5-99C1
			452 *	3	83B7-83D2

Table 5.1: A list of deficiency lines screened for proportion emergence during morning and evening hours. Single stars by the side of the Deficiency Line ID indicate lines for which at least three replicates were obtained (data shown). Double stars indicate lines for which at least two replicates were obtained (data not shown).

Adult emergence assay

The adult emergence assay for morning and evening hours was done for all the lines listed (Table 5.1). Adults that were used to initiate the next generation were transferred to fresh food vials with yeast paste and are allowed to lay eggs for 2-3 days. On the 3^{rd} - 4^{th} day these adults are transferred to another fresh set of vials with yeast paste. This process is repeated such that there are at least four replicate vials for each of the deficiency lines. All these vials were kept under LD12:12 (~70 lux), 25 °C and ~60-70% RH. When emergence was about to begin, the assay was initiated. Flies emerging from each vial in every 6 h interval (ZT20, ZT02, ZT08 and ZT14) were recorded for 3-4 days consecutively. Only vials which had at least 10 flies in each cycle and that showed at least two such robust cycles were included in the analyses. The morning window was defined as the duration between ZT20 and ZT02 and the evening window was defined as the duration between ZT08 and ZT14. Out of all the lines screened only 13 lines could be used for analyses. These are the lines that show robust rhythms in a minimum of three replicate vials.

Statistical procedures

Proportion of emergence in the morning and evening windows of the total number of flies emerging throughout the day was our dependent variable and this was *arcsine* square root transformed before performing any analyses (Zar, 1999). Multiple one factor analyses of variances (ANOVA) were performed on proportion emergence in the morning and evening windows, where deletion line was treated as a fixed factor using STATISTICA v5.0 (StatSoft Inc., Tulsa, OK). A Tukey's Honestly Significant Difference test was performed for multiple comparisons where required (from Zar, 1999). All results were considered statistically

significant at alpha=0.05.

Results

Proportion emergence in the morning window

ANOVA revealed no significant difference among deficiency lines ($F_{13,32}=1.43$, $p>0.05$) in the mean proportion of flies emerging in the morning window when each of the lines tested for had at least three replicate vials (Table 5.2a; Figure 5.1a). ANOVA revealed no significant difference among deficiency lines ($F_{24,43}=1.67$, $p>0.05$) in the mean proportion of flies emerging in the morning window even when each of the lines tested for had at least two replicate vials (data not shown).

Proportion emergence in the evening window

ANOVA revealed no significant difference among deficiency lines ($F_{13,32}=1.12$, $p>0.05$) in the mean proportion of flies emerging in the evening window as well when each of the lines tested for had at least three replicate vials (Table 5.2b; Figure 5.1b). ANOVA revealed no significant difference among deficiency lines ($F_{24,43}=1.3$, $p>0.05$) in the mean proportion of flies emerging in the evening window even when each of the lines tested for had at least two replicate vials (data not shown).

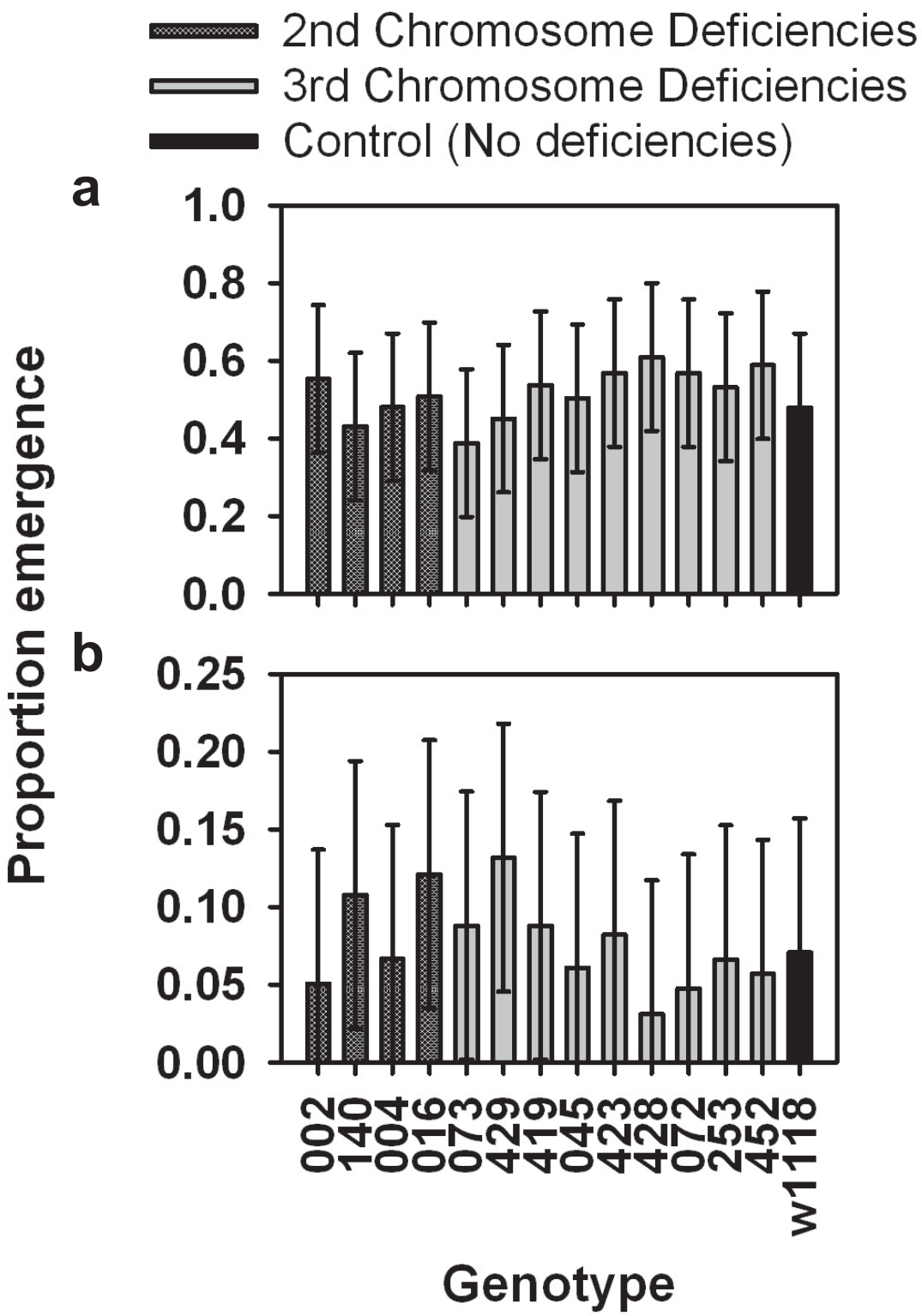

Figure 5.1: Deficiency screening for proportion emergence. (a) Mean proportion of emergence in the morning window. (b) Mean proportion of emergence in the evening window. Error bars are 3xSD of mean across lines. Non overlapping error bars, thus, indicate outliers.

a

	df Effect	MS Effect	df Error	MS Error	*F*	*p-level*
Deficiency Line	13.00	0.01	32.00	0.01	1.43	0.20

b

	df Effect	MS Effect	df Error	MS Error	*F*	*p-level*
Deficiency Line	13.00	0.01	32.00	0.01	1.12	0.38

Table 5.2: Results of ANOVA performed on proportion emergence of Deficiency Lines after performing an *arcsine* square root transformation. (a) ANOVA done on proportion of emergence in the morning window. (b) ANOVA done on proportion of emergence in the evening window. Italicised effects are significant at alpha=0.05.

Discussion

After more than 200 generations of selection on the timing of emergence the *early* and *late* stocks have evolved differences in the proportion emergence of flies during the morning and evening windows respectively. This is seen in all four independently maintained, replicate populations suggesting that such changes are due to imposed selection. Also we argued earlier that the phase of entrainment is a quantitative trait (Kim et al., 2007, Kumar et al., 2007) and therefore the variation underlying this trait is likely to be due to small contributions made by several alleles segregating at multiple loci simultaneously (Falconer and Mackay, 1996). Due to this, studying the effects of mutations on the phenotype of highly inbred strains may not represent the phenotypic variation in natural conditions (Garland and Rose, 2009). Therefore, studying the contributions of different loci to the variation in phenotypes in the GATE stocks is the closest approximation to studying the genetic bases for phenotypic variation in a natural population.

Initial studies in order to identify the genetic bases for adaptive variation in circadian clock properties of the GATE populations revealed that the underlying genetic architecture is complex and primarily comprises of dominance and epistatic effects (Vaze et al., 2013). We used deficiency lines to test loci that contribute to this divergence via dominance effects alone. The deficiency lines used in our experiments are heterozygous and are maintained over balancer chromosomes. Consequently, the regions of the chromosome that are absent in any line could have potentially housed a dominant allele that affects morning or evening emergence. The absence of such an allele could lead to reduced morning or evening emergence. From our preliminary studies the regions tested do not seem to house any

dominant allele affecting morning or evening emergence. This is likely due to the small number of deficiency screens. A larger deficiency screen covering ~77% of the whole *Drosophila* genome is underway and is likely to yield insights into the loci contributing to the divergence between the *early* and the *late* stocks.

Chapter 6

The way forward?

