A Comparative Study of Circadian Behaviours and the Underlying Neuronal Correlates of Five Drosophilid Species

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By
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Dedicated to

Amma & Achan

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Declaration

I declare that the matter presented in my thesis entitled *"A Comparative Study of Circadian Behaviours and the Underlying Neuronal Correlates of Five Drosophilid Species"* is the result of studies carried out by me at the Evolutionary and Organismal Biology Unit of the Jawaharlal Nehru Centre for Advanced Scientific Research, Bangalore, India, under the supervision of Dr. Sheeba Vasu and that this work has not been submitted elsewhere for any other degree.

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 investigators. Any omission, which might have occurred by oversight or misjudgement, is regretted.

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<u>Certificate</u>

This is to certify that the work described in the thesis entitled "*A Comparative Study of Circadian Behaviours and the Underlying Neuronal Correlates of Five Drosophilid Species*" is the result of investigations carried out by **Ms. Priya M. P** in the Evolutionary and Organismal Biology Unit of the Jawaharlal Nehru Centre for Advanced Scientific Research, Bangalore, India, under my supervision, and that the results presented in the thesis have not previously formed the basis for the award of any other diploma, degree or fellowship.

Sheeba Vasu

Faculty Fellow

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List of publications

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4. Prabhakaran PM and Sheeba V (2014) Temperature sensitivity of circadian clocks is conserved across *Drosophila* species *melanogaster*, *malerkotliana* and *ananassae*. *Chronobiol Int* (in press).

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Summary

Circadian behaviours and the neuronal circuit controlling such behaviours in fruit flies *Drosophila melanogaster* have been studied extensively. In an attempt to better understand the functional significance of circadian organization using a comparative approach, we examined behaviours which exhibited a daily rhythm and the neuronal circuitry of wild-caught Drosophilids including *D. melanogaster* and four other sympatric species *D. malerkotliana*, *D. ananassae*, *D. nasuta* and *Zaprionus indianus*.

Daily cycles of environmental factors regulate rhythmic behaviours controlled by circadian clocks in most organisms including *D. melanogaster*. Under 12:12 hr laboratory light: dark (LD) cycles *D. melanogaster* shows bimodal activity pattern with most of its activity occurring within peaks coinciding with lights-ON and lights-OFF. Unlike *D. melanogaster*, *D. ananassae* exhibited predominantly unimodal activity with a distinct morning peak, restricting majority of its activity to the light phase with no apparent 'siesta' during midday. Under a range of photoperiods this predominant morning activity of *D. ananassae* is reflected in the persistence and phasing of the morning peak. *D. melanogaster* exhibits an evening peak that is considered to be more dominant compared to the morning peak under a range of photoperiods and under constant darkness (DD). In comparison, in *D. ananassae*, morning peak was the most dominant and persistent peak under long and short photoperiods. Circadian clocks regulating activity/rest rhythm of *D. ananassae* has a significantly shorter period and its activity is more consolidated compared to *D. melanogaster*. Thus we hypothesized that these two sympatric species occupy distinct temporal niches due to differences in their underlying circadian clocks and speculated that they would occupy different spatial microenvironments in the wild. We also characterized the circadian activity/rest rhythm

of *D. malerkotliana*, another sympatric species to *D. melanogaster* under laboratory LD cycles, constant darkness, constant light and different photoperiods. In all these conditions *D. malerkotliana* exhibited circadian activity/rest behaviour similar to *D. melanogaster*. The results of this study are described in the second chapter of my thesis.

To examine if temperature as a time giver has differential effects on *D. melanogaster*, *D. malerkotliana* and *D. ananassae*, we studied the activity/rest rhythms of these three closely related species under thermal cycles. We studied the entrainment properties of their circadian clocks under 12:12 hr thermophase: cryophase cycles of low and high contrasts, in the laboratory. Under both low and high amplitude cycling *D. melanogaster* and *D. malerkotliana* showed predominant evening activity, whereas *D. ananassae* continued to show predominantly morning activity similar to their behaviour under laboratory LD cycles. *D. melanogaster* and *D. malerkotliana* showed consolidation of activity under high amplitude cycles compared to low amplitude cycles. However, *D. ananassae* showed higher activity with proper anticipation to morning only under low amplitude cycles. The ability of circadian clock period to be compensated for changing temperatures was confirmed across a range of temperatures (19-29 ℃) for *D. melanogaster*, *D. malerkotliana* and *D. ananassae*. Further we found that temperature sensitivity of circadian clocks of *D. melanogaster* and *D. ananassae* was similar by comparing phase-shifts elicited at phases of maximum advance and delay by temperature pulses. Results of these experiments are described in the third chapter of my thesis.

We studied the circadian activity/rest rhythm of *D. melanogaster*, *D. malerkotliana*, *D. ananassae* and *Z. indianus* in an outdoor enclosure under more natural conditions (semi-natural; SN) where multiple time cues could alter activity rhythms of different species thus enabling them to adopt different temporal patterns. To

find out to what extent features of activity/rest rhythm of flies are conserved across species under changing environmental conditions encountered across seasons, we studied activity of these species over a span of 1.5 yrs. The results of this study form the fourth chapter of my thesis. We found that these species exhibited inter-species differences and seasonality in activity/rest patterns. *D. ananassae* was active mostly during the day, whereas *D. melanogaster* and *D. malerkotliana* exhibited almost similar activity patterns across seasons with predominantly morning and evening peaks. Under standard laboratory conditions *Z. indianus* displayed poor rhythmic activity compared to *D. melanogaster*. However *Z. indianus* showed more robust rhythm under SN condition throughout the year.

Unlike their bimodal activity pattern under standard laboratory protocols, under SN conditions *D. melanogaster* showed an additional activity peak in the afternoon (A-peak) and this additional peak has previously been reported to be temperature dependent. We studied activity/rest pattern of four Drosophilid species *D. melanogaster*, *D. malerkotliana*, *D. ananassae* and *Z. indianus* under various simulated gradual natural light and/or temperature conditions in the laboratory. The results of these studies form the fifth chapter of my thesis. We could reproduce the SN activity pattern of all the species under laboratory conditions. *D. ananassae* persistently showed their predominant morning activity under these conditions. We found that gradually changing light intensities with a maximum reaching ~ 3000 lux can elicit A-peak in *D. melanogaster*, *D. malerkotliana* and *D. ananassae*. High amplitude gradually changing temperature cycles alone can induce A-peak in all species and only A-peak was present for *D. ananassae* and *Z. indianus* during constant light conditions showing that A-peak could be a mere stress response to harsh environmental conditions.

Adult emergence of *D. melanogaster* shows rhythmic pattern and under SN conditions daily fluctuations in temperature, humidity and light at dawn have been shown to influence this rhythm. We studied the adult emergence pattern of *D. melanogaster*, *D. malerkotliana* and *D. ananassae* populations first in the laboratory to examine whether the difference in activity/rest rhythm among species extends to other circadian behaviours. We also examined adult emergence pattern of these species under SN conditions and found that even though inter-species differences were seen under laboratory conditions, under SN all three species showed similar adult emergence pattern. Our results suggest that seasonal changes in temperature and humidity have major role in the differences in adult emergence pattern. In *D. melanogaster*, *D. malerkotliana* and *D. ananassae* adult emergence rhythm became less tightly gated, with low amplitude peak and high day-to-day variation in timing of the peak of emergence during cooler and wetter seasons. There was strong influence of environmental factors under SN on the emergence rhythm of *D. melanogaster*, *D. malerkotliana* and *D. ananassae* such that in a given season all of them exhibit similar adult emergence pattern and therefore respond to the changing seasons in a similar manner. These results are described in the sixth chapter of my thesis.

D. melanogaster and *D. ananassae* showed difference in activity pattern both under laboratory and SN conditions. To verify whether the activity pattern of *D. ananassae* observed under various laboratory and SN conditions obtained from Drosophila Activity Monitoring system (DAM) are artefacts of the method, we performed visual observations on *D. melanogaster* and *D. ananassae* under various arenas under laboratory and SN conditions. We found that the visual observations corroborate the findings from the DAM system. *D. ananassae* preferred to remain

active during the day and limited their activity at night to extremely low levels. It is likely that *D. melanogaster* avoid activity during midday when minimal humidity, maximum temperature, and maximum light levels occur to minimize desiccation. Therefore, it is puzzling that *D. ananassae*, a close relative of *D. melanogaster*, is most active at this time. Therefore, we hypothesized that *D. ananassae* may be more desiccation tolerant compared to *D. melanogaster*. However, our experiments showed that *D. ananassae* is less tolerant to desiccation than *D. melanogaster*. The results of these studies comprise the seventh chapter of my thesis.

In *D. melanogaster,* the circadian clock neuronal network is distributed across few clusters of neurons. Studies on *D. melanogaster* have suggested that distinct groups of neurons regulate the morning and evening peaks in activity. A neuropeptide Pigment dispersing factor (PDF) has been shown to be an important synchronizing agent of the clock neuronal network. In *D. melanogaster*, PDF is expressed in four small ventral lateral neurons (sLNvs) and four to five large ventral lateral neurons (lLNvs). Period (PER) is one among the core clock proteins and is expressed in all known clock neurons including the sLNv and lLNv neurons, dorsal neurons (DN) and dorsal lateral neurons (LNd). Most studies have traditionally focused on laboratory strains of *D. melanogaster*. Of late, the view that we can assign distinct anatomical identities to the so-called morning (M) and evening (E) oscillators is being seriously challenged. We studied the expression of PDF and PER in the circuitry of *D. malerkotliana*, *D. ananassae*, *D. nasuta* and *Z. indianus* along with *D. melanogaster* and the results are described in chapter eight of my thesis. Even though the overall organisation of circadian neuronal cell types of *D. ananassae* was similar to that of *D. melanogaster*, there was reduction in the number of so-called 'evening-cell' groups. Expression

pattern of PDF in *D. malerkotliana* was similar to that of *D. melanogaster*; however, there was reduction in the number of PER expressing DN3s and we could not detect PDF^{-ve} 5th sLNv cell in any of the *D. malerkotliana* brains sampled. *D. nasuta* also showed expression of PDF and PER similar to that of *D. melanogaster*, except that there were more number of dorsal neurons (DN2 and DN3) which expressed PER. *Z. indianus* showed expression of PDF in cells other than LNvs both in the 3rd larval instar stage and adult stage. These results suggest that difference in the circadian circuitry may have resulted in the observed change in behaviours of these Drosophilid species.

In summary, this comparative study showed that the bimodality of activity pattern exhibited by *D. melanogaster* is not characteristic of all Drosophilid species. *D. ananassae* which is a sympatric species to both *D. melanogaster* and *D. malerkotliana* showed a unimodal activity pattern consistently under a range of laboratory conditions including different photoperiods, temperature cycles and semi-natural conditions. Expression of clock protein PER in *D. ananassae* is suggestive of this behaviour being mediated by a similar neuronal network as that of *D. melanogaster*, with less number of 'evening-cell' groups in *D. ananassae*. *D. malerkotliana* showed activity pattern almost similar to *D. malerkotliana* and we could not detect any difference in their circadian neurons in our preliminary studies. In the future, more detailed studies on the underlying neuronal circuits including temporal pattern of oscillations of known circadian proteins among these neuronal subsets, under different cyclic environments may reveal greater insight into the nature of circadian organisation among these sympatric Drosophilid species.

Chapter 1

Introduction

Circadian rhythms

Many organisms show rhythmicity in physiological processes, metabolism, behaviours and reproduction. Depending upon the period, rhythms are classified as - ultradian rhythms (milliseconds to hr), circadian rhythms (24 hr), tidal rhythms, annual rhythms etc. The rotation of the earth on its axis, its revolution around the sun and the revolution of the moon around the earth subject organisms to geophysical cycles such as daily cycles (24 hr) of light, temperature and to seasonal changes. Organisms appear either to cope with the daily changes in the external environment or even to make use of these periodic changes. Many organisms from simple unicellular beings to complex mammals exhibit daily rhythms (DeCoursey, 2004) and this 24 hr rhythmic pattern is not merely a response to the external environment and is in fact produced endogenously as was first demonstrated by Jacques de Mairan, in 1729 in the heliotrope plant (de Mairan, 1729). These daily rhythms are characterised by several features: 1) they are endogenously generated (produced within the organism) self-sustaining (continue to oscillate in the absence of any external time cues) with a free running periodicity of ~ 24 hr, hence the name circadian (from Latin 'circa' – approximately and 'diem' – a day) 2) they are entrainable (can be synchronized to periodic external conditions like light, temperature etc) and 3) they are temperature compensated (circadian period is not significantly altered by physiologically tolerable changes in temperature). The circadian system can be conceptualised as consisting of input pathways, which synchronize the clock to the environment; central oscillator, which maintains time; and output pathways, which convey information from central oscillator to temporally organize physiology and behaviour.

Circadian behaviours

Circadian clocks control many rhythmic physiological processes and behaviours in a variety of organisms as exemplified below. Prokaryotic cyanobacterium *Synechococcus*

shows circadian rhythm in photosynthesis and nitrogen fixation (Huang et al, 1990). The fungus *Neurospora crassa* exhibits circadian rhythm in the production of asexual spores (Merrow et al, 2001). Plants also show circadian rhythmicity in numerous behaviours such as stomatal opening, leaf movements, photosynthetic activity, flower opening, fragrance emission etc (reviewed in McClung, 2006). In sea anemone *Nematostella vectensis* the locomotor activity follows a daily pattern that persists in constant conditions (Hendricks et al., 2012). Circadian behaviours of many insect species have been studied extensively (reviewed in Helfrich Förster, 1998). Cockroaches show circadian rhythm in their locomotor activity that can be monitored for several months under constant conditions (reviewed in Helfrich Förster, 1998). Cricket species *Teleogryllus commodus* and *Gryllus bimaculatus* show circadian rhythmicity in locomotion and singing (reviewed in Helfrich Förster, 1998). Moths, beetles, grass hoppers, fruit flies, house flies and mosquitoes also show circadian rhythmicity in several behaviours (reviewed in Helfrich Förster, 1998). The snails *Bulla gouldiana* and *Aplysia californica* have been used extensively in the study of circadian rhythmicity (reviewed in Block et al., 1993). The zebrafish (*Danio rerio*) exhibits circadian rhythms in locomotor activity and they are being used as a simple model system to study circadian rhythmicity in vertebrates (reviewed in Cahill et al., 2002). Birds and mammals also exhibit circadian rhythmicity in various behaviours (such as sleep/wake, locomotor activity, feeding etc) and physiological processes (cortisol levels, metabolism etc). Thus circadian rhythms have been seen in almost all organisms examined thus far, pointing towards the universality and possible adaptive significance of circadian clocks.

Evolution of circadian rhythms

In most of the organisms studied by circadian biologists, the mechanisms controlling circadian behaviours share several conserved features such as the presence of feedback loops which involve either transcriptional- translational steps and / or post-translational processes.

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Thus it is reasonable to assume that circadian clocks may have arisen very early in the evolution of life-forms and has been preserved over time perhaps due to the adaptive advantage that it confers. Circadian clocks have been speculated to have originated in water in early microorganisms, having developed as an escape mechanism from DNA damaging effects of UV radiation of sun light (Gehring and Rosbash, 2003). Thus, it is believed that circadian rhythms evolved as a direct response to the rhythmic external environment enabling organisms to adjust to the cyclic external environment for their survival. This phenomenon of synchronization to daily external stimuli has been studied in various organisms and among the earliest reports are the diurnal rhythms in leaf movements exhibited by many plants (Darwin and Darwin, 1880; reviewed in Vaze and Sharma, 2013). The ubiquitous nature of circadian rhythms is often used as evidence for their adaptive value to organisms. Possessing circadian clocks may help organisms to cope with the predictable changes in external environmental conditions thus conferring an extrinsic adaptive value of circadian clocks (Aschoff, 1964; Sharma, 2003). Circadian clocks also synchronize several rhythms within an organism thereby providing an intrinsic adaptive value (reviewed in Vaze and Sharma, 2013). However, circadian clocks have been shown to persist in several organisms inhabiting relatively constant environmental conditions (no detectable daily rhythms in the environment) suggesting an intrinsic adaptive significance of circadian clocks. For example among troglobitic (cave restricted) catfishes, *Trichomycterus* sp., *Pimelodella kronei* and *Imparfinis* sp. some proportion of fishes assayed showed circadian rhythm in locomotor activity while the rest of them showed ultradian or infradian rhythms (Trajano and Menna-Barreto, 1995, 1996). In laboratory studies where the fruit fly *Drosophila melanogaster* were raised and spent all their lives in aperiodic environments, flies were shown to retain their rhythmicity in locomotion, eclosion and oviposition even after ~ 600 generations (Sheeba et al., 1999, 2001,

2002). Thus the above studies infer an intrinsic adaptive significance in possessing circadian rhythms.

Organization of circadian clocks

In unicellular organisms mostly populational rhythms have been studied and it is believed that circadian rhythms produced by these organisms are due to cellular interactions through signalling molecules among members of the population. For example in the dinoflagellate *Gonyaulax*, secretion of 'gonyauline' molecule has been shown to shorten the period of its circadian clock (Roenneberg et al., 1991). However, only very few unicellular organisms have been shown to exhibit circadian rhythm in single cells which persist for more than one cycle. In *Gonyaulax* circadian rhythms have also been observed in single cells (Ronnenberg and Morse, 1993). The unicellular alga *Acetabularia* exhibits circadian rhythm in photosynthesis (evolution of oxygen) in single cells and they continue to show rhythmicity even when the nucleus is removed (Sweeney and Haxo, 1961). Studies revealed the existence of various rhythms in *Gonyaulax* which are desynchronized under specific light and temperature conditions (Roenneberg and Morse, 1993). Thus it is likely that single cells may possess multiple circadian oscillators.

In case of multicellular organisms ranging from insects to humans studies have shown that circadian clocks may exist in most cells of the body, which are capable of functioning without input from any other cells- cell autonomous (Welsh et al., 1995). However, the period of these clocks are not identical and need to be synchronized to each other and to the environment. For many organisms light can directly entrain rhythms of each cell (Plautz et al., 1997; Whitmore et al., 2000). In multicellular organisms specific cell groups assume the role of central pacemakers as they set the pace of behavioural rhythms, are important for synchronization of the many clocks distributed throughout the body and are present in anatomically discrete locations in many living organisms. These central pacemakers include

the suprachiasmatic nuclei (SCN) in the hypothalamus of mammals (Ralph et al., 1990) and the optic lobes of cockroach, cricket and *Drosophila* (Page, 1982; Tomioka and Chiba, 1984; Ewer et al., 1992; Frisch et al., 1994; reviewed in Helfrich-Förster, 1998). In zebrafish, even though many cells and tissues contain circadian clocks, a clear hierarchy with a central clock is not yet known (Cahill, 2002). It has been suggested very early on that 'the organism comprises a population of quasi-autonomous oscillatory systems' (Pittendrigh, 1960). Experiments in various organisms have demonstrated the existence of functionally separable oscillators over a single central pacemaker including studies showing the existence of self sustained oscillators in the absence of daily entrainment (Moore-Ede et al., 1982).

In insects, it had been shown that circadian oscillators are present in peripheral organs in addition to the central nervous system (Giebultowicz, 1999; Plautz et al., 1997). Reproductive system of a moth *Lymantria dispar* contains a photoreceptive self sustained clock which can control the sperm release from testes (Giebultowicz et al., 1989) and in cockroaches a piece of epidermis in culture showed rhythm in cuticle secretion (Weber, 1995). Clock gene expression in different organs in *Drosophila* and many vertebrates points toward the existence of peripheral oscillators (Hall, 1995; Giebultowicz, 2000; Yamazaki et al, 2000). Oscillation of clock molecules in tissues involved in various physiological processes such as reproduction, excretion etc., suggests that circadian clocks may be involved in coordinating many physiological processes in a tissue autonomous manner. The molecular and physiological evidence for self- sustaining multi-oscillatory circadian systems suggests many possible organizations: 1) hierarchical control by central pacemakers which synchronise all oscillators, 2) peripheral clocks operating independently and their synchrony results due to entrainment by external environmental cycles (i.e., occurrence of multiple clocks rather than a central clock) or 3) a non-hierarchical organization where each oscillator in the system can

receive and give information for synchrony and none of these oscillators are acting as a central oscillator.

Circadian rhythms in *Drosophila melanogaster*

Circadian behaviours

Drosophila exhibits several behaviours which are under circadian control. Among several organisms studied under circadian biology, *Drosophila melanogaster* has received greatest attention in the past few decades due to the availability of genetic tools that have helped in unravelling the genetic, molecular and cellular bases of circadian behaviours. Before the appearance of *D. melanogaster* as a popular model system, the act of emergence (eclosion) of adults from pupae of another fly species *Drosophila pseudoobscura* was shown to be under circadian control such that most flies eclose during early morning (Pittendrigh, 1954). Later studies showed that *D. melanogaster* also exhibit daily rhythms in eclosion and peripheral clocks in prothoracic glands of *D. melanogaster* control this rhythm (Myers et al., 2003). One of the best studied circadian behaviour in *D. melanogaster* is its activity/rest rhythm. Under 'standard' laboratory conditions of 12:12 hr light/dark cycles (LD) *D. melanogaster* exhibits bimodal activity pattern with a morning peak around lights-ON and an evening peak around lights-OFF (Hamblen-Coyle et al., 1992). Under constant dark conditions (DD) this rhythm persists with a periodicity close to 24 hr. Temperature cycles have also been shown to entrain activity/rest rhythm (Wheeler et al., 1993; Yoshii et al., 2002). Light avoidance behaviour of *D. melanogaster* larvae shows circadian rhythmicity with a peak around late night/early morning (Mazzoni et al., 2005). Flies show feeding behaviour at specific time of the day and thus food consumption is also under circadian control (Xu et al., 2008). Courtship and mating of *D. melanogaster* exhibit daily pattern and it had been shown that these behaviours are regulated by circadian clock (Sakai and Ishida, 2001; Fujii et al., 2007). *D. melanogaster* flies also exhibits circadian rhythmicity in egg-

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laying (Howlader and Sharma, 2006). Flies use sensillae in antennae and maxillary palps to sense different odorants. Electroantennogram responses of fly antennal neuron to different odorants revealed that these responses peaks during night under LD and continue to show rhythmicity under DD (Krishnan et al., 1999). Survival rates of *D. melanogaster* flies to *Pseudomonas aeruginosa* infection showed a daily peak during middle of the night and trough during early daytime and thus in immune responses also flies exhibit a circadian pattern (Lee and Edery, 2008). Short-term associative memory formation through olfactory learning in *D. melanogaster* was found to be regulated by circadian clocks in such a way that flies exhibited a performance peak during early subjective night (Lyons and Roman, 2009). Other than these behaviours *D. melanogaster* flies also exhibit circadian rhythmicity in certain physiological processes (Ito et al., 2008; Krishnan et al., 2008; Mehnert et al., 2007).

Molecular mechanism of circadian clock

Studies in *D. melanogaster* flies have shown that the transcriptional − translational feedback loops are the molecular bases of circadian clock. In a screen for mutants which show altered free running rhythm of eclosion, the first clock gene *period* (*per*) was identified (Konopka and Benzer, 1971). Later on it was found that *per* mRNA and PER protein cycle in a circadian manner and PER protein is required for *per* mRNA cycling, suggested that this mechanism functions via a feedback loop (Hardin et al, 1990). PER-dependent inhibition of *per* mRNA expression further refined the role of PER in this feedback loop as a transcriptional repressor (Hardin et al., 1992; Zeng et al., 1994). Subsequent studies identified many other clock genes *timeless-tim* (Sehgal et al., 1994), *clock- clk* (Allada et al., 1998), *cycle- cyc* (Rutila et al., 1998), *doubletime- dbt* (Price et al., 1998), *cryptochrome- cry* (Stanewsky et al., 1998), *shaggy- sgg* (Martinek et al., 2001), casein kinase 2- CK2 (Akten et al., 2003) etc. Our current understanding of the mechanisms that generate circadian oscillations in the core-clock proteins is briefly summarised below.

CLOCK and CYCLE have a protein-protein interaction domain (PAS domain) and a basic Helix-Loop-Helix (bHLH) domain for DNA binding. CLOCK and CYCLE proteins promote the transcription of *per* and *tim* along with other clock genes (Kyriacou and Rosato, 2000). PER and TIM protein levels also oscillate. PER and TIM start to accumulate 6-8 hr after their mRNA accumulation which is due to the phosphorylation and degradation of PER by DBT and stabilization of PER-DBT complex by TIM (Kloss et al., 1998; Price et al., 1995; Price et al., 1998). PER is also phosphorylated by CK2. Phosphorylation of PER by DBT and CK2 affects the repressor activity of PER (Nawathean and Rosbash, 2004). TIM is also phosphorylated by SGG and CK2 (Meissner et al., 2008). Nuclear localization of phosphorylated PER and TIM are delayed due to the degradation by proteins SLIMB and SGG respectively (Grima et al., 2002; Ko et al., 2002; Martinek et al., 2001). TIM is also degraded after phosphorylation in a light dependent manner by a protein called JETLAG (Koh et al., 2006). PER and TIM are dephosphorylated by protein phosphatase 2a (PP2a) and protein phosphatase 1 (PP1) respectively, which stabilize PER and TIM (Fang et al., 2007; Sathyanarayanan et al., 2004). PER and TIM proteins heterodimerise and translocate into the nucleus (Gekakis et al., 1995; Curtin et al., 1995) and inhibits their own gene transcription by suppressing CLK-CYC complex (Darlington et al., 1998). After lights-ON, inside the nucleus TIM gets degraded and this leads to the degradation of PER (Hunter-Ensor et al., 1996; Lee et al., 1996; Myers et al., 1996; Zeng et al., 1996). Photoreceptor molecule CRYPTOCHROME (CRY) binds directly to TIM in a light-dependent manner leading to the degradation of TIM (Busza et al., 2004; Ceriani et al., 1999; Dissel et al., 2004; Naidoo et al., 1999). PER is progressively phosphorylated by DBT and finally leads to the degradation process by SLIMB (Grima et al., 2002; Kloss et al., 2001; Ko et al., 2002; Naidoo et al., 1999). After PER degradation, repression over CLK-CYC is removed which initiates *per* and *tim* transcription. Other than this core feedback loop CLK-CYC complex activates transcription of *vrille* (*vri*),

pdp1 and *clockwork orange* (*cwo*). As the levels of VRI protein increases, VRI binds to the VRI/PDP1- boxes in the *clk* promoter and represses the transcription of *clk* (Cyran et al., 2003; Glossop et al., 2003). PDP1 protein level accumulates several hours after VRI and PDP1 bind to the VRI/PDP1- box and activates *clk* transcription (Cyran et al., 2003). CWO protein inhibits CLK-CYC transcription (Kadener et al., 2007; Lim et al., 2007; Matsumoto et al., 2007). Thus cycling of mRNAs and proteins in feedback loops constitutes the *D. melanogaster* circadian oscillator (reviewed in Hardin, 2011).

Anatomical identity of circadian clocks

Even though autonomous circadian oscillators are present throughout the body of the fly (Plautz et al., 1997), the central circadian clock of *D. melanogaster* is thought to be comprised of about 150 neurons in the brain. These neurons are divided into different groups based on anatomical position- the ventrolateral neurons (LNv), six dorsal lateral neurons (LNd), three lateral posterior neurons (LPN), dorsal neurons 1 (DN1), DN2 and DN3. These neuronal groups are further subdivided depending on their size and gene expression. The LNv are divided into five small LNv (sLNv) and four to five large LNvs (lLNv). Four out of five sLNv and all the lLNv express the neuropeptide pigment-dispersing factor (PDF) and the 5th sLNv is PDF^{-ve}. Three to four LNds express CRY. DN1s are divided into two anterior DN1s (DN1a) which express the neuropeptide IPNamide and around 15 posterior DN1s (DN1p). All of these neuronal subgroups express the circadian protein PER. The DN1a and a subset of DN1p express CRY protein. There are two DN2 and around 40 DN3 cells (reviewed in Dubruille and Emery, 2008).

The circuitry by which these circadian neurons communicate to each other is not fully understood. Mutants and ablation studies showed that the neuropeptide PDF is essential for normal circadian functioning (Renn et al., 1999). The PDF expressing sLNv send projections to the dorsal region and rhythmically express PDF in their terminals which arborise near DN1

(Park et al., 2000). These projections show rhythmic change in the structure of their arborisations (Fernandez et al., 2008). In the circadian pacemaker neurons of null mutants of *pdf* (*pdf ^{01}*), oscillations of *per* and *tim* mRNA dampen faster than wild type flies indicating that PDF is essential for the communication between circadian neurons (Peng et al., 2003). Another interesting study showed that PDF induced rise in cAMP levels occurs in clock neurons (LNd, DN1, DN2, DN3) including sLNv through PDF receptor PDFR, indicating that PDF is a direct modulator of most of the neurons in the *Drosophila* circadian clock network (Shafer et al., 2008). Even though loss of PDF expression and ablation of PDF expressing neurons produce abnormal locomotor behaviour, the fact that flies were still able to entrain suggests that there exists transmitters other than PDF which are required for normal circadian functioning (Taghert et al., 2001). Circadian locomotor activity of *D. melanogaster* has been shown to be controlled by multiple neuropeptides (Taghert et al., 2001). The DN1 neurons express a neuropeptide called IPNamide (Shafer et al., 2006). Subsets of LNd express neuropeptide F (Lee et al., 2006). Neuropepetide ion transport peptide (ITP) is expressed in one LNd and PDF^{-ve} sLNv (Johard et al., 2009). The mushroom bodies are also shown to affect locomotor behaviour (Martin et al., 1998). The dual oscillator model initially proposed for mammals proposes a mechanism by which activity during dawn is controlled by a morning (M) oscillator and during dusk by an evening (E) oscillator (Pittendrigh and Daan, 1976). In *D. melanogaster* also distinct neuronal subgroups have been postulated as M and E oscillators although, the identity of these groups has been equivocal among fly researchers. PDF expressing sLNvs have been proposed to control the morning peak of activity while the PDF^{-ve} CRY^{+ve} cells are thought to control the evening activity (Grima et al., 2004; Stoleru et al., 2004), while another group suggests a possible role of the 5th sLNv to be a part of the E oscillator (Hermann et al., 2012).

The current state of knowledge leads to an understanding of the circadian neuronal architecture in *D. melanogaster* as consisting of a network of cells with characteristic neuropeptide expression and distinct connectivity with each other that can function as independent oscillators and can independently drive rhythmic behaviour (Yao and Shafer, 2014). Thus *D. melanogaster* flies have proven to be a very useful model system to study circadian rhythms providing a means to address questions at the level of both behaviour and underlying physiological processes while also enabling us to address questions of the functional significance of circadian rhythms. Yet, we must bear in mind that a sole focus on one species may very well prove to be misleading if we wish to understand the general principles that govern a certain process or phenomenon since it is quite likely that the species of intensive study may be an evolutionary exception in some respects and hence may be unique in terms of how that process is controlled.

Comparative studies of species

In the past it has been shown that behavioural studies comparing a particular behaviour across species gives a better understanding of the characteristics of that behaviour. It has been long suggested that introduced species have better seed dispersal than native plant species. However, one study which used data for 51 introduced and 360 native plant species revealed that is not the case and concluded that better spread-rate of introduced plant species is not because of their better seed dispersal but could be driven by differences in post-dispersal processes (Flores-Moreno et al., 2013). In humans, response to music includes a tendency to entrain or align movement to auditory pulses and the prevalent notion was that this behaviour is unique to humans. But, comparison of behaviours in response to music of non-human vocal mimicking species led to the hypothesis that entrainment evolved as a by-product of selection for vocal mimicry (Schachner et al., 2009). Comparative analysis of decoration behaviour of crabs to camouflage suggests that the costs of decoration

maintenance for larger crab species may limit the evolutionary distribution of decoration

camouflage among the spider crabs and lead them to adopt other camouflage mechanisms

(Hultgren and Stachowicz, 2009). A comparative study on sexual and asexual species of

evening primroses by Johnson et al (2009) provided experimental evidence to support

Recombination-Mating System hypothesis which posits that reduced sexual reproduction

limits adaptive evolution of plant defences against arthropod herbivores (Johnson et al.,

2009). Thus comparative studies provide insights into the significance of several behaviours.

Studies of circadian rhythms in *Drosophila* species

Genus *Drosophila* consists of several species (~ 1500) and this genus provides a

model system to undergo comparative experimental research with its well defined phylogeny

and extensive literature in various fields of biology such as genetics, ecology, neurobiology,

development, physiology and behaviour. Circadian behaviours of several species of

Drosophila other than *D. melanogaster* have been studied. Many decades ago, the daily

pattern in adult emergence rhythm from pupae of *D. pseudoobscura* was shown to be

independent of temperature (Pittendrigh, 1954). Another study which used 12 strains of *D.

subobscura* showed geographical variability in adult emergence rhythm with properties of this

particular circadian behaviour (period and phase) changing with the latitude of the strain

(Lankinen, 1993). This study showed the existence of latitudinal cline (north and south strain

difference) in the emergence rhythm properties as previously shown for a number of other

Drosophilid species (Pittendrigh and Takamura, 1989; Lankinen, 1985; Lankinen, 1987).

Latitude dependent change was also shown in another circadian behaviour - activity/rest

rhythm. A study which compared the activity rhythms of eleven Drosophild species that are

not human commensals inhabiting a range of latitudes (~19° N and ~60° S) in the North

American continent found that species from more temperate latitudes exhibited relatively

greater midday activity as compared to the Southern species, and even within similar

latitudinal ranges, species which breed and live in wet microhabitats exhibit greater midday activity (Simunovic and Jaenike, 2006).

However a study on adult emergence rhythm of *D.ananassae* revealed that even the strains of same latitude exhibited difference in their behaviour depending upon altitude (Khare et al., 2002). High altitude Himalayan strains of *D.ananassae* showed arrhythmicity in their adult emergence behaviour when the temperature was lowered to 13 or 17 ºC as compared to 21 ºC under light/dark cycles and under different temperature cycles (along with light/dark cycles, constant light, constant darkness). Under these low temperatures (13 or 17 ºC) emergence was dependent only on the thermophase rather than the light/dark condition. Even though low altitude strain showed temperature dependent changes in the periodicity and phasing of the rhythm, they were rhythmic under 13 and 17 ºC light/dark cycles (Khare et al., 2002). The authors suggested that under high altitude conditions of the Himalayas these *D.ananassae* strains cannot rely on photoperiod or light intensity in the wild where the temperature can be below 0 ºC in winter and therefore may have led to natural selection favouring temperature over light (Khare et al., 2002). Altitude-dependent effect was shown in the activity/rest rhythm of another Drosophilid species *D. helvetica*, high altitude *D. helvetica* strain (haH) exhibited a unimodal activity pattern with a delayed onset of activity with reference to lights-ON where as low altitude strain (laH) showed bimodal activity (Vanlalhriatpuia et al., 2007). One explanation for the difference in the activity pattern between these two strains is the effect of environmental conditions, especially temperature. In high altitudes where the temperature during morning is too low to exhibit activity whereas in low altitudes flies were active in the morning and evening which are the relatively cooler time of the day and avoid the high temperature during middle of the day (Vanlalhriatpuia et al., 2007). Taken together, these studies imply that circadian behaviours of these species adapted to the existing environmental conditions which they inhabit.

Comparative study of activity/rest behaviours of *D. melanogaster* and a distantly related species *D. virilis* showed that *D.virilis* behaved like the null mutant of the *pdf* gene (pdf^{01}) in *D. melanogaster*, which is an important circadian output molecule of *D. melanogaster*. Like the pdf^{01} mutant, *D. virilis* flies restricted most of their activity to the light phase of LD and were arrhythmic under DD (Bahn et al., 2009). PDF of *D.virilis* (DvPdf) was not expressed in sLNv cells and DvPdf was showed to be capable of directing their expression in all endogenous PDF neurons of *D. melanogaster* (Bahn et al., 2009). This study revealed how differential expression of PDF can affect activity behaviour not just in *D. melanogaster* but in other *Drosophila* species thus showing the conserved role of PDF in circadian clock controlling activity/rest rhythm (Bahn et al., 2009). *D. melanogaster* flies show prolonged midday inactivity under high temperature and thermosensitive splicing of the 3′-terminal intron (dmpi8) from the key clock gene *period* (*per*) was found to be important for this behaviour (Majercak et al., 1999). However flies of another species *D. yakuba* did not show thermal calibration in the splicing mechanism of 3′-terminal introns of *per* gene and fluctuation in temperature did not affect their distribution of activity (Low et al., 2008). *D. yakuba* flies have a more ancestral distribution in Afro-equatorial regions where the temperature and day length variation is minimal unlike *D. melanogaster* which are widely distributed including temperate regions. Thus, this comparative study suggested that natural selection in the splicing mechanism plays an important role in the temperature dependent behaviours (Low et al., 2008). *D. montana* flies unlike *D. melanogaster* lack morning component of activity and they show rhythmicity under constant light condition which renders *D. melanogaster* flies arrhythmic (Kauranen et al., 2012). Furthermore, comparative study of the circadian neuronal circuitry of *D. melanogaster* and *D.montana* revealed that there are differences in the expression pattern of PDF and CRY in circadian neurons and this may account for their behavioural differences. It was suggested that the environment where
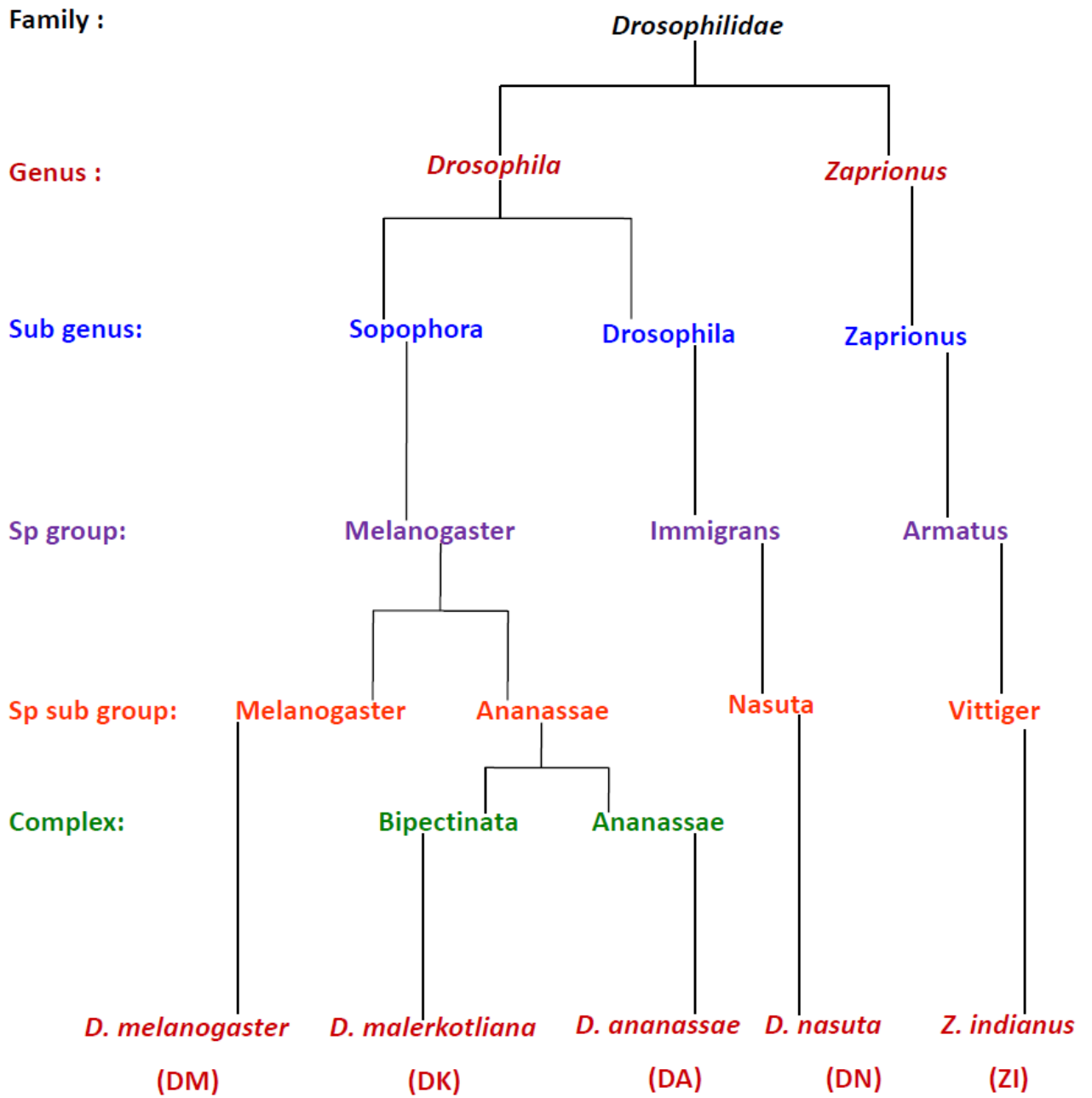
the flies inhabit influence their activity pattern, in *D.montana* the peculiar activity pattern may be due to adaptation to Northern high latitudes where they are found (Kauranen et al., 2012). A recent study which compared the neurons expressing clock proteins VRI, PDP1, CRY and PDF among ten *Drosophila* species revealed that the anatomy of circadian clock network is highly conserved among species of the *Drosophila* genus which inhabit different habitats (Hermann et al., 2012). Thus comparing different Drosophilid species by examining the relationship between rhythmic behaviours and the expression of known clock proteins will provide greater understanding towards the mechanism of these behaviours.

In the present study, I compared circadian behaviours and circadian neurons of *D. melanogaster* Meigen 1830 with other Drosophilid species *Drosophila malerkotliana* Parshad and Paika 1964, *D. ananassae* Doleschall 1858,. *D. nasuta* Lamb 1914 and *Zaprionus indianus* Gupta 1970 to examine a) whether rhythmic behaviours are conserved across species, b) whether there are differences in the pattern of rhythmicity in various behaviours c) and whether such differences in behaviours are also accompanied by differences in the underlying neuronal network that controls these behaviours. We rationalised that such an approach may provide greater insight into the functional significance of these rhythmic behaviours. Taxonomic relationship of these five species is shown in Fig. 1. *D. melanogaster*, *D. malerkotliana* and *D. ananassae* belong to the sub genus Sopophora and species group *melanogaster* (Fig. 1). *D. melanogaster* is a cosmopolitan species with an Afrotropical origin and distributed world-wide except in the extreme altitudes or latitudes (David and Tsacas, 1981; David and Capy, 1988). *D. malerkotliana* is a tropical species distributed throughout Southeast Asia and first reported from Punjab, India (Kopp and Barmina, 2005). *D. malerkotliana* and *D. ananassae* belong to species sub group *ananassae*. *D. ananassae* is distributed in the tropical, subtropical, and mildly temperate regions and is thought to have originated in Southeast Asia (Das et al., 2004; Dobzhansky and Dreyfus,

1943; Tobari, 1993). *D. nasuta* was first reported and characterized from Seychelles, Africa and distributed in Southeast Asia and Africa (Bachtrog, 2006; Kitagawa et al., 1982). *Z. indianus* is of African origin and distributed in the tropical regions of the world (da Conceição Galego and Carareto, 2010). All the five species (*D. melanogaster*, *D. malerkotliana*, *D. ananassae*, *D. nasuta* and *Z. indianus*) were caught using fruit traps and net sweeps from Bangalore, India (12°58′N, 77°38′E) during 2004-2005. These species were maintained in plexi-glass cages as large random mating populations of ~1200 individuals under laboratory LD (~1.5 W/m^2) conditions at constant temperature (~25 ºC) and relative humidity (~70%) on cornmeal medium with charcoal. A discrete generation cycle of 21 days was followed.

TAXONOMIC RELATIONSHIP BETWEEN THE STUDY SPECIES

Family : *Drosophilidae*

Genus : *Drosophila*   *Zaprionus*

Sub genus: Sopophora   Drosophila   Zaprionus

Sp group: Melanogaster   Immigrans   Armatus

Sp sub group: Melanogaster   Ananassae   Nasuta   Vittiger

Complex: Bipectinata   Ananassae

D. melanogaster   *D. malerkotliana*   *D. ananassae*   *D. nasuta*   *Z. indianus*

(DM)   (DK)   (DA)   (DN)   (ZI)

Figure 1

Differential responses in activity pattern of three Drosophilid species to varying photoperiods

Introduction

Circadian clocks are believed to enable organisms to time their physiology and behaviour in a manner that is most adaptively advantageous to them. It is plausible therefore to assume that timing of various behaviours is phased to enable organisms to gain maximum fitness benefit with minimum risk. For example, in *Drosophila melanogaster*, the timing of emergence of adults from pupae under natural conditions coincides with dawn, which is also the time of highest humidity and lowest temperature (De et al., 2012). This is believed to enable flies to expand their wings upon emergence (Saunders, 2002). While emergence is predominantly unimodal in *D. melanogaster*, activity/rest rhythm shows distinct bimodality with large proportion of activity occurring within peaks coinciding with lights-ON and lights-OFF under symmetric 12:12 hr laboratory light/dark (LD) cycles (Saunders, 2002). Such a preponderance of activity during twilight hours is thought to enable flies to escape harsh conditions during the middle of the day although there is no empirical evidence for the same (Pittendrigh, 1993). Another line of thought has been that bimodality in behaviour is simply a reflection of the nature of circadian timing systems, which are comprised of two oscillators, one of which is coupled to dawn and the other to dusk, and show differential sensitivity to light and temperature (Helfrich-Förster, 2009). In recent years, many studies on *D. melanogaster* have tried to examine the neuronal correlates of the dual-oscillator organization and its so-called 'morning' and 'evening' oscillator cells (Grima et al., 2004; Stoleru et al., 2004; Rieger et al., 2006). However, it is increasingly becoming evident that such bimodality in behaviour is governed by a plastic network comprising a large number of neurons (reviewed in Sheeba et al., 2008) and is largely dependent on photoperiod, light intensity and temperature (Rieger et al., 2003; Miyasako et al., 2007; Yoshii et al., 2009; Zhang et al.,

2010). Another study revealed that two strains of *D. melanogaster* from Northern and

Southern latitudes show differences in their ability to entrain to long-photoperiods and that

this behaviour is dependent on temperature and twilight conditions (Rieger et al., 2012).

Furthermore, under natural conditions, there appear to be three, rather than two peaks of

activity in *D. melanogaster* (Vanin et al., 2012).

Even though several insect species have been used to study circadian rhythms

(reviewed in Helfrich-Förster et al., 1998), *D. melanogaster* has provided the highest traction

on several aspects such as the genetic, molecular and cellular bases of circadian behaviour due

to its genetic amenability. We reason that we can gain greater insight into the functional

significance of rhythmic behaviours if we compared *D. melanogaster* with another closely

related sympatric species, *D. ananassae*. Both *D. melanogaster* and *D. ananassae* are

cosmopolitan species and belong to the species-group *melanogaster* (subgenus *Sophophora*)

and show ~83% similarity in their genomes (*Drosophila* 12 genome consortium, 2007). *D.*

ananassae is thought to have originated in Southeast Asia (Tobari, 1993), later having

invaded more temperate regions (Dobzhansky and Dreyfus, 1943; Das et al., 2004), and now

both species are sympatrically distributed in several tropical regions. *D. melanogaster* flies

are distributed widely across temperate regions, whereas *D. ananassae* flies are rarely found

in more temperate latitudes. Previous studies in *D. ananassae* have suggested that it is a good

model organism for genetical, behavioural and evolutionary studies (reviewed in Singh, 2010;

Singh and Singh, 2008; Sisodia and Singh, 2012). Along with *D. melanogaster* and *D.*

ananassae we also studied another species *D. malerkotliana* which also belongs to the

Sophophora subgenus. *D. malerkotliana* is a tropical species, distributed in Southeast Asia

and was first reported from Punjab (India) (Kopp and Barmina, 2005). Taxonomically, it is

thought to be more closely related to *D. ananassae* and its current world-wide distribution also closely overlaps that of *D. ananassae*. It is thought to be a recent invasive that has come to occupy large areas due to greater movement of products across the world.

A recent study comparing *D. melanogaster* and *D. ananassae* species has demonstrated divergence in courtship behaviour and its neuronal basis that may have contributed to their reproductive-isolation (Riabinina et al., 2011). Yet another study reveals that synaptic structure at the neuromuscular junction of *D. melanogaster* and *D. ananassae* are significantly diverged from each other to extents that are not explained by their phylogenetic relationships (Campbell and Ganetzky, 2012). Although circadian behaviours of both *D. ananassae* and *D. melanogaster* have previously been studied, no systematic comparisons between the two, providing insights into their circadian organization have been carried out until very recently (Hermann et al., 2013). This recent study found a high degree of similarity in the neuroanatomy of circadian neurons in the brains of *D. ananassae* and *D. melanogaster* in terms of cell number and expression of core circadian proteins such as VRILLE (VRI) and Par Domain Protein 1 (PDP1) and an important circadian neuropeptide Pigment Dispersing Factor (PDF). The protein sequence identity of circadian photoreceptor CRYPTOCHROME and neuropeptide Ion Transport Peptide (ITP) were also found to be as high as 86.9 and 98.7% respectively (Hermann et al., 2013) while there was no difference in PDF sequence between the two species.

Our studies were focused on overt activity/rest rhythmic behaviour, which is the outcome of the cellular and molecular machinery of the circadian clock organization, under a wide range of photoperiods, in the three species *D. melanogaster*, *D. ananassae* and *D. malerkotliana*. We found that while *D. melanogaster* and *D. malerkotliana* displayed the

expected bimodal activity pattern with peaks around dawn and dusk, *D. ananassae* were predominantly day-active, with maximum activity after lights-ON and weak evening activity peak suggesting that these species show significant differences in their preference for timing of activity/rest behaviour.

Materials and methods

Activity recording. 2-3-day old virgin males (except when specified) of each species were placed individually into glass tubes (3 or 5 mm diameter and 65 mm long) and recorded using *Drosophila* activity monitors (TriKinetics, Waltham, USA) at light intensity ~0.28W/m^2; temperature 25 ± 0.5ºC. To estimate free-running period (τ), flies were first exposed to 2 days of LD12:12 after which they were released into constant darkness (DD) in an incubator (Sanyo, MIR-154, Japan). Activity in constant light (LL) was assayed after 4 days of LD12:12, when lights remained ON throughout. DD experiments were repeated at least four times with similar results (Table 1). Photoperiods LD20:4, 18:6, 16:8, 14:10, 12:12, 10:14, 8:16, 6:18 and 4:20 were created in an incubator (DR-36VLC8, Percival Scientific Inc, USA).

Analysis of activity. Activity was recorded in 5 min bins. Raw time series data from individual flies further binned into 15 min were used to obtain average actograms. For Figure 1 and 4, activity profiles were obtained by averaging raw activity counts across 7 days for each fly and averaging across flies. Morning and evening anticipation indices were estimated only for LD12:12 for each species since true-peaks coincided with D/L and L/D transitions only in this regime. Data of individual flies were averaged across 7 days and ratio of activity in the final 3 hr prior-to lights-ON and -OFF, to that which occurs in the 6 hr preceding the transitions (Harrisingh et al., 2007) were used as indices of anticipation and compared across species using Student's *t*-test.

Sleep analysis. Based on previous studies on *D. melanogaster*, sleep was defined as any duration of uninterrupted immobility (0 counts/min) lasting ≥ 5 min (Andretic and Shaw, 2005). Sleep profiles for individual flies were plotted by averaging minutes of sleep per hour across 7 days. These profiles were then averaged across individual flies to obtain average sleep profiles. The interval of time between sleep bouts was considered as a bout of waking. Average number of sleep bouts per 30 min, average duration of sleep-bouts and wake-bouts were estimated for individual flies for each day using a macro in Excel (MS–Office) written by Paul Shaw and modified by the Cirelli lab. Activity and sleep levels were analyzed using 2-way ANOVA with species and time interval as fixed factors followed by post-hoc Tukey's HSD test.

Free running period. Raw time series data obtained under DD was analyzed using Lomb-Scargle (LS) periodogram method in ClockLab, Actimetrics, USA, with $p = 0.05$ as threshold for rhythmicity. To avoid transients, only data from the last 7 days were used for analysis. The τ and robustness (amplitude of LS periodogram) (Table 1) were compared using 2-way ANOVA followed by Tukey's HSD test.

Photoperiod analysis. Average activity profiles (mean $\pm$ SEM) were plotted using 5 min bin data for both species by first averaging across days for individual flies and then averaging across flies. To avoid transients, data from the last 7 days of entrainment were used for the analysis (except in case of LD4:20 where only the last 6 days were used, due to larger number of transients). We first manually scanned activity profiles of individual flies obtained after averaging across 7 days and identified 15 min time windows which corresponded to 'true' (having either anticipation and/or gradual decline in activity) peaks and also startle responses associated with morning and evening transitions (Table 2). In cases where startle-peak was indistinguishable from true-peak, they were considered as true-peak. The true-peak phase

values thus obtained for individuals were averaged across flies to obtain mean phases of the peaks for each species in each photoperiod (Fig. 10B).

To quantify 'morning' and 'evening' preference for activity in *D. melanogaster* and *D. ananassae* under different photoperiods, we compared levels of activity during different parts of the day or night (Figs. 6, 7). For long photoperiods, we estimated forenoon (FN) and afternoon (AN) indices during the light part of the day when flies were most active. We divided the light duration into four equal quarters, excluding 30 min after lights-ON to avoid startle activity. FN index was calculated as the ratio of activity during the first quarter to that during the first half of the light phase. The AN index was calculated as ratio of activity during the last quarter of light phase to that during the second half of light phase.

Under short photoperiods since flies were mostly active at night, we first excluded 30 min data just after lights-OFF to eliminate the startle response. We then divided the entire night into four equal quarters. We defined an index of activity during the pre-dawn segment (Pr–DN) as the ratio of activity in the last quarter of the night to that during the second half of the night. To examine activity patterns after L/D transition, we defined post-dusk (Po–DK) index as ratio of activity in first quarter of night to that in the first half of night.

To compare distribution of activity of the two species *D. melanogaster* and *D. ananassae* across both light and dark durations within each photoperiod, the 24 hr day was divided into 2 hr intervals and lights-ON was considered as ZT0 in all photoperiods. Normalized mean activity in each 2 hr interval was estimated and 2-way ANOVA with species and time as fixed factors, followed by Tukey's HSD were performed to evaluate differences. Pr-DN, Po-DK, FN, AN indices, phase of morning and evening peaks were derived from 15 min binned activity profiles and compared between *D. melanogaster* and *D. ananassae* using Student's *t*-test. Total activity levels of *D. malerkotliana* during different

photoperiods were compared with total activity during LD 12:12 by doing one-way ANOVA followed by post-hoc multiple comparisons using Tukey's HSD test. All statistical tests were done using STATISTICA-7 (StatSoft Inc., USA) with level of significance set to $p < 0.05$.

Results

D. ananassae exhibited unimodal activity pattern compared to bimodal activity of D. melanogaster and D. malerkotliana under LD12:12. Comparison of activity profiles of the two species under LD12:12 revealed that unlike *D. melanogaster*, *D. ananassae* showed distinct unimodal morning preference in activity (Fig. 1A-C). As expected, virgin male *D. melanogaster* showed two peaks of activity that coincided with lights-ON and lights-OFF, with anticipation to both lights-ON and lights-OFF and reduced activity both during midday and midnight (Fig. 1A left and middle panels, Fig. 1C). In contrast, *D. ananassae* males showed a single peak of activity coinciding with lights-ON following which activity counts gradually tapered-off (Fig. 1B left and middle panels, Fig. 1C). Although both species showed anticipation to lights-ON, the index was significantly higher for *D. ananassae* compared to *D. melanogaster* (0.75 ± 0.09 versus 0.65 ± 0.03, mean ± 95%CI; Fig. 1C). This was due to steeper increase in activity in *D. ananassae* immediately before lights-ON and very little nighttime activity compared to *D. melanogaster* (Fig. 1A-B left and middle panels, Fig. 1C). On the other hand, anticipation to L/D transition in *D. ananassae* was very low and could be visualized only at higher resolution (15 min bins, Fig. 1C) which became undetectable when data was binned in 1 hr intervals (Fig. 1B, middle panel). Compared to *D. melanogaster* it was significantly lower as estimated by evening anticipation indices (0.44 ± 0.07 versus 0.69 ± 0.05, mean ± 95%CI, Fig. 1C). While daytime activity levels did not differ between the two species (*D. melanogaster* = 499.4 ± 40 and *D. ananassae* = 397.1 ± 41

counts/12 hr), *D. ananassae* showed significantly lower nighttime activity (65.68 ± 7.6

counts/12 hr compared to 432.8 ± 38, mean ± 95%CI; 2-way ANOVA, Tukey's HSD). Such

morning preference was also seen in females (Fig. 2A-B). When male flies were allowed to

free-run under DD after 2-4 days of LD, we found that *D. ananassae* activity in DD followed

from the morning peak in LD, whereas the DD activity of *D. melanogaster* predominantly

followed from the evening peak (Fig. 2C). Thus, we find that these two species exhibit

different temporal preferences for activity under LD cycles.

Next, we examined whether the altered phasing of activity in these two species is

achieved because of differences in τ. *D. ananassae* showed significantly shorter τ compared

to *D. melanogaster* ($F_{1,164} = 69.32$, $p < 0.05$; Fig. 1A-B right panels, Table 1) with no

significant difference in robustness or percentage rhythmicity. Moreover, even under DD, *D.

ananassae* showed single narrow band of activity whereas *D. melanogaster* activity was much

broader and appeared to be composed of elements from both morning and evening

components (Fig. 1A-B right panels). The average wake-bout duration of *D. ananassae* under

DD was only 3.1 ± 0.2 hr while that of *D. melanogaster* was 7.6 ± 0.4 hr. Thus these two

species with modest differences in τ, adopt distinct activity patterns under LD12:12 and DD.

