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Analysis of light pulse induced phase shift locomotor activity rhythms in *vg* and *cry*^{b} mutants of *Drosophila melanogaster*

R. Sivaperumal[a], P. Subramanian[a*], Pankaj Yadav[b] and Vijay Kumar Sharma[b]

[a]Department of Biochemistry and Biotechnology, Faculty of Science, Annamalai University, Annamalainagar – 608 002, Tamil Nadu, India.

[b]Chronobiology Laboratory, Evolutionary and Organismal Biology Unit, Jawaharlal Nehru Centre for Advanced Scientific Research, Jakkur, Bangalore - 560 064, Karnataka, India.

Abstract

Circadian rhythms are governed by endogenous oscillators (clocks) in a wide variety of organisms can be phase shifted (i.e., delayed or advanced) by brief exposure to light and changes in temperature. The present study was to determine the influence of single light pulse (~ 100 lux; 15 minutes) at four circadian time points (CT 00, CT 06, CT 12 and CT 18) in wild type (WT; Oregon R$^+$); and *vestigial* (*vg*) and *cryptochrome depleted* (*cry*^{b}) mutants of *Drosophila melanogaster* (fruit fly) under constant darkness (DD) condition. Single light pulse in *vg* and *cry*^{b} mutants showed significant ($p<0.05$) phase shift of the free running circadian locomotor rhythms under these circadian time points as compared to WT flies. From this study, we suggest that blue-light component *cryptochrome* as well as wing structure play an important role for free-running circadian rhythms in *D. melanogaster* and single light pulse effectively alters the phase of free-running circadian rhythms in *vg* and *cry*^{b} mutants at these circadian time points.

Keywords: Circadian, *cryptochrome depleted*, *Drosophila melanogaster*, light pulse, phase shift, *vestigial*.

Introduction

Among the many biological rhythms, circadian rhythms perhaps the most evident and they regulate the daily cycles in most of the living organisms. Circadian rhythms (~24h oscillations), serve to anticipate the daily changes of the external world and are widely present in Neurospora [1, 2], Arabidopsis [3], *Drosophila* [4, 5] and mouse [6, 7]. One of the most important *zeitgebers* in nature are the daily light-dark (LD) cycles, which are perceived by specialized photoreceptors which then convey this information to the clock. Fruitfly (*Drosophila*) serves as an important model organism for understanding the circadian clock at the behavioural, cellular and molecular level. *Drosophila* circadian system receives photic information for entrainment from four different photoreceptors and/or photopigments, i.e. the external photoreceptors (the compound eyes and ocelli), the Hofbauer–Buchner's (H–B) eyelet, the blue-light photopigment *Cryptochrome* (CRY), and unknown photopigments in the dorsal neurons (DNs) [8 - 11]

Circadian rhythms are genetic and cell autonomous in nature [12, 13]. Mutations affecting circadian rhythms were described in *Drosophila* [14], Neurospora [15] and numerous organisms [16 - 18]. A number of mutations that influence circadian rhythms have been also reported in *Drosophila* [19, 20]. Genetic and molecular analyses reveal that *vestigial* (*vg*) regulates wing identity by forming a complex with the *Scalloped* protein that binds sequence specifically to essential sites in wing-specific enhancers [21] and *vg* is known to be essential for wing development in *D. melanogaster* [22]. *vg* protein is able to reprogram some cells in leg and other imaginal discs to adopt a wing fate [23]. Furthermore, Vg is required in all wing cells and loss of Vg function eliminates wing formation [24]. *vg* mutant is characterized by severely reduced wings and loss of the wing-margin structures [25, 26], and this phenotype is being associated with extensive cell death in the wing pouch of the third instar larval imaginal discs [27, 28]. Previous reports showed that alteration of wing size and cell density in *D. melanogaster* modified the periodicities of locomotor activity [29].

Light entrains the oscillator through a blue-light-responsive pterin/flavin-binding protein, *cryptochrome* (CRY) [30]. *cryptochrome depleted* (cry^b) flies express functionless CRY protein (Asp – replaced by Asn at 410 position) [31, 32] and CRY appears to be both photoreceptor and a core clock protein in antennae, probably the eyes [33]. *Drosophila* has one *cryptochrome* (dCRY) that acts as a cell autonomous photoreceptor and is sufficient for most aspects of circadian light sensitivity and entrainment to LD cycles [34].

