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Under warm ambient conditions, *Drosophila melanogaster* suppresses nighttime activity via the neuropeptide pigment dispersing factor

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Abstract

Rhythmic locomotor behaviour of flies is controlled by an endogenous time-keeping mechanism, the circadian clock, and is influenced by environmental temperatures. Flies inherently prefer cool temperatures around 25°C, and under such conditions, time their locomotor activity to occur at dawn and dusk. Under relatively warmer conditions such as 30°C, flies shift their activity into the night, advancing their morning activity bout into the early morning, before lights-ON, and delaying their evening activity into early night. The molecular basis for such temperature-dependent behavioural modulation has been associated with core circadian clock genes, but the neuronal basis is not yet clear. Under relatively cool temperatures such as 25°C, the role of the circadian pacemaker ventrolateral neurons (LNvs), along with a major neuropeptide secreted by them, pigment dispersing factor (PDF), has been showed in regulating various aspects of locomotor activity rhythms. However, the role of the LNvs and PDF in warm temperature-mediated behavioural modulation has not been explored. We show here that flies lacking proper PDF signalling or the LNvs altogether, cannot suppress their locomotor activity resulting in loss of sleep during the middle of the night, and thus describe a novel role for PDF signalling and the LNvs in behavioural modulation under warm ambient conditions. In a rapidly warming world, such behavioural plasticity may enable organisms to respond to harsh temperatures in the environment.

KEYWORDS
activity suppression, circadian clock, *Drosophila melanogaster*, midnight activity, pigment dispersing factor, ventral-lateral neurons, warm temperature

1 | INTRODUCTION

In the fly brain, a group of ~150 neurons house circadian clocks that give rise to rhythms in physiology and behaviour, with periodicities of ~24 h. At the molecular level, so-called 'circadian clock-proteins' show oscillations in their abundance and undergo several post-translational modifications.[1] One behavioural output of this clock is rhythmic locomotion. Under standard laboratory conditions of 12:12 h::light:dark, 25°C (LD25), activity-rest behaviour has a bimodal profile across time, with two peaks of activity, around the transitions

between day and night, and periods of rest in the interim.[2] The coordinated action of the circadian clock neurons and the neuropeptides and neurotransmitters secreted by them largely contributes to this stereotyped behaviour, and is well described.[3] In the wild, especially in tropical environments, ambient temperatures can remain around 30°C or higher for much of the day. However, flies are known to prefer temperatures of 23–25°C and avoid temperatures above 27°C when exposed to a thermal gradient of 18–37°C under lab conditions.[4] In order to mitigate the harsh effects of warm temperatures in the wild, flies likely utilise behavioural strategies.[5–7] In response to potentially stressful conditions such as ambient temperatures of 29 or 30°C (LD29 or LD30) in the lab,[8,9] the circadian clock modulates activity-rest behaviour of flies such that the peaks of activity shift into the dark, that is, morning activity advances into early dawn and evening activity delays into the early night. An opposite effect is seen when flies are exposed to relatively cooler temperatures of 18°C (LD18), where flies shift their peaks of activity into the daytime.[8,10] Majercak and colleagues showed that the molecular bases of modulation of phasing of behaviour with respect to ambient temperature are the transcript levels of two canonical clock genes, *period* and *timeless*.[8,10] They found that there is an increase in *per* mRNA accumulation and a decrease in *tim* mRNA accumulation under cooler conditions of LD18 as compared with relatively warmer LD25 or LD29. Moreover, there is an increase in splicing of the *per* transcript under LD18, which causes a specific splice variant of *per*, type B' to increase in abundance, which corresponds to earlier accumulation of the PER protein. Earlier PER accumulation likely allows the molecular clock-protein loop to complete an oscillation sooner, resulting in visibly more diurnal activity patterns in flies. Little is known about the neurons involved in such behavioural modulation. Our previous work showed that the thermosensory ion channel, *Drosophila* Transient Receptor Potential-A1 (*dTRPA1*) and the neurons that express it are necessary for modulation of phasing under LD30.[9] However, the identity of *circadian* neurons involved in such plasticity and the underlying mechanisms that modulate *phasing* of behaviour under these conditions has remained unclear.

Most studies that have shed light on modulation of activity-rest behaviour in response to temperature have come from flies exposed to temperature *cycles* of alternating thermophase and cryophase (TC, 12 h each) which are known to be *entraining* cues.[11] Entrainment refers to the active and stable synchronisation of the internal clock of an organism with external time-cues[12] such as cyclic light or temperature cycles (or both). The Dorsal Neuron 1 s (DN1s), DN2s and the small ventrolateral neurons (sLNvs) play roles in enabling stable activity-rest behaviour under TCs. For instance, presence of a molecular clock in the DN1s alone is sufficient to give rise to morning and evening bouts of activity under cycles of 25–20°C TC.[13] On the other hand, when 25–20°C TC cycles are coupled with light cycles but with advanced phase (LD + TC), another subset, the DN2s, have been shown to enable entrainment.[14] Here, the DN2 molecular clock follows the phase of the TC, indicating temperature-entrainment, while the rest of the circadian circuit follows the LD phase. A conspicuous activity bout corresponds with this molecular oscillation of the DN2s, suggesting a neuron-to-behaviour mapping. Interestingly, both the DN1s and DN2s can respond to acute increase or decrease in temperatures when flies are affixed to a Peltier module and neuronal activity is recorded in vivo.[15] Apart from the DNs, lack of axonal projections from the sLNvs disrupts the ability of flies to entrain to 28–20°C TCs,[16] suggesting a role for the pacemaker LNvs in modulating activity-rest behaviour under *cyclic* temperature conditions.

Under *constant* temperature conditions and LD cycles like LD25, the LNv subgroup of the fly's clock neurons is known to regulate the morning phase of activity.[17,18] The LNvs secrete a neuropeptide, called pigment dispersing factor (PDF).[19] A lack of PDF, seen in Pdf^{01}null-mutant flies, results in the absence of morning anticipatory activity and advancement of the evening bout[20] (also see Figure 1A, arrowhead). Morning anticipation is a hallmark of a properly ticking clock's ability to predict the light transition at the beginning of the day. The phasing defect of the evening bout in Pdf^{01} flies under LD25 suggests a role for PDF in communicating with the canonical evening cells (E-cells), which are thought to require 'delaying' signals via PDF to appropriately phase behaviour, as they inherently possess faster running clocks.[20–22] Despite these behavioural defects, molecular rhythms in clock neurons of Pdf^{01} flies under LD25 are not severely affected and hence the advanced E-peak can be attributed to a stably entrained clock.[23] However, when Vaze and Helfrich-Forster utilised environmental cycles (T-cycles) ranging in periodicities between 22–32 h (T22-LD11:11 h and so on), they observed large desynchrony among molecular rhythms in the clock neurons of Pdf^{01} flies under T32 (LD16:16 h). There were also severe defects such as: (i) an extremely advanced E-phase under T32, which was unexplained by PDF-mediated phase-delays of E-cells, and (ii) an unchanging phase-relationship of the E-peak with lights-OFF across T-cycles, indicating lack of stable entrainment under LD. Thus, there seem to be complex ways in which PDF modulates the phasing of behaviour across different environmental conditions. Given this, we explored if PDF or the LNvs themselves may have additional roles in phasing activity-rest behaviour, such as when the ambient temperature is relatively warm (30°C).

We examined the activity-rest behaviour of flies where PDF levels or the LNv system were modified and found that experimental flies show heightened activity in the middle of the night under relatively warm ambient conditions. Thus, we were able to uncover a previously unreported role for PDF signalling and the LNvs in distribution of activity through the night under relatively warm ambient conditions, specifically, in suppression of activity during midnight.

2 | MATERIALS AND METHODS

2.1 | Fly strains

All genotypes were reared on standard cornmeal medium under LD (12 h Light: 12 h Dark) and 25°C. The lines *Pdf-GAL4*, *tim-GAL4 (A3)*, *UAS-Dti* and *UAS-Kir2.1* were obtained from Todd Holmes, the PDF mutant yw^{0}; Pdf^{01} (*w15*) and its background control (*w33*) from Paul