To examine whether light promotes and/or darkness inhibits activity of *D. ananassae* and also

to verify whether circadian clocks of this species are also susceptible to constant light (LL),

we assayed activity of males of both species under LL and found that like *D. melanogaster*,

D. ananassae were also arrhythmic in this regime (Fig. 2D).

We expected that the difference in activity patterns under LD would also be reflected

in their patterns of sleep. Since previous studies in *D. melanogaster* have demonstrated that 5

min of immobility can be considered as sleep, we reasoned that the same criterion is likely to

provide a reasonable estimate of sleep-like state in *D. ananassae* also, although we have not

performed experiments to validate the same. Indeed *D. ananassae* showed distinctly altered

sleep patterns under LD12:12 compared to *D. melanogaster* with very little sleep in the early

half of the day and lack of midday siesta (Fig. 1D). Daytime sleep of *D. ananassae* peaks

around ZT10 with a minor dip at ZT11 (which corresponds with low level of evening

anticipation, Fig. 1C) whereas *D. melanogaster* sleep levels fell between ZT8-11 (Fig. 1D).

Moreover, *D. ananassae* slept more at night, remaining asleep for most part of the dark phase

and only beginning to wake within about 1 hr prior-to dawn (Fig. 1B, D). Analysis of sleep

levels showed significantly higher nighttime sleep in *D. ananassae* although no significant

difference in overall daytime sleep was detectable (Fig. 3A). Comparison of sleep bout

numbers between the two species revealed that *D. ananassae* had significantly higher number

of daytime sleep-bouts and significantly lower number of nighttime sleep-bouts (Fig. 3B, 2-

way ANOVA, Tukey's HSD). These differences are expectedly accompanied by longer

average nighttime sleep-bout duration in *D. ananassae*, although mean daytime sleep-bout

duration did not differ from *D. melanogaster* (Fig. 3C, 2-way ANOVA, Tukey's HSD).

Taken together it appears that unlike *D. melanogaster*, which are considered crepuscular, *D. ananassae* clearly preferred to be active during daytime and exhibited a more consolidated

sleep at night.

Activity profiles of *D. malerkotliana* virgin male flies showed bimodal pattern like *D. melanogaster* under conditions of LD 12:12. These flies exhibited a morning activity peak

around lights-ON and an evening activity peak around lights-OFF with anticipation to both

the light transitions (Fig. 4A left and middle panel, B). They were inactive during midday

and midnight (Fig. 4A left and middle panel, B). Virgin female flies showed activity pattern

similar to male flies with two peaks (Fig. 4B). Male and female flies showed anticipation to

both the light transitions, however anticipation to lights-OFF was significantly higher for

males compared to females (0.74 ± 0.04 *vs* 0.56 ± 0.07, mean ± 95% CI) (Fig. 4B). Sleep profiles of males and females revealed midday siesta which peaks around ZT6 and nighttime sleep which peaks around ZT14 (Fig. 4C). Although the sleep profiles were similar in both sexes, females exhibited comparatively lesser sleep, most prominently during the daytime. When the male flies were under DD at 25 ºC, they showed free-running periodicity close to 24 hr (Fig 4A right panel, Table 2). Under LL similar to *D. melanogaster* and *D. ananassae*, *D. malerkotliana* flies also became arrhythmic (Fig. 2E).

Unimodal activity of D. ananassae persisted under short- and long-photoperiods. We asked if such preference for daytime activity in *D. ananassae* persists under different photoperiods and examined if oscillators that regulate activity/rest rhythm in these two species have differential sensitivity to light duration. We subjected flies to a range of photoperiods from extremely short (LD4:20) to extremely long (LD20:4; Fig. 5). We found that morning preference for activity in *D. ananassae* was further accentuated under short and long-photoperiods. With increasing day length, *D. melanogaster* gradually shifted most of its activity towards lights-OFF, while *D. ananassae* strongly preferred morning (Fig. 5A). This was most evident in LD20:4 (Figs. 5A, 6) where evening activity peak of *D. melanogaster* occurred about 6 hr ahead of lights-OFF whereas *D. ananassae* activity peak, while being damped, still remained mostly close to lights-ON with very little activity towards the end of light phase. Interestingly, at intermediately long-photoperiods (LD14:10 and LD16:8), *D. ananassae* showed a small increase in evening activity just before lights-OFF, which disappeared when day length increased beyond 18 hr (Figs. 5A, 6).

With decreasing day length, activity peak of *D. melanogaster* occurred under darkness and invariably a blunted pre-dawn peak occurred although they continued to show significant evening peak coinciding lights-OFF and acquired more prominence in contrast to morning

activity (Figs. 5B, 7). In contrast, *D. ananassae* continued to show a preference for D/L transition even when day length was reduced to 4 hr. *D. ananassae* timed majority of its activity to pre-dawn duration with a prominent morning peak, up to 4-6 hr ahead of lights-ON in the most extreme short-photoperiod (LD4:20; Figs. 5B, 7). Thus, over a range of photoperiods, *D. ananassae* shows preference for morning activity suggesting that oscillators controlling this behaviour are tightly coupled to dawn. This also suggests that circadian organization of *D. ananassae* is quite different from *D. melanogaster* thus providing a novel model system for comparative analysis with the canonical model on which most of our current understanding of invertebrate circadian circuit organization is based.

D. ananassae distributed majority of its activity around the morning transition under both long- and short-photoperiods. In order to quantify morning and evening preference, we carried out finer analyses of activity in *D. melanogaster* and *D. ananassae* by examining average activity profiles of individual flies under each photoperiod and assigning true or startle tags to the peaks (Table 3; Figs. 6, 7). Next, we compared activity levels between these two species during various parts of the day/night under long (Fig. 6) and short-photoperiods (Fig. 7). Under long-photoperiods majority of activity and differences between species were seen during daytime, hence we estimated forenoon and afternoon activity indices (FN and AN, see methods). Under LD12:12, although both species exhibited high activity during early half of the day (Fig. 6A), *D. ananassae* showed significantly higher FN and lower AN compared to *D. melanogaster*. Comparison of activity in 2 hr bins showed that *D. ananassae* exhibited significantly greater activity during the light phase for the first 8 hr, whereas both in the last 2 hr of day and for most of the night it displayed significantly lower activity compared to *D. melanogaster* (Fig. 6B). Upon increasing day length to 14 hr, similar pattern of differences between species persisted (Fig. 6B). Comparison of 2 hr bins showed that the

differences in activity are significant for most of the day except the first 2 hr bin, which likely included the startle response in both species (Fig. 6B). As seen in LD12:12, *D. ananassae* became significantly more active compared to *D. melanogaster* during forenoon and less active compared to *D. melanogaster* during afternoon (Fig. 6B). In the intermediately long-photoperiod LD16:8, both species appeared to show similar patterns of activity, with *D. ananassae* showing a hint of evening activity (Fig. 6A; Table 3), while *D. melanogaster* showed bimodal but blunted morning and evening peaks. Comparison of 2 hr bin activity showed no difference between species during most of the day and night in this photoperiod except in the bin immediately preceding lights-OFF where *D. ananassae* was less active (Fig. 6B). With increasing day length (LD18:6 and LD20:4), the evening activity peak became predominant in *D. melanogaster* whereas *D. ananassae* continued to phase most of its activity to the early part of the day. Under LD18:6, neither *D. melanogaster* nor *D. ananassae* appeared to anticipate lights-ON, but a phase-delayed, true morning peak was detectable (Fig. 6A) in ~90% flies in both species (Table 3). While true evening peak of both species were shifted well into the day and advanced compared to L/D transition, *D. ananassae* evening peak was of very low amplitude (Fig. 6A). Comparison of 2 hr bins showed that *D. melanogaster* exhibited significantly lower activity in the forenoon while in the last 6 hr of day the pattern was reversed - *D. melanogaster* became significantly more evening-active as evidenced by presence of the prominent evening peak that re-appeared in this photoperiod (Fig. 6B). Under LD20:4, *D. melanogaster* showed prominent evening peak that was as high as the morning startle (Fig. 6A). Comparisons of 2 hr activity bins showed that *D. ananassae* had significantly higher activity up to 10 hr after lights-ON whereas this trend was reversed between ZT12-18. Under all long-photoperiods FN and AN indices showed completely opposite trends in the two species, *D. ananassae* exhibiting significantly high FN and low AN

activity compared to *D. melanogaster* (Fig. 6A). Thus under increasing photoperiods, evening activity became predominant in *D. melanogaster*, whereas in *D. ananassae*, morning component continued to persist while any signs of evening activity became undetectable under extreme long-photoperiod suggesting that morning oscillator is the dominant entity in *D. ananassae*.

We quantified the difference in activity profiles under short-photoperiods using another set of indices - pre-dawn (Pr-DN) and post-dusk (Po-DK, see methods). Under LD12:12 (Fig. 7A), Pr-DN activity of *D. ananassae* was significantly lower than that of *D. melanogaster*, whereas Po-DK activity did not differ. When day length was made less than 12 hr (LD10:14, 8:16, 6:18) *D. ananassae* shifted most of its activity to the interval preceding dawn such that Pr-DN became significantly higher than that of *D. melanogaster* (Fig. 7A). Under LD8:16 and 6:18, activity of *D. ananassae* preceding dawn lasted for even longer durations than in LD10:14 (Fig. 7). However, no difference in Po-DK activity was detectable between species, although *D. ananassae* appeared to show very little activity during that interval (Fig. 7A). Comparing 2 hr data in LD10:14 revealed that during early morning, i.e., up to 6 hr after lights-ON, *D. ananassae* was more active than *D. melanogaster* (Fig. 7B). This trend was reversed towards the end of the day and continued through most of the night when *D. melanogaster* was more active compared to *D. ananassae* except for the bin immediately preceding lights-ON. Under LD8:16 and 6:18, *D. melanogaster* switched from a clear bimodal pattern of activity to showing a prominent evening peak, with Pr-DN activity becoming flattened and spread out over a period of 4-6 hr (Fig. 7A). Here, although all flies showed a low amplitude true morning peak, less than 43% showed morning startle (Table 3) with large inter-individual variance in peak phase (Fig. 10B). While some degree of flattening of morning peak occurred even in *D. ananassae*, 100% flies exhibited high-

amplitude true morning peak (Fig. 7A, Table 3). In all short-photoperiods, it was not possible to distinguish the evening startle activity from a potential true-peak in either species, since they all appeared to have some degree of anticipatory activity except under LD4:20. Under extremely short photoperiod (LD4:20), no difference in Pr-DN was detectable between species although *D. ananassae* showed a distinct peak compared to flattened morning activity of *D. melanogaster* (Fig. 7A). In contrast, Po-DK activity of *D. melanogaster* was significantly higher than *D. ananassae* (Fig. 7A). The sharper pre-dawn peak of *D. ananassae* was detected as significantly higher values during ZT18-22 (Fig. 7B). Thus, our analyses point towards morning preference for activity in *D. ananassae* compared to *D. melanogaster* even under extremely short-photoperiod and the possibility of oscillator controlling diurnal activity of *D. ananassae* being strongly coupled to dawn.

We also assayed *D. malerkotliana* under different photoperiods. Under LD 12:12 *D. malerkotliana* exhibited only one peak each for morning and evening, where startle and true peaks were inseparable (Figs. 8, 9). As the day length increased, unlike their LD 12:12 behaviour, flies started to show two peaks during morning and evening time, one true peak and one startle peak (Figs. 8, 9 left panels). This was more evident in the extreme long photoperiods (LD 18:6 and LD 20:4) and their evening peak became more predominant during this long day condition (Figs. 8, 9 left panels). With decreasing photoperiod, *D. malerkotliana* flies shifted most of their activity to the dark phase (Figs. 8, 9 right panels). They showed only one peak each for morning and evening time (Fig. 9 right panel). Startle responses were absent for lights-ON and they exhibited a blunted morning peak during pre-dawn (Fig. 9 right panel). True evening peak was indistinguishable from lights-OFF startle response and they showed considerable amount of activity after lights-OFF, this was more

evident in the extreme short photoperiods LD 6:18 and LD 4:20 (Fig. 9 right panel). Activity levels were also affected by varying day lengths. During photoperiods similar to LD 12:12 - 10:14, 8:16 and 14:20, *D. malerkotliana* flies showed lower activity levels compared to LD 12:12 (Fig. 10A). Under extreme long photoperiod LD 20:4 also flies exhibited reduced activity levels compared to LD 12:12 (Fig. 10A).

We compared the phasing of true-peaks of *D. melanogaster* and *D. ananassae* under both increasing and decreasing photoperiods (irrespective of amplitude) to examine whether there may be differences among species in coupling strengths of the circadian clocks with either morning or evening transitions (Fig. 10B). Under LD12:12, *D. melanogaster* showed tight coupling of both morning and evening peaks with D/L and L/D transitions respectively. Morning peak of *D. ananassae* was significantly delayed while evening peak was advanced compared to *D. melanogaster* (Fig. 10B). Under LD14:10, morning peak of *D. ananassae* was phase-delayed compared to *D. melanogaster* while evening peaks of both species were similarly phased. Under day lengths longer than 14 hr, both species showed similar phase-relationships with both transitions, except in LD20:4, where true evening peak was shown only by < 30% *D. ananassae* flies (Table 3). Under short-days (LD10:14, 8:16 and 6:18), *D. ananassae* was consistently less phase-advanced compared to *D. melanogaster* (by 0.7 hr, 1.4 hr and 1.1 hr respectively) and showed lesser inter-individual variation (Fig. 10B). Interestingly, under extreme short-day (LD4:20) both species have similarly advanced morning peaks (Fig. 10B). True evening peaks of both species were indistinguishable from startle under short-days or LD12:12.

Phasing of morning and evening peak of *D. malerkotliana* also showed difference in their coupling with lights-ON and lights-OFF respectively during long and short photoperiods (Fig. 10B). Flies showed strong coupling to lights-ON and lights-OFF during LD 12:12, true

morning and evening peak coincided with lights-ON and lights-OFF respectively (Fig. 10B). As the day length increased flies showed a morning peak after lights-ON (except 14:10) and an evening peak prior to lights-OFF, thus both peaks were uncoupled from the light transitions. Interestingly under short photoperiods only morning peak was uncoupled from lights-ON and evening peak coincided with lights-OFF transition (Fig. 10B).

Thus, *D. ananassae* is clearly a morning preferring species compared to *D. melanogaster* and *D. malerkotliana* whose activity although symmetrically distributed around both morning and evening transitions under LD12:12 conditions reveal themselves to be predominantly evening preferring flies when examined under either long- or short-photoperiods. The above results underscore separation of *D. melanogaster* and *D. ananassae* on a temporal axis and suggest that these two sympatric species probably adopt different physiological measures to deal with the environmental challenges under different photoperiods.

Discussion

Our studies suggest that two sympatric species of *Drosophila* – *D. melanogaster* and *D. ananassae* may occupy distinct temporal niches under identical environmental conditions. We show that *D. ananassae* prefers to remain active during the day and limits its activity at night to extremely low levels. Interestingly the time at which *D. ananassae* was most active under LD12:12 (ZT1-6) also coincided with the time of day when *D. melanogaster* rapidly reduced its activity (Figs. 1, 2). It is likely that *D. melanogaster* avoids activity during midday when minimal humidity, maximum temperature and maximum light levels occur to minimize chances of desiccation (Low et al., 2008). Therefore, it is puzzling that *D. ananassae*, a close relative of *D. melanogaster* in fact prefers to be most active at this time

(Figs. 1, 2). Moreover, *D. melanogaster* showed a distinct bimodality similar to many crepuscular animals that are thought to use the relatively milder conditions of twilight to minimize the effects of environments at other harsher times of the day. Such preference for twilight was clearly lacking in *D. ananassae*. It is possible that on a local scale, the two species occupy different habitats even though they occur in the same geographical areas. Anecdotal evidence suggests that *D. ananassae* is more often found in areas around human habitation such as inside homes, kitchens, garbage dumps, markets and grocery stores whereas *D. melanogaster* is more likely to be caught around orchards and open fields. We speculate that systematic net sweep studies done in a time-logged manner may reveal that the two species experience very different micro-climates while living in the same area.

Compared to *D. melanogaster*, *D. ananassae* exhibit much more consolidated sleep at night. During the day, even though mean sleep-bout duration of *D. ananassae* does not differ from *D. melanogaster*, the former exhibits greater number of daytime sleep-bouts suggesting that these flies do not have sustained activity during their preferred active phase and that activity is interspersed with short sleep-bouts. In contrast, their nighttime sleep is more consolidated compared to *D. melanogaster* as they exhibit significantly fewer sleep-bouts and also significantly longer mean sleep-bout duration. Such differences also persist under DD where only one narrow band of activity is exhibited by *D. ananassae* compared to more spread-out activity of *D. melanogaster* (Figs. 1, 2C). *D. melanogaster* may have the required physiology and sensory apparatus that allows activity even in the absence of light. Nights are accompanied by cooler temperatures, hence it is possible that *D. ananassae* avoids activity at night and that may partly be the reason for their distribution being restricted to warmer, more tropical clines compared to *D. melanogaster* (Das et al., 2004). Future studies on other

physiological and behavioural aspects of sleep in *D. ananassae* such as the arousal threshold, sensitivity to temperature and light intensity may help in a better understanding of *D. ananassae* sleep characteristics. One of the limitations of our studies is that they were all done under laboratory conditions, thus, future studies that examine rhythmic behaviours under cycling temperature cycles and/or semi-natural conditions may provide further clues towards the ecological significance of this temporal preference.

Our studies show that phasing of morning peak with D/L transition is modulated by photoperiod in both species (Fig. 10B). Under LD12:12 it is tightly coupled with lights-ON especially in *D. melanogaster* and both species show a significant advancement of phase under short-day conditions - *D. melanogaster* being more advanced compared to *D. ananassae*. This suggests that if indeed dual oscillators which are coupled to dawn and dusk regulate the two peaks in these two species, then, the morning oscillator of *D. ananassae* has a stronger coupling to lights-ON compared to *D. melanogaster*. On the other hand, in *D. melanogaster*, the persistence of evening peak, especially under long-photoperiods suggests the presence of a dominant evening oscillator. The coupling of evening oscillator to dusk in *D. melanogaster* is further confirmed upon release into DD (from LD12:12), by the persistence of a major evening activity component and its phasing (Fig. 2C). This suggests that strong morning and evening oscillators regulate *D. melanogaster* activity behaviour (Grima et al., 2004; Stoleru et al., 2004, reviewed by Helfrich-Förster, 2009*), whereas *D. ananassae* probably has only a dominant morning oscillator and a much weaker evening oscillator. Our results do not rule out the existence of evening oscillator in *D. ananassae* since these flies show small bouts of evening anticipatory activity under several regimes, especially becoming evident under intermediately long-photoperiods (LD16:8 and 18:6; Fig.

6A). Based on the coupled-oscillator model of Pittendrigh and Daan (1976), previous studies have hypothesized that in *D. melanogaster*, the morning oscillator is dominant under short-photoperiod while the evening oscillator has greater effect under long-photoperiod (Stoleru et al., 2007). Under long-photoperiod, *D. ananassae* shows high variation in evening peak phase both among individual flies and across days (Figs. 6A, 10B) suggesting that under long-photoperiod, even in *D. ananassae*, the otherwise dominant morning oscillator coexists with a strengthened evening oscillator. In comparison with the neuronal substrates of the morning and evening oscillators that have been unraveled in *D. melanogaster*, *D. ananassae* would be expected to have either greater number of so-called 'morning' cells (which was not seen in a recent study by Hermann et al., 2013), or that 'morning' cells exert a stronger influence on the motor circuits controlling activity rhythm. Alternatively, lesser number or weaker coupling of evening cells with the circuit can result in such a dominance of the morning oscillator in the organization of circadian pacemakers of *D. ananassae*.

It is also possible that the cellular identity of the morning oscillator in *D. ananassae* is different from that of *D. melanogaster*, thus, our studies point towards potential differences in the nature of the underlying circadian pacemaker circuit and/or its outputs in *D. ananassae* compared to *D. melanogaster*. Interestingly, the morning preference in *D. ananassae* is also accompanied by a faster ticking clock suggesting that the molecular clockwork may also differ from that of *D. melanogaster*. Hermann and colleagues (2013) find no difference between *D. melanogaster* and *D. ananassae* in terms of cell number among PDF, VRI and PDP1 expressing cells, but minor differences were seen in the projection patterns of ventral lateral neurons. They speculate that in contrast to *D. melanogaster* where only one subset - the small ventral lateral neurons, which modulate morning activity, sends projections towards

the dorsal protocerebrum, in *D. ananassae* the second subset, the large ventral lateral neurons also project dorsally. Another significant anatomical difference between the two species was seen in the number of neuropeptide ITP expressing cells, ipc-2 and ipc-4, although the role of these neurons in regulating diurnal activity/rest behaviour is as yet unknown. In *D. ananassae,* compared to *D. melanogaster,* fewer neurons of ipc-2 subset were seen, while ipc-4 subtype was undetectable. Taken together with our own results, this suggests that in these two closely related species with high homology in the sequences of the genes and proteins involved in circadian clocks and also similar neuroanatomical features of circadian neuronal network, the striking divergence in temporal preference for activity/rest behaviour probably arises from some subtle differences in the coupling among those neurons. We propose that *D. ananassae* may serve as a useful model to conduct comparative studies of the neuronal circuitry and underlying genetic basis of circadian clocks, given that the *D. ananassae* genome has been fully sequenced (*Drosophila* 12 genome consortium, 2007). In future, modern tools of transgenesis that have proven to be invaluable for *D. melanogaster* could also be modified to be used in *D. ananassae*. Furthermore, down regulating or enhancing neuropeptide (or its receptor) expression or manipulating electrical properties by ectopic expression of ion channels within the circadian clock neurons may reveal features of circadian circuit organization that have not been discovered through studies on *D. melanogaster*.

Photoperiod dependent changes in the phasing of morning and evening peaks of *D. malerkotliana* strengthen the hypothesis of coupled-oscillator model (Pittendrigh and Daan, 1976). Similarity in the phasing of morning and evening peaks under different photoperiods and other circadian activity behaviours in *D. malerkotliana* and *D. melanogaster* suggest that these two species may share similar circadian organization. Further, it will be interesting to

find out whether the proposed cellular correlates of morning and evening oscillator cells of *D. melanogaster* is present in *D. malerkotliana* also and whether there is any difference in the expression pattern of important clock proteins PDF, PERIOD, TIM etc. Our studies suggest an interesting temporal separation of behaviour between two sympatric species *D. melanogaster* and *D. ananassae*, which may allow better understanding of the functional significance of timing of activity. Functionally, this preferential timing could imply better competitive advantage, foraging and mating related movements and/or exploratory behaviour. We propose that comparison of closely related sympatric species under natural conditions in parallel with laboratory studies will allow additional insights into mechanistic details and functional significance of circadian clocks and the behaviours that they control.

Table 1. *D. ananassae* (DA) exhibits shorter free-running period compared to *D. melanogaster* (DM) under DD. * $p < 0.05$ (two-way ANOVA; Tukey's HSD).

Experiment	Genotype	*n*	Period (hr) ± 95 % CI	Robustness Periodogram Amplitude ± 95 % CI	Rhythmicity %
1	DM	14	24.49 ± 0.37	53.63 ± 15.54	100
	DA	11	23.46 ± 0.55	57.24 ± 17.54	100
2	DM	16	24.12 ± 0.33	69.17 ± 22.92	100
	DA	23	23.30 ± 0.22*	60.02 ± 13.28	93.5
3	DM	30	24.25 ± 0.19	57.14 ± 07.98	100
	DA	25	23.67 ± 0.18*	52.12 ± 10.82	100
4	DM	24	24.00 ± 0.19	63.76 ± 14.96	100
	DA	29	23.56 ± 0.19*	79.21 ± 10.60	100

Table 2. Free running periodicity of *D. malerkotliana* under constant darkness (DD) at 25 ºC.

Experiment	*n*	Period (h) ± SEM	Robustness Periodogram Amplitude ± SEM	Rhythmicity %
1	30	23.89 ± 0.08	81.43 ± 5.74	100
2	30	24.40 ± 0.10	43.78 ± 4.13	100
3	13	24.69 ± 0.21	56.77 ± 9.69	100
4	15	24.26 ± 0.15	60.23 ± 6.29	100

Table 3. Number of *D. melanogaster* (DM) and *D. ananassae* (DA) flies showing true peaks (T) and startle peaks (S) for morning and evening transitions. *n* = total number of flies analysed. Asterisks indicate peaks where true and startle components are indistinguishable and hence considered as true peaks.

| Photoperiod	Morning			Evening			*n*
	True Peaks (%)	Startle Peaks (%)	T/S or Both	True Peaks (%)	Startle Peaks (%)	T/S or Both	
DM							
20:04	24 (77.4)	31 (100)	31	31 (100)	20 (64.5)	31	31
18:06	29 (90.7)	32 (100)	32	30 (93.8)	29 (90.6)	32	32
16:08	22 (68.8)	32 (100)	32	30 (93.8)	31 (96.9)	32	32
14:10	26 (100)*	0	26	24 (85.7)	14 (50)	28	28
12:12	30 (100)*	0	30	30 (100)*	0	30	30
10:14	19 (73.1)	11 (42.3)	26	26 (100)*	0	26	26
08:16	27 (100)	2 (7.4)	27	28 (100)*	0	28	28
06:18	28 (100)	12 (42.9)	28	29 (100)*	0	29	30
04:20	30 (100)	22 (73.3)	30	30 (100)*	0	30	30
DA							
20:04	29 (90.6)	32 (100)	32	7 (29.2)	24 (77.4)	24	32
18:06	25 (89.3)	27 (96.4)	28	18 (69.2)	26 (92.9)	28	28
16:08	17 (65.4)	25 (96.2)	26	24 (100)	24 (92.3)	26	29
14:10	22 (100)*	0	22	13 (65)	9 (45)	20	23
12:12	28 (100)*	0	28	28 (100)*	0	28	28
10:14	17 (56.7)	13 (43.3)	30	24 (100)*	0	24	30
08:16	27 (100)	7 (25.9)	27	25 (100)*	0	25	27
06:18	28 (100)	17 (60.7)	28	25 (100)*	0	25	30
04:20	19 (79.2)	16 (66.7)	24	24 (100)*	0	24	26
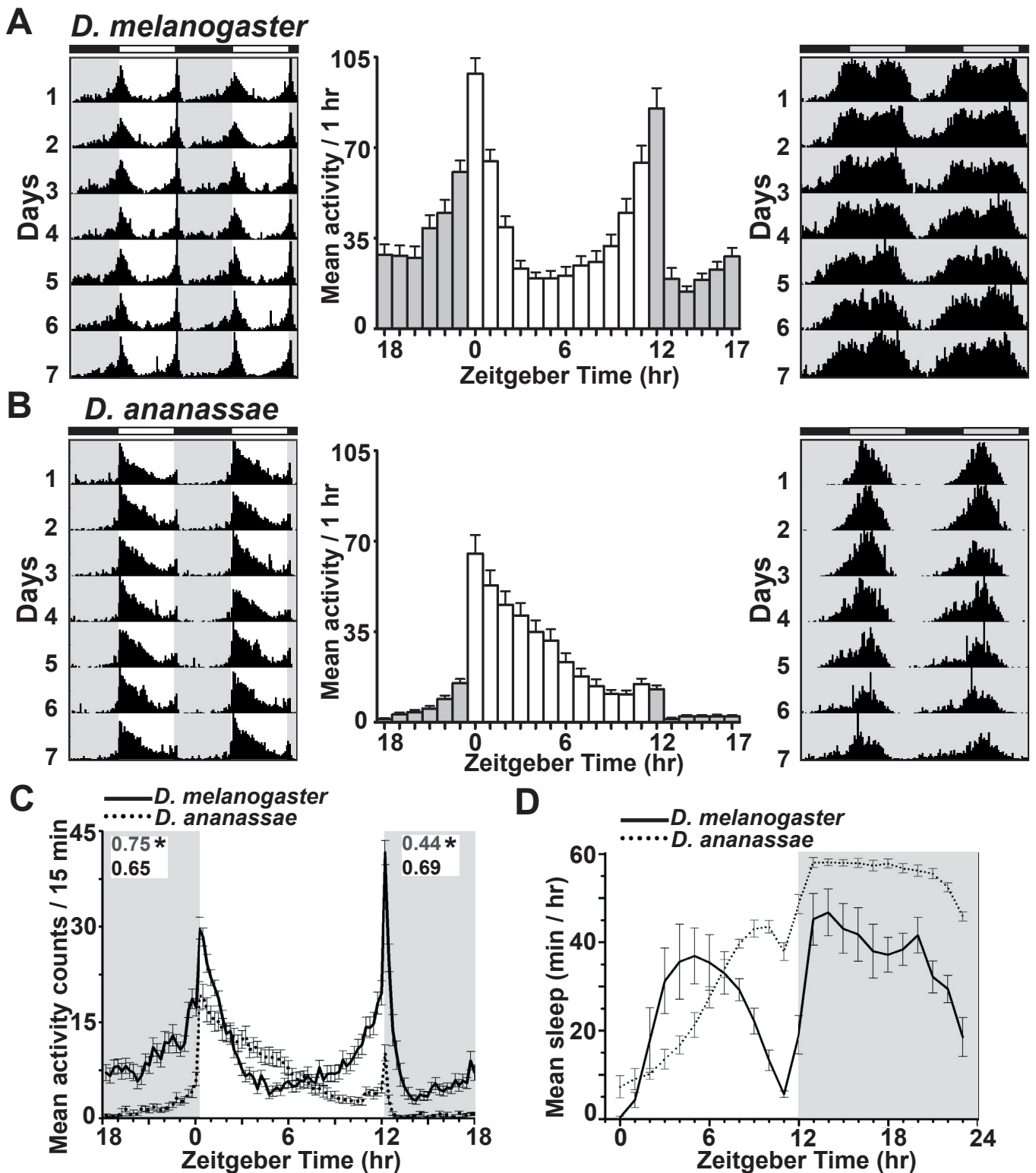
Figure 1. ***D. ananassae*** **showed preference for morning activity compared to *D. melanogaster* under LD12:12.** (**A**) Average double plotted actograms of virgin male *D. melanogaster* (*n*=28) under LD12:12 at 25°C (left) and DD (right, *n*=25). The x-axis represents time of day from 0-48 hr, consecutive days are plotted along y-axis. Histograms show average activity in 1hr bins across 7 days under LD12:12 (mean ± SEM). Grey shaded areas in actograms represent darkness while grey bars in the histogram (middle panel) denote activity in the dark phase under LD12:12. Black and white bars above the actograms indicate the dark and light phases respectively under LD cycles. (**B**) Actograms and activity profiles of male *D.ananassae* (*n*=28) in LD and DD (*n*=30). All other details are same as panel A. (**C**) Raw activity counts in 15 min bins under LD12:12 averaged across 7 days for both DA and DM. Morning anticipation (top left values) of DA is significantly higher than that of DM (*$p < 0.05$). Evening anticipation (top right values) of DA is significantly lower than DM (*$p < 0.05$). Error bars are SEM. (**D**) Sleep profiles under LD12:12 show midday peak in sleep for DM (*n*=28) while DA shows a gradual increase in sleep throughout the day phase with highest sleep just before dusk (*n*=28). Error bars are 95%CI. *$p < 0.05$.

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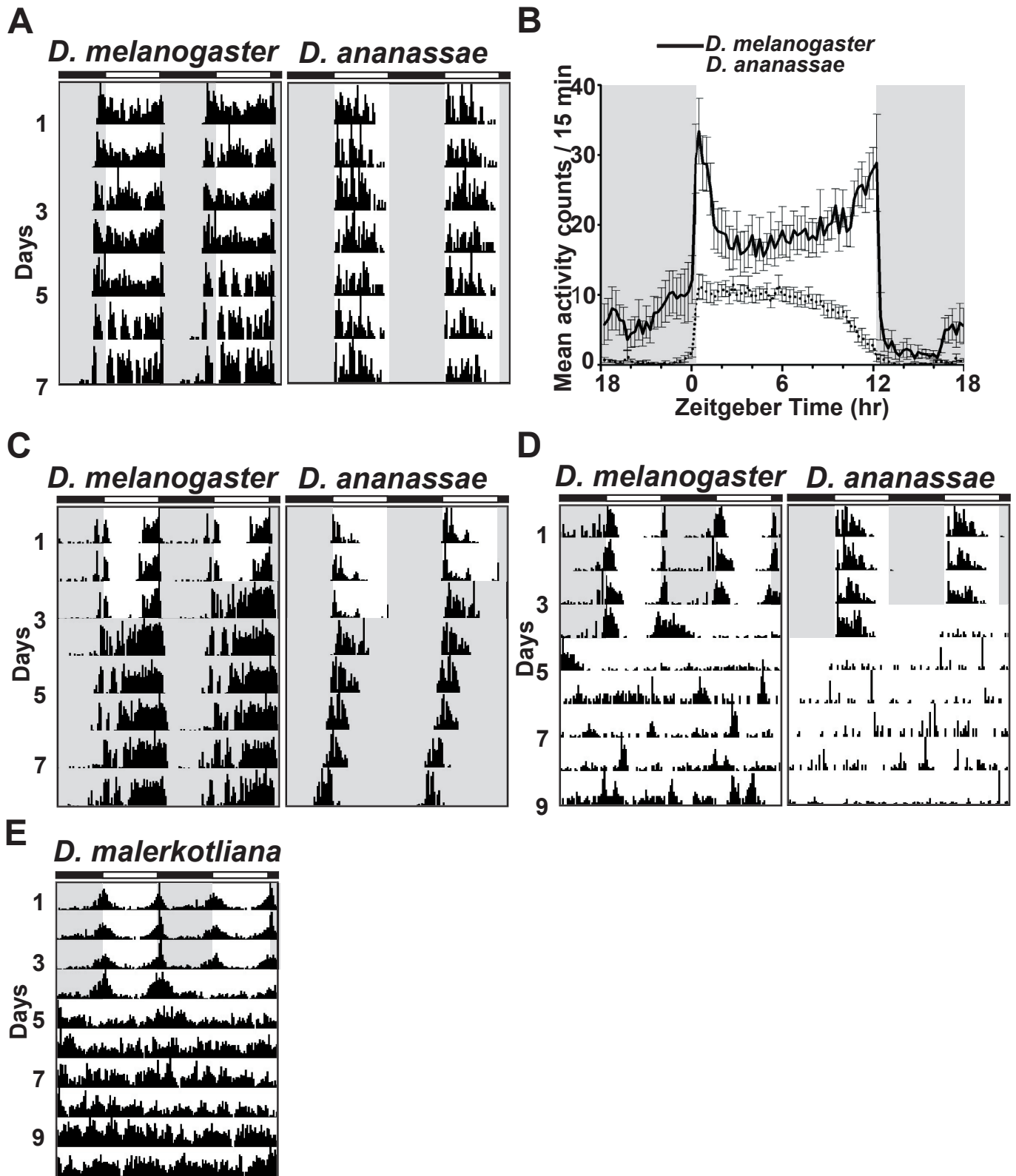
Figure 2. *D. ananassae* females show morning preference in activity/rest pattern similar to males. (**A**) Representative actograms of *D. melanogaster* and *D. ananassae* virgin female flies under LD12:12. (**B**) Raw Activity counts (15 min bin) averaged across 7 days for both DM and DA virgin female flies (mean ± SEM). (**C**) Representative actograms of *D. melanogaster* and *D. ananassae* virgin male flies under 3 days of LD12:12 followed by DD. (**D**) Representative actograms of *D. melanogaster* and *D. ananassae* virgin male flies under 3 days of LD12:12 followed by LL. (**E**) Average actogram of *D. malerkotliana* flies under 3 days of LD12:12 followed by LL. Grey shaded areas indicate darkness. Black and white bars above the actograms indicate the dark and light phases respectively under LD cycles.

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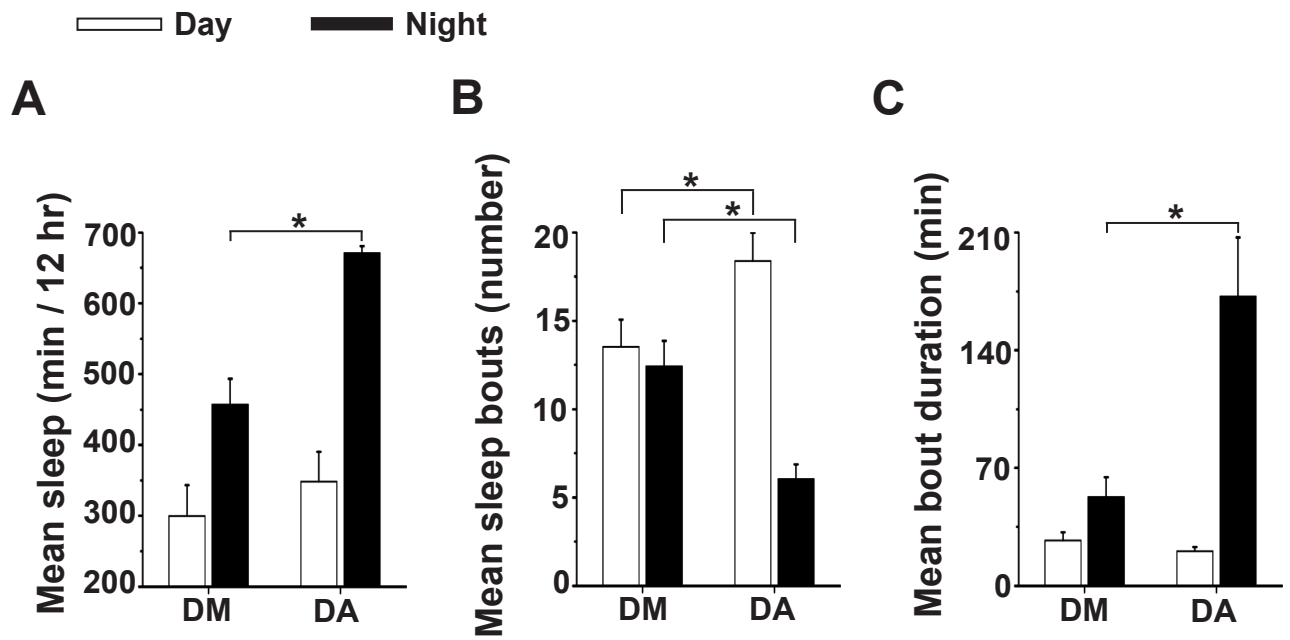
Figure 3. *D. ananassae* **(DA) exhibited higher levels of consolidated night time sleep compared to** *D. melanogaster* **(DM).** (**A**) No significant difference in mean daytime sleep between species but nighttime sleep is significantly higher in DA. (**B-C**) Decreased mean night sleep bout number and increased mean bout duration of nighttime sleep in DA. Asterisks indicate significant differences across species (2-way ANOVA; Tukey's HSD). Error bars are 95% CI.
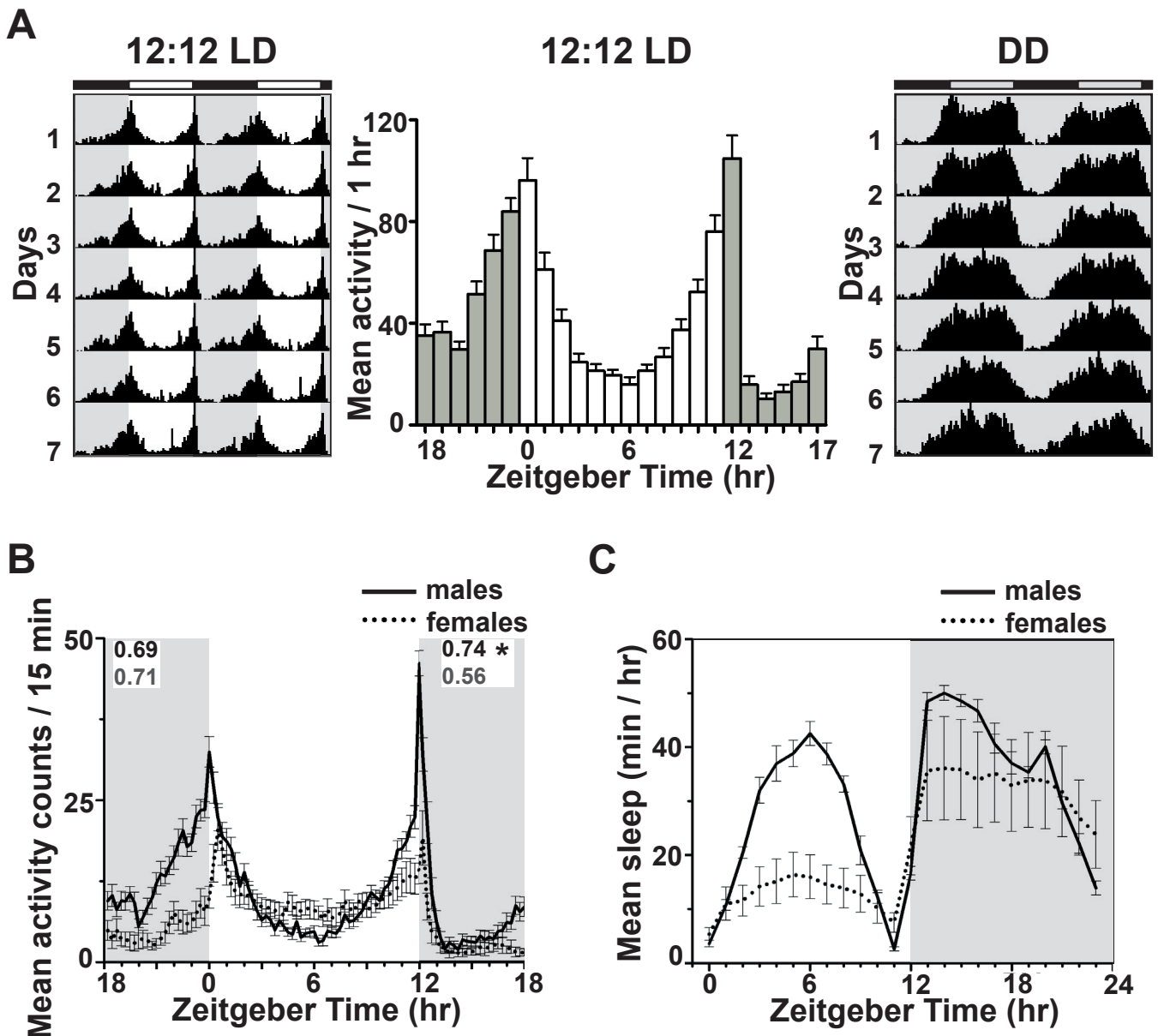
Figure 4. Bimodality in the activity pattern of *D. malerkotliana* under LD12:12. (**A**) Double plotted average actograms of virgin male *D. malerkotliana* flies under LD12:12 at 25°C (left, *n*=29) and DD (right, *n*=30). The x-axis represents time of day from 0-48 hr, consecutive days are plotted along y-axis. Histograms (middle panel) show average activity in 1 hr bins across 7 days under LD12:12 (mean ± SEM). Grey shaded areas in actograms represent darkness and grey bars in the histogram (middle panel) denote activity in the dark phase under LD12:12. Black and white bars above the actograms indicate the dark and light phases respectively under LD cycles. (**B**) Raw activity counts in 15 min bins averaged across 7 days for virgin males and females under LD12:12. Error bars are SEM. Top left values shows morning anticipation of males (black) and females (grey). Evening anticipation (top right values) of males is significantly higher than females (*$p < 0.05$). (**C**) Sleep profiles of males and females under LD12:12. Error bars are SEM.
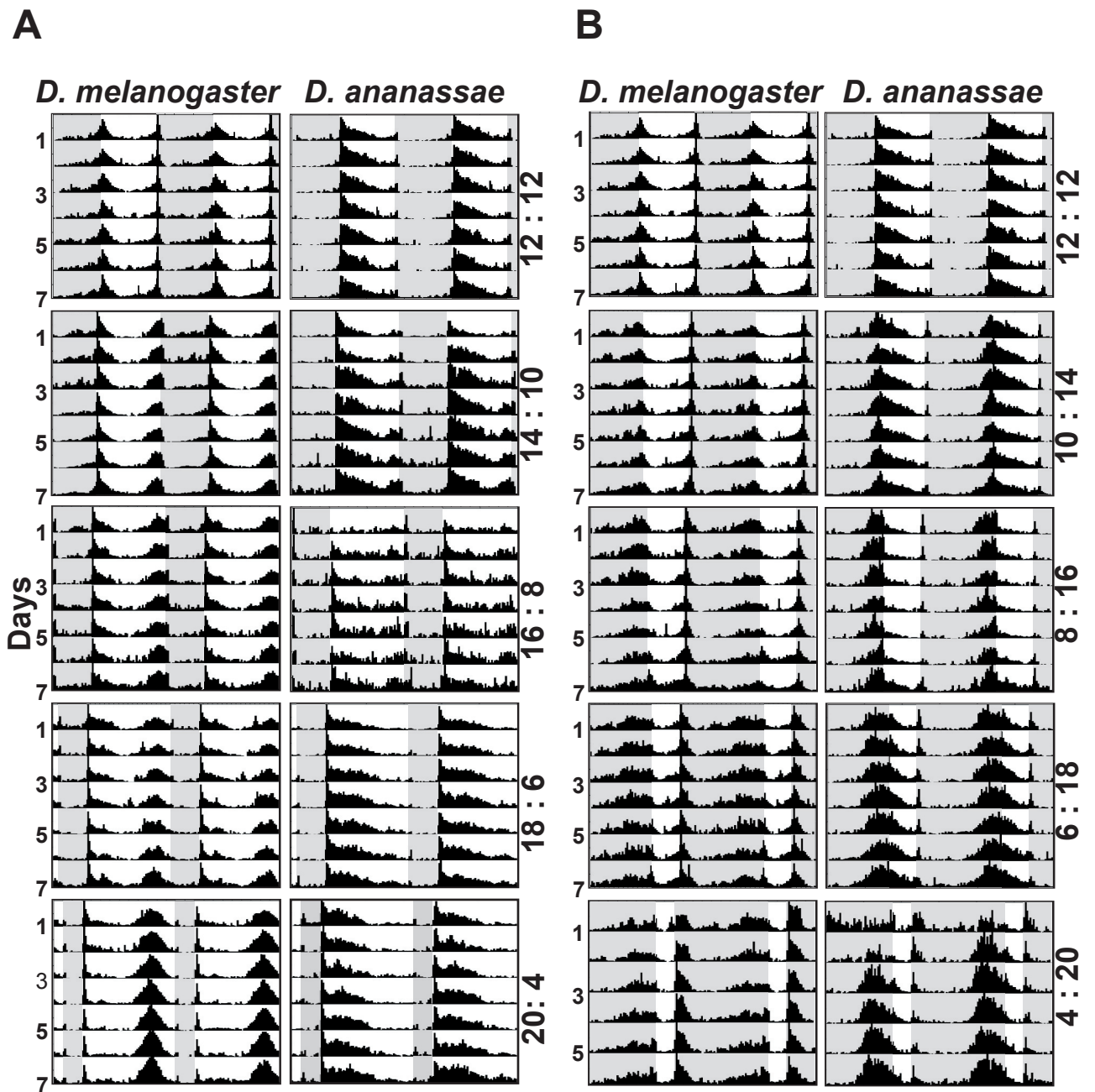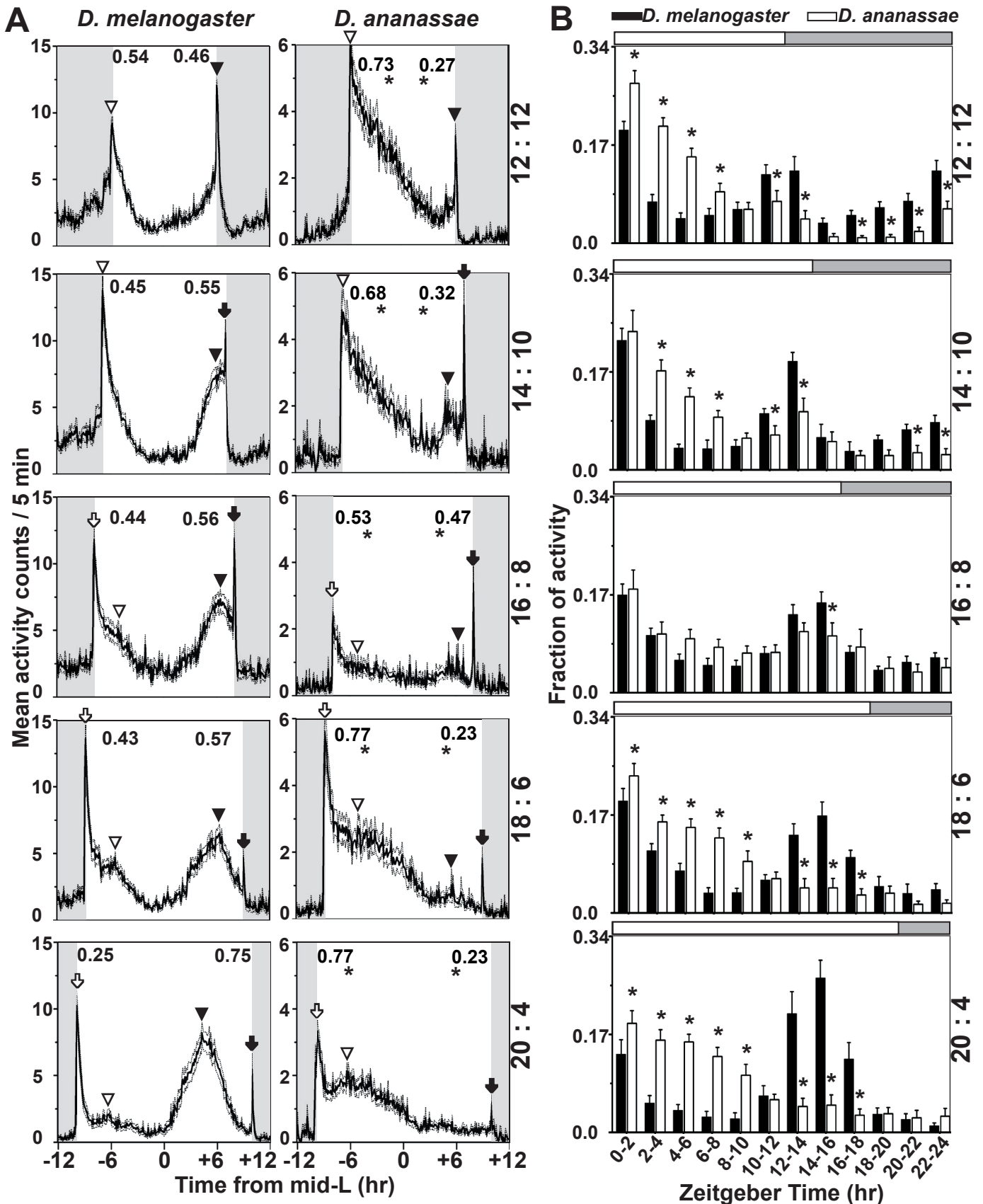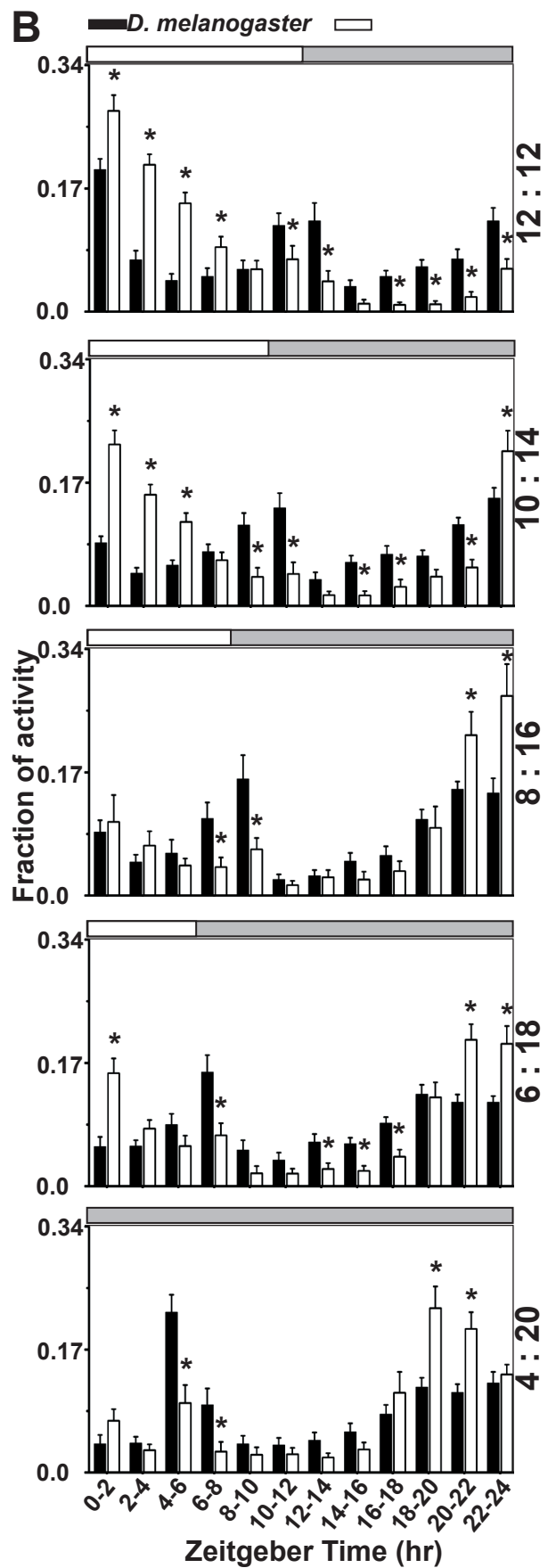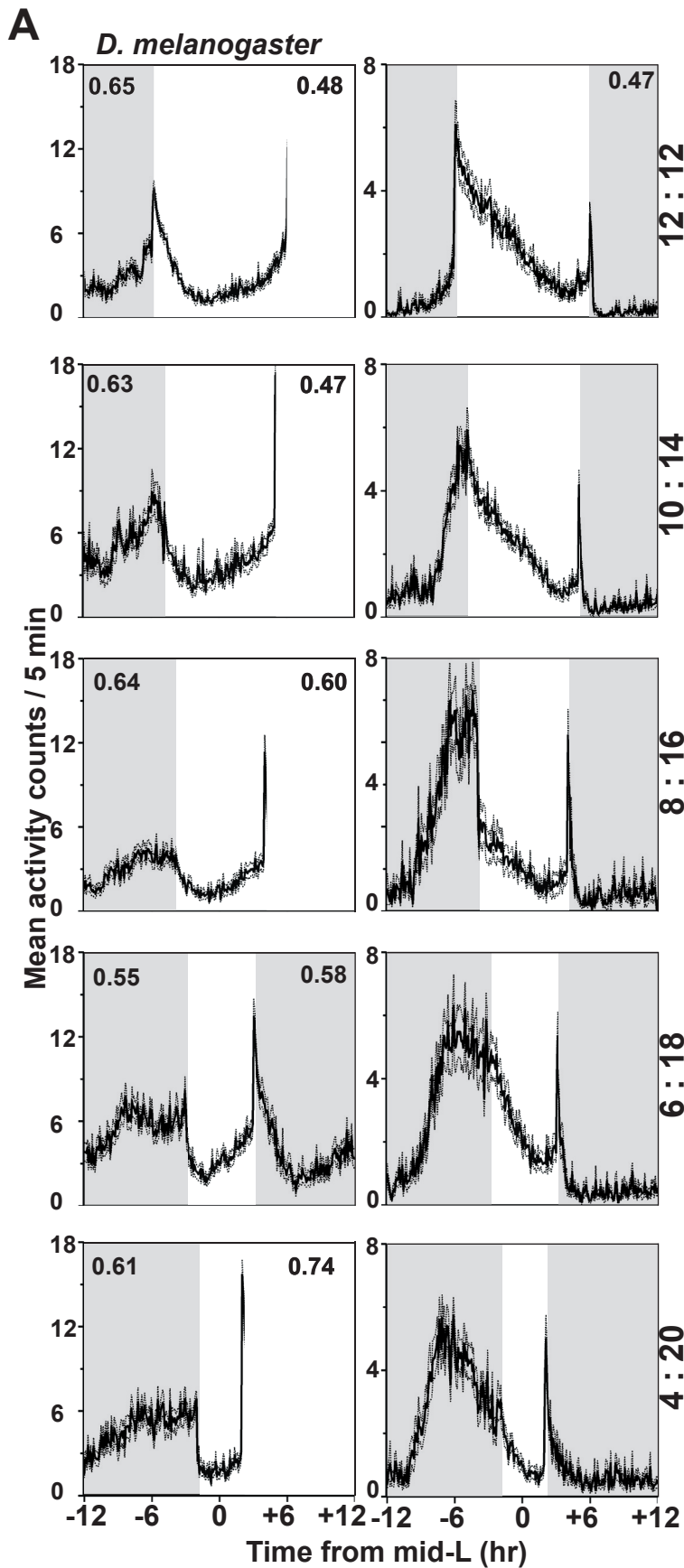
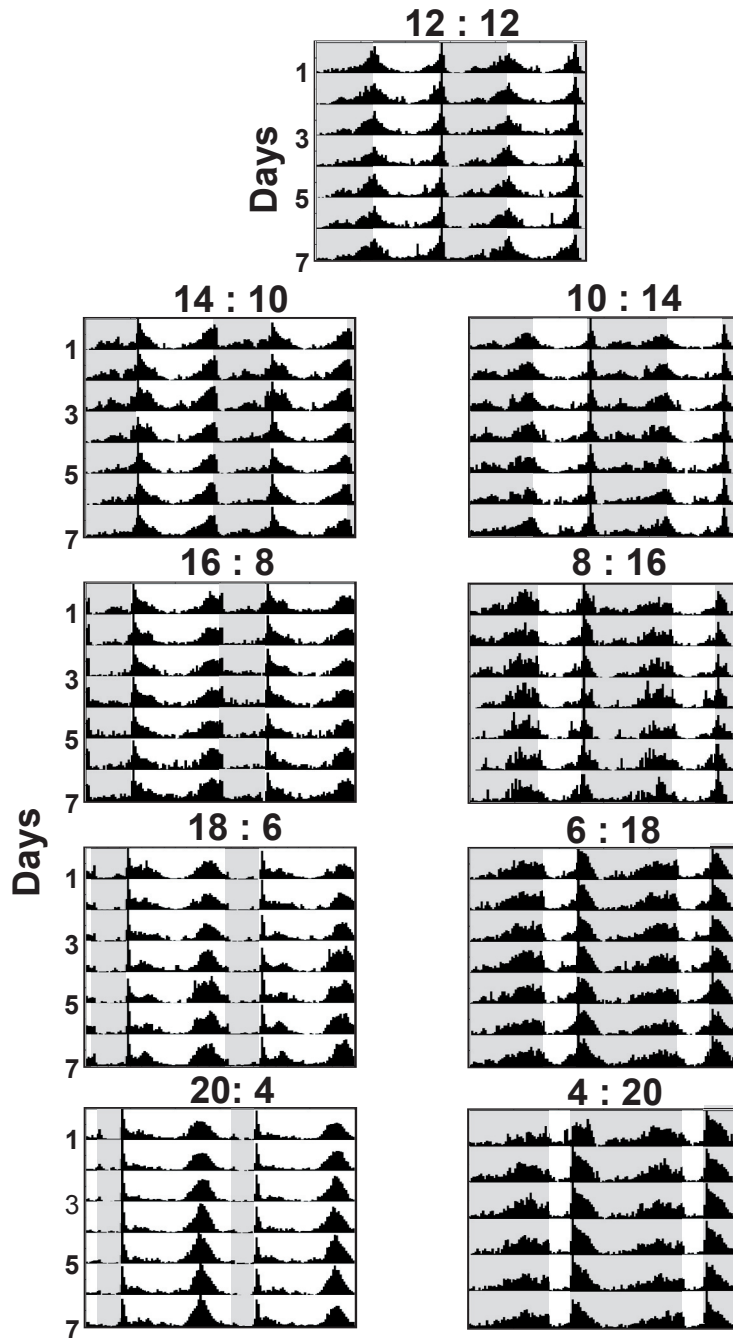**Figure 5. Prominent evening peak persisted in *D. melanogaster* across multiple photoperiods, whereas *D. annassae* showed persistent morning activity.** Double plotted normalized average actograms ($n \geq 23 \leq 32$ flies) for *D. melanogaster* and *D. annassae* under (**A**) long-photoperiods and (**B**) short-photoperiods. All other details are similar to Fig. 1.
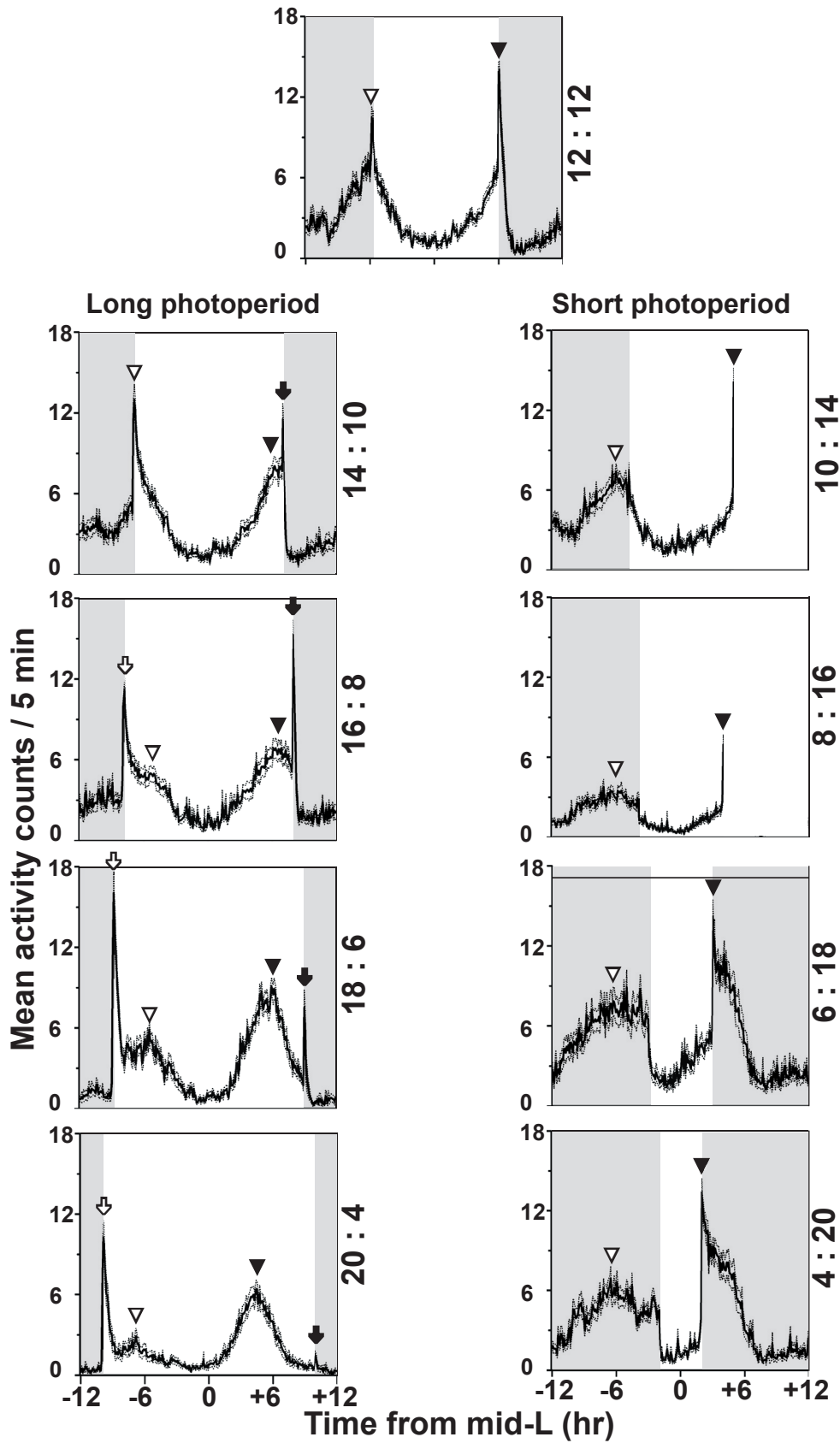
Figure 6. *D. ananassae* **exhibited higher forenoon activity while *D. melanogaster* exhibited higher afternoon activity under long-photoperiods.** (**A**) Activity profiles with mean activity counts in 5 min bins (±SEM) averaged across 7 days. In each profile, morning and evening preference estimated by FN (top left) and AN (top right) activity indices are indicated for both species. Under all long photoperiods, *D. ananassae* show significantly higher morning preference compared to *D. melanogaster*. *D. melanogaster* show higher evening preference compared to *D. ananassae*. Arrowheads indicate presence of true peaks while arrows indicate startle peaks when shown by more than 50% flies (Table 2). Unfilled (white) symbols denote morning peaks and black symbols, evening peaks. (**B**) Quantification of mean activity (2 hr bin) normalized to total activity (±95%CI). Horizontal white and grey bar above each graph denotes duration of light and dark respectively in all photoperiods. All other details as Fig. 1.

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Figure 8. Prominent evening peak persisted in *D. malerkotliana* across different photoperiods. Double plotted normalized average actograms of *D. malerkotliana* ($n \geq 23 \leq 32$ flies) under LD12:12, long-photoperiods and short-photoperiods. All other details are similar to Fig. 1.

Long photoperiod

Short photoperiod

12 : 12

14 : 10

10 : 14

16 : 8

8 : 16

18 : 6

6 : 18

20 : 4

4 : 20

Mean activity counts / 5 min

Time from mid-L (hr)

51
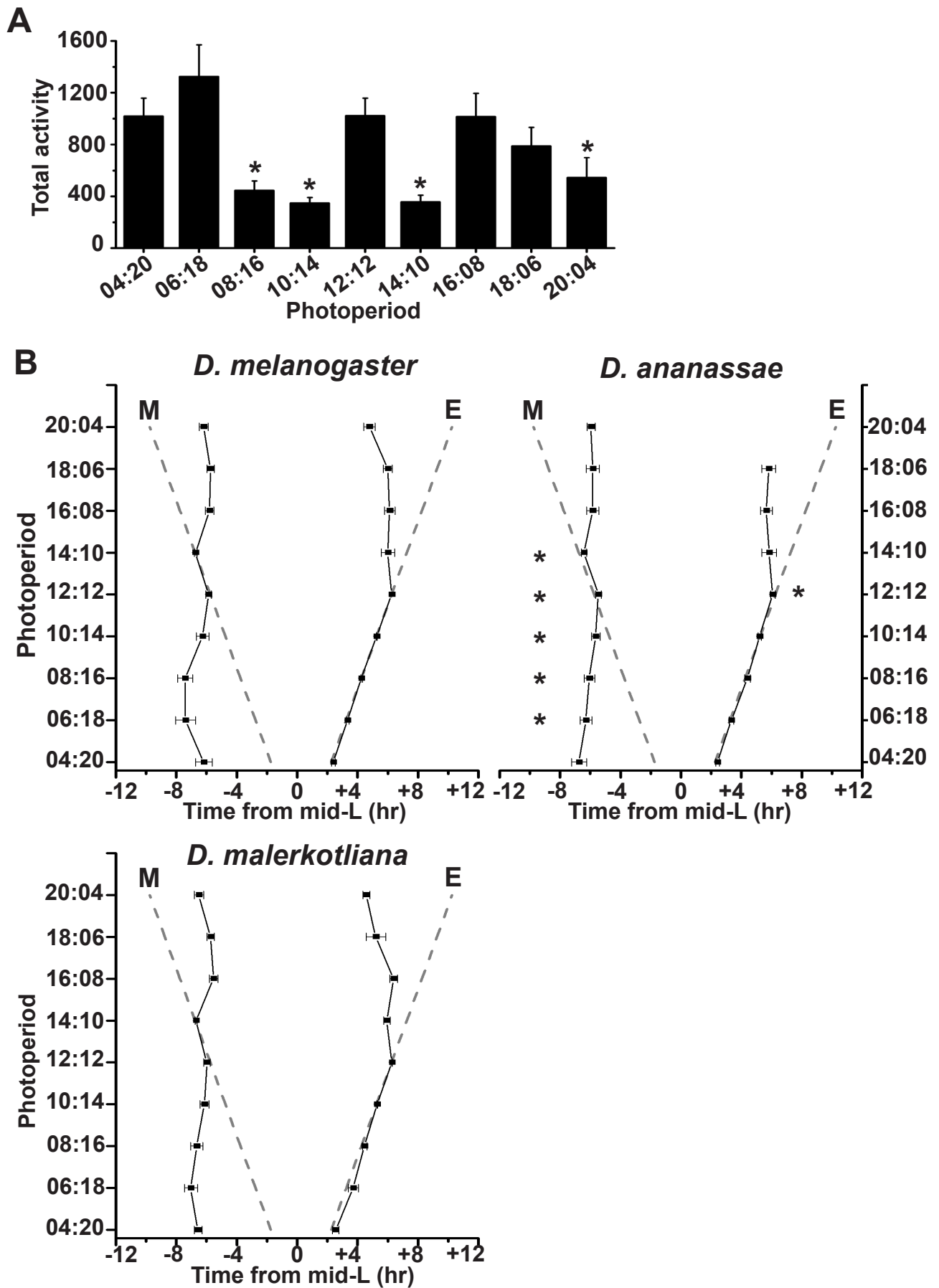
Figure 10. Phasing of true-peaks of *D. melanogaster*, *D. ananassae* and *D. malerkotliana* under different photoperiods. (**A**) Total activity counts of *D. malerkotliana* (±95%CI) averaged across 7 days under each photoperiod compared with that of LD 12:12. (**B**) Phase plots of true morning and evening peaks under all photoperiods for *D. melanogaster, D. ananassae* and *D. malerkotliana* (mean ± 95%CI). Dashed grey lines indicate phases of lights-ON and lights-OFF in each photoperiod. *$p < 0.05$.

Temperature sensitivity of circadian clocks in *Drosophila* species *melanogaster*, *malerkotliana* and *ananassae*

Introduction

Light and temperature cycles are known to be the strongest zeitgebers for circadian timekeeping. While circadian entrainment to light has been extensively investigated, entrainment to temperature is less understood. Several studies clearly show that temperature can also act as a Zeitgeber for the circadian clocks of *D. melanogaster* (reviewed in Dubruille and Emery, 2008). Under laboratory 12:12 hr light/dark (LD) cycles, male *D. melanogaster* flies exhibit bimodal activity pattern with morning and evening peaks coinciding with lights-ON and lights-OFF, respectively (Helfrich-Förster, 2000). Similar to their LD behaviour, under thermophase/cryophase (TC) cycles, *D. melanogaster* shows bimodality in activity pattern with a peak in the morning (thermophase onset) and a prominent peak in the evening (cryophase onset), which is slightly advanced with respect to dusk (cryophase onset). Temperature cycles can entrain the activity rhythm of *D. melanogaster* under constant darkness (DD) (Busza et al., 2007; Stanewsky et al., 1998; Tomioka et al., 1998; Wheeler et al., 1993; Yoshii et al., 2002), and under constant light (LL) (Glaser and Stanewsky, 2005; Tomioka et al., 1998; Yoshii et al., 2002; Yoshii et al., 2005, Yoshii et al., 2007) - which otherwise causes arrhythmicity (Konopka et al., 1989). Cycles of temperature with a difference as low as 3 °C have been shown to entrain activity rhythm of *D. melanogaster* (Wheeler et al., 1993). Temperature has also been shown to influence activity rhythm in the presence of LD cycles (Majercak et al., 1999). Ambient temperature modulates the distribution of activity, in what appears to be an adaptive manner. With increasing temperature, *D. melanogaster* shifts its activity into the night with a decrease in midday activity (Majercak et al., 1999). Further, depending on the time of the day, warm temperature pulses have been shown to induce advance, delay or no response to the circadian activity rhythm of *D. melanogaster* (Busza et al., 2007). Although TC cycles can entrain circadian clocks, its period is temperature compensated, i.e., their period does not change drastically

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with increase or decrease of temperature within the physiological range (Bruce and Pittendrigh, 1956; Hastings and Sweeney, 1957; Pittendrigh, 1954). This ability was also exhibited by the circadian clocks of *D. melanogaster* regulating activity/rest rhythm (Dunlap et al., 2004). More recent studies have shown that the dual-oscillator model, initially proposed to explain adaptation to seasonal variation in mammals and later validated in fruitflies for adaptation to photoperiods, can also explain entrainment to thermoperiods (Bywalez et al., 2012). The study showed that under temperature cycles the two oscillators that govern morning and evening activity peaks are more strongly coupled to each other than under photoperiods. Thus, temperature seems to have a major role in the temporal distribution of activity in *D. melanogaster*.

Previously we have shown that another *Drosophila* species *D. ananassae* differs from *D. melanogaster* and *D. malerkotliana* in several features of their activity/rest rhythm (chapter 2). *D. melanogaster* and *D. malerkotliana* exhibit a bimodal activity pattern while *D. ananassae* is predominantly active in the morning across a range of photoperiods in the laboratory. From the results of our previous studies we hypothesized that *D. ananassae* may occupy different temporal niches from that of *D. melanogaster* and *D. malerkotliana* due to the differences in their underlying circadian clocks. In the present study, we examined whether like *D. melanogaster*, the activity/rest rhythm of *D. malerkotliana* and *D. ananassae* show entrainment to TC cycles and whether the unique features exhibited by *D. ananassae* under LD cycles extends to TC cycles as well since both temperature and light are robustly varying environmental factors that could potentially influence the rhythm in different ways. We also compared the effect of temperature perturbation on the circadian clocks controlling activity/rest rhythm of *D. melanogaster* and *D. ananassae*, at two circadian phases, known to respond by resetting the clocks by eliciting maximum delays and advances, to understand

whether they differ in their sensitivity to temperature. Further, to confirm that the circadian

clocks of *D. malerkotliana* and *D. ananassae* are temperature compensated, we calculated the

periodicity of their activity/rest rhythm under a range of constant temperatures. We report

that the unimodal and predominantly morning activity pattern of *D. ananassae* persisted

under TC cycles, whereas *D. melanogaster* and *D. malerkotliana* showed bimodal activity

pattern. Warm temperature pulses caused similar phase-shifts in *D. melanogaster* and *D.

ananassae*, suggesting that their underlying circadian clocks are similarly sensitive to

temperature in terms of their phase-resetting ability. We show evidence for the circadian

clocks controlling activity/rest rhythm of *D. melanogaster*, *D. malerkotliana* and *D.

ananassae* being temperature compensated.

Materials and methods

Fly strains. Wild flies of *D. melanogaster*, *D. malerkotliana* and *D. ananassae* were caught

between 2004-2005 from Bangalore, India (12°58'N, 77°38'E). Each species was maintained

as random mating populations of ~1200 individuals under LD12:12 hr (~1.5 W/m^2)

conditions at constant temperature (~25 ºC) and humidity (~70%) on cornmeal medium.

Activity recording. Virgin male flies of age 2-3 days were placed individually into glass

tubes and their locomotor activity behaviour was recorded using *Drosophila* activity monitors

(DAM2, TriKinetics, Waltham, USA). All the experiments were done inside an incubator

(Sanyo, MIR-154, Japan) with programmable temperature and light conditions.

Analysis of activity. Activity was recorded in 5 min bins. Average actograms of each species

were obtained by 15 min binning of the raw time series data of individual flies. Light

intensity of ~0.28 W/m^2 was used for LD and LL experiments. For 28:25 ºC

thermophase:cryophase (TC) cycles, flies were initially exposed to 3 days of LD and then

shifted to TC (TC1) which was in phase with the LD cycles. Average activity profiles of

activity during TC 1 were obtained by first averaging across days for each fly and then averaging across flies. After visually estimating that the activity had synchronised to the TC cycles, separate sets of flies were then subjected to 6 hr delayed or 6 hr advanced TC cycles (TC2). In a separate assay, a higher amplitude TC of 29:21 ºC (TC1) was imposed, prior to which flies were exposed to 1 day of LL.

After visual estimation of synchronization of activity under TC1, separate sets of flies were exposed to either 6 hr delay, 10 hr delay or 6 hr advance (TC2). For quantification of activity under TC1 only the last 4 days data were taken to avoid transients. To quantify the amount of activity during morning and evening under TC1, the 24 hr day was divided into 4 hr intervals, and starting of thermophase was considered as ZT00. Total activity count in each 4 hr interval was calculated, and one-way ANOVA was carried out to test the null hypothesis that activity is equally distributed across the day. ANOVA revealed a main effect of time interval on activity counts for all species, and so this was followed by post-hoc analyses for each species using Tukey's HSD test to determine the time intervals during which activity differed from each other. We visually estimated the daily offset of activity for each fly under each TC and when the regression line across these time points was parallel to the y-axis in the actogram we considered that the activity rhythm has reached a steady state. The number of days that individual flies took to reach a steady phase of activity offset following a phase-shift in TC cycles was estimated as the number of transient cycles and this was averaged across flies. One-way ANOVA was carried out to evaluate statistically significant differences across species under each TC cycle. We estimated the periodicity of activity/rest rhythm of flies in each TC after the transients and if the periodicity was in the range of 24 ± 0.5 hr we considered the rhythm to be entrained to the TC cycle. Phase-relationship (ψ) between the TC cycle and the entrained rhythm was defined as the time from thermophase offset to the

activity offset (thermophase offset- activity offset). We calculated the ψ of *D. melanogaster* and *D. ananassae* under 29:21 ºC TC (TC1) and compared them using Student's *t*-test.

For experiments measuring the phase-shift effects of warm temperature pulses, *D. melanogaster* and *D. ananassae* flies were first entrained to LD cycles at a constant temperature of 21 ºC for 5 days and then transferred to DD with a constant temperature of 21 ºC. On the first day of DD, separate sets of flies were exposed to 12 hr of 29 ºC - one batch at Circadian Time 09 (CT09, CT00 being the phase of onset of activity) and another batch at CT19. For both time points, separate sets of handling controls, not exposed to any warm pulse but similarly handled, were also maintained. After the warm pulse, flies were kept under DD at 21 ºC. To calculate phase-shifts we avoided 3 to 4 days of transients after the temperature pulse and estimated offsets of the free-running rhythm. From these offset values we drew a regression line extrapolating back to the phase on the day of warm pulse. These phases, calculated for the control flies were averaged and the effective phase of experimental flies was calculated by subtracting the extrapolated phase of each experimental fly from the average phase of the respective controls. Phase-shifts of *D. melanogaster* and *D. ananassae* were compared using Student's *t*-test separately at CT09 and CT19.

To estimate free-running periodicities under different constant temperatures, raw time series data obtained under DD at each temperature were analyzed using Lomb-Scargle (LS) periodogram method in ClockLab (Actimetrics, Wilmette, IL), with $p = 0.05$ as a threshold for rhythmicity. Flies were reared under LD- 25 ºC (12: 12 hr) and exposed to respective constant temperatures for at least 10 days starting at the age of 2-3 days. To avoid transients, only data from the last 7 days were used for analysis. Periods were compared using 2-way ANOVA with species and temperature as factors followed by post-hoc analysis using Tukey's HSD test. To calculate Q_{10} values of each species, average periodicities of *D. melanogaster*,

D. malerkotliana, and *D. ananassae* under 19 and 29 ºC were used - Q_{10} = period at 29 ºC / period at 19 ºC. Total activity levels (± 95%CI) were obtained by averaging activity counts of individual flies across last 7 days under DD at each temperature and then averaging across flies. Activity counts were compared using 2-way ANOVA with species and temperature as factors. STATISTICA-7 (StatSoft Inc., USA) was used for all statistical tests with level of significance set to $p < 0.05$. Experimental protocols performed in this manuscript conform to international ethical standards (Portaluppi et al., 2010).

Results

Temperature cycles were able to entrain circadian clocks of D. malerkotliana and D. ananassae. To investigate whether TC cycles can entrain activity/rest pattern of *D. malerkotliana* and *D. ananassae* flies which were initially under LD (12:12 hr) were exposed to TC cycles of 28:25 ºC (TC1) (Fig. 1). Like *D. melanogaster*, *D. malerkotliana* and *D. ananassae* also showed activity pattern that was synchronized to this TC cycle. When TC cycles were phase delayed or advanced by 6 hr, these three species exhibited synchronization to the newly imposed cycles with few transient cycles (Fig. 1A, B). Since it is likely that the previously experienced LD cycle which was in-phase with the TC cycle may have been responsible for synchronisation to TC cycles, we conducted another experiment where flies were exposed to one day of LL prior to TC cycle. LL is known to render *D. melanogaster* flies arrhythmic both in terms of its activity/rest rhythm and the underlying molecular clock (Konopka et al., 1989), and our own studies suggested that this was true also for *D. ananassae* and *D. malerkotliana* (chapter 2). In this experiment a higher contrast of temperatures were provided – 29:21 ºC to examine whether a higher amplitude Zeitgeber (with a temperature difference of 8 ºC as compared to 3 ºC) may differentially modify the pattern of entrainment (Fig. 2). When the thermophase was delayed by 6 hr or10 hr or advanced by 6 hr (TC2), after

few transient cycles *D. melanogaster*, *D. malerkotliana* and *D. ananassae* showed

entrainment (Figs. 2, 3). Thus in all three species most flies (> 91 %) entrained to TC1 and

TC2 in both low and high amplitude TC experiments, as determined by the similarity of

period to 24 hr (see method). Furthermore, activity rhythm free-ran under constant

temperature from the phase of the previously imposed TC cycles, suggesting true entrainment

to TC and not masking (Figs. 1, 2A, 3). Under both low and high amplitude TC (28:25 ºC

and 29:21 ºC), *D. ananassae* exhibited day time (thermophase) preference for activity as we

have observed previously under LD cycles (Prabhakaran and Sheeba, 2012). In case of *D.

ananassae*, most activity occurred during the first 4 hr of the thermophase both in 28:25 ºC

(~64% of total activity) ($F_{5,162} = 25.04$, $p < 0.00001$) and 29:21 ºC cycles (~59% of total

activity) ($F_{5,180} = 52.97$, $p < 0.00001$), whereas *D. melanogaster* and *D. malerkotliana*

exhibited a normal morning (thermophase onset) peak and a prominent evening (around

cryophase onset) peak with greater activity in the last 4 hr of the thermophase (28:25 ºC , *D.

melanogaster* - $F_{5,186} = 36.01$, $p < 0.00001$; *D. malerkotliana* - $F_{5,168} = 13.63$, $p < 0.00001$;

29:21 ºC , *D. melanogaster* - $F_{5,186} = 39.6$, $p < 0.00001$; *D. malerkotliana* - $F_{5,156} = 25.9$, $p <

0.00001$; Fig. 4A, B). However, these three species did not differ statistically in the time

taken to entrain to advanced or delayed TC cycles - on average, they exhibited 5 to 7 transient

cycles except under 29:21 ºC- 6 hr delay where *D. ananassae* took fewer transient cycles to

re-entrain to the newly imposed TC cycle compared to *D. melanogaster* and *D. malerkotliana*

(Figs. 1, 2, 4C). *D. melanogaster* and *D. malerkotliana* showed lowest number of transient

cycles under 29:21 ºC- 6 hr advance, where as *D. ananassae* showed lowest number of

transient cycles under 29:21 ºC- 6 hr delay and there was no consistent trend in the number of

transient cycles based on amplitude of TC cycles (Fig. 4C). Interestingly, *D. ananassae*

showed pre-dawn (before thermophase onset) activity only under low amplitude 28:25 ºC TC

cycles as reflected by the activity counts 4 hr prior to thermophase (Figs. 4A, B). This is

likely to be due to the tendency of *D. ananassae* to exhibit reduced morning activity under low temperatures. *D. melanogaster* and *D. malerkotliana* showed anticipation to both morning and evening transitions under 28:25 ºC and 29:21 ºC cycles as evident from their high activity 4 hr prior to thermophase and cryophase (Figs. 1, 2, 3, 4A, B). Thus even under TC cycles *D. ananassae* is predominantly active post dawn (after thermophase onset) and is clearly different from both *D. melanogaster* and *D. malerkotliana* where most of the activity occurred around evening (around cryophase onset). Since *D. ananassae* differed from *D. melanogaster* and *D. malerkotliana* in the profile of activity (Fig. 2A, right panel) it was not meaningful to compare phases of morning (thermophase onset) or evening (cryophase onset) peaks across species, hence we used the offset of activity as a phase marker to compare the relative phases of *D. melanogaster* and *D. ananassae* with cryophase onset, and found that the activity offset for *D. ananassae* was significantly phase advanced (+4. 6 ± 0.15 hr) compared to that of *D. melanogaster* (-1.07 ± 0.03 hr) under 29:21 ºC TC cycles, thus indicating differences in the phase of entrainment to TC cycles between *D. ananassae* and *D. melanogaster*.

Both D. melanogaster and D. ananassae showed similar phase-shifts in response to temperature pulses. Since the phase of the offset of activity/rest rhythm in *D. ananassae* was significantly advanced compared to *D. melanogaster* (Fig. 1) and our previous study showed that the period of the rhythm was significantly shorter than that of *D. melanogaster* under DD 25 ºC (Prabhakaran and Sheeba, 2012), we asked whether the circadian clocks of these two species are differently sensitive to temperature. We examined the circadian period of *D. melanogaster*, *D. ananassae* and *D. malerkotliana* flies under a range of different ambient temperatures (19, 21, 23, 25, 27 and 29 ºC). As reported previously, periodicity of *D. ananassae* was shorter at 25 ºC compared to *D. melanogaster* ($F_{10,466} = 10.8$, $p < 0.00001$;

Figs. 5A, 7A). Except for two temperatures (21 ℃ and 27 ℃) all three species showed

similar periods under a given temperature (Figs. 5A, 7A). We calculated the Q_{10} values for

each species which are considered as indicators of the temperature compensating ability of

circadian clocks, using the periodicities at the two extreme temperatures 19 and 29 ℃, and

found that *D. melanogaster*, *D. malerkotliana* and *D. ananassae* have values close to 1 (Q_{10}

value *D. melanogaster* = 0.96, *D. malerkotliana* = 0.98, and *D. ananassae* = 0.96). Thus,

even though the circadian periods were different under moderately warm and cool

temperatures, in more extreme conditions, circadian clocks controlling activity rhythm of

these three species are temperature compensated. Furthermore, we found that the activity

levels of the three species were not affected by temperature (Fig. 5B). Consistent with

previous studies *D. ananassae* showed significantly lower activity counts compared to *D.

melanogaster* and *D. malerkotliana* under all temperatures tested ($F_{10,466}$ = 230.7, *p* <

0.00001; Fig. 5B).

Next we asked whether the ability of the circadian clocks controlling activity/rest

rhythm of *D. ananassae* to be phase-shifted by temperature is different from that of *D.

melanogaster*. Since previous studies on *D. melanogaster* have shown that maximum

advance and delay phase-shifts occur at CT09 and CT19 (Busza et al., 2007), we chose these

two phases for our studies. Flies were first entrained to LD cycles at 21 ℃ and then released

into DD at a constant temperature of 21 ℃. On the first day of DD, flies were subjected to

high temperature of 29 ℃ for 12 hr at CT09 or CT19. Flies were allowed to remain under

DD-21 ℃ for another10 days following which actograms of individual flies exhibiting free-

running rhythms were examined and phase-shifts were estimated (see methods). Temperature

pulse at CT09 caused phase delay in both *D. melanogaster* and *D. ananassae* (Figs. 6, 7B).

Phase advance occurred for *D. melanogaster* and *D. ananassae* for a temperature pulse of 29

ºC given at CT19 (Figs. 6, 7B). Delays and advances caused by temperature pulse for *D. melanogaster* and *D. ananassae* were similar, thus it appears that the circadian clocks of these two species respond similarly to temperature perturbations at CT09 and CT19, and is in agreement with the lack of significant differences in the number of transient cycles taken for re-entrainment to shifted temperature cycles.

Discussion

Previously we have shown that *Drosophila* species *malerkotliana* and *ananassae* exhibit daily rhythms in activity/rest pattern in presence of LD cycles and that the rhythm persists under DD with periodicities close to 24 hr (chapter 2). Our present study shows that circadian clocks of these two species can be entrained by another important Zeitgeber namely TC cycles. Similar to the behaviour under standard laboratory LD 12:12 hr at constant 25 ºC and under various photoperiods, *D. ananassae* was consistently more active during the early hours of the day even under TC cycles. This is interesting because, even though *D. melanogaster* and *D. malerkotliana* exhibits bimodal activity under TC cycles, predominantly, they are active in the evening. Unlike their LD behaviour, where under some photoperiods a small evening peak in activity is exhibited by *D. ananassae* (Prabhakaran and Sheeba, 2012), under TC cycles, no such peak was seen (Figs. 1-3). Although under TC cycles activity pattern of *D. ananassae* is different from *D. melanogaster* and *D. malerkotliana*, the fact that there are no significant difference among species in the number of transient cycles taken to re-synchronize to either delayed or advanced TC cycles (Fig. 4C), suggests that their circadian clocks are similarly sensitive to TC cycles. Alternatively, the speed of resetting to shifted TC cycles is better conserved than their phase with respect to TC cycles.

Previous studies have suggested that in *D. melanogaster* different subsets of circadian pacemaker neurons are involved in circadian entrainment to light and temperature (reviewed

in Peschel and Helfrich-Förster, 2011). One study proposed that a subset of these neurons called the small ventral lateral neurons (sLNv), also referred to as the morning or M-cells and a subset of the evening or E-cells are mostly light sensitive and less responsive to temperature while another subset of circadian neurons namely the lateral posterior neurons and dorsal neurons are the strongly temperature sensitive cells that regulate circadian entrainment to TC cycles (Miyasako et al., 2007). On the other hand, another report suggested that while E-cells are highly sensitive to TC cycles, M-cells can also detect and respond to TC cycles and that another yet unknown group of cells that they term the TS cells are involved in temperature entrainment (Busza et al., 2007). The latter study showed that fewer transient cycles are required to synchronize to phase-shifted TC cycles when the M-cells are absent and proposed that a strong M-cell oscillator prevents other oscillators from responding excessively to temperature changes in *D. melanogaster* (Busza et al., 2007). Our studies also show that when released to DD after TC cycles, the activity of *D. melanogaster* and *D. malerkotliana* followed predominantly from the evening bout of activity whereas in *D. ananassae* activity under DD followed from the morning bout (Figs. 1, 2). Taken together with the inferences from previous studies we propose that *D. ananassae* probably posses a strong M-oscillator regardless of the type of environmental cue. However, we cannot rule out presence of E-oscillator cells in *D. ananassae* because under certain photoperiods this species shows small bouts of evening activity (Prabhakaran and Sheeba, 2012). Compared with the M and E oscillator neuronal substrates that have been found in *D. melanogaster*, previous studies on *D. ananassae* did not detect any difference in the anatomical location of M-cells and E-cells (Hermann et al., 2013).

Previously it has been noted that under TC cycles with night temperature as low as 20 ºC both morning anticipation and morning peak of activity are suppressed and that morning activity is also suppressed under LD cycles at low temperatures (Busza et al., 2007; Miyasako

et al., 2007). This is probably because 25 ºC is the preferred temperature for *D. melanogaster* (Sayeed and Benzer, 1996) and night time temperature as low as 20 ºC represses their activity. If this assumption is correct, then we hypothesise that the reduction in activity seen in *D. ananassae* under cool nights of 21 ºC (where pre-dawn activity and morning anticipation is almost negligible) indicates that the cold temperature threshold for *D. ananassae* which causes it to reduces its activity is higher than that of *D. melanogaster*, and must be tested in future studies. The temperature preference profile of *D. ananassae* is likely to be across a higher range compared to *D. melanogaster*, whereas *D. malerkotliana* which is more closely related to *D. ananassae* than to *D. melanogaster* (van der Linde et al., 2010) appears to have a similar temperature preference as that of *D. melanogaster*. It is important to note however that in absence of cyclic temperature or light cues, total activity levels are not significantly altered by temperature even in *D. ananassae* (Fig. 5B). Thus it appears that flies inhibit activity under cool temperatures only under cyclic conditions when a warm phase is also available. Based on the phase-shifting effect of warm temperature pulses at two time points (CT09 and CT19) which was not different for the two species, clock sensitivity to temperature does not appear to differ in *D. melanogaster* and *D. ananassae* (Fig. 7B). The ability to maintain a constant period under extreme temperatures is an important property of circadian clocks, which expectedly and similar to *D. melanogaster*, appears to be conserved in both *D. ananassae* and *D. malerkotliana*. Nevertheless we note that at slightly cooler temperature of 21 ºC or slightly warmer temperature of 27 ºC, circadian period of *D. ananassae* appears to be undercompensated whereas in our hands both *D. melanogaster* and *D. malerkotliana* clocks appear to be over-compensated for small decrease in temperature.

A recent study has shown high degree of similarity in the anatomy of circadian neurons in *D. melanogaster* and *D. ananassae*, in terms of cell number and expression of an important circadian neuropeptide, Pigment Dispersing Factor (PDF) and some circadian

proteins such as VRILLE (VRI) and Par Domain Protein 1 (PDP1) (Hermann et al., 2013).

They also showed protein sequence identity of *D. melanogaster* and *D. ananassae* as high as

86.9% and 98.7% for circadian photoreceptor CRYPTOCHROME and neuropeptide Ion

Transport Peptide (ITP) respectively (Hermann et al., 2013). Therefore, it will be interesting

and informative to study species which show differences in rhythmic activity pattern and to

conduct comparative studies of the neuronal circuitry and underlying genetic basis of

circadian clocks of such species. Since the *D. ananassae* genome has been fully sequenced it

will be feasible to do these types of comparative studies in the future.
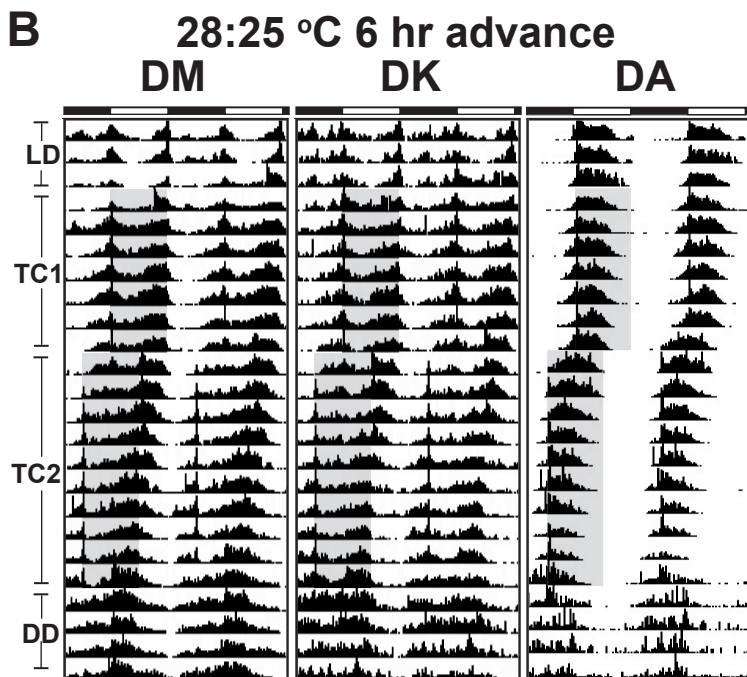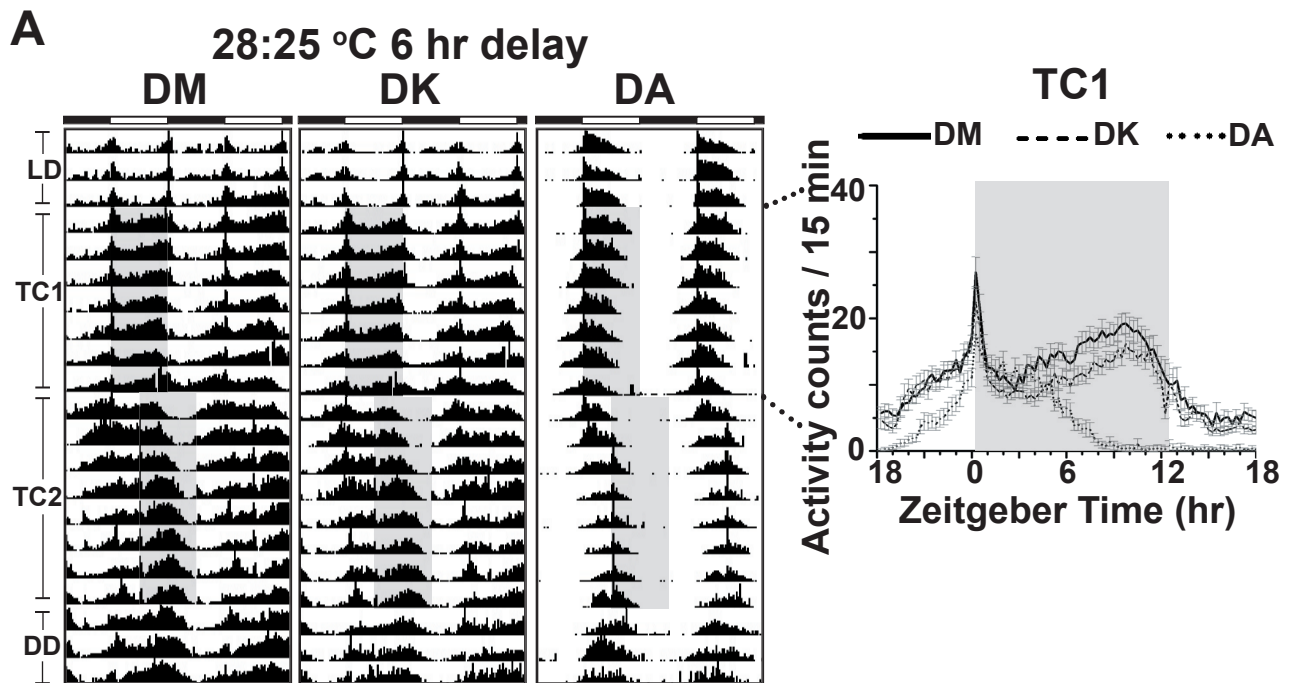
Figure 1. Temperature cycles of 28:25 ºC entrain circadian activity rhythm of *D. melanogaster*, *D. malerkotliana* and *D. ananassae*. (A) Average double plotted actograms of virgin male flies of *D. melanogaster* (DM, n = 25), *D. malerkotliana* (DK, n = 26) and *D. ananassae* (DA, n = 26). Flies were first exposed to LD at 25 ºC for 3 days followed by temperature cycles of 28:25 ºC in DD for 7 days (TC1). Temperature cycles were shifted (TC2) such that the thermophase was delayed by 6 hr compared to TC1 and then the flies were released into DD at constant temperature of 25 ºC. The x-axis represents time of the day from 0-48 hr, consecutive days are plotted along y-axis. Black and white bars above the actograms indicate the dark and light phases respectively under LD cycles. Average activity profiles of DM, DK and DA under TC1 alone are shown on the extreme right panel. Grey shaded areas in actograms and activity profiles represent thermophase of TC (28 ºC) (B) Average double plotted actograms of virgin male flies of DM (n = 23), DK (n = 25) and DA (n = 23). All the details are same as that of panel A, except that thermophase was 6 hr advanced under TC2.
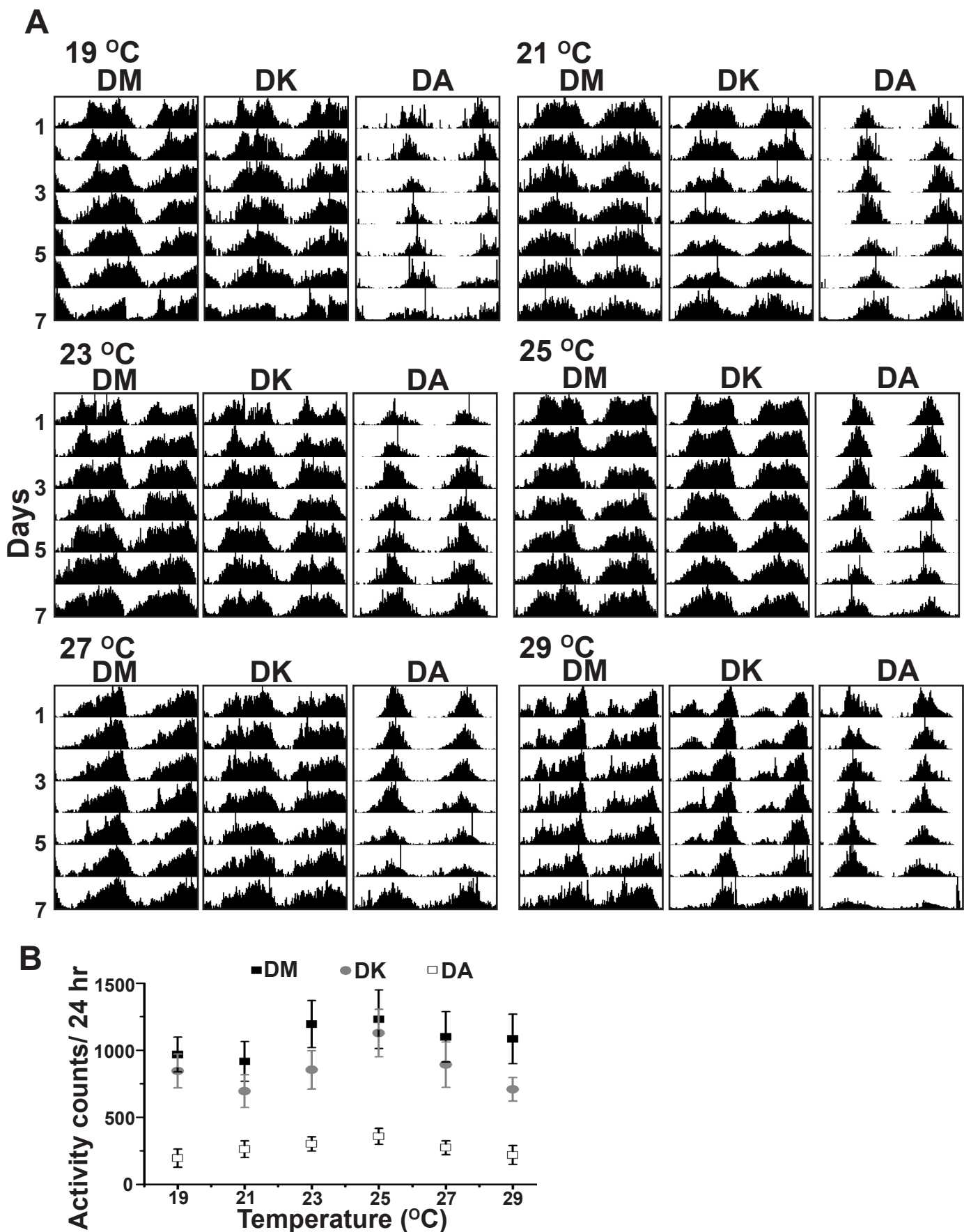
67
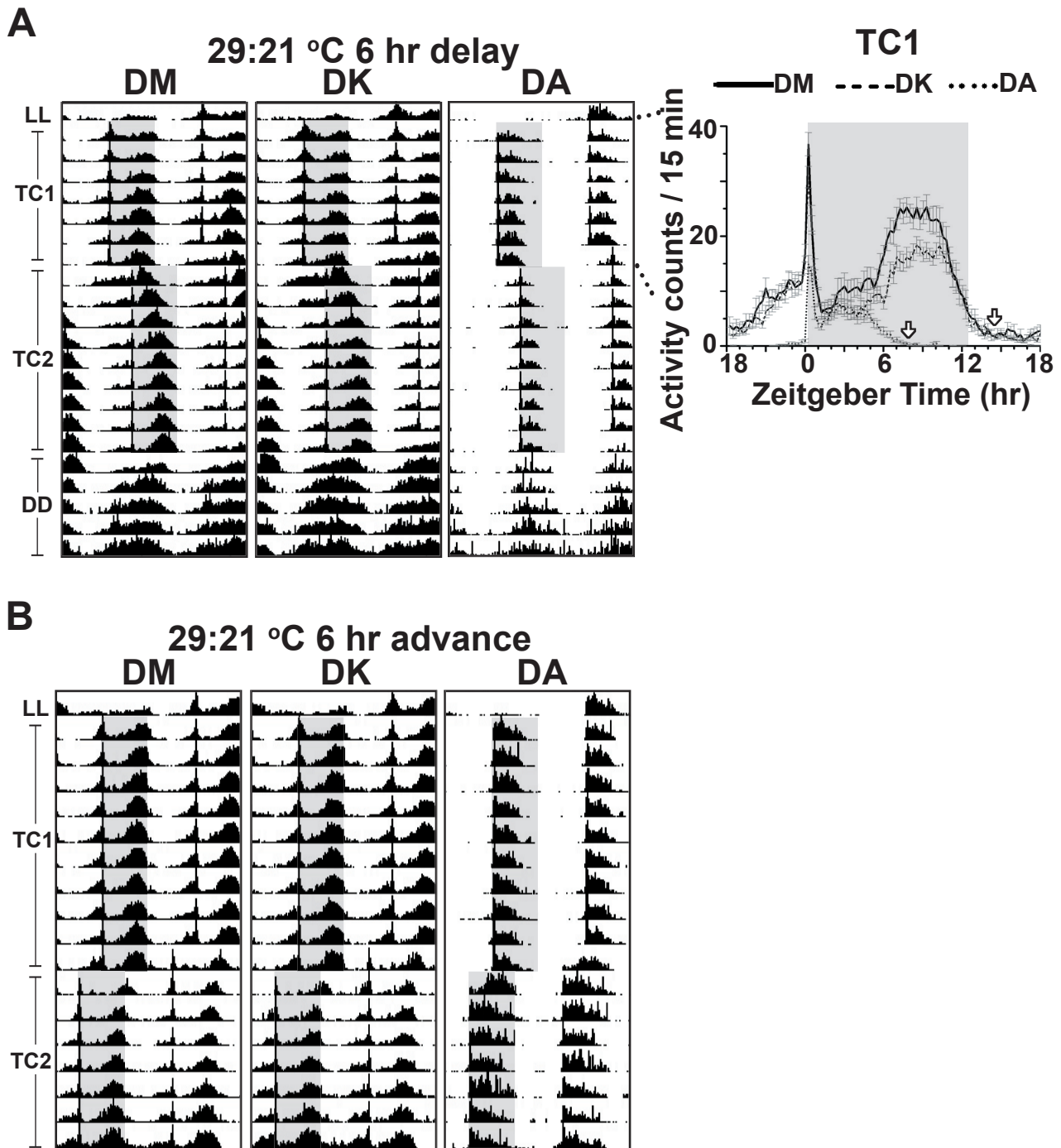
Figure 2. High amplitude temperature cycles of 29:21 ºC entrained activity rhythm of *D. mela-nogaster*, *D. malerkotliana* and *D. ananassae*. (**A**) Average double plotted actograms of virgin male flies of *D. melanogaster* (DM, n = 26), *D. malerkotliana* (DK, n = 25) and *D. ananassae* (DA, n = 24). Flies were first exposed to LL at 21 ºC for 1 day followed by temperature cycles of 29:21 ºC in DD (TC1). Then the flies were exposed to temperature cycles of 29:21 ºC (TC2) where the thermophase was delayed by 6 hr from TC1 and then the flies were released into DD at constant temperature of 21 ºC. The x-axis represents time of day from 0-48 hr, consecutive days are plotted along y-axis. Average activity profiles of DM, DK and DA under TC1 alone are shown on the extreme right panel. Unfilled arrows denote offset of activity. Grey shaded areas in actograms and activity profiles represent thermophase of TC (29 ºC). (**B**) Average double plotted actograms of virgin males of DM (n = 22), DK (n = 24) and DA (n = 23). All other details are same as that of panel A, except that thermophase was 6 hr advanced under TC2.

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29:21 ºC 10 hr delay
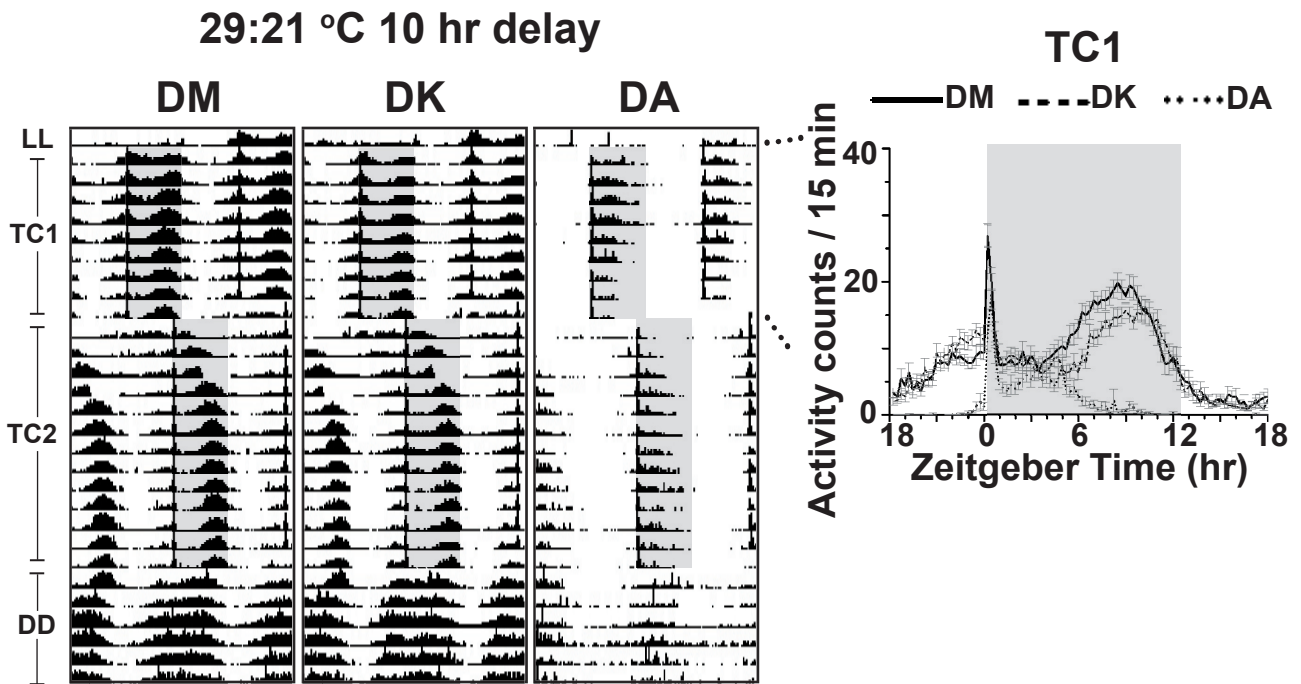
Figure 3. Temperature cycles of 29:21 ºC with 10 hr delay entrained activity rhythms of *D. mela-nogaster*, *D. malerkotliana* and *D. ananassae*. Average double plotted actograms of virgin male flies of *D. melanogaster* (DM), *D. malerkotliana* (DK) and *D. ananassae* (DA). Flies were first exposed to LL at 21 ºC for 1 day followed by temperature cycles of 29:21 ºC in DD for 9 days (TC1). Then the flies were exposed to temperature cycles of 29:21 ºC (TC2) where the thermophase was delayed by 10 hr and after 13 days flies were released into DD at constant temperature of 21 ºC. Average activity profiles of DM, DK and DA under TC1 alone are shown on the extreme right panel. The *x*-axis represents time of day from 0-48 hr, consecutive days are plotted along *y*-axis. Grey shaded areas represent thermophase of TC (29 ºC).
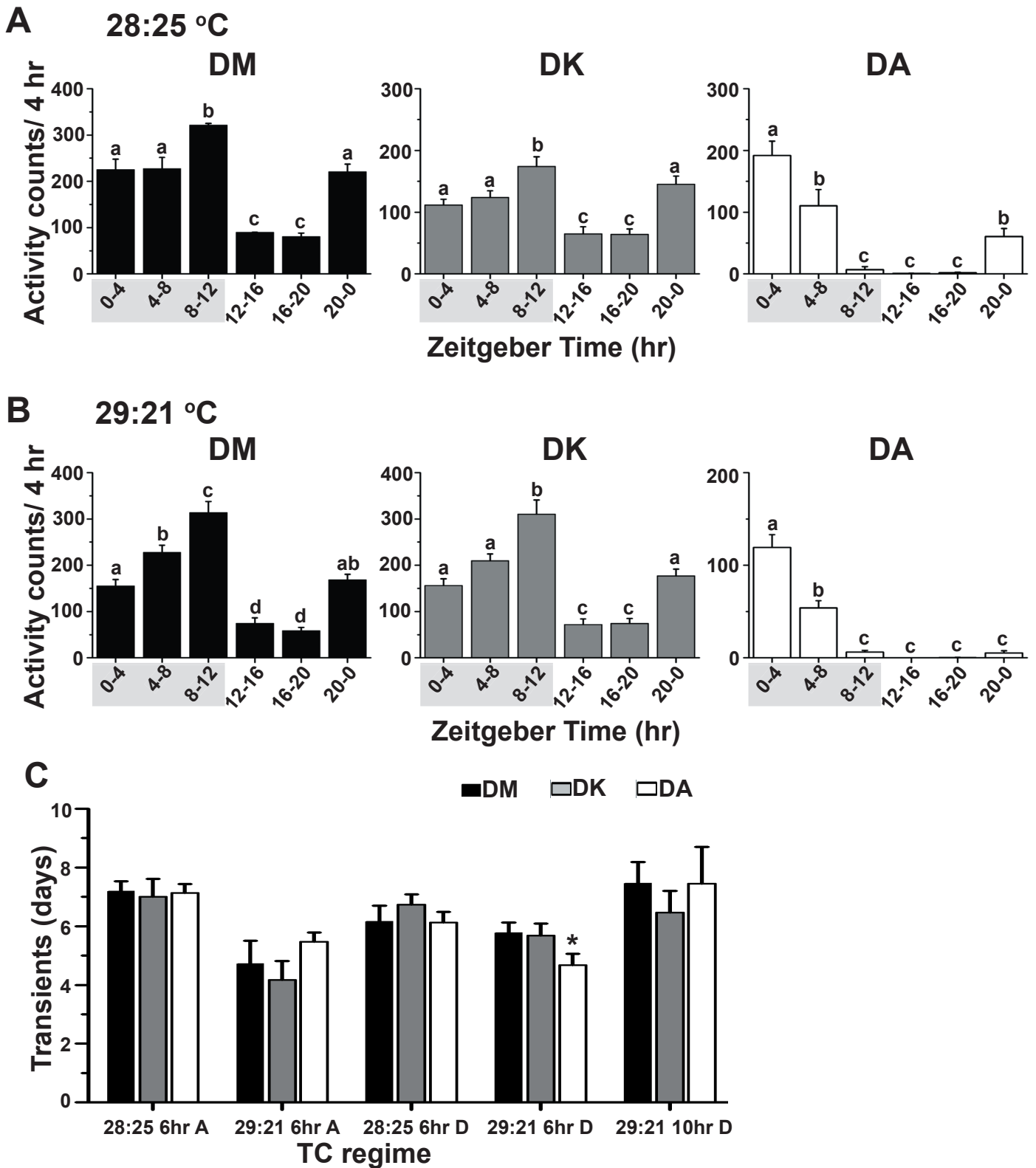
Figure 4. Predominant morning activity of *D. ananassae* under temperature cycles as opposed to evening activity of *D. melanogaster* and *D. malerkotliana*. (**A**) Quantification of mean activity counts (4 hr bin) (±95%CI) averaged for last 4 days under TC1 for *D. melanogaster* (DM), *D. malerkotliana* (DK) and *D. ananassae* (DA) under 28:25 ºC. Horizontal grey shading on x - axis label denotes thermophase under temperature cycles. (**B**) Quantification of mean activity counts (4 hr bin) (±95%CI) averaged for last 4 days under TC1 for DM, DK and DA under 29:21 ºC. Horizontal grey shading on x-axis label denotes thermophase. Different letters above bars indicate values that are significantly different from each other ($p < 0.05$). (**C**) Average number of transient cycles (±95%CI) under TC2 for advance (A) and delay (D) shifted TC regimes for each species. * indicates significant difference across species at $p < 0.05$.