We have discussed earlier that phases of entrainment may be adaptive (Cloudsley-Thompson, 1960; Fleury et al., 2000; O'Donell et al., 2011; reviewed in Vaze and Sharma, 2013). If they were so, then mechanisms of entrainment as we know today may have some limitations in explaining the functional organisation of circadian clocks. Entrainment, we know, is defined as the process by which the length of internal and external cycles match each other. The way a circadian clock does this is by phasing its rhythm such that appropriate phases of the cycle are exposed to the zeitgeber thereby giving rise to a deterministic relationship between τ and φ (reviewed in Daan, 2000). If phases of entrainment were adaptive then, expecting such a covariation of period and phase would be counter intuitive. Our studies on the *early*, *control* and *late* stocks revealed that there is no correlation between period and phase, at least, for locomotor activity rhythm under LD cycles. Moreover, across a range of period values the phases attained seem similar. This is not surprising if we may deem phases of entrainment as adaptive. The interesting question, however, was how organisms or populations maintain the same phase despite having different periodicities. We hypothesised that this can happen only if the functional organisation of the circadian system had two components, one that sets τ (τSC) and one that sets the φ (φSC) and there was another component that coupled these two. Under certain environmental conditions the coupling component is expressed and under others it is not. Under LD cycles it is likely that the coupling component was OFF and thus allowed for maintenance of phase values despite the period differences. But in *period* mutants of *D.melanogaster* it was observed that the τ-φ relationship existed even under LD cycles (Hamblen-Coyle et al., 1992; Manishi et al., unpublished data). This prompted us to add another condition to the expression of this coupling component viz., it gets turned ON or OFF only within a specific range of period values. The dynamics is explained in greater detail in

Chapter 2 (Figure 2.7). In order to further find evidence in favour of the presence of dual components, one may use single oscillator models other than the one used here that are forced under different kinds of zeitgeber and simulate the τ-φ relationship to ask if they can be uncorrelated if both are outputs of the same oscillator. The other approach is to genetically screen for regions of the chromosome that may regulate only period or only phase. If such regions are found, one can be certain that there are dual components. To test the postulated dynamics between the τSC and φSC under different ranges of period values *period* mutants could be used to study eclosion and locomotor activity rhythms under LD and temperature cycles.

Despite all this, a mechanism of entrainment still remained elusive. We then asked if the circadian system is capable of entrainment using the CIRC. We found that it is not. The CIRCs for locomotor activity rhythm of the *early*, *control* and *late* stocks are not different. We believe that the circadian system is entraining and matching its frequency to that of the LD cycle but not because the rhythm phases itself appropriately. This is likely to occur via a different mechanism in maybe a parametric fashion but the phases are preserved at what could be presumed as an optimal time. If temperature cycles are provided then phases are not preserved at their optimal time (data not shown). They seem to diverge governed by their periodicities. The coupling component here seems to be ON and this is likely to be dependent on the association of temperature with something of ecological relevance for the fly. In such a case one may ask if entrainment is governed by the CIRC. In order to test this, we plan to do thermoperiod experiments on eclosion and locomotor activity rhythms using the *early*, *control* and *late* stocks. Our data suggests that it is time we move towards a new model of

entrainment incorporating the individual contributions of period and phase to it and by acknowledging the fact that they are not likely to be coupled under all kinds of environmental conditions.

The other module of this thesis was to identify the genetic bases for divergence in the *early* and *late* emergence phenotypes with respect to their *control* stocks. Results reported here have not revealed any candidate loci but that is likely due to small regions of the genome sampled. A more comprehensive analysis using deficiency lines to map these phenotypes is underway. Furthermore, owing to the underlying complexity of traits such as emergence it would be useful to study the genetic bases of this behaviour using alternative approaches as well. Regions of the genome that are detected as candidate loci via all the used approaches would be major contributors to the phenotype and would extend our confidence in predicting the underlying genetic architecture of the emergence phenotype. In order to do this one could use techniques such as chromosome substitution. These studies done simultaneously along with deficiency mapping could be powerful methods of detecting loci responsible for variation in quantitative traits (Pasyukova et al., 2000; Vaze et al., 2013).

Appendices

Appendix A1

Code to create a function file where the function comprises of the first order components of the van der Pol oscillator.

```
function [dy]=vdpol_sin(t,y)
global e w1 w0 E

dy=zeros(2,1);

dy(1)=y(2);
dy(2)=e*(1-(y(1))^2)*y(2)-(w0.^2)*y(1)+(E.*cos(w1*t));
end
```