Circadian systems are entrained and phase shifted by light is well documented [35]. Action spectrum for light-induced phase shifts of the circadian rhythm of adult emergence (eclosion) in *Drosophila pseudoobscura* were well documented [36]. Locomotor activity of insects is an important behavioural feature essential for behavioural traits. Research in chronobiology demonstrated that light stimuli can be used to delay or advance the phase of the oscillator, allowing it to influence the underlying physiological processes. 3–5 consecutive days of exposure to intermittent pulses of bright light (3,000–11,000 lux) as brief as 5 min in duration have been shown to induce phase advances of the endogenous circadian pacemaker in humans [37].

The effect of light pulses delivered at different times in the circadian cycle has been studied extensively in many circadian systems [38]. Previous report from our lab indicates that *vg* and cry^b mutants under LD (12:12) condition influence the rhythmic expression patterns of locomotor activity patterns under different wavelengths of light [39]. However, light pulse induced locomotor activity rhythms of these mutants under free-running condition have not been investigated so far. Hence the present study was carried out to investigate the influence of single white light pulse (100 lux; 15 min) at different circadian time points (CT 00, CT 06, CT 12 and CT 18) under DD in *vg* and cry^b mutants of *D. melanogaster*..

Materials and Methods

Rearing and maintenance of flies

Cultures of *D. melanogaster* **[**wild type (WT-Oregon R^+), *vestigial* (*vg)* and *cryptochrome depleted* (cry^b*)* mutants] were reared and maintained in standard corn food medium in the vial (9.0 mm height × 2.4 mm diameter) containing agar, yeast, corn flour, sucrose, antifungal agent (methyl ρ-hydroxy benzoate) and propionic acid under standard laboratory conditions such as temperature and relative humidity at 24 ± 1^0C and 80 (±5%) respectively.

Experimental set up

The newly eclosed male virgin flies of WT and mutants were collected in the separate food vials and kept under LD (12:12) chronocubicle with a cool white fluorescent lamp (~100 lux) in the environment-controlled room connected to the programmed electrical timer (Frontier digital timer, India). Then placing a single fly (newly eclosed virgin male) in a small transparent locomotor monitoring silicon glass tube (dimension 3 X 3 X 70 mm); food sealed with paraffin wax at one end and non-absorbent cotton at the other end of the locomotor tube. Then, the tube was placed in DAM2 *Drosophila* activity monitor with 32-channel capacity (TriKinetics Inc, Waltham, Massachusetts, United States) containing an IR emitter-detector pair setup.

Light pulse induction in WT and mutants

WT and mutants of *Drosophila melanogaster* (n=12 in each genotypes) in DAM2 *Drosophila* activity monitors were entrained for LD (12:12) cycle for 2 days and then kept for constant darkness for 3 days. An IR beam breaks every time the fly moves up and down the tube, and the responses triggered for 15 min were recorded during this experiment in WT and mutants using a computerized system

[40]. A dim red light lamp (~λ 640 nm) in the dark phase of LD cycles was used to facilitate observation during assay.

Onset of activity of flies (CT 00) under DD was predicted and which is used for light pulse treatment on the next day. Light (~ 100 lux white fluorescent) pulse was administered to these flies on the next day for 15 minutes at different circadian time (CT) points such as CT 00, CT 06, CT 12 and CT 18 (separate sets of WT and mutants for each CT points); whereas control (without light pulse) was also maintained for each CT points for WT as well as mutants. Then light pulse and control groups of WT and mutant flies were kept under DD for several (~ 11 days) cycles and locomotor activity was recorded.

Measurement of light pulse phase shift and statistical analysis

Locomotor activities of individual flies were monitored and activity data were summed and stored to disc at 15 min intervals, and were inspected visually in the form of standard 'actograms'. The analysis was done with a signal processing toolbox implemented in MATLAB (Mathworks; http://www.mathworks.com) as described [41]. Phase shift of control and experimental flies was calculated by determining the phase (ϕ1) in the cycle at which the light pulse occurred by forward extrapolation from the rhythm before the light pulse and the phase (ϕ2) of the same event calculated by backward extrapolation from the rhythm after the light pulse. The phase shift was simply calculated as $\Delta\phi = (\phi2 - \phi1)$ [42].

Statistical analyses of phase shift in WT and mutants were analyzed by analysis of variance (ANOVA) using SPSS version 15.0 (SPSS, CARY, NC, USA) and the individual comparisons were obtained by Duncan's multiple range test (DMRT) [43]. Phase shift values were considered statistically significant when $p<0.05$.

Results

Double plots of representative activity-rest actograms of experimental (light pulse ~100 lux; 15 min) and control flies of wild-type (WT), *vestigial* (*vg*), and *cryptochrome depleted* (cry^b) mutants of *D. melanogaster* under different circadian time points (CT 00, CT 06, CT 12 and CT 18) were shown in Figures 1-4 respectively. In general, WT as well as mutants (*vg* and cry^b) showed marked differences of phase shift as compared to their respective control flies in all circadian time points (Figure 5).

vg and cry^b flies exhibited significant ($p<0.05$) light induced phase shift of free running circadian locomotor activity rhythms at CT 00, CT 06, CT 12 and CT 18 as compared to WT flies (Figure 6). At CT 00, *vg* and cry^b mutants showed advance in the phase of its activity rhythm of about 1.5 h and 2.0 h respectively as compared to WT. About 0.5 h and 4.0 h advanced activity rhythms in *vg* and cry^b flies were shown at CT 06 as compared to WT. *vg* and cry^b flies showed about 3.3 h and 0.7 h phase advanced its activity rhythms at CT 12 as compared to WT. AT CT 18, about 2.0 h and 2.5 h advanced activity of *vg* and cry^b were observed as compared to WT.

Discussion

Entrainment of circadian clocks by LD cycles is believed to be due to daily discrete phase shifts equal in magnitude to the difference between the periodicity of LD cycles and the period of the circadian rhythms [44]. Under entrained state, circadian clocks adjust their phase and/or period to synchronize their 'internal time' with the phase and period of the geophysical world, and thus acquire a stable phase relationship with the environment [45, 46]. Thus, entrainment of circadian clocks by appropriate *zeitgebers* appears to have served as the proximal and ultimate mechanism for their evolution. Behavioural, genetic, and molecular