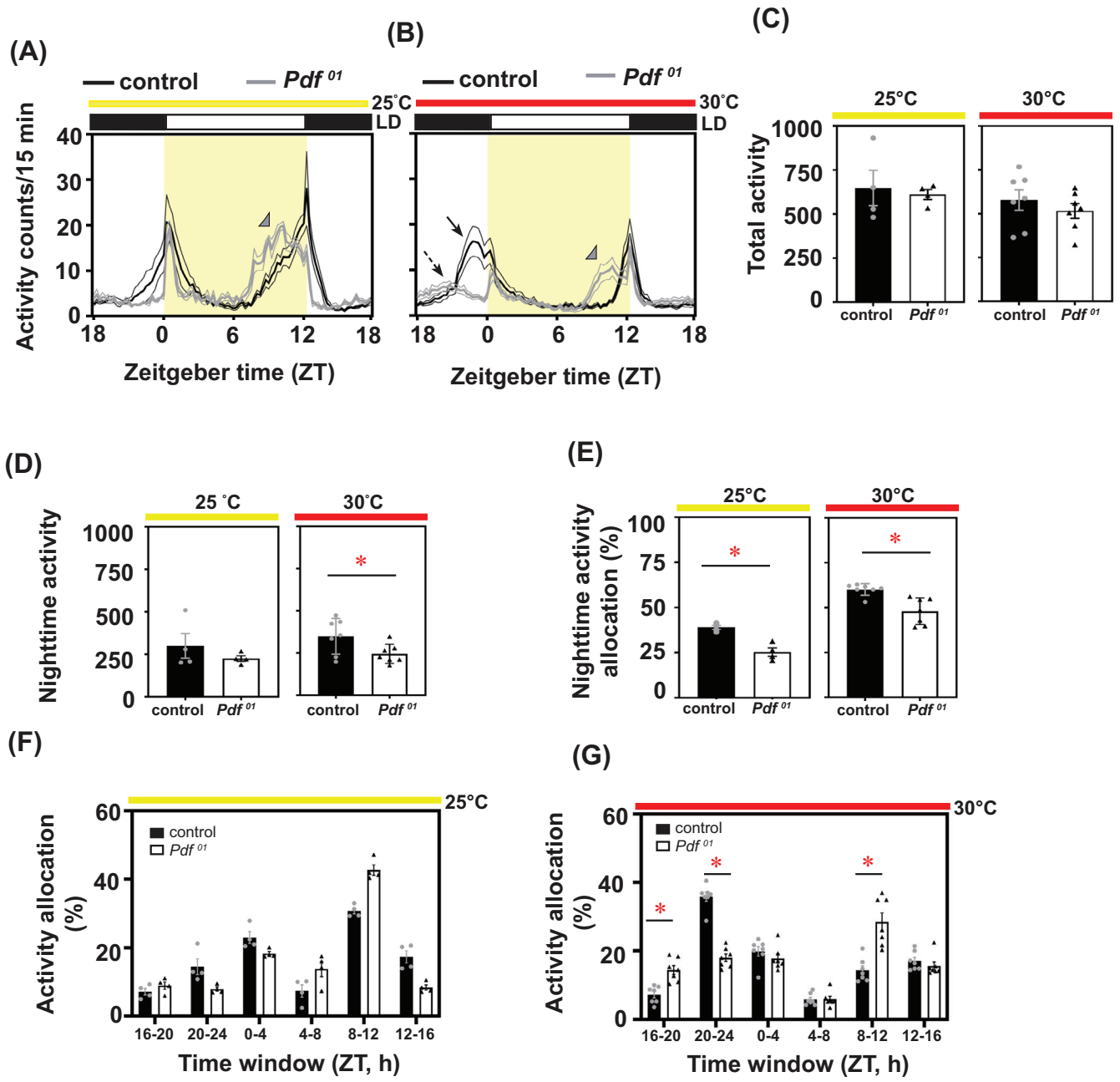

FIGURE 1 Under LD 12:12 and warm ambient temperature of 30°C (LD30), lack of PDF in Pdf^{01} flies results in an increased midnight activity (ZT16-20), as compared with controls. (A, B) Activity profiles for control (bold black trace) and Pdf^{01} (bold grey trace) flies, averaged across means of replicate experiments and plotted against Zeitgeber time (ZT). Faint traces indicate SEM. Black bar on top indicates dark (night) and white bar and shaded region indicates light (day); constant temperature is indicated with a solid bar above. (A) Under LD25, control flies display two bouts of activity around the light transitions, while Pdf^{01} flies display a morning bout reduced to a startle and an advanced Evening peak (E-peak) (arrowhead). (B) Under LD30, control flies display an advanced phase of Morning (M)-peak (solid arrow), while Pdf^{01} flies show increased activity during the night (dashed-arrow) and an advanced E-peak (arrowhead). (C–G) Quantification of activity levels for control (black bar) and Pdf^{01} flies (white bar), averaged across replicate experiments under LD25 or LD30: (C) The total activity level is not significantly different between genotypes under LD25 or LD30. (D) The activity level during nighttime (12 h) is not significantly different between genotypes under LD25, but is significantly decreased in Pdf^{01} flies, under LD30. (E) The percentage of activity allocated to nighttime is significantly decreased in Pdf^{01} flies under LD25 as well as LD30. (F) The percentage of activity allocated to individual time-windows is not significantly different between control and Pdf^{01} flies under LD25 (Midnight, ZT 16–20, Dawn, ZT 20–24; Morning, ZT 0–4; Afternoon, ZT 4–8; Evening, ZT 8–12 and Dusk, ZT 12–16). (G) The percentage of activity allocated to individual time-windows under LD30 is significantly different between control and Pdf^{01} flies during three time-windows, such that the Pdf^{01} flies show decreased levels during Dawn (ZT20-24) and increased levels during Midnight (ZT16-20) and Evening (ZT8-12), as compared with controls. There is no difference between genotypes during the remaining three time-windows (Morning, ZT8-12; Afternoon, ZT12-16 and Dusk, ZT16-20). See text for comparison across temperatures. Error = *SEM* across experiments for LD25 ($N = 4$) and LD30 ($N = 7$), number of biological replicates, $n = 16–32$ for control and $n = 20–32$ for Pdf^{01}per replicate (Details in Table S1). In (C–G) symbols indicate means of replicate experiments; asterisks indicate statistically significant comparisons

Taghert,[20] and the following lines from Bloomington Drosophila Stock Centre (NIH P40OD018537): *Pdfr*^{3369} (#33069), *Pdfr*^{5304} (#33068), *UAS-dicer* (#24650) and *UAS-Pdf-RNAi* (#25802).

2.2 | Behavioural assays

Locomotor assay in brief: Flies were reared and collected at 25°C. Virgin males were used for the study, except for Figure S1 where virgin females were also tested. 2–3-day old flies were housed individually, inside glass tubes (6.5 cm × 0.7 cm), with standard corn food (filled ~1/5th of their length) at one end that was sealed with wax and plugged with cotton at the other end. These tubes were loaded into Drosophila activity monitors (DAM, RRID:SCR_021798)[24] Trikinetics, Waltham) which were placed in incubators manufactured by Sanyo (MIR-134, Japan) under constant temperature and light intensity (~200–250 lux) during daytime. A tray of water was kept inside the incubator to maintain humidity to ensure that all behavioural responses resulted from temperature changes alone. Zeitgeber time or ZT is the timestamp of the environmental time-cue. In these experiments, strict alternating light: dark (LD) cycles served as time cues and by convention, time of lights-ON is defined as ZT0. For flies in an LD30 experiment, the first experience of high temperature always coincided with the phase of lights-ON, following which there was no change in temperature until the end of the experiment. The number of IR beam-interruptions acts as a proxy for activity levels and is recorded in notepad files (DAM System3 software, version v3.03) (RRID:SCR_021809) on a computer connected to the DAMSystem.[24] The data used for analysis were collected between days 2 and 6 post-introduction into assay tubes (age: 4–8 days post-eclosion).

2.3 | Analysis

For a genotype, raw activity counts per fly were binned into 15-min bins (DAMFileScan software, version v1.10) (RRID:SCR_021817), were averaged across 5 days and normalised to average total activity per day for a given experiment. These are henceforth referred to as 'percentage activity levels'. Data were similarly binned into 4-h bins to calculate raw activity levels and percentage activity allocated to different time windows. *M* and *E-phases* were calculated for an individual by marking average phase across days from activity profiles. *Actograms*: Individual actograms were normalised to average maximum activity per individual and batch actograms were obtained by averaging across individuals of a genotype. These were plotted using ClockLab (RRID:SCR_014309) in Matlab. *Sleep*: Sleep profiles, total sleep and sleep during the midnight window (ZT16-20) were calculated using MS Excel. Activity per waking min (ZT16-20): Total duration spent waking during ZT16-20 was calculated by subtracting sleep (min) during this window from the total duration of the window (240 min). Dividing raw activity counts during this window by the above, yielded activity counts per waking minute which estimated how active the flies were, when awake. All figures were plotted using GraphPad Prism (RRID:SCR_002798, version 9.0.0) for Windows (www.graphpad.com) and arranged using Adobe InDesign CS (RRID: SCR_021799, version 3.0).

2.4 | Statistical analysis

Mean values were obtained for a replicate experiment by averaging across individual flies and these were used as data points for analysis. In cases where the experiment was performed only once, analysis was performed utilising individual fly data. Details of sample sizes are provided in Table S1. *Activity levels*: In a pilot experiment with *Pdf*^{01} and its background control, activity levels were compared across time-windows and genotypes using a repeated-measures analysis of variance (ANOVA). As the sphericity assumption tested by Maucheley's test was violated, Tukey's HSD could not be used as a post hoc test. Instead, a paired *t* test was performed between *Pdf*^{01}and control for individual time-windows and the family-wise error was adjusted using the Bonferroni corrected $\alpha = 0.008$. In experiments that followed, we performed comparisons for only those time-windows where we observed differences in the *Pdf*^{01} experiments. For this, we employed the mixed-model ANOVA with *genotype* as fixed and *replicate* experiment as a random factor and implemented Tukey's HSD as a post hoc test, keeping $\alpha = 0.05$. All tests were performed using STATISTICA™ software (RRID:SCR_014213, version 7) by StatSoft Inc., USA, except Tukey's HSD which was performed in MS Excel, using the MSE and *df* from ANOVA Tables. *M-phase*: A single-sample *t* test was performed to test if the average phase of the M-peak for a genotype was different from ZT0. *E-phase*: A paired *t* test or mixed-model ANOVA (as described above) was performed where appropriate to test if the average phase of the E-peak was different across genotypes. *Frequency of midnight bout*: Raw activity profiles were categorised as displaying an advanced M-peak or midnight activity bout following visual observation of activity profiles. Data were pooled across experiments and compared between genotypes using chi-square tests of independence. *Comparisons across temperature regimes*: Experiments were performed separately for LD25 and LD 30 regimes. Different strategies were employed to make comparisons between these regimes depending on the number of replicate experiments performed. Experiments where N≥1 for LD25: These data were analysed using within-subjects ANOVA (i.e., repeated measures ANOVA) where *temperature* was incorporated as a fixed factor and *genotype* was the within-subject factor. Post hoc differences were identified as described above (Activity levels). Experiments where $N = 1$ for LD25: Here, population-level estimates were obtained for each genotype at LD30 by averaging across means of replicate LD30 experiments. Individual-level measurements under LD25 were subtracted from population means for the corresponding genotypes to obtain individual-level deviation values. These data were analysed using one-way ANOVA, followed by Tukey's HSD (or a Welch ANOVA when the Levene's test of homogeneity was violated) to identify pair-wise differences between genotypes. *Sleep analysis*: Statistical comparisons were performed separately for each replicate experiment using *t*-tests owing to large variation in sleep levels across replicate experiments.