Appendix A2

Code to simulate the period phase relationship.

```
% % % range of e=0.35, 0.45, 0.55, 0.65;

clear all; clc; clf; close all;

tic

global e E w1 w0

% input variables
save_file_name='sin_e0.65';
save_file_name_corr='sin_e0.65_corr';

e=0.65;

w1=0.2618;
n_ind=300;

interval=0.1;
n_subdivisions=24000;
tspan=0.1:interval:interval*n_subdivisions;

l_dset_1day=24/interval;

tau=19+rand(1,n_ind)*(29-19);
w0_array=(2*pi)./tau;
l_w0_array=length(w0_array(1,:));

w0_mean=mean(w0_array);

E_l=(sqrt(2)*((w0_mean^2)-(w1^2)))+(e*w1/sqrt(2));

E_min=2*E_l;
E_max=3*E_l;

E_array_choose=E_min+rand(1,1000)*(E_max-E_min);

E_array=[min(E_array_choose) max(E_array_choose)];

alpha=zeros(length(tspan),length(w0_array(1,:)),length(E_array(1,:)));
beta=zeros(length(tspan),length(w0_array(1,:)),length(E_array(1,:)));
```

```
41
42  alpha_cycles=zeros(l_dset_1day,l_w0_array,length(tau(1,:)),length(E_array(1,:)));
43  beta_cycles=zeros(l_dset_1day,l_w0_array,length(tau(1,:)),length(E_array(1,:)));
44  zeitgeber_cycles=zeros(l_dset_1day,l_w0_array,length(E_array(1,:)));
45
46  alpha_avg_cycles=zeros(l_dset_1day,length(tau(1,:)),length(E_array(1,:)));
47  beta_avg_cycles=zeros(l_dset_1day,length(tau(1,:)),length(E_array(1,:)));
48  zeitgeber_avg_cycles=zeros(l_dset_1day,1,length(E_array(1,:)));
49  data=zeros(l_dset_1day,length(tau(1,:)),length(E_array(1,:)));
50
51  for n=1:length(w0_array(1,:))
52
53      for i=1:length(E_array(1,:))
54
55          w0=w0_array(1,n);
56          E=E_array(1,i);
57
58          [t,y]=ode45(@vdpol_sin,tspan,[2, 0.5]);
59
60          alpha(1:length(y(:,2)),n,i)=y(:,2);
61          beta(1:length(y(:,1)),n,i)=y(:,1);
62
63          zeitgeber=transpose(E*cos(w1*tspan)/abs(cos(w1*tspan)))+E;
64
65
66  alpha_cycles_array=mat2cell(alpha((l_dset_1day*10+1):((l_dset_1day*10)+(l_dset_1day*
67  30)),n,i),repmat(l_dset_1day,1,length(alpha((l_dset_1day*10+1):((l_dset_1day*10)+(l_dse
68  t_1day*30)),n,i))/l_dset_1day));
69
70  beta_cycles_array=mat2cell(beta((l_dset_1day*10+1):((l_dset_1day*10)+(l_dset_1day*30
71  )),n,i),repmat(l_dset_1day,1,length(beta((l_dset_1day*10+1):((l_dset_1day*10)+(l_dset_1
72  day*30)),n,i))/l_dset_1day));
73
74          s=size(alpha_cycles_array);
75
76          for j=1:s(1,1)
77              alpha_cycles(:,j,n,i)=alpha_cycles_array{j};
78              beta_cycles(:,j,n,i)=beta_cycles_array{j};
79          end
80
81          for j=1:l_dset_1day
82              alpha_avg_cycles(j,n,i)=mean(alpha_cycles(j,:,n,i));
83              beta_avg_cycles(j,n,i)=mean(beta_cycles(j,:,n,i));
84          end
85
86          data(:,:,i)=E_array(1,i)+beta_avg_cycles(:,:,i)+randn;
```

```
87
88      end
89  end
90
91  zt=[6.1:0.1:23.9 0:0.1:6];
92  time_deg=transpose((zt.*360)/24);
93
94  % converting to radians
95  rad=time_deg.*(pi/180);
96  % sin and cos components of the angle in radians
97  sinr=sin(rad);
98  cosr=cos(rad);
99
100 % create dummy matrices
101 fsinr=zeros(length(data(:,1)),length(beta_avg_cycles(1,:,1)),length(E_array(1,:)));
102 fcosr=zeros(length(data(:,1)),length(beta_avg_cycles(1,:,1)),length(E_array(1,:)));
103 sumsin=zeros(1,length(beta_avg_cycles(1,:,1)),length(E_array(1,:)));
104 sumcos=zeros(1,length(beta_avg_cycles(1,:,1)),length(E_array(1,:)));
105 n1=zeros(1,length(beta_avg_cycles(1,:,1)),length(E_array(1,:)));
106 xbar=zeros(1,length(beta_avg_cycles(1,:,1)),length(E_array(1,:)));
107 ybar=zeros(1,length(beta_avg_cycles(1,:,1)),length(E_array(1,:)));
108 r=zeros(1,length(beta_avg_cycles(1,:,1)),length(E_array(1,:)));
109 costheta=zeros(1,length(beta_avg_cycles(1,:,1)),length(E_array(1,:)));
110 sintheta=zeros(1,length(beta_avg_cycles(1,:,1)),length(E_array(1,:)));
111 tantheta=zeros(1,length(beta_avg_cycles(1,:,1)),length(E_array(1,:)));
112 theta=zeros(1,length(beta_avg_cycles(1,:,1)),length(E_array(1,:)));
113 thetad=zeros(1,length(beta_avg_cycles(1,:,1)),length(E_array(1,:)));
114
115 for i=1:length(beta_avg_cycles(1,:,1))
116     for j=1:length(E_array(1,:))
117         fsinr(:,i,j)=data(:,i,j).*sinr;
118         fcosr(:,i,j)=data(:,i,j).*cosr;
119         % summing up over all sin and cos frequency multiplied components and finding y
120 and x
121         sumsin(:,i,j)=sum(fsinr(:,i,j));
122         sumcos(:,i,j)=sum(fcosr(:,i,j));
123         n1(:,i,j)=sum(data(:,i,j));
124         xbar(:,i,j)=(sumcos(:,i,j)/n1(:,i,j));
125         ybar(:,i,j)=(sumsin(:,i,j)/n1(:,i,j));
126
127         % compute r
128         r(:,i,j)=sqrt((xbar(:,i,j).^2)+(ybar(:,i,j).^2));
129
130         % compute mean angle
131         costheta(:,i,j)=(xbar(:,i,j)./r(:,i,j));
132         sintheta(:,i,j)=(ybar(:,i,j)./r(:,i,j));
```

```
133         tantheta(:,i,j)=(sintheta(:,i,j)./costheta(:,i,j));
134
135         if xbar(:,i,j)>0
136             theta(:,i,j)=atan(tantheta(:,i,j));
137         else
138             theta(:,i,j)=pi+(atan(tantheta(:,i,j)));
139         end
140
141         %     convert to degrees
142         thetad(:,i,j)=theta(:,i,j).*(180/pi);
143
144         for ii=1:length(thetad(1,:,1))
145             for jj=1:length(thetad(1,1,:))
146                 if thetad(1,ii,jj)<0
147                     thetad(1,ii,jj)=360+thetad(1,ii,jj);
148                 else
149                     thetad(1,ii,jj)=thetad(1,ii,jj);
150                 end
151             end
152         end
153
154         phase_time_prelim=(thetad.*24)./360;
155
156     end
157 end
158
159 phase_time=zeros(1,length(beta_avg_cycles(1,:,1)),length(E_array(1,:)));
160
161 for i=1:length(phase_time_prelim(1,:,1))
162     for j=1:length(phase_time_prelim(1,1,:))
163         if phase_time_prelim(1,i,j)<10
164             phase_time(1,i,j)=24+phase_time_prelim(1,i,j);
165         else
166             phase_time(1,i,j)=phase_time_prelim(1,i,j);
167         end
168     end
169 end
170
171 phase_time_E=zeros(length(phase_time(1,:,1)), length(phase_time(1,1,:)));
172 tau_final=zeros(length(phase_time(1,:,1)),1);
173
174 for i=1:length(phase_time(1,:,1))
175     for j=1:length(phase_time(1,1,:))
176         phase_time_E(i,j)=phase_time(1,i,j);
177         tau_final(i,1)=tau(1,i);
178     end
```

```
end

tau_phi_matrix=[tau_final phase_time_E];

threshold=sortrows(tau_phi_matrix);

threshold_short=zeros(length(threshold(:,1)),length(threshold(1,:)));
threshold_int=zeros(length(threshold(:,1)),length(threshold(1,:)));
threshold_long=zeros(length(threshold(:,1)),length(threshold(1,:)));

for i=1:length(threshold(:,1))
    if threshold(i,1)>19 && threshold(i,1)<22.5
        threshold_short(i,:)=threshold(i,:);
    elseif threshold(i,1)>22.5 && threshold(i,1)<25
        threshold_int(i,:)=threshold(i,:);
    else
        threshold_long(i,:)=threshold(i,:);
    end
end

threshold_short(threshold_short==0)=[];
threshold_int(threshold_int==0)=[];
threshold_long(threshold_long==0)=[];

threshold_short_final=reshape(threshold_short,[length(threshold_short)/length(threshold(1,
:)),length(threshold(1,:))]);
threshold_int_final=reshape(threshold_int,[length(threshold_int)/length(threshold(1,:)),leng
th(threshold(1,:))]);
threshold_long_final=reshape(threshold_long,[length(threshold_long)/length(threshold(1,:)
),length(threshold(1,:))]);

threshold_short_phase_mean=zeros(1,length(E_array));
threshold_short_phase_sd=zeros(1,length(E_array));

threshold_int_phase_mean=zeros(1,length(E_array));
threshold_int_phase_sd=zeros(1,length(E_array));

threshold_long_phase_mean=zeros(1,length(E_array));
threshold_long_phase_sd=zeros(1,length(E_array));

phase_threshold_short=zeros(length(threshold_short_final(:,1)),length(threshold_short_fin
al(1,:))-1);
phase_threshold_int=zeros(length(threshold_int_final(:,1)),length(threshold_int_final(1,:))-
1);
phase_threshold_long=zeros(length(threshold_long_final(:,1)),length(threshold_long_final(
1,:))-1);
```

```
225
226 for i=2:length(threshold_short_final(1,:))
227     threshold_short_phase_mean(1,i-1)=mean(threshold_short_final(:,i));
228     threshold_short_phase_sd(1,i-1)=sqrt(var((threshold_short_final(:,i))));
229     threshold_int_phase_mean(1,i-1)=mean(threshold_int_final(:,i));
230     threshold_int_phase_sd(1,i-1)=sqrt(var((threshold_int_final(:,i))));
231     threshold_long_phase_mean(1,i-1)=mean(threshold_long_final(:,i));
232     threshold_long_phase_sd(1,i-1)=sqrt(var((threshold_long_final(:,i))));
233
234     phase_threshold_short(:,i-1)=threshold_short_phase_mean(1,i-
235 1)+randn(length(phase_threshold_short(:,1)),1).*threshold_short_phase_sd(1,i-1).*0.5;
236     phase_threshold_int(:,i-1)=threshold_int_phase_mean(1,i-
237 1)+randn(length(phase_threshold_int(:,1)),1).*threshold_int_phase_sd(1,i-1).*0.5;
238     phase_threshold_long(:,i-1)=threshold_long_phase_mean(1,i-
239 1)+randn(length(phase_threshold_long(:,1)),1).*threshold_long_phase_sd(1,i-1).*0.5;
240 end
241
242 tau_phi_single=tau_phi_matrix;
243 tau_phi_cond_pooled=[threshold_short_final(:,1)
244 phase_threshold_short;threshold_int_final(:,1)
245 phase_threshold_int;threshold_long_final(:,1) phase_threshold_long];
246 tau_phi_cond_short=[threshold_short_final(:,1) phase_threshold_short];
247 tau_phi_cond_int=[threshold_int_final(:,1) phase_threshold_int];
248 tau_phi_cond_long=[threshold_long_final(:,1) phase_threshold_long];
249
250 r_single=zeros(1,length(E_array(1,:)));
251 p_single=zeros(1,length(E_array(1,:)));
252
253 r_cond_pooled=zeros(1,length(E_array(1,:)));
254 p_cond_pooled=zeros(1,length(E_array(1,:)));
255
256 r_cond_short=zeros(1,length(E_array(1,:)));
257 p_cond_short=zeros(1,length(E_array(1,:)));
258 r_cond_int=zeros(1,length(E_array(1,:)));
259 p_cond_int=zeros(1,length(E_array(1,:)));
260 r_cond_long=zeros(1,length(E_array(1,:)));
261 p_cond_long=zeros(1,length(E_array(1,:)));
262
263
264 for i=1:length(E_array(1,:))
265
266 [r_single(1,i),p_single(1,i)]=circ_corrcl(((tau_phi_single(:,i+1).*24)./360).*(pi/180),tau_ph
267 i_single(:,1));
268
269 [r_cond_pooled(1,i),p_cond_pooled(1,i)]=circ_corrcl(((tau_phi_cond_pooled(:,i+1).*24)./3
270 60).*(pi/180),tau_phi_cond_pooled(:,1));
```

```
271
272     [r_cond_short(1,i),p_cond_short(1,i)]=circ_corrcl(((tau_phi_cond_short(:,i+1).*24)./360).*
273     (pi/180),tau_phi_cond_short(:,1));
274
275     [r_cond_int(1,i),p_cond_int(1,i)]=circ_corrcl(((tau_phi_cond_int(:,i+1).*24)./360).*(pi/180
276     ),tau_phi_cond_int(:,1));
277
278     [r_cond_long(1,i),p_cond_long(1,i)]=circ_corrcl(((tau_phi_cond_long(:,i+1).*24)./360).*(
279     pi/180),tau_phi_cond_long(:,1));
280     end
281
282     data_save=[tau_phi_single tau_phi_cond_pooled];
283     correlation=[E_array;r_single;p_single;r_cond_pooled;p_cond_pooled;r_cond_short;p_con
284     d_short;r_cond_int;p_cond_int;r_cond_long;p_cond_long];
285
286     save(save_file_name,'data_save','-ASCII');
287     save(save_file_name_corr,'correlation','-ASCII');
288
289     toc
```