Figure 5. Activity rest/rhythms of *D. melanogaster*, *D. malerkotliana* and *D. ananassae* under constant temperatures under DD. (A) Average double plotted actograms of virgin male flies of *D. melanogaster* (DM), *D. malerkotliana* (DK) and *D. ananassae* (DA). Flies were exposed to various constant temperatures and period was calculated from the last 7 days under each regime. The *x*-axis represents time of day from 0-48 hr, consecutive days are plotted along *y*-axis. **(B)** Total activity counts (±95%CI) of DM, DK and DA under various constant temperatures.
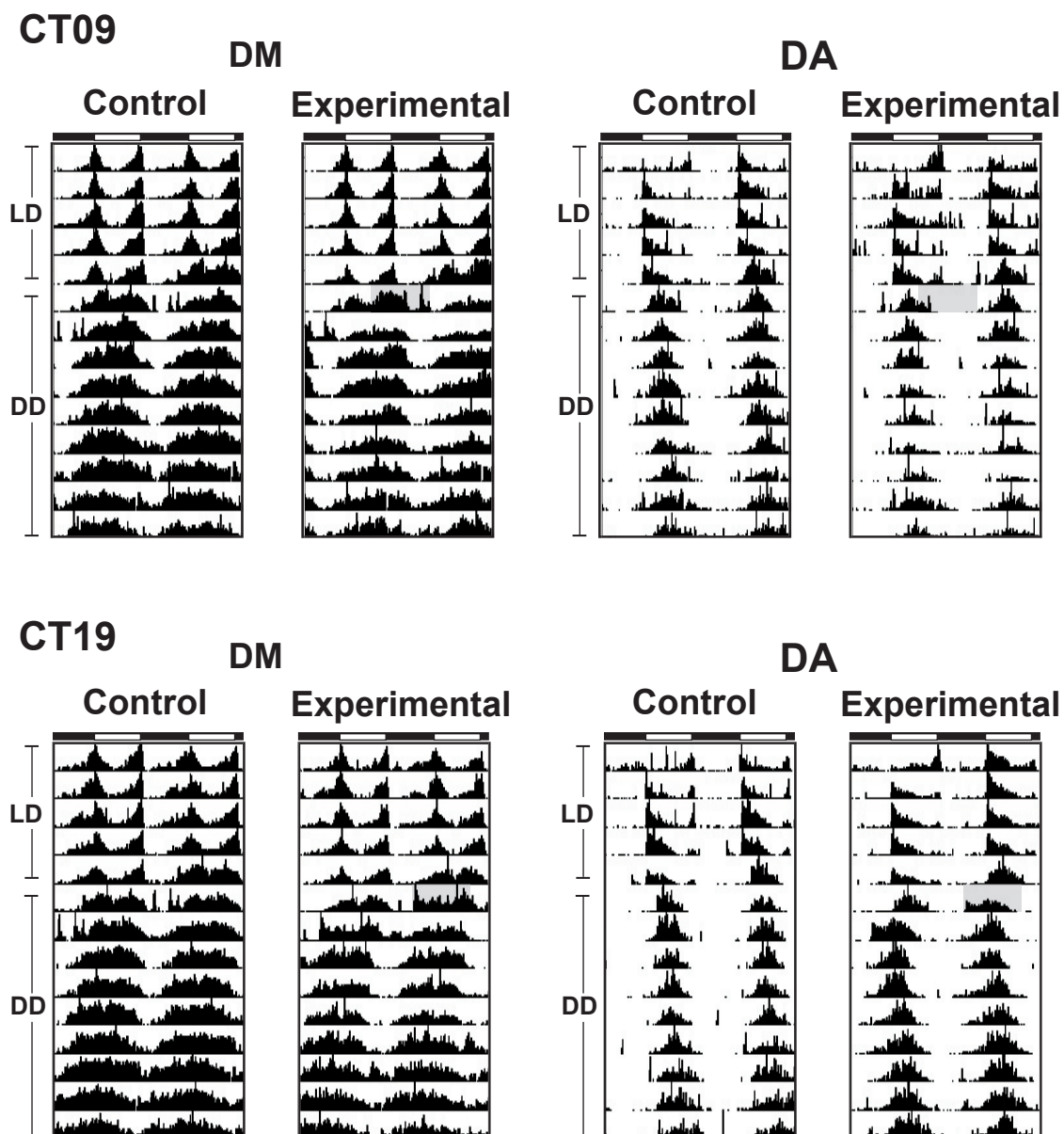
Figure 6. Effect of temperature pulses in activity/rest rhythm of *D. melanogaster* and *D. ananassae*. Average double plotted actograms of virgin male flies of *D. melanogaster* (DM) and *D. ananassae* (DA). Flies were first exposed to LD cycles at 21 °C and then released to DD at 21 °C. On the first day of DD a temperature pulse of 29 °C for 12 hr (grey shaded area) was given at CT09 or CT19. Black and white bars above the actograms indicate the dark and light phases respectively under LD cycles. The *x*-axis represents time of day from 0-48 hr, consecutive days are plotted along *y*-axis.
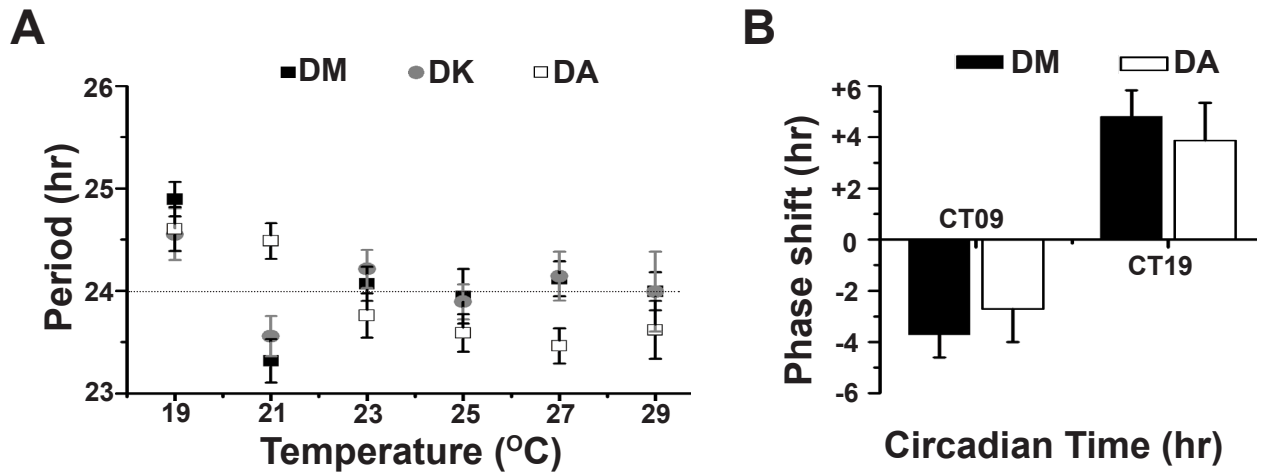
Figure 7. *D. melanogaster* **and** *D. ananassae* **showed similar phase-shift to temperature pulses.** (**A**) Circadian periodicities (±95%CI) of *D. melanogaster* (DM), *D. malerkotliana* (DK) and *D. ananassae* (DA) under DD and various constant temperatures (n ≥ 22). Flies were reared under LD- 25 °C and exposed to respective constant temperatures for at least 10 days, of which, only data from the last 7 days were used for analysis. Dotted horizontal line indicates periodicity of 24 hr. (**B**) Phase-shifts (±95%CI) exhibited by DM and DA to temperature perturbation at CT09 and CT19 (n ≥ 26). Flies were first exposed to LD cycles at 21 °C and then released to DD at 21 °C. On the first day of DD a temperature pulse of 29 °C for 12 hr was given at CT09 or CT19.

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Chapter 4

Temporal patterns of activity across four Drosophilid species under semi-natural conditions

Introduction

Circadian rhythms in *D. melanogaster* have mostly been studied under controlled laboratory conditions until recently (De et al., 2012; De et al., 2013; Menegazzi et al., 2012; Menegazzi et al., 2013; Vanin et al., 2012). Studies under semi-natural (SN) conditions revealed that many features of activity/rest rhythm differed from those seen under 'standard' laboratory conditions probably due to the influence of multiple environmental time-cues in nature (Vanin et al., 2012). While crepuscular activity patterns are seen in the laboratory, under SN conditions flies were reported to show a temperature-dependent third peak in the middle of the day termed as the afternoon peak or 'A-peak' (De et al., 2013; Menegazzi et al., 2012; Vanin et al., 2012). Furthermore, oscillation of circadian protein expression in circadian pacemaker neurons (Menegazzi et al., 2013) differed from the laboratory. When compared across seasons, the occurrence of the A-peak was proposed to be determined by daytime temperature in two studies (Menegazzi et al., 2013; Vanin et al., 2012). A separate study which examined another rhythmic behaviour - adult emergence revealed enhanced robustness under SN compared to the laboratory with no dependence on the canonical clock gene *period*, unlike the laboratory studies (De et al., 2012). These reports have collectively pointed towards the limitations of laboratory-based studies and have attempted to understand how rhythmic behaviours are modulated by natural environmental cycles. Yet, we have made little progress in this direction, due to the fact that there are only small differences in behavioural patterns across genotypes (regardless of whether a functional clock is present or not) in either the occurrence or the phasing of peaks.

Previous studies on activity of wild-type flies under SN or simulated natural conditions in the laboratory have used two strains obtained from the mixing of isofemale lines caught from the wild, WT_{ALA} from Alto Adige in Italy 46°N and *Hu* from Houten, Netherlands

52°N in 2004 (Vanin et al., 2012). Comparison of the standard laboratory strain *Canton-S* with *WT_ALA* and *Hu* showed that the three strains exhibit variations in how they entrain to long photoperiods especially when nature-like twilight conditions were provided (Rieger et al., 2012). Although a clear latitudinal cline was not detectable, the behaviour of the southern strain *WT_ALA* was partially explained by the fact that flies of this strain carried two alleles of the core clock gene *timeless (tim) - ls-tim* and *s-tim* unlike the Northern strains (Rieger et al., 2012). The authors concluded that there is a need to examine more wild-caught strains to understand the nature of adaptations to local climatic conditions. Although activity/rest behaviour of wild-type and circadian mutant strains of *D. melanogaster* have been studied recently under SN conditions (Menegazzi et al., 2012; Menegazzi et al., 2013; Vanin et al., 2012), thus far there have been no reports on species other than *D. melanogaster.*

D. ananassae is a sympatric species with *D. melanogaster*, which our previous studies under laboratory light/dark (LD) cycles have shown to have a distinct activity/rest profile from *D. melanogaster* (Prabhakaran and Sheeba, 2012). *D. ananassae* flies are predominantly day-active while *D. melanogaster* display the expected bimodal activity pattern and this difference in their activity pattern persists under varying photoperiods, suggesting that these two species have significant differences in their preference for timing of activity/rest behaviour. We reasoned that by comparing the behaviour of two species *D. melanogaster* and *D. ananassae*, assayed in parallel under SN conditions, across different seasons, we may discover features of rhythmic behaviours that are conserved *vis-a-vis* those that vary, across species and across seasons, thus revealing features of circadian clocks that are likely to be most hardwired or plastic and how different species cope with changing environmental conditions encountered in different seasons. Along with flies from a wild-caught population of *D. melanogaster* and *D. ananassae* we assayed under SN two other Drosophilid species, *D. malerkotliana* and *Zaprionus indianus*, in 12 assays spread over a

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period of 1.5-years. *D. malerkotliana* was first reported from Punjab, India and is distributed

throughout Southeast Asia (Kopp and Barmina, 2005). *D. malerkotliana* and *D. ananassae*

which belong to the same species group *ananassae* have not been systematically examined

with reference to behavioural phenotypes, but anecdotal evidence suggests that they exhibit

differences from *D. melanogaster* in their preference for feeding and mating sites (Sharmila

Bharathi et al., 2003). *Z. indianus* is believed to have originated in Africa and is currently

distributed throughout the tropical regions (da Conceição Galego and Carareto, 2010). We

found that while each species exhibits variation in their activity pattern across the year, *D.

ananassae* confined most of its activity to daytime and its activity was highest during the

afternoon window. These four species show interesting differences from one another that

may be due to a combination of differences in its sensitivity to ecological factors and the

differences in underlying cellular or molecular machinery controlling circadian behaviours.

Materials and methods

Fly strains. All the four species *D. ananassae*, *D. melanogaster*, *D. malerkotliana* and *Z.

indianus* were wild-caught within Bangalore, India (12°58'N, 77°38'E), using fruit-traps as

bait and net sweeps between 2004-2005 and maintained as large random mating populations

of ~1200 individuals (with roughly 1:1 sex ratio) to prevent random genetic drift and founder

effects from influencing behavioural phenotypes. A discrete-generation stock maintenance

cycle of 21 days on cornmeal medium under LD12:12 (~1.5-W/m^2) conditions at constant

temperature (~25 ºC) and humidity (~70%) was followed.

Activity recording. 2-3 day old, virgin males of each species, reared under laboratory

conditions of LD12:12 were used for the assays. Individual flies were placed into glass tubes

(5 mm diameter and 65 mm long) and locomotor movement along the length of the tube of

each fly was recorded using *Drosophila* activity monitors (DAM2, TriKinetics, Waltham,

USA). Monitors were then placed inside an iron enclosure ($122 \times 122 \times 122$ cm^3) with grids (6×6 cm^2) allowing free flow of air, and covered only on top with a sloping translucent plastic sheet (whose spectral characteristics are unknown). While this reduced the light intensity reaching the monitors, we expect that the nature of diffused sunlight which reached monitors from all four sides of the enclosure was not affected. The enclosure is situated within the JNCASR campus in Bangalore, below a dense canopy to avoid exposure to direct sunlight. Daily profiles of light, temperature, and relative humidity were also monitored simultaneously using an environmental monitor (DEnM, Trikinetics, USA). Humidity values recorded may not reflect values inside the glass tubes since they are sealed and contain fly food medium. Although at this latitude, photoperiod does not vary much throughout the year, seasons are marked by changes in temperature maxima (T_{max}) and minima (T_{min}) as well as variation in relative humidity (Table 1). The harshest conditions were marked by low humidity and high midday temperature (eg. April 2011) or low T_{min} (eg. January 2012), whereas during moderate seasons variation in day/night temperature and humidity was least (eg. August 2011).

Analysis of activity. Activity was recorded in 5 min bins. Activity profiles (mean ± SEM) were obtained by binning raw time series data of individual flies into 15 min intervals. In our studies fly-to-fly variation in activity levels was higher than day-to-day variation and the environmental variables measured did not vary much across days (Table 1). 15 min binned data was averaged across all flies for 6 days (Figs. 1, 3, 5, 9). Profiles of light, temperature and humidity were also obtained by 15 min binning. From the 15 min binned light profile, phase of the first bin showing values greater than 0 lux during the morning interval was considered as sunrise and the phase of the first bin showing 0 lux during the evening interval was considered as sunset. An interval of ±3 hr around sunrise was considered as the morning window (M-window). Similarly, the evening window (E-window) was defined as the interval

of 3 hr before and after sunset, and afternoon window (A-window) as the duration intervening morning and evening windows. Presence of peak in morning, afternoon and evening windows was qualitatively determined (if there was a gradual increase in activity leading to a peak and a gradual decline in activity from a peak) from 15 min binned average profiles across 6 days for each fly in each assay and the phase of the highest activity counts (peak) within each of the respective windows were taken as the phase of M, A and E peaks. To compare the distribution of activity during day, fraction of activity to the total activity during M, A and E windows were taken for each fly separately and averaged across 6 days and further averaged across all flies. Two-way ANOVA followed by post-hoc multiple comparisons using Tukey's HSD test was performed to evaluate statistically significant differences across assay and species separately for each window. Total activity levels ($\pm$95% CI) were plotted along with T_{min} and H_{min} by averaging activity counts of individual flies across 6 days and averaging across all flies. One-way ANOVA followed by post-hoc multiple comparisons using Tukey's HSD test was performed to evaluate statistically significant differences across assays. To compare the total activity during daytime, fraction of daytime activity to total activity counts of individual flies were averaged across 6 days and those were averaged across all flies ($\pm$95% CI). Two-way ANOVA followed by post-hoc multiple comparisons using Tukey's HSD test was performed to evaluate statistically significant differences across assays and species. Statistical analysis of the phase of peaks was done for only those assays where at least 20% of flies exhibited a peak. The phase of the morning (M) and evening (E) activity peaks was estimated by scanning activity profiles of individual flies and peak phase values thus obtained were averaged across flies to obtain the mean phases of the peaks ($\pm$95% CI) for each species in each assay. One-way ANOVA followed by post-hoc multiple comparisons using Tukey's HSD test were performed to evaluate differences across assays. Non-parametric Spearman's rank order correlation test

was applied on the following pairs of day-wise data points: fraction of daytime activity versus average day temperature, nighttime activity counts versus average nighttime humidity ($H_{avg\ night}$), nighttime activity counts versus average nighttime temperature ($T_{avg\ night}$), fraction of M-window activity versus T_{min}, fraction of A-window activity versus T_{max}, fraction of E-window activity versus T_{max}, fraction of E-window activity versus average day temperature, fraction of E-window activity versus H_{min}, fraction of E-window activity versus average day humidity, M-peak phase versus timing of sunrise; M-peak phase versus timing of temperature T_{min}; E-peak phase versus timing of sunset. Separate one-way ANOVA were performed to evaluate statistically significant differences between separate assays within a species for the M, A and E-peaks followed by post-hoc multiple comparisons using Tukey's HSD test. We separately analysed between-species differences in onset of activity during January-2012 using one-way ANOVA, followed by post-hoc multiple comparisons using Tukey's HSD test. Regression analysis was performed on day-wise proportion of flies showing A-peak with average daytime temperature, T_{max}, average daytime light intensity and light intensity maximum (L_{max}). All statistical tests were done using STATISTICA-7 (StatSoft Inc., USA) with level of significance set to $p < 0.05$.

Results

Persistence of daytime activity of D. ananassae under SN. When *D. ananassae* flies were subjected to SN conditions, we found that across assays *D. ananassae* confined most of its activity to the light phase (Figs. 1, 2). Among them, in some assays, *D. ananassae* appeared to show three peaks of activity corresponding to morning, afternoon and evening intervals (Fig. 1). The afternoon activity was the most consistent, detected in 10 out of 12 assays, and overall *D. ananassae* flies showed greater activity in the afternoon window (Figs. 1, 2, 11B

bottom left). *D. ananassae* almost always started activity after sunrise with negligible activity at night (Figs. 1, 2) and in one assay during January-2012 when the average nighttime temperature fell to 12.7 ^{o}C, *D. ananassae* showed delay in the onset of activity with respect to sunrise (Fig. 1). This delay in onset of morning activity was greater compared to two other species examined (phase with reference to sunrise *D. melanogaster* = -0.6, *D. malerkotliana* = -0.8 and *D. ananassae* = -0.95 hr; $F_{2,84}$ = 52.84, p = 0.0001, Figs. 1-7). Evening activity was low and a small evening bout of activity was detectable in about half the assays (Figs. 1, 2, 11B bottom right).

When we examined the behaviour of *D. melanogaster* in parallel with *D. ananassae*, in most assays only M and E-peaks were seen prominently and a distinct A-peak similar to that seen in previous studies (Menegazzi et al., 2013; Vanin et al., 2012) was rarely detected (Fig. 3). Whenever the A-peak was detected, it was of smaller amplitude and was highly variable in phase, among flies within a single assay. Table 2 shows the fraction of flies that exhibit A-peak based on the criteria applied by previous studies and described in the methods section. During April-2011 assay, when temperature rose to ~35°C with very high intensity midday light and humidity dropping to as low as 29.8%, making the environmental conditions relatively harsh (Table 1), a prominent A-peak was seen along with M and E-peaks (Figs. 3, 4; Table 2). Nevertheless, the A-peaks of *D. melanogaster* seen in our studies were of comparatively lower amplitude than that observed at more temperate latitudes (>45°N) reported by Vanin et al. (2012) and Menegazzi et al. (2012). When environmental conditions were relatively moderate with low light intensity and little variation in temperature and humidity across the day (eg. August 2011), low levels of uniformly distributed daytime activity were seen with very few flies showing A-peak (Figs. 3, 4; Table 1, 2). This suggests that possibly a combination of high light and temperature induces the A-peak. Furthermore,

as can be predicted from laboratory studies, flies tend to shift their activity into daytime under low ambient temperatures (Majercak et al., 1999), we find that when T_{min} dipped (eg. December-2011), *D. melanogaster* showed very little nighttime activity with a prominent E-peak and a relatively blunted M-peak (Figs. 3, 4; Table 1). During January-2012, where T_{min} dropped to 12.7 ^{o}C and humidity was low, we could not detect pre-dawn activity and M-peak was delayed with respect to sunrise similar to *D. ananassae* (Figs. 3, 4). Thus, *D. ananassae* that was previously shown to exhibit a temporal preference distinct from *D. melanogaster* under laboratory LD cycles continued to exhibit such divergence under a wide range of SN conditions ranging from the harsh cold dry days of January-2012 or warm dry days of April-2011 and March-2012 to mild conditions of August-2011 (Fig. 7).

D. malerkotliana flies, except for some subtle differences, showed similar activity/rest profiles as that of *D. melanogaster*, in all assays. In brief, *D. malerkotliana* exhibited clear A-peak only during April-2011 and June-2011 whereas in most other assays small bursts of activity were shown by a small fraction of flies (Figs. 5, 6). However, during December-2011, unlike *D. melanogaster*, *D. malerkotliana* exhibited startle responses corresponding to the two peaks in the light profile (Figs. 3- 6).

In addition to the three species discussed above, we also examined in parallel, the activity/rest rhythm of a more distantly related Drosophilid species (*Z. indianus*) that was also caught from the same area as the other three species. In the laboratory under LD12:12, *Z. indianus* exhibited very low activity levels, with low anticipation to both lights-ON and OFF and only ~40% flies were robustly rhythmic under constant darkness (DD) at 25 ºC (Fig. 8). Therefore, we asked if under more natural time-cues it may be possible to visualise activity rhythms in this species also, since rhythmic activity has otherwise been observed across a wide range of insects (reviewed in Helfrich-Förster et al., 1998). We found that across assays

under SN, activity levels of *Z. indianus* were low compared to the other three species but they showed three activity peaks in most assays (Figs. 1-6, 9, 10; Table 2). In June-2011, when environmental conditions were milder than that of April 2011, *Z. indianus* exhibited three distinct peaks unlike *D. melanogaster* and almost all of its activity occurred during the light phase (Figs. 3, 4, 9, 10), suggesting that *Z. indianus* may be more sensitive to high temperature or light intensity. Similar to *D. melanogaster*, *Z. indianus* also showed a delayed morning activity onset in January-2012 compared to other assays, but surprisingly exhibited a small but distinct midday activity peak coinciding with L_{max} (Figs. 9, 10). Yet in the assay during September-2012, *Z. indianus* exhibited a clear bimodal distribution of activity with distinct build-up of activity prior to dawn and dusk unlike other species (Figs. 9, 10).

Activity levels of the four species varied across seasons. In addition to the distribution pattern of activity being modulated by environmental factors, there was a significant difference in the total activity counts of *D. melanogaster* seen across assays ($F_{11, 317} = 6.83$, $p < 0.05$; Fig. 11A). Overall, we found that under moderate conditions, when H_{min} and T_{min} was relatively high, *D. melanogaster* showed significantly higher levels of activity compared to most other times of the year (Fig. 11A; Table 1). During harsh conditions when H_{min} reached as low as 23.3%, *D. melanogaster* exhibited lowest level of activity (Fig. 11A; Table 1). *D. malerkotliana* also showed a similar trend with a significant drop in activity levels on dry days although the differences were not as dramatic ($F_{11, 324} = 4.09$, $p < 0.05$; Fig. 11A). In sharp contrast to this variation in activity of *D. melanogaster*, *D. ananassae* flies exhibited significantly higher levels of activity during March-2012 ($F_{11, 325} = 6.29$, $p < 0.05$; Fig. 11A). On the other hand, even though *Z. indianus* showed seasonal variation in total activity counts ($F_{9, 252} = 7.99$, $p < 0.05$; Fig. 11A), they did not show any similarity in pattern with any of the other three species under study exhibiting highest activity during December-2011 and

September-2012 suggesting that some other environmental factors are likely to influence activity levels in this species. Overall, all the four species exhibited lower activity in January-2012, when the nighttime temperature was lowest (Table 1). Thus, while the two related species *D. melanogaster* and *D. malerkotliana* exhibited similarities in terms of total activity, another related species *D. ananassae* showed quite contrasting behaviours especially under the most extreme warm and dry conditions. These results strengthen our hypothesis that these two recently diverged sympatric species (*D. melanogaster* and *D. ananassae*) probably occupy different micro-habitats and are therefore differently affected by daily variations in temperature and light.

D. ananassae confined most of its activity to daytime across seasons. All the four species showed significant differences in their relative distribution of activity during day and night across assays. Daytime activity was higher in *D. ananassae* during all experiments (Fig. 11B, top left), and they showed little or no nighttime activity. During March-2012 and April 2011, when daytime temperatures were highest accompanied by low humidity, *D. ananassae* showed high fraction of daytime activity in contrast to *D. melanogaster* and *D. malerkotliana* (Fig. 11B, top left). During January-2012, when T_{min} was as low as 12.7 ^{o}C, *D. melanogaster* showed significantly higher daytime activity compared to most other time of the year suggesting that nighttime activity is suppressed at cooler temperatures (Fig. 11B top left; Table 1). *D. malerkotliana* also showed such high daytime activity during January-2012. *D. melanogaster* and *D. malerkotliana* showed a negative correlation of daytime activity with average daytime temperature (*D. melanogaster* - $r = -0.69$; *D. malerkotliana* - $r = -0.69$, $p < 0.05$), whereas *D. ananassae* showed no correlation, and *Z. indianus* showed a positive correlation (*Z. indianus* - $r = +0.64$, $p < 0.05$). Interestingly, *D. melanogaster* and *D. malerkotliana* exhibited high nocturnal activity during some assays when both average

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nighttime humidity and temperature was high (Figs. 3, 5; $H_{avg\ night}$ *D. melanogaster* - $r =$ +0.26; *D. malerkotliana* - $r =$ +0.27; $T_{avg\ night}$ *D. melanogaster* - $r =$ +0.26; *D. malerkotliana* - $r =$ +0.36, $p < 0.05$). While *D. ananassae* showed no such correlation, *Z. indianus* appeared to show nocturnal activity during low night temperatures (Fig. 9; $T_{avg\ night}$ *Z. indianus* - $r =$ -0.5 $p < 0.05$). Thus, the distribution of activity of *D. melanogaster* and *D. malerkotliana* was similar and their daytime activity levels were reduced as the average daytime temperature increased, whereas *D. ananassae* confined most of its activity to daytime, irrespective of the seasonal variation in temperature.

Higher midday activity in D. ananassae irrespective of environmental variations. In order to quantify the distribution pattern of activity among the species across assays we compared the fraction of activity in the three windows (M, A and E). Irrespective of the assay condition, *D. ananassae* exhibited highest activity during the A-window and lowest during E-window (Fig. 11B, bottom), once again confirming their preference for activity during midday. Compared to *D. melanogaster* and *D. malerkotliana*, *D. ananassae* showed significantly lower activity during M and E-windows (M-window $F_{2,\ 968} = 22.4$, E-window $F_{2,\ 968} = 884.4$, $p < 0.05$; Fig. 11B), and higher activity during the A-window ($F_{2,\ 968} = 2046$, $p < 0.05$; Fig. 11B, bottom left). For each window, two-way ANOVA for activity levels across assays and species, showed a significant interaction between species and season for the M-window ($F_{22,\ 968} = 11.9$), A-window ($F_{22,\ 968} = 14.5$) and E-window ($F_{22,\ 968} = 4.8$, $p < 0.05$). *D. ananassae* flies showed significantly different allocation of activity into each of these windows across seasons compared to the other two species which were similar to each other (Fig. 11B). Activity in the M-window was positively correlated with T_{min} for *D. melanogaster*, *D. malerkotliana* and *Z. indianus*, whereas such a correlation was not detected for *D. ananassae* (*D. melanogaster* - $r = 0.61$; *D. malerkotliana* - $r = +0.58$; *Z. indianus* - $r =$

+0.67; $p < 0.05$; Fig. 11B). High daytime temperatures was associated with low activity in *D. melanogaster* and *D. malerkotliana* –activity in A-window of *D. melanogaster* and *D. malerkotliana* showed negative correlation with T_{max} (*D. melanogaster* - $r = -0.73$; *D. malerkotliana* - $r = -0.65$, $p < 0.05$; Fig. 11B). The same did not show any correlation with T_{max} in case of *D. ananassae* and *Z. indianus*. Moreover, we could not detect any correlation between activity in E-window with any of the measured environmental variables for any of the species. Thus *D. ananassae* showed highest activity during middle of the day across all seasons and was unaffected by temperature variation, whereas *D. melanogaster* and *D. malerkotliana* exhibited reduced activity as the midday temperature increased.

Timing of M and E-peaks depends on environmental factors. While all the four species exhibited M-peaks they showed significant difference in phase across assays (Fig. 12A; Table 2; *D. melanogaster* - $F_{11, 304} = 42.6$, *D. malerkotliana* - $F_{11, 313} = 14.7$, *D. ananassae* - $F_{11, 290} = 39.5$ and *Z. indianus* - $F_{9, 221} = 18$, $p < 0.05$). *D. melanogaster* and *D. malerkotliana* timed their M-peak to coincide sunrise and showed a positive correlation with time of sunrise (*D. melanogaster* - $r = +0.77$; *D. malerkotliana* - $r = +0.73$, $p < 0.05$; Fig. 12A). However, during January-2012, M-peak occurred at a later phase compared to all other assays in all four species (Fig. 12A). This delay was probably due to lower nighttime and dawn temperatures (Table 1). Throughout the year, *D. ananassae* delayed their M-peak with respect to sunrise (Fig. 12A). Unlike *D. melanogaster* and *D. malerkotliana*, the phase of M-peak in *D. ananassae* and *Z. indianus* showed a negative correlation with T_{min} (*D. ananassae* - $r = -0.63$; *Z. indianus* - $r = -0.66$, $p < 0.05$; Fig. 12A). Phase of M-peak in *Z. indianus* was similar to that of *D. ananassae* in most cases (Fig. 12A). However, *Z. indianus* showed a phase-delayed M-peak during two assays (June 2011 and July-2012) wherein the peaks coincided the time when light intensity reached its maximum (Figs. 9, 10, 12A).

E-peak was displayed by *D. melanogaster*, *D. malerkotliana* and *Z. indianus* during all the assays, whereas *D. ananassae* sometimes showed small bouts of activity in the evening (Figs. 1, 12B; Table 2). The phase of E-peak showed a positive correlation with time of sunset in all the species (*D. ananassae* - $r = +0.95$; *D. melanogaster* - $r = +0.65$; *D. malerkotliana* - $r = +0.75$; *Z. indianus* - $r = +0.69$, $p < 0.05$; Fig. 12B). E-peak was exhibited by ~50% *D. ananassae* flies during August-2011, while less than 25% showed E-peak during August-2012, although the only difference in environmental factors was higher daytime light intensity. This suggests that when temperature is high and humidity levels are low *D. ananassae* restricts its activity to midday (Figs. 1, 12B). *D. ananassae* exhibited A-peak in ten out of twelve assays which was the most compared to the other three species except *D. malerkotliana* (10/12) (*D. melanogaster* = 7/12 and *Z. indianus* = 7/10) (Table 2).

Occurrence of A-peak is influenced by both light and temperature. A previous study has suggested that average daytime temperature elicits the A-peak in *D. melanogaster* and not light intensity (Vanin et al., 2012), and that circadian clocks partially influence the occurrence and amplitude of this peak (Menegazzi et al., 2012). However, another study showed that bright light in the afternoon is indispensible for the occurrence of the A-peak (De et al., 2013). We examined the association between the proportion of flies exhibiting A-peak and average daytime temperature and light intensity to determine if such a pattern exists in these four species. Regression analysis using day-wise proportion of flies exhibiting A-peak revealed that for all species there was a significant association with L_{max} and T_{max} (data not shown). Similar analysis with average daytime temperature showed a significant association for all species except *D. ananassae*, whereas the average daytime light was found to be associated with A-peak occurrence for all species except *Z. indianus* (Fig. 13). Thus our study which included more than ten assays in four species conducted at a more southern latitude than some

of the previous reports suggest that both light intensity and temperature influences the A-peak occurrence.

Discussion

Since most previous studies on circadian rhythms of Drosophilids including those that examined rhythmic behaviour under SN conditions, have focussed on *D. melanogaster*, we aimed to conduct a comparative study across species based on the rationale that it might reveal how pliable or conserved, features of the rhythm are, across species and seasons. Our studies were carried out on four species of Drosophilids that have been relatively recently (2004-2005) caught from the wild, in locations within a radius of 10 km and can be considered as sympatric, although, it is likely that they occupy different spatial niches or micro-habitats. Thus, all four species are expected to have evolved rhythmic behaviours in response to similar photoperiods, temperature and other climatic features. At this latitude, flies do not experience large variation in photoperiods across seasons, hence, light intensity, temperature and relative humidity levels are likely to be more crucial features of the environment that influence rhythmic behaviours. Across assays, the seasons varied from moderate to harsh, with harsh conditions implying combination of low humidity and warm midday temperatures (April-2011, March-2012) or low humidity and cool night temperatures (January-2012). Most other assay conditions were relatively mild (Table 1).

Two species, *D. melanogaster* and *D. malerkotliana* showed almost similar activity/rest pattern throughout the year (Figs. 3- 6). Although there are no studies thus far that reveal the extent of phylogenetic relationship or the approximate time of divergence between these two species, from our studies it is clear that these two species share similar circadian organization. This is particularly interesting because *D. melanogaster* (*melanogaster* subgroup) and *D. malerkotliana* (*ananassae* subgroup) belong to different

species subgroups, and phylogenetically *D. malerkotliana* is more closely related to *D. ananassae* than *D. melanogaster* (Crosby et al., 2007; Yang et al., 2012).

We show that *D. ananassae* is in fact a diurnally active species compared to *D. melanogaster*, which from our studies is found to be predominantly crepuscular (Figs. 1, 3, 11B) with clear temporal separation of activity (Fig. 7). This is in agreement with our previous studies where *D. melanogaster* and *D. ananassae* showed temporal separation of activity under a variety of photoperiods in the laboratory (Prabhakaran and Sheeba, 2012) confirming morning preference for activity in *D. ananassae*. An interesting contrast between ours and previous studies (Vanin et al., 2012) is that the afternoon component of activity contributed to less than 25% of the total activity, in almost all assays for *D. melanogaster* and all species except *D. ananassae* (Fig. 11B, bottom left). Another novel finding from our studies is the enhanced nocturnal activity exhibited by *D. melanogaster* and *D. malerkotliana* during some assays when both temperature and humidity levels were high. Although we cannot rule out the possibility that flies in activity tubes may not experience the same humidity levels as that recorded by the DEnM, we speculate that these flies probably find the combination of warm and humid nights conducive for activity.

In our studies *D. melanogaster* rarely exhibited a distinct A-peak (April 2011, July 2011) (Figs. 3, 4) and it was not as prominent as M and E-peaks. Since it is counter-intuitive to expect flies to exhibit locomotion during a time of day when they are most likely to face the risk of desiccation, we propose that this behaviour may be an artefact of the experimental protocol. A recent study by our group conducted in the same outdoor location on the *Canton-S* (CS) strain of *D. melanogaster* which is considered as 'wild-type' by convention also supports this view (De et al., 2013). Two other studies also suggest that the A-activity could be an escape response from harsh conditions (Menegazzi et al., 2012; Vanin et al., 2012). One difference between our studies and that of others, which is likely to cause a smaller A-

peak, is the diameter of the glass tubes used for assaying locomotor activity. We used a larger version of the recording apparatus (DAM7), which uses tubes of 5 mm inner diameter, while other studies (De et al., 2013) used tubes of 3 mm diameter which probably makes flies more sensitive to warm temperature. It is reasonable to assume that flies may prefer to be active during twilight, when the environmental conditions are favourable, and therefore, the bimodal activity of *D. melanogaster*, *D. malerkotliana* and *Z. indianus* may reflect courtship and foraging behaviours. On the other hand, *D. ananassae* appears to have evolved mechanisms that enable them to occupy the diurnal temporal niche. The studies reported here is the first attempt, to the best of our knowledge, which has compared circadian behaviours both across seasons and closely related species under SN conditions. They reveal that bimodality of activity is a robust characteristic feature of some species of Drosophilids and likely reflects evolved features of the underlying circadian clocks to adapt to local cyclic environmental factors. Nevertheless, the lack of robust bimodality and a clear diurnality in at least one species out of the four suggests the existence of circadian clocks with alternate type of organization which remains to be explored. Our study also reveals that even among the species that exhibit crepuscular behaviour under SN, there are differences in the environmental factors with which the activity peaks are associated. This suggests species-specific variation in Zeitgeber-dependence of circadian clocks.

Table 1A. Details of light profile (mean of 6 days ± SEM) across different assays.

| Assays	Time (hr)		Light (lux)	
	Sunrise	Sunset	Max	Day Average
April 2011	6.25 ± 0	18.75 ± 0	2512 ± 0	1301.6 ± 20.7
June 2011	6.00 ± 0	18.75 ± 0	506.0 ± 15.8	204.5 ± 12.6
July 2011	6.00 ± 0	18.75 ± 0	435.7 ± 14.8	189.4 ± 5
August 2011	6.25 ± 0	18.75 ± 0	157.3 ± 19.3	86.6 ± 4.7
November 2011	6.50 ± 0.6	18.50 ± 0	259.2 ± 4.8	92.4 ± 4.7
December 2011	6.75 ± 0	18.00 ± 0	777.5 ± 58.1	179.2 ± 11.2
January 2012	7.00 ± 0	18.00 ± 0	152.0 ± 5.1	90.5 ± 0.8
February 2012	7.00 ± 0	18.25 ± 0	270.8 ± 8	131.1 ± 1.6
March 2012	6.50 ± 0	18.75 ± 0	242 ± 8	150.1 ± 2.8
July 2012	6.00 ± 0	18.75 ± 0	1035 ± 140	527.3 ± 100.1
August 2012	6.25 ± 0	18.50 ± 0	1410 ± 56.1	618.2 ± 32.4
September 2012	6.25 ± 0	18.25 ± 0	904.8 ± 72.9	498.2 ± 27.5

Table 1B. Details of temperature and humidity profile (mean of 6 days ± SEM) across different assays.

Assays	Temperature (oC)			Humidity (%)	
	Min	Max	Day average	Min	Max
April 2011	22.6 ± 0.4	35.4 ± 0.2	30 ± 0.1	29.8 ± 1.2	95.3 ± 0
June 2011	21.4 ± 0.2	29.1 ± 0.2	26.0 ± 0.1	49.7 ± 1.7	85.7 ± 0.5
July 2011	21.5 ± 0.2	28.2 ± 0.5	25.4 ± 0.4	59.2 ± 2.3	91.5 ± 02.7
August 2011	21.2 ± 0.1	26.1 ± 0.4	24.2 ± 0.2	70.5 ± 1.3	90.3 ± 01.9
November 2011	21.0 ± 0.3	26.5 ± 0.3	24.1 ± 0.3	71.3 ± 5.8	98.0 ± 0.3
December 2011	17.5 ± 0.6	27.3 ± 0.5	23.6 ± 0.3	50.2 ± 2.9	94.3 ± 1.1
January 2012	12.7 ± 0.6	26.9 ± 0.4	22.6 ± 0.5	30.3 ± 3	87.8 ± 2.2
February 2012	19.4 ± 0.4	29.7 ± 0.3	26.1 ± 0.2	36.5 ± 2.3	84.5 ± 02.7
March 2012	21.5 ± 1	33.6 ± 0.1	29.4 ± 0.2	23.3 ± 1.9	69.7 ± 5.8
July 2012	21.3 ± 0.2	28.0 ± 0.8	25.1 ± 0.7	63.2 ± 5.7	90.5 ± 2.3
August 2012	20.8 ± 0.2	26.3 ± 0.6	23.9 ± 0.3	70.3 ± 2.4	88.8 ± 1.4
September 2012	21.3 ± 0.3	28.0 ± 0.2	25.1 ± 0.2	63.5 ± 1.5	91.0 ± 1.5

Table 2. Number of flies showing morning, afternoon and evening peaks. n = total number of flies analyzed.

Species	Assay	Morning (%)	Afternoon (%)	Evening (%)	n
DA	April 2011	25 (89.3)	28 (100)	11 (39.3)	28
	June 2011	30 (100)	30 (100)	3 (10)	30
	July 2011	20 (71.4)	28 (100)	14 (50)	28
	August 2011	30 (100)	1 (3.33)	17 (56.7)	30
	November 2011	20 (100)	0	0	20
	December 2011	24 (85.7)	28 (100)	14 (50)	28
	January 2012	11 (52.4)	21 (100)	20 (95.2)	21
	February 2012	26 (100)	20 (76.9)	19 (73.1)	26
	March 2012	23 (100)	19 (82.6)	21 (91.3)	23
	July 2012	24 (100)	6 (25)	16 (66.7)	24
	August 2012	14 (48.3)	29 (100)	7 (24.1)	29
	September 2012	22 (100)	13 (59.1)	9 (40.9)	22
DM	April 2011	28 (100)	28(100)	28(100)	28
	June 2011	28 (96.5)	10 (34.5)	29 (100)	29
	July 2011	29 (93.6)	21 (67.7)	31 (100)	31
	August 2011	29 (100)	13 (44.8)	29 (100)	29
	November 2011	15 (100)	7 (46.7)	15 (100)	15
	December 2011	30 (93.8)	2 (6.3)	32 (100)	32
	January 2012	22 (68.8)	3 (9.4)	32 (100)	32
	February 2012	30 (96.8)	2 (6.5)	31 (100)	31
	March 2012	25 (100)	0	25 (100)	25
	July 2012	27 (100)	0	27 (100)	27
	August 2012	18 (100)	7 (38.9)	17 (94.4)	18
	September 2012	27 (100)	12 (44.4)	27 (100)	27
DK	April 2011	25 (100)	20 (80)	25 (100)	25
	June 2011	30 (96.8)	30 (96.8)	31 (100)	31
	July 2011	27 (96.4)	13 (46.4)	28 (100)	28
	August 2011	28 (100)	6 (21.4)	28 (100)	28
	November 2011	16 (100)	3 (18.8)	16 (100)	16
	December 2011	27 (96.4)	6 (21.4)	28 (100)	28
	January 2012	21 (75)	7 (25)	28 (100)	28
	February 2012	30 (93.8)	5 (15.6)	32 (100)	32
	March 2012	29 (100)	10 (34.5)	29 (100)	29
	July 2012	30 (100)	7 (23.3)	30 (100)	30
	August 2012	26 (100)	11 (42.3)	26 (100)	26
	September 2012	28 (96.6)	12 (41.4)	29 (100)	29
ZI	June 2011	32 (100)	32 (100)	28 (87.5)	32
	July 2011	21 (100)	10 (47.6)	20 (95.2)	21
	August 2011	24 (92.3)	1 (3.8)	26 (100)	26
	November 2011	20 (100)	6 (30)	19 (95)	20
	December 2011	19 (79.2)	2 (8.3)	24 (100)	24
	January 2012	22 (88)	21 (68)	25 (100)	25
	February 2012	18 (85.7)	17 (81)	21 (100)	21
	March 2012	26 (100)	24 (92.3)	26 (100)	26
	July 2012	23 (100)	15 (62.2)	20 (87)	23
	September 2012	26 (100)	0	26 (100)	26

D. ananassae
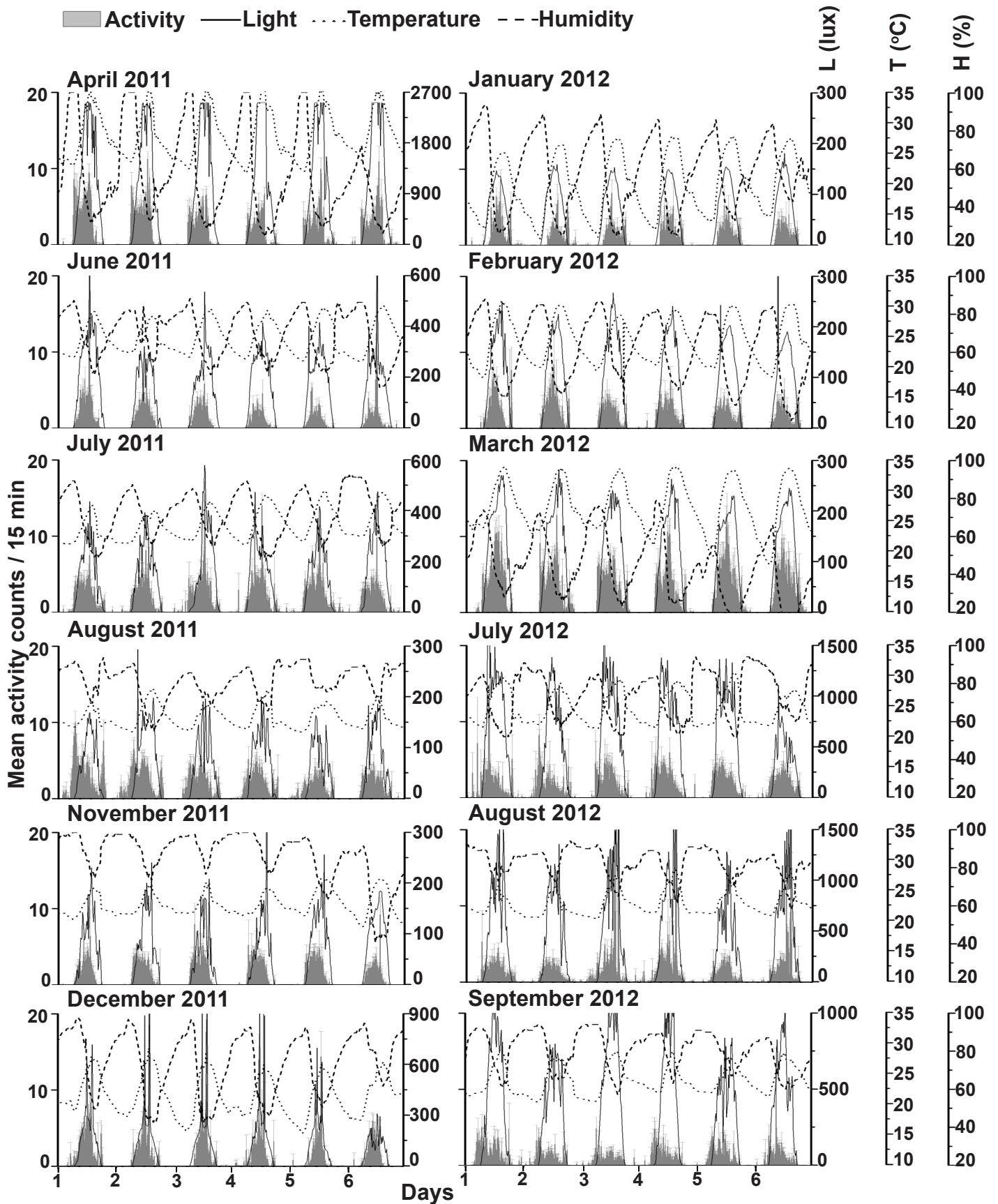
Figure 1. *D. ananassae* restricted most of its activity to the light phase across different seasons- day wise pattern. Average activity/rest profiles of virgin male *D. ananassae* flies across different assays in semi-natural condition. Mean activity counts, in 15 min bins (± SEM) averaged across all flies is plotted along with environmental factors light (L-solid curve), temperature (T -dotted curve) and humidity (H- dashed curve).
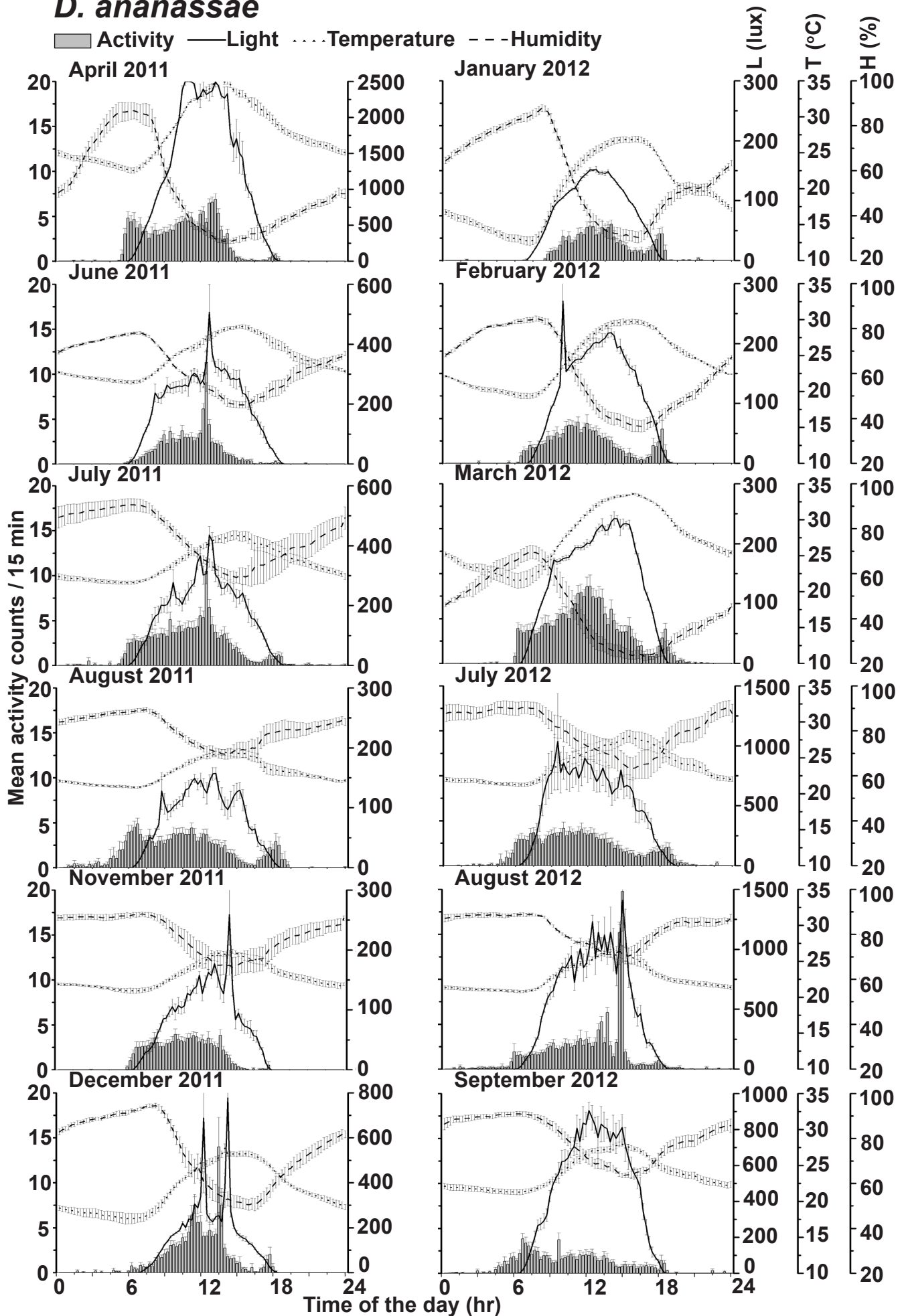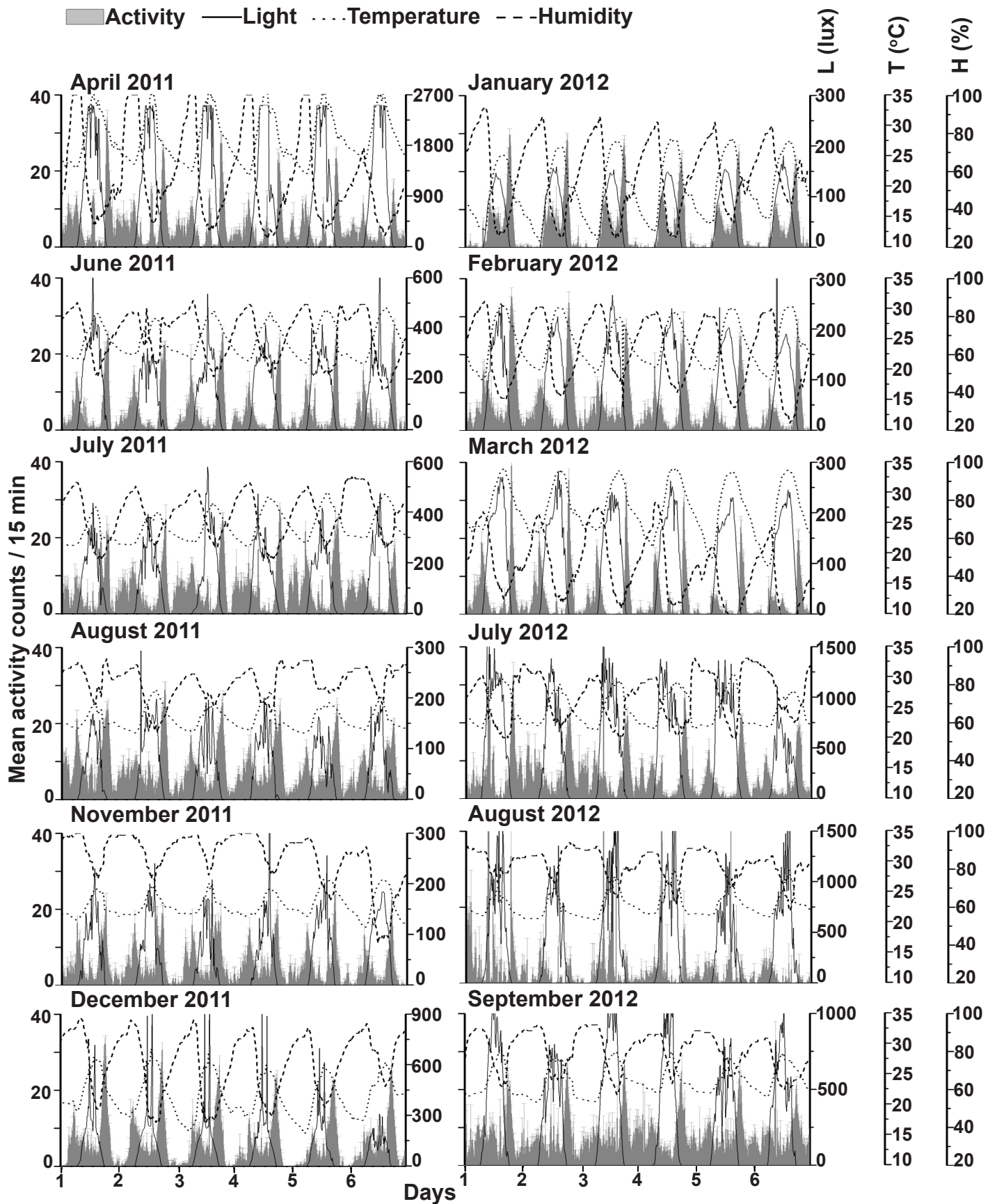
Figure 2. *D. ananassae* restricted most of its activity during light phase across different seasons. Average activity/rest profiles of virgin male flies *D. ananassae* across different assays in semi-natural condition. Mean activity counts, in 15 min bins (±SEM) averaged across flies over 6 days is plotted along with environmental factors light (L-solid curve), temperature (T-dotted curve) and humidity (H-dashed curve) whose values were averaged across 6 days.

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D. melanogaster

Activity —Light ⋯Temperature – – Humidity

Figure 3. Activity/rest pattern of *D. melanogaster* varied across different seasons- day wise pattern. Average activity/rest profiles of virgin male *D. melanogaster* flies across different assays in semi-natural condition. All other details are same as Fig. 1.
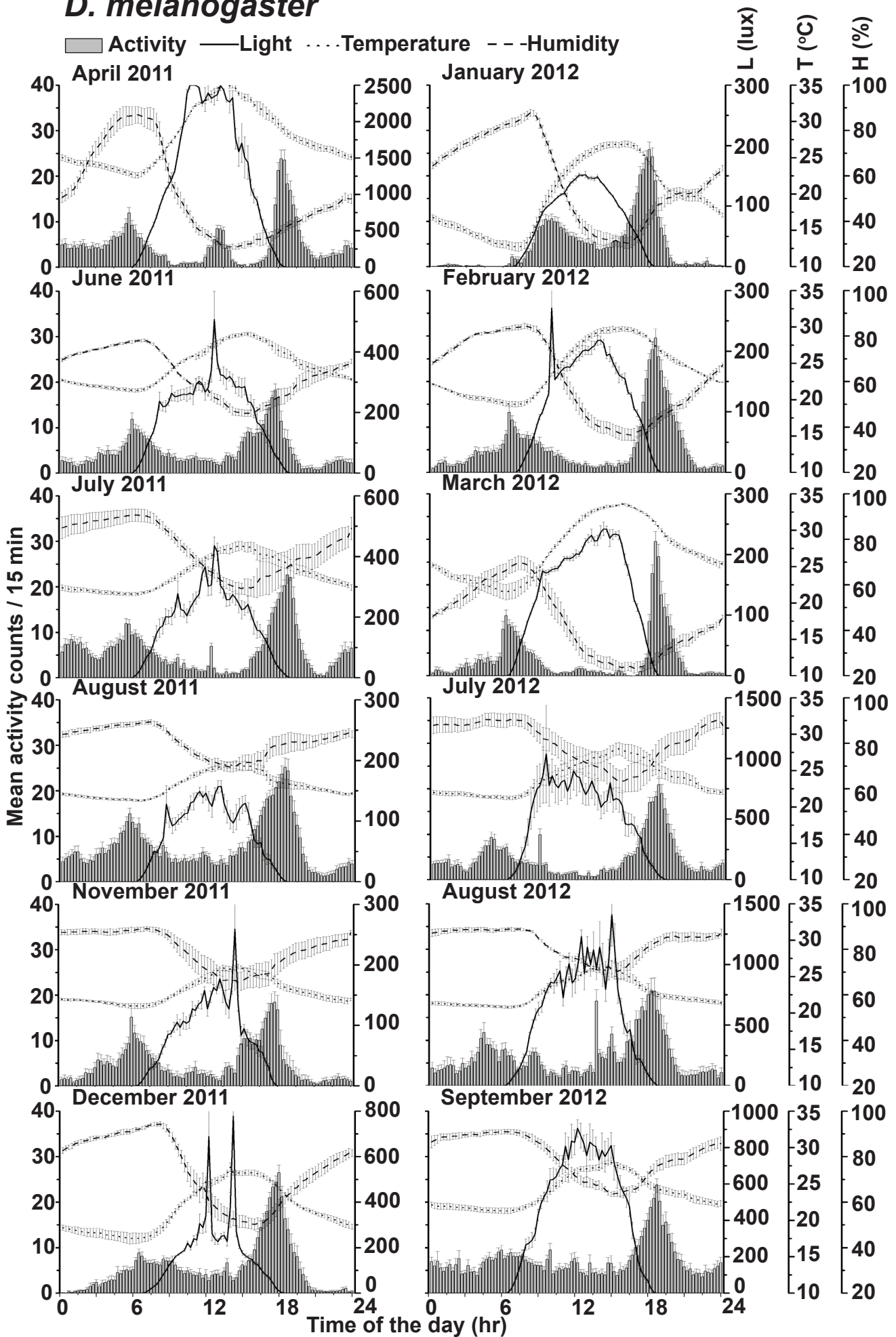
95

Figure 4. Activity/rest pattern of *D. melanogaster* varied with varying environmental factors across different seasons. Average activity/rest profiles of virgin male flies *D.melanogaster* across different assays in semi-natural condition. All other details are the same as Fig. 2.

D. malerkotliana
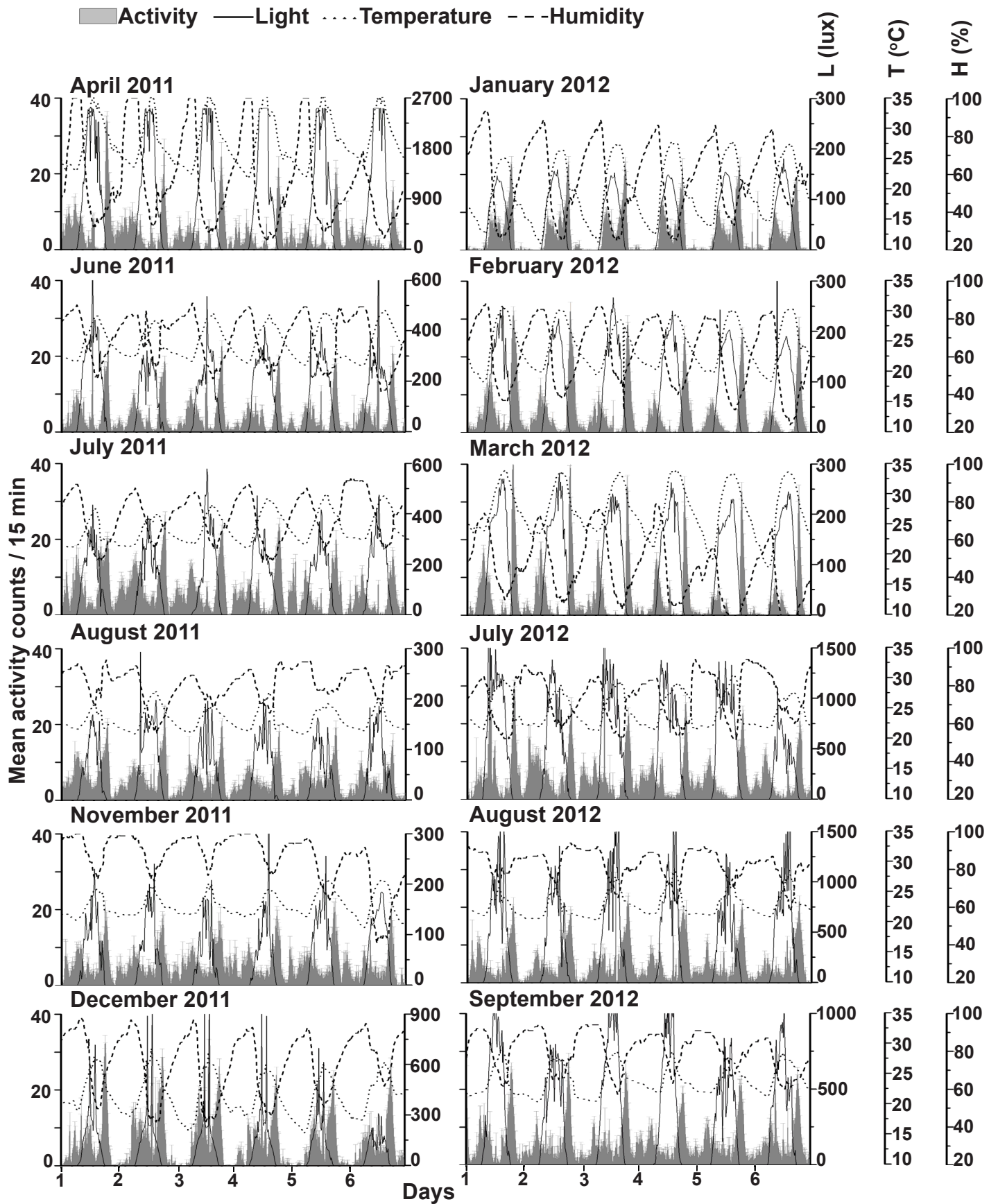
Activity ——Light ····Temperature – – ‑Humidity

Figure 5. *D. malerkotliana* exhibited almost similar activity/rest pattern as that of *D. melanogaster* across different seasons- day wise pattern. Average activity/rest profiles of virgin male *D. malerkotliana* flies across different assays in semi-natural condition. All other details are same as Fig. 1.
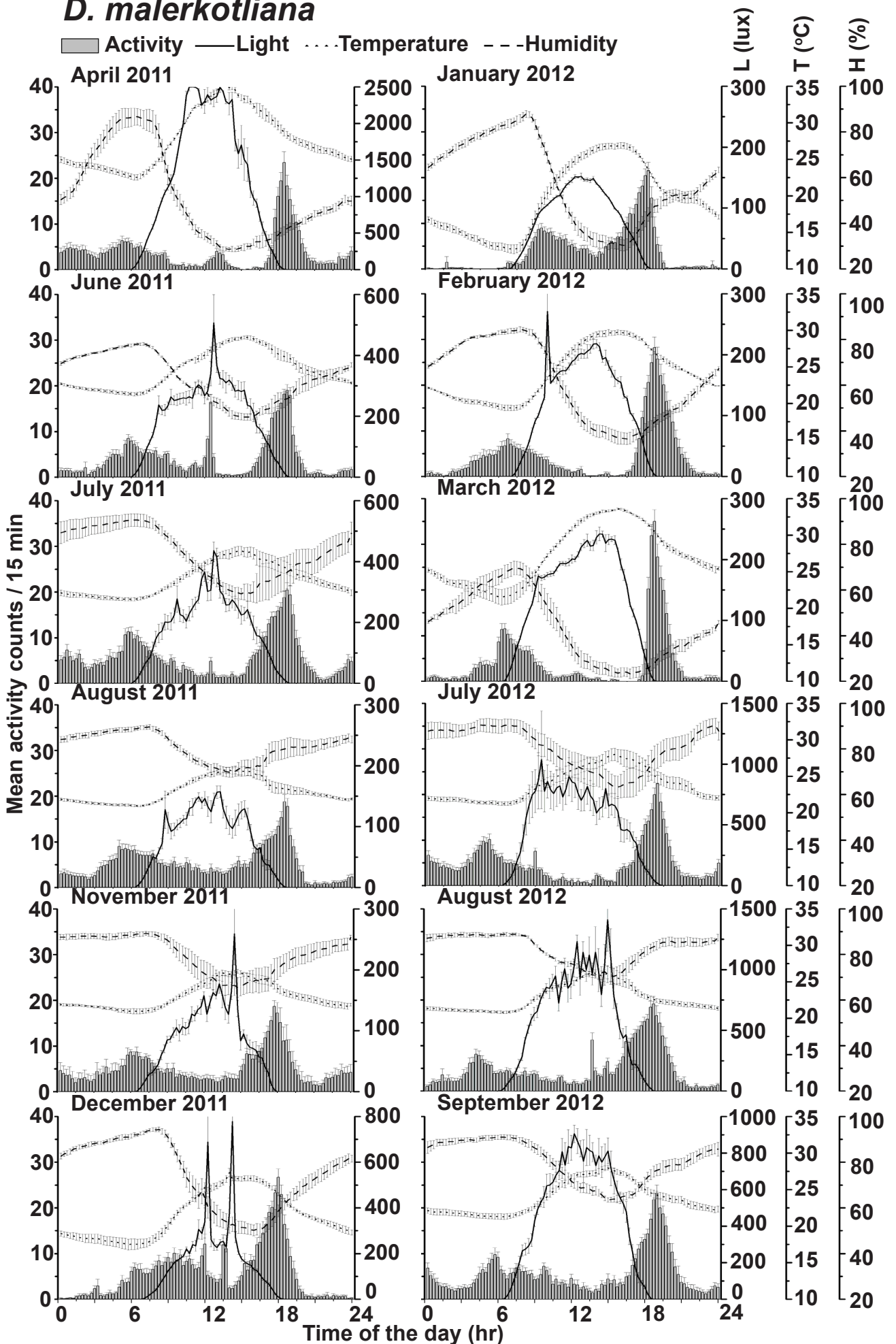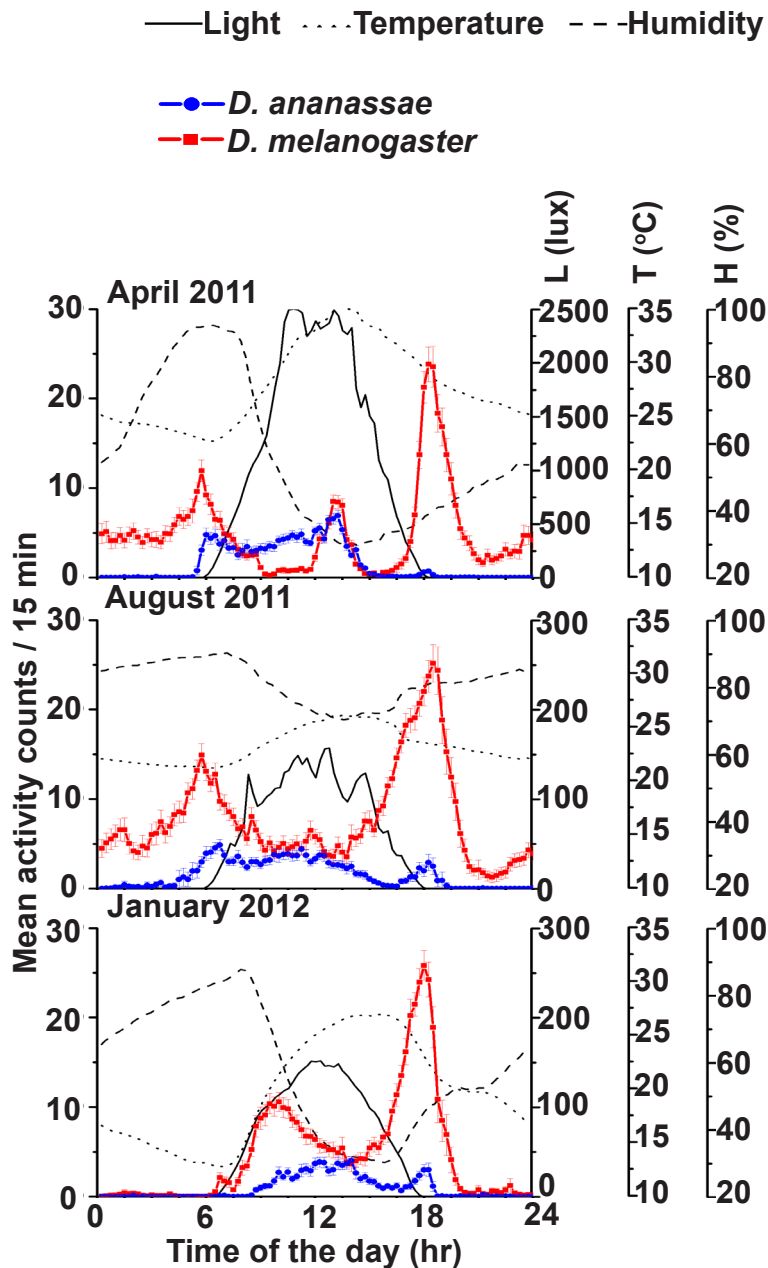
97

D. malerkotliana

Figure 6. D. malerkotliana exhibited almost similar activity/rest pattern as that of D. melanogaster across different seasons. Average activity/rest profiles of virgin male flies *D.malerkotliana* across different assays in semi-natural condition. All other details are the same as Fig. 2.

Figure 7. Divergence in activity/ rest pattern between *D. melanogaster* and *D. ananassae*. Average activity/rest profiles of virgin male flies *D. ananassae* (blue) and *D. melanogaster* (red) under warm dry days of April 2011 or cold dry days of January 2012 to the mild and least varying August 2011. Mean activity counts, in 15 min bins (±SEM) averaged across 6 days is plotted along with environmental factors light (L-solid curve), temperature (T-dotted curve) and humidity (H-dashed curve) whose values were averaged across 6 days.
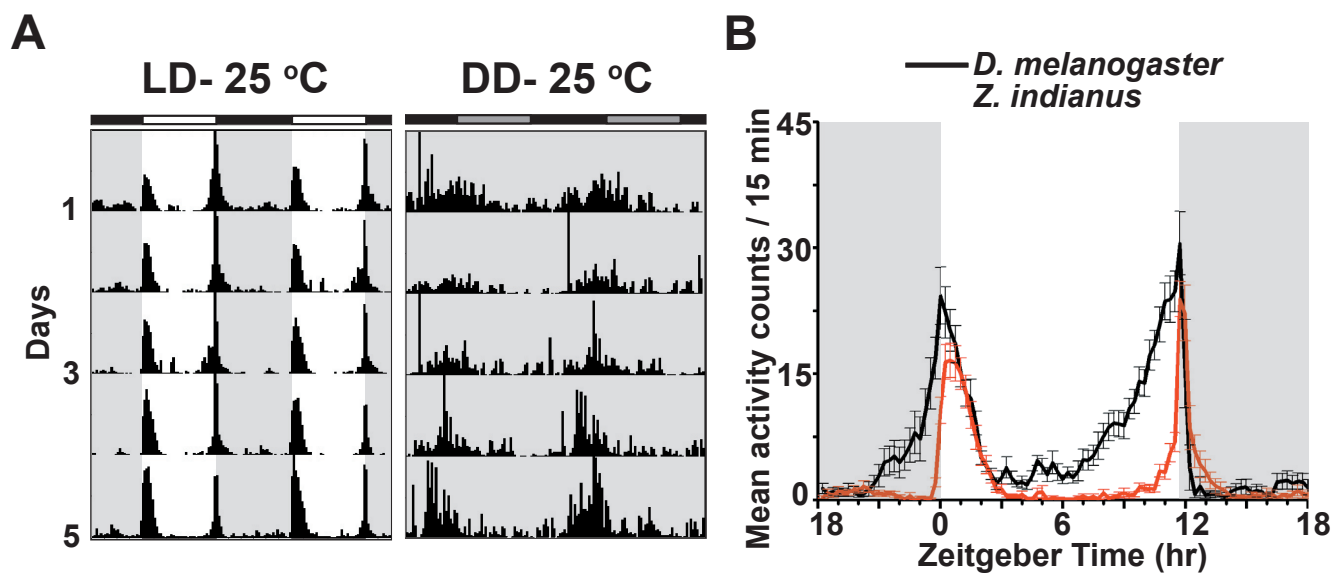
Figure 8. *Z. indianus* **showed bimodal activity pattern under LD12:12 and poor rhythmicity under constant darkness (DD).** **(A)** Average double plotted actograms of male *Z. indianus* under LD12:12 at 25 ºC (left) and DD (right). The x-axis represents time of day from 0-48 hr, consecutive days are plotted along y-axis. **(B)** Raw Activity counts (15 min bin) averaged across 5 days for both *D. melanogaster* and *Z. indianus* virgin male flies (mean ± SEM). Grey shaded areas in actograms represent darkness.

Z. indianus
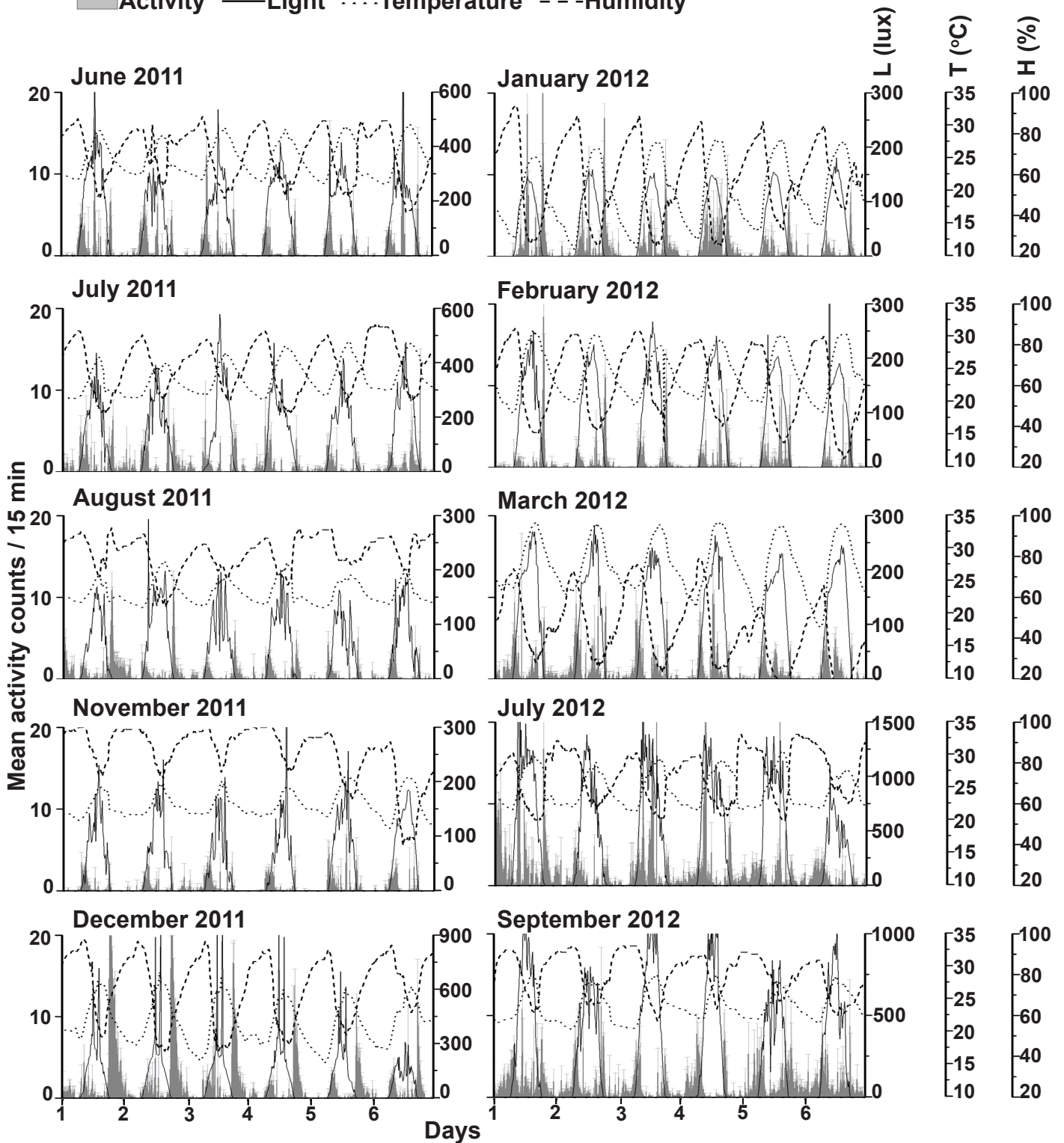
Figure 9. *Z. indianus* **showed variation in its activity/rest pattern across different seasons even though its activity levels were low compared to** *D. melanogaster*- **day wise pattern.** Average activity/rest profiles of virgin male *Z. indianus* flies across assays in semi-natural condition. All other details are same as Fig. 1.

Z. indianus
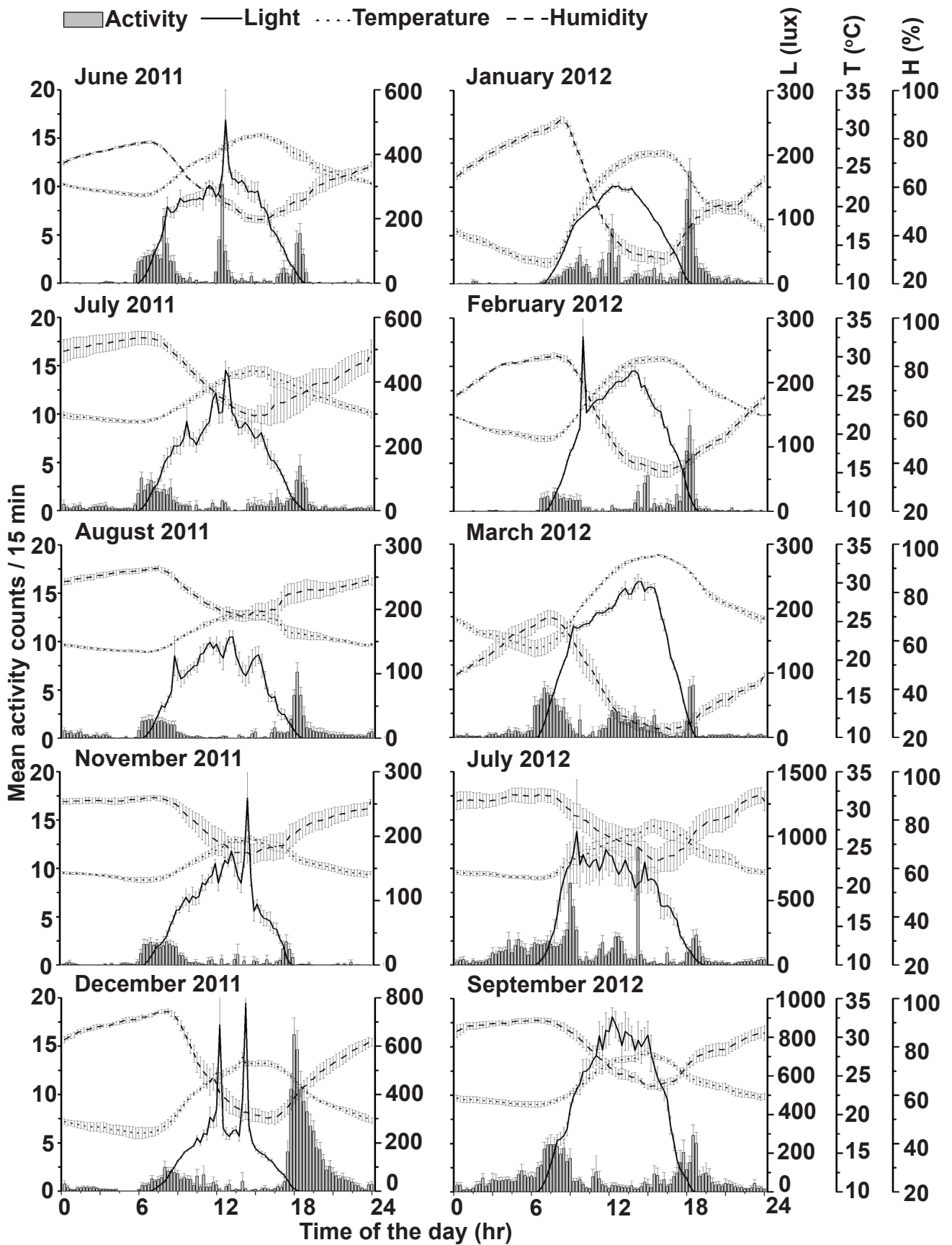
Figure 10. ***Z. indianus*** **showed variation in its activity/ rest pattern across different seasons.** Average activity/rest profiles of virgin male flies *Z. indianus* across assays in semi-natural condition. All other details are the same as Fig. 2.
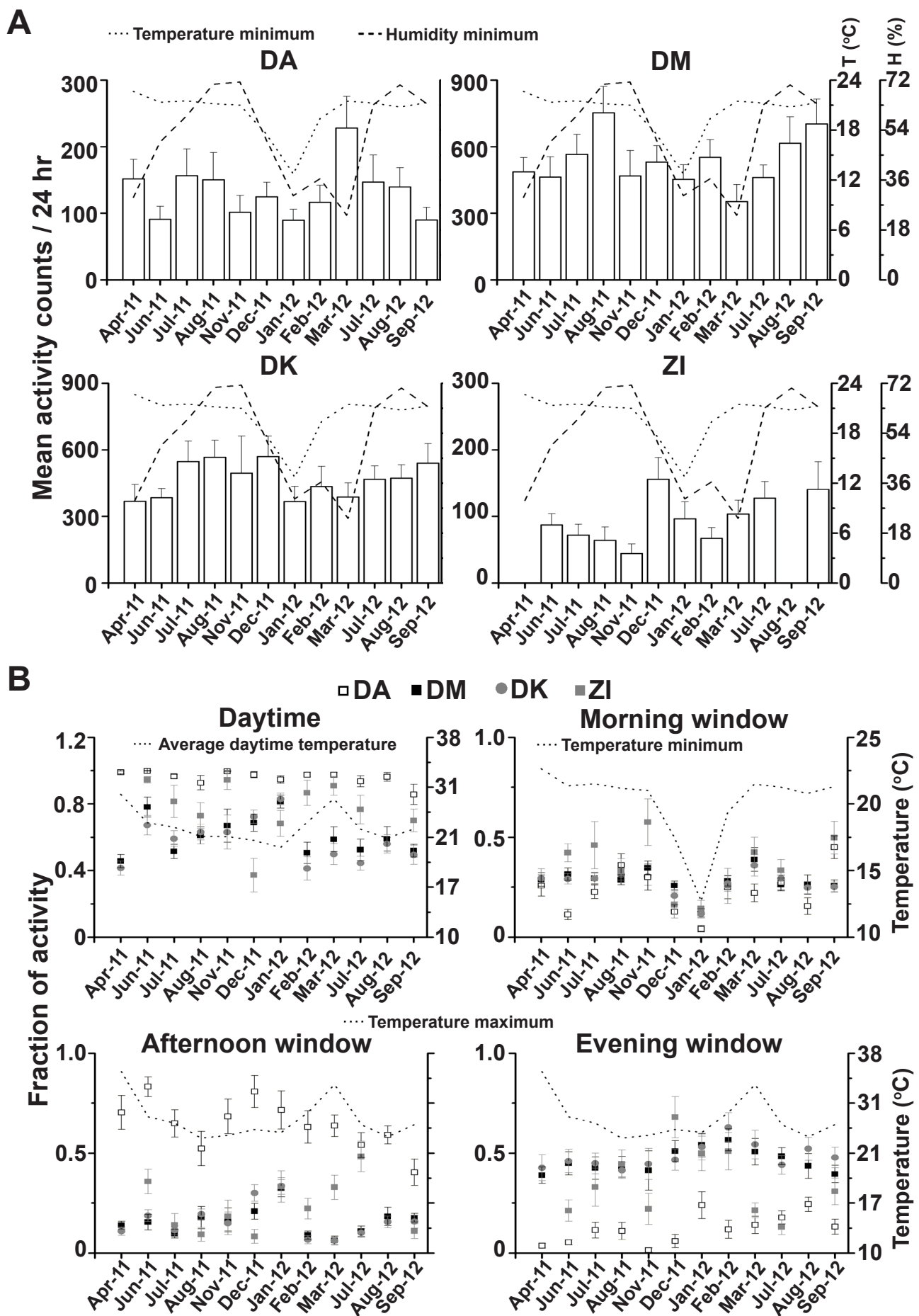
Figure 11. Total and proportion of day activity varies within species across different seasons. (**A**) Mean activity counts during 24 hr averaged across 6 days is plotted along with minimum temperature (T-dotted curve) and minimum humidity (H-dashed curve). (**B**) Mean activity counts during 12 hr of day (as a fraction of total activity) averaged across 6 days is plotted along with average day temperature (dotted curve), mean activity counts during M-window, A-window and E-window (as a fraction of total activity) averaged across 6-days are plotted along with minimum and maximum temperatures (dotted curve) during each season. DA- *D. ananassae,* DM- *D. melanogaster,* DK- *D. malerkotliana,* ZI- *Z. indianus.* Error bars are 95% CI.

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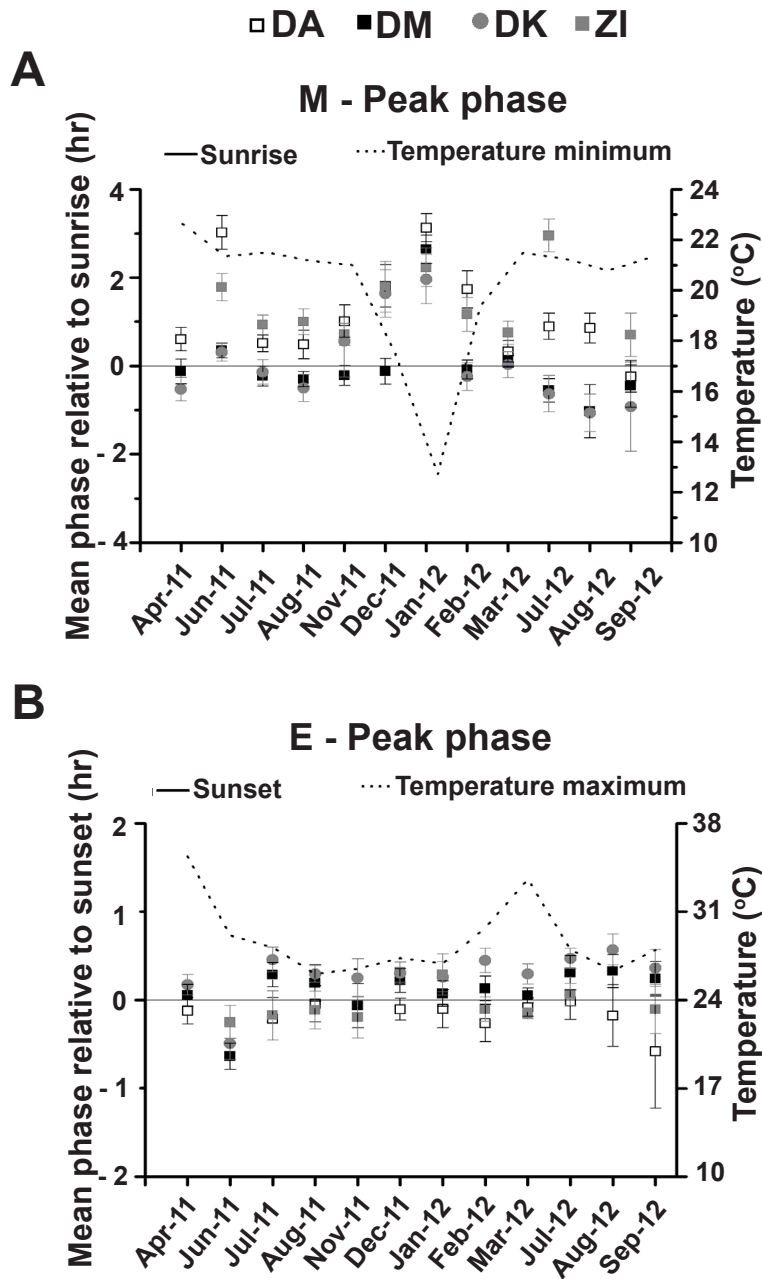
Figure 12. Phase of morning and evening peak of activity exhibited correlation with different environmental factors. (**A**) Mean phase of morning activity peak relative to sunrise is plotted along with sunrise (solid line) and minimum temperature (dotted curve). (**B**) Mean phase of evening activity peak relative to sunset is plotted along with sunset (solid line) and maximum temperature (dotted curve). DA- *D. ananassae,* DM- *D. melanogaster,* DK- *D. malerkotliana,* ZI- *Z. indianus.* Error bars are 95% CI.

A - Peak occurrence

□DA ■DM ●DK ■ZI

Average day temperature

DA $R^2 = 0.02$

DM $*R^2 = 0.13$

DK $*R^2 = 0.18$

ZI $*R^2 = 0.33$

Average day light intensity

DA $*R^2 = 0.08$

DM $*R^2 = 0.37$

DK $*R^2 = 0.21$

ZI $R^2 = 0.06$
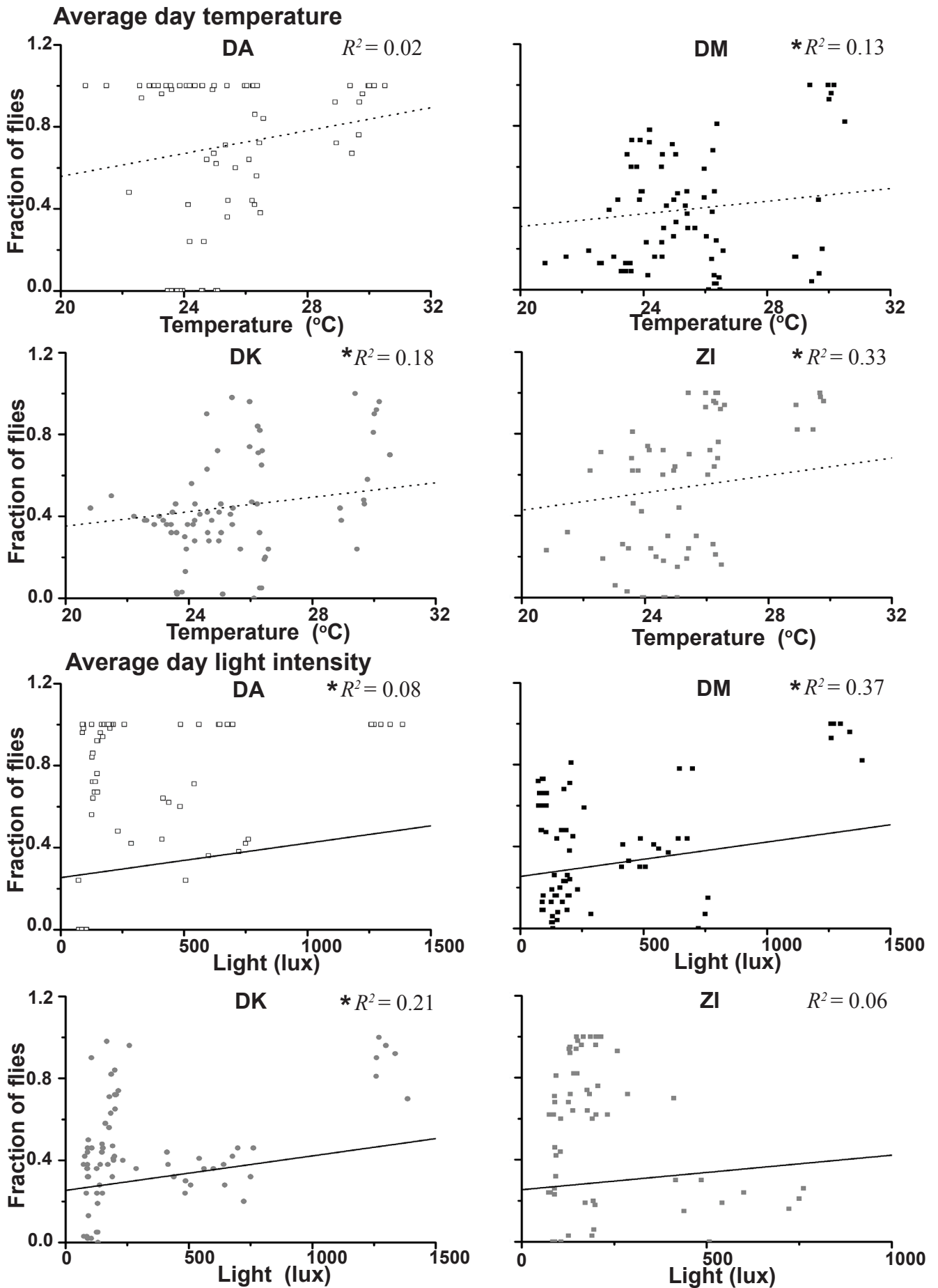
Figure 13. Occurrence of A-peak is influenced by both light and temperature. Day-wise proportion of *D. anan-assae* (DA), *D. melanogaster* (DM), *D. malerkotliana* (DK) and *Z. indianus* (ZI) flies showing A-peak is plotted against average day temperature and average day light intensity. *$p < 0.05$.

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Simulating natural light and temperature cycles in the laboratory reveals differential effects on activity/rest rhythm of four Drosophilids

Introduction

Most studies to understand the circadian entrainment have been performed in the laboratory, wherein two zeitgebers- light and temperature, have been provided separately, or simultaneously to study their synergistic effect (Foster and Helfrich-Förster, 2001; Yoshii et al., 2009). Recent studies on mice and fruit flies in natural conditions where multiple, gradually changing time cues are present, revealed several interesting features of the daily activity pattern which were not seen under 'standard' laboratory conditions (Daan et al., 2011; De et al,. 2013; Menegazzi et al., 2012; Prabhakaran and Sheeba, 2013; Vanin et al., 2012), thus revealing lacunae in our understanding of circadian entrainment. Unlike the bimodal activity pattern seen under standard laboratory protocols (12:12 hr light/dark cycle or LD12:12), *Drosophila melanogaster* when studied in outdoor enclosures (henceforth referred to as semi-natural (SN) conditions), also showed an additional peak in the afternoon (A-peak) under certain environmental conditions (Vanin et al., 2012). It was suggested that intact circadian clocks enable flies to prevent high activity during harsh afternoon conditions and that flies with circadian dysfunction are unable to reduce afternoon activity thus providing evidence for an adaptive value of the clock in the real world (Menegazzi et al., 2012). High temperature and light intensity have been postulated to induce the occurrence of the A-peak (De et al., 2013; Menegazzi et al., 2012; Vanin et al., 2012). The underlying molecular components of circadian clocks - of which PERIOD and TIMELESS proteins are considered to be crucial, were also found to show interesting deviations from what is seen under standard LAB conditions – the oscillations in protein levels were decoupled under summer conditions and were found to be strongly influenced by photoperiod (Menegazzi et al., 2013). This is in contrast to the activity/rest rhythm of *D. melanogaster* which was found to be strongly affected by temperature (Menegazzi et al., 2013).

Besides *D. melanogaster*, the circadian behaviours of very few among 1,500 species of *Drosophila* have been examined thus far (Bahn et al., 2009; Kauranen et al., 2012; Khare et al., 2002; Low et al., 2008; Pittendrigh et al., 1954; Prabhakaran and Sheeba, 2012; Prabhakaran and Sheeba, 2013; Simunovic and Jaenike., 2006). We have previously studied the behaviour of four species of Drosophilids including wild caught *D. melanogaster*, and three other species *D. malerkotliana*, *D. ananassae* and *Zaprionus indianus* under SN conditions across a span of 1.5 years and reported that these four species possibly adopt different temporal niches (Prabhakaran and Sheeba, 2013). Unlike *D. melanogaster*, *D. ananassae* showed predominant daytime activity and this difference in activity between *D. melanogaster* and *D. ananassae* persisted under different laboratory photoperiods and under SN conditions (Prabhakaran and Sheeba, 2012; Prabhakaran and Sheeba, 2013). Another species *D. malerkotliana* although more closely related to *D. ananassae* (based on phylogenetic studies -van der Linde et al., 2010) exhibited activity pattern almost identical to *D. melanogaster* (Prabhakaran and Sheeba, 2013). *Z indianus*, which is comparatively much more distantly related to *D. melanogaster*, *D. malerkotliana* and *D. ananassae*, showed enhanced and a more consolidated activity pattern compared to their behaviour in the LAB where they were poorly rhythmic and responded only to lights-ON and OFF transitions (Prabhakaran and Sheeba, 2013).

Our previous study under SN described in chapter 4 showed that for the two species *D. melanogaster* and *D. malerkotliana*, among 12 assays, a prominent A-peak was present only in very few assays (Prabhakaran and Sheeba, 2013), and when present, the amplitude of the A-peak in *D. melanogaster* was lower than that observed by others (De et al., 2013; Menegazzi et al., 2012; Vanin et al., 2012). We speculated that this may be due to the fact that we used larger glass tubes (5 mm diameter) and thus flies were less affected by temperature (Prabhakaran and Sheeba, 2013). This is in line with another previous study

which showed that midday activity of wild caught (WT_{ALA}) flies in glass vials (30 mm diameter) monitored under nature-like temperature cycles, was lower compared to glass tubes (3 mm diameter) (Menegazzi et al., 2012). In our study, the profile of *D. ananassae* was such that high activity occurred during the daytime across most of the 12 assays, although the pattern of this activity was not similar to *D. melanogaster* (Prabhakaran and Sheeba, 2013).

However, assays under SN conditions while providing us with an opportunity to study behaviours in the presence of multiple simultaneously varying zeitgebers also presents several challenges, in that there are several other environmental factors that could co-vary in ways that are not perceived and hence not accounted for by the experimenter. To enable a cleaner dissection of the contribution of two of the major cyclic environmental variables in nature we conducted a series of experiments in the laboratory inside Drosophila environmental chambers where we separately, or simultaneously provided cycles of gradually changing light intensity and/or temperature, in a manner that closely mimicked what was observed in our own previous studies under SN (Prabhakaran and Sheeba, 2013).

We reasoned that by doing so we can examine (1) the species-specific effects of gradually varying light or temperature cues alone, (2) synergic effect of nature-like temperature and light on each species and (3) reproducibility of the A-peak under LAB conditions in the different species. We were able to reproduce under LAB conditions, the SN activity pattern of all the species using gradually varying light and temperature cycles, which suggests that they are indeed the major determinants of activity patterns in nature. Moreover, different species exhibited differential responses to rhythmic temperature and/or light cues. *D. ananassae* flies yet again displayed strong preference for activity during the early part of the day under a variety of simulated regimes. High amplitude gradual temperature cycles by themselves were able to induce the A-peak in all species and for the most part, the A-peak

alone was present in *Z. indianus* in combination with constant light suggesting that it is merely a response to stressfully warm temperatures.

Materials and methods

Fly strains. Four species *D. melanogaster*, *D. malerkotliana*, *D. ananassae*, and *Z. indianus* were collected using fruit traps and net sweeps between 2004-2005 within Bangalore, India (12°58'N, 77°38'E). They were maintained as large random mating populations of ~1200 individuals on cornmeal medium under LD12:12 (~1.5 W/m^2) conditions at constant temperature (~25 ºC) and relative humidity (~70%).

Activity recording. Virgin male flies (2 to 3 day old) of each species reared in the LAB under LD12:12 were placed individually into glass tubes (5 mm inner diameter and 65 mm long) containing cornmeal medium at one end and a sponge plug at the other end. Locomotor movement of the fly along the length of the tube was recorded using *Drosophila* activity monitors (DAM2, TriKinetics, Waltham, USA). Monitors were placed inside incubators (DR-36VLC8, Percival Scientific, Perry, IA or MIR-154, Sanyo, Tokyo, Japan), where light and temperature conditions were maintained as described below. Profiles of light and temperature were monitored in parallel using an environmental monitor (DEnM, Trikinetics, USA).

Regimes:

1. LD12:12 cycles at constant temperatures - 21 ºC, 25 ºC or 30 ºC denoted as LD T-21, LD T-25 and LD T-30. The light intensity for both LD T-25 and LD T-30 was at constant low of 350 lux or 0.96 W/m^2 whereas for LD T-21 it was higher (2000 lux or 5.53 W/m^2).

2. Gradually changing (ramped) light intensity cycles (light intensity peak 2000 lux or 5.6 W/m^2) at constant temperatures 25 ºC or 30 ºC denoted as Lr T-25 and Lr T-30. In

one additional ramped light regime denoted as Lr_h T-25, a higher light intensity peak (3000 lux or 8.9 W/m^2) was imposed to ask whether under moderate and constant ambient temperatures, high light intensity alone can elicit A-peak.

3. Ramped temperature cycles of low (range 21-28 ºC) or high (17-32 ºC) amplitudes under LD denoted as LD Tr Lo, LD Tr Hi and under constant dark (DD) conditions- DD Tr Lo and DD Tr Hi to compare the effect of gradually changing temperature cycles of a mild or stressful amplitude in combination with standard laboratory LD cycles or by itself.

4. Ramped light intensity and temperature cycles of low (range 17-28 ºC) or high (17-32 ºC) amplitudes denoted as Lr Tr Lo and Lr Tr Hi to compare the effect of the combined action of gradually changing cycles of both light and temperature.

5. Ramped temperature cycles of low and high amplitudes (17-28 ºC or 17-32 ºC respectively) at constant light intensities of 100 or 1000 lux (0.28 or 2.92 W/m^2 respectively) denoted as LL_{100} Tr Lo, LL_{100} Tr Hi and LL_{1000} Tr Lo, LL_{1000} Tr Hi to examine the effect of temperature cycles under constant light where the circadian clock is assumed to be disrupted in *D. melanogaster*.

6. Standard step-shift temperature cycles under constant light of 100 lux intensity denoted as LL_{100} TC 21-29 to examine the effect of abruptly changing temperature cycles in the absence of other time cues.

Analysis of activity. Activity was recorded in 5 min bins. Activity profiles (mean ± SEM) were obtained by binning raw time series data of individual flies into 15 min intervals. These 15 min binned data of individual flies were averaged across 6 days for each fly and averaged across flies. Profiles of light and temperature were also obtained by averaging across 6 days. From the 15 min binned light profile, phase of the first bin showing values greater than 0 lux during morning was considered as dawn or beginning of the day and the phase of the first bin

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showing 0 lux during evening interval was considered as dusk or beginning of night. In regimes where temperature was the only time cue, based on the LD regime during development, the step at which the first increase in temperature occurred was taken as dawn and the step at which temperature dropped to a steady low level as dusk. An interval of ± 3 hr around dawn was considered as the morning window. Similarly, the interval of 3 hr before and after dusk was considered as evening window, and the duration intervening morning and evening as afternoon window. Onset of activity peak in morning and evening windows was visually determined as the point at which there was a gradual increase in activity leading to a peak (from 15 min binned average profiles across 6 days) for each fly in each assay. For further analysis of phase of peaks, only assays where more than 20% flies exhibited a particular peak were considered. To estimate the proportion of flies exhibiting A-peak a similar visual examination of average profiles of each fly was done. Total activity levels (± 95%CI) were obtained by averaging activity counts of individual flies across 6 days and averaging across flies. To compare the total activity during daytime, fraction of daytime activity to total activity counts of individual flies were averaged across 6 days and further averaged across flies (± 95%CI). Pre-dawn activity was estimated as the ratio of activity occurring 3 hr before lights-ON to the sum of activity 3 hr before and 3 hr after lights-ON, on data of individual flies averaged across 6 days and then averaged across flies. One-way ANOVA followed by post-hoc multiple comparisons using Tukey's HSD test was performed to evaluate statistically significant differences across different regimes for total activity, daytime activity and pre-dawn activity. Anticipation indices for lights-ON transition under LD T-25 and Lr T-25 were calculated as the ratio of activity occurring 3 hr prior to lights-ON to that of 6 hr prior to lights-ON (Harrisingh et al., 2007) and compared across regimes using Student's t-test. For each species, regression analyses were performed on proportion of flies showing A-peak with various environmental factors of the different regimes - average

daytime temperature ($T_{ave\ day}$), maximum temperature (T_{max}), average daytime light intensity ($L_{ave\ day}$) and maximum light intensity (L_{max}). The phase of morning (M) and evening (E) activity onset was estimated by scanning activity profiles of individual flies and onset phase values thus obtained were averaged across flies to obtain mean phases ($\pm$ 95% CI) for each species in each regime. One-way ANOVA were performed to evaluate statistically significant differences between separate assays within a species for M and E activity onsets followed by post-hoc multiple comparisons using Tukey's HSD test. All statistical tests were done using STATISTICA-7 (StatSoft Inc., USA) with level of significance set to $p < 0.05$.

Results

Warm and cool ambient temperatures differentially modulate behaviours of Drosophilid species in the presence of light/dark cycles. Our previous laboratory studies on the four species were conducted under a moderate temperature of 25 ℃ which has been demonstrated to be the 'preferred' temperature for *D. melanogaster* (Sayeed and Benzer., 1996). Since it is not known that the other species under study have a similar temperature preference we explored the possibility that cooler and warmer ambient temperatures may differentially modify the activity patterns of the other three species compared to *D. melanogaster*. Under LD T-25, three of the species studied (*D. melanogaster*, *D. malerkotliana*, and *Z. indianus*) showed bimodal activity pattern (Fig. 1A), whereas, as we have reported previously *D. ananassae* was predominantly day active and exhibited a much smaller E- peak (Fig. 1A) (Prabhakaran and Sheeba, 2012; Prabhakaran and Sheeba, 2013). When temperature was lowered to 21 ℃ (LD T-21), *D. melanogaster* and *D. malerkotliana* showed almost similar activity profiles (Fig. 1A) and total activity counts as that under 25 ℃ (Fig. 1B), but with relatively higher daytime activity (Fig. 1C). Under such cool ambient temperature, in addition to the fact that *D. ananassae* and *Z. indianus* have overall lower activity levels compared to *D. melanogaster*, they exhibited an even greater reduction in total activity

compared to LD T-25 (Fig. 1B) and little or no activity during night (Fig. 1A). When the temperature was increased to 30 ºC (LD T-30) all the four species showed lower activity levels compared to LD T-25 (Fig. 1B). Under warm ambient conditions, *D. melanogaster* and *D. malerkotliana* advanced their morning activity such that the peak occurred before lights-ON (Fig. 1A, right panels) and thereby reduced their daytime activity (Fig. 1C). Such a preference for nighttime activity under warm ambient temperatures has been reported previously for *D. melanogaster* and is thought to enable flies to avoid harsh conditions caused due to the combination of bright light and high temperature (Majercak et al., 1999). In all the three regimes, activity profiles of *D. malerkotliana* were similar to *D. melanogaster* (Fig. 1A). In contrast to *D. melanogaster* and *D. malerkotliana*, even under high temperature, *D. ananassae* maintained preference for daytime activity and *Z. indianus* did not show any advancement of morning activity into the night phase (Fig. 1A). Although total activity of *D. melanogaster* and *D. malerkotliana* at LD T-21 was not different from LD T-25, fraction of daytime activity was higher under cooler, and lower under warmer regimes compared to standard laboratory condition of LD T-25 (Fig. 1C). Thus total activity of *D. ananassae* and *Z. indianus* was lowered by either increase or decrease in temperature from 25 $^{\circ}$C, whereas only higher temperature of 30 ºC affected *D. melanogaster* and *D. malerkotliana*. Interestingly, *D. ananassae* whose activity was predominantly restricted to daytime in all the three regimes showed a significantly lower fraction of daytime activity at LD T-25 compared to the cooler and warmer regimes (Fig. 1C). Furthermore, *Z. indianus* showed a significantly higher fraction of daytime activity at the cool temperature although under warm temperature, no reduction in activity occurred (Fig. 1C). These assays showed that under constant, moderately cool (LD T-21) or harsh warm temperatures (LD T-30), when time cues were provided in the form of step-LD cycles, the four species under consideration exhibited differences in behavioural response in terms of phasing of rhythmic activity. Overall it

appears that for all four species warm temperatures causes inhibition of activity, whereas cool

temperature of 21°C causes inhibition of activity only for *D. ananassae* and *Z. indianus*.

In the light of results from our previous studies under SN conditions where we found

that L$_{max}$ had a significant impact on the rhythmic behaviour of these species (Prabhakaran

and Sheeba, 2013), we attempted to simulate a gradually changing light intensity similar to

SN condition under harsh summer days such that a maximum of either 2000 or higher

intensity of 3000 lux was achieved at midday through a series of 9 to 10 steps (Lr, 0-2000 lux;

Lr $_h$, 0-3000 lux) while temperature was kept at a constant 25 ºC (Fig. 2A, middle and left

panels). In these regimes, *D. melanogaster* exhibited higher activity levels around dawn and

dusk and greater anticipation to dawn under Lr T-25 (0.72 ± 0.05) compared to LD T-25 (0.65

± 0.03) (Figs. 1A, 2A). When the maximum light intensity reached was 3000 lux, even at the

preferred ambient temperature of 25 ºC (Lr $_h$ T- 25) most *D. melanogaster* flies (81%) showed

midday activity with a small A-peak, although this was not clearly visible in the averaged

activity profile (Fig. 2A). This was due to between-fly variation in the phase of the peak and

a representative profile of an individual *D. melanogaster* fly showed a much clearer A-peak

(Fig 2B, left). We also verified that this behaviour is exhibited by another more commonly

used *D. melanogaster* strain *Canton-S* (CS) (Fig 2B, middle and right panels). Thus, we find

that high light intensity when reached in a gradual manner can induce midday activity in *D.

melanogaster* flies. Similar to the behaviour at LD T-30, *D. melanogaster* flies advanced

their morning activity and reduced the overall activity levels under Lr T-30 (Figs. 1A, 2A, C),

thus constant high ambient temperature alone can cause this reduction in activity. *D.

malerkotliana* flies also showed a similar pattern of behaviour as *D. melanogaster*, in the

regimes described thus far, including the propensity to exhibit A-peak under Lr $_h$ T -25 (86%),

suggesting that these two species have evolved similar behavioural and possibly physiological

approaches to deal with these environmental factors (Fig. 2A). In the case of *D. ananassae*

also, the overall activity was reduced under Lr T-30 and was significantly different from total activity under Lr $_h$ T-25 (Fig. 2C). Unlike the other three species which responded to warm ambient temperatures by reducing their total activity, *Z. indianus* exhibited activity levels throughout the day and night under Lr T-30 suggesting that in this species, locomotion that is detected in the DAM monitors is more likely to be a response to stressfully warm conditions (Fig. 2A, C).

We compared the fraction of pre-dawn activities of all species across all the six regimes described above and found that when the ambient temperature was increased to 30 ℃ under LD, *D. melanogaster* and *D. malerkotliana* shifted their morning activity to the pre-dawn window and this shift was significantly different from lower constant temperature LD and Lr conditions (*D. melanogaster* -$F_{5, 175} = 26.8$, *D. malerkotliana* -$F_{5, 166} = 23.3$, $p \ll 0.0001$) (Figs. 1A, 2A, D). Overall pre-dawn activity of both *D. ananassae* and *Z. indianus* was low in all the regimes described thus far whereas *D. melanogaster* and *D. malerkotliana* showed overall higher pre-dawn activity compared to the other two species (Fig. 2D). Especially under constant high ambient temperature conditions *D. melanogaster* and *D. malerkotliana* re-distribute their activity into the night (with reference to lights-ON) whereas *D. ananassae* and *Z. indianus* do not employ such behavioural modification (Fig. 2D).

High amplitude gradual temperature cycles alone can induce the A-peak. Some previous studies under SN conditions have suggested that daytime temperature influences activity pattern in Drosophilids (Prabhakaran and Sheeba, 2013; Vanin et al., 2012). Since it is not possible to separate out the contribution of other environmental factors in experiments conducted under SN, we carried out experiments in the laboratory where flies were exposed to gradual temperature cycles of either low, Tr 17-28 ℃ (Tr Lo) or high, Tr 17-32 ℃ (Tr Hi) amplitude in presence of gradually changing light intensity (Lr) conditions. The average daytime temperatures in these two types of regimes were 25 and 27 ^{o}C respectively. To

enable comparisons with laboratory studies a separate set of experiments were also done under step-shift LD cycles. We found that in all four species, high amplitude temperature cycle (Tr Hi) induced afternoon activity with a prominent A-peak irrespective of whether light came ON abruptly or its intensity was increased in a gradual manner (Fig. 3). In comparison with LD Tr regimes, when both temperature and light were varied gradually (Lr Tr Lo and Lr Tr Hi) all four species showed enhanced morning activity, especially *D. ananassae* and *Z. indianus* (Fig. 3). Furthermore, while *D. melanogaster*, *D. malerkotliana* and *Z. indianus* showed consolidation of activity in the form of three peaks during Tr Hi regimes (both LD and Lr), the activity of *D. ananassae* remained mostly restricted to the first half of the day (Fig. 3).

Since the above results suggest that temperature during midday is most critical for the occurrence of the A-peak irrespective of the type of light regime, we next conducted studies under constant dark (DD) while providing gradually changing temperature cycles of either low or high amplitude (Fig. 4A, left panels). Overall we found a broadening of activity for *D. melanogaster* and *D. malerkotliana* in these regimes. High amplitude gradual temperature cycles (DD Tr Hi) induced afternoon activity with a prominent A-peak. Under low amplitude temperature cycles (DD Tr Lo), since activity was high and dispersed throughout the day for *D. melanogaster* and *D. malerkotliana* (Fig. 4A, left panel) the A-peak was not distinguishable. Instead, a series of bumps in activity were seen suggesting that flies moved as an immediate response to each rising step of temperature, followed by a drop in activity (Fig. 4A, left panel). They also showed such a startle response to the first downward temperature step (Fig. 4A, left panel). On the other hand, *D. ananassae* restricted their activity to the rising phase of the temperature cycle and activity levels tapered down even as temperatures rose to 28 ºC (Fig. 4A, left panel). Under these regimes *Z. indianus* showed very little activity, being restricted to the 'day' phase of the Tr cycles and only under Tr Hi

did they show some activity mostly during the higher temperature steps after which they abruptly stopped being active (Fig. 4A, left panel).

We also subjected flies to gradually changing temperature cycles in LL, which is known to disrupt the canonical circadian clock machinery and cause arrhythmic activity in *D. melanogaster* (Konopka et al., 1989). Under LL, high amplitude gradual temperature cycles were able to produce rhythmic consolidated activity pattern with three peaks (M, A and E) in two out of four species (*D. melanogaster* and *D. malerkotliana*), while *D. ananassae* showed prominent A-peak (Fig. 4A, right panel). Interestingly the M-peak of *D. melanogaster* and *D. malerkotliana* appeared to be merely a startle response to the first temperature step-up without any anticipation to the same, whereas the E-peak appeared to be a more gradual build-up and fall of activity suggesting that oscillators underlying the M-peak are more susceptible to damage by constant light. Activity patterns of *D. melanogaster* and *D. malerkotliana* were similar to each other under LL irrespective of the light intensity (Figs. 4A, 5). Previous studies on *D. melanogaster* under LL have shown that in presence of temperature cycles flies exhibit a startle M-peak, an E-peak which is slightly phase-advanced relative to dusk, and a startle response to lights-OFF (Yoshii et al., 2005; Yoshii et al., 2007). *D. melanogaster* and *D. malerkotliana* exhibited such behaviour in our studies also, whereas *D. ananassae* once again restricted their activity only to the first half of the thermophase, with very little activity during other parts of the day and night (Fig. 5). High amplitude temperature cycles (Tr Hi) induced A-peak in all the species; however, for *D. ananassae* and *Z. indianus*, A-peak was the only clear activity peak present under these regimes (Fig. 4A).

During the low amplitude gradual temperature cycles under LL conditions (LL_{100} Tr Lo, LL_{1000} Tr Lo) and LL_{100} TC 21-29, activity was almost completely abolished for *Z. indianus* while *D. ananassae* showed very low levels of activity compared to all other regimes (Figs. 4A, 5). Irrespective of the intensity of light in LL, the A-peak was elicited

only under high amplitude temperature cycles. Thus, as reported previously occurrence of A-peak depends on the daytime temperature (Vanin et al. 2012), we also found that this is the case in the absence of light or under LL conditions. Overall we find that with increasing temperature the proportion of flies exhibiting A-peak increased in all the four species (Fig. 4B). Regression analyses revealed that proportion of flies exhibiting A-peak was associated with T_{max} for all the four species (*D. melanogaster* - $r = +0.57$; *D. malerkotliana* - $r = +0.52$; *D. ananassae* - $r = +0.56$; *Z. indianus* - $r = +0.82$, $p < 0.05$) and not with $T_{ave\ day}$, (as reported by Vanin et al. 2012) or L_{max} or $L_{ave\ day}$.

Onset of M-peak is modulated by light and temperature while E-peak onset is less flexible.
Since previous studies on *D. melanogaster* under SN conditions showed that the phase of onset of M-peak depends on temperature and twilight- the phase being inversely correlated with temperature (Vanin et al., 2012), we asked whether such a relationship is conserved across species. *D. melanogaster*, *D. malerkotliana* and *D. ananassae* clearly exhibited advanced onset of M-peak under constant temperature conditions, both in presence of LD and Lr cycles (Fig. 6A), thus appearing to anticipate lights-ON. Low amplitude gradual temperature cycles under DD also resulted in the M-peak of *D. melanogaster* and *D. malerkotliana* to be advanced compared to dawn (Fig. 6A). In contrast, under LL with ramped temperature cycles all the species showed M-peak almost coinciding with dawn (Fig. 6A), suggesting that this peak is likely to be a startle response to a critical, small, temperature up-shift. Yet, when a step-shift TC cycle was paired with LL, flies were able to anticipate dawn and show an advanced M-peak (Fig. 6A). Under almost all the regimes tested, M-peak of *D. ananassae* was delayed compared to that of *D. melanogaster* and *D. malerkotliana*, similar to their SN behaviour (Prabhakaran and Sheeba, 2013; Fig. 6A). In the case of *Z. indianus* we could not detect any pattern in the timing of M-peak onset across regimes; it was either advanced or occurred close to dawn (Fig. 6A).

Across all the four species, whenever the E-peak was present, it showed an advanced onset with reference to lights-OFF (Fig. 6B). *D. melanogaster* and *D. malerkotliana* exhibited E-peak under all the regimes and when a step temperature cycle was imposed in LL (LL_{100} TC 21-29 ºC), the onset of E-peak was significantly advanced, occurring few hours before lights-OFF (cold temperature onset) as shown previously for DM under both DD and LL (Busza et al. 2007; Glaser and Stanewsky, 2005; Yoshii et al., 2005) (Fig. 6B). *D. ananassae* flies did not exhibit an E-peak in 9 out of 17 regimes. In the other regimes when cyclic light cues of either the gated or ramped intensity type was provided, a small E-peak was seen, suggesting that it is a light-modulated behaviour (Figs. 1, 2A, 3, 6B) in this species. LL conditions abolished the E-peak of *Z. indianus* (Figs. 4A, 5, 6B). Thus unlike the M-peak, E-peak when present, always occurred before dusk in all four species and under all regimes that we tested (Fig. 6).

Discussion

Previously we showed that four Drosophilid species *D. melanogaster*, *D. malerkotliana*, *D. ananassae* and *Z. indianus*, caught from various locations in Bangalore within a 10 km radius, exhibit differences in activity patterns and that these differences persist across different months of the year (Prabhakaran and Sheeba, 2013). While some studies conducted under natural conditions have been performed under temperate latitudes where seasonal changes are quite large (Menegazzi et al., 2012; Menegazzi et al., 2013; Vanin et al., 2012), in our previous studies conducted in Bangalore (12°58'N, 77°38'E) photoperiods remained fairly invariant (11.25- 12.75 hr) throughout the year and it was mostly temperature and humidity maxima and minima that varied across the months (Prabhakaran and Sheeba, 2013). Hence, in our present study we simulated the gradually changing light intensity and/ or temperature maxima and minima of nature in a series of experiments either in tandem or separately. The results of our study revealed that species-specific features of the rhythmic activity under

natural conditions can be reproduced in the laboratory with gradually changing temperature or light conditions.

Various simulated light and temperature conditions elicited the diurnal activity pattern of *D. ananassae* previously observed under various LAB photoperiods and SN conditions (Prabhakaran and Sheeba, 2012; Prabhakaran and Sheeba, 2013). Under the simulated conditions *D. ananassae* did not show significant nocturnal activity, evening activity was negligible whenever it was present and the E-peak was absent when there was no cyclic light information (DD/LL) (Figs. 1-6). Even when daytime temperature was as high as 30 ºC, these flies preferred to be active only during the day and did not push their activity into the night like the other two species *D. melanogaster* and *D. malerkotliana* (Fig. 1). Furthermore, it appears that either gradual light or temperature cycles are sufficient to induce the natural activity profile of *D. ananassae* particularly during the early hours of the day (Figs. 3, 4). Thus, these results strengthen our previous hypothesis of *D. ananassae* having a dominant morning oscillator (Prabhakaran and Sheeba, 2012) compared to the dual oscillator model proposed for *D. melanogaster* (Grima et al., 2004; reviewed in Helfrich-Förster, 2009; Stoleru et al., 2004; Yao and Shafer, 2014).

A previous study showed that very low intensity light cycles can entrain circadian clocks of *D. melanogaster* and that flies mostly use changing light profile during dawn and dusk for circadian entrainment (Reiger et al., 2007). We compared activity of flies under conditions wherein light intensity was kept at a constant high level during the day (LD) or increased in a step-wise manner (Lr) and paired them with either low or high amplitude temperature cycles (Tr Lo or Tr Hi), and found that irrespective of the light condition, A-peak was elicited in *D. melanogaster*, *D. malerkotliana* and *Z. indianus* only under high amplitude ramped temperature cycles. Interestingly, the afternoon activity of *D. ananassae* flies appears to be modulated by light in ramped temperature conditions such that under gated LD and low

amplitude ramped temperature conditions, flies showed a distinctly diurnal activity with activity peak occurring 6 hr after dawn, whereas in presence of Lr under the same low amplitude Tr, peak activity occurred at dawn followed by a tapering of levels during the day and a very small E-peak at dusk.

Although LL is known to induce arrhythmicity in *D. melanogaster* flies (Konopka et al., 1989) temperature cycles under LL can produce rhythmic activity/rest pattern in *D. melanogaster* (Glaser and Stanewsky, 2005; Tomioka et al., 1998; Yoshii et al., 2005). *D. melanogaster* and *D. malerkotliana* exhibited rhythmic activity pattern in all the LL regimes tested, with an advanced E-peak under LL_{100} TC 21-29 (Fig. 6B) as reported previously for *D. melanogaster* (Yoshii et al., 2005). High temperature LD cycles are known to shift the activity of *D. melanogaster* to night (Majercak et al., 1999). Similarly, we find that *D. melanogaster* and *D. malerkotliana* under LD T-30 shifted their morning activity to pre-dawn; however, there was no such shift in their evening activity (Figs. 1, 2D).

Previously, based on our studies under SN conditions, we proposed that both light intensity and temperature contributes to the occurrence of the A-peak (Prabhakaran and Sheeba, 2013) unlike some other previous reports including one from our own lab which suggest that either light or temperature is sufficient for the occurrence of A-peak (De et al., 2013; Vanin et al., 2012). In agreement with our studies under SN conditions we find that under simulated natural conditions, high light intensity (~ 3000 lux) or high temperature (> 29 °C) during the middle of the day can produce the A-peak (Figs. 2- 5). Comparing across species and regimes, we find that the proportion of flies exhibiting an A-peak has an overall similarity of pattern for all species except in the case of *Z. indianus* where high light intensity in the middle of the day failed to elicit the A-peak under ambient temperature 25 °C (Lr_h T-25, Fig. 4B), and the case of *D. ananassae* flies exhibiting the A-peak even under low intensity LL and low amplitude Tr (LL_{100} Tr Lo, Fig. 4B), suggesting that perhaps the

threshold for light sensitivity to elicit the A-peak is higher in *Z. indianus* flies and lower in *D. ananassae* flies, compared to *D. melanogaster* and *D. malerkotliana*. While our studies do not address the question of whether flies exhibit an A-peak in the wild, when not restrained by the experimental apparatus, and whether they seek shade during that time, the results suggest caution in interpreting the physiological significance of this behaviour and re-emphasize the need for field studies.

Interestingly, *Z. indianus* exhibited a consistent rhythmic activity/rest pattern only in the presence of light cycles. Under LL, they showed consolidated bouts of activity only when temperature rose above 30 ºC, suggesting a high temperature induced response rather than a circadian clock regulated behaviour (Figs. 4, 5). Furthermore for *Z. indianus*, cyclic light condition seems to be indispensible for the regulation of rhythmic activity/rest pattern. The onset of M-peak was close to dawn under all the LL conditions (except LL_{100} TC 21-29 ºC) in *D. melanogaster* and *D. malerkotliana*, suggesting reduced anticipation to dawn (Fig. 6A), thus the M-peak, under natural conditions, is probably a light and temperature driven response (Vanin et al., 2012). Our studies suggest that the regulation of M-peak of *Z. indianus* to be different from the other species under comparison, whereas the E-peak appears to be circadian clock controlled. Nevertheless we do not know whether this species has an intrinsically low level of activity compared to the other three species or whether the assay conditions or food provided to these flies is inappropriate or sub-optimal. Future studies which use other methods to record activity or other types of food along with visual observation of flies are likely to shed more light on rhythmic behaviours of this species.

The two species *D. melanogaster* and *D. malerkotliana* are thought to be phylogenetically more divergent than *D. melanogaster* and *D. ananassae*, whereas *D. ananassae* and *D. malerkotliana* are thought to be closely related (van der Linden et al., 2010), yet except for slight differences, *D. melanogaster* and *D. malerkotliana* exhibited

similar activity pattern under all the light and temperature regimes examined, just as previously seen under SN conditions. The only consistent difference was in their morning activity pattern during LD T-25, where *D. malerkotliana* showed higher pre-dawn activity compared to *D. melanogaster* (Figs. 1A, 2D) and the onset of M-peak was delayed in *D. melanogaster* compared to *D. malerkotliana* (Fig. 6A). At this point we cannot speculate on the probable reasons for this variation under standard LAB condition, especially when both show similar activity pattern in all the other simulated and SN conditions (Prabhakaran and Sheeba, 2013), and future studies which investigate the highly invasive tropical *D. malerkotliana* species may reveal the underlying neuronal or molecular basis for the same. Thus, the results reported here show that several aspects of species-specific difference in activity/rest pattern of four Drosophilid species under SN can be elicited by natural-like conditions in the laboratory using light or temperature cycles alone.
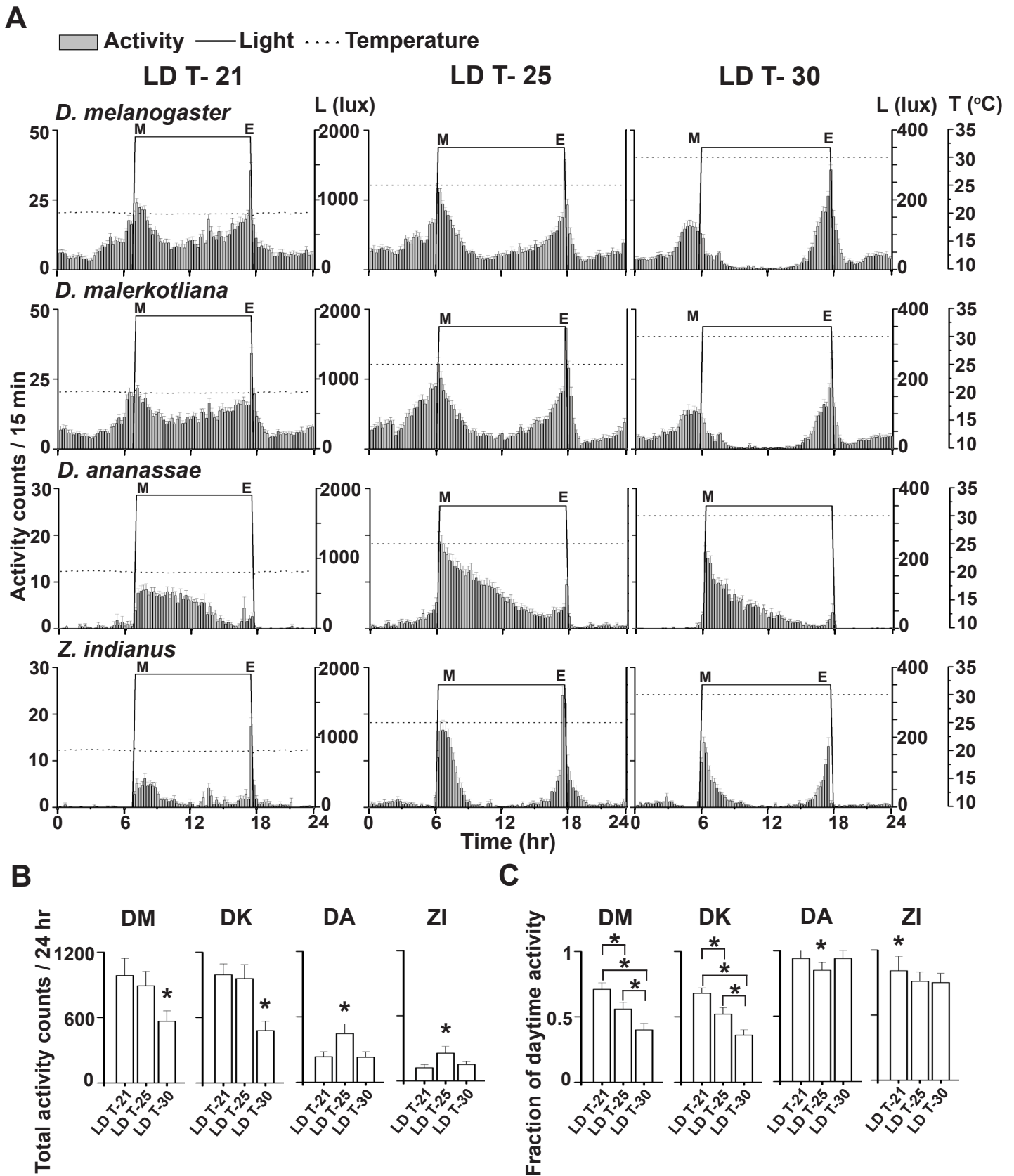
Figure 1. **Activity/rest pattern of Drosophilids is modulated by ambient temperature under light/dark cycles.**
(A) Average activity/rest profiles of virgin male *D. melanogaster*, D. *malerkotliana*, *D. ananassae* and *Zaprionus indianus* flies under LD 12:12 cycles of temperatures 21, 25 and 30 ºC is plotted along with environmental factors light intensity (L, black-solid line) and temperature (T, black-dotted line). Presence of morning and evening peaks are indicated by M and E respectively. **(B)** Total activity counts of *D. melanogaster* (DM), *D. malerkotliana* (DK), *D. ananassae* (DA) and *Zaprionus indianus* (ZI) flies during 24 hr averaged across 6 days (± 95% CI). **(C)** Fraction of activity counts during 12 hr of day over total activity averaged across 6 days (± 95% CI). Asterisks denote significant differences between regimes for each species at *p* < 0.05.
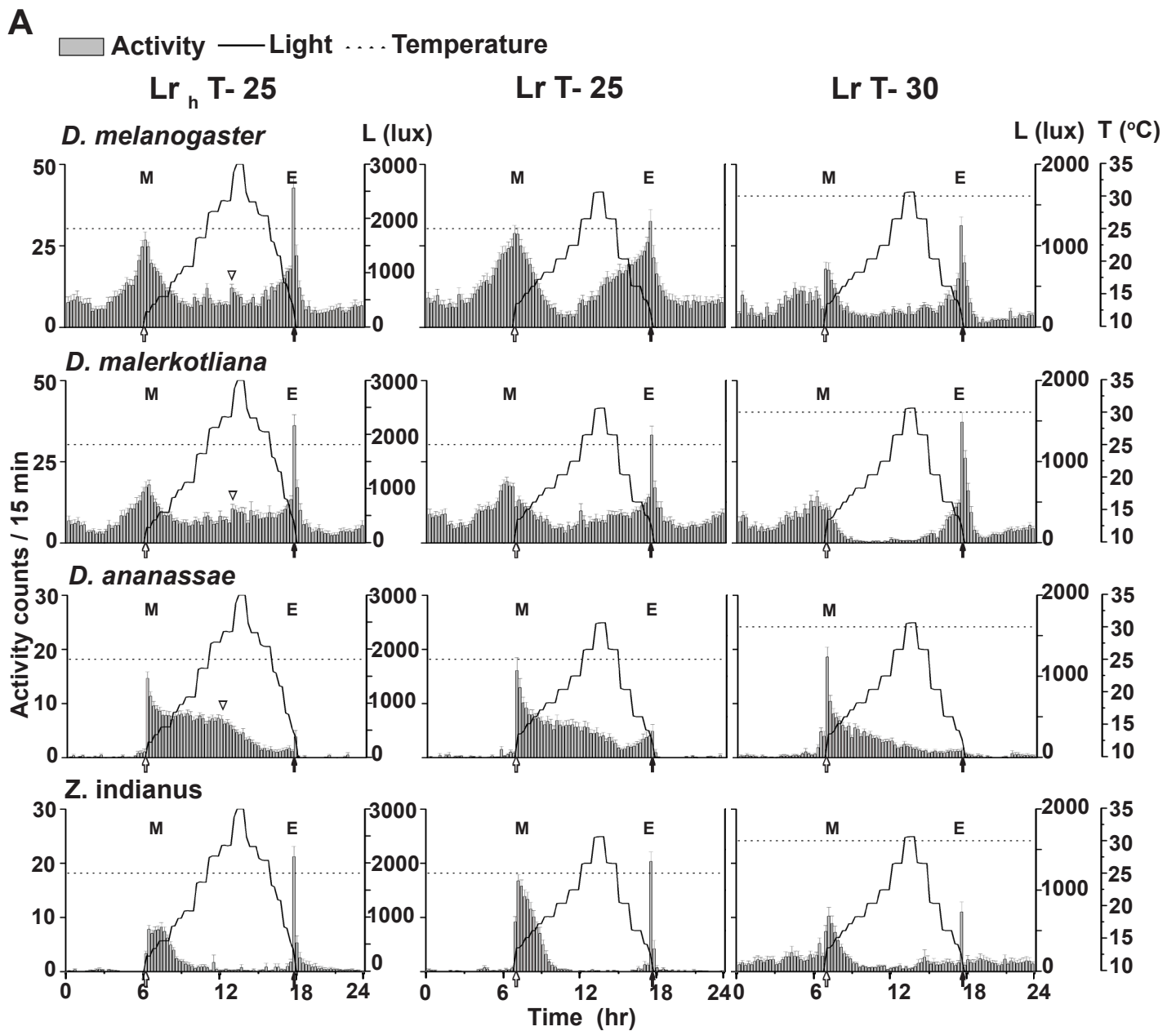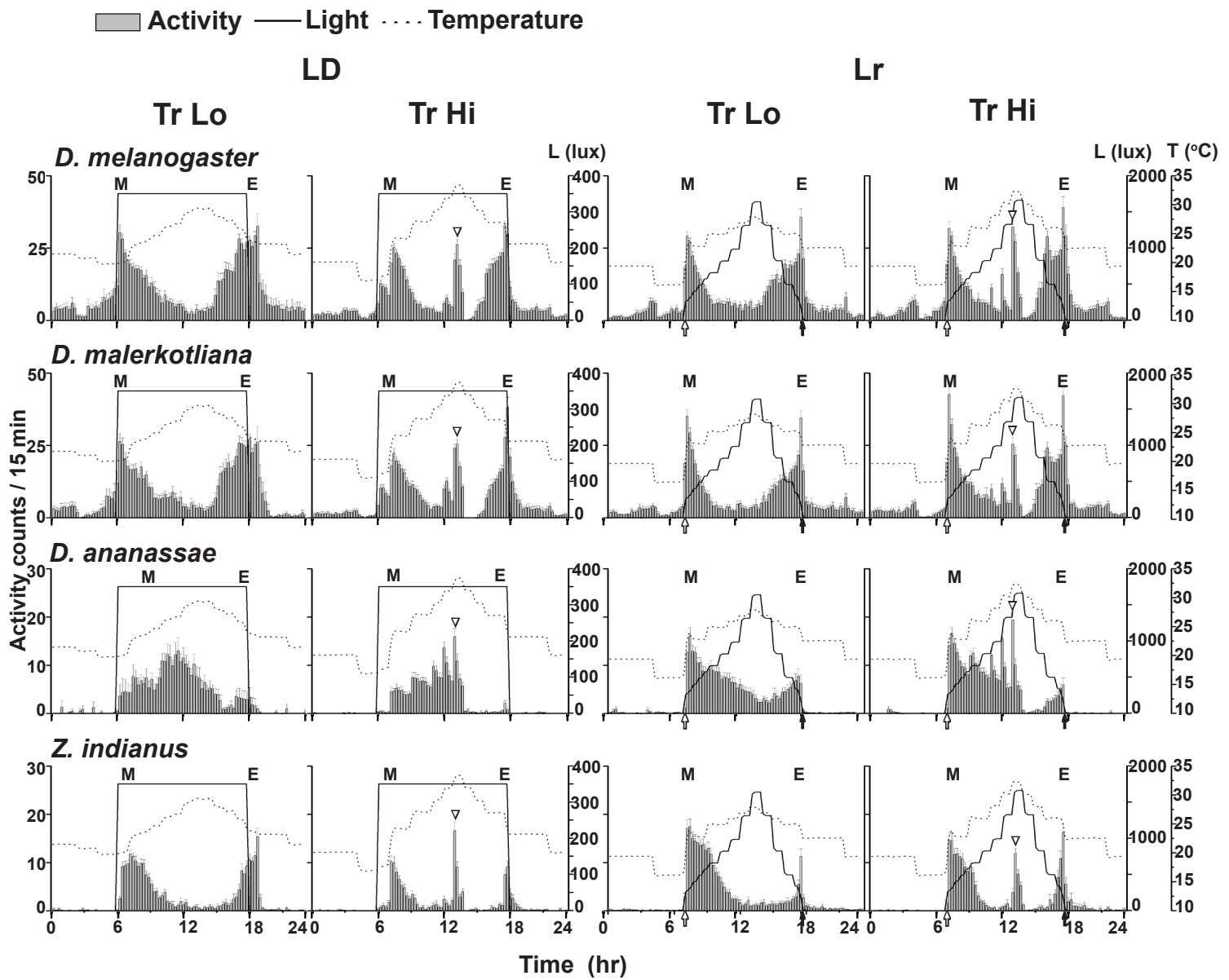
Figure 2. High light intensity during mid-day can elicit A-peak in three Drosophilid species. (A) Average activity/rest profiles of virgin male *D. melanogaster*, *D. malerkotliana*, *D. ananassae* and *Z. indianus* flies under ramped light intensity cycles of temperature 25 and 30 ºC is plotted along with environmental factors light intensity (L, black-solid line) and temperature (T, black-dotted line). Presence of morning and evening peaks are indicated by M and E respectively. Lr h – light ramp with high light intensity- 3000 lux. Lr- light ramp with light intensity 2000 lux. Triangles indicate occurrence of A-peak. Below the x-axis unfilled arrow indicates dawn and filled arrow indicates dusk.
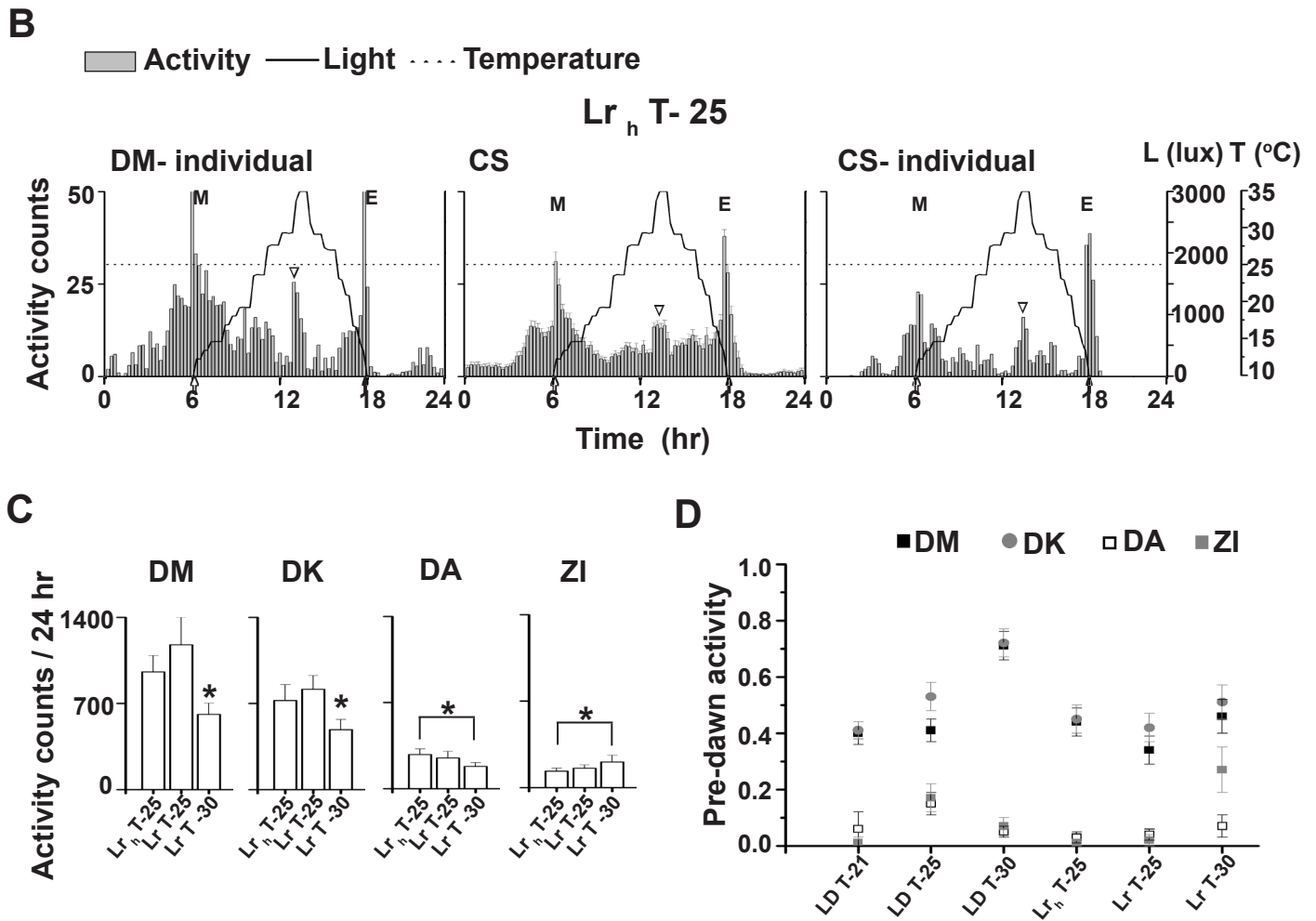
Figure 2. **High light intensity during mid-day can elicit A-peak in three Drosophilid species.** (**B**) Activity/rest profile of virgin male *D. melanogaster* (DM) individual fly (left panel), average activity/rest profile of virgin male CS flies (middle panel) and activity/rest profile of virgin male CS individual fly (right panel) under ramped high light intensity cycles of temperature 25 ºC (Lr h T-25). (**C**) Total activity counts of DM, *D. malerkotliana* (DK), *D. ananassae* (DA) and *Zaprionus indianus* (ZI) flies during 24 hr averaged across 6 days (± 95% CI). Asterisks denote significant differences between regimes for each species at $p < 0.05$ (**D**) Pre-dawn activity of DM, DK, DA and ZI flies under LD and Lr conditions (± 95% CI).

Figure 3. Natural-like light and temperature conditions could elicit activity patterns similar to semi-natural conditions. Average activity/rest profiles of virgin male *D. melanogaster*, *D. malerkotliana*, *D. ananassae* and *Z. indianus* flies under gradual natural-like temperature in LD and gradual light intensity conditions is plotted along with environmental factors light intensity and temperature. Lr- light ramp, Tr Lo- temperature ramp up to 28 ºC, Tr Hi- temperature ramp up to 32 ºC. All other details are similar to Fig. 2.
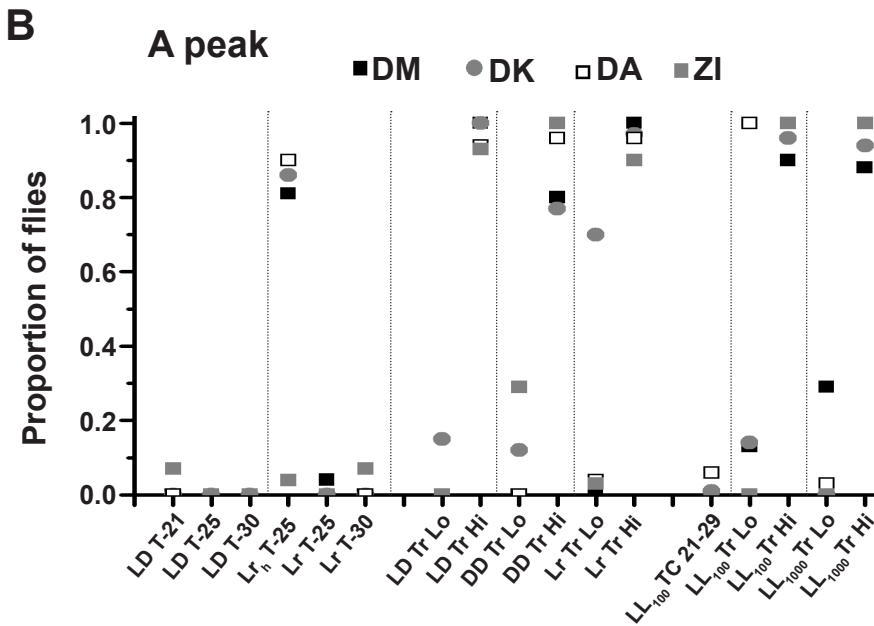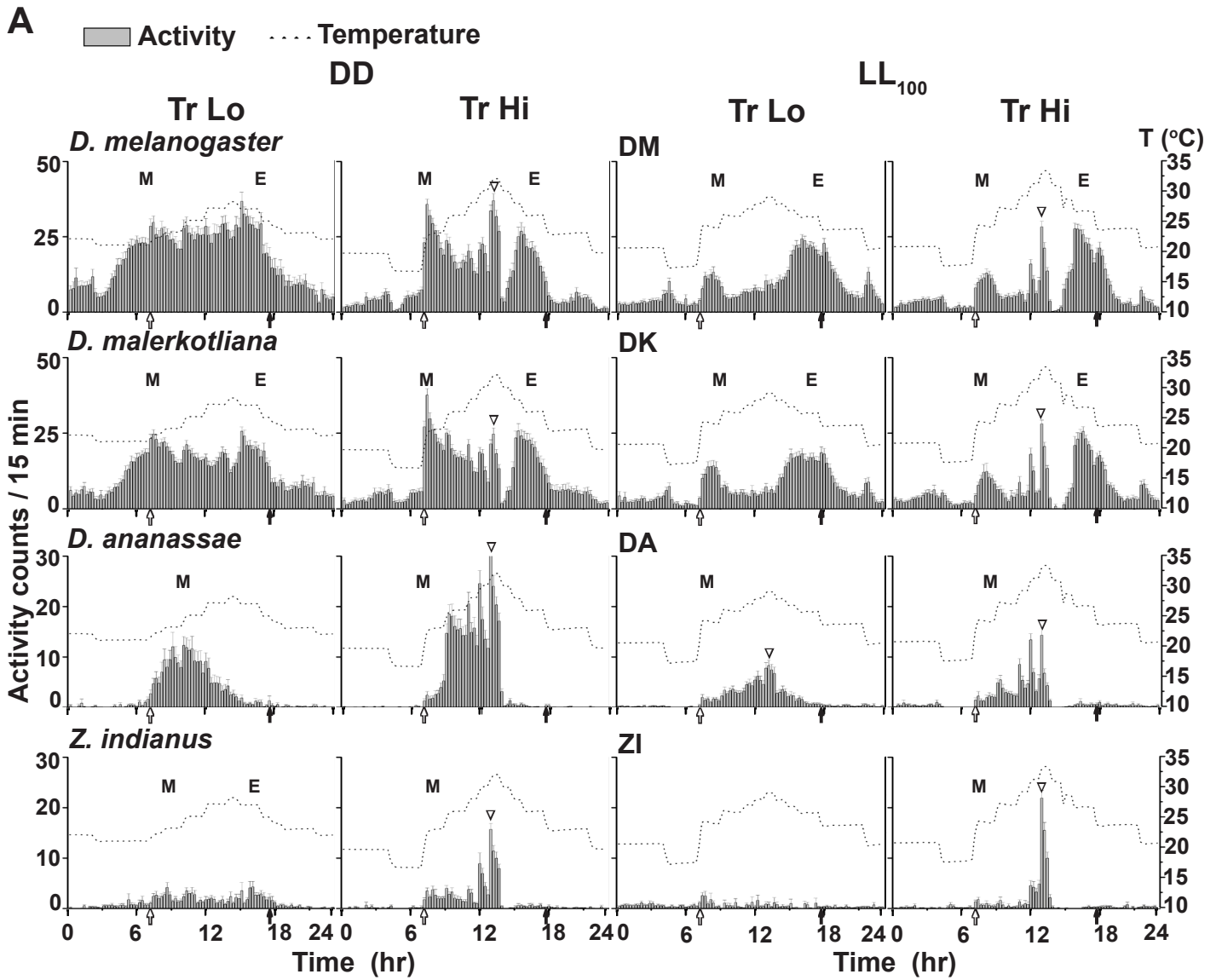
Figure 4. **High amplitude temperature cycles alone can induce mid-day activity in all four species.** (**A**) Average activity/rest profiles of virgin male *D. melanogaster*, *D. malerkotliana*, *D. ananassae* and *Z. indianus* flies under gradual temperatures conditions under DD (constant darkness) and LL100 (constant light of intensity 100 lux) is plotted along with temperature. All other details are similar to Fig. 2. (**B**) Proportion of *D. melanogaster* (DM), *D. malerkotliana* (DK), *D. ananassae* (DA) and *Zaprionus indianus* (ZI) flies showing A-peak. Vertical dotted lines are used as separators for easier visualisation.
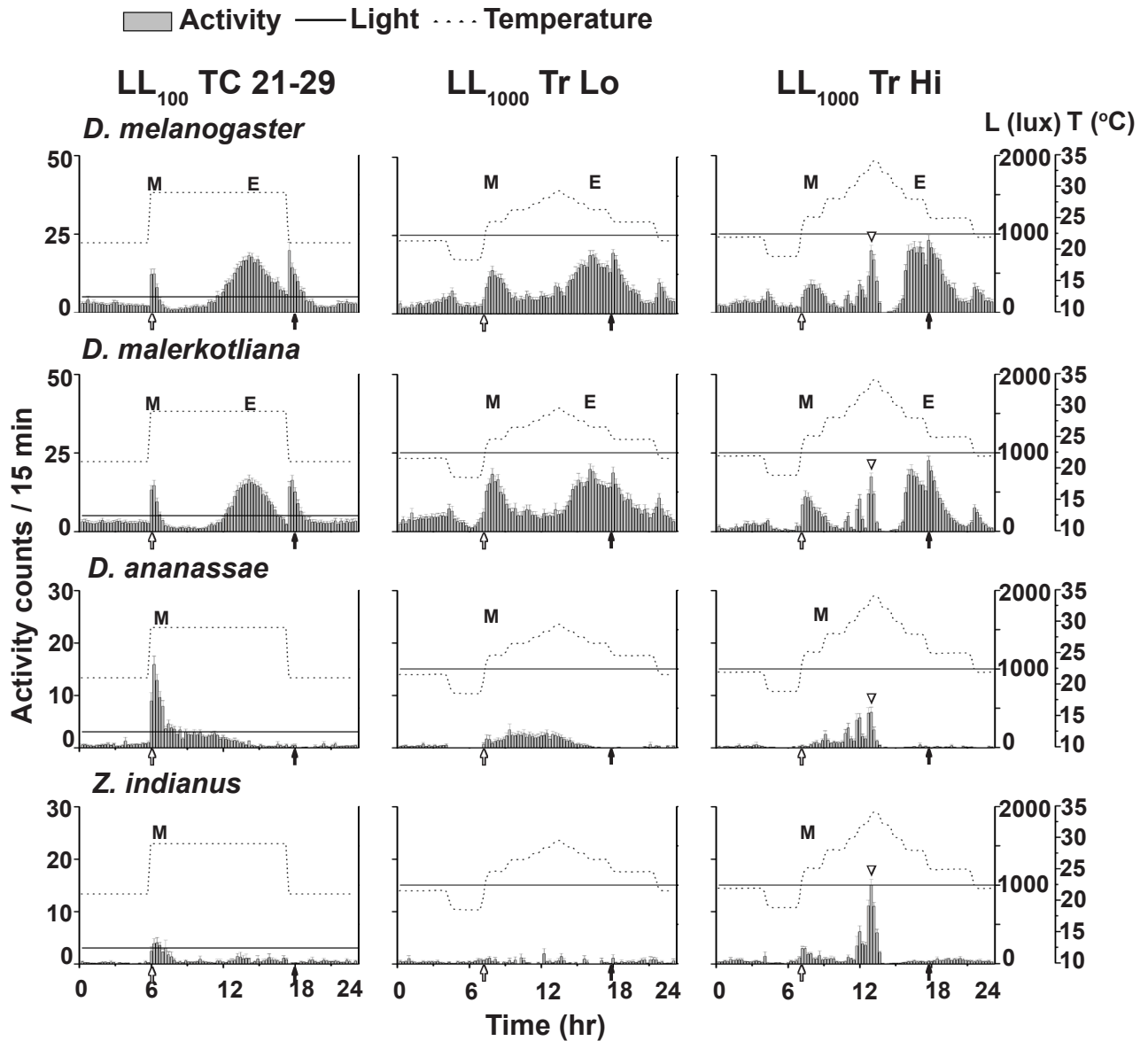
Figure 5. Under constant light intensity conditions high amplitude temperature cycles can induce mid-day activity. Average activity/rest profiles of virgin male *D. melanogaster*, *D. malerkotliana*, D. *ananassae* and *Z. indianus* flies under constant light intensity conditions (LL) of temperature cycles 21- 29 ºC, ramped temperature cycles of amplitude Lo (17-28 ºC) and Hi (17-32 ºC) is plotted along with environmental factors light intensity and temperature. All other details are similar to Fig. 2.
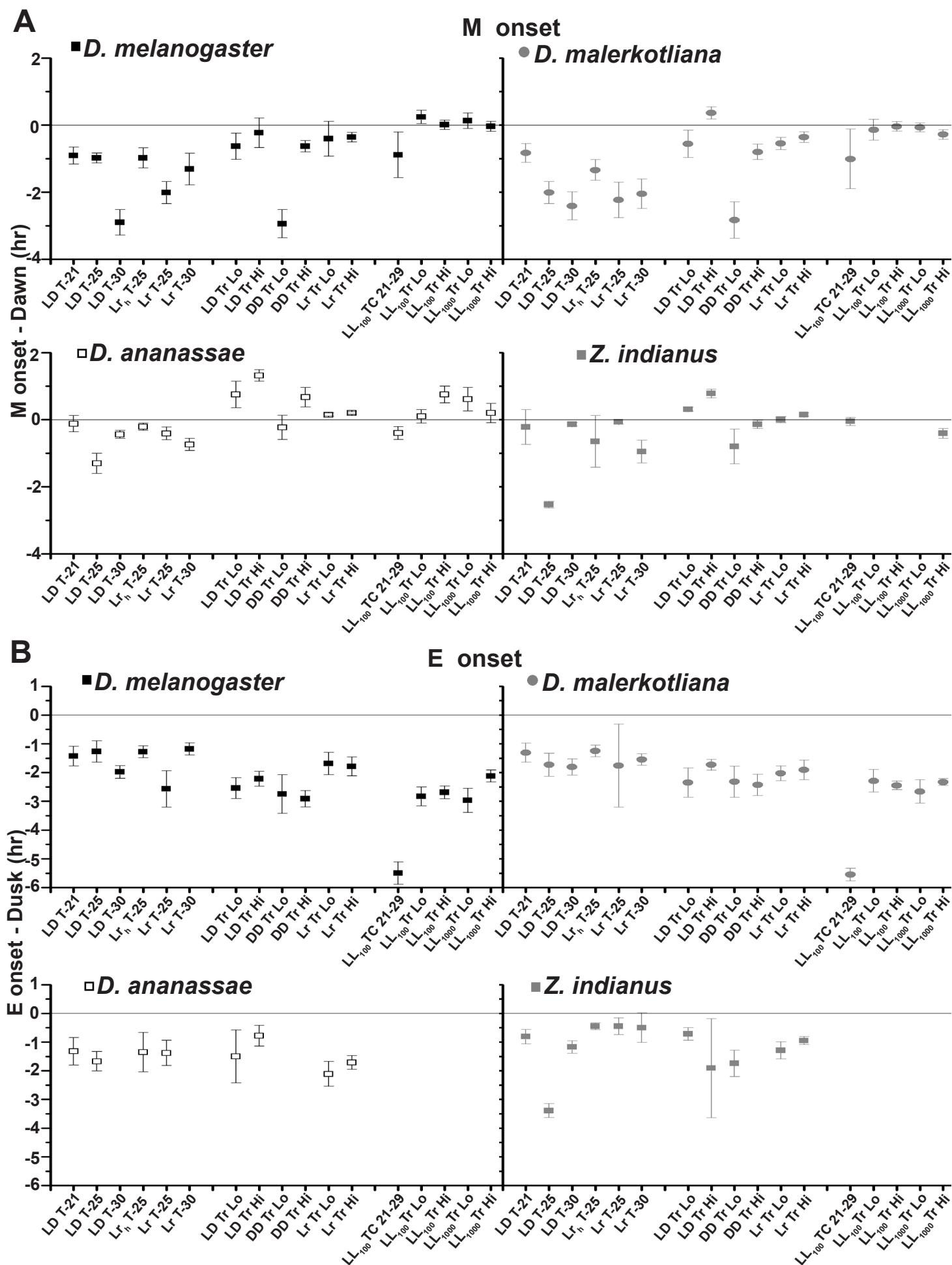
Figure 6. Phase of morning and evening activity onset under different regimes. (**A**) Mean phase of onset of morning activity peak relative to dawn. Horizontal solid line indicates dawn. (**B**) Mean phase of onset of evening activity peak relative to dusk. Horizontal solid line indicates dusk. Error bars are 95% CI.

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Population rhythm of adult emergence in three *Drosophila* species under laboratory and semi-natural conditions

Introduction

In most organisms daily environmental cycles modulate the rhythmic behaviours controlled by circadian clocks, thus enabling them to maximally exploit resources and to minimize the effects of adverse conditions (Pittendrigh, 1993; Saunders, 2002). In the previous chapters I have described studies that show sympatric species *Drosophila melanogaster* and *D. ananassae* differ in several features of their activity/rest rhythm (Prabhakaran and Sheeba, 2012). *D. melanogaster* exhibits a bimodal activity pattern whereas activity of *D. ananassae* is skewed towards morning, which persists under a range of photoperiods in the laboratory. Under laboratory conditions, *D. ananassae* is most active at the beginning of the light phase after which its activity tapers off as the day progresses. Thus, unlike *D. melanogaster*, *D. ananassae* does not exhibit 'siesta' during midday (Prabhakaran and Sheeba, 2012). Such differences also persisted across a range of seasons when assayed under semi-natural conditions (Prabhakaran and Sheeba, 2013). We hypothesized that these two relatively recently diverged sympatric species of *Drosophila* occupy different temporal niches due to the differences in their underlying circadian clocks. In this chapter I present the results of studies that were aimed at examining whether the above mentioned differences in activity/rest rhythm extends to another circadian behaviour - adult emergence rhythm. Although it has long been hypothesised that emergence of fruit flies peaks at dawn to coincide with maximum humidity levels (Pittendrigh, 1954), there is no clear evidence for such an adaptive response. Furthermore, it is known that there are other insects whose emergence is restricted to daytime when humidity levels are low (Saunders, 2002). Hence we examined the pattern of adult emergence of *D. ananassae* whose activity is phased predominantly towards the early part of the day, a time during which *D. melanogaster* activity falls dramatically. These studies were carried out along with another sympatric species *D. malerkotliana* under laboratory conditions

(LAB) and under semi-natural conditions (SN) that were created in an outdoor facility (De et al., 2012). *D. malerkotliana* flies exhibited an activity/rest rhythm very similar to *D. melanogaster* flies under LAB 12:12 hr light/dark cycles (LD) and constant darkness (DD) regimes (Chapter 2). For each species large random mating populations that have been relatively recently caught from localities in Bangalore (12°59'N 77°35'E) and maintained in the LAB were used.

We also attempted to explore how environmental factors across seasons shape adult emergence of Drosophilids by conducting studies at five different times of the year. Although at this location we do not experience large changes in photoperiod, seasons are marked by changes in absolute values of temperature and relative humidity as well as in the day/night variation of these environmental variables. We refer to the studies done outside the laboratory as semi-natural conditions throughout, since we acknowledge that our method does not capture the behaviour under truly natural conditions. In *D. melanogaster*, the act of adult emergence has been shown to be clock-controlled and entrainable to daily cycles of light and temperature (Saunders, 2002). Under standard LAB protocols (LD), emergence is gated in such a manner that it is largely restricted to daytime with a sharp peak around dawn (Kumar et al., 2007). One popular hypothesis regarding the circadian regulation of emergence at dawn stresses upon the importance of temperature and humidity as key factors (Pittendrigh, 1993). Recently we have demonstrated that under SN most of the emergence occurs during early morning which is also the time when temperature is low and relative humidity is high (De et al., 2012). Similar to what has been seen with activity/rest rhythm (Bhutani, 2009; De et al., 2013; Menegazzi et al, 2012; Prabhakaran and Sheeba, 2013; Vanin et al., 2012) several features of emergence rhythm also differ between SN and LAB (De et al., 2012). Under SN, the gate-width of adult emergence rhythm and nighttime emergence of flies decreased

significantly compared to LAB, which suggests that natural environmental cycles probably exert greater pressure upon the gating of this rhythm than those of the LAB. This may be due to the presence of multiple, gradually varying time-cues (Zeitgebers) of relatively higher amplitude in SN unlike LAB, where the only Zeitgeber is white light (ON/OFF) of relatively low intensity (~100 lux), whose wavelength composition is also constant.

A preliminary attempt to examine the effect of seasonal variations in environmental factors on adult emergence of *Canton-S* (CS) strain of *D. melanogaster* flies revealed that during harsh conditions, much of the emergence occurs between late night to early morning, whereas during milder weather conditions, emergence continues until afternoon (De et al., 2012). The study also found that under milder conditions, the number of CS flies emerging during the day was correlated to daily changes in the light intensity but not with temperature or humidity (De et al., 2012). During harsh conditions, the same was correlated to daily changes in humidity and temperature but not to light (De et al., 2012). A careful inspection of emergence during dawn revealed that the number of flies emerging during the morning hours (between 4-10 hr) is positively correlated with changes in the average light intensity (De et al., 2012). Since these correlations were based on only one strain of *D. melanogaster* (w^{1118}) it has only a limited value in revealing how environmental variables influence the emergence rhythm of flies. Therefore, to obtain greater insight on how natural environment influences emergence, we used a comparative approach and examined this rhythm in three Drosophilid species under SN. The assays were spread across six months with greater variation in environmental factors than before (De et al., 2012), including the coolest and warmest times of the year in Bangalore, India.

Previous studies have shown that differences among strains of *D. melanogaster* in activity/rest and emergence rhythm were significantly reduced when studied under SN

compared to LAB (De et al., 2012; Vanin et al., 2012). Flies carrying a mutation in the

period gene (*per[0]*), an important circadian clock gene showed rhythmic emergence in SN, much like wild-type strain (De et al., 2012), and their activity/rest pattern was also very similar - especially with respect to the morning component (Vanin et al., 2012). However, *D. melanogaster* flies that have evolved precise circadian clocks showed greater divergence in emergence pattern from their controls when assayed under SN (Kannan et al., 2012). A similar enhancement of difference in phasing of the peak of emergence was seen under SN between two sets of populations of flies selected to emerge either in the morning or late in the evening when studied under SN (Vaze et al., 2012). We asked whether there is any difference in the emergence rhythm among closely related species of *Drosophila* in the LAB, if yes, whether that extends to different seasons in SN as well. We found that even though the three species (*D. melanogaster*, *D. malerkotliana* and *D. ananassae*) showed differences in their emergence rhythm in the LAB, under SN such differences were considerably reduced. These results suggest that factors in the natural environment that influence emergence have an overriding effect on this behaviour which nullifies any functional difference in rhythmic behaviour that each species is able to exhibit in the LAB.

Materials and Methods

Fly strains. *D. melanogaster*, *D. malerkotliana* and *D. ananassae* flies were caught from wild using fruit-traps as bait and net sweeps within Bangalore, India, between 2004-2005. To prevent random genetic drift and founder effects, these flies were maintained as large random mating populations with roughly 1:1 sex ratio of ~1200 individuals. Stocks were maintained under LD12:12 (~1.5 W/m^2) conditions at constant temperature (~25 ℃) and humidity (~70%) with a discrete-generation cycle of 21 days on cornmeal medium.

Adult emergence assay. Assays were conducted in three different conditions- laboratory light 12 hr: dark 12 hr (LD) at 25 ℃, laboratory constant darkness (DD) at 25 ℃ and semi-natural (SN) conditions. From population cages of *D. melanogaster*, *D. malerkotliana* and *D. ananassae*, approximately 300 eggs were collected and placed into each glass vial with ~10 ml of food. Ten such vials were used per species per condition. Vials were monitored for darkening of pupae and emergence of the first fly at approximately 6 hr intervals. Upon emergence of the first few flies, the vials were monitored at 2 hr intervals and adults were cleared from the vials and counted. Assays under SN were conducted in an outdoor enclosure kept under a canopy within JNCASR campus (De et al., 2012) during five different months- March, April, November and December-2012 and February-2013. In parallel the daily profiles of light, temperature, and humidity under SN were monitored using DEnM, Trikinetics, USA. Unlike light intensity and temperature, humidity profile outside the vials is likely to be different from what the developing flies experience inside the vials, which were also plugged with cotton.

Analysis of emergence data. Emergence profiles of each species were plotted by averaging daily profiles of 10 replicate vials for successive days. To compare emergence rhythm across species in laboratory conditions, we quantified several properties of the rhythm - gate-width, onset of emergence, peak phase, variance in peak timing and percentage of nighttime emergence (LD) for each vial. Gate-width was estimated as the time-interval between start and end of emergence in one complete cycle (using 5% of total emergence in that cycle as cut-off). The onset of emergence was determined from the daily profiles of each vial as the first bin above the 5% cut-off. Peak(s) of emergence were determined from daily profiles of each vial using analysis of variance (ANOVA) with time-point as fixed factor, followed by post-hoc multiple comparisons using Tukey's HSD test. Period was estimated as the cycle-to-cycle time interval between two emergence peaks. Variance in peak-timing was estimated as

day-to-day variation in timing of emergence-peak in each vial, averaged over replicate vials. One-way ANOVA followed by post-hoc multiple comparisons using Tukey's HSD test was performed to evaluate statistically significant differences across species in LD and DD separately for gate width, onset of emergence, period, peak phase, peak amplitude and nighttime emergence. Under SN conditions, to compare emergence rhythm across species and months, we quantified several parameters of the rhythm - gate-width, phase of onset of emergence, peak timing, % nighttime emergence and variance in peak timing. The duration from 22:00 hr to 4:00 hr was considered as 'nighttime' since the DEnM monitor did not register values above 0 lux light intensity. Percentage nighttime emergence was averaged across vials and cycles. The gate-width, phase of onset of emergence, peak phase, day-to-day variance in peak phase, peak amplitude and nighttime emergence data were subjected to separate two-way ANOVA to examine the main effect and interaction of species and assay month. Non-parametric Spearman's rank order correlation test was applied on the following pairs of datasets : gate width versus maximum, minimum, average day and average night temperature and humidity (T_{max}, T_{min}, $T_{ave\ day}$, $T_{ave\ night}$, H_{max}, H_{min}, $H_{ave\ day}$ and $H_{ave\ night}$); gate width versus maximum and average day light intensity (L_{max} and $L_{ave\ day}$); onset phase of emergence versus temperature, humidity and light values (T_{max}, T_{min}, $T_{ave\ day}$, $T_{ave\ night}$, H_{max}, H_{min}, $H_{ave\ day}$, $H_{ave\ night}$, L_{max} and $L_{ave\ day}$); peak phase of emergence versus temperature, humidity and light values (T_{max}, T_{min}, $T_{ave\ day}$, $T_{ave\ night}$, H_{max}, H_{min}, $H_{ave\ day}$, $H_{ave\ night}$, L_{max} and $L_{ave\ day}$). Error bars shown in the emergence profiles are ± SEM. Error bars in all other graphs are 95% Confidence Interval (± 95% CI). All statistical tests were done using STATISTICA-7 (StatSoft Inc., USA) with level of significance set to $p < 0.05$.

Results

Under laboratory conditions adult emergence rhythm of the three Drosophilid species show differences in temporal distribution. This being the first report of emergence rhythm for the

two species *D. ananassae* and *D. malerkotliana*, we began by examining whether this behaviour is indeed rhythmic and whether the rhythm is similar to the well-studied species *D. melanogaster.* All three species *D. melanogaster*, *D. malerkotliana* and *D. ananassae* showed robust entrainment of emergence rhythm to LD cycles with period indistinguishably close to 24 hr (*D. melanogaster* - 23.8 ± 0.2 hr, *D. malerkotliana* - 23.7 ± 0.1 hr, *D. ananassae* - 24.2 ± 0.2 hr; Fig. 1A), and the rhythms persisted under DD with no species-specific difference in free-running period (Fig. 1B, C). Under LD, the onset of emergence was significantly delayed for *D. malerkotliana* compared to *D. melanogaster* and *D. ananassae* ($F_{2,27} = 11.3$, $p < 0.0003$; Fig. 1A, D) as determined from vial-wise data (see methods). Both under LD and DD conditions *D. ananassae* exhibited a significantly narrower gate-width of adult emergence than *D. melanogaster* (LD-$F_{2, 27} = 7.1$, $p < 0.003$; DD-$F_{2,27} = 7.2$, $p < 0.003$; Fig. 1E), while this difference from *D. malerkotliana* was statistically not significant. *D. ananassae* also exhibited advanced peak of emergence compared to *D. melanogaster* and *D. malerkotliana* ($F_{2,27} = 14.1$, $p << 0.001$; Fig. 1A, F). Nighttime emergence was significantly different in the three species and *D. ananassae* showed highest nocturnal emergence (LD-$F_{2,27} = 74.4$, $p << 0.001$; Fig. 1G). We also estimated the intra-species measure of day-to-day variation in phase of emergence peak as a read-out of the accuracy of the emergence peak and did not detect any difference in this measure among the three species (Fig. 1H). The assay was conducted under LD twice with similar outcome (data not shown). Thus under LAB conditions the emergence rhythm of these three species differed from each another in terms of the onset of emergence, peak of emergence, nighttime emergence and gate-width, although many other features of the rhythm were similar across species.

Seasonal variations in temperature and humidity. Since the natural environment contains a large number of simultaneously varying time cues, we asked whether the emergence rhythm of these three species may adopt different phase-relationships with such cues thus exhibiting temporal separation between the species. We assayed the rhythm under SN during five different months between 2012 and 2013 representing summer and winter conditions at this latitude. During this study the weather conditions varied especially in terms of temperature and humidity although light intensity at the study site was not different in four out of the five assays. The extreme high intensity light in one of the assays was due to the clearing of canopy above the enclosure and does not reflect a season-specific change. The temperature and humidity conditions in the five months during which our study was performed are summarized in Table 1. March and April-2012 were the warmest with maximum temperatures above 30 °C, and average daytime temperatures between 25 and 30 °C. These months also had lowest humidity levels. In November, humidity remained high throughout with average day and nighttime humidity above 80%. On the contrary, the average day and nighttime humidity in the other three months were around 60%. Due to a technical fault, humidity was not recorded in the month of December-2012. As expected the amplitude of daily oscillation in temperature was low in winter compared to summer, whereas phase of light onset and humidity trough remained mostly unaffected by season (Table 1, Fig. 2). Since light intensity varied greatly depending on the extent of canopy it was not used to assess how harsh or mild the weather was in a particular month. Based on the temperature and humidity values, March and April conditions were considered as harsh and November, December and February as mild.

The three species responded similarly to seasonal variations in the natural environment.
We measured several properties of emergence rhythm in the three species across five different

months under SN (Figs. 2, 3). *D. melanogaster*, *D. malerkotliana* and *D. ananassae* exhibited similar emergence pattern under SN except that during certain months all the three species showed some variations with respect to the number of cycles of emergence (Fig. 2). Furthermore, a delay in initiation of emergence was seen in some months for *D. melanogaster* (March, November, December) and *D. malerkotliana* (December, February). We did not see any consistent pattern in this delay based on the three environmental factors that we monitored. Unlike LAB assays, gate-width of emergence was not different among species but under SN, differed across months (narrower under harsh seasons; $F_{4,124} = 15.5$, $p << 0.001$; Figs. 2, 3A). We found that increase in temperature is associated with narrower gate-width of emergence in *D. malerkotliana* and *D. ananassae* - gate-width in *D. malerkotliana* and *D. ananassae* showed negative correlation with T_{max} (*D. malerkotliana* - $r = -0.7$; *D. ananassae* - $r = -0.5$, $p < 0.05$). High humidity levels probably enabled *D. malerkotliana* and *D. ananassae* flies to emerge in a broader window during the day- gate-width in *D. malerkotliana* and *D. ananassae* showed positive correlation with H_{max} (*D. malerkotliana* - $r = 0.6$; *D. ananassae* - $r = 0.6$, $p < 0.05$). Although humidity is usually inversely correlated with temperature we cannot rule out the combined action of the two. For *D. melanogaster*, such correlations of temperature and humidity with gate-width did not reach statistical level of significance. Onset of emergence was clearly affected by season ($F_{4,124} = 69.7$, $p << 0.001$), as evidenced by the delayed onset in February-2013 for all the three species with no difference between them (Figs. 2, 3B). As temperature increased, there was an advance in the phase of onset of emergence in all the three species- onset of emergence showed a negative correlation with T_{min} and $T_{ave\ night}$ (T_{min} *D. melanogaster* - $r = -0.6$, *D. malerkotliana* - $r = -0.6$; *D. ananassae* - $r = -0.7$; $T_{ave\ night}$ *D. melanogaster* - $r = -0.6$; *D. malerkotliana* - $r = -0.5$; *D. ananassae* - $r = -0.7$, $p < 0.05$). Similar to onset of emergence, peak of emergence

was also affected by season ($F_{4,124} = 73.9.7$, $p << 0.001$) and there was no difference among the three species (Figs. 2, 3C). Peak of emergence was also advanced with increase in nighttime temperature- peak of emergence showed negative correlation with T_{min} and $T_{ave\ night}$ (T_{min} *D. melanogaster* - $r = -0.7$; *D. malerkotliana* - $r = -0.8$; *D. ananassae* - $r = -0.8$; $T_{ave\ night}$ *D. melanogaster* - $r = -0.8$; *D. malerkotliana* - $r = -0.7$; *D. ananassae* - $r = -0.7$, $p < 0.05$). We found that increased nighttime humidity was associated with an advance in the phase of emergence-peak ($H_{ave\ night}$ *D. melanogaster* - $r = -0.7$; *D. malerkotliana* - $r = -0.7$; *D. ananassae* - $r = -0.7$, $p < 0.05$) although we cannot conclude a causal role for humidity levels in modulating emergence from these results. During November, December-2012 and February-2013, the peak of emergence shifted towards the day probably because favourable conditions persisted past dawn (Figs. 2, 3C). Our studies show that nighttime emergence was greater across species during comparatively warmer and drier days (except in *D. ananassae* during December-2012) (Figs. 2, 3D). There was no difference among species in the nighttime emergence which differed among months ($F_{2,129} = 36.6$, $p << 0.001$; Fig 3D). Fraction of flies emerging in the nighttime was significantly higher in the month of April-2012 compared to all other months (including March-2012) in case of *D. melanogaster* and *D. malerkotliana*, while *D. ananassae* had similar fraction of flies emerging during nighttime across months (Fig. 3D).

Day-to-day variation in peak timing was greater in relatively milder conditions especially for *D. melanogaster* and *D. malerkotliana* (Figs. 2, 3E) and does not differ among species in any given month, although it did differ across months ($F_{4,116} = 16.16$, $p << 0.001$). A significant interaction between species and months ($F_{8,125} = 3.64$, $p < 0.001$) was detected probably due to the fact that unlike *D. melanogaster* and *D. malerkotliana* flies, *D. ananassae* did not show any reduction in variance in the harsher months of March and April (Figs. 2,

3E). Since the reduction in day-to-day variance in phase of peak emergence could be a trivial consequence of reduced variation in environmental conditions, we examined variance in the $T_{ave\ day}$ during all the five months and found that there was no such reduction during the harsh months (similar SEM values, Table 1). As the weather conditions became milder between November-2012 to February-2013, the amplitude of peak of emergence was reduced ($F_{4,129} = 74.97$, $p < 0.001$). Amplitude of the peak also differed across species ($F_{2,129} = 4.38$, $p < 0.01$) with significant interaction between species and months ($F_{8,129} = 6.92$, $p < 0.001$). This reduction in the peak amplitude can be considered as a by-product of broadening of the gate-width of emergence under milder conditions.

In summary, adult emergence rhythm of the three species (*D. melanogaster*, *D. malerkotliana* and *D. ananassae*) differ under LAB (LD and DD), however, such differences were not detectable in SN. This is possibly due to the presence of stronger and richer time cues in nature.

Discussion

Although early studies on circadian rhythms in insects employed a wide variety of species (Saunders, 2002), over the past few decades *D. melanogaster* has become the most widely used Drosophilid to study circadian clocks due to the development of various genetic tools and the availability of mutants. More recently few studies have explored other Drosophilids which shed some light on how they differ among each other in terms of their clock properties and rhythmic behaviour (Bahn et al., 2009; Hermann et al., 2013; Prabhakaran and Sheeba, 2012; Prabhakaran and Sheeba, 2013). We investigated adult emergence behaviour in three closely related Drosophilids (Crosby et al., 2007; Yang et al., 2012), under various environmental conditions in both LAB (LD and DD) and SN (harsh and mild seasons) to investigate whether there is any inter-species difference in their emergence patterns.

Although differences in mating or feeding are more likely to promote speciation, we reasoned that the differences in activity may be a reflection of the ability of *D. ananassae* flies to tolerate harsh environmental conditions of midday and therefore *D. ananassae* may have also differed in their emergence pattern. Our studies were carried out on three species of Drosophilids that have been relatively recently (2004-2005) caught from the wild, from locations within Bangalore, India and therefore can be considered sympatric; however, the possibility that they occupy different micro-habitats cannot be ruled out. Previously we have reported the temporal separation of activity rhythm in *D. melanogaster* and *D. ananassae* under LAB and SN (Prabhakaran and Sheeba, 2012; Prabhakaran and Sheeba, 2013), here we report that the adult emergence rhythm differs only in the LAB. Furthermore, while we had reported earlier that free-running period of activity/rest rhythm of *D. melanogaster* is greater than *D. ananassae* (Prabhakaran and Sheeba, 2012), this difference did not extend to the period of adult emergence rhythm (Fig. 1C). Moreover, *D. melanogaster* and *D. malerkotliana* showed almost similar adult emergence pattern both under LD and DD (Fig. 1) much like their activity/rest pattern (Prabhakaran and Sheeba, 2013). Even though there are no studies to the best of our knowledge that unravel the phylogenetic relationship between *D. melanogaster* and *D. malerkotliana*, it is clear from our studies that these two species may have similar circadian organization.

When studied under SN at five different months, the interspecies differences in the adult emergence rhythm reduced to a great extent. However, they all showed changes in their emergence rhythm consistent with variations in environmental conditions and they responded to changes in the environment very similarly. This is not surprising in the light of recent studies on activity/rest rhythm in which factors in natural environment was shown to dominate the behaviour more than the genotype and even the circadian clock mutant flies *per^0* showed activity/rest pattern very similar to the wild type flies (De et al., 2013; Menegazzi et

al, 2012; Vanin et al., 2012). In case of adult emergence also, the arrhythmicity in per^0 mutants seen in the LAB was partly rescued under SN (De et al., 2012). Here we show that even if such differences in emergence rhythm exist among the three related species of *Drosophila*, they are overridden by natural environmental factors. However, in another long-term study in which we assayed the activity/rest rhythm of these three species under SN across seasons over a span of 1.5 yrs revealed that *D. ananassae* continued to be diurnal similar to their LAB behaviour, suggesting that the overwhelming effect of natural environment cannot not be generalised to all circadian behaviours (Prabhakaran and Sheeba, 2013).

Temperature appears to play a major role in gating adult emergence rhythm in *Drosophila*, and under harsh or high temperature-low humidity conditions, flies of all three species avoid emerging during the later part of the day similar to previous reports on *D. melanogaster* (De et al., 2012). We find that gate-width of only *D. malerkotliana* and *D. ananassae* was reduced with increasing temperature while this was not apparent in *D. melanogaster*. It is likely that this reduction is due to the high amplitude cycling of temperature during the warmer months of March and April in contrast to November. While a previous LAB study on *D. melanogaster* has shown that low amplitude warm/cold cycle (29/25 ^{o}C) does not alter the gate-width from that of a constant 25 ^{o}C regime (Kannan et al., 2012), higher amplitude cycles (28/18°C) can reduce gate-width by ~4 hr (Nikhil KL and Sharma VK, personal communication). Yet another study has shown both theoretically and empirically that gate-width of *D. melanogaster* is likely to widen with increase in ambient temperature (Mukherjee et al., 2012). Our studies reveal that under natural conditions, across months where temperature fluctuations were as high as13 ^{o}C (March, February) or as low as 5 ^{o}C (November), gate-width was not significantly altered. Onset and peak of emergence was also affected by temperature and humidity in such a way that during drier and hotter days,

flies emerged earlier perhaps to avoid harsh conditions (De et al., 2012). Even though humidity levels showed significant correlation with the emergence properties, we acknowledge that its values recorded from the enclosure may not reflect those inside the glass vials in which the pupae developed, due to the constant presence of food medium. Unlike previous studies under SN, our study did not show correlation of light with any of the features of the emergence rhythm (De et al., 2012). This could be because in our assays, light intensity did not vary much across the months (except February-2013, Table 1).

Thus our studies performed under both LAB and SN on the adult emergence rhythm of three closely related Drosophilids - *D. melanogaster*, *D. malerkotliana* and *D. ananassae*, suggests that (1) inter-species differences in the properties of one circadian behaviour need not be reflected in another, (2) the difference in a particular rhythmic behaviour seen under the simplified LAB environment may not manifest under SN due to overriding effects of strong natural time cues. This also underscores the point that while studying behaviour of species under more natural-like conditions one must exercise caution in interpreting the results as it is not easy to separate the clock-controlled phenotypes from mere masking due to the presence of multiple strong environmental factors.

**Table 1A.** Maximum and minimum of environmental factors across days (mean ± SEM).

Assay	Light (lux)	Temperature (^{o}C)		Humidity (%)	
	Max	Max	Min	Max	Min
March 2012	482.0 ± 16.5	31.7 ± 0.1	18.7 ± 0.6	79.3 ± 2.2	27.3 ± 2.7
April 2012	237.7 ± 5.8	33.5 ± 0.2	22.2 ± 0.9	79.7 ± 2.0	34.3 ± 1.8
November 2012	359.0 ± 16.9	25.1 ± 0.8	20.2 ± 0.2	94.3 ± 1.1	69.5 ± 4.5
December 2012	484.2 ± 17.6	27.2 ± 0.2	16.6 ± 0.3	-	-
February 2013	2375.0 ± 28.5	28.7 ± 0.3	15.7 ± 0.9	84.4 ± 1.9	30.0 ± 2.9

-Humidity values for December 2012 were not collected due to a technical fault.

Table 1B. Average values of environmental factors during day and nighttime across days (mean ± SEM).

Assay	Light (lux)	Temperature (^{o}C)		Humidity (%)	
	Average day	Average day	Average night	Average day	Average night
March 2012	257.4 ± 16.8	27.0 ± 0.1	23.1 ± 0.2	42.4 ± 2.6	61.8 ± 5.5
April 2012	142.2 ± 4.8	29.6 ± 0.3	25.5 ± 0.2	51.3 ± 0.9	63.9 ± 1.6
November 2012	171.7 ± 18.2	21.8 ± 0.4	22.3 ± 0.6	81.2 ± 2.4	87.3 ± 0.6
December 2012	239.2 ± 3.7	23.1 ± 0.3	19.6 ± 0.1	-	-
February 2013	1138.8 ± 23.5	24.0 ± 0.3	19.9 ± 0.7	49.9 ± 3.2	61.8 ± 3.3

-Humidity values for December 2012 were not collected due to a technical fault.
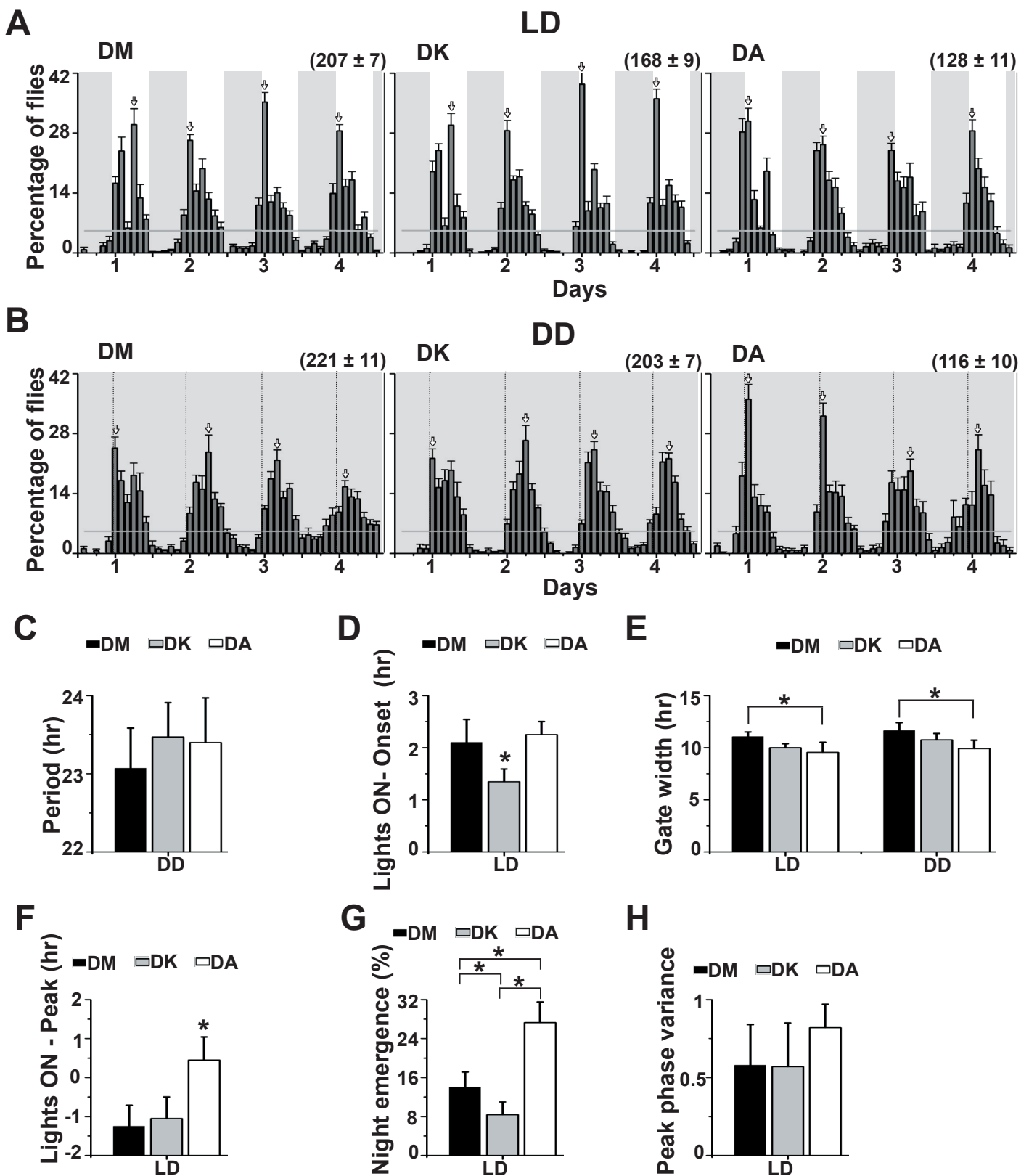
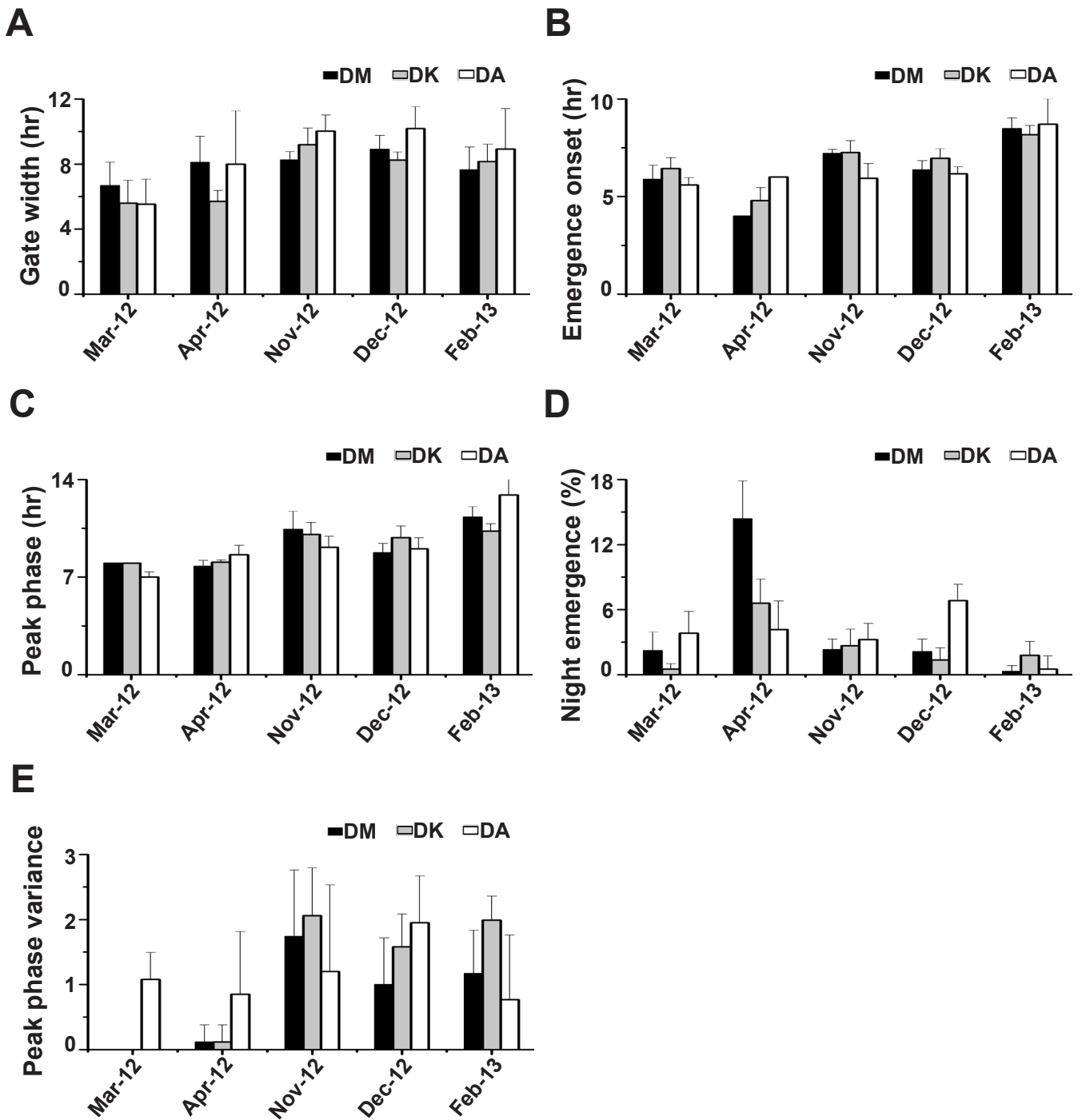**Figure 1. Adult emergence rhythm of *D. melanogaster* differed from *D. ananassae* under laboratory conditions.** (**A**) Average adult emergence profiles (% of flies emerged/ 2hr ± SEM, for each species averaged across 10 vials) of *D. mela-nogaster* (DM), *D. malerkotliana* (DK) and *D. ananassae* (DA) under LD 12:12. Grey shaded areas in the average profiles indicate darkness and 5% of the emergence is denoted by the grey horizontal line. Arrows indicate the peak for each cycle in this average profile. Values in parentheses indicate the total number of flies emerged averaged across 10 vials (± SEM). (**B**) Average adult emergence profiles of DM, DK and DA under DD (averaged across 10 vials ± SEM). Dotted lines indicate phase of lights-ON in the previously experienced LD regime. All other details are same as in panel A. (**C**) Average period based on onset of emergence (± 95% CI, averaged across vial) of DM, DK and DA under DD. (**D**) Average phase of onset of emer-gence (Time of onset - lights-ON ± 95% CI, averaged across 10 vials) of DM, DK and DA under LD. (**E**) Average gate-width of emergence (± 95% CI, averaged across 10 vials) of DM, DK and DA under LD and DD. (**F**) Average phase of the peak of emergence (Time of peak - lights-ON ± 95% CI, averaged across 10 vials) of DM, DK and DA under LD. (**G**) Average per-centage of nighttime emergence (± 95% CI, averaged across 10 vials) of DM, DK and DA under LD. (**H**) Average day-to-day variation in peak emergence under LD estimated for each vial (n = 10 vials). *p < 0.05.
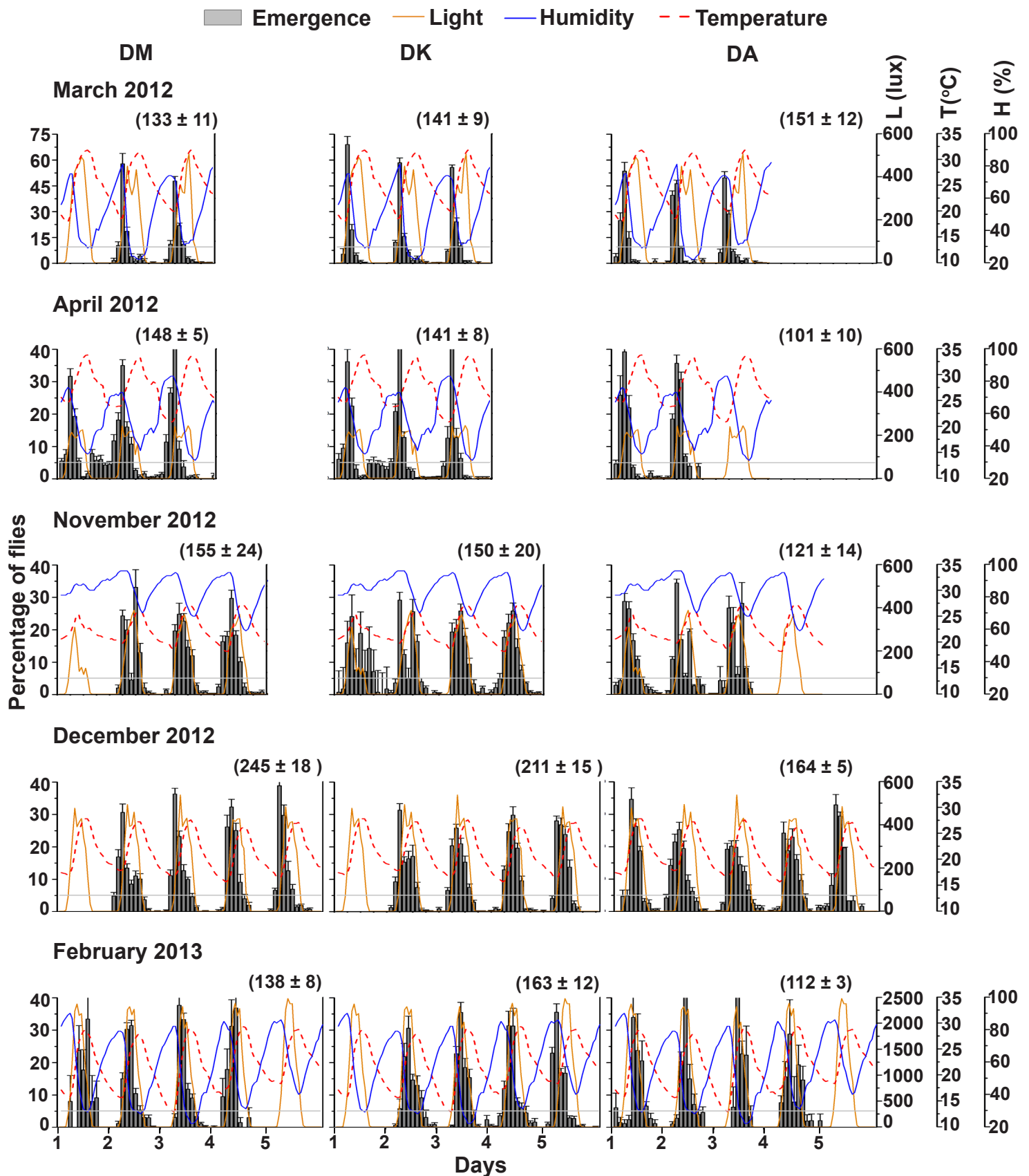
Figure 2. Seasonal variation in adult emergence rhythm of three Drosophilids under semi-natural conditions (SN). Average profiles of adult emergence rhythm (percentage of flies emerged/ 2hr ± SEM, for each species averaged across 10 vials) of DM, DK and DA during five different months of the years 2012 (March, April, November and December) and 2013 (February) under SN is plotted along with environmental factors light (L, orange-solid curve), temperature (T, red-dashed curve) and humidity (H, blue-solid curve). Values in parentheses indicate the total number of flies emerged averaged across 10 vials (± SEM).

Figure 3. Properties of adult emergence rhythm of three Drosophilid species under semi-natural conditions (SN). (**A**) Average gate-width of emergence (averaged across 10 vials) of DM, DK and DA. (**B**) Average phase of onset of adult emergence (external time, averaged across 10 vials) of DM, DK and DA. (**C**) Average phase of adult emergence peak (external time, averaged across 10 vials) of DM, DK and DA. (**D**) Average percentage of nighttime emergence of flies (averaged across 10 vials) of DM, DK and DA. (**E**) Day-to-day variance in peak phase of emergence (averaged across 10 vials) of DM, DK and DA. Error bars are 95% Confidence Interval (± 95% CI).

Visual observation of behaviours of *Drosophila* species *melanogaster* and *ananassae*

Introduction

Drosophila melanogaster display bimodal pattern of locomotor activity under 12:12 hr laboratory light/dark conditions (LD 12:12), with two peaks, coinciding with lights-ON and lights-OFF (Hamblen-Coyle et al., 1992). Several organisms ranging from invertebrates to mammals display such bimodality in activity patterns (reviewed in Saunders, 2002; Dunlap et al., 2004). One explanation that can be offered for such bimodality is that activity at these times correspond to dawn and dusk in the real world, which is also the time when organisms are least likely to experience potentially harmful high temperatures and low humidity. A previous study found differences in the ability to entrain to long photoperiods in the laboratory at different ambient temperatures among strains of *D. melanogaster* caught from the wild from Northern and Southern latitudes (Rieger et al., 2012). This difference in their ability to entrain, estimated on the basis of the ability of the evening activity peak to flexibly shift in response to ambient temperature and photoperiod, was attributed to the fact that Northern and Southern strains have probably evolved to deal with different environmental conditions (Rieger et al., 2012). Nevertheless, all strains showed the bimodal activity pattern previously described for the laboratory strain *Canton-S*, suggesting that the pattern is typical of this species. On the other hand, a distantly related species *D. virilis*, thought to have diverged from *D. melanogaster* about 63 million years ago (Tamura et al., 2004) showed significantly reduced morning activity compared to *D. melanogaster* (Bahn et al., 2009). It was suggested that this difference in activity pattern of *D. virilis* and *D. melanogaster* may be due to the evolution of circadian clocks which best suit their respective habitats (*D. virilis* - East Asian origin; *D. melanogaster* - Afro-tropical origin; Bahn et al., 2009).

A comparison of *D. melanogaster* with a sympatric and closely related species *D. simulans* showed high degree of similarity in behavioural rhythm under a range of ambient temperatures, exhibiting a prolonged siesta under warm conditions and greater daytime

activity under cool conditions (Low et al., 2008). However, two other species *D. yakuba* and *D. santomea*, which are, also sister species within the *melanogaster* subgroup showed distinct pattern in their activity rhythm such that they continue to exhibit a significant midday siesta even under cool ambient temperatures (Low et al., 2008). This difference in behaviour was attributed to the geographic distribution of the latter two species, which are restricted to equatorial tropical regions where temperatures do not vary much. *D. melanogaster* and *D simulans*, although of similar origin are both cosmopolitan and currently also occupy temporal latitudes and hence are likely to encounter extremes of cold and warm temperatures during the course of a year whereas *D. yakuba* and *D. santomea* due to their equatorial distribution do not experience such extremes and probably therefore did not evolve behavioural and molecular mechanisms to deal with such contingencies (Low et al., 2008). Yet another study which examined activity rhythms of eleven non-cosmopolitan Drosophild species that are not human commensals inhabiting a range of latitudes (~19° N and ~60° S) in the North American continent found that species from more temperate latitudes exhibited relatively greater midday activity as compared to the southern species, and interestingly even within similar latitudinal ranges, species which breed and live in swampy microhabitats exhibit greater midday activity (Simunovic and Jaenike, 2006). The authors concluded that latitude and breeding site correlate with behavioural patterns, which may be a reflection of differences in desiccation stresses that these species experience (Simunovic and Jaenike, 2006). Thus, even within the genus *Drosophila*, daily activity pattern varies considerably, suggesting that the bimodal daily activity pattern of *D. melanogaster* is only one among the various temporal programmes that flies may adopt in the face of various ecological and evolutionary constraints.

We have also shown previously that a close relative and a sympatric species of *D. melanogaster*, *D. ananassae* displays unimodal activity pattern with maximum activity after

lights-ON and a weak evening activity peak (Prabhakaran and Sheeba, 2012). This preference for morning activity of *D. ananassae* persisted under temperature cycles in the laboratory (chapter 3) and under a range of semi-natural conditions (Prabhakaran and Sheeba, 2013). Thus, *D. ananassae* clearly shows temporal separation of activity from *D. melanogaster*. Interestingly, a previous study has shown that the mating rhythms of these two species are also differently phased (Nishinokubi et al., 2006). Unlike *D. melanogaster*, most *D. ananassae* flies mated during subjective day under constant darkness (DD) (Nishinokubi et al., 2006). Even though *D. melanogaster* and *D. ananassae* displayed differences in yet another rhythmic behaviour namely adult emergence under laboratory LD12:12 regime, their emergence pattern under semi-natural conditions were largely similar (Prabhakaran et al., 2013), suggesting that in nature, environmental factors can cause the behaviours of these species to become synchronised.

Here we report the results of our studies which were aimed to confirm that differences in activity pattern of *D. ananassae* obtained using automated Drosophila Activity Monitors (DAM system) (Prabhakaran and Sheeba, 2012; Prabhakaran and Sheeba, 2013) are not artefacts of the assay method, by conducting visual observations of behaviours. Both species were observed at specific intervals throughout the day and/ or night both in the laboratory and under semi-natural conditions and several aspects of their behaviour were noted (chronoethogram, see method). We also asked whether *D. ananassae* differs from *D. melanogaster* in sensitivity to high light intensity or desiccation, thus allowing the former to exhibit higher midday activity. Our study using visual observations confirmed the results obtained from the DAM system; however, *D. ananassae* flies were, contrary to our expectations, found to be less tolerant to desiccation compared to *D. melanogaster*.

Materials and methods

Fly strains. *D. melanogaster* and *D. ananassae* flies were caught from the wild during 2005 from Bangalore, India (12°59'N 77°35'E) and maintained as large random mating populations in the laboratory under 12:12 hr light:dark cycles (henceforth LD12:12) at constant temperature ~25 ℃ and ~70% relative humidity.

Automated activity recording. For all experiments virgin male flies of age 2-3 days were used (except when specified). Flies were placed individually into glass tubes (3 mm inner diameter and 65 mm long) and recorded using *Drosophila* activity monitors (DAM IV) that record locomotor movement of flies across the length of the glass tube, particularly movement in the middle of the tube where an infra-red (IR) emitter-sensor pair is situated such that movement of the fly breaks the IR beam (TriKinetics, Waltham, USA; www.trikinetics.com). For studies under semi-natural conditions (SN), DAM monitors were placed in an outdoor enclosure ($122\times122\times122$ cm^3) with grids (6×6 cm^2) within the JNCASR campus in Bangalore (De et al., 2012). This enclosure allows free flow of air, and only the top portion is covered with a translucent plastic sheet. An environmental monitor (DEnM, Trikinetics, USA) was used to record daily profiles of light, temperature and relative humidity. For a subset of experiments, a shelf within the enclosure which received high levels of light and another shelf where the canopy cover was highest were chosen to record activity under high and low light intensities respectively (Fig. 5A).

Analysis of locomotor activity from DAM system. Activity was recorded in 5 min bins. Raw time series data from individual flies were further binned into 15 min and activity profiles were obtained by averaging raw activity counts across days for each fly and then averaging across flies (Figs. 3A, 4A, 5A). For assays testing light intensity effects in the laboratory, average activity counts were estimated across 6 days. We then considered activity counts during 3 hr prior to lights-ON as pre-dawn activity, counts during 3 hr after lights-ON as post-dawn activity and counts during 3 hr before lights-OFF as pre-dusk activity for each

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individual fly. Pre-dawn, post-dawn, and pre-dusk activities under 0.1 lux and 1000 lux LD

12:12 were compared using Student's *t*-test, separately for each species. Under SN, activity

profiles and profiles of light, temperature, and relative humidity were obtained from 15 min

binned DEnM data and averaged across 4 days. Daytime, nighttime and proportion of

daytime activity to total activity were estimated by averaging activity counts of individual

flies during that time interval across 4 days and then averaging across flies. Separate 2-way

ANOVA were performed for daytime, nighttime and proportion of daytime activity with

species and light regime as fixed factors.

Visual observation of behaviours. Visual observation of behaviours was done either in glass

tubes (similar to those used for DAM system activity monitoring) or petri plates (Fig. 1A, B)

across the day at equal intervals of time as detailed below (chronoethogram). Three nearly

equally spaced zones were marked out on the glass tubes – 'near food', 'middle', and 'near

plug'. The tubes were placed horizontally on a flat tray and the location of the fly in the tube

(zone), and whether it was active or resting (1 or 0) was scored once every 2 hr throughout the

day and night for 5 consecutive days under LD12:12 at light intensity of 100 lux (~0.28

W/m^2) and temperature 25 ± 0.5 °C ($n = 16$ flies). A similar experiment was conducted under

SN (in parallel with DAM system recording) where flies were observed every 1 hr, only

during daytime for 5 consecutive days ($n = 32$ flies). For petri plate experiments, plastic

plates (90 mm diameter) were filled with a base of 2% agar up to about one-third its depth,

and a cube of standard cornmeal fly medium (~$2 \times 2 \times 0.5$ cm^3) placed in the centre of the

plate. Three types of plate assays were conducted with observations made every 2 hr for 5

days: (i) single virgin male fly was housed per plate ($n = 5$ flies), (ii) 3 males and 3 females

per plate ($n = 5$ plates), and (iii) 6 virgin males in a plate, where half the lid was shaded with

black chart paper such that half of the plate received light while the other half did not receive

direct light ($n = 5$ plates). Whether the fly was active or at rest and the location of the resting

fly (agar or food) was scored for single male fly assays. In the second assay type (male + female group) fraction of flies (out of 6) showing courtship related activities (wing expansion, chasing, and copulation) were scored in addition to fraction of flies showing locomotor activity or rest at a particular location. In the third type of assay the fraction of flies (out of 6 flies) spotted in the uncovered region of the plate was scored.

Analysis of visual behaviours. *Glass tube assays:* We estimated 'locomotion index' as the propensity of flies to show locomotion at a given time point. This was calculated by first assigning individual flies scores of 0 or 1 (resting or active) at a given time point and then averaging scores for a fly across five days. The value thus obtained for single flies were then averaged across flies ($n \sim 16$) for each time point (Figs. 1C, 3B). We assume that this locomotion index can serve as a proxy for activity levels. A 'zone index' was calculated as the propensity of a fly to occupy a particular zone along the length of the tube at a given time point, in a manner similar to the locomotion index. All fractional data were subjected to arcsine transformation. Separate 2-way ANOVA were carried out for each behaviour/zone (activity/ food, middle and plug zone) to determine time-of-day and species effects followed by Tukey's HSD test.

Single-fly plate assays: We estimated locomotion index in petri plates similar to the glass tube assay. Zone index was calculated only for resting flies because flies that were locomoting moved too frequently from one zone to other (agar/food) such that it did not reflect a tendency to occupy a particular zone, unlike in the tube assays. Separate 2-way ANOVA with time and species as fixed factors followed by Tukey's HSD test was performed to examine time-dependent and species-dependent effects on activity, rest-on-agar and rest-on-food (Fig. 1B, D). This experiment was conducted twice.

Grouped-flies plate assays: Fraction of flies (out of 6 flies) in a plate showing a particular behaviour - locomotion, rest-on-agar, rest-on-food and courtship were scored at each time

point and averaged across 5 days for a plate and then averaged across plates (Fig. 2). Each behaviour was analyzed separately using 2-way ANOVA with time and species as fixed factors followed by Tukey's HSD test (Fig. 2). *Shaded plate assays*: Fraction of flies (out of 6 flies) visible in the uncovered region of the half-shaded plate were scored at each time point and averaged across 5 days for a plate and then averaged across plates (Fig. 5C). This experiment was repeated twice. Two-way ANOVA with time and species as fixed factors was performed to analyze time-dependent preference of species for the uncovered region.

Desiccation tolerance assay. This assay was carried out on 4-day-old virgin male flies of *D. melanogaster* and *D. ananassae* under LD12:12 at light intensity of 100 lux (~0.28 W/m^2) and temperature 25 ± 0.5 °C. There were three treatments – (1) starvation, (2) desiccation + starvation and (3) severe desiccation + starvation. All the three treatments were carried out in glass vials (2 cm diameter and 9 cm length) with a circular sponge disc placed at a height of 6 cm from the base and the mouth of the vial sealed with parafilm. Each vial housed 7 virgin flies of one species with 5 replicate vials for each treatment per species. Flies were kept in vials with 1 ml of 2% agar medium for treatment type 1. For type 2, empty vials were used. For type 3, severe desiccation was imposed by adding 2 g of $CaCl_2$ above the sponge disc in an empty vial. The flies were checked for deaths (no movement of limbs on gentle tapping) and the number of flies alive in each vial was noted every hour or 30 min in cases of rapid deaths. The checks continued until all the flies in every vial of treatment type 2 (desiccation + starvation) for both species were dead. In each treatment, percentage of flies alive in each vial was calculated and averaged across vials for each time point and then averaged across vials. We estimated the time taken to reach 100% death in each vial in treatment type 2 and 3 for both the species. Two-way ANOVA with treatment type and species as fixed factors was performed to analyze the species and treatment dependent effect on survivorship. All

statistical tests were performed using STATISTICA-7 (StatSoft Inc., USA) with level of significance set to $p < 0.05$.

Results

Visual observation of activity pattern under LD confirmed daytime activity of D. ananassae.
To confirm the results of our previous studies that were obtained using the automated DAM system, we visually observed *D. melanogaster* and *D. ananassae* flies which were kept in glass tubes (similar to that used in DAM system) under LD12:12 (Fig. 1A). Two-way ANOVA revealed a statistically significant effect of species and time (species- $F_{1, 324} = 210.5$, $p < 0.00001$; time- $F_{11, 324} = 8.21$, $p < 0.0001$) and a statistically significant effect of species $\times$ time interaction ($F_{11, 324} = 4.04$, $p < 0.0001$) in locomotion (Fig. 1C). Similar to our findings using the automated DAM system, our visual observations suggested that overall, *D. melanogaster* flies exhibited higher activity compared to *D. ananassae* (Locomotion index: *D. melanogaster* = 0.7 ± 0.02, *D. ananassae* = 0.33 ± 0.02; Fig. 1C, orange solid lines). *D. melanogaster* flies exhibited greatest locomotor activity around lights-ON and lights-OFF with a dip in the middle of the day (ZT04 < ZT22 and ZT10) and midnight (ZT14-18 < ZT10 and ZT22; Fig. 1C, left). *D. ananassae* showed high activity coinciding with lights-ON following which levels gradually tapered-off (ZT00 > ZT02-22) with a small increase in activity around evening (ZT12 > ZT14; Fig. 1C, right). We detected very little nighttime activity of *D. ananassae* flies (ZT14-22; Fig. 1C, right).

To test for any species-specific tendency of flies to be located at a certain part of the experimental tube in a time-of-day dependent manner, a 2-way ANOVA was performed on each type of zone-index. There was no difference between the two species in the propensity of flies to be in the food zone; however, there seemed to be an effect of time of the day on the propensity of flies to occupy the food zone ($F_{11, 324} = 1.84$, $p = 0.046$; Fig. 1C, blue shaded bar). A significant species $\times$ time interaction ($F_{11, 324} = 3.7$, $p < 0.0001$) was seen, mostly due

to lowered food zone index of *D. melanogaster* flies during evening as compared to *D. ananassae* flies (ZT12). *D. melanogaster* showed greater propensity to occupy the middle zone compared to *D. ananassae* (*D. melanogaster* = 0.5 ± 0.02, *D. ananassae* = 0.3 ± 0.01; $F_{1, 324}$ = 38.3, $p < 0.00001$; Fig. 1C, cyan shaded bar). *D. ananassae* flies tended to spend more time in the plug region compared to *D. melanogaster* (*D. melanogaster* = 0.3 ± 0.02, *D. ananassae* = 0.5 ± 0.02; $F_{1, 324}$ = 43.2, $p < 0.00001$; Fig. 1C, hashed red bar). Both species showed difference in the propensity to occupy plug region during different times of the day (species × time interaction: $F_{11, 324}$ = 4.3, $p < 0.0001$; Fig. 1C). This was mostly due to reduced propensity of *D. melanogaster* flies to occupy the plug region during early nighttime (ZT14-20) compared to *D. ananassae*. Thus in addition to corroborating the findings using the DAM system under LD cycles we also obtained additional information regarding the preference for different regions within the activity tubes across time for these two species.

Since it is possible that the differences seen between the two species is merely an artifact of flies being placed in narrow glass tubes, and that *D. ananassae* flies are therefore exhibiting activity under potentially more stressful conditions than *D. melanogaster* for reasons unknown to us, we tested the flies in larger spatial arena (Fig. 1B). Two-way ANOVA on data from visual observations of flies in petri plates revealed a statistically significant effect of species ($F_{1, 72}$ = 14.7, $p < 0.001$), time ($F_{11, 72}$ = 2.21, $p < 0.05$), and species × time interaction ($F_{11, 72}$ = 2.2, $p < 0.05$). Similar to what was seen in the tubes, overall activity of *D. melanogaster* flies was observed to be higher than *D. ananassae* (*D. melanogaster* = 0.7 ± 0.04, *D. ananassae* = 0.52 ± 0.04; Fig. 1D). *D. melanogaster* flies exhibited higher activity around lights-ON and lights-OFF with a dip in the middle of the day (ZT00, ZT10 > ZT06) similar to what was observed in the tubes (Fig. 1D, left, orange solid line). *D. ananassae* flies displayed an overall greater activity during daytime compared to night (ZT00-12 > ZT14-20; Fig. 1D). A dip in activity occurred at ZT10 with a small

increase after lights-OFF (ZT12; Fig. 1D). While resting, *D. ananassae* spent more time on

agar compared to *D. melanogaster* ($F_{1, 72} = 49.5$, $p < 0.00001$), and there was no difference

among species in terms of time spent on food ($F_{1, 72} = 2.6$, $p = 0.11$; Fig. 1D). However, we

detect a statistically significant time-of-day effect ($F_{11, 72} = 2.0$, $p < 0.05$) and species × time

interaction ($F_{11, 72} = 3.1$, $p < 0.05$), which was due to *D. melanogaster* flies resting on food

mostly after dusk (ZT12-16), whereas *D. ananassae* flies spent very little time on food during

those times, resting there mostly between midnight and dawn (ZT20; Fig. 1D).

Since it would be reasonable to expect that behaviours of flies studied in mixed-sex

groups are remarkably different from flies studied in isolation, we asked how the activity

rhythm of the two species under study is altered under group conditions. We have shown

previously for a laboratory strain of *D. melanogaster* that visual observation of males and

females housed in groups and exposed to natural conditions was different from the pattern of

activity of isolated flies (De et al., 2013). Furthermore, this type of assay allowed us to

estimate the contribution of specific behaviours such as those related to courtship towards the

overall activity pattern (De et al., 2013). We used a similar method to compare the two

species under a more simplified laboratory LD condition (see methods) (Fig. 2). The

differences in locomotor activity pattern among the species were detectable even under

grouped conditions (species × time interaction: $F_{11, 96} = 3.7$, $p < 0.001$; Fig. 2A). *D.*

melanogaster flies as a group also displayed overall higher locomotor activity compared to *D.*

ananassae (*D. melanogaster* = 0.43 ± 0.02, *D. ananassae* = 0.22 ± 0.02; $F_{1, 96} = 99.9$, $p <$

0.00001; Fig. 2A). In case of *D. melanogaster*, higher proportion of flies exhibited activity

before dawn and dusk (ZT22 and ZT10), which reduced at midday (ZT06) and at night (ZT

14-18; Fig. 2A, left). In *D. ananassae*, higher proportion of flies exhibited activity at ZT22

and ZT12 with a dip at ZT10 and during night (ZT14-18; Fig. 2A, right). While resting, *D.*

ananassae flies spent more time on agar and food compared to *D. melanogaster* (Agar: $F_{1, 96}$ = 57.4, $p < 0.00001$; Food: $F_{1, 96}$ = 45.2, $p < 0.00001$; Fig. 2A).

We did not detect any difference between the two species in overall incidence of courtship related activities (species: $F_{1, 96}$ = 0.87, $p = 0.4$) while a significant time of day effect was seen across species (time: $F_{11, 96}$ = 15.93, $p < 0.0001$) due to higher proportion of flies from both species exhibiting courtship related activities around dawn (ZT00-06; Fig. 2B). Thus, we found that the overall activity pattern of *D. melanogaster* and *D. ananassae* was conserved under each of the arenas tested with *D. ananassae* being predominantly day-active and *D. melanogaster* showing bimodal activity pattern. Even though there were slight differences in the pattern of rest between single fly and group of flies, overall *D. ananassae* displayed greater rest compared to *D. melanogaster*. Furthermore, preliminary studies suggest that the difference in activity pattern between the two species is not due to difference in timing of their courtship activities.

***D. melanogaster* and *D. ananassae* exhibited difference in their activity pattern under SN conditions similar to that observed under LD as confirmed by visual observation.** We have shown previously that the activity patterns of *D. ananassae* and *D. melanogaster* differ even when they are studied in an outdoor enclosure where they receive naturally varying environmental cycles that are likely to be stronger time cues and could potentially reduce the differences between species (Prabhakaran and Sheeba, 2013). We asked whether the patterns of activity exhibited by *D. ananassae* under such SN conditions correspond to actual movement of flies along the length of the tube and whether they occupy specific regions of the glass tube at different times of the day by conducting visual observations at 1 hr intervals (only during daytime -6:00 to 18:00 hr). We simultaneously monitored activity of *D. melanogaster* and *D. ananassae* using the DAM under SN. We found that for both *D. melanogaster* and *D. ananassae* the activity peaks detected by DAM system correspond to

increased activity detected by visual observations (Fig. 3). *D. melanogaster* showed bimodal activity pattern with a morning and a prominent evening peak with a period of inactivity during midday (DAM system, Fig. 3A; visual observation real time 6-8 and 16-18 hr, Fig. 3B, left). DAM system data for *D. ananassae* detected most activity during daytime when light was present with considerable amount of morning activity and a small evening peak (Fig. 3A, right). Visual observations, which were limited to the daytime, also detected time-dependent differences in locomotion across species ($F_{12, 780} = 12.8$, $p < 0.00001$; Fig. 3B). Overall, *D. melanogaster* flies showed higher locomotor activity compared to *D. ananassae* (*D. melanogaster* = 0.53 ± 0.01, *D. ananassae* = 0.42 ± 0.01; $F_{1, 780} = 38.6$, $p < 0.0001$; Fig. 3B). Visual observation data of *D. ananassae* revealed significantly higher activity at midday (between 11-14 hr compared to 15-17 hr).

Unlike laboratory LD12:12 where the species did not differ in their propensity to remain near food, under SN, *D. melanogaster* occupied the food region of the tube more than *D. ananassae* especially during middle of the day (*D. melanogaster* = 0.23 ± 0.01, *D. ananassae* = 0.15 ± 0.01; $F_{1, 793} = 34.4$, $p < 0.0001$; Fig. 3B). Similar to LD12:12, under SN also *D. melanogaster* spent more time in the middle zone compared to *D. ananassae* (*D. melanogaster* = 0.31 ± 0.01, *D. ananassae* = 0.2 ± 0.01; $F_{1, 793} = 7.3$, $p < 0.01$; Fig. 3B), where as *D. ananassae* spent more time in the plug region (*D. melanogaster* = 0.46 ± 0.01, *D. ananassae* = 0.65 ± 0.01; $F_{1, 793} = 137.6$, $p < 0.00001$; Fig. 3B). Thus visual observation results of activity pattern of *D. melanogaster* and *D. ananassae* closely matched that obtained by DAM system under SN condition and more importantly they revealed differences between *D. ananassae* and *D. melanogaster* in their propensity to occupy certain regions of the activity tube, which was consistent with what was observed in the laboratory. Furthermore, it was clear from these observations that the high midday activity in *D. ananassae* detected by the automated DAM system was not due to flies merely occupying the middle zone. Instead, we

find that they more frequently occupied the plug region compared to *D. melanogaster*. Thus, our studies verified that *D. ananassae* flies do indeed exhibit higher locomotion compared to *D. melanogaster* flies during midday irrespective of type of spatial arena (tubes or petri-plates) or environmental cycles (laboratory LD or SN).

Light intensity did not significantly alter species-specific activity pattern of D. ananassae under SN. The robust bimodal activity pattern of *D. melanogaster* seen under laboratory LD12:12 is believed to be an adaptive response of flies to be active during times of the day when the environment is least stressful (Pittendrigh, 1993). The fact that *D. ananassae* exhibits relatively greater activity during daytime in contrast to *D. melanogaster* which suppresses its activity during that time, led us to speculate that perhaps in the wild, *D. ananassae* occupies microhabitats different from *D. melanogaster*. We asked whether *D. ananassae*, which is predominantly a human commensal, found mostly inside homes and shaded habitats such as fruit and vegetable markets, differs from *D. melanogaster* in its sensitivity to light (Sharmila Bharathi et al., 2003). Under laboratory conditions, we provided LD12:12 with either of four light intensities during the day – 0.1 lux, 10 lux, 100 lux or 1000 lux and recorded activity using DAM system. *D. melanogaster* showed a shift of activity into the pre-dawn duration (3 hr before lights-ON) under very high light intensity accompanied by a reduction in activity in the pre-dusk window (3 hr before lights-OFF) (Fig. 4A, B). On the other hand, even at the highest intensity of 1000 lux, *D. ananassae* did not shift their activity into the night (Fig. 4A, B). Instead, they reduced activity levels in the post-dawn window (3 hr after lights-ON) with increasing light intensity (Fig. 4A, B). Thus, the two species appear to differ in their responses to daytime light intensity such that *D. melanogaster* reallocates activity to different parts of the day/night whereas *D. ananassae* simply suppresses activity with increasing daytime light levels.

We then compared the activity levels of the two species under SN using DAM system under either high (peak intensity of ~2600 lux) or low (peak intensity of ~370 lux) light intensity while other measured environmental variables – temperature and relative humidity did not differ significantly between the two treatments. *D. melanogaster* displayed bimodal activity pattern with a prominent evening peak under both light intensities (Fig. 5A). Although, the activity profile of *D. ananassae* appeared to differ between the two light intensities (Fig. 5A), we did not detect statistically significant differences in either day or night activity counts between the two (day- $F_{1, 44} = 0.00048$, $p = 0.98$; night- $F_{1, 44} = 0.23$, $p = 0.63$). We also compared the relative proportion of daytime activity under these two conditions to examine whether high light intensity in SN inhibits activity of either species. We found that light intensity did not affect fraction of daytime activity ($F_{1, 44} = 0.009$, $p = 0.92$, Fig. 5B) in either *D. ananassae* or *D. melanogaster*, and that *D. ananassae* continued to exhibit higher fraction of daytime activity across both conditions suggesting that at least under the conditions we tested, light intensity does not seem to differentially affect *D. ananassae versus D. melanogaster*.

To test the hypothesis that *D. ananassae* prefers low intensity microhabitats we conducted an experiment in which we provided flies with an arena which consisted of a petri-plate, one half of which had very low intensity light compared to the other half (see methods). Our visual observations during the daytime revealed that irrespective of time of day, neither species showed any preference for the shaded region of the plate ($F_{11, 96} = 1.92$, $p > 0.01$; Fig. 5C). This experiment was repeated twice with similar results (data not shown). From this experiment we make the limited interpretation that *D. ananassae* does not actively avoid light or seek shade nor do the two species show any time of day dependent preference for shade.

D. ananassae flies are less tolerant to desiccation compared to D. melanogaster. The relatively higher locomotion of *D. ananassae* compared to *D. melanogaster* during the day,

especially during times when temperature is high and relative humidity is low verified using different methodologies (automated and manual) led us to ask if *D. ananassae* flies are capable of being active during potentially more stressful times of the day because they are equipped with physiological mechanisms to deal with environmental stress. We therefore compared the ability of *D. ananassae* and *D. melanogaster* to resist severe dryness by estimating their desiccation tolerance. For both species, flies that were subjected to starvation alone (treatment 1) did not die until the termination of the experiment (36 hr), thus we eliminated the possibility of death due to starvation during the first 36 hr of the experiment (Fig. 6). Both *D. melanogaster* and *D. ananassae* flies died fastest under severe desiccation (treatment 3) compared to desiccation + starvation (treatment 2) ($F_{1, 16} = 123.9$, $p < 0.0001$; 100% death occurred 19 hr earlier for *D. melanogaster* and 16 hr earlier for *D. ananassae*) (Fig. 6). Under both desiccation treatments (type 2 and 3) *D. ananassae* flies died faster compared to *D. melanogaster* ($F_{1, 16} = 31.8$, $p < 0.0001$; Fig. 6). Thus, contrary to our expectation we found, that *D. ananassae* flies were less tolerant to desiccation compared to *D. melanogaster* (Fig. 6).

Thus, irrespective of spatial arena, *D. ananassae* is clearly day active compared to *D. melanogaster* whose activity is bimodal with prominent evening peak and our visual observations confirms this differential activity pattern of the two species. *D. ananassae* did not exhibit any preference for shady region, neither did they show reduced activity at high light intensity under SN. Desiccation tolerance level of *D. ananassae* was also lower compared to *D. melanogaster*. The above results confirm the temporal separation of activity of the two species and suggest that the former species probably adopt physiological measures other than tolerance to desiccation to deal with the environmental challenges during midday.

Discussion

Our studies were aimed to verify whether closely related species *D. melanogaster* and *D. ananassae* have different temporal profiles of locomotor activity and also to validate our previous results which were obtained using an automated activity recording system (Prabhakaran and Sheeba, 2012; Prabhakaran and Sheeba, 2013). It seemed odd that while one species inhibits its activity during midday and limits it to dawn and dusk intervals- a behaviour which intuitively appears adaptive considering that dry afternoons and cold nights are likely more stressful, another closely related and sympatric species exhibits greatest activity during the day. We wished to rule out any possible interaction between species and locomotor activity assay apparatus leading to misleading conclusions and hence we conducted visual observations of behaviour throughout the day and night. Our studies under two different spatial arenas demonstrate that *D. ananassae* exhibits relatively higher activity in the morning and middle of the day.

The 'siesta' observed in *D. melanogaster* has been postulated to be a mechanism to avoid high temperature during middle of the day (Hamblen-Coyle et al., 1992). Temperature has a major role in controlling the midday activity in *D. melanogaster* - with increase in ambient temperature, activity is shifted into the night and an increased 'siesta' is seen (Majercak et al., 1999). *D. melanogaster* also shows afternoon activity when assayed under certain SN conditions (De et al., 2013; Menegazzi et al., 2012; Prabhakaran and Sheeba, 2013; Vanin et al., 2012). However, this activity in the afternoon has been suggested to be an escape response of flies rather than clock driven activity (De et al., 2013; Prabhakaran and Sheeba, 2013). One possibility of *D. ananassae* being active in the daytime may be a mere response to light or temperature during the day. However, when *D. ananassae* flies are subjected to short photoperiods their activity became shifted to pre-dawn interval, moreover, they showed some anticipation to morning, and when subjected to DD from LD, their activity followed from the morning activity of the previous LD cycles (Prabhakaran and Sheeba,

2012). Thus, the morning activity showed by *D. ananassae* flies is not a masking response and it persists in different spatial arenas.

In case of a species distantly related to *D. melanogaster*, *D. virilis*, the differential expression of an important circadian neuropeptide- pigment dispersing factor (PDF) has been postulated to mediate differences in activity pattern from *D. melanogaster* (Bahn et al., 2009). PDF is not expressed in the morning oscillator cells- small ventral lateral neurons of *D. virilis* and this variation in expression was suggested to be because of the difference in the regulatory mechanisms of *pdf* transcription. In the case of *D. ananassae*, our previous results suggest presence of strong morning oscillator cells compared to *D. melanogaster* (Prabhakaran and Sheeba, 2012). However, there is no difference in the anatomy of circadian neurons in the brains of *D. ananassae* and *D. melanogaster* in terms of cell number based on the expression of PDF and some other core circadian proteins VRILLE and Par Domain Protein 1 (Hermann et al., 2013). Furthermore, PDF sequence of *D. melanogaster* and *D. ananassae* were also identical (Hermann et al., 2013). Another important clock protein of *D. melanogaster* is PERIOD (PER), and thermo-sensitive splicing in the 3′-terminal intron (dmpi8) of *per* gene has been shown to control temperature dependent activity pattern in the middle of the day (Low et al., 2008). In two other species *D. yakuba* and *D. santomea* such thermo-sensitive splicing of 3′-terminal intron of *per* gene is absent and thus they do not show temperature dependent activity pattern as shown by *D. melanogaster* (Low et al., 2008). It is also possible that variation in the splicing efficacy of *per* contributes to the differential activity pattern of *D. ananassae*, a hypothesis that awaits future studies.

Out of many environmental stressors, one important factor in the middle of the day is desiccation. We attempted to test the role of higher desiccation tolerance in this increased diurnality of *D. ananassae*. Since *D. ananassae* flies were active in the middle of the day, we expected it to be more tolerant to desiccation than *D. melanogaster*. However, we found that

D. ananassae is less tolerant to desiccation and thus more prone to death due to water loss compared to *D. melanogaster*. Another possibility is that *D. ananassae* flies can remain active in the middle of the day by preferring shaded regions to avoid high temperature and light intensity. Our preliminary studies did not show any such preference for shaded region by *D. ananassae*. Thus, our results thus far confirm the daytime activity of *D. ananassae* although the mechanisms that underlie the ability to deal with the environmental stress during that time remain unclear. We speculate that *D. ananassae* flies may inhabit a microclimate where the fluctuation in daily temperature is relatively low, thus allowing them to be active during daytime. Systematic collection of *D. ananassae* flies from wild during different times of the day may reveal whether they prefer to inhabit microclimates where flies experience less environmental stress.
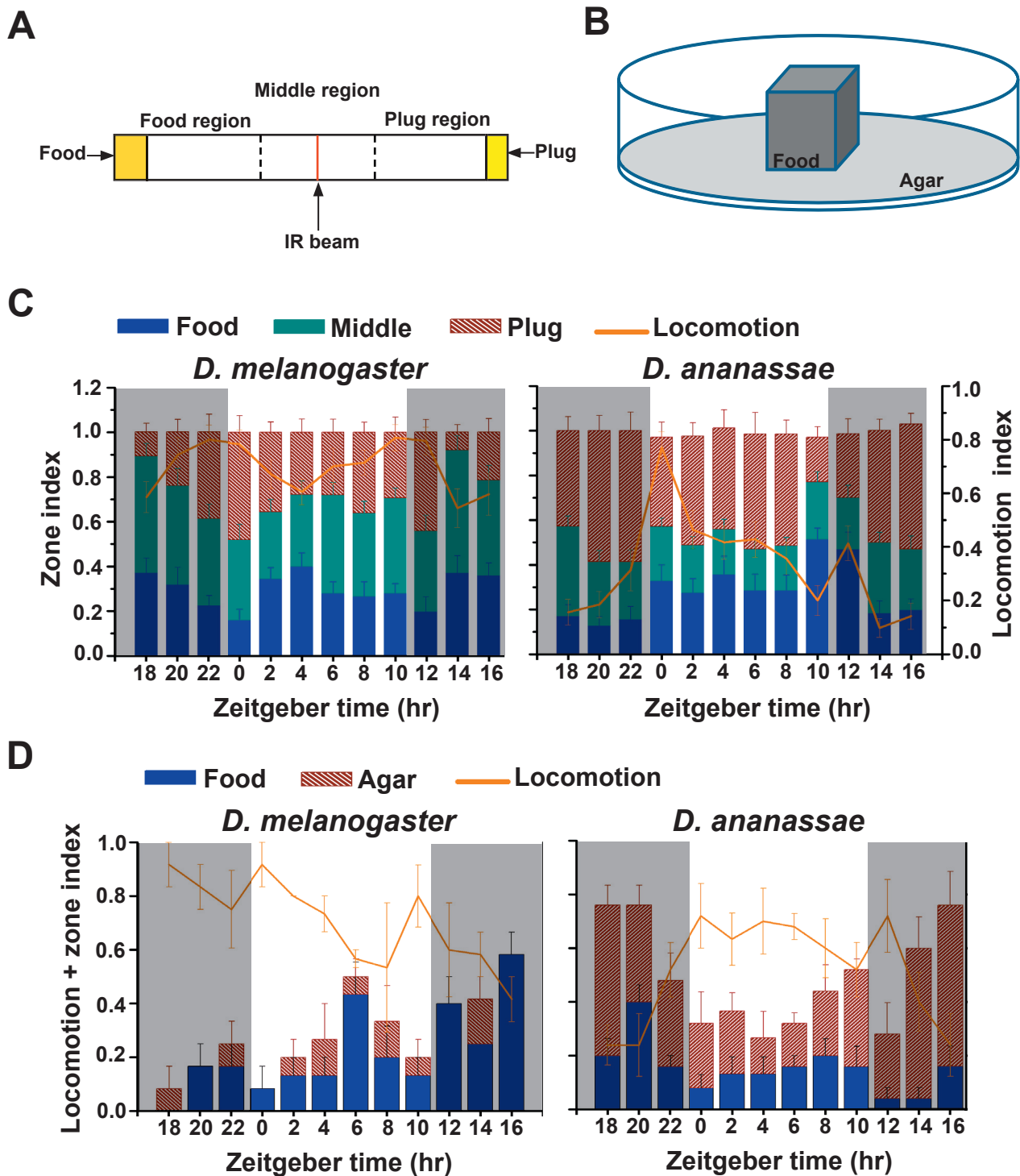
Figure 1. *D. ananassae* **showed relatively high daytime activity in different spatial arenas. (A)** Diagrammatic sketch of experimental set up in glass tubes. **(B)** Diagrammatic sketch of experimental set up in petri-plates. **(C)** Average values of zone index in tube assays, which indicates propensity of flies to occupy a particular zone of the locomotor activity tube: dark blue shade represents food region, cyan represents middle region and hashed red represents the plug region of the tube. Average values of locomotion index, which indicates propensity of flies to exhibit locomotor activity is shown by solid orange line. The x-axis represents Zeitgeber time (ZT00 = lights-on) and left and right y-axes represent zone and locomotion indices respectively. Grey shaded areas represent darkness under LD12:12. **(D)** Average values of zone and locomotion indices in petri-plate assays: which indicates propensity of flies to rest on food (blue shade) or agar (hashed red) or exhibit locomotor activity (orange solid line). Other details same as panel C. Error bars are SEM.
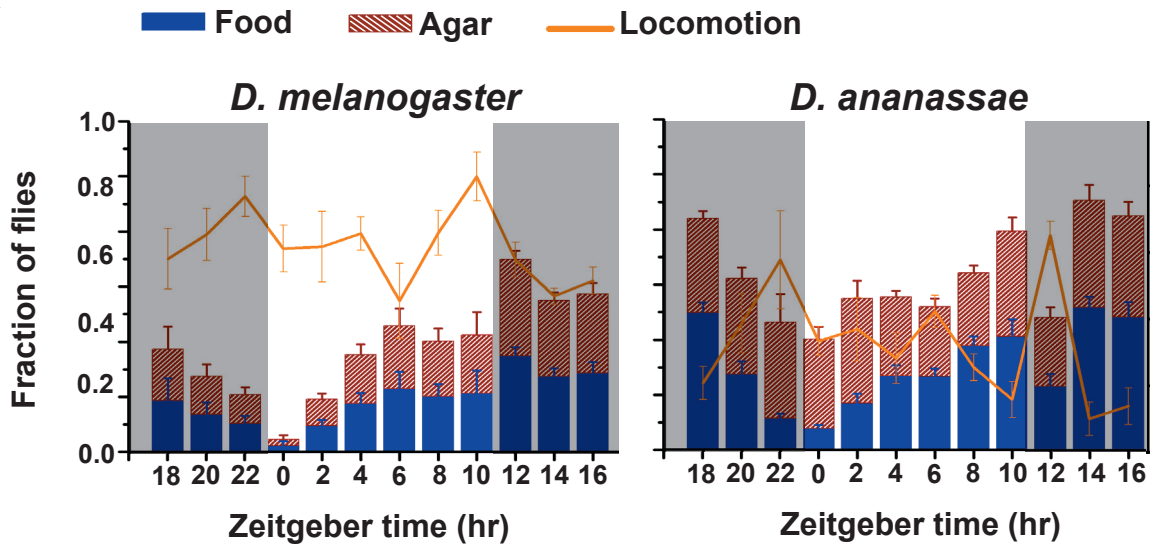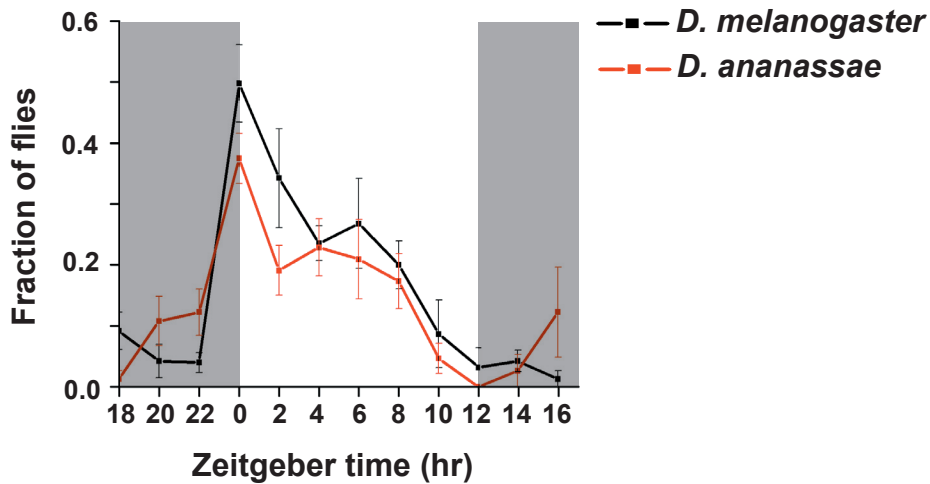
Figure 2. ***D. melanogaster*** **and** ***D. ananassae*** **exhibited similar time dependant courtship related activities under LD.** **(A)** Average proportion of flies occupying a particular zone of the petri-plate for rest (food rest- dark blue, agar rest- hashed red) and proportion of flies showing locomotor activity (orange solid line). **(B)** Average proportion of *D. melanogaster* (black solid line) and *D. ananassae* (red solid line) flies showing courtship related activities. All other details are similar to Fig. 1C.
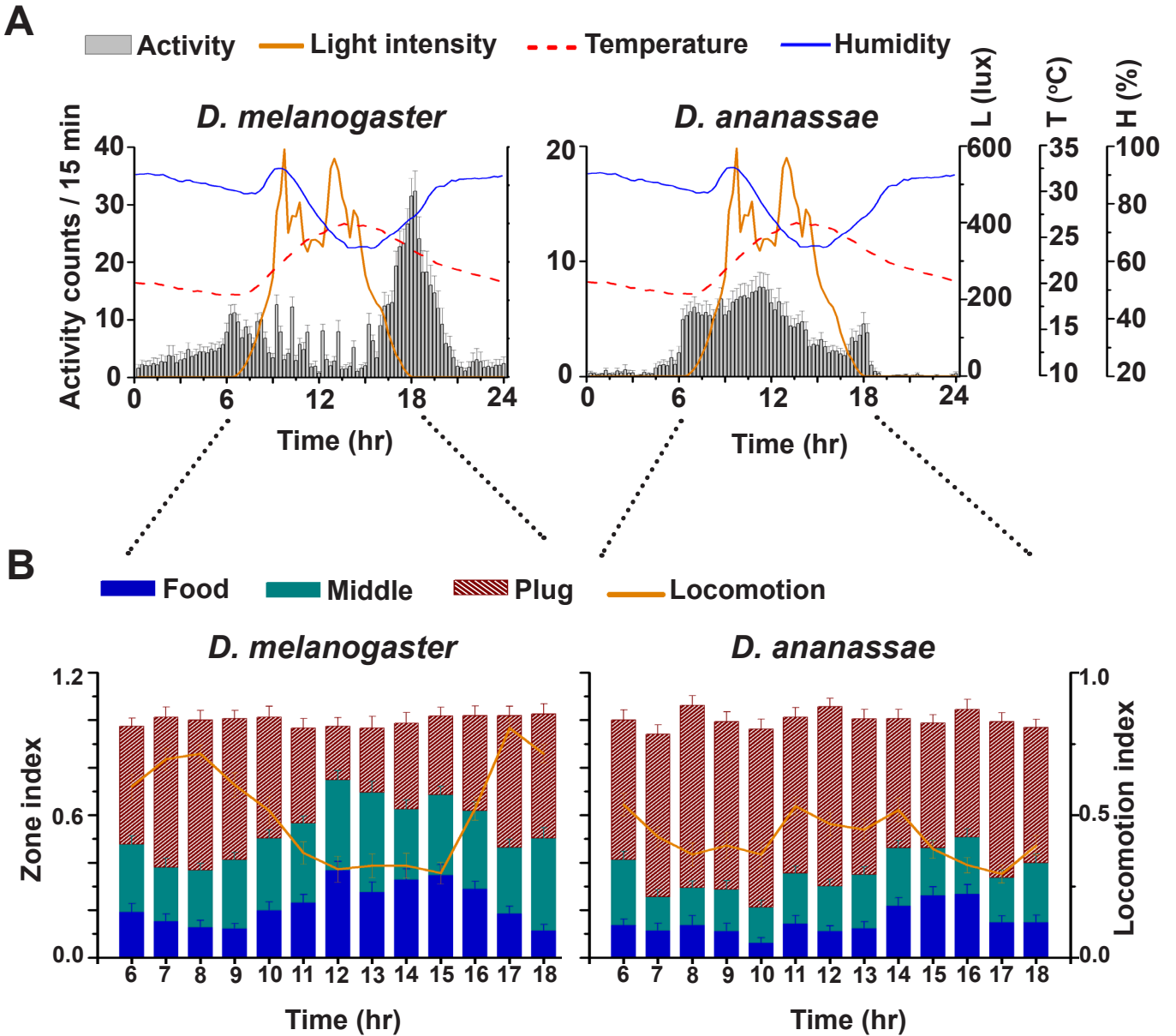
Figure 3. Under SN visual observations confirmed the species-specific differences in activity patterns obtained using the DAM system. **(A)** Average activity profiles of *D. melanogaster* and D. *ananassae* under SN obtained from DAM system recording. Mean activity counts in 15 min bins averaged across 4 days are plotted along with light intensity (orange solid curve), temperature (red dashed curve) and relative humidity (blue solid curve). **(B)** Average zone and locomotion indices in glass tubes only during the daytime (06.00 to 18.00 hr). Other details are similar to Fig. 1C.
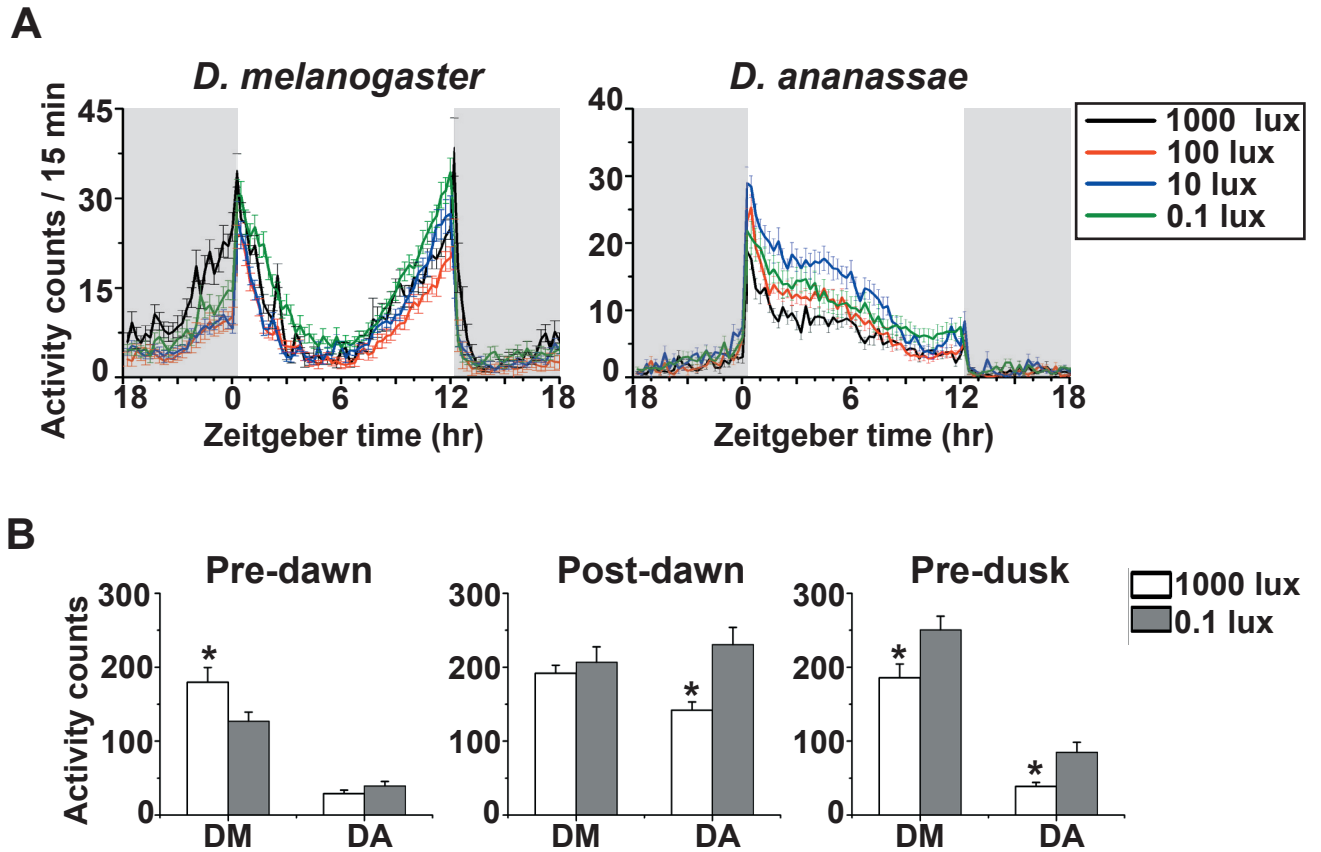
Figure 4. Under laboratory light intensity affected activity patterns of *D. melanogaster* and *D. ananassae*. **(A)** Under laboratory LD12: 12, average activity profiles of D. *melanogaster* and *D. ananassae* under different daytime light intensities (0.1, 10, 100 and 1000 lux) obtained using DAM system. **(B)** Average pre-dawn, post-dawn and pre-dusk activity counts of *D. melanogaster* (DM) and *D. ananassae* (DA) under 0.1 and 1000 lux. Error bars are SEM. Asterisks denote significant differences in activity counts between light intensities within a species.
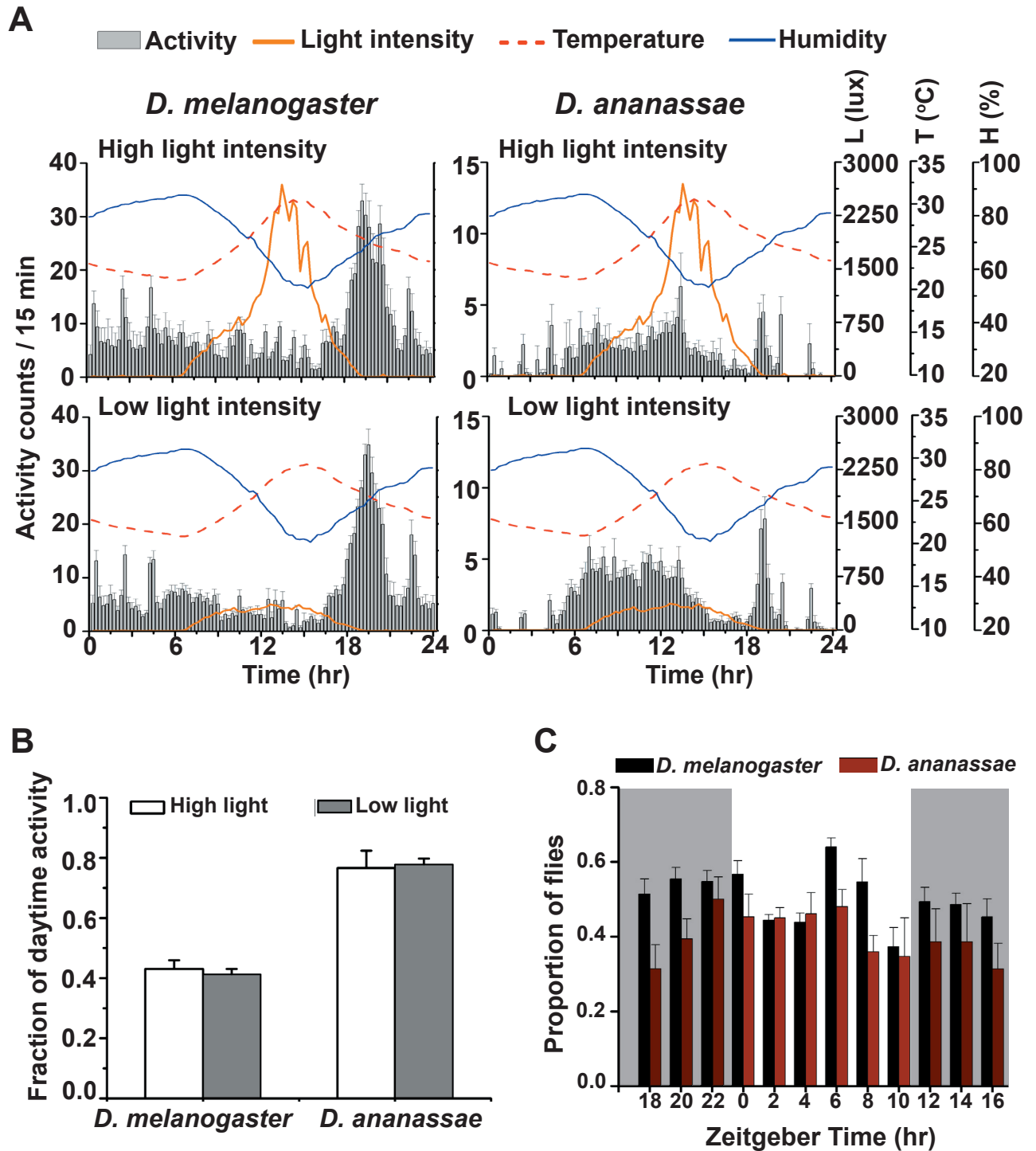
Figure 5. Under SN high light intensity did not significantly alter activity patterns of *D. melanogaster* and *D. ananassae*. (A) Average activity profiles of *D. melanogaster and D. ananassae* under SN with high and low light intensities obtained from DAM system recording. Mean activity counts in 15 min bins averaged across 4 days are plotted along with light intensity (L- orange solid curve), temperature (T- red dashed curve) and relative humidity (H- blue solid curve). Error bars are SEM. (B) Proportion of daytime activity to the total activity under high and low light intensity conditions under SN. Error bars are 95% CI. (C) Proportion of flies spotted in the uncovered region of petri-plate in visual observation experiments with partially shaded petri-plates under LD12:12. Error bars are SEM. Grey shaded areas represent darkness under LD12:12.
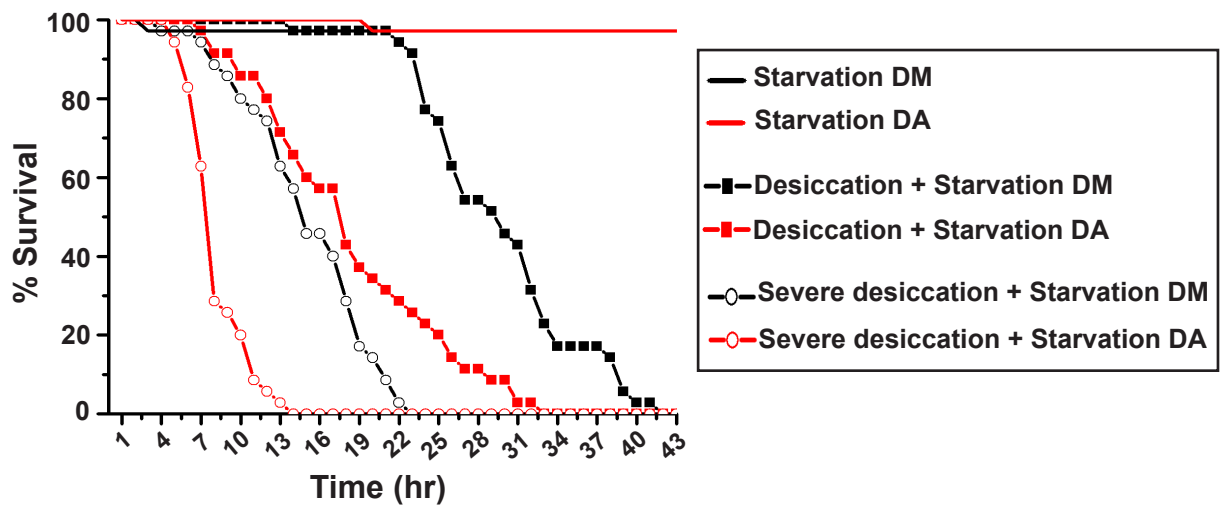
Figure 6. *D. ananassae* is less tolerant to desiccation compared to *D. melanogaster*. Percentage of flies alive during experimental conditions of starvation, desiccation + starvation and severe desiccation (with CaCl2) + starvation for *D. melanogaster* (DM-black lines) and *D. ananassae* (DA-red lines).

Chapter 8

Circadian neuronal circuitry of five Drosophilid species

Introduction

Circadian behaviours and the neuronal network controlling these behaviours have been examined in several organisms. The organisation of circadian clocks shows interesting similarities across a wide range of metazoans. The so-called central clocks are connected to various receptive organs (to receive light, temperature etc.), thus enabling entrainment to environmental cycles and they produce neurotransmitters and other inter-cellular signalling molecules that communicate with downstream cells that allow modulation of various behavioural functions (reviewed in Helfrich-Förster, 2004). Across organisms, the master clocks controlling circadian rhythms are composed of neuronal cells organized in different clusters with distinct identity in their anatomical position, morphology, and the neurotransmitters they release (reviewed in Helfrich-Förster, 2004). Previously, many attempts have been made to examine the clock network of many insects and the optic lobes, more specifically the accessory medulla (AMe) have been implicated as the location of circadian network (reviewed in Helfrich-Förster, 1998). The AMe is a small neuropil at the anterior base of the medulla and has been associated with circadian pacemaker activity in several insects, including *Drosophila melanogaster* and the cockroach *Leucophaea maderae* (Homberg et al., 2003; Stanewsky, 2002). Cellular clocks are constituted in clock neurons capable of sustained autonomous circadian rhythmicity, and these neurons are organized into clock circuits. Neurons within neuronal clock circuits require to communicate with each other to synchronize their phase through intercellular signals, such as neurotransmitters, neuromodulators and neuropeptides.

Among ~1500 *Drosophila* species, circadian circuitry of *D. melanogaster* has been studied most extensively (reviewed in Peschel and Helfrich-Förster, 2011). Circadian clock network of *D. melanogaster* consists of around 150 cells per brain and they are divided into seven groups based on their anatomical position. Four sets of lateral neurons - small and

large ventral lateral neurons (sLNv and lLNv), dorsal lateral neurons (LNd) and lateral posterior neurons (LPN). Dorsal neurons are divided into three sets- dorsal neurons 1 (DN1), DN2 and DN3.

In addition to their anatomical positions, the circadian pacemaker neurons are also distinguished based on the types of proteins they express. The neuropeptide Pigment dispersing factor (PDF) is expressed in four sLNv and four to five lLNv and has been shown to be an important synchronizing agent of the clock neuronal circuit (Renn et al., 1999; Lin et al., 2004). Period (PER) is one among the core clock proteins that is expressed in all known clock neurons except the LPN. On the other hand, the expression of another important core-clock protein TIMELESS overlaps with PER in all neurons and is also expressed in the LPN (Kaneko and Hall, 2000; Shafer et al., 2006). A blue light photopigment CRYPTOCHROME (CRY) functions as a photoreceptor to entrain circadian oscillators to light-dark cycles and as a transcription factor and is expressed in LNv, a subset of LNd and DN1 (Benito et al., 2008). Other neuropeptides used by the clock neurons are IPNamide (coded by the gene *neuropeptide- like precursor 1*) expressed in two anterior cells in the DN1 neuronal group - DN1a (Shafer et al., 2006), neuropeptide F (NPF) expressed in a subset of LNd cells (3 out of 6) (Lee et al., 2006), short neuropeptide F (sNPF) expressed in PDF$^+$ sLNv and two LNd cells, and ion transport peptide (ITP) expressed in PDF$^-$ 5th sLNv and one LNd (Johard et al., 2009). Thus, in addition to the anatomy the neuronal circuitry of *D. melanogaster* has been further characterised by unique expression patterns of several other proteins. This pattern also suggests functional distinction between the neuronal groups. Distinct neuronal subgroups have been postulated to regulate the peaks in activity levels coinciding with lights-ON (morning) and OFF (evening) in *D. melanogaster*. Such bimodality has also been observed using other time cues such as temperature thus, suggesting that distinct groups of neurons

regulate the morning (M) and evening (E) peaks in activity (Grima et al., 2004; Stoleru et al., 2004).

Only recently the circadian circuit of other species of *Drosophila* have been reported (Bahn et al., 2009; Hermann et al., 2013). Comparison of ten different *Drosophila* species found in distinct habitats revealed striking similarity in the overall anatomy of circadian circuit (Hermann et al., 2013). However there were differences among species in the expression of circadian photoreceptor CRY and PDF (Hermann et al., 2013). On the other hand, expression of ITP was consistently seen in the 5th sLNv and one LNd neuron among the ten *Drosophila* species suggesting that its function is likely to be highly conserved in the circadian network of *Drosophila* (Hermann et al., 2013). A recent paper confirms the role of ITP in the circuit of *D. melanogaster* (Hermann-Luibl et al., 2014) and it remains to be seen whether such a role persists across other species. In *D. virilis* the cells corresponding to the M cells of *D. melanogaster* (sLNv) were absent and the authors of the study postulated this to be a potential cause for the absence of morning activity in this species (Bahn et al., 2009).

We have shown earlier that *D. ananassae* differs from *D. melanogaster* in many aspects of its circadian activity pattern (Prabhakaran and Sheeba, 2012). *D. ananassae* unlike *D. melanogaster* is found to be active during the first half of the day and exhibits very little evening activity under a variety of regimes (Prabhakaran and Sheeba, 2012). This had led us to speculate that perhaps *D. ananassae* flies have a stronger M oscillator and a weaker E oscillator compared to *D. melanogaster*. The study by Hermann and colleagues concluded that the two species have similar neuronal architecture based on expression of circadian protein VRILLE (VRI), Par Domain Protein 1 (PDP1) and PDF (Hermann et al., 2013). Another *Drosophila* species *D. malerkotliana* showed almost similar activity pattern in the laboratory and semi-natural conditions (Prabhakaran and Sheeba, 2013). *Zaprionus indianus* showed slightly different activity pattern from *D. melanogaster* and exhibited less rhythmicity

under constant darkness (Prabhakaran and Sheeba, 2013). Thus there are species which show similarities and dissimilarities in circadian behaviours from *D. melanogaster* and we wanted to know whether these behaviours could be attributed to differences in their circadian neuronal organization. We studied expression of PDF and PER in four other Drosophilid species *D. malerkotliana*, *D. ananassae*, *D. nasuta* and *Z. indianus* along with *D. melanogaster*. We found that although they differed in the number of neuronal subsets, overall organization of circadian neurons was similar in all the five species with respect to the proteins we labelled.

Materials and methods

Immunohistochemistry. Flies that were reared in LD 12:12 at ~25ºC and ~70% humidity were used for dissections. Adult male fly brains were dissected in 1% PBS solution during third or fourth day of LD between ZT 23 and 1 (one hr before and one hr after lights –ON respectively). Larval brains were dissected at the third instar stage. Brains were fixed with 4% paraformaldehyde (PFA) at room temperature, rinsed and washed several times with PBS-0.5% Triton X-100. 10% horse serum was used for blocking. Incubation with primary antibodies rat anti-PDF (1:3,000) and pre-absorbed rabbit anti-PER (1:20,000) was done overnight at 4 ºC. This was followed by several washes with PBS-0.5% Triton X-100, incubation with anti-rat alexa 633 and anti-rabbit alexa 488 overnight at 4 ºC followed by more washes with PBS-0.5% Triton X-100. Samples were mounted on slides using 50% glycerol PBS medium with the ventral side facing upward. Images were acquired with a Zeiss LSM510 confocal imaging system. We classified the neuronal subsets in *D. malerkotliana*, *D. ananassae*, *D. nasuta* and *Z. indianus* based on their anatomical position as designated for *D. melanogaster*. For quantification of cell numbers 12-14 brain hemispheres were used for each species.

Results

Expression of PDF and PER in the 3rd instar larval stage. In *D. melanogaster* during 3rd instar larval stage PDF is expressed in the four lateral neurons- sLNv and these neurons project dorsally (Fig.1). We also found similar PDF expression pattern in *D. malerkotliana*, *D. ananassae* and *D. nasuta* (Fig.1). PDF was expressed in some other dorsally located cells in *Z. indianus* (Figs. 1, 3A). PDF was also expressed in few cells in the ventral ganglion region of all the species. In contrast, in *Z. indianus* in addition to the cells in the basal region of ventral ganglion PDF was expressed in few more cells arranged in two rows which were located marginally (Fig. 3B). PER protein was expressed in four sLNv, one or two DN1 and one or two DN2 cells in the larval brain of *D. melanogaster* (Fig.1). Four species *D. malerkotliana*, *D. ananassae*, *D. nasuta* and *Z. indianus* also showed similar expression of PER protein in their larval brain (Fig.1; DN cells were not so clearly visible due to high background issue in anti-PER staining). Dorsally located cells of *Z. indianus* which expressed PDF did not express PER protein (Figs.1, 3A).

Expression of PDF and PER in the adult fly brain. *D. melanogaster* showed expression of PDF in four sLNv and 4-5 lLNv cells (Fig. 2A). Similarly *D. malerkotliana*, *D. ananassae*, *D. nasuta* and *Z. indianus* showed PDF expression in 4 sLNv and 4-5 lLNv cells (Fig. 2A). In all these five species sLNv cells projected to the dorsal protocerebral area and lLNv cells projected ipsilaterally and contralaterally (Figs. 2A, 3C). *Z. indianus* expressed PDF additionally in another cluster of cells which were located dorsally (Fig. 3C). PDF staining in these cells did not overlap with PER staining (Fig. 2A). PER staining in *D. melanogaster*, *D. malerkotliana*, *D. ananassae* and *Z. indianus* revealed anatomically similar type of neuronal clusters- sLNv, lLNv, LNd, DN1, DN2 and DN3 (Fig. 2A, Table 1). There was considerable variation in the number of cells within each group among these species (Fig. 2, Table 1). With PER staining we did not detect PDF⁻ 5th sLNv cell in any of the *D. malerkotliana* brains

sampled (Fig. 2, Table 1). The number of DN3 cells as observed by PER staining was also lower compared to *D. melanogaster* (Fig. 2, Table 1). All other cell groups were similar in *D. melanogaster* and *D. malerkotliana* (Fig. 2, Table 1). *D. ananassae* showed expression of PER only in five LNd cells, DN1 and DN3 cell numbers were less compared to *D. melanogaster* and they expressed PER in 3 cells in the DN2 cluster as opposed to 2 cells in *D. melanogaster* (Fig. 2, Table 1). *D. nasuta* brains also expressed PER in fewer LNd cells, but the numbers of DN2 and DN3 cells which express PER were more in *D. nasuta* compared to *D. melanogaster* (Fig. 2, Table 1). In *Z. indianus* the numbers of LNd, DN1 and DN3 cells were lower compared to that of *D. melanogaster* (Fig. 2, Table 1). Thus these five Drosophilid species show almost similar circadian neuronal clusters as revealed by PDF and PER staining with variation in the number of cells in neuronal clusters.

Discussion

Several insect species have been studied in the field of circadian rhythm and the master clocks controlling these rhythms are also the topic of investigation. Many *Drosophila* species show strikingly similar circadian organization (Hermann et al., 2013). Our studies were intended to compare the circadian organization of five Drosophilid species whose circadian behaviours were compared previously (Prabhakaran and Sheeba, 2012; Prabhakaran and Sheeba, 2013; Prabhakaran et al., 2013). We were interested to know whether the differences in circadian behaviours among species are correlated to their neuronal architecture. We found that even though these species show difference in the number of cells in different neuronal subsets, all four of them (*D. malerkotliana*, *D. ananassae*, *D. nasuta* and *Z. indianus*) had similar neuronal subsets as that of *D. melanogaster*. Our results suggest that the overall organisation of the master clocks controlling the circadian behaviours were conserved among Drosophilid species.

Circadian behaviours of *D. malerkotliana* were similar to *D. melanogaster* under laboratory and semi-natural conditions and these results led us to suggest that these two species share similar circadian organization. Our preliminary studies on the circadian neurons based on the expression of PDF and PER proteins revealed presence of similar neuronal subsets in *D. melanogaster* and *D. malerkotliana*. However we could not detect PDF⁻ 5th sLNv cell in *D. malerkotliana* brain. In *D. melanogaster* this cell has been proposed to have a role in controlling evening activity along with other PDF⁻ cells (Grima et al., 2004; Stoleru et al., 2004). Since there was no difference in the evening activity of *D. melanogaster* and *D. malerkotliana*, this finding is puzzling and we speculate that in *D. malerkotliana* the role of PDF⁻ 5th sLNv is being executed by other cells that are considered to be part of the E-oscillator. Interestingly *D. ananassae* whose activity pattern differs strikingly from *D. melanogaster* also showed difference in the number of cells in neuronal subsets. Based on the behavioural differences we hypothesized that *D. ananassae* may possess a strong M-oscillator and a weak E-oscillator. We found no difference in the number of cells controlling morning activity (Fig. 2, Table 1). *D. ananassae* showed fewer LNd (5 *vs* 6) and DN1 cells compared to *D. melanogaster* which are part of the E-oscillator of *D. melanogaster* (Grima et al., 2004; Stoleru et al., 2004; Stoleru et al., 2007). *D. ananassae* showed expression of PER protein in three DN2 like cells as opposed to 2 cells in *D. melanogaster* wherein these cells have been shown to have role in temperature entrainment (Picot et al., 2009). We did not find any difference in the temperature entrainment properties of *D. melanogaster* and *D. ananassae*. Thus our results suggest that there are possibly subtle differences in the neuronal network between the two species. It appears that the differences in behaviour are brought about not by large differences in neuronal subtypes or location, but possibly by the nature and strength of connections among them.

Although we studied behaviours of *D. nasuta* along with the other species under 12:12 hr laboratory light: dark cycles / or temperature cycles, we could not detect a clear rhythm in either the activity/rest behaviour or in adult emergence (data not shown). Under constant darkness also *D. nasuta* showed very low percentage of rhythmic flies (18%). Based on immunostaining data the subtypes and number of circadian clock cells in the brains of *D. nasuta* did not differ drastically from *D. melanogaster* or the other species, hence it is unlikely that they differ dramatically in terms of the circadian neuronal network. Based on the fact that most organisms studied so far, including close relatives of *D. nasuta* are rhythmic in many behaviours including activity/rest it is unlikely that *D. nasuta* flies are intrinsically arrhythmic. Our working hypothesis as of now is that the experimental methods have been standardized for *D. melanogaster* and it is likely that the conditions are not optimal for *D. nasuta*. Further studies are needed to reveal the existence of any possible rhythmic pattern in circadian behaviours of *D. nasuta*.

Among all the species studied, *Z. indianus* flies are thought to be most distantly related to *D. melanogaster*, yet they only differed slightly from *D. melanogaster* in terms of the number of cells within the circadian neuronal subtypes in the brain. One clear difference between *D. melanogaster* and *Z. indianus* was the presence of few clusters of PDF^{+} cells other than LNv in *Z. indianus* (Figs. 1, 3). Previously in some other *Drosophila* species also these additional PDF^{+} cells were detected (Hermann et al., 2013). Since PER expression was not detected in these additional PDF[+]cells, they might be not part of the clock network (Figs. 2A, 3) (Hermann et al., 2013).

We used antibodies that were raised against *D. melanogaster* proteins to detect PDF and PER in the other species. Cross reactivity of anti-PER gave rise to high background in the other species. Thus, we were unable to quantify the levels of PER across the time of day. This could have revealed any differences that may exist in terms of molecular oscillations of

the circadian clock in the four species. We also chose a time point when the nuclear localization of the PER protein is known to be relatively high in *D. melanogaster* (ZT 23- 1). Therefore we cannot rule out the possibility that the reduction in PER expressing neuronal cell numbers in other species may be due to the difference in the cycling pattern of PER protein. We also attempted to stain the brains of *D. malerkotliana*, *D. ananassae*, *D. nasuta* and *Z. indianus* with antibodies against TIM and CRY protein, but failed to obtain clear images due to high non-specific background, a problem that was reported previously for other *Drosophila* species also (Hermann et al., 2013). Thus our preliminary studies reveal that general anatomy of the clock network among Drosophilid species is well conserved. Future studies, possibly using more specific antibodies raised against the circadian proteins of each species are required to obtain a clearer picture of the circadian organisation.

Table 1. Average number of cells/ brain hemisphere in different neuronal subsets ±

SEM estimated by PDF and PER staining in adult brains of Drosophilids.

Cell type	D.melanogaster	D. malerkotliana	D. ananassae	D. nasuta	Z. indianaus
sLNv	3.9 ± 0.10	4.0 ± 0	4.0 ± 0	4.0 ± 0	4.0 ± 0
5thsLNv	1 ± 0	0	0.9 ± 0.1	1 ± 0	1 ± 0
lLNv	4.3 ± 0.15	4.5 ± 0.22	4.27 ± 0.15	4.6 ± 0.22	4.5 ± 0.17
LNd	5.8 ± 0.2	6.00 ± 0.0	4.93 ± 0.23	4.1 ± 0.1	5.0 ± 0
DN1	14.6 ± 0.34	14.75 ± 0.31	5.75 ± 0.22	15.4 ± 0.23	7.9 ± 0.31
DN2	2.0 ± 0.0	2.0 ± 0.11	3.0 ± 0	3.86 ± 0.14	2.0 ± 0
DN3	29.6 ± 0.58	10.25 ± 0.31	4.5 ± 0.15	32.5 ± 0.43	13.2 ± 0.44
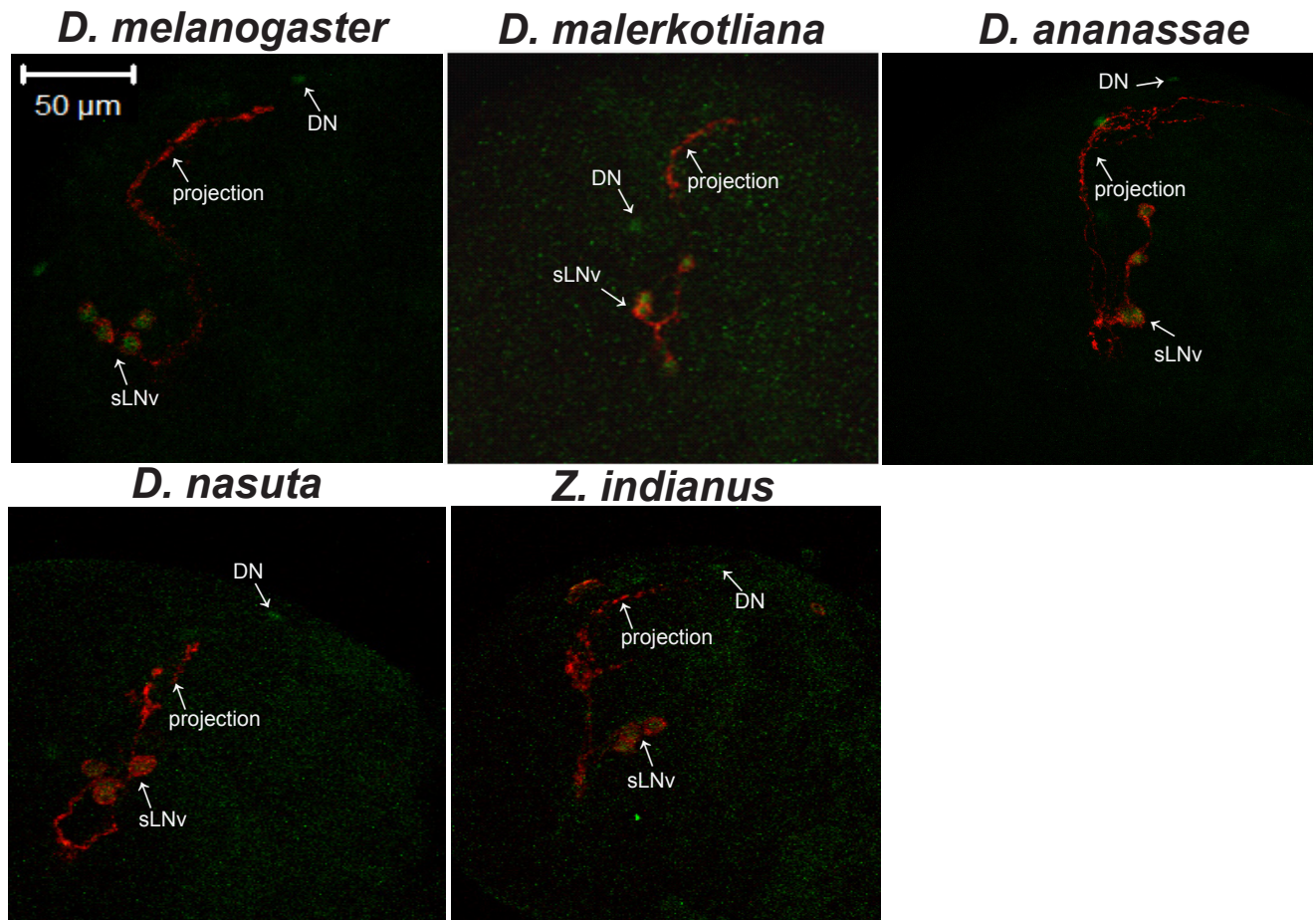
Figure 1. PDF and PER expression in larval brains of five Drosophilids. Anti-PDF (in red) and anti-PER (in green) staining in 3rd instar larval brains of *D. melanogaster, D. malerkotliana, D. ananassae*, *D. nasuta* and *Z. indianus.*. Double staining revealed similar clusters of clock neurons in all the five Drosophilid species. Scale bars in all the images = 50 µm.
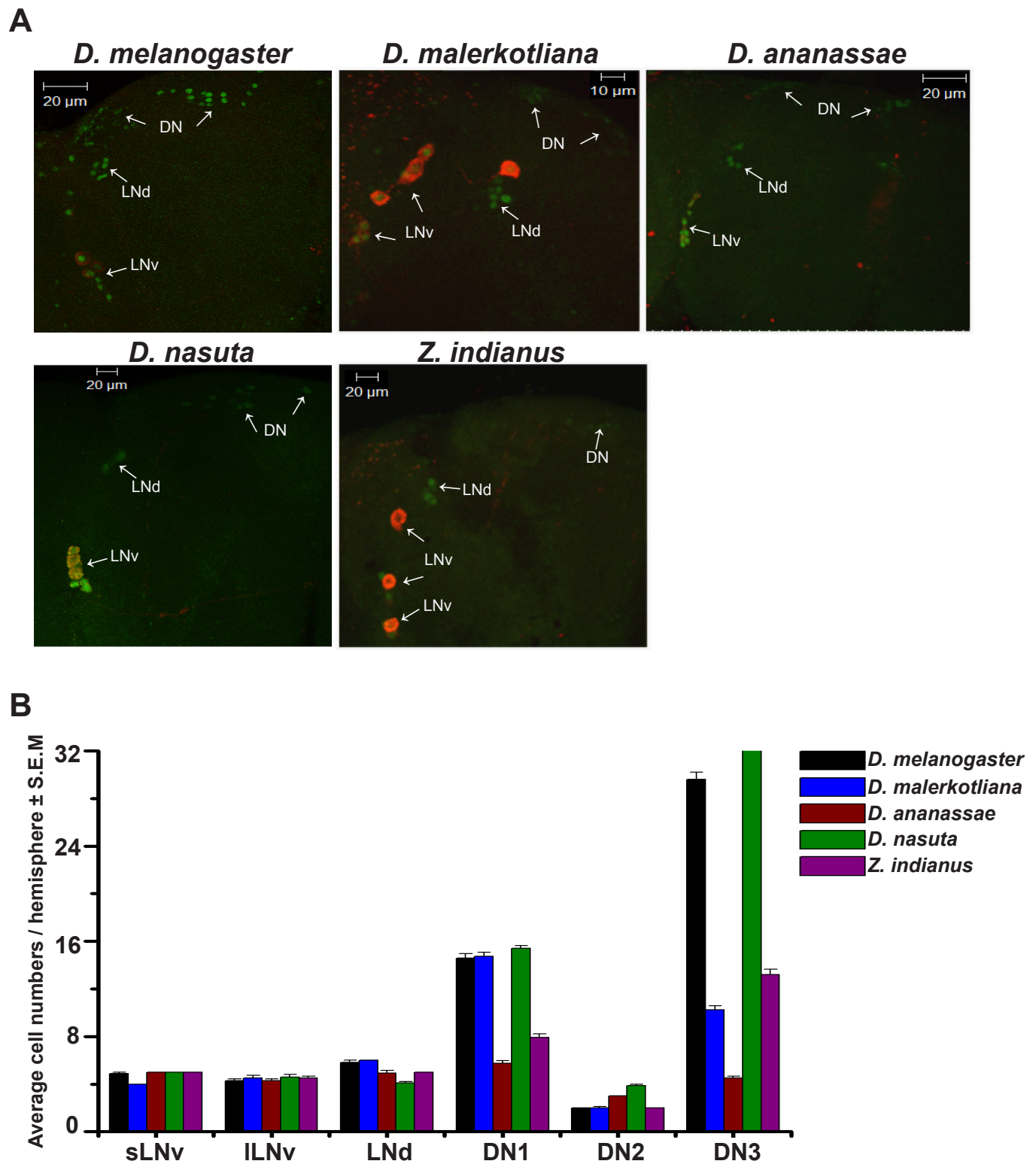
Figure 2. PDF and PER expression in adult brains of Drosophilids. (A) Anti-PDF (in red) and anti-PER (in green) staining in the adult brains of *D. melanogaster, D. malerkotliana, D. ananassae*, *D. nasuta* and *Z. indianus*. **(B)** Quantification of cells in each neuronal subset per hemisphere in the adult brains of *D. melanogaster , D. malerkotliana, D. ananassae, D. nasuta* and *Z. indianus*.
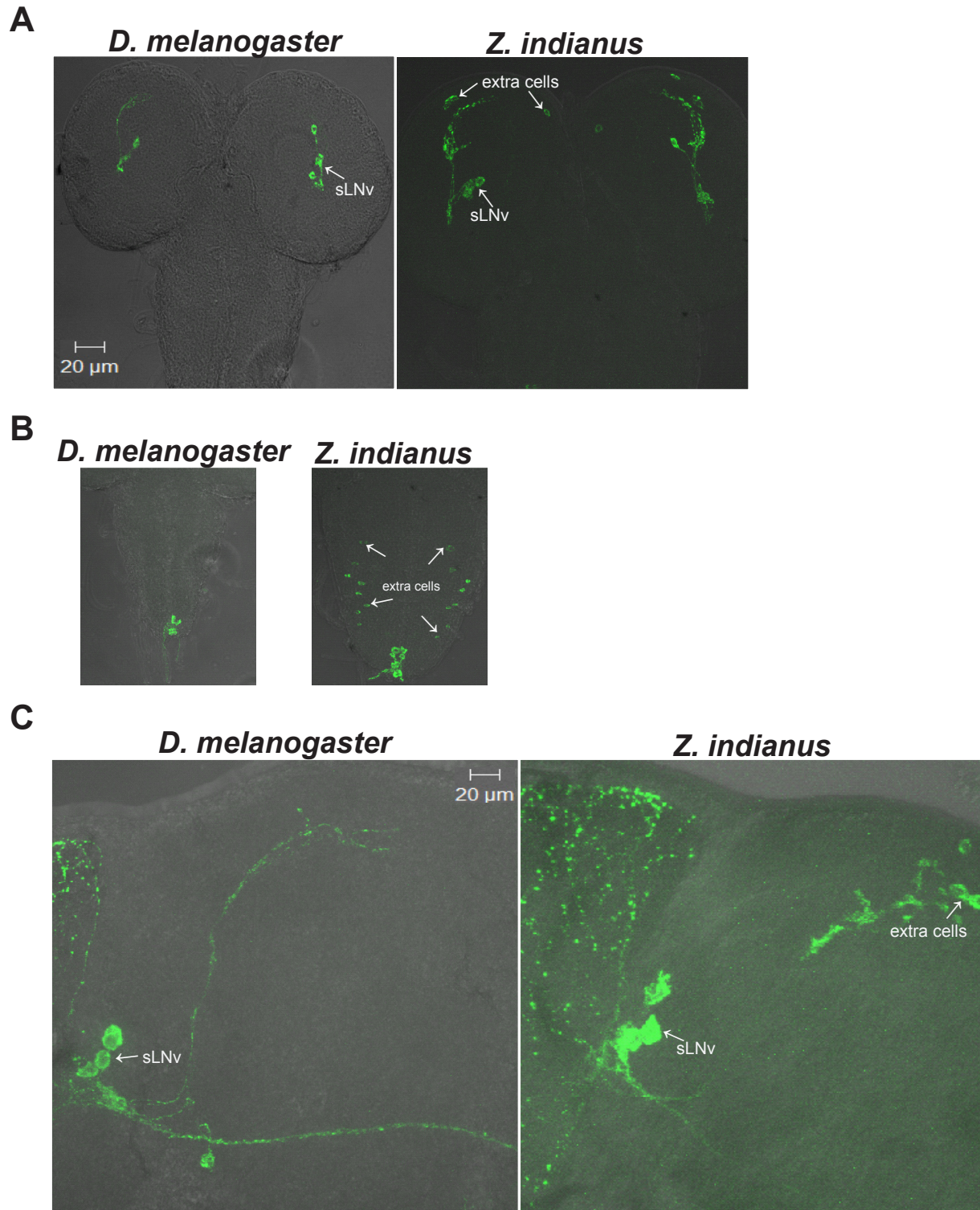
Figure 3. Extra neuronal clusters expressing PDF in larval and adult brains of Z. *indianus*. **(A)** Anti-PDF (in green) staining in the 3rd instar larval brains of *D. melanogaster* and *Z. indianus*. **(B)** Anti-PDF (in green) staining in the 3rd instar larval ventral ganglion of *D. melanogaster* and *Z. indianus*. **(C)** Anti-PDF (in green) staining in the adult brain of *D. melanogaster* and *Z. indianus*.

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