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RESEARCH ARTICLE

Insights into differential activity patterns of drosophilids under semi-natural conditions

Priya M. Prabhakaran and Vasu Sheeba*

Behavioural Neurogenetics Laboratory, Evolutionary and Organismal Biology Unit, Jawaharlal Nehru Centre for Advanced Scientific Research, Jakkur, Bangalore-560064, India

*Author for correspondence (sheeba@jncasr.ac.in)

SUMMARY

We showed recently that *Drosophila ananassae*, a closely related and sympatric species of the commonly studied fruitfly *D. melanogaster*, shows distinctly deviant patterns in circadian activity/rest rhythm from the latter under a variety of laboratory conditions. To examine whether such differences extend to more natural conditions where a variety of time cues and similar environmental pressures might force different species to adopt similar temporal patterns, we examined these two species under semi-natural conditions over a span of 1.5 years. Furthermore, we asked to what extent features of activity/rest rhythm of flies are conserved across species under changing environmental conditions encountered across seasons, and to do so, we studied two more drosophilid species. We found that while each species exhibits seasonality in activity patterns, this seasonality is marked by interesting inter-specific differences. Similar to laboratory studies, *D. ananassae* showed activity mostly during the day, while *D. melanogaster* and *D. malerkotliana* exhibited almost similar activity patterns across seasons, with predominantly two peaks of activity, one in the morning and another in the evening. Throughout the year, *Zaprionus indianus* displayed very low levels of activity compared with *D. melanogaster*, yet, compared with those seen in standard laboratory assays, this species exhibited more robust rhythms under semi-natural conditions. We hypothesise that different ecological factors may have influenced these species to adopt different temporal niches.

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Key words: circadian, season, semi-natural, *Drosophila melanogaster*, *Drosophila ananassae*, *Drosophila malerkotliana*, *Zaprionus indianus*.

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INTRODUCTION

The genus *Drosophila* consists of more than 1500 species, of which the circadian rhythmicity of very few have been studied thus far. Among them, *D. melanogaster* Meigen 1830 (DM) has received most attention because of historical reasons and the consequent availability of genetic tools. In a laboratory study on another drosophilid species, *D. virilis*, certain circadian behaviours and the expression of neuropeptide pigment dispersing factor in the circadian neurons was examined in comparison with DM (Bahn et al., 2009). Although a detailed neuroanatomical description of circadian circuits across 10 species was published recently (Hermann et al., 2013), surprisingly, such a comparative study for circadian behaviours does not exist. *Drosophila ananassae* Doleschall 1858 (DA) is a sympatric species with DM that our previous studies under laboratory light:dark cycles have shown to have a distinct activity/rest profile from DM (Prabhakaran and Sheeba, 2012). DA flies are predominantly day-active, while DM display the expected bimodal activity pattern; 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.

Circadian rhythms in DM 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 because of 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 the afternoon (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 that seen in 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 that examined another rhythmic behaviour – adult emergence – revealed enhanced robustness under SN conditions compared with 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) in either the occurrence or the phasing of peaks.

Previous studies on the 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 in 2004: WTALA from Alto Adige, Italy, 46°N, and Hu from Houten, The Netherlands, 52°N (Vanin et al., 2012). Comparison of the standard laboratory strain Canton-S with WTALA 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 WTALA was partially explained by the fact that flies of this strain carried two alleles of the core clock gene *timeless* – *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 DM 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 DM.

We reasoned that by comparing the behaviour of two species, DM and DA, 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 wild-caught populations of DM and DA, we assayed two other drosophilid species, *Drosophila malerkotliana* Parshad & Paika 1965 (DK) and *Zaprionus indianus* Gupta 1970 (ZI), under SN conditions in 12 assays spread over a period of 1.5 years. DK was first reported from Punjab, India, and is distributed throughout Southeast Asia (Kopp and Barmina, 2005). DK and DA, 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 DM in their preference for feeding and mating sites (Sharmila Bharathi et al., 2003). ZI 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, DA 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 four species (DA, DM, DK and ZI) were wild-caught between 2004 and 2005 within Bangalore, India (12°58′N, 77°38′E), using fruit traps as bait and net sweeps, 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 12 h:12 h light:dark (LD) (~1.5 W m^{-2}) conditions at constant temperature (~25°C) and humidity (~70%) was followed.

Activity recording

Virgin male flies (age 2–3 days), reared under laboratory conditions of 12 h:12 h LD, were used for the assays. Individual flies were placed into glass tubes (5 mm diameter, 65 mm length) and locomotor movement along the length of the tube of each fly was recorded using *Drosophila* activity monitors (DAM2, TriKinetics, Waltham, MA, USA). Monitors were then placed inside an iron enclosure (122×122×122 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 that reached monitors from all four sides of the enclosure was not affected. The enclosure is situated within the Jawaharlal Nehru Centre for Advanced Scientific Research campus in Bangalore, below a dense canopy to avoid exposure to direct sunlight; the maximum light intensity reached is ~2500 lx, probably as a result of the canopy and the plastic sheet on top. Daily profiles of light, temperature and relative humidity were also monitored simultaneously using an environmental monitor (DEnM, Trikinetics). Humidity values recorded may not reflect values inside the glass tubes as 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 (Tables 1, 2). The harshest conditions were marked by low humidity and high mid-day temperature (e.g. April 2011) or low T_{min} (e.g. January 2012), whereas during moderate seasons variation in day/night temperature and humidity was lowest (e.g. August 2011). Raw data may be obtained by writing to the corresponding author.

Analysis of activity

Activity was recorded in 5 min bins. Activity profiles (mean ± s.e.m.) were obtained by binning raw time series data of individual flies

Table 1. Details of light profiles across different assays

	Time (h)		Light (lx)	
Assay	Sunrise	Sunset	Maximum	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±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±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

Data are means ± s.e.m. of 6 days.

Table 2. Details of temperature and humidity profiles across different assays

Assay	Temperature (°C)			Humidity (%)	
	Minimum	Maximum	Day average	Minimum	Maximum
April 2011	22.6±0.4	35.4±0.2	30.0±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±2.7
August 2011	21.2±0.1	26.1±0.4	24.2±0.2	70.5±1.3	90.3±1.9
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±3.0	87.8±2.2
February 2012	19.4±0.4	29.7±0.3	26.1±0.2	36.5±2.3	84.5±2.7
March 2012	21.5±1.0	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

Data are means ± s.e.m. of 6 days.

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 (Tables 1, 2). Binned data were averaged across all flies for 6 days (Figs 1–4). Profiles of light, temperature and humidity were also obtained by 15 min binning. From the 15 min binned light profile, the phase of the first bin showing values greater than 0 lx during the morning interval was considered as sunrise and the phase of the first bin showing 0 lx during the evening interval was considered as sunset. An interval of ±3 h around sunrise was considered as the morning window (M window). Similarly, the evening window (E window) was defined as the interval of 3 h before and after sunset, and the afternoon window (A window) as the duration intervening the M and E windows. The presence of a 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 was taken as the phase of M, A and E peaks.

To compare the distribution of activity during the day, the proportion of activity during the M, A and E windows was measured 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 honestly significant difference (HSD) test was performed to evaluate statistically significant differences across assays and species separately for each window. Total activity levels (±95% CI) were plotted along with T_{min} and H_{min} by averaging activity counts of individual flies across 6 days and 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 the daytime, the proportion of daytime activity of individual flies was averaged across 6 days, and these averages were averaged across all flies (±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 performed for only those assays where at least 20% of flies exhibited a peak. The phase of the M and E activity peaks was estimated by scanning activity profiles of individual flies, and these peak phase values were averaged across flies to obtain the mean phases of the peaks (±95% CI) for each species in each assay. One-way ANOVA followed by *post hoc* multiple comparisons using Tukey's HSD test was performed to evaluate differences across assays.

Non-parametric Spearman's rank order correlation test was applied on the following pairs of daywise data points: proportion of daytime activity *versus* average day temperature; nighttime activity counts *versus* average nighttime humidity ($H_{avg,nit}$); nighttime activity counts *versus* average nighttime temperature ($T_{avg,nit}$); proportion of M-window activity *versus* T_{min}; proportion of A-window activity *versus* T_{max}; proportion of E-window activity *versus* T_{max}; proportion of E-window activity *versus* average day temperature; proportion of E-window activity *versus* H_{min}; proportion of E-window activity *versus* average day humidity; M-peak phase *versus* timing of sunrise; M-peak phase *versus* timing of T_{min}; and E-peak phase *versus* timing of sunset. Separate one-way ANOVAs 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 the daily proportion of flies showing A peaks with average daytime temperature, T_{max}, average daytime light intensity and light intensity maximum (L_{max}). All statistical tests were performed using STATISTICA-7 (StatSoft Inc., Tulsa, OK, USA) with the level of significance set to $P<0.05$.

RESULTS

Species-specific differences in activity under SN conditions

When DA flies were subjected to SN conditions, we found that across assays they confined most of their activity to the light phase (Fig. 1, supplementary material Fig. S1). In some assays, DA appeared to show three peaks of activity corresponding to M, A and E intervals (Fig. 1). The afternoon activity was the most consistent, detected in 10 out of 12 assays, and overall DA flies showed greater activity in the A window (Fig. 1, supplementary material Fig. S1). DA almost always started activity after sunrise, with negligible activity at night (Fig. 1, supplementary material Fig. S1), and in one assay during January 2012 when the average nighttime temperature fell to 12.7°C, DA showed delay in the onset of activity with respect to sunrise (Fig. 1). This delay in onset of morning activity was greater compared with two other species examined (phase with reference to sunrise, DM=–0.6, DK=–0.8 and DA=–0.95 h; $F_{2,84}=52.84$, $P=0.0001$; Figs 1–3, supplementary material Fig. S5). Evening activity was low and a small evening bout of activity was detectable

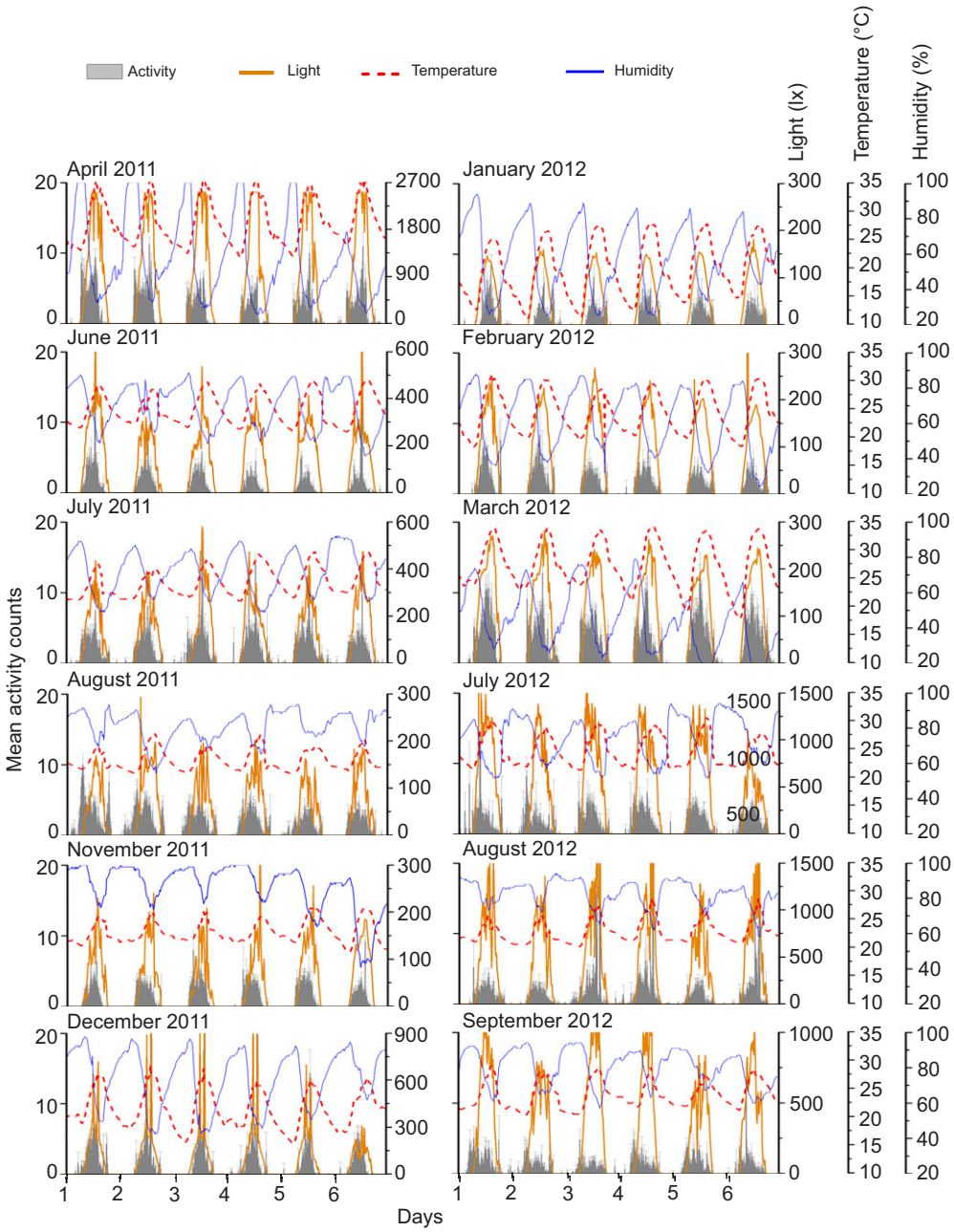

Fig. 1. *Drosophila ananassae* restricted most of its activity to the light phase across different seasons. Average activity/rest profiles of virgin male flies across different assays in semi-natural conditions. Mean (±s.e.m.) activity counts, in 15 min bins averaged across all flies, are plotted along with the environmental factors light (orange solid curve), temperature (red dashed curve) and humidity (blue solid curve).

in approximately half of the assays (Fig. 1, supplementary material Fig. S1).

When we examined the behaviour of DM in parallel with DA, 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. 2). Whenever the A peak was detected, it was of smaller amplitude, and was highly variable in phase among flies within a single assay. Table 3 shows the proportion of flies that exhibit A peak based on the criteria applied by previous studies and described in Materials and methods. During the 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 (Tables 1, 2), a prominent A peak was seen along with M and E peaks (Fig. 2, Table 3, supplementary material Fig. S2). Nevertheless, the A peaks of DM seen in our studies were of comparatively lower amplitude than that observed at more temperate latitudes (>45°N) reported by Vanin et al. (Vanin et al., 2012) and Menegazzi et al. (Menegazzi et al., 2012). When environmental conditions were relatively moderate with low light intensity and little variation in temperature and humidity across the day (e.g. August 2011), low levels of uniformly distributed daytime activity were seen with very few flies showing A peak (Fig. 2, Tables 1–3, supplementary material Fig. S2). This suggests that a combination of high light and temperature may induce the A peak. Laboratory studies show that flies tend to shift their activity into daytime under low ambient temperatures (Majercak et al., 1999). Accordingly, we found that when T_{min} dipped (e.g. December 2011), DM showed very little nighttime activity with a prominent E peak and a relatively blunted M peak (Fig. 2, Table 2, supplementary material Fig. S2). During January 2012, where T_{min} dropped to 12.7°C and humidity was low, we could not detect pre-dawn activity and M peak was delayed with respect to sunrise, similar to DA (Fig. 2, supplementary material Fig. S2). Thus, DA, which was

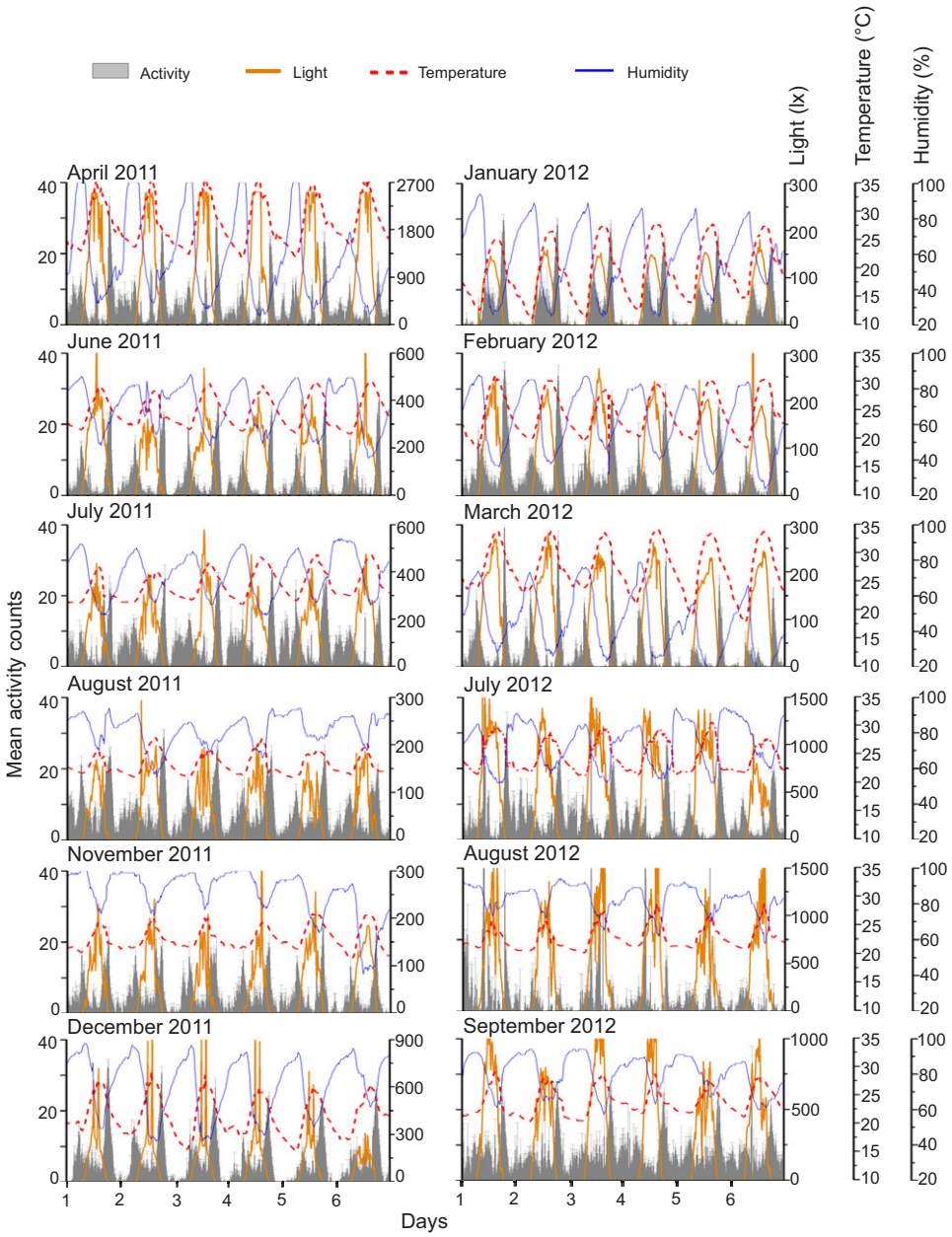

Fig. 2. Activity/rest pattern of *Drosophila melanogaster* varied across different seasons. Average activity/rest profiles of virgin male flies across different assays in semi-natural conditions. All other details are the same as in Fig. 1.

previously shown to exhibit a temporal preference distinct from DM 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 the mild conditions of August 2011 (supplementary material Fig. S5).

DK flies, except for some subtle differences, showed activity/rest profiles similar to those of DM in all assays. In brief, DK 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 proportion of flies (Fig. 3, supplementary material Fig. S3). However, during December 2011, unlike DM, DK exhibited startle responses corresponding to the two peaks in the light profile (Figs 2, 3, supplementary material Figs S2, S3).

In addition to the three species discussed above, we also examined in parallel the activity/rest rhythm of a more distantly related drosophilid species (ZI) that was caught from the same area as the other three species (Fig. 4, supplementary material Fig. S4). In the laboratory under 12 h:12 h LD, ZI exhibited very low activity levels, with low anticipation to both lights on and lights off, and only ~40% flies were robustly rhythmic under constant dark at 25°C (supplementary material Fig. S6). Therefore, we asked whether under more natural time cues it may be possible to visualise activity rhythms in this species, because rhythmic activity has been observed across a wide range of insects (reviewed in Helfrich-Förster et al., 1998). We found that across assays under SN conditions, activity levels of ZI were low compared with the other three species (Figs 1–4) but they showed three activity peaks in most assays (Fig. 4, Table 3, supplementary material Fig. S4). In June 2011, when environmental conditions were milder than that of April 2011, ZI exhibited three distinct peaks, unlike DM, and almost all of its activity occurred during the light phase (Figs 2, 4, supplementary material Figs S2, S4), suggesting that ZI may be more sensitive to high temperature or light intensity. Similar to DM, ZI also showed a delayed morning activity onset in January 2012 compared with other assays, but surprisingly exhibited a small but distinct midday

Table 3. Number of flies analyzed, showing morning, afternoon and evening peaks

Species	Assay	Morning (%)	Afternoon (%)	Evening (%)	*N*
Drosophila ananassae (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
Drosophila melanogaster (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
Drosophila malerkotliana (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
Zaprionus indianus (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 2011	26 (100)	0	26 (100)	26

N, total number of flies analyzed.

activity peak coinciding with L_{max} (Fig. 4, supplementary material Fig. S4). Yet in the assay during September 2012, ZI exhibited a clear bimodal distribution of activity, with a distinct build-up of activity prior to dawn and dusk, unlike the other species (Fig. 4).

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 DM seen across assays ($F_{11,317}$=6.83, P<0.05; Fig. 5A). Overall, we found that under moderate conditions, when H_{min} and T_{min} were relatively high, DM showed significantly higher levels of activity compared with most other times of the year (Fig. 5A, Table 2). During harsh conditions when H_{min} reached as low as 23.3%, DM exhibited the lowest level of activity (Fig. 5A, Table 2). DK 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. 5A). In sharp contrast to this variation in activity of DM, DA flies exhibited significantly higher levels of activity during March 2012 ($F_{11,325}$=6.29, P<0.05; Fig. 5A). However, even though ZI showed seasonal variation in total activity counts ($F_{9,252}$=7.99, P<0.05; Fig. 5A), they did not show any similarity in pattern with any of the other three species under study, exhibiting the 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 four species exhibited lower activity in January 2012, when the nighttime temperature was lowest (Table 2). Thus, while the two related species DM and DK exhibited similarities in terms of total activity, another related species (DA) showed quite contrasting behaviours, especially under the most extreme warm and dry conditions. These

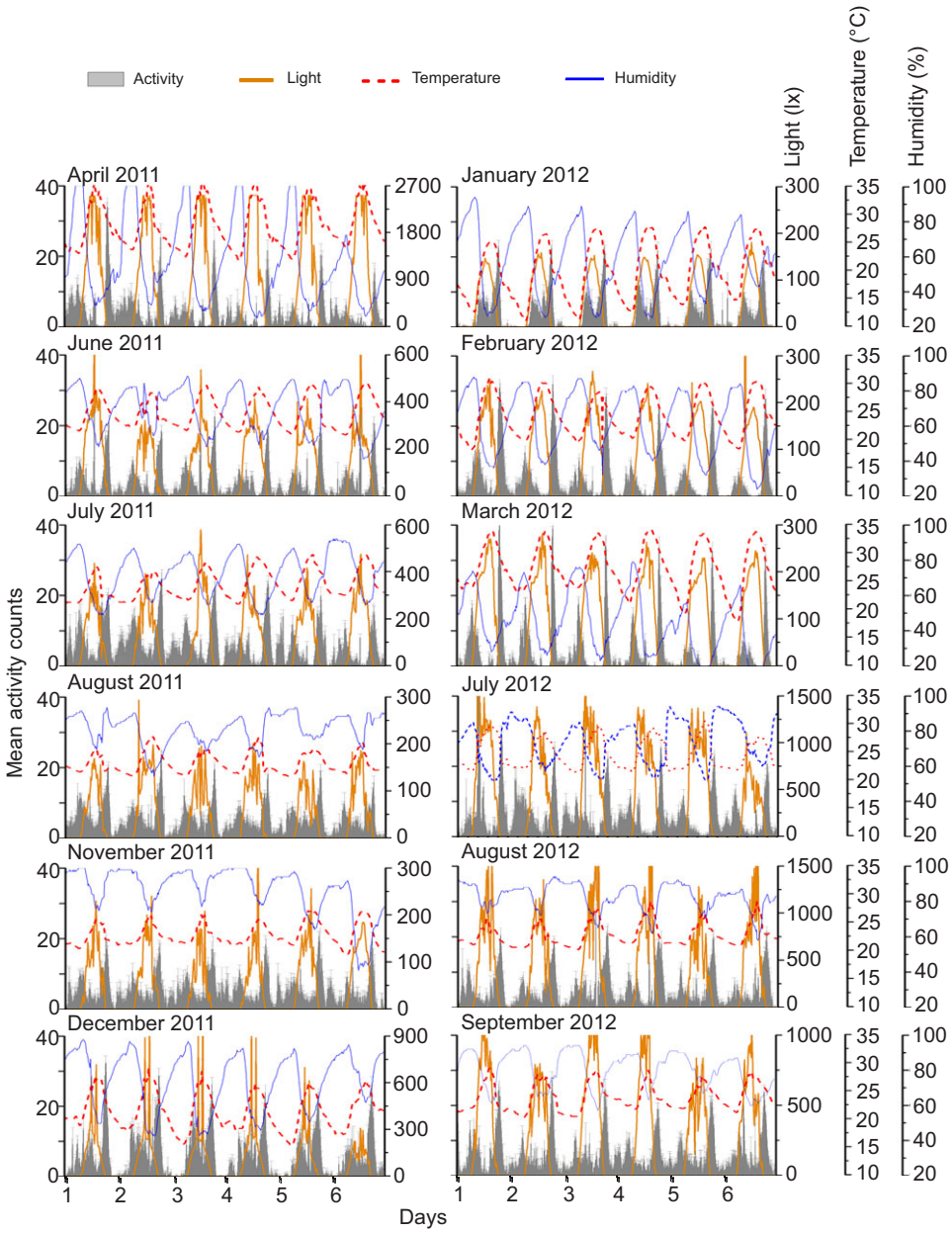

Fig. 3. *Drosophila malerkotliana* exhibited almost similar activity/rest patterns as those of *D. melanogaster* across different seasons. Average activity/rest profiles of virgin male flies across different assays in semi-natural conditions. All other details are the same as in Fig. 1.

results strengthen our hypothesis that these two recently diverged sympatric species (DM and DA) probably occupy different micro-habitats and are therefore differently affected by daily variations in temperature and light.

DA confined most of its activity to daytime across seasons

All four species showed significant differences in their relative distribution of activity during day and night across assays. Daytime activity was higher in DA during all experiments (Fig. 5B, top left), and they showed little or no nighttime activity. During April 2011 and March 2012, when daytime temperatures were highest and accompanied by low humidity, DA showed a high proportion of daytime activity in contrast to DM and DK (Fig. 5B, top left). During January 2012, when T_{min} was as low as 12.7°C, DM showed significantly higher daytime activity compared with most other times of the year, suggesting that nighttime activity is suppressed at cooler temperatures (Fig. 5B, top left, Table 2). DK also showed high daytime activity during January 2012. DM and DK showed a negative correlation of daytime activity with average daytime temperature (DM, r=−0.69, DK, r=−0.69, P<0.05), whereas DA showed no correlation, and ZI showed a positive correlation (ZI, r=+0.64, P<0.05). Interestingly, DM and DK exhibited high nocturnal activity during some assays when both average nighttime humidity and temperature was high ($H_{avg,nit}$, DM, r=+0.26, DK, r=+0.27; $T_{avg,nit}$, DM, r=+0.26, DK, r=+0.36; P<0.05; Figs 2, 3). While DA showed no such correlation, ZI appeared to show nocturnal activity during low night temperatures ($T_{avg,nit}$, ZI, r=–0.5, P<0.05; Fig. 4). Thus, the distribution of activity of DM and DK was similar and their daytime activity levels were reduced as the average daytime temperature increased, whereas DA confined most of its activity to daytime, irrespective of the seasonal variation in temperature.

Higher midday activity in DA irrespective of environmental variation

In order to quantify the distribution pattern of activity among the species across assays, we compared the proportion of activity in the three windows (M, A and E). Irrespective of assay condition,

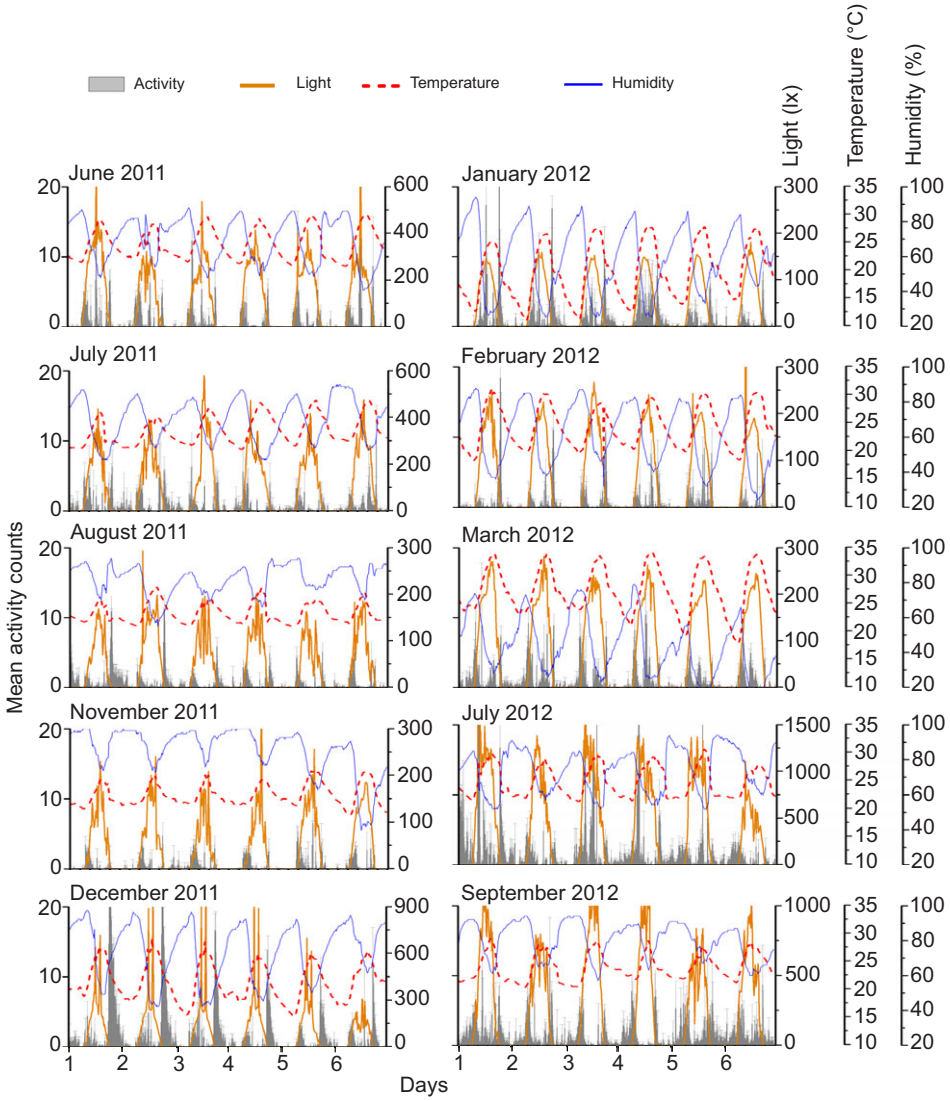

Fig. 4. *Zaprionus indianus* showed variation in its activity/rest pattern across different seasons even though its activity levels were low compared to DM. Average activity/rest profiles of virgin male flies across assays in semi-natural conditions. All other details are the same as in Fig. 1.

DA exhibited highest activity during the A window and lowest during the E window (Fig. 5B, bottom), once again confirming their preference for activity during midday. Compared with DM and DK, DA 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. 5B), and higher activity during the A window ($F_{2,968}$=2046, P<0.05; Fig. 5B, bottom left). For each window, two-way ANOVA for activity levels across assays and species showed a significant interaction between species and season (M window, $F_{22,968}$=11.9; A window, $F_{22,968}$=14.5; E window, $F_{22,968}$=4.8; P<0.05). DA flies showed significantly different allocation of activity into each of these windows across seasons compared with the other two species, which were similar to each other (Fig. 5B). Activity in the M window was positively correlated with T_{min} for DM, DK and ZI, whereas such a correlation was not detected for DA (DM, r=0.61; DK, r=+0.58; ZI, r=+0.67; P<0.05; Fig. 5B). High daytime temperatures were associated with low activity in DM and DK: activity in the A window of DM and DK showed a negative correlation with T_{max} (DM, r=−0.73; DK, r=−0.65, P<0.05; Fig. 5B). The same was not the case for DA and ZI. Moreover, we could not detect any correlation between activity in the E window with any of the measured environmental variables for any of the species. Thus DA showed the highest activity during middle of the day across all seasons and was unaffected by temperature variation, whereas DM and DK exhibited reduced activity as the midday temperature increased.

Timing of M and E peaks depends on environmental factors

Although all four species exhibited M peaks, they showed significant differences in phase across assays (DM, $F_{11,304}$=42.6, DK, $F_{11,313}$=14.7, DA, $F_{11,290}$=39.5 and ZI, $F_{9,221}$=18, P<0.05; Fig. 6A, Table 3). DM and DK timed their M peak to coincide with sunrise and showed a positive correlation with time of sunrise (DM, r=+0.77; DK, r=+0.73, P<0.05; Fig. 6A). However, during January 2012, M peak occurred at a later phase compared with all other assays in all four species (Fig. 6A). This delay was probably due to lower nighttime and dawn temperatures (Table 2). Throughout the year, DA delayed their M peak with respect to sunrise (Fig. 6A). Unlike DM and DK, the phase of M peak in DA and ZI showed a negative correlation with T_{min} (DA, r=−0.63; ZI, r=−0.66; P<0.05; Fig. 6A). The phase of M peak in ZI was similar to that of DA in most cases (Fig. 6A). However, ZI showed a phase-delayed M peak during two assays (June 2011 and July 2012) wherein the peaks coincided with the time at which light intensity reached its maximum (Fig. 4, Fig. 6A, supplementary material Fig. S4).

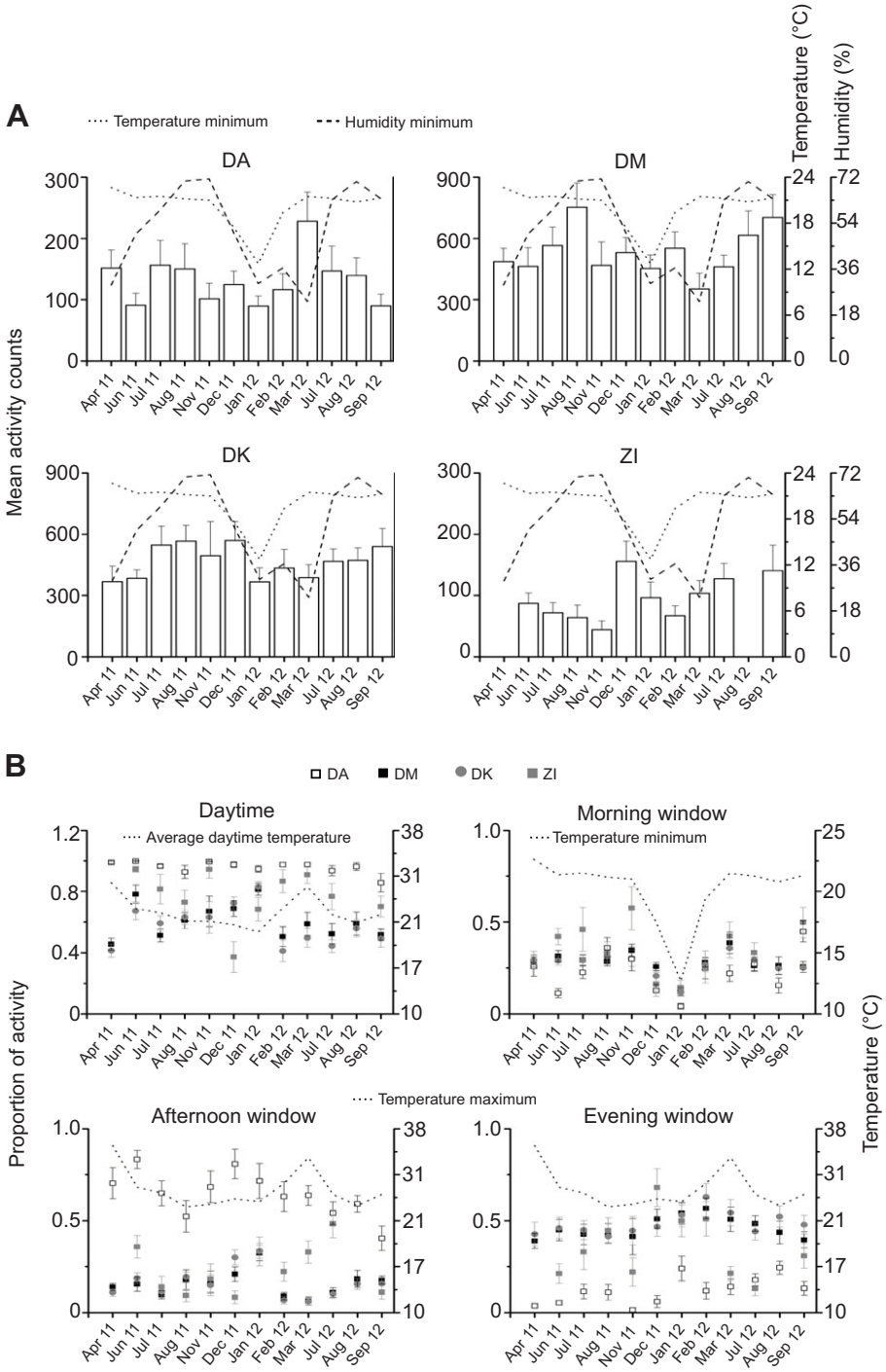

Fig. 5. Total and proportion of daytime activity varies within species across different seasons. (A) Mean activity counts during 24 h averaged across 6 days plotted with minimum temperature (dotted curve) and minimum humidity (dashed curve). (B) Mean activity counts during 12 h of day (as a proportion of total activity) averaged across 6 days is plotted along with average day temperature (dotted curve), mean activity counts during morning, afternoon and evening windows (as a proportion of total activity) averaged across 6 days are plotted along with minimum and maximum temperatures (dotted curve) during each season. DA, *Drosophila ananassae*; DM, *Drosophila melanogaster*; DK, *Drosophila malerkotliana*; ZI, *Zaprionus indianus*. Error bars are 95% CI.

E peak was displayed by DM, DK and ZI during all assays, whereas DA sometimes showed small bouts of activity in the evening (Fig. 1, Fig. 6B; Table 3). The phase of E peak showed a positive correlation with time of sunset in all species (DA, r=+0.95; DM, r=+0.65; DK, r=+0.75; ZI, r=+0.82; P<0.05; Fig. 6B). E peak was exhibited by ~50% of DA 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, DA restricts its activity to midday (Fig. 1, Fig. 6B). DA exhibited A peak in 10 out of 12 assays, which was the same as DK but more compared with the other two species (DM=7/12 and ZI=7/10; Table 2).

Occurrence of A peak is influenced by both light and temperature

Previous studies have suggested that average daytime temperature and not light intensity elicits the A peak in DM (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 indispensable 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 whether such a pattern exists in these four species. Regression analysis using daily proportion of flies exhibiting A peak revealed

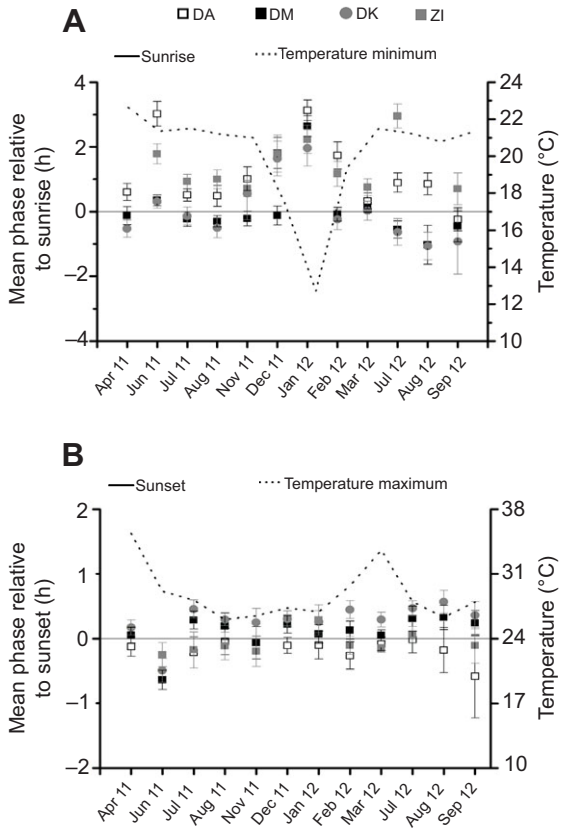

Fig. 6. Phase of morning and evening peak of activity exhibited correlation with different environmental factors. (A) Mean phase of morning activity peak relative to sunrise is plotted along with sunrise (solid line) and minimum temperature (dotted curve). (B) Mean phase of evening activity peak relative to sunset is plotted along with sunset (solid line) and maximum temperature (dotted curve). Error bars are 95% CI.

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 DA, whereas the average daytime light was found to be associated with A-peak occurrence for all species except ZI (Fig. 7). Thus our study, which included more than 10 assays in four species conducted at a more southern latitude than some previous reports, suggests that both light intensity and temperature influences the A-peak occurrence.

DISCUSSION

As most previous studies on circadian rhythms of drosophilids, including those that examined rhythmic behaviour under SN conditions, have focused on DM, 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 photoperiod 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 mid-day 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, DM and DK showed almost similar activity/rest patterns throughout the year (Figs 2, 3, supplementary material Figs S2, S3). 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 DM (*melanogaster* subgroup) and DK (*ananassae* subgroup) belong to different species subgroups, and phylogenetically DK is more closely related to DA than to DM (Crosby et al., 2007; Yang et al., 2012).

We show that DA is in fact a diurnally active species compared with DM, which from our study is found to be predominantly crepuscular (Figs 1, 2, Fig. 5B) with clear temporal separation of activity (supplementary material Fig. S5). This is in agreement with our previous study, where DM and DA showed temporal separation of activity under a variety of photoperiods in the laboratory (Prabhakaran and Sheeba, 2012) confirming a morning preference for activity in DA. An interesting contrast between ours and a previous study (Vanin et al., 2012) is that the A component of activity contributed to less than 25% of the total activity in almost all assays for DM and all species except DA (Fig. 5B, bottom left). Another novel finding from our study is the enhanced nocturnal activity exhibited by DM and DK 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 the present study, DM rarely exhibited a distinct A peak (April 2011, July 2011; Fig. 2, supplementary material Fig. S2) and when it occurred it was not as prominent as the M or E peaks. As 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 strain of DM, which is considered ‘wild-type’ by convention, also supports this view (De et al., 2013). Two other studies also suggest that the afternoon activity could be an escape response from harsh conditions (Menegazzi et al., 2012; Vanin et al., 2012). One difference between our studies and others that is likely to result in a smaller A peak is the diameter of the glass tubes used to assay locomotor activity. We used a larger version of the recording apparatus (DAM7), which uses tubes of 5 mm inner diameter, while other studies (e.g. 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 DM, DK and ZI may reflect courtship and foraging behaviours. However, DA appears to have evolved mechanisms that enable them to occupy the diurnal temporal niche. The experiments reported here are the first attempt, to the best of our knowledge, to compare circadian behaviours both across seasons and among closely related species under SN conditions. They reveal that bimodality of activity is a robust characteristic feature of some species of drosophilids and that it likely reflects evolved features of the underlying circadian

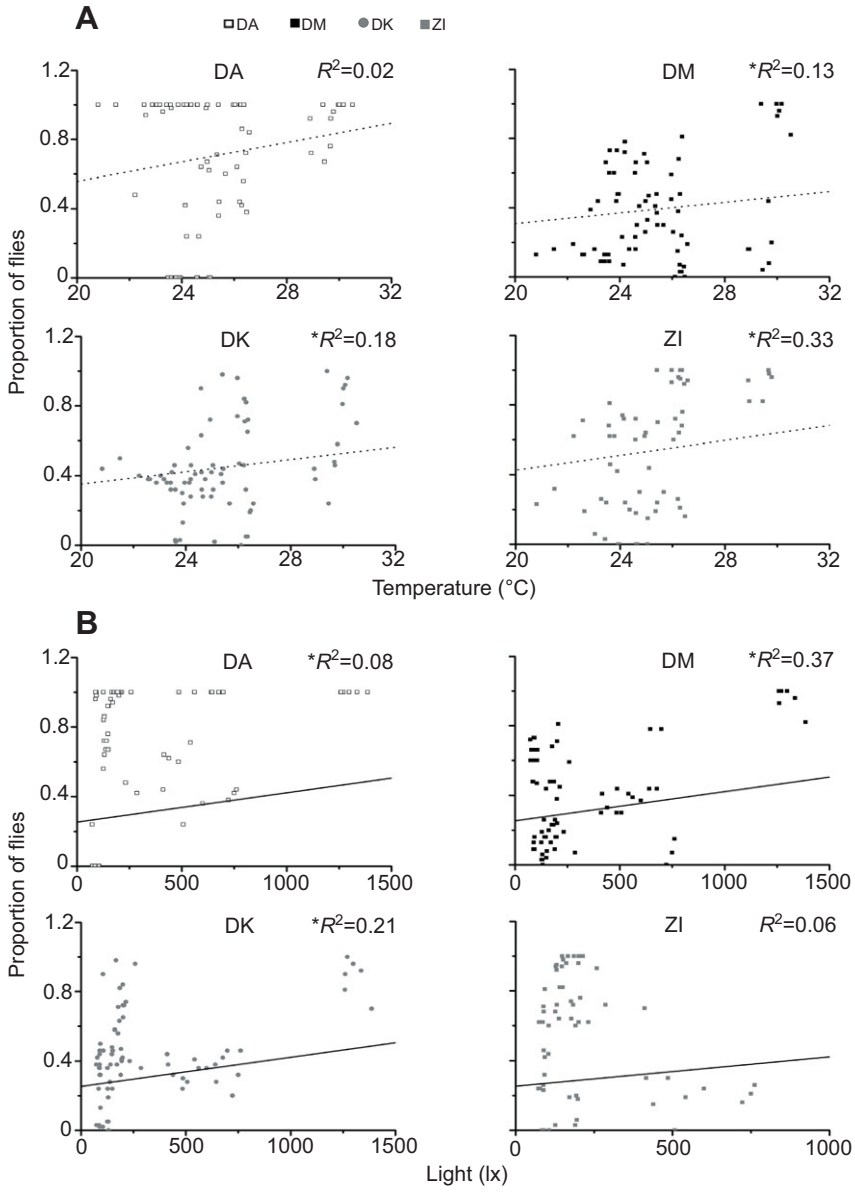

Fig. 7. Occurrence of afternoon peak (A peak) is influenced by both light and temperature. Day-wise proportion of DA, DM, DK and ZI flies showing A peak is plotted against (A) average day temperature and (B) average day light intensity. *P<0.05.

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 an alternate type of organization, which remains to be explored. Our study also reveals that even among the species that exhibit crepuscular behaviour under SN conditions, there are differences in the environmental factors with which the activity peaks are associated. This suggests species-specific variation in the zeitgeber dependence of circadian clocks.

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We thank two anonymous reviewers for critical comments on an earlier version of the manuscript; Vijay Kumar Sharma and Sheetal Potdar for suggestions; and Avani Mital and Pawas Singh for help in execution of some of the experiments. Sudeshna Das is acknowledged for the collection of flies. We thank N. Rajanna and M. Muniraju for technical assistance.

AUTHOR CONTRIBUTIONS

P.M.P. and V.S. conceived of, designed and interpreted the findings of the study. P.M.P. executed and analysed the results of the study. P.M.P. and V.S. wrote and revised the article.

COMPETING INTERESTS

No competing interests declared.

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Supplementary Online Material

Insights into differential activity patterns of Drosophilids under semi-natural conditions

*Priya M Prabhakaran, Vasu Sheeba**

Affiliation: Behavioural Neurogenetics Laboratory, Evolutionary and Organismal Biology Unit, Jawaharlal Nehru Centre for Advanced Scientific Research, Bangalore

Running title: *Activity/rest rhythm in nature.*

***Corresponding author:** Vasu Sheeba

Address: Behavioural Neurogenetics Laboratory, Evolutionary and Organismal Biology Unit, Jawaharlal Nehru Centre for Advanced Scientific Research, Jakkur P.O., Bangalore – 560 064, India

Email: sheeba@jncasr.ac.in, Ph. No. - +9180 2208 2987, Fax. +9180 2208 2766

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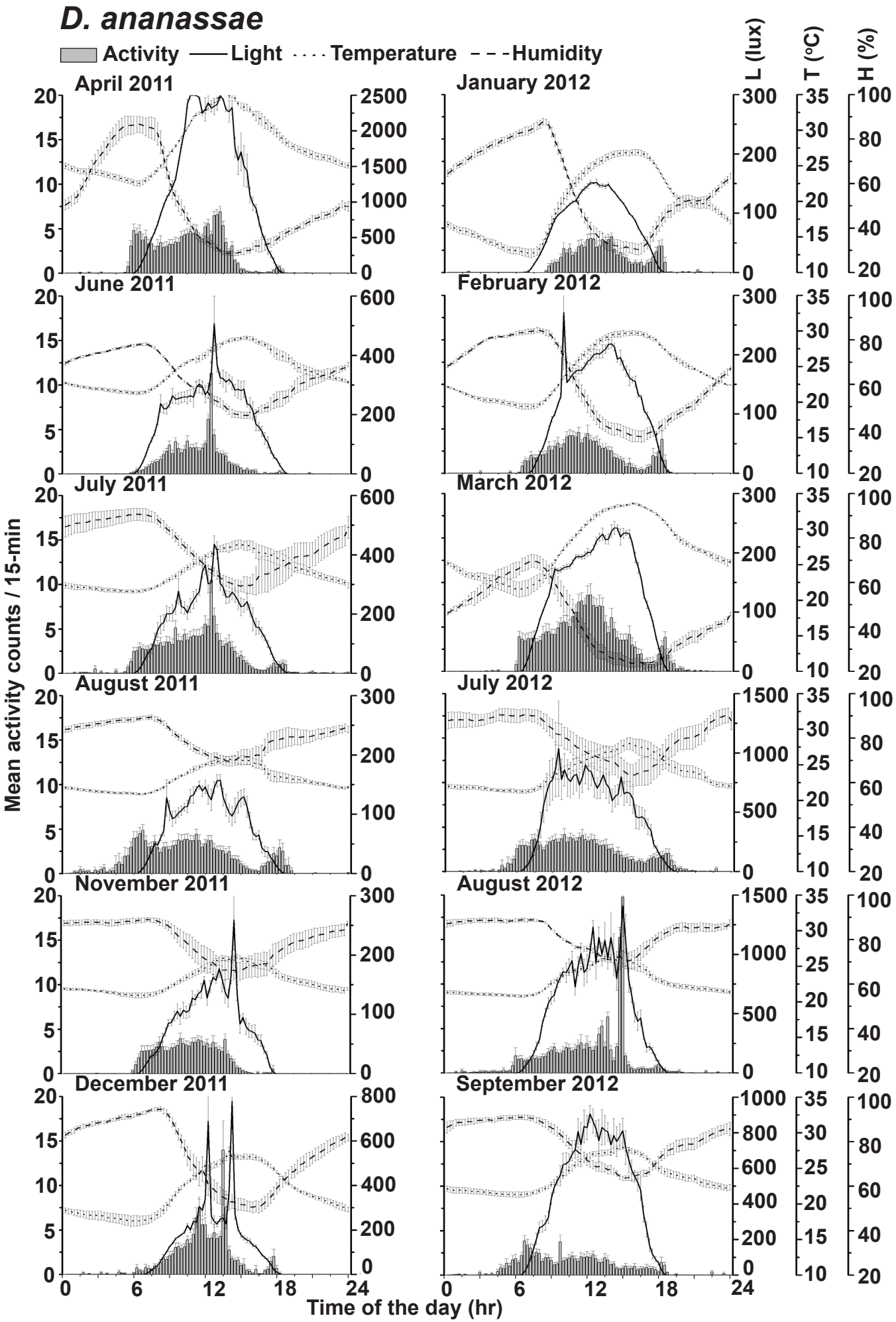

Figure S1. D. ananassae restricted most of its activity during light phase across different seasons. Average activity/rest profiles of virgin male flies *D. ananassae* (DA) across different assays in semi-natural condition. Mean activity counts, in 15-min bins (±SEM) averaged across flies over 6-days is plotted along with environmental factors L-light (solid curve), T-temperature (dotted curve) and H-humidity (dashed curve) whose values were averaged across 6-days.

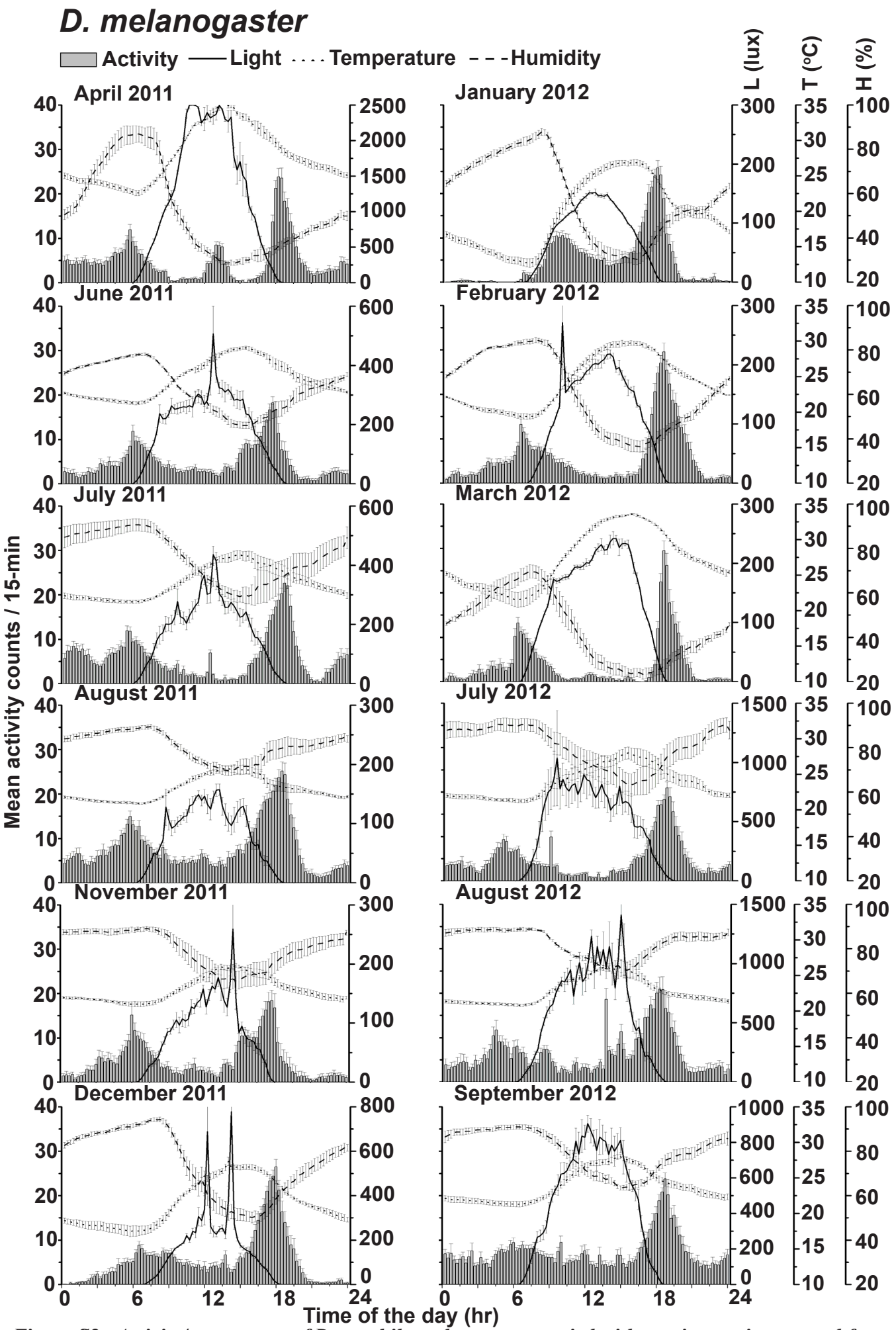

Figure S2. Activity/rest pattern of Drosophila melanogaster varied with varying environmental factors across different seasons. Average activity/rest profiles of virgin male flies *D.melanogaster* (DM) across different assays in semi-natural condition. All other details are the same as Fig. S1.

Prabhakaran and Sheeba, Figure S2

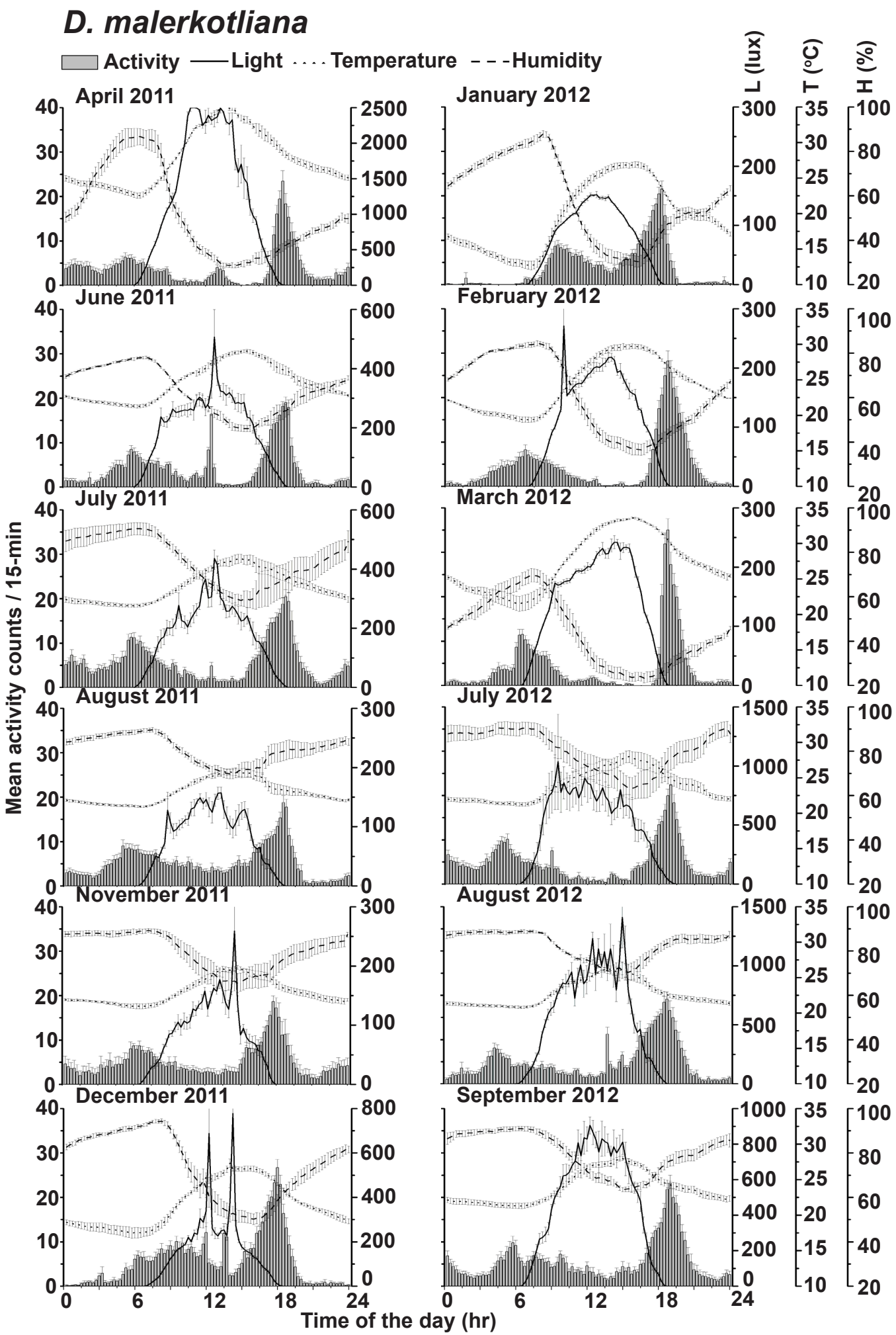

Figure S3. D. malerkotliana exhibited almost similar activity/rest pattern as that of DM across different seasons. Average activity/rest profiles of virgin male flies *D.malerkotliana* (DK) across different assays in semi-natural condition. All other details are the same as Fig. S1.

Z. indianus

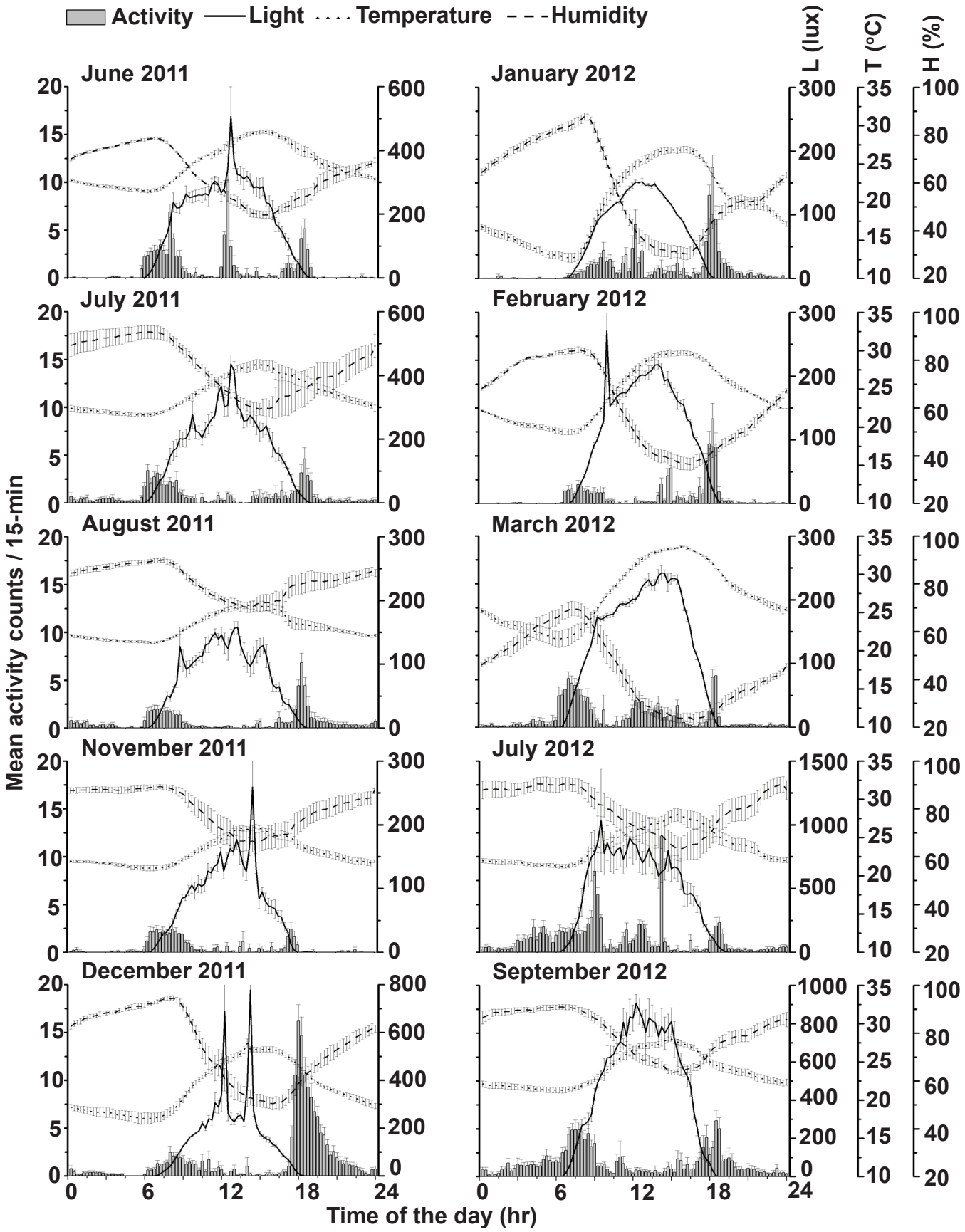

Figure S4. Z. indianus showed variation in its activity/ rest pattern across different seasons even though its activity levels were low compared to DM. Average activity/rest profiles of virgin male flies *Z. indianus* (ZI) across assays in semi-natural condition. All other details are the same as Fig. S1.

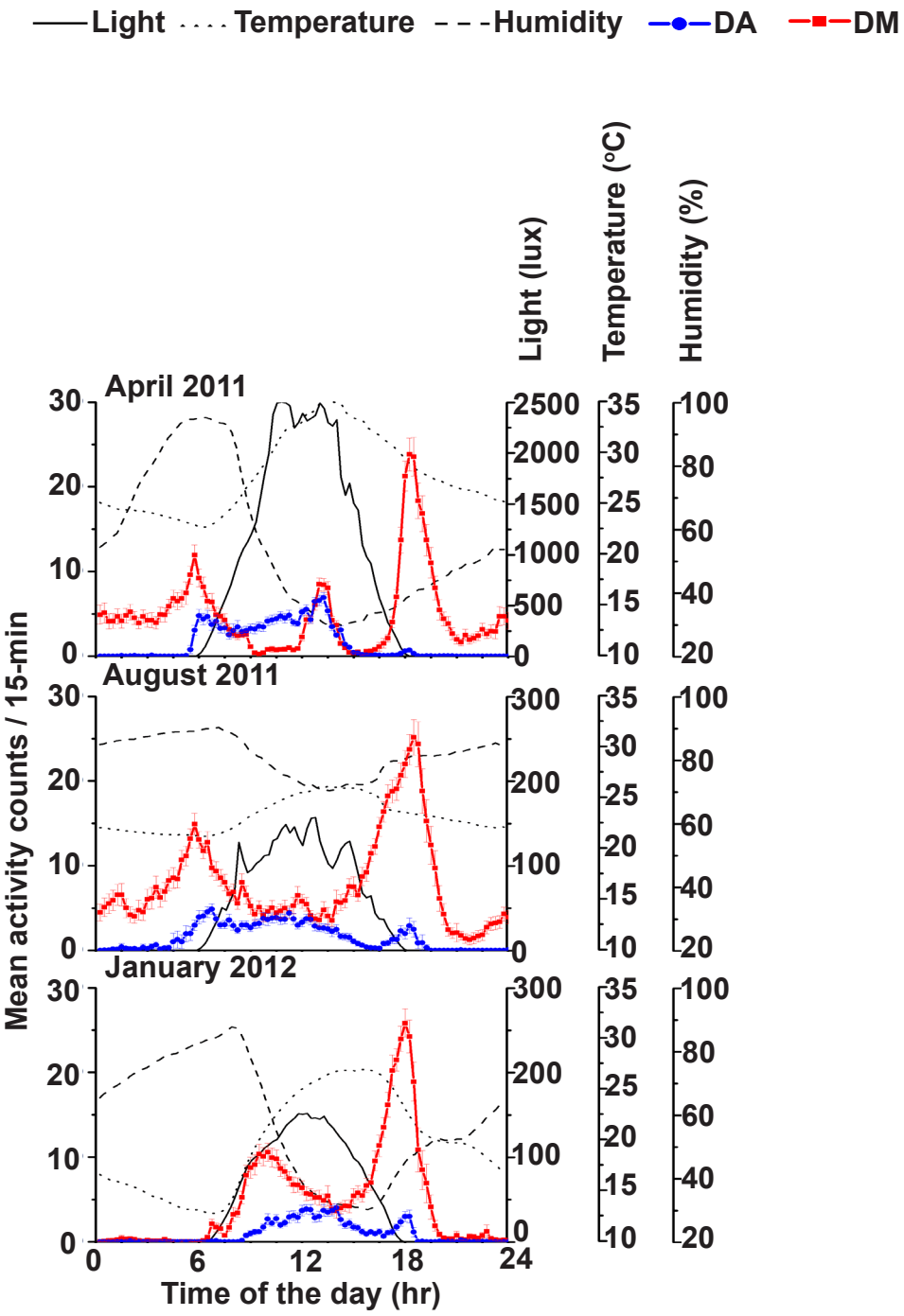

Figure S5. Divergence in activity/ rest pattern between DM and DA. Average activity/rest profiles of virgin male flies DA (blue) and DM (red) under warm dry days of April 2011 or cold dry days of January 2012 to the mild and least varying August 2011. Mean activity counts, in 15-min bins (±SEM) averaged across 6-days is plotted along with environmental factors light (solid curve), temperature (dotted curve) and humidity (dashed curve) whose values were averaged across 6-days.

Prabhakaran and Sheeba, Figure S5

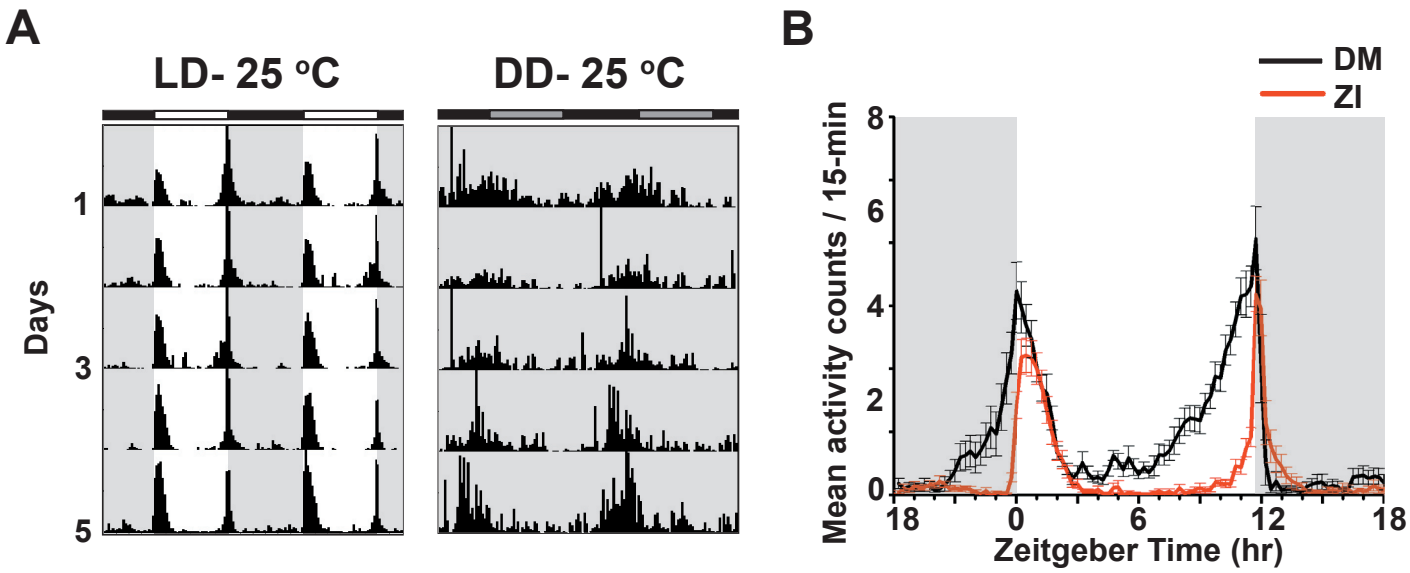

Figure S6. ZI showed bimodal activity pattern under LD12:12 and poor rhythmicity under constant darkness (DD). **(A)** Average double plotted actograms of male ZI under LD12:12 at 25 °C (left) and DD (right). The x-axis represents time of day from 0-48 hr, consecutive days are plotted along y-axis. **(B)** Raw Activity counts (15-min bin) averaged across 5 days for both DM and ZI virgin male flies (mean ± SEM). Grey shaded areas in actograms represent darkness.

Prabhakaran and Sheeba, Figure S6