Appendix B

Code to model the CIRC

```
% % % 1. Generate CIRCs for all possible combinations of a and s
% % % 2. Create an LD 12:12 and LD 18:06 cycle and position them at
% % %       different phases of the CIRCs
% % % 3. Multiply CIRC responses to LD profile at each phase
% % % 4. Compute net integral under the CIRC curve for different LD phases
% % % 5. Find out for which phase of the LD cycle is the integral value
% % %       closest to tau_e-T for each combination of a and s (for this
% % %       [[tau_e-t]-net integral] is computed)
% % % 6. Compute the phase of Centre of Mass for each CIRC (for this the
% % %       CIRC responses are squared)
% % % 7. Then compute the phase angle difference between Lights ON and CoM
% % %       of the CIRC for all combinations of a and s
% % % 8. Then compute the simulated phase angle for all combinations of a,
% % %       s, and photoperiod
% % % 9. Then compute the difference between experimental and simulated
% % %       phase for each photoperiod
% % % 10. Square this difference and then sum of the squared difference is
% % %       computed
% % % 11. Find the combination of a and s for the given tau_e and
% % %       experimental phases that provide best fits



clear all; clf;clc;
tic


% % % input variables
save_file_name='gc_loc_parms';
figure_title='gc_loc';
save_circ_name='gc_loc_circ';

input_data=importdata('gc_loc_input.txt');

tau_e_1212_b1=input_data(1,1);
tau_e_1212_b2=input_data(2,1);
tau_e_1212_b3=input_data(3,1);
tau_e_1212_b4=input_data(4,1);

tau_e_1806_b1=input_data(5,1);
```

```
tau_e_1806_b2=input_data(6,1);
tau_e_1806_b3=input_data(7,1);
tau_e_1806_b4=input_data(8,1);

phase_angle_exp_ld1212_b1=0-input_data(1,2); %phase of LON=ZT0=0 radians
phase_angle_exp_ld1212_b2=0-input_data(2,2);
phase_angle_exp_ld1212_b3=0-input_data(3,2);
phase_angle_exp_ld1212_b4=0-input_data(4,2);

phase_angle_exp_ld1806_b1=0-input_data(5,2);%phase of LON=ZT0=0 radians
phase_angle_exp_ld1806_b2=0-input_data(6,2);
phase_angle_exp_ld1806_b3=0-input_data(7,2);
phase_angle_exp_ld1806_b4=0-input_data(8,2);



% % % define a and s
a=linspace(0.1,2,25);
s=linspace(0.1,0.5,25);


% % % create phi array for sine function
phi=transpose(linspace(0,2*pi,240));


% % % define dummy matrices for CIRCs
c1=zeros(240,length(a),length(s));
c_max=zeros(1,length(a),length(s));
c=zeros(240,length(a),length(s));


% % % create zeitgeber arrays in accordance to LON LD 12:12
zeitgeber_1212=zeros(240,240);
zeitgeber_1212(:,1)=[ones(120,1);zeros(120,1)];


% % % for all combinations of a and s create CIRC responses and create
% % % zeitgeber arrays
for i=1:length(a)
    for j=1:length(s)

        c1(:,i,j)=sin(phi)+s(:,j)*sin(2*phi);

        for jj=1:floor(length(c1(:,1,1))/2)
            if c1(jj,i,j)<0
                c1(jj,i,j)=0;
```

```
            end
            if a(:,i)<1
                c1(jj,i,j)=c1(jj,i,j).*a(:,i);
            end
        end

        for kk=(ceil(length(c1(:,1,1))/2)):length(c1(:,1,1))
            if c1(kk,i,j)>0
                c1(kk,i,j)=0;
            end
            if a(:,i)>1
                c1(kk,i,j)=c1(kk,i,j)./a(:,i);
            end
        end

        if a(:,i)<1
            c_max(:,i,j)=abs(min(c1(:,i,j)));
            elseif a(:,i)>=1
                c_max(:,i,j)=max(c1(:,i,j));
        end

        for o=1:length(c1(:,1,1))
            c(o,i,j)=c1(o,i,j)/c_max(1,i,j);
        end

        for ii=2:length(zeitgeber_1212(1,:))
            zeitgeber_1212(:,ii)=circshift(zeitgeber_1212(:,ii-1),1);
        end
    end
end


% % % compute the square of CIRC responses and compute CoM 1212
circ=c.^2;
zt1212=[transpose(18:0.1:23.9);transpose(0:0.1:17.9)];
zt_deg1212=(zt1212.*360)/24;
zt_rad1212=(zt_deg1212.*pi)./180;
sin_comp1212=sin(zt_rad1212);
cos_comp1212=cos(zt_rad1212);

sin_comp_circ1212=zeros(240,length(a),length(s));
cos_comp_circ1212=zeros(240,length(a),length(s));

for i=1:length(a)
    for j=1:length(s)
        sin_comp_circ1212(:,i,j)=sin_comp1212.*circ(:,i,j);
```

```
        cos_comp_circ1212(:,i,j)=cos_comp1212.*circ(:,i,j);
        sum_circ_resp1212=sum(circ);
        x1212=sum(cos_comp_circ1212);
        y1212=sum(sin_comp_circ1212);
        xbar1212=x1212./sum_circ_resp1212;
        ybar1212=y1212./sum_circ_resp1212;
        tantheta1212=atan(ybar1212./xbar1212);

        if xbar1212(:,i,j)<0
            tantheta1212(:,i,j)=pi+tantheta1212(:,i,j);
        else
            tantheta1212(:,i,j)=tantheta1212(:,i,j);
        end
    end
end

for i=1:length(a)
    for j=1:length(s)
        if tantheta1212(:,i,j)<0
            tantheta1212(:,i,j)=2*pi+tantheta1212(:,i,j);
        else
            tantheta1212(:,i,j)=tantheta1212(:,i,j);
        end
    end
end


% % % create dummy matrices for CIRC responses multiplied by zeitgeber
% % % values at different phases of the LD cycle and the net integral uder the
% % % CIRC curve
int_resp1212=zeros(240,length(a),length(s),length(zeitgeber_1212(1,:)));
integral1212=zeros(length(zeitgeber_1212(1,:)),length(a),length(s));


% % % compute the net integral under the CIRC curve when positioned
% % % differently under LD cycles
for i=1:length(a)
    for j=1:length(s)
        for ii=1:length(zeitgeber_1212(1,:))
            int_resp1212(:,i,j,ii)=c(:,i,j).*zeitgeber_1212(:,ii);
            integral1212(ii,i,j)=sum(int_resp1212(:,i,j,ii));
        end
    end
end
```

```
% % % define T value and the difference between tau_e and T
T=24;
tau_e_minus_T_1212_b1=tau_e_1212_b1-T;
tau_e_minus_T_1212_b2=tau_e_1212_b2-T;
tau_e_minus_T_1212_b3=tau_e_1212_b3-T;
tau_e_minus_T_1212_b4=tau_e_1212_b4-T;

% % % compute the difference between tau_e-T and net_integral at all
% % %   computed phases
tau_e_minus_T_1212_minus_integral_b1=zeros(length(zeitgeber_1212(1,:)),length(a),leng
th(s));
tau_e_minus_T_1212_minus_integral_b2=zeros(length(zeitgeber_1212(1,:)),length(a),leng
th(s));
tau_e_minus_T_1212_minus_integral_b3=zeros(length(zeitgeber_1212(1,:)),length(a),leng
th(s));
tau_e_minus_T_1212_minus_integral_b4=zeros(length(zeitgeber_1212(1,:)),length(a),leng
th(s));

for i=1:length(a)
    for j=1:length(s)
        for k=1:length(tau_e_minus_T_1212_minus_integral_b1(:,1,1))
            tau_e_minus_T_1212_minus_integral_b1(k,i,j)=tau_e_minus_T_1212_b1-
integral1212(k,i,j);
            tau_e_minus_T_1212_minus_integral_b2(k,i,j)=tau_e_minus_T_1212_b2-
integral1212(k,i,j);
            tau_e_minus_T_1212_minus_integral_b3(k,i,j)=tau_e_minus_T_1212_b3-
integral1212(k,i,j);
            tau_e_minus_T_1212_minus_integral_b4(k,i,j)=tau_e_minus_T_1212_b4-
integral1212(k,i,j);
        end
    end
end


% % % square the difference
tau_e_minus_T_minus_integral_sq_1212_b1=tau_e_minus_T_1212_minus_integral_b1.^2
;
tau_e_minus_T_minus_integral_sq_1212_b2=tau_e_minus_T_1212_minus_integral_b2.^2
;
tau_e_minus_T_minus_integral_sq_1212_b3=tau_e_minus_T_1212_minus_integral_b3.^2
;
tau_e_minus_T_minus_integral_sq_1212_b4=tau_e_minus_T_1212_minus_integral_b4.^2
;


% % % create array of phase of zeitgeber cycle
```

```
phase_lon=zeros(240,241);
phase_lon(:,1)=transpose(0:0.0262:2*pi);
phase_lon(:,241)=-3+ones(240,1);

for i=2:length(phase_lon(1,:))-1
   phase_lon(:,i)=circshift(phase_lon(:,i-1),1);
end


% % % only the first row of the phase_lon matrix is of interest to us
% % %   because it is by that value that the CIRC shifts to the left
circ_phi=0-phase_lon(1,:);


% % % create dummy matrices to store minimum integral value and how many of
% % %   them exist
min_integral1212_b1=zeros(1,length(a),length(s));
min_integral1212_b2=zeros(1,length(a),length(s));
min_integral1212_b3=zeros(1,length(a),length(s));
min_integral1212_b4=zeros(1,length(a),length(s));

n_min_integral1212_b1=zeros(1,length(a),length(s));
n_min_integral1212_b2=zeros(1,length(a),length(s));
n_min_integral1212_b3=zeros(1,length(a),length(s));
n_min_integral1212_b4=zeros(1,length(a),length(s));


% % % find the values of closest to 0 and find how many such values exist
% % %   and the maximum number of such values (this basically means that
% % %   one can get best entrainment at multiple phases of the LD cycle)
for i=1:length(a)
   for j=1:length(s)
      min_integral1212_b1(:,i,j)=min(tau_e_minus_T_minus_integral_sq_1212_b1(:,i,j));

n_min_integral1212_b1(1,i,j)=sum(tau_e_minus_T_minus_integral_sq_1212_b1(:,i,j)==mi
n_integral1212_b1(1,i,j));
      max_n_min_integral1212_b1=max(n_min_integral1212_b1(:));

      min_integral1212_b2(:,i,j)=min(tau_e_minus_T_minus_integral_sq_1212_b2(:,i,j));

n_min_integral1212_b2(1,i,j)=sum(tau_e_minus_T_minus_integral_sq_1212_b2(:,i,j)==mi
n_integral1212_b2(1,i,j));
      max_n_min_integral1212_b2=max(n_min_integral1212_b2(:));

      min_integral1212_b3(:,i,j)=min(tau_e_minus_T_minus_integral_sq_1212_b3(:,i,j));
```

```
270
271 n_min_integral1212_b3(1,i,j)=sum(tau_e_minus_T_minus_integral_sq_1212_b3(:,i,j)==mi
272 n_integral1212_b3(1,i,j));
273         max_n_min_integral1212_b3=max(n_min_integral1212_b3(:));
274
275         min_integral1212_b4(:,i,j)=min(tau_e_minus_T_minus_integral_sq_1212_b4(:,i,j));
276
277 n_min_integral1212_b4(1,i,j)=sum(tau_e_minus_T_minus_integral_sq_1212_b4(:,i,j)==mi
278 n_integral1212_b4(1,i,j));
279         max_n_min_integral1212_b4=max(n_min_integral1212_b4(:));
280       end
281 end
282
283
284 % % % create dummy matrices to store the coordinates of tau_e-T-integral
285 % % %   values closest to 0 and then to store the phases determined by
286 % % %   these coordinates
287 phase_coord1212_b1=zeros(max_n_min_integral1212_b1,length(a),length(s));
288 phase_coord1212_b2=zeros(max_n_min_integral1212_b2,length(a),length(s));
289 phase_coord1212_b3=zeros(max_n_min_integral1212_b3,length(a),length(s));
290 phase_coord1212_b4=zeros(max_n_min_integral1212_b4,length(a),length(s));
291
292 phase_val1212_b1=zeros(max_n_min_integral1212_b1,length(a),length(s));
293 phase_val1212_b2=zeros(max_n_min_integral1212_b2,length(a),length(s));
294 phase_val1212_b3=zeros(max_n_min_integral1212_b3,length(a),length(s));
295 phase_val1212_b4=zeros(max_n_min_integral1212_b4,length(a),length(s));
296
297
298 % % % find the coordinates for which the values are closest to 0 and the
299 % % %   phases determined by these coordinates
300 for i=1:length(a)
301     for j=1:length(s)
302
303         if n_min_integral1212_b1(1,i,j)==1
304
305 phase_coord1212_b1(1,i,j)=find(tau_e_minus_T_minus_integral_sq_1212_b1(:,i,j)==min_
306 integral1212_b1(1,i,j));
307         elseif n_min_integral1212_b1(1,i,j)==2
308
309 phase_coord1212_b1(1:2,i,j)=find(tau_e_minus_T_minus_integral_sq_1212_b1(:,i,j)==mi
310 n_integral1212_b1(1,i,j));
311         elseif n_min_integral1212_b1(1,i,j)==3
312
313 phase_coord1212_b1(1:3,i,j)=find(tau_e_minus_T_minus_integral_sq_1212_b1(:,i,j)==mi
314 n_integral1212_b1(1,i,j));
315         elseif n_min_integral1212_b1(1,i,j)==4
```

```
316
317     phase_coord1212_b1(1:4,i,j)=find(tau_e_minus_T_minus_integral_sq_1212_b1(:,i,j)==mi
318     n_integral1212_b1(1,i,j));
319         elseif n_min_integral1212_b1(1,i,j)==5
320
321     phase_coord1212_b1(1:5,i,j)=find(tau_e_minus_T_minus_integral_sq_1212_b1(:,i,j)==mi
322     n_integral1212_b1(1,i,j));
323         end
324
325         if n_min_integral1212_b2(1,i,j)==1
326
327     phase_coord1212_b2(1,i,j)=find(tau_e_minus_T_minus_integral_sq_1212_b2(:,i,j)==min_
328     integral1212_b2(1,i,j));
329         elseif n_min_integral1212_b2(1,i,j)==2
330
331     phase_coord1212_b2(1:2,i,j)=find(tau_e_minus_T_minus_integral_sq_1212_b2(:,i,j)==mi
332     n_integral1212_b2(1,i,j));
333         elseif n_min_integral1212_b2(1,i,j)==3
334
335     phase_coord1212_b2(1:3,i,j)=find(tau_e_minus_T_minus_integral_sq_1212_b2(:,i,j)==mi
336     n_integral1212_b2(1,i,j));
337         elseif n_min_integral1212_b2(1,i,j)==4
338
339     phase_coord1212_b2(1:4,i,j)=find(tau_e_minus_T_minus_integral_sq_1212_b2(:,i,j)==mi
340     n_integral1212_b2(1,i,j));
341         elseif n_min_integral1212_b2(1,i,j)==5
342
343     phase_coord1212_b2(1:5,i,j)=find(tau_e_minus_T_minus_integral_sq_1212_b2(:,i,j)==mi
344     n_integral1212_b2(1,i,j));
345         end
346
347         if n_min_integral1212_b3(1,i,j)==1
348
349     phase_coord1212_b3(1,i,j)=find(tau_e_minus_T_minus_integral_sq_1212_b3(:,i,j)==min_
350     integral1212_b3(1,i,j));
351         elseif n_min_integral1212_b3(1,i,j)==2
352
353     phase_coord1212_b3(1:2,i,j)=find(tau_e_minus_T_minus_integral_sq_1212_b3(:,i,j)==mi
354     n_integral1212_b3(1,i,j));
355         elseif n_min_integral1212_b3(1,i,j)==3
356
357     phase_coord1212_b3(1:3,i,j)=find(tau_e_minus_T_minus_integral_sq_1212_b3(:,i,j)==mi
358     n_integral1212_b3(1,i,j));
359         elseif n_min_integral1212_b3(1,i,j)==4
```

```
360
361     phase_coord1212_b3(1:4,i,j)=find(tau_e_minus_T_minus_integral_sq_1212_b3(:,i,j)==mi
362     n_integral1212_b3(1,i,j));
363         elseif n_min_integral1212_b3(1,i,j)==5
364
365     phase_coord1212_b3(1:5,i,j)=find(tau_e_minus_T_minus_integral_sq_1212_b3(:,i,j)==mi
366     n_integral1212_b3(1,i,j));
367         end
368
369         if n_min_integral1212_b4(1,i,j)==1
370
371     phase_coord1212_b4(1,i,j)=find(tau_e_minus_T_minus_integral_sq_1212_b4(:,i,j)==min_
372     integral1212_b4(1,i,j));
373         elseif n_min_integral1212_b4(1,i,j)==2
374
375     phase_coord1212_b4(1:2,i,j)=find(tau_e_minus_T_minus_integral_sq_1212_b4(:,i,j)==mi
376     n_integral1212_b4(1,i,j));
377         elseif n_min_integral1212_b4(1,i,j)==3
378
379     phase_coord1212_b4(1:3,i,j)=find(tau_e_minus_T_minus_integral_sq_1212_b4(:,i,j)==mi
380     n_integral1212_b4(1,i,j));
381         elseif n_min_integral1212_b4(1,i,j)==4
382
383     phase_coord1212_b4(1:4,i,j)=find(tau_e_minus_T_minus_integral_sq_1212_b4(:,i,j)==mi
384     n_integral1212_b4(1,i,j));
385         elseif n_min_integral1212_b4(1,i,j)==5
386
387     phase_coord1212_b4(1:5,i,j)=find(tau_e_minus_T_minus_integral_sq_1212_b4(:,i,j)==mi
388     n_integral1212_b4(1,i,j));
389         end
390
391     % % %       because the dummy matrices have 0s the coordinates defined by
392     % % %         such values will produce an error. therefore all of them have been
393     % % %         replaced by 241 which is a redundant coordinate index and therefore can
394     % % %         be removed later
395         phase_coord1212_b1(phase_coord1212_b1==0)=241;
396         phase_coord1212_b2(phase_coord1212_b2==0)=241;
397         phase_coord1212_b3(phase_coord1212_b3==0)=241;
398         phase_coord1212_b4(phase_coord1212_b4==0)=241;
399
400         for k=1:length(phase_val1212_b1(:,1,1))
401            phase_val1212_b1(k,i,j)=circ_phi(:,phase_coord1212_b1(k,i,j));
402            phase_val1212_b2(k,i,j)=circ_phi(:,phase_coord1212_b2(k,i,j));
403            phase_val1212_b3(k,i,j)=circ_phi(:,phase_coord1212_b3(k,i,j));
404            phase_val1212_b4(k,i,j)=circ_phi(:,phase_coord1212_b4(k,i,j));
405         end
```

```
406         end
407     end
408
409
410     % % % compute phase angle difference now
411     phase_of_entrainment1212_b1=zeros(max_n_min_integral1212_b1,length(a),length(s));
412     phase_of_entrainment1212_b2=zeros(max_n_min_integral1212_b2,length(a),length(s));
413     phase_of_entrainment1212_b3=zeros(max_n_min_integral1212_b3,length(a),length(s));
414     phase_of_entrainment1212_b4=zeros(max_n_min_integral1212_b4,length(a),length(s));
415
416     for i=1:length(a)
417         for j=1:length(s)
418             for k=1:length(phase_of_entrainment1212_b1(:,1,1))
419                 phase_of_entrainment1212_b1(k,i,j)=phase_val1212_b1(k,i,j)-tantheta1212(1,i,j);
420                 phase_of_entrainment1212_b2(k,i,j)=phase_val1212_b2(k,i,j)-tantheta1212(1,i,j);
421                 phase_of_entrainment1212_b3(k,i,j)=phase_val1212_b3(k,i,j)-tantheta1212(1,i,j);
422                 phase_of_entrainment1212_b4(k,i,j)=phase_val1212_b4(k,i,j)-tantheta1212(1,i,j);
423             end
424         end
425     end
426
427     for i=1:length(a)
428         for j=1:length(s)
429             for k=1:length(phase_of_entrainment1212_b1(:,1,1))
430                 if phase_of_entrainment1212_b1(k,i,j)>2*pi
431                     phase_of_entrainment1212_b1(k,i,j)=phase_of_entrainment1212_b1(k,i,j)-2*pi;
432                 elseif phase_of_entrainment1212_b1(k,i,j)<-2*pi
433                     phase_of_entrainment1212_b1(k,i,j)=phase_of_entrainment1212_b1(k,i,j)+2*pi;
434                 else
435                     phase_of_entrainment1212_b1(k,i,j)=phase_of_entrainment1212_b1(k,i,j);
436                 end
437
438                 if phase_of_entrainment1212_b2(k,i,j)>2*pi
439                     phase_of_entrainment1212_b2(k,i,j)=phase_of_entrainment1212_b2(k,i,j)-2*pi;
440                 elseif phase_of_entrainment1212_b2(k,i,j)<-2*pi
441                     phase_of_entrainment1212_b2(k,i,j)=phase_of_entrainment1212_b2(k,i,j)+2*pi;
442                 else
443                     phase_of_entrainment1212_b2(k,i,j)=phase_of_entrainment1212_b2(k,i,j);
444                 end
445
446                 if phase_of_entrainment1212_b3(k,i,j)>2*pi
447                     phase_of_entrainment1212_b3(k,i,j)=phase_of_entrainment1212_b3(k,i,j)-2*pi;
448                 elseif phase_of_entrainment1212_b3(k,i,j)<-2*pi
449                     phase_of_entrainment1212_b3(k,i,j)=phase_of_entrainment1212_b3(k,i,j)+2*pi;
450                 else
451                     phase_of_entrainment1212_b3(k,i,j)=phase_of_entrainment1212_b3(k,i,j);
```

```
            end

            if phase_of_entrainment1212_b4(k,i,j)>2*pi
                phase_of_entrainment1212_b4(k,i,j)=phase_of_entrainment1212_b4(k,i,j)-2*pi;
            elseif phase_of_entrainment1212_b4(k,i,j)<-2*pi
                phase_of_entrainment1212_b4(k,i,j)=phase_of_entrainment1212_b4(k,i,j)+2*pi;
            else
                phase_of_entrainment1212_b4(k,i,j)=phase_of_entrainment1212_b4(k,i,j);
            end
        end
    end
end


% % % create the phase angle difference in a format that can be plotted
phase_angle_sim_1212_b1=zeros(length(a),length(s),max_n_min_integral1212_b1);
phase_angle_sim_1212_b2=zeros(length(a),length(s),max_n_min_integral1212_b2);
phase_angle_sim_1212_b3=zeros(length(a),length(s),max_n_min_integral1212_b3);
phase_angle_sim_1212_b4=zeros(length(a),length(s),max_n_min_integral1212_b4);

for ii=1:length(phase_of_entrainment1212_b1(1,1,:))
    for jj=1:length(phase_of_entrainment1212_b1(:,1,1))
        phase_angle_sim_1212_b1(:,ii,jj)=transpose(phase_of_entrainment1212_b1(jj,:,ii));
        phase_angle_sim_1212_b2(:,ii,jj)=transpose(phase_of_entrainment1212_b2(jj,:,ii));
        phase_angle_sim_1212_b3(:,ii,jj)=transpose(phase_of_entrainment1212_b3(jj,:,ii));
        phase_angle_sim_1212_b4(:,ii,jj)=transpose(phase_of_entrainment1212_b4(jj,:,ii));
    end
end



% % % NOW FOR LD 18:06


% % % compute the square of CIRC responses and compute CoM 1212
zt1806=[transpose(21:0.1:23.9);transpose(0:0.1:20.9)];
zt_deg1806=(zt1806.*360)/24;
zt_rad1806=(zt_deg1806.*pi)./180;
sin_comp1806=sin(zt_rad1806);
cos_comp1806=cos(zt_rad1806);

sin_comp_circ1806=zeros(240,length(a),length(s));
cos_comp_circ1806=zeros(240,length(a),length(s));

for i=1:length(a)
    for j=1:length(s)
```

```
        sin_comp_circ1806(:,i,j)=sin_comp1806.*circ(:,i,j);
        cos_comp_circ1806(:,i,j)=cos_comp1806.*circ(:,i,j);
        sum_circ_resp1806=sum(circ);
        x1806=sum(cos_comp_circ1806);
        y1806=sum(sin_comp_circ1806);
        xbar1806=x1806./sum_circ_resp1806;
        ybar1806=y1806./sum_circ_resp1806;
        tantheta1806=atan(ybar1806./xbar1806);

        if xbar1806(:,i,j)<0
            tantheta1806(:,i,j)=pi+tantheta1806(:,i,j);
        else
            tantheta1806(:,i,j)=tantheta1806(:,i,j);
        end
    end
end

for i=1:length(a)
    for j=1:length(s)
        if tantheta1806(:,i,j)<0
            tantheta1806(:,i,j)=2*pi+tantheta1806(:,i,j);
        else
            tantheta1806(:,i,j)=tantheta1806(:,i,j);
        end
    end
end


% % % create zeitgeber arrays in accordance to LON LD 18:06
zeitgeber_1806=zeros(240,240);
zeitgeber_1806(:,1)=[ones(180,1);zeros(60,1)];


% % % create zeitgeber arrays
for ii=2:length(zeitgeber_1806(1,:))
    zeitgeber_1806(:,ii)=circshift(zeitgeber_1806(:,ii-1),1);
end


% % % create dummy matrices for CIRC responses multiplied by zeitgeber
% % % values at different phases of the LD cycle and the net integral uder the
% % % CIRC curve
int_resp1806=zeros(240,length(a),length(s),length(zeitgeber_1806(1,:)));
integral1806=zeros(length(zeitgeber_1806(1,:)),length(a),length(s));
```

```
% % % compute the net integral under the CIRC curve when positioned
% % % differently under LD cycles
for i=1:length(a)
    for j=1:length(s)
        for ii=1:length(zeitgeber_1806(1,:))
            int_resp1806(:,i,j,ii)=c(:,i,j).*zeitgeber_1806(:,ii);
            integral1806(ii,i,j)=sum(int_resp1806(:,i,j,ii));
        end
    end
end

% % % define T value and the difference between tau_e and T
T=24;
tau_e_minus_T_1806_b1=tau_e_1806_b1-T;
tau_e_minus_T_1806_b2=tau_e_1806_b2-T;
tau_e_minus_T_1806_b3=tau_e_1806_b3-T;
tau_e_minus_T_1806_b4=tau_e_1806_b4-T;


% % % compute the difference between tau_e-T and net_integral at all
% % %   computed phases
tau_e_minus_T_minus_integral1806_b1=zeros(length(zeitgeber_1806(1,:)),length(a),lengt
h(s));
tau_e_minus_T_minus_integral1806_b2=zeros(length(zeitgeber_1806(1,:)),length(a),lengt
h(s));
tau_e_minus_T_minus_integral1806_b3=zeros(length(zeitgeber_1806(1,:)),length(a),lengt
h(s));
tau_e_minus_T_minus_integral1806_b4=zeros(length(zeitgeber_1806(1,:)),length(a),lengt
h(s));

for i=1:length(a)
    for j=1:length(s)
        for k=1:length(tau_e_minus_T_minus_integral1806_b1(:,1,1))
            tau_e_minus_T_minus_integral1806_b1(k,i,j)=tau_e_minus_T_1806_b1-
integral1806(k,i,j);
            tau_e_minus_T_minus_integral1806_b2(k,i,j)=tau_e_minus_T_1806_b2-
integral1806(k,i,j);
            tau_e_minus_T_minus_integral1806_b3(k,i,j)=tau_e_minus_T_1806_b3-
integral1806(k,i,j);
            tau_e_minus_T_minus_integral1806_b4(k,i,j)=tau_e_minus_T_1806_b4-
integral1806(k,i,j);
        end
    end
end

tau_e_minus_T_minus_integral1806_sq_b1=tau_e_minus_T_minus_integral1806_b1.^2;
```

```
tau_e_minus_T_minus_integral1806_sq_b2=tau_e_minus_T_minus_integral1806_b2.^2;
tau_e_minus_T_minus_integral1806_sq_b3=tau_e_minus_T_minus_integral1806_b3.^2;
tau_e_minus_T_minus_integral1806_sq_b4=tau_e_minus_T_minus_integral1806_b4.^2;


% % % create dummy matrices to store minimum integral value and how many of
% % %   them exist
min_integral1806_b1=zeros(1,length(a),length(s));
min_integral1806_b2=zeros(1,length(a),length(s));
min_integral1806_b3=zeros(1,length(a),length(s));
min_integral1806_b4=zeros(1,length(a),length(s));

n_min_integral1806_b1=zeros(1,length(a),length(s));
n_min_integral1806_b2=zeros(1,length(a),length(s));
n_min_integral1806_b3=zeros(1,length(a),length(s));
n_min_integral1806_b4=zeros(1,length(a),length(s));

% % % find the values of closest to 0 and find how many such values exist
% % %   and the maximum number of such values (this basically means that
% % %   one can get best entrainment at multiple phases of the LD cycle)
for i=1:length(a)
    for j=1:length(s)
        min_integral1806_b1(:,i,j)=min(tau_e_minus_T_minus_integral1806_sq_b1(:,i,j));

n_min_integral1806_b1(1,i,j)=sum(tau_e_minus_T_minus_integral1806_sq_b1(:,i,j)==min
_integral1806_b1(1,i,j));
        max_n_min_integral1806_b1=max(n_min_integral1806_b1(:));

        min_integral1806_b2(:,i,j)=min(tau_e_minus_T_minus_integral1806_sq_b2(:,i,j));

n_min_integral1806_b2(1,i,j)=sum(tau_e_minus_T_minus_integral1806_sq_b2(:,i,j)==min
_integral1806_b2(1,i,j));
        max_n_min_integral1806_b2=max(n_min_integral1806_b2(:));

        min_integral1806_b3(:,i,j)=min(tau_e_minus_T_minus_integral1806_sq_b3(:,i,j));

n_min_integral1806_b3(1,i,j)=sum(tau_e_minus_T_minus_integral1806_sq_b3(:,i,j)==min
_integral1806_b3(1,i,j));
        max_n_min_integral1806_b3=max(n_min_integral1806_b3(:));

        min_integral1806_b4(:,i,j)=min(tau_e_minus_T_minus_integral1806_sq_b4(:,i,j));

n_min_integral1806_b4(1,i,j)=sum(tau_e_minus_T_minus_integral1806_sq_b4(:,i,j)==min
_integral1806_b4(1,i,j));
        max_n_min_integral1806_b4=max(n_min_integral1806_b4(:));
    end
```

```
636 end
637
638
639 % % % create dummy matrices to store the coordinates of tau_e-T-integral
640 % % %   values closest to 0 and then to store the phases determined by
641 % % %   these coordinates
642 phase_coord1806_b1=zeros(max_n_min_integral1806_b1,length(a),length(s));
643 phase_coord1806_b2=zeros(max_n_min_integral1806_b2,length(a),length(s));
644 phase_coord1806_b3=zeros(max_n_min_integral1806_b3,length(a),length(s));
645 phase_coord1806_b4=zeros(max_n_min_integral1806_b4,length(a),length(s));
646
647 phase_val1806_b1=zeros(max_n_min_integral1806_b1,length(a),length(s));
648 phase_val1806_b2=zeros(max_n_min_integral1806_b2,length(a),length(s));
649 phase_val1806_b3=zeros(max_n_min_integral1806_b3,length(a),length(s));
650 phase_val1806_b4=zeros(max_n_min_integral1806_b4,length(a),length(s));
651
652
653 % % % find the coordinates for which the values are closest to 0 and the
654 % % %   phases determined by these coordinates
655 for i=1:length(a)
656    for j=1:length(s)
657
658        if n_min_integral1806_b1(1,i,j)==1
659
660 phase_coord1806_b1(1,i,j)=find(tau_e_minus_T_minus_integral1806_sq_b1(:,i,j)==min_i
661 ntegral1806_b1(1,i,j));
662        elseif n_min_integral1806_b1(1,i,j)==2
663
664 phase_coord1806_b1(1:2,i,j)=find(tau_e_minus_T_minus_integral1806_sq_b1(:,i,j)==min
665 _integral1806_b1(1,i,j));
666        elseif n_min_integral1806_b1(1,i,j)==3
667
668 phase_coord1806_b1(1:3,i,j)=find(tau_e_minus_T_minus_integral1806_sq_b1(:,i,j)==min
669 _integral1806_b1(1,i,j));
670        elseif n_min_integral1806_b1(1,i,j)==4
671
672 phase_coord1806_b1(1:4,i,j)=find(tau_e_minus_T_minus_integral1806_sq_b1(:,i,j)==min
673 _integral1806_b1(1,i,j));
674        elseif n_min_integral1806_b1(1,i,j)==5
675
676 phase_coord1806_b1(1:5,i,j)=find(tau_e_minus_T_minus_integral1806_sq_b1(:,i,j)==min
677 _integral1806_b1(1,i,j));
678        end
679
680        if n_min_integral1806_b2(1,i,j)==1
```

```
681
682     phase_coord1806_b2(1,i,j)=find(tau_e_minus_T_minus_integral1806_sq_b2(:,i,j)==min_i
683     ntegral1806_b2(1,i,j));
684         elseif n_min_integral1806_b2(1,i,j)==2
685
686     phase_coord1806_b2(1:2,i,j)=find(tau_e_minus_T_minus_integral1806_sq_b2(:,i,j)==min
687     _integral1806_b2(1,i,j));
688         elseif n_min_integral1806_b2(1,i,j)==3
689
690     phase_coord1806_b2(1:3,i,j)=find(tau_e_minus_T_minus_integral1806_sq_b2(:,i,j)==min
691     _integral1806_b2(1,i,j));
692         elseif n_min_integral1806_b2(1,i,j)==4
693
694     phase_coord1806_b2(1:4,i,j)=find(tau_e_minus_T_minus_integral1806_sq_b2(:,i,j)==min
695     _integral1806_b2(1,i,j));
696         elseif n_min_integral1806_b2(1,i,j)==5
697
698     phase_coord1806_b2(1:5,i,j)=find(tau_e_minus_T_minus_integral1806_sq_b2(:,i,j)==min
699     _integral1806_b2(1,i,j));
700         end
701
702         if n_min_integral1806_b3(1,i,j)==1
703
704     phase_coord1806_b3(1,i,j)=find(tau_e_minus_T_minus_integral1806_sq_b3(:,i,j)==min_i
705     ntegral1806_b3(1,i,j));
706         elseif n_min_integral1806_b3(1,i,j)==2
707
708     phase_coord1806_b3(1:2,i,j)=find(tau_e_minus_T_minus_integral1806_sq_b3(:,i,j)==min
709     _integral1806_b3(1,i,j));
710         elseif n_min_integral1806_b3(1,i,j)==3
711
712     phase_coord1806_b3(1:3,i,j)=find(tau_e_minus_T_minus_integral1806_sq_b3(:,i,j)==min
713     _integral1806_b3(1,i,j));
714         elseif n_min_integral1806_b3(1,i,j)==4
715
716     phase_coord1806_b3(1:4,i,j)=find(tau_e_minus_T_minus_integral1806_sq_b3(:,i,j)==min
717     _integral1806_b3(1,i,j));
718         elseif n_min_integral1806_b3(1,i,j)==5
719
720     phase_coord1806_b3(1:5,i,j)=find(tau_e_minus_T_minus_integral1806_sq_b3(:,i,j)==min
721     _integral1806_b3(1,i,j));
722         end
723
724         if n_min_integral1806_b4(1,i,j)==1
```

```
725
726 phase_coord1806_b4(1,i,j)=find(tau_e_minus_T_minus_integral1806_sq_b4(:,i,j)==min_i
727 ntegral1806_b4(1,i,j));
728         elseif n_min_integral1806_b4(1,i,j)==2
729
730 phase_coord1806_b4(1:2,i,j)=find(tau_e_minus_T_minus_integral1806_sq_b4(:,i,j)==min
731 _integral1806_b4(1,i,j));
732         elseif n_min_integral1806_b4(1,i,j)==3
733
734 phase_coord1806_b4(1:3,i,j)=find(tau_e_minus_T_minus_integral1806_sq_b4(:,i,j)==min
735 _integral1806_b4(1,i,j));
736         elseif n_min_integral1806_b4(1,i,j)==4
737
738 phase_coord1806_b4(1:4,i,j)=find(tau_e_minus_T_minus_integral1806_sq_b4(:,i,j)==min
739 _integral1806_b4(1,i,j));
740         elseif n_min_integral1806_b4(1,i,j)==5
741
742 phase_coord1806_b4(1:5,i,j)=find(tau_e_minus_T_minus_integral1806_sq_b4(:,i,j)==min
743 _integral1806_b4(1,i,j));
744         end
745
746
747 % % %         because the dummy matrices have 0s the coordinates defined by
748 % % %           such values will produce an error. therefore all of them have been
749 % % %           replaced by 241 which is a redundant coordinate index and therefore can
750 % % %           be removed later
751         phase_coord1806_b1(phase_coord1806_b1==0)=241;
752         phase_coord1806_b2(phase_coord1806_b2==0)=241;
753         phase_coord1806_b3(phase_coord1806_b3==0)=241;
754         phase_coord1806_b4(phase_coord1806_b4==0)=241;
755
756         for k=1:length(phase_val1806_b1(:,1,1))
757             phase_val1806_b1(k,i,j)=circ_phi(:,phase_coord1806_b1(k,i,j));
758             phase_val1806_b2(k,i,j)=circ_phi(:,phase_coord1806_b2(k,i,j));
759             phase_val1806_b3(k,i,j)=circ_phi(:,phase_coord1806_b3(k,i,j));
760             phase_val1806_b4(k,i,j)=circ_phi(:,phase_coord1806_b4(k,i,j));
761         end
762     end
763 end
764
765
766 % % % compute phase angle difference now
767 phase_of_entrainment1806_b1=zeros(max_n_min_integral1806_b1,length(a),length(s));
768 phase_of_entrainment1806_b2=zeros(max_n_min_integral1806_b2,length(a),length(s));
769 phase_of_entrainment1806_b3=zeros(max_n_min_integral1806_b3,length(a),length(s));
770 phase_of_entrainment1806_b4=zeros(max_n_min_integral1806_b4,length(a),length(s));
```

```
771
772 for i=1:length(a)
773     for j=1:length(s)
774         for k=1:length(phase_of_entrainment1806_b1(:,1,1))
775             phase_of_entrainment1806_b1(k,i,j)=phase_val1806_b1(k,i,j)-tantheta1806(1,i,j);
776             phase_of_entrainment1806_b2(k,i,j)=phase_val1806_b2(k,i,j)-tantheta1806(1,i,j);
777             phase_of_entrainment1806_b3(k,i,j)=phase_val1806_b3(k,i,j)-tantheta1806(1,i,j);
778             phase_of_entrainment1806_b4(k,i,j)=phase_val1806_b4(k,i,j)-tantheta1806(1,i,j);
779         end
780     end
781 end
782
783 for i=1:length(a)
784     for j=1:length(s)
785         for k=1:length(phase_of_entrainment1806_b1(:,1,1))
786             if phase_of_entrainment1806_b1(k,i,j)>2*pi
787                 phase_of_entrainment1806_b1(k,i,j)=phase_of_entrainment1806_b1(k,i,j)-2*pi;
788             elseif phase_of_entrainment1806_b1(k,i,j)<-2*pi
789                 phase_of_entrainment1806_b1(k,i,j)=phase_of_entrainment1806_b1(k,i,j)+2*pi;
790             else
791                 phase_of_entrainment1806_b1(k,i,j)=phase_of_entrainment1806_b1(k,i,j);
792             end
793
794             if phase_of_entrainment1806_b2(k,i,j)>2*pi
795                 phase_of_entrainment1806_b2(k,i,j)=phase_of_entrainment1806_b2(k,i,j)-2*pi;
796             elseif phase_of_entrainment1806_b2(k,i,j)<-2*pi
797                 phase_of_entrainment1806_b2(k,i,j)=phase_of_entrainment1806_b2(k,i,j)+2*pi;
798             else
799                 phase_of_entrainment1806_b2(k,i,j)=phase_of_entrainment1806_b2(k,i,j);
800             end
801
802             if phase_of_entrainment1806_b3(k,i,j)>2*pi
803                 phase_of_entrainment1806_b3(k,i,j)=phase_of_entrainment1806_b3(k,i,j)-2*pi;
804             elseif phase_of_entrainment1806_b3(k,i,j)<-2*pi
805                 phase_of_entrainment1806_b3(k,i,j)=phase_of_entrainment1806_b3(k,i,j)+2*pi;
806             else
807                 phase_of_entrainment1806_b3(k,i,j)=phase_of_entrainment1806_b3(k,i,j);
808             end
809
810             if phase_of_entrainment1806_b4(k,i,j)>2*pi
811                 phase_of_entrainment1806_b4(k,i,j)=phase_of_entrainment1806_b4(k,i,j)-2*pi;
812             elseif phase_of_entrainment1806_b4(k,i,j)<-2*pi
813                 phase_of_entrainment1806_b4(k,i,j)=phase_of_entrainment1806_b4(k,i,j)+2*pi;
814             else
815                 phase_of_entrainment1806_b4(k,i,j)=phase_of_entrainment1806_b4(k,i,j);
816             end
```

```
        end
    end
end


% % % create the phase angle difference in a format that can be plotted
phase_angle_sim_1806_b1=zeros(length(a),length(s),max_n_min_integral1806_b1);
phase_angle_sim_1806_b2=zeros(length(a),length(s),max_n_min_integral1806_b2);
phase_angle_sim_1806_b3=zeros(length(a),length(s),max_n_min_integral1806_b3);
phase_angle_sim_1806_b4=zeros(length(a),length(s),max_n_min_integral1806_b4);

for ii=1:length(phase_of_entrainment1806_b1(1,1,:))
    for jj=1:length(phase_of_entrainment1806_b1(:,1,1))
        phase_angle_sim_1806_b1(:,ii,jj)=transpose(phase_of_entrainment1806_b1(jj,:,ii));
        phase_angle_sim_1806_b2(:,ii,jj)=transpose(phase_of_entrainment1806_b2(jj,:,ii));
        phase_angle_sim_1806_b3(:,ii,jj)=transpose(phase_of_entrainment1806_b3(jj,:,ii));
        phase_angle_sim_1806_b4(:,ii,jj)=transpose(phase_of_entrainment1806_b4(jj,:,ii));
    end
end


% % % now compute the difference between exp and simulation
fit_1212_b1=phase_angle_exp_ld1212_b1-phase_angle_sim_1212_b1;
fit_1212_b2=phase_angle_exp_ld1212_b2-phase_angle_sim_1212_b2;
fit_1212_b3=phase_angle_exp_ld1212_b3-phase_angle_sim_1212_b3;
fit_1212_b4=phase_angle_exp_ld1212_b4-phase_angle_sim_1212_b4;

fit_1806_b1=phase_angle_exp_ld1806_b1-phase_angle_sim_1806_b1;
fit_1806_b2=phase_angle_exp_ld1806_b2-phase_angle_sim_1806_b2;
fit_1806_b3=phase_angle_exp_ld1806_b3-phase_angle_sim_1806_b3;
fit_1806_b4=phase_angle_exp_ld1806_b4-phase_angle_sim_1806_b4;

fit_1212_sq_b1=fit_1212_b1.^2;
fit_1212_sq_b2=fit_1212_b2.^2;
fit_1212_sq_b3=fit_1212_b3.^2;
fit_1212_sq_b4=fit_1212_b4.^2;

fit_1806_sq_b1=fit_1806_b1.^2;
fit_1806_sq_b2=fit_1806_b2.^2;
fit_1806_sq_b3=fit_1806_b3.^2;
fit_1806_sq_b4=fit_1806_b4.^2;

ss_fit=fit_1212_sq_b1+fit_1806_sq_b1+fit_1212_sq_b2+fit_1806_sq_b2+fit_1212_sq_b3
+fit_1806_sq_b3+fit_1212_sq_b4+fit_1806_sq_b4;
```

```
% % % find a and s for best fit
min_fit=min(ss_fit);
min_coord_s=find(min_fit==min(min_fit));
min_coord_a=find(ss_fit(:,min_coord_s)==min(ss_fit(:,min_coord_s)));

best_a=a(1,min_coord_a);
best_s=s(1,min_coord_s);

lowest_ssd=min(ss_fit(:));

phi_entrainment_1212_time_b1=((phase_of_entrainment1212_b1(:,min_coord_a,min_coor
d_s)*180)/(pi))*(24/360);
phi_entrainment_1212_time_b2=((phase_of_entrainment1212_b2(:,min_coord_a,min_coor
d_s)*180)/(pi))*(24/360);
phi_entrainment_1212_time_b3=((phase_of_entrainment1212_b3(:,min_coord_a,min_coor
d_s)*180)/(pi))*(24/360);
phi_entrainment_1212_time_b4=((phase_of_entrainment1212_b4(:,min_coord_a,min_coor
d_s)*180)/(pi))*(24/360);

phi_entrainment_1806_time_b1=((phase_of_entrainment1806_b1(:,min_coord_a,min_coor
d_s)*180)/(pi))*(24/360);
phi_entrainment_1806_time_b2=((phase_of_entrainment1806_b2(:,min_coord_a,min_coor
d_s)*180)/(pi))*(24/360);
phi_entrainment_1806_time_b3=((phase_of_entrainment1806_b3(:,min_coord_a,min_coor
d_s)*180)/(pi))*(24/360);
phi_entrainment_1806_time_b4=((phase_of_entrainment1806_b4(:,min_coord_a,min_coor
d_s)*180)/(pi))*(24/360);

phi_com_best_circ_1212_time=((tantheta1212(:,min_coord_a,min_coord_s)*180)/(pi))*(2
4/360);
phi_com_best_circ_1806_time=((tantheta1806(:,min_coord_a,min_coord_s)*180)/(pi))*(2
4/360);

data=[best_a;best_s;lowest_ssd;phi_entrainment_1212_time_b1;phi_entrainment_1212_ti
me_b2;phi_entrainment_1212_time_b3;phi_entrainment_1212_time_b4;phi_entrainment_
1806_time_b1;phi_entrainment_1806_time_b2;phi_entrainment_1806_time_b3;phi_entrai
nment_1806_time_b4;phi_com_best_circ_1212_time;phi_com_best_circ_1806_time];
best_circ=c(:,min_coord_a,min_coord_s);

format_spec1='a is %4.2f\n';
format_spec2='s is %4.2f\n';

disp('Abhilash, here are the results for the input variables that you gave me. Cheers!!');

fprintf(format_spec1,best_a);
fprintf(format_spec2,best_s);
```

```
909
910     % save(save_file_name,'data','-ASCII');
911     % save(save_circ_name,'best_circ','-ASCII');
912
913
914     % % % draw figures of interest
915     % figure(1), hold on
916     % plot(c(:,min_coord_a,min_coord_s),'r-*','LineWidth',2);
917     % plot(zeitgeber_1212(:,zeitgeber_coord),'k-o', 'LineWidth',2);
918     % title('Best CIRC-Zeitgeber Alignment','FontSize',22,'FontWeight','bold');
919     % xlabel('Time of Day (in radians)')
920     % ylabel('CIRC Responses (in red) Zeitgeber Responses (in black)')
921     % axis tight
922     % hold off
923
924     % fig=figure(2); hold on
925     % [aa,ss]=meshgrid(linspace(0.1,2,1.9000e+03),linspace(0.1,0.5,1.9000e+03));
926     % fit_interpolated=interp2(a,s,ss_fit,aa,ss,'linear');
927     % q=surf(aa,ss,transpose(fit_interpolated));
928     % set(q,'edgecolor','none');
929     % set(gca,'FontSize',24,'FontWeight','bold');
930     % set(gcf,'color','white');
931     % grid off
932     % colormap(hsv);
933     % h=colorbar;
934     % set(h,'FontSize',24,'FontWeight','Bold');
935     % title(figure_title,'FontSize',32,'FontWeight','bold');
936     % xlabel('Asymmetry Factor (a)','FontSize',28,'FontWeight','bold');
937     % ylabel('Size Factor (s)','FontSize',28,'FontWeight','bold');
938     % zlabel('Fit Values')
939     % axis tight
940     % % saveas(fig,save_file_name,'jpg');
941     % hold off
942
943     toc
```

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