2.5 | Immunocytochemistry

Flies were reared under LD25 and maintained under LD30 for 3–4 days (age: 5–6 days post-eclosion) before dissection. Flies were dissected around ZT2-4 in ice-cold Phosphate buffered saline (PBS) and brains fixed in 4% Paraformaldehyde (PFA) for 30–40 min. Brain samples were treated with blocking solution (10% horse serum) for 1-h at room temperature and additional 6-h at 4°C to reduce non-specific binding of antibodies. This was followed by incubation with primary antibodies at 4°C for 24–48 h (anti-PER, host: rabbit, 1:20,000, gift from Jeffrey Hall, Brandeis University) and anti-PDF (RRID: AB_2315084) (host: mouse, 1:5000, DSHB). After incubation, brain samples were given 6–7 washes with 0.5% PBS + Triton-X (PBT), after which they were incubated with Alexa-fluor conjugated secondary antibodies for 24-h at 4°C (goat anti-rabbit 488 and goat anti-mouse at 1:3000, Invitrogen). The brain tissue was further washed 6–7 times with 0.5% PBT, and finally cleaned and mounted on a glass slide in mounting medium (7:3 glycerol: PBS). Images were acquired using a Zeiss LSM 880 microscope.

Quantification of anti-PDF intensity: Brain samples were imaged at ×40 magnification. Maximum intensity projections are provided for visualisation. *Statistical analysis of imaging data*: Analysis was performed on raw images using Fiji software[25] (RRID:SCR_002285). Staining intensity was estimated for each observable large ventrolateral neuron (lLNVs) by measuring its pixel intensity and subtracting the average of three background measurements from it. Mean value for a brain was arrived at by averaging staining intensity across all lLNVs detected in a single hemisphere, and then averaging across both hemispheres of a brain sample. Since this dataset violated the assumption of variance homogeneity, these means were compared across genotypes using a Welch ANOVA. Data were plotted using the values for pixel intensity normalised to the average value for control (Figure 2H) to provide an estimate of fold-change.

3 | RESULTS

3.1 | PDF is necessary to suppress midnight activity levels, under warm ambient conditions of LD30

Unlike behaviour under LD25 (Figure 1A), the morning peak (M-peak) of control flies was advanced relative to lights-ON under LD30, (Figure 1B, solid arrow). We observed that only males displayed this behaviour under LD30 (Figure S1A–C) and that the phasing of the M-peak was not different across temperatures for females (Figure S1A–D). The M-peak of control males occurred around ZT23 (Figure S2A, left). On the other hand, Pdf^{01} male flies only show a startle to lights-ON occurring at ZT0 (Figure 1A,B; Figure S2A, left) without anticipation. The evening peak (E-peak) in Pdf^{01}flies showed an advance as compared with control (Figure 1A,B, arrowhead), occurring around ZT10 (Figure S2A, right).[17] The startle to lights-ON and the advance in E-peak under LD30 is consistent with the behaviour of Pdf^{01} flies under LD25 as reported previously.[20] However, certain features about the behaviour under LD30 (Figure 1B), become apparent upon further examination.

We compared the activity levels in a day (24 h) across genotypes under LD25 and LD30. Under relatively cool LD25, there was no difference in overall activity levels between the genotypes (LD25: $t_{(3)} = 0.459$, $p = 0.677$, LD30: $t_{(6)} = 1.051$, $p = 0.333$, Figure 1C), suggesting that lack of PDF did not affect overall activity levels. However, as is visible in the average profile of activity under LD30, Pdf^{01} flies show about of activity a little after midnight (Figure 1B, dashed arrow; Figure S2B, blue arrow) that is not seen under LD25 (Figure 1A). We observed a significant effect of genotype on the frequency of this behaviour, with a greater proportion of Pdf^{01} flies displaying midnight activity as compared with control (chi-square test of independence, $\chi^2_{(1,1)} = 302.707$, $p << 0.001$, Figure S2C) across seven replicate LD30 experiments.

Next, we quantified raw activity levels and the proportion (or percentage) of activity allocated to the daytime (12 h) and nighttime (12 h) under cooler LD25 and warmer LD30 to estimate the impact of lack of PDF-neuromodulation on apportioning of activity into day and night. Comparing raw activity levels during daytime or nighttime between the two genotypes showed time-of-day-specific and temperature-specific differences. While there was no difference between genotypes during daytime under either regime (LD25: $t_{(3)} = -1.825$, $p = 0.165$; LD30: $t_{(6)} = -1.188$, $p = 0.279$, Figure S2D), Pdf^{01} flies exhibited significantly lower raw activity counts during nighttime under LD30 ($t_{(6)} = 3.149$, $p = 0.019$) but not under LD25 ($t_{(3)} = 1.909$, $p = 0.152$, Figure 1D). Thus, average nighttime activity levels were affected in flies that lack PDF but only under warm conditions. The *proportion* or *percentage* of total activity allocated to the nighttime differed significantly between control and Pdf^{01}flies. Irrespective of ambient temperature, Pdf^{01} flies allocated a lower proportion of activity to nighttime (LD25: $t_{(3)} = -6.062$, $p = 0.009$, LD30: $t_{(6)} = 3.585$, $p = 0.011$, Figure 1E), probably on account of increased activity during the evening time window. Taken together, these results indicate a role for PDF in allocating activity through the day (Percentage of activity during nighttime, Figure 1E) without affecting overall total activity levels (Figure 1C).

Next, we carried out detailed quantification of activity allocation to different times of the day (percentage of total activity), by dividing the day (24 h) into six time-windows (4 h each): Three daytime windows: Morning (ZT0-4), Afternoon (ZT4-8) and Evening (ZT8-12), and three nighttime windows: Dusk (ZT12-16), Midnight (ZT16-20) and Dawn (ZT20-24) (Figure 1F,G). We compared activity levels between the two genotypes using a paired *t* test, with Bonferroni correction $\alpha = 0.008$, for each time-window under LD25 or LD30 and found increased activity allocation in Pdf^{01} flies to one daytime window, Evening (ZT8-12), under cooler conditions of LD25 ($t_{(3)} = -6.982$, $p = 0.006$, Figure 1F) as well as under relatively warmer LD30 ($t_{(6)} = -6.733$, $p = 0.000$, Figure 1G). This could be attributed to the advanced evening bout during the daytime in Pdf^{01}flies (Figure 1A,B, arrowhead). Further, while there was no significant difference between genotypes across the three nighttime windows under LD25

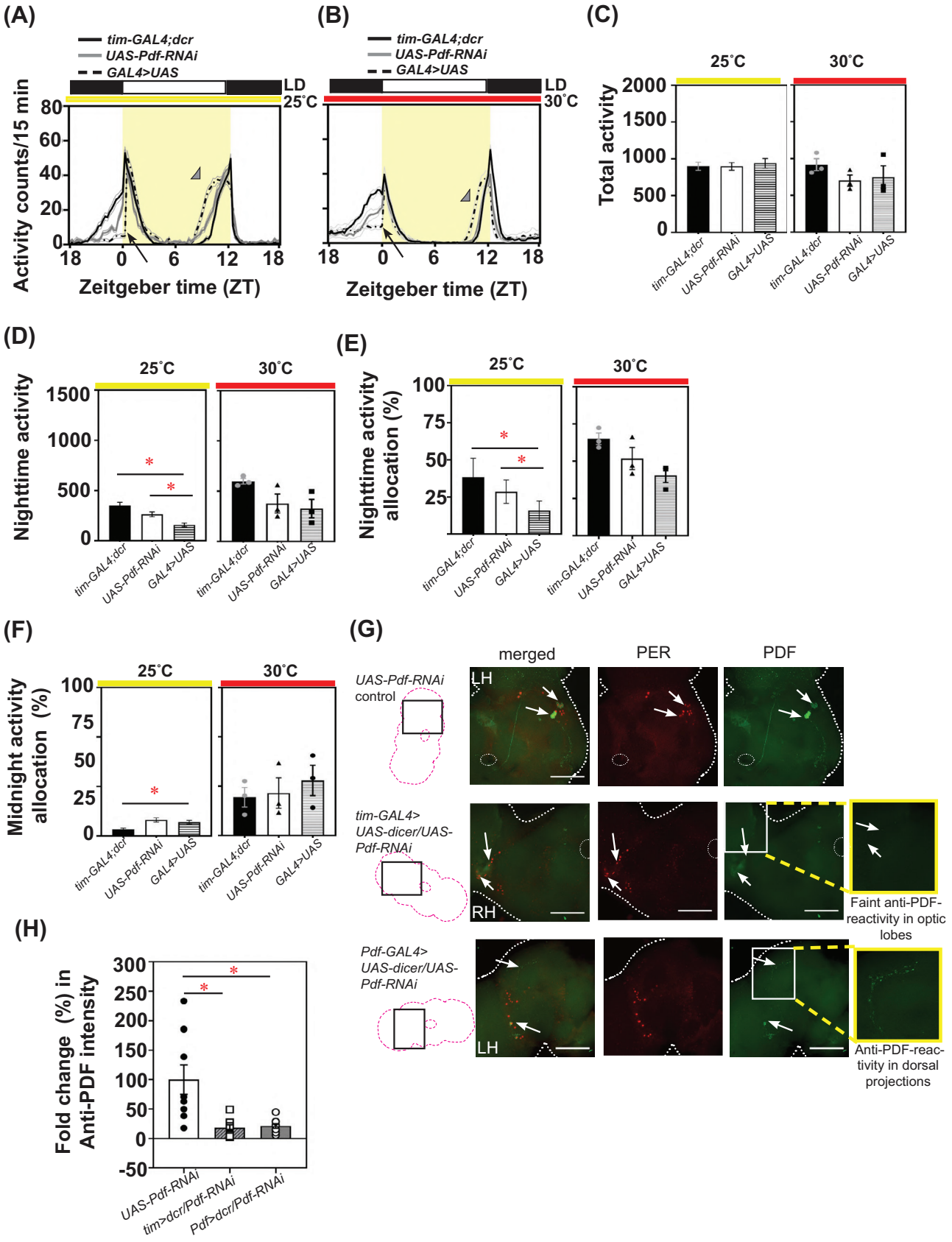

FIGURE 2 Legend on next page.