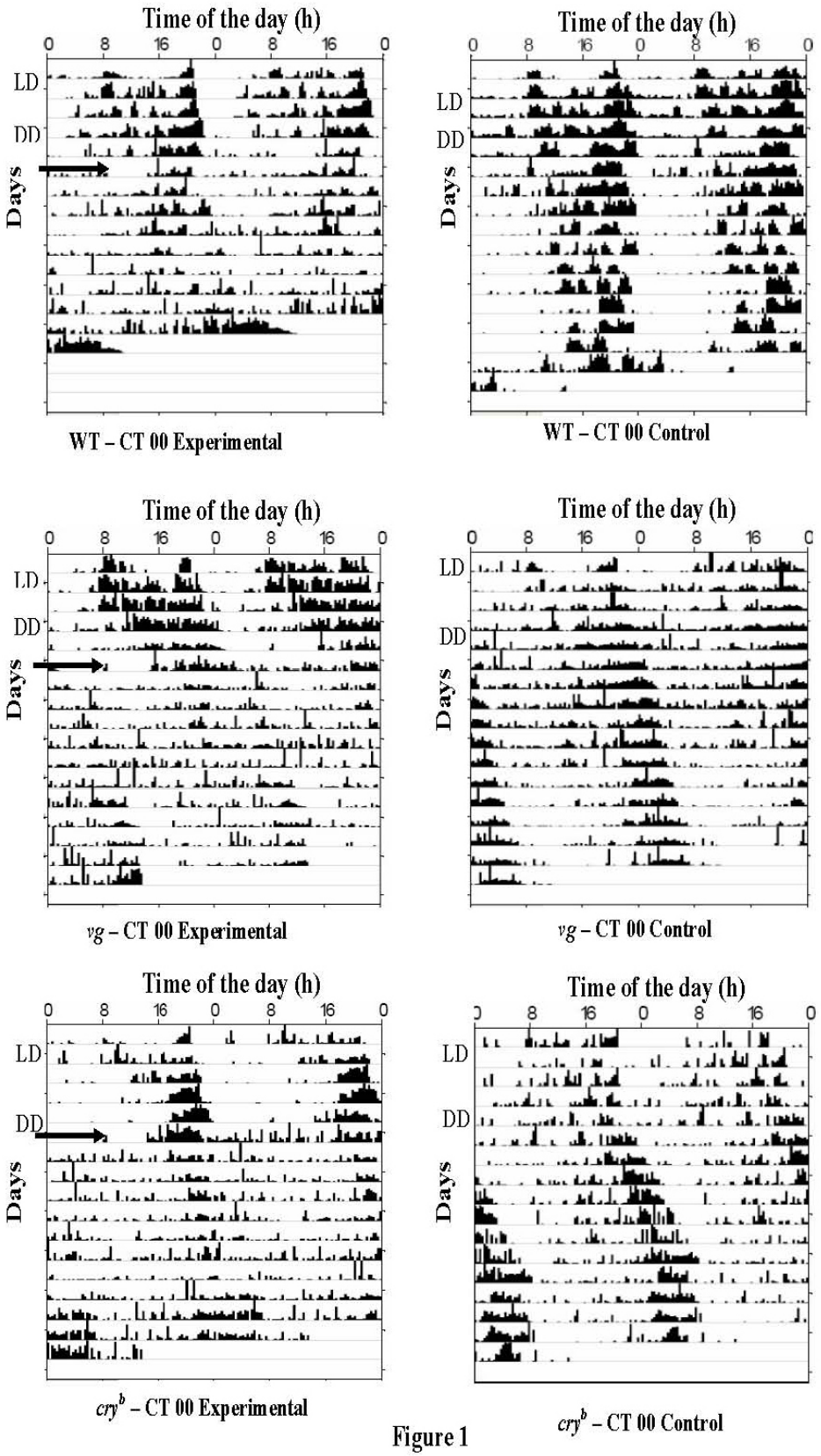

Figure 1 Representative double plotted activity–rest actograms of experimental (~100 lux; 15 min light pulse) and control flies of wild type (WT), *vestigial* (*vg)* and *cryptochrome depleted* (*cry*^{b}*)* mutants of *D. melanogaster* at CT 00. Arrow indicates the time of light pulse.

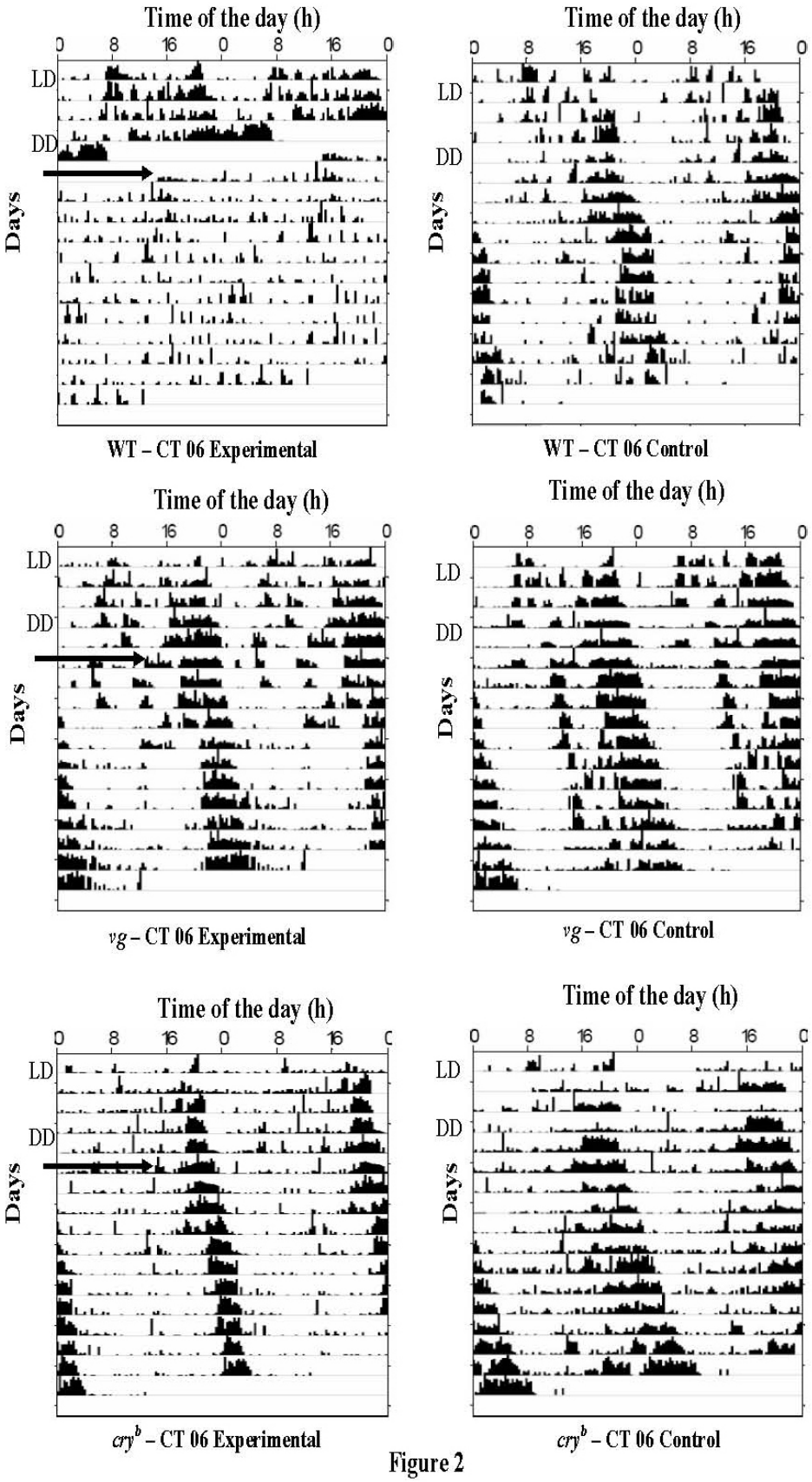

Figure 2

Figure 2 Representative double plotted activity–rest actograms of experimental (~ 100 lux; 15 min light pulse) and control flies of WT, *vg* and *cry*^{b} mutants of *D. melanogaster* at CT 06. Arrow indicates the time of light pulse.

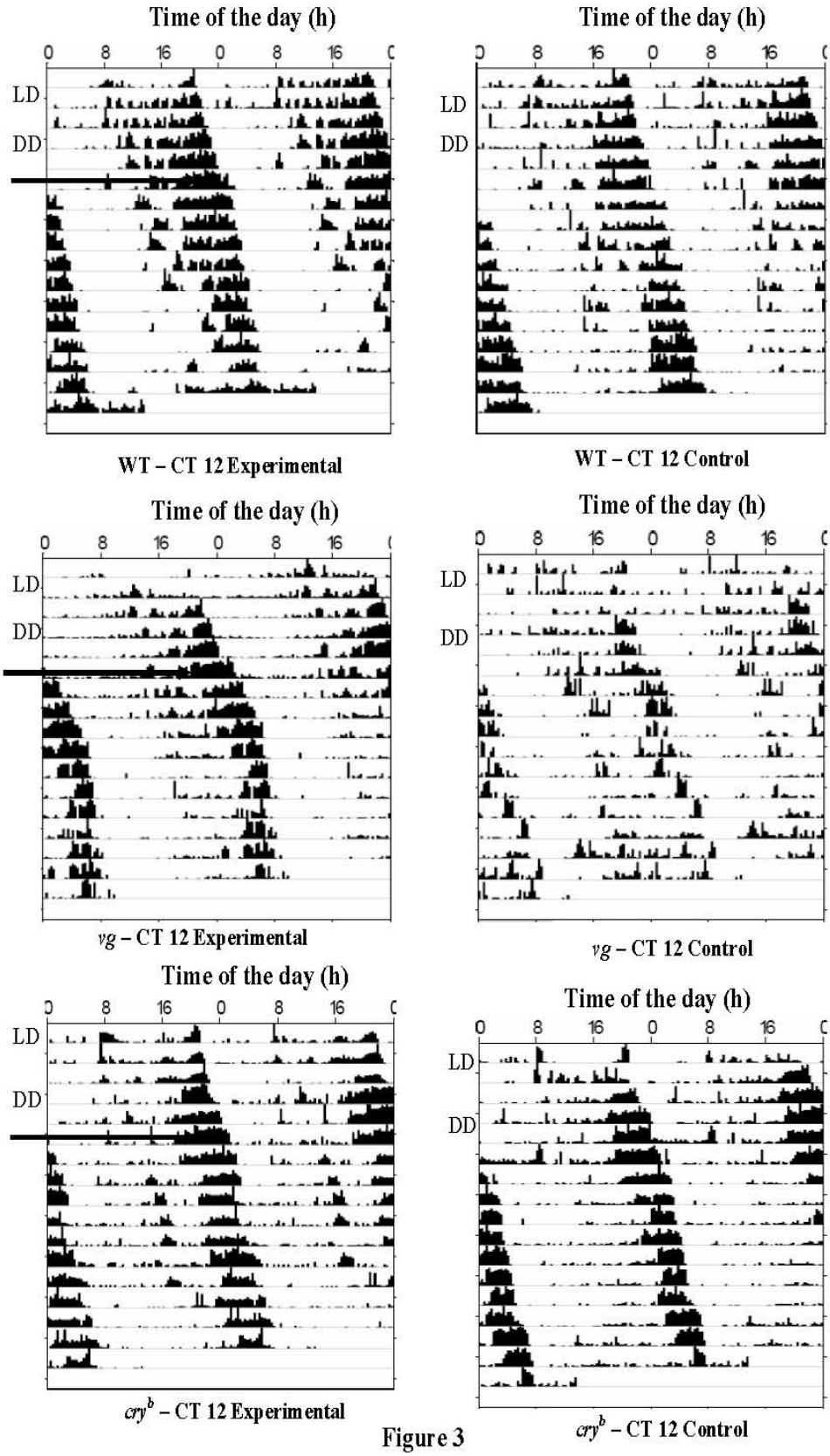

Figure 3

Figure 3 Representative double plotted activity–rest actograms of experimental (~ 100 lux; 15 min light pulse) and control flies of WT, *vg* and *cry*^{b} mutants of *D. melanogaster* at CT 12. Arrow indicates the time of light pulse.

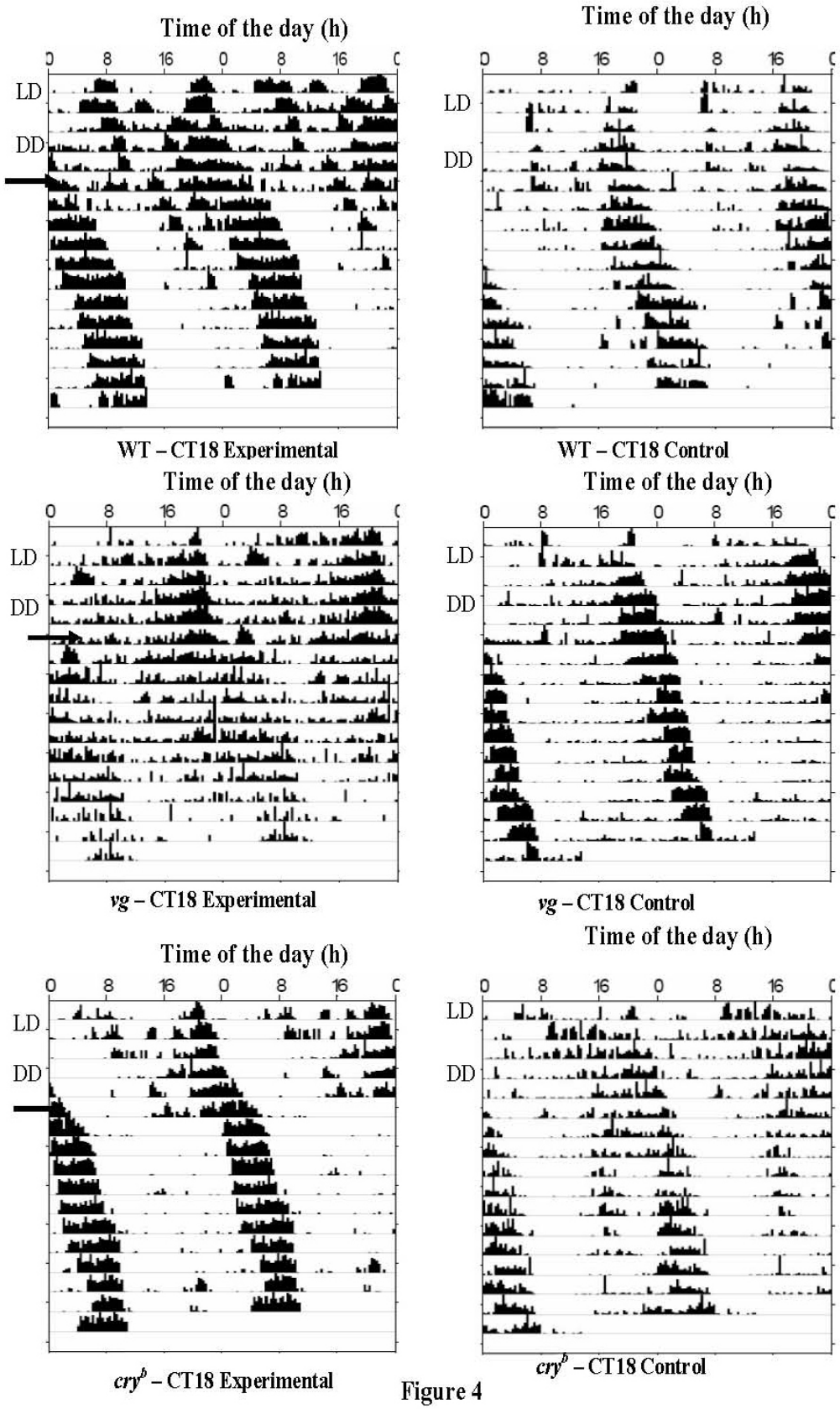

Figure 4 Representative double plotted activity–rest actograms of experimental (~ 100 lux; 15 min light pulse) and control flies of WT, *vg* and *cry*^{b} mutants of *D. melanogaster* at CT 18. Arrow indicates the time of light pulse.

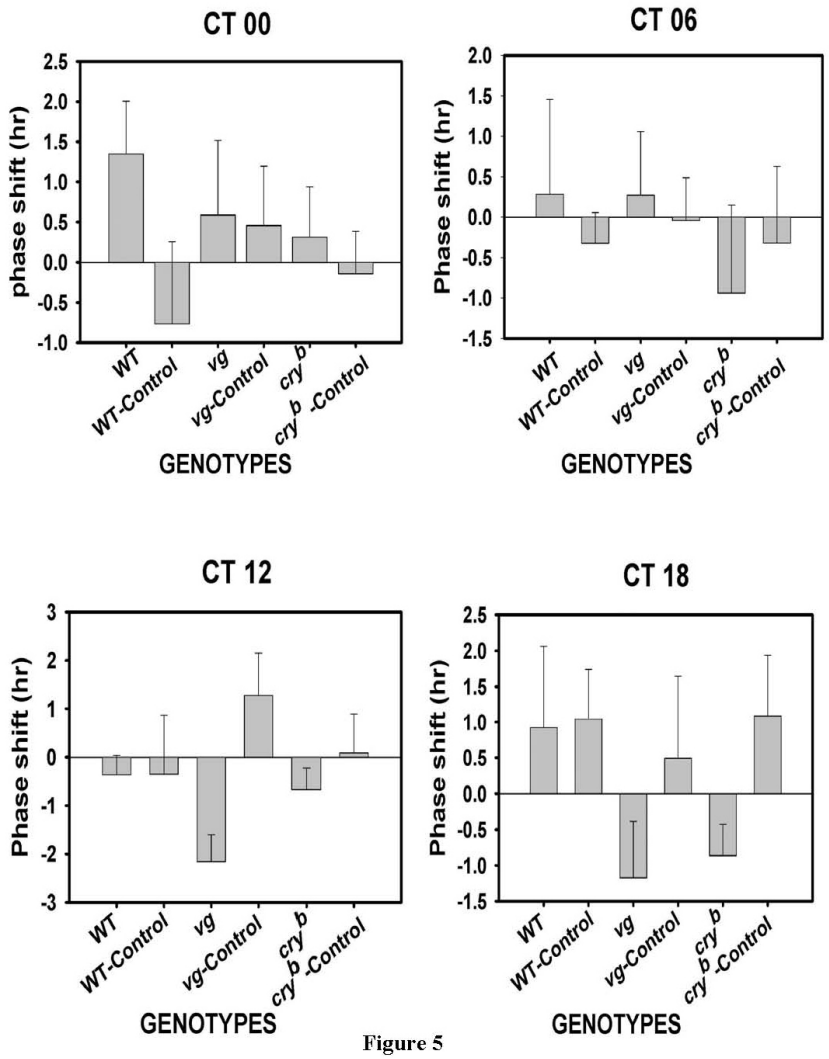

Figure 5

Figure 5 Light pulse (100 lux; 15 min) induced phase shift at CT00, CT 06, CT 12 and CT 18 of control and experimental flies of WT, *vg* and *cry*^{b} mutants of *D. melanogaster*. Phase delays and phase advances are plotted (± SEM) as negative and positive values respectively.

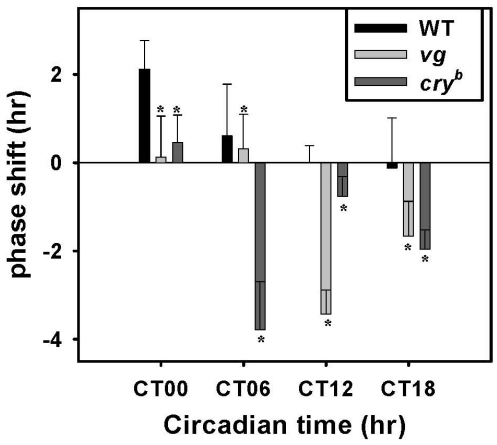

Figure 6

Figure 6 Light pulse (100 lux; 15 min) induced phase shift at CT 00, CT 06, CT 12 and CT 18 of WT, *vg* and *cry*^{b} mutants of *D. melanogaster*.

studies in the little fly have aided in understanding the bases of circadian time keeping and rhythmic behaviours not only in *Drosophila*, but also in other organisms, including mammals. Bright light pulses applied during the early night delay the phase of the clock; light pulses during the late night advance it [47]. Numerous studies indicates that single pulses of bright light are capable of generating modest phase delay and phase advance shifts of the human circadian pacemaker [48, 49].

In *Drosophila melanogaster*, lateral neurons in the brain have been thought to play an essential role in controlling the circadian locomotor activity rhythms [50]. *period* (*per*) is a central component of the circadian oscillator and oscillations of *per* and *timeless (tim)* gene products are an integral part of the feedback loop that controls the locomotor activity of the fly [51]. Light transduction pathways mediated by the rhodopsins and the dedicated circadian blue light photoreceptor *cryptochrome* are also critical in providing the circadian clock with entraining light signals from the environment [52].

Circadian rhythms can be generated by endogenous generator, hardly modified by external disturbances [53]. In animals, it is under debate whether natural, dim, nocturnal light affects the endogenous clock. The process of entrainment is based on differential phase and period responses of the circadian systems to light depending on the phase at which the stimulus is applied. The major entraining cue for circadian rhythms is light, which causes rapid TIM protein degradation; Photoreceptors trigger the ubiquitin-proteasome dependent degradation of TIM via tyrosine phosphorylation [54].

The relationship between phase shifts induced by light pulses of varying duration and intensity is of particular importance for understanding of the mechanisms of entrainment [55]. Stimuli capable of altering the phase of free-running circadian rhythms are presumed to act either on the underlying circadian pacemaker, or on its entrainment pathways, rather than on effector systems downstream from the pacemaker [56]. In our study, single light pulse (~100 lux; 15 min) in WT and mutants (*vg* and cry^b) showed marked changes of phase shift of free running locomotor rhythms as compared to their respective control (without light pulse) flies at CT 00, CT 06, CT 12 and CT 18. This might be due to pulses of light during the dark period rapidly reducing the level of TIM through an ubiquitin-proteasome mechanism [57]. Differential light induced phase shift of WT and mutants in all circadian time points might be corroborated with the previous findings that, the timing and duration of light exposure can also phase shift the circadian clock [58]. In addition, light pulse at these circadian times could modulates/affects several features of circadian oscillations such as phase, period, and amplitude. But, the exact mechanism about how light pulse affects molecular aspects of pacemaker function is still unclear.

In our study, *vg* flies exhibited significant light induced phase shift of free running circadian rhythms at all circadian time points as compared to WT flies. It is believed that mutation causes behavioural synchronization defects in addition to molecular cycling and affects molecules functioning within the circadian clock [59]. *VG* is a selector protein for wing identity and development in *Drosophila*. Previous report suggests that nuclear localization of *vg* could be involved in regulating numerous gene expression [60]. In addition, clock genes such as *per, tim* [61, 62] and *vrille* [63] expressions are weaker in *vg* mutants as compared to WT. Hence, reduced wing structure modulates the circadian locomotor

rhythms in *vg* mutants causes significant phase shift of free running locomotor activity rhythms during light pulse treatment as compared with WT in all circadian time points in this study**.** However, the molecular mechanism for significant light pulse phase shift in *vg* mutants is not exactly known.

To keep the circadian system in synchrony with the outside world, information about ambient light is communicated to the oscillator via input pathways. In *Drosophila*, these input pathways impinge on TIM protein: degradation of TIM in the light alters the level of this clock component and thus sets the phase of the oscillator. CRY has been shown to contribute to entrainment and dCRY is thought to signal light information to the clock through light-dependent interactions with the integral clock proteins dTIM and dPER, regulating the ability of the dPER-dTIM complex to inhibit CLOCK-mediated transcription [64]. *cry* as a key intracellular mediator between light information and the core circadian machinery within *Drosophila* brain neurons. CRY could act both as a photoreceptor and core clock protein in the body and probably in eyes [13]. In our study, cry^b mutants showed significant light induced phase shift of free running circadian locomotor activity rhythms as compared to WT in all circadian time points. This might be due to cry^b mutant flies (a missense mutation in the conserved flavin binding region) causes the protein to degrade and PER and TIM oscillations and it reduces the light sensitivity of the fly's clock [31]; this causes phase shift of free running locomotor rhythms during light pulse at these circadian time points.

Conclusion

Light is a more potent *zeitgeber* for circadian rhythms in *Drosophila melanogaster* and the light stimuli phase shift the clock might cause rapid changes in one or more clock components, such as PER and TIM. From this study, we suggest that blue-light component *cryptochrome* as well as wing structure play an important role for free-running circadian rhythms in *D. melanogaster* and single light pulses significantly phase shift free-running circadian locomotor rhythms in *vg* and cry^b mutants.

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