(Dusk: $t_{(3)} = 5.172$, $p = 0.014$; Midnight: $t_{(3)} = 2.888$, $p = 0.063$; Dawn: $t_{(3)} = 2.559$, $p = 0.080$, Figure 1F), under relatively warmer LD30, control flies displayed a significantly higher activity during Dawn ($t_{(6)} = 12.392$, $p = 0.000$) as compared with *Pdf*^{01}flies (Figure 1G). This could be attributed to the advancement of the morning peak under LD30 in control flies (Figure 1B, solid arrow), and the inability of *Pdf*^{01}flies to do the same. In addition to these differences, *Pdf*^{01} flies also displayed significantly higher activity than controls in the Midnight window (ZT16-20) specifically under LD30 (LD25: $t_{(3)} = 2.888$, $p = 0.063$, LD30: $t_{(6)} = -4.888$, $p = 0.002$, Figure 1G and Figure 1B, solid arrow). To test how *Pdf*^{01} and control flies responded across temperatures, we utilised a design that incorporated temperature regime as a fixed factor (see methods) and were able to identify a significant interaction between genotype and temperature in their effects on the proportion of activity allocated to midnight. Supporting our above observations, *Pdf*^{01} flies significantly increased the proportion of activity in the midnight under LD30 as compared with LD25 ($F_{(1,9)} = 7.013$, $p = 0.026$). This suggested a temperature-specific modulation of behaviour mediated via PDF. Raw activity counts in individual time windows for the two regimes were also quantified and the results are summarised in Figure S2E,F.

We tested if the observed increase in midnight activity (ZT16-20) in mutant flies affected sleep levels (Figure S3A–E). Overall sleep levels increased in *Pdf*^{01} mutants under LD25, as previously reported,[26] but showed no differences between genotypes in three out of four experiments under LD30 (Figure S3C). Sleep levels during midnight (ZT16-20) were not different between genotypes under LD25 but were significantly lower for *Pdf*^{01}flies ($p < 0.01$) in 3 out of 4 experiments under LD30 (details in Table S2). This reduced sleep was not accompanied by hyperactivity (Activity/waking min: $p >> 0.05$, Figure S3D). With these results, we conclude that lack of PDF causes allocation of significantly higher proportion of midnight activity (ZT16-20) under warm LD30 (Figure 1G), possibly because of losing sleep in this window (Figure S3C).

3.2 | Even a small amount of PDF is sufficient to suppress midnight activity levels under warm ambient conditions of LD30

We examined the role of PDF in modulation of locomotor activity under LD30 by tissue-specific downregulation of PDF via RNA interference. We used two separate drivers, the *tim-GAL4 (A3)* driver, which can target a broad set of the circadian neurons[27,28] and the *Pdf-GAL4* driver that targets only the PDF-expressing LNvs.[20] Under relatively cool LD25, experimental flies, *tim-GAL4 (A3)>UAS-dicer/UAS-Pdf-RNAi* displayed behavioural defects similar to *Pdf*^{01}flies, that is, a reduced morning peak (Figure 2A,B, arrow) and an advanced evening peak (Figure 2A,B, arrow head) as reported previously.[27] Overall activity levels under LD25 ($N = 1$) and LD30 ($N = 3$) were comparable across genotypes (LD25: $F_{(2,90)} = 0.1662$, $p = 0.847$; LD30: $F_{(2,2)} = 1.278$, $p = 0.372$, Figure 2C), similar to our observations with *Pdf*^{01} flies (Figure 1C). Activity levels in experimental flies were significantly higher through daytime under LD25, but not LD30 (LD25: $F_{(2,90)} = 8.503$, $p = 0.004$; LD30: $F_{(2,2)} = 2.583$, $p = 0.190$; Figure S4A) and lower through nighttime under LD25, but not under LD30 (LD25: $F_{(2,90)} = 14.879$, $p = 0.000$; LD30: $F_{(2,2)} = 2.945$, $p = 0.163$ Figure 2D). Further, although a trend was visible for percentage of activity allocated to nighttime which was similar across both temperatures, we observed significantly lower levels in experimental flies only under LD25 (Figure 2E, LD25: $F_{(2,90)} = 43.869$, $p = 0.000$; LD30: $F_{(2,2)} = 3.797$, $p = 0.119$). Despite these similarities, we failed to identify differences in midnight activity allocation across genotypes at LD30 although a trend in the appropriate direction was observed. When we compared data across temperatures, we found that all genotypes increased their activity allocation to the midnight under LD30 ($F_{(2,57.45)} = 15.002$, $p = 0.000$). However, the experimental genotype showed significantly larger increases relative to the parental controls. We also made observations with a second set of experimental flies under LD30 ($N = 3$), *Pdf-GAL4>UAS-dicer/UAS-Pdf-*

FIGURE 2 Under LD30, even a small amount of PDF in clock neurons is sufficient to suppress midnight activity (ZT16-20). (A,B) Average activity profiles for parental controls, *tim-GAL4;UAS-dicer* (black trace) and *UAS-Pdf-RNAi* (grey trace) and experimental flies, *tim-GAL4>UAS-dicer/UAS-Pdf-RNAi*, (dashed trace) under (A) LD25 and (B) LD30. All other details same as Figure 1. Control flies display two bouts of activity around the light transitions, while experimental flies display a reduced morning peak (arrow) and an advanced evening bout (arrowhead). (C–H) Quantification of activity levels for *tim-GAL4;UAS-dicer* (black bar), *UAS-Pdf-RNAi* (white bar) and experimental flies, *tim-GAL4>UAS-dicer/UAS-Pdf-RNAi*: (C) There is no significant difference across genotypes in total activity under LD25 or LD30. (D, E) There are significant differences between genotypes in (D) activity during nighttime and (E) percentage of activity allocated to nighttime. (F) However, activity allocated to midnight (ZT16-20) did not show significant differences under LD25 or LD30. See text for comparison across temperatures. Error = SEM across individuals (LD25, $N = 1$) or means of replicate experiments (LD30, $N = 3$); number of biological replicates-*tim-GAL4;UAS-dicer*: $n = 15–31$;*UAS-Pdf-RNAi*: $n = 16–30$; experimental flies, $n = 23–32$). (G) Brain tissue stained (scale bar = 100 μm) for PERIOD protein (red) and PDF neuropeptide (green) in control (w>*UAS-Pdf-RNAi*, above) and experimental flies, *tim-GAL4>UAS-dicer/UAS-Pdf-RNAi* (middle) and *Pdf-GAL4>UAS-dicer/UAS-Pdf-RNAi* (below). PDF$^+$ neurons were identified based on location and co-staining with anti-PER. PDF$^+$ LNvs and their axonal projections are observed in the *w>UAS-Pdf-RNAi* control (arrows) and are stained by both, anti-PER and anti-PDF antibody. In the experimental genotype, *tim-GAL4>UAS-dicer/UAS-Pdf-RNAi*, while staining for anti-PER is detected, staining for anti-PDF is faint, indicating reduced level of PDF. In the second genotype, *Pdf-GAL4>UAS-dicer/UAS-Pdf-RNAi*, staining for anti-PER is detected, and staining for anti-PDF is reduced in the cell bodies but is visible in the dorsal projections (inset). (H) Quantification of anti-PDF staining for control and experimental genotypes: Scatter plot depicts mean PDF-staining intensity in large LNvs for brain samples, averaged across hemispheres and then normalised to average intensity in control (100%), to obtain percentage fold change. The PDF-staining is ~80% lower and significantly reduced for the experimental genotypes as compared with control. (Error = *SEM* across brains, $n = 10$ whole brain samples per genotype, $N = 1$; * = significant difference from control, symbols indicate mean for a single brain sample)

RNAi, and found that these individuals displayed behaviour comparable to controls. Neither were there any differences in phasing of the morning peak (Figure S5A,B), nor were any major differences in activity levels observed (Figure S5C–G). We speculated that incomplete downregulation of PDF using the RNAi approach may be a possible reason for these observations. To test this, we performed PDF antibody staining on brain samples of experimental genotypes, *tim-GAL4 (A3)>dicer/Pdf-RNAi* and *Pdf-GAL4>dicer/Pdf-RNAi* and compared mean staining intensities of PDF^+ large LNvs with that of *UAS-Pdf-RNAi* parental control (Figure 2G–H). The PDF-staining intensity in both experimental genotypes was reduced by ~80% as compared with control samples ($F_{(2,15)} = 4.968$, $p = 0.028$). However, we observed faint anti-PDF reactivity in the optic lobes of some *tim-GAL4 (A3)>dicer/Pdf-RNAi* samples (inset) and in the dorsal projections of the PDF^+ small LNvs of *Pdf-GAL4>dicer/Pdf-RNAi* samples (inset). The existence of low levels of PDF in the experimental genotypes may explain why we failed to observe increases in midnight activity allocation in *Pdf-GAL4>dicer/Pdf-RNAi* flies and diminished, albeit still significant, increases in *tim-GAL4 (A3)>dicer/Pdf-RNAi* flies.

3.3 | The midnight activity bout in Pdf^{01} flies under LD30 may be clock-controlled

Why does activity in Pdf^{01} mutants under relatively warm temperature manifest only during a specific time, that is, the middle of the night? In other words, is it time-of-day and thus, clock-dependent? To answer this, we subjected control and Pdf^{01} flies to two different photoperiods (Figure S6). When exposed to a photoperiod other than equinox (12:12), the circadian clock is expected to track the phase of lights-OFF and to achieve stable phase-relationships between circadian behaviour and the zeitgeber.[29,30] We hypothesised that if the midnight bout of activity is circadian clock-dependent, then advancing or delaying the phase of the lights-OFF (pink dotted line, Figure S6A) should correspondingly advance or delay the phase of the midnight bout (blue dashed line, Figure S4A). Keeping temperature at 30°C, we advanced the phase of lights-OFF such that it occurred 4 hours earlier (LD 08:16, short photoperiod) or delayed it such that it occurred 4 h later (LD 16:08, long photoperiod) than the reference LD cycle (LD 12:12, equinox photoperiod).[30] As expected, the phase of the morning peak from lights-ON in control flies was advanced under the short photoperiod and delayed under long photoperiod when compared with the phase under equinox ($F_{(1,2)} = 4262.6$, $p = 0.001$; Figure S4B, top [yellow circle]). In Pdf^{01} flies, we observed that the phase of the midnight bout was advanced under short photoperiod relative to its phase under equinox ($t_{(56)} = 2.528$, $p = 0.014$; Figure S6B, below [blue symbol]; Figure S6C, top [arrow]), like the control morning peak, indicative of clock-control. However, under a relatively longer photoperiod, we observed only one bout of activity in Pdf^{01} flies during the dark phase, which coincided with the phase of lights-ON (Figure S6C, bottom). This made it difficult to determine if it was the midnight bout or the morning startle and assess its clock-dependence. We speculate based on results from the short photoperiod experiment that the timing of increase, or lack of suppression of activity in the Pdf^{01}flies, is indeed clock-controlled. Nevertheless, this hypothesis requires further validation using other photoperiod regimes.

3.4 | PDFR is necessary to suppress midnight activity levels, under warm ambient conditions of LD30

We reasoned that the phenotype detected in the Pdf^{01} mutant must be elicited via the action of PDF on its only identified receptor—PDF receptor (PDFR), *Han* (*or groom-of-Pdf*, *gop*),[31] which is present on several cells within and outside the circadian pacemaker circuit in the brain. Flies that possess a truncated *Pdfr* gene show a defect in activity-rest behaviour under LD25 (Figure 3A), thus phenocopying Pdf^{01} flies[31–33] and in temperature preference behaviour in the middle of the night.[32] With this prior knowledge, we tested the behaviour of two known mutants of the receptor gene, $Pdfr^{3369}$ and $Pdfr^{5304}$, to understand the role of signalling via the PDFR in bringing about distribution of activity under LD30.

For control flies under LD30, the M-peak was advanced relative to the phase of lights-ON (Figure 3A), occurring around ZT23 (Figure S1A, left). On the other hand, $Pdfr^{3369}$ and $Pdfr^{5304}$ mutants showed only a startle to lights-ON at ZT0 (Figure 3A,B). E-peak in the mutants was advanced compared with controls (arrowhead, Figure 3A,B; Figure S1A, right). As with Pdf^{01}, we observed a significant effect of genotype on the incidence of midnight activity, with a greater proportion of *Pdfr* mutant flies displaying the midnight activity bout (Figure 3B, solid arrow), as compared with control (chi-square test of independence, $\chi^2_{(1,1)} = 302.707$, $p << 0.001$, Figure S7A,B).

We compared overall activity levels (total activity counts, Figure 3C) under LD25 ($N = 1$) and LD30 ($N = 4$) and found a significant effect of genotype under LD30 ($F_{(2,3)} = 19.321$, $p = 0.008$), brought about by a difference between the two mutant genotypes, $Pdfr^{3369}$ and $Pdfr^{5304}$, but not between control and either mutant. There were no differences across genotypes under LD25 ($t_{(30)} = -0.966$, $p = 0.327$). There was no significant effect of genotype under LD25 or LD30 on raw activity levels during nighttime (LD25: $t_{(31)} = -0.314$, $p = 0.755$; LD30: $F_{(2,3)} = 5.73$, $p = 0.06$, Figure 3D) or on the percentage of activity allocated to nighttime (LD25: $t_{(29)} = 0.062$, $p = 0.950$; LD30: $F_{(2,4)} = 2.295$, $p = 0.216$, Figure 3E), unlike our observations with the Pdf^{01}flies and its control (Figure 1). Further, there was a main effect of genotype on raw daytime activity levels under LD30, between $Pdfr^{5304}$ and control (Figure S7C, $F_{(2,2)} = 9.829$, $p = 0.02$), but not under LD25 (LD25: $t_{(31)} = -1.227$, $p = 0.228$).

To quantify activity differences that were visible in activity profiles for the $Pdfr^{3369}$ and $Pdfr^{5304}$ mutants, we estimated the percentage of midnight activity (ZT16-20) (Figure 3F). There was no

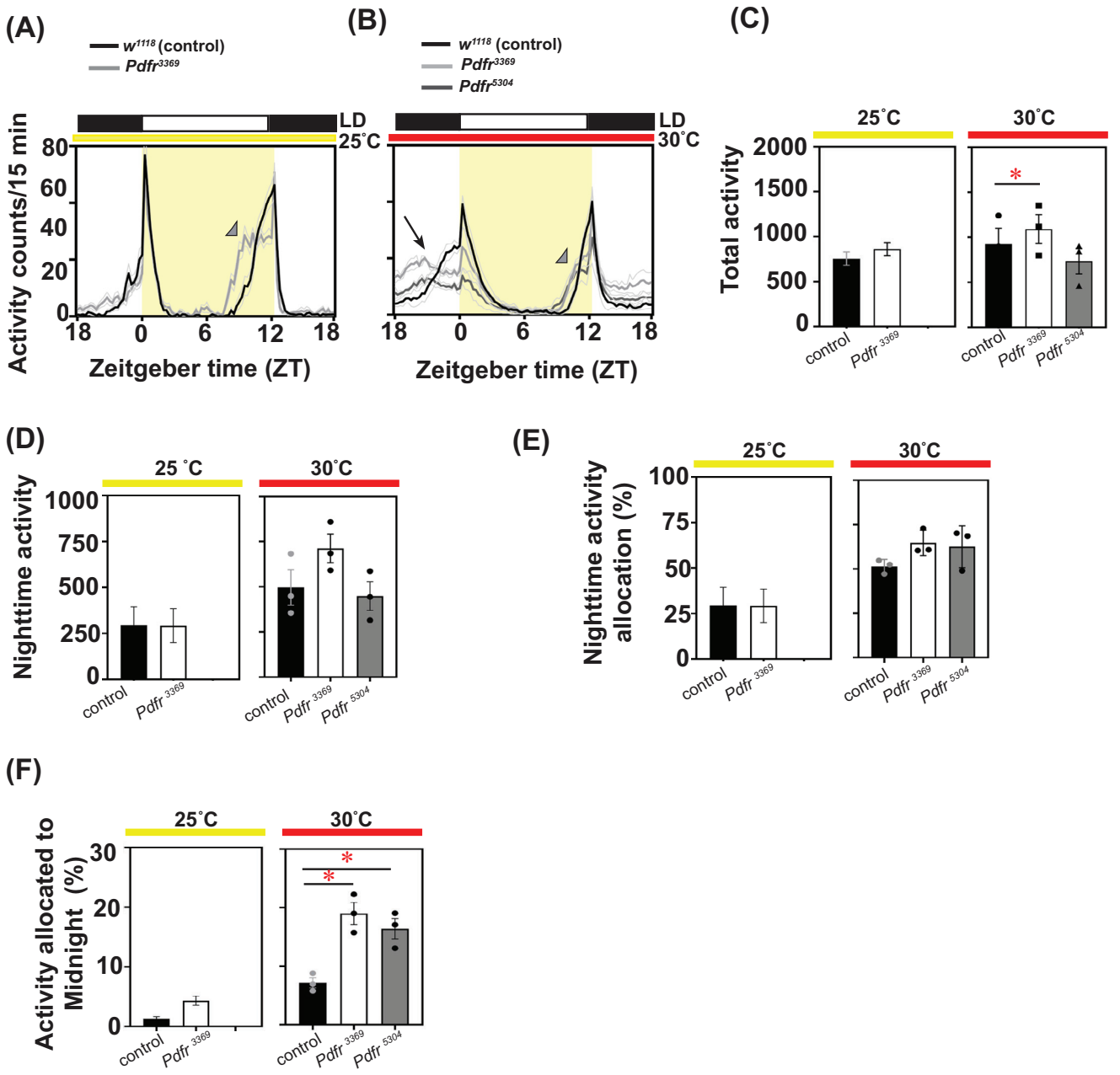

FIGURE 3 Under LD30, mutation in the *Pdf receptor* results in increased midnight activity (ZT16-20) as compared with control. (A, B) Activity profiles for control (bold black trace), $Pdfr^{5304}$ (bold dark grey) and $Pdfr^{3369}$ (bold light grey), is plotted against Zeitgeber time (ZT) under LD25 or LD30. All other details same as Figure 1. Under LD30, control flies display an advanced M-peak, while $Pdfr^{5304}$ and $Pdfr^{3369}$ flies show increased activity (arrow) before lights-ON, a morning startle, and an advanced E-peak (arrowhead). (C) Overall activity levels are not significantly different between $Pdfr^{3369}$ mutant and control under LD25 and is different between only this mutant and control under LD30. (D, E) There is no difference between genotypes in (D) nighttime activity levels or (E) percentage of activity allocated to night under LD25 or LD30. (F) The percentage of activity allocated to midnight (ZT16-20) is significantly higher in both *Pdfr* mutants as compared with control under LD30, but not under LD25. See text for comparison across temperatures. Error = *SEM* across individuals (LD25, $N = 1$) or means of replicate experiments (LD30, $N = 3$); $n = 16–32$ for control, $n = 18–32$ for $Pdfr^{3369}$ and $n = 22–32$ for $Pdfr^{5304}$ per experiment. Details in Table S1. Symbols indicate means of technical replicates; asterisk indicates significant differences between genotypes

difference between genotypes under LD25 (LD25:$t_{(31)} = -1.12$, $p = 0.261$), but significantly higher values were seen for the *Pdfr* mutants compared with control under LD30 ($F_{(2,4)} = 13.103$, $p = 0.017$). Comparing across temperatures, we found that the $Pdfr^{3369}$ mutant exhibited significantly greater allocation of activity to the midnight window under LD30, while the controls showed only minor increases with temperature ($F_{(1,45)} = 128.686$, p <<0.0001). This supports the role of PDF signalling in the suppression of midnight activity levels under warm ambient temperature and is consistent with our observations with Pdf^{01}flies (Figure 1).

3.5 | The PDF$^+$ ventrolateral neurons (LNvs) are necessary to suppress midnight activity levels, under warm ambient conditions of LD30

We hypothesised that if PDF modulates temperature-dependent behaviours, then modifying properties of PDF-expressing ventrolateral neurons (LNvs) must impact lack of suppression of midnight activity under LD30. To this end, we targeted the LNvs using the *Pdf-GAL4* driver and manipulated them by: (a) reducing neuronal firing that is, hyperpolarising the LNvs using the *UAS-Kir2.1* construct,[34] and (b) ablating the LNvs by expressing the Diphtheria Toxin (*UAS-Dti*).[35] Hyperpolarisation of the LNvs, heightened activity of experimental

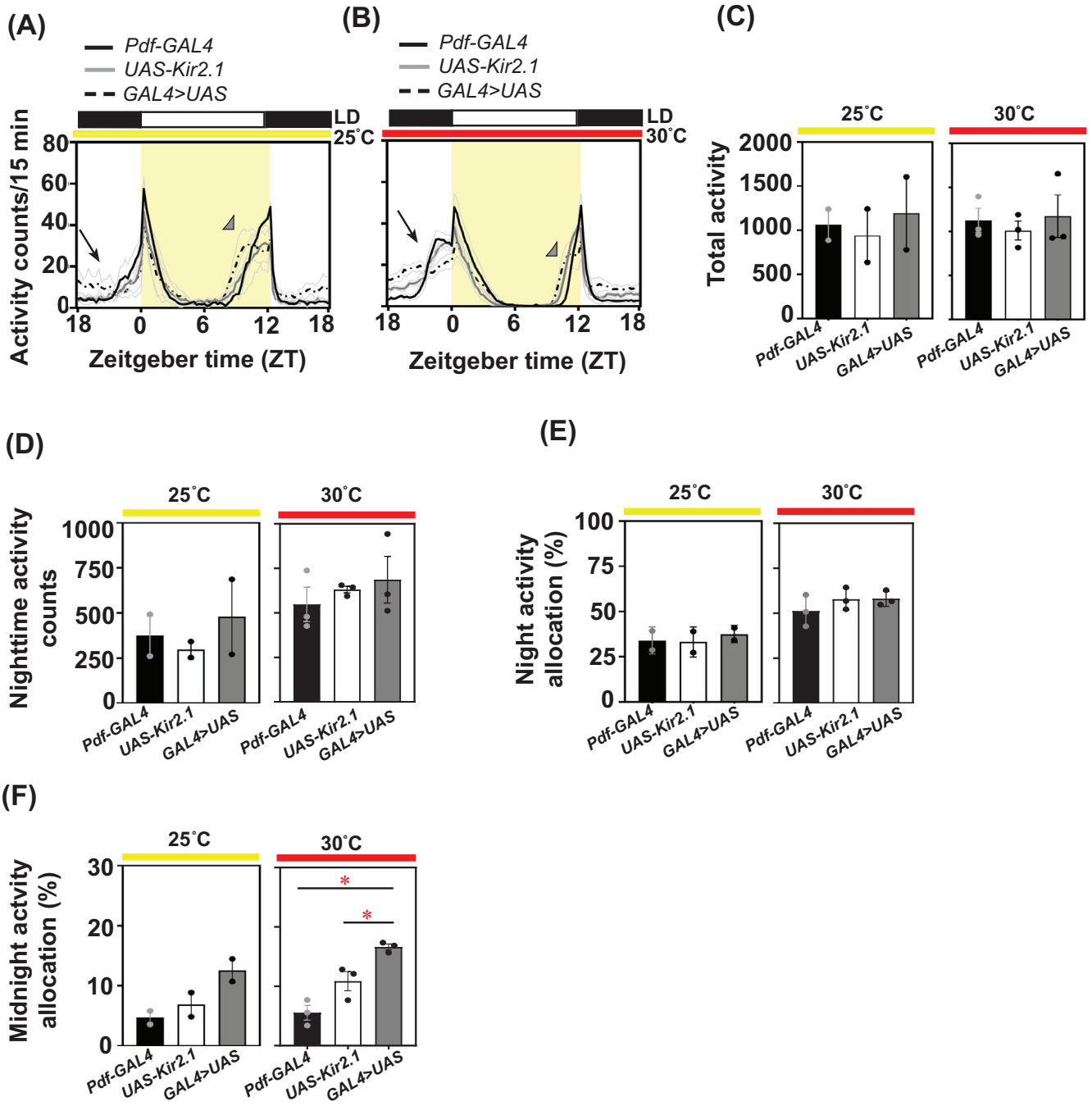

FIGURE 4 Reduced firing of LNvs results in increased midnight activity (ZT16-20). (A,B) Average activity profiles for parental controls, *Pdf-GAL4* (black trace) and *UAS-Kir2.1* (grey trace) and experimental flies (*Pdf>Kir2.1*) (dashed line) under LD25 and LD30 and is plotted against Zeitgeber time (ZT). All other details same as Figure 1. Under LD25 and LD30, experimental flies display heightened night activity (arrow) and an advanced evening bout (arrowhead). (C–F) Quantification of activity levels for parental control flies, *Pdf-GAL4* (black bar) and *UAS-Kir2.1* (white bar), and experimental flies *(Pdf>Kir2.1)* (grey bar): Under LD25 or LD30, there is no significant difference between either controls and experimental flies in (C) total activity, (D) activity during nighttime, or (E) activity allocated to nighttime. (F) Midnight activity allocation is higher in experimental flies as compared with controls under LD30, although a similar trend is observed under LD25 as well (see text for details). Error = *SEM* across means of replicate experiments (LD25, $N = 2$; LD30, $N = 3$); $n = 16–32$ for *Pdf-GAL4*, $n = 16–32$ for *UAS-Kir2.1* and $n = 16–48$ for experimental genotype, per experiment. Details in Table S1. Symbols indicate means of technical replicates; asterisk indicates significant differences between genotypes

flies under LD25 ($N = 2$) as well as LD30 ($N = 3$) (Figure 4A,B, arrow) visualised in the activity profiles. Quantification showed no significant effect of genotype on most parameters of activity levels under LD25 or LD30 (Total activity: LD25:$F_{(2,1)} = 1.143$, $p = 0.466$; LD30: $F_{(2,2)} = 0.955$, $p = 0.457$; Night activity: LD25: $F_{(2,1)} = 1.236$, $p = 0.447$; LD30: $F_{(2,2)} = 0.553$, $p = 0.613$; Nighttime Allocation:

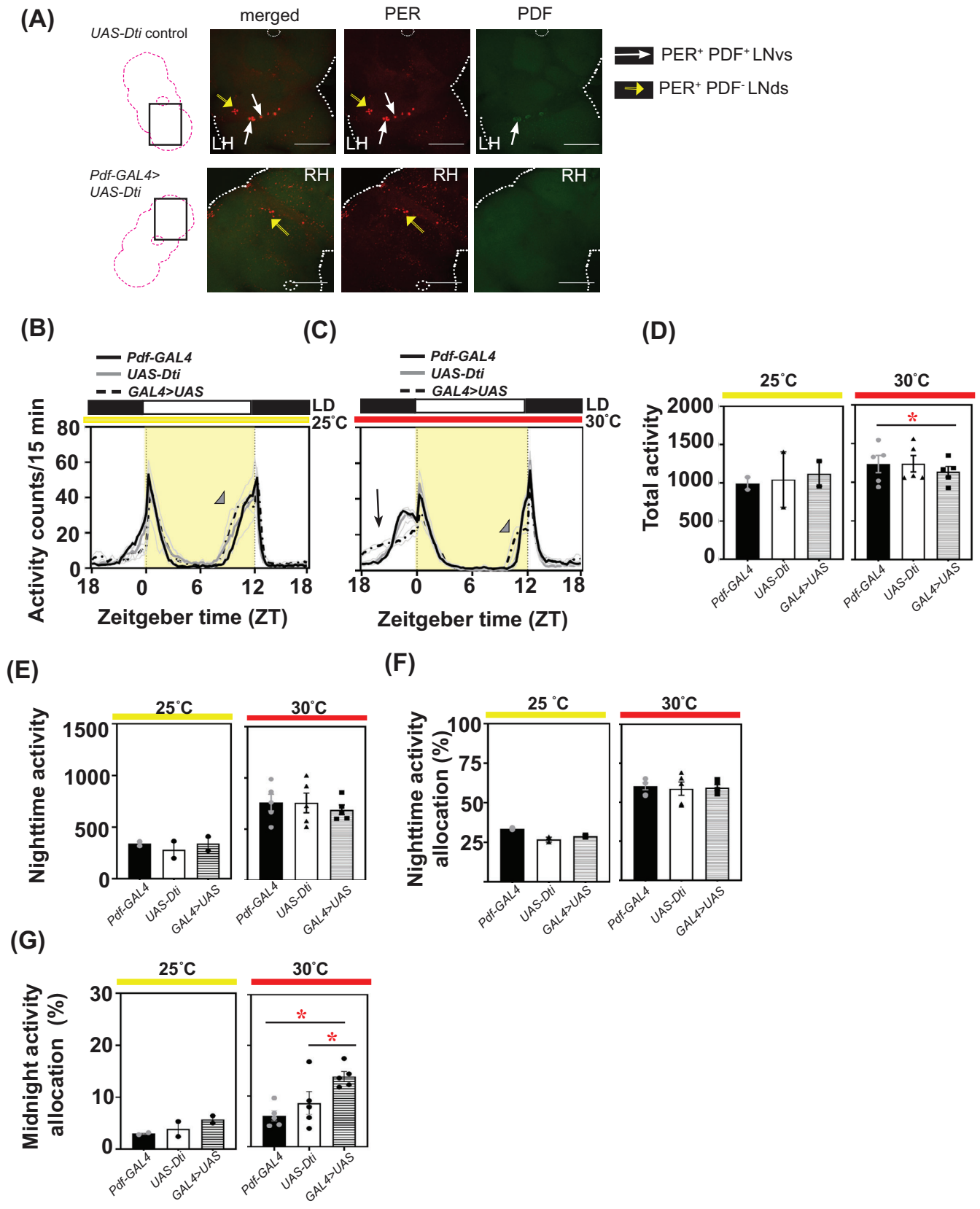

FIGURE 5 Legend on next page.

LD25: $F_{(2,1)} = 0.148$, $p = 0.870$; LD30: $F_{(2,2)} = 1.511$, $p = 0.324$, Figure 4C,E; Daytime Activity: LD25: $F_{(2,1)} = 1.231$, $p = 0.890$; LD30: $F_{(2,2)} = 5.617$, $p = 0.068$, Figure S8A). Midnight activity allocation experienced significant effects of genotype only under LD30, but not under LD25 (LD25: $F_{(2,1)} = 3.88$, $p = 0.204$; LD30: $F_{(2,2)} = 25.622$, $p = 0.005$). However, we note that midnight activity levels appear to be higher in experimental flies even under LD25 and speculate that the absence of statistical significance for these differences may result from lower replication under LD25. This was reflected in comparisons across temperatures which showed a significant effect of genotype in midnight activity allocation ($F_{(1,2)} = 20.919$, $p = 0.001$) but no significant interaction effect between genotype and temperature ($F_{(1,2)} = 0.780$, $p = 0.499$). Overall, these data suggest that reduced firing from the LNvs, resulted in experimental flies displaying increased levels of activity irrespective of temperature.

On the other hand, ablation of the LNvs (confirmed by staining PDF in adult brain tissue, Figure 5A) showed warm temperature-specific changes in behaviour of experimental flies. For both parental controls (*Pdf-GAL4>w* and *w>UAS-Dti*), the M-peak was advanced relative to the phase of lights-ON (Figure 5C), occurring around ZT23 under LD30 (Figure S1A, left). The experimental flies (*Pdf-GAL4>UAS-Dti*) showed a broad bout of activity before lights-ON (Figure 5B,C; Figure S9A, arrow), which was different from Pdf^{01} flies under LD30, while the E-peak of experimental flies was advanced as compared with controls, like Pdf^{01} flies under LD30 (Figure S1A, right). We found no significant effect of genotype under LD25 ($N = 2$) or LD30 ($N = 5$), when comparing overall activity levels (LD25: $F_{(2,1)} = 0.050$, $p = 0.956$; LD30: $F_{(2,4)} = 1.177$, $p = 0.356$, Figure 5D), activity levels during daytime (LD25: $F_{(2,1)} = 0.105$, $p = 0.901$; LD30: $F_{(2,4)} = 1.049$, $p = 0.393$, Figure S9B), nighttime (LD25: $F_{(2,1)} = 0.190$, $p = 0.841$; LD30: $F_{(2,4)} = 0.911$, $p = 0.439$, Figure 5E) or in the percentage of activity allocated to nighttime (Figure 5F, LD25: $F_{(2,1)} = 10.113$, $p = 0.089$; LD30: $F_{(2,4)} = -0.275$, $p = 0.765$).

To understand the role of the LNvs in modulating distribution of activity and the observed behavioural defects under LD30, we compared percentage of midnight activity across genotypes (ZT16-20) (Figure 5G) and found that experimental flies showed significantly higher activity levels than both parental controls under LD30 ($F_{(2,4)} = 22.694$, $p = 0.000$), but not under LD25 ($F_{(2,10)} = 2.903$, $p = 0.256$). Analyses incorporating temperature as a factor showed that there were main effects of genotype ($F_{(2,10)} = 9.302$, $p = 0.005$), but no significant interaction effects ($F_{(2,10)} = 2.142$, $p = 0.168$). While it is possible that ablation of the LNvs increases midnight activity even under LD25, we speculate that the absence of a significant interaction effect may result from lower replication under LD25 ($N = 2$) compared with LD30 ($N = 5$). Taken together, our results present an unreported role of the LNvs in modulation of activity allocation over a day under warm ambient conditions like LD30 and support our previous results about the role of PDF signalling in suppression of midnight activity under warm LD30.

4 | DISCUSSION

Flies can modulate their activity-rest behaviour in response to environmental cues. For instance, when environmental temperatures are elevated, flies can redistribute activity levels through a day and modulate timing of their behaviour. This idea garners support from studies that have investigated the behaviour of clock-mutants under more natural conditions. Utilising whole-body clock-gene mutants, Helfrich-Forster and colleagues explained that a crucial role of a functional clock under relatively high temperature may be to suppress 'unproductive' activity in order to perhaps save energy for other activities like foraging or escaping predators.[36,37] But the neuronal basis for such behaviour remained known. Here, we report the role of a subset of core clock neurons in flies, the LNvs, and their signalling via the neuropeptide PDF, in modulating activity levels when exposed to a warm ambient temperature which is thought to be stressful for flies. Specifically, we find that flies lacking PDF, proper PDF signalling or the PDF^+ LNvs, display an increase in activity levels in the middle of the night under LD30 (summarised in Table 1), when control flies are quiescent.

In contrast to laboratory incubator settings, light and temperature conditions change considerably through the day and night in nature. Under such conditions, PDF and the LNvs have been shown to

FIGURE 5 Under LD30, lack of PDF-expressing LNvs results in increased allocation of midnight activity (ZT16-20) as compared with controls. (A) Confirmation of ablation of PDF^+ neurons, using immunocytochemistry of control (*UAS-Dti*, above) and experimental (*Pdf-GAL4>UAS-Dti*, below): Brain tissue stained for PERIOD protein (red) and PDF neuropeptide (green). PDF^+ neurons were identified based on location and co-staining with anti-PER; Scale bar = 100 μm. Detection of the clock subset, dorsolateral neurons, LNds (yellow arrow), acted as a positive control for PER detection. PDF^+ LNvs are observed in *w>UAS-Dti* control (white arrow) and are stained by both anti-PER and anti-PDF antibody. In the experimental genotype, while staining for anti-PER is detected in LNds, staining for anti-PDF is absent, indicating lack of PDF^+ neurons. (B, C) Average activity profiles for parental controls, *Pdf-GAL4* (black trace) and *UAS-Dti* (grey trace) and experimental flies (*Pdf>Dti*) (dashed line) under (B) LD25 or (C) LD30 and is plotted against Zeitgeber time (ZT). All other details same as Figure 1. (C) Under LD30, experimental flies display a broad bout of activity (arrow). (D–G) Quantification of activity levels for parental control flies, *Pdf-GAL4* (black bar) and *UAS-Dti* (white bar), and experimental flies *(Pdf>Dti)* (grey bar): Under LD25, there is no difference between both controls and experimental flies in (D) total activity and under LD30 there is significant difference between experimental flies and only one control. (E, F) Under LD25 and LD30, there is no difference between both controls and experimental flies in (E) activity during nighttime or (F) activity allocated to nighttime. (G) There is a significant increase in activity allocated to midnight in experimental flies as compared to controls under LD30, but there is no difference across genotypes under LD25. See text for comparison across temperatures. Error = *SEM* across means of replicate experiments (LD25, $N = 2$; LD30, $N = 5$); $n = 24$–32 for *Pdf-GAL4*, $n = 16$–32 for *UAS-Dti* and $n = 31$–64 for experimental genotype, per experiment. Details in Table S1. Symbols indicate means of technical replicates; asterisk indicates significant differences between genotypes

TABLE 1 Summary table of genetic manipulations utilised in the study

Genotype	PDF$^+$ LNv cell-body and projection status	PDF status	Midnight activity (%) under LD30
Control	Present	Present	-
Pdf^{01}	Present (with possible arborisation and remodelling defects[61])	Absent[20]	High[a]
tim-GAL4 (A3)>UAS-dcr/ UAS-Pdf-RNAi	Present (with possible arborisation and remodelling defects[61])	Reduced[27]	Comparable to control[a]
Pdf-GAL4>UAS-dcr/ UAS-Pdf-RNAi	Present (with possible arborisation and remodelling defects[61])	Reduced[27]	Comparable to control[b]
Pdf-GAL4>UAS-Kir2.1	Present (with normal arborisation[55])	Present (normal levels[55] but secretion may be affected)	Comparable to control[c]
Pdf-GAL4>UAS-Dti	Absent	Absent	High[c]
Pdfr^{3369}	Present	Present	High[a]
Pdfr^{5304}	Present	Present	High[b]

[a]Across temperature comparison showed greater allocation of activity to midnight under LD30.
[b]Across temperature comparison not made.
[c]Across temperature comparison showed no effect on allocation of activity to midnight under LD30.

regulate activity-rest behaviour.[16,38] Vanin and colleagues studied flies in a quasi-natural set-up with natural light and temperature cycles where average temperature was 28°C, with the maximum being as high as 34.4°C during midday. The phase of onset of morning activity (M-onset) in *Pdf^{01}* flies was significantly delayed compared with wildtype strains and was found to track temperature. This suggests that the M-onset may depend on PDF and that it is sensitive to temperature. In another study, *Pdf^{01}* flies and flies with ablated PDF$^+$ LNvs were exposed to laboratory conditions of constant darkness accompanied with Thermophase-Cryophase (TC) cycles of 28:20°C, that *gradually* ramped-up (day) or down (night) over the course of 12 h. Here, the authors examined the entrainment ability of flies and concluded that axonal projections of the LNvs, but not PDF, were necessary for entrainment to more naturalistic temperature conditions.[16] While we have not modelled temperature in our experiments in a naturalistic fashion, we believe that our experimental design with constant warm temperature simulates conditions where mean daily temperatures are high. This approach has also been utilised previously to understand phasing of behaviour in flies during seasonal adaptation,[8,10] and has aided the understanding of how neuro-sensory systems and organisms as a whole respond to such conditions. Our experiments extend this knowledge by demonstrating that PDF+ LNvs also regulate behaviour in response to constant ambient temperature.

High temperatures are correlated with low humidity levels in nature but in our experiments, relatively high humidity levels were maintained irrespective of ambient temperature in the incubators. Thus, the behavioural responses reported by us are expected to be responses to temperature differences alone.

4.1 | PDF, activity and sleep levels and temperature

Short-term exposure to warm temperature can influence sleep levels in female flies, such that exposure to a single day of constant warm temperature alters the distribution of sleep through the day via increases in daytime sleep and decreases in nighttime sleep.[26,39,40] In a study conducted on males, activation of the circadian pacemaker neurons, the large LNvs (lLNvs), could recapitulate the decrease in nighttime sleep under warm temperatures even under ambient conditions of LD25 and also altered the distribution of activity levels.[41] In contrast, lack of PDF or PDFR in female flies can also result in increased total sleep.[26] These results suggest a role for PDF and the lLNvs in distribution of sleep as well as activity through a day under relatively cool conditions of LD25 in both sexes. In our experiments (Figure 1 and S3), we observed no difference between genotypes in total activity or sleep levels under cool LD25 or relatively warm LD30 in male flies that lack PDF, but do observe reduced sleep during the midnight time-window under LD30, which was associated with increased midnight activity. We also note a significant change in the *percentage of activity* allocated to the daytime or nighttime in *Pdf^{01}* flies compared with controls, supporting a role of PDF in distribution of activity levels through a day. Work by Choi and colleagues in male flies showed that the cAMP signalling pathway in the small LNv (sLNvs) is important for allocation of daily activity under LD25 and that increasing the levels of cAMP can lead to increase in what they term as 'morningness' or morning activity levels.[42] They also put forth the role of PDFR$^+$ neurons as necessary and sufficient to increase this morningness in flies under LD25. Our results support and add to the role of the LNvs in distribution of activity under warm LD30 as reduced PDF or PDFR signalling and ablation of LNvs resulted in increased activity allocated to the middle of the night, possibly via loss of sleep.

4.2 | Downstream targets of PDF can regulate activity levels

The increase in activity we observe in flies lacking proper PDF signalling is seen *only* under constant warm ambient temperature, and

specifically during the middle of the night, suggesting a temperature-dependent modulation by a circuit controlling locomotor behaviour in flies. Which PDFR^{+} neurons may be involved in this? Choi and colleagues note that activation of autoreceptors of PDFR on the LNvs is able to increase 'morningness' (or activity levels during the morning) and activation of PDFR in non-LNv circadian neurons is necessary and sufficient in bringing about this morningness.[42] Our observations with the two mutants of *Pdfr* also support the role of these receptors and hence the PDFR^{+} neurons, in modulating activity allocation, specifically to the middle of the night under warm ambient temperature (Figure 3). However, when we downregulated PDFR levels in the LNvs (autoreceptors) using RNA-interference (data not shown) we did not observe any defect in behaviour under warm ambient temperature. It remains to be seen if other PDFR-expressing circadian and non-circadian neurons in the brain and outside regulate the behavioural observations we report here. The ellipsoid body (EB) of the central complex is one non-circadian cluster of neurons that is known to modulate sleep and locomotion in the fly.[26,43,44] Activation of a subset of EB neurons promotes sleep in female flies,[43] although it is not clear if these same neurons also express PDFR.[45]

4.3 | LNv-DN1p circuit can potentially modulate temperature-dependent activity-rest behaviour

Another subset of clock neurons that could have a potential role in modulating behaviour under warm temperature is that of the Dorsal Neurons on the posterior side (DN1ps). Under warm ambient temperature of 31°C, disrupting the molecular clock in the DN1ps causes flies to remain awake at times of the night when control flies kept at 22°C are asleep, indicating a role for the DN1p clock in timing of circadian sleep–wake behaviour under warm ambient temperature. On the other hand, silencing of the DN1ps inhibits delay of daytime sleep which is seen in controls under warm ambient temperature.[46] Further, under LD25, the DN1ps are most active between ZT20-4 (late-night to early morning),[47] firing more before lights-ON.[47,48] Thus, DN1ps can promote wakefulness when activated. Based on these reports, it would be interesting to see if the DN1ps contribute to the phenotypes that we report here. There is evidence for the modulation of the DN1ps by PDF or the LNvs. The DN1ps lie close enough to the LNv projections to form synaptic contacts[49] and also respond to PDF resetting,[50,51] most likely via PDF receptors.[52] Thus, the DN1ps are potential post-synaptic partners to the LNvs. On the other hand, targeted activation of the glutamatergic DN1ps can inhibit the firing of the LNvs,[50] while downregulation of glutamate receptors on the sLNvs can reduce the amplitude of locomotor behaviour.[16] These results indicate an additional pre-synaptic role of the DN1ps for the sLNvs. Considering together the timing of increased night activity levels (ZT16-20) in *Pdf*^{01} flies under LD30 and the timing of the peak of neuronal activity known in DN1ps under LD25,[47] we can speculate a role for DN1ps in promoting activity in *Pdf*^{01} flies during the middle of the night under LD30. More recent reports based on recordings of endogenous calcium levels and membrane potential of these neurons also confirm their response to acute temperature changes.[15,50] Collectively, these results suggest a reciprocal relationship between the LNvs and the DN1ps that could potentially restructure locomotor behaviour of flies in response to environmental cues, importantly temperature. Interestingly, the DN1s could also contribute to the sexual dimorphism in behaviour that we report here (Figure S1) since Guo and colleagues showed that DN1s fire more in males than females under warm temperature and this coincides with differences in locomotor activity and sleep across sexes.[49] Further, the dominance of the DN1ps among other circadian neurons under warm ambient conditions has been suggested by Lamaze and colleagues, who reported the role of a clock in these cells for phasing of sleep (discussed above, see Ref (43)). It would be interesting to know if constant warm ambient temperature reorganises timing of firing of the DN1ps in normal flies, in turn influencing the functioning of the LNvs and other circadian neuronal clusters like the dorsolateral neurons (LNds) and, consequently, behaviour. Additionally, it remains to be seen whether firing of the DN1ps is altered under LD30 in flies lacking PDF, where modulation of the DN1ps by the LNvs may be potentially modified.

Putting all the above in perspective, we acknowledge that an alternate way of viewing the behaviour we describe here would be to interpret the 'midnight activity' under LD30 in *Pdf*^{01}as an extremely phase-advanced M-peak. In this scenario, this PDF-independent 'M-peak' must be generated by the other clock neurons, possibly the DN1ps, as discussed above. Indeed, there is some evidence to support this model, specifically under summer-like conditions.[53] Flies that had been exposed to 35-25°C TCs displayed molecular PER oscillations in the DNs that were advanced compared with the canonical LNv clock. Moreover, as discussed previously, the DN1p clock is sufficient to generate the M-peak in clock-less flies.[13] We aimed to address this conflict in interpretation between 'advanced M-peak' or 'midnight activity bout' under LD30 through our photoperiod experiments (Figure S6). After carefully inspecting individual profiles for *Pdf*^{01} flies, we found that the midnight activity bout was advanced under short photoperiod and hence appeared to be clock-controlled, implying that it may be the M-peak. However, it could not be distinguished from the morning startle under long photoperiod, which prevented any inference regarding clock-control of the activity bout. Such behavioural deficits in *Pdf*^{01} flies across photoperiods have also been reported by others.[30,54] Future experiments and analyses using other photoperiods or T-cycles will be needed to confirm the clock-control of this behaviour, and in the absence of confirmatory results, we conservatively call this behaviour increased midnight activity. It is important to note that this alternative explanation does not change our current conclusion that PDF or the LNvs are necessary to suppress midnight activity at high environmental temperatures.

If the PDF-system integrates environmental temperature into behavioural changes under warm conditions, it could potentially do so by changing aspects of its own oscillation such as phase or amplitude. This could be explored in the future by characterising the molecular oscillation of PDF accumulation under LD30 in wildtype animals which yet remains unknown.

4.4 | Manipulation of LNvs can influence behaviour in addition to PDF-related effects

Using constant warm ambient temperature, we have been able to uncover a previously unreported role of PDF signalling and the LNvs in suppression of activity, specifically affecting the middle of the night. Under relatively cool LD25, electrical silencing of the LNvs causes behavioural deficits like increased nocturnal activity without disrupting the molecular clock in these neurons.[55] Altered membrane properties can potentially affect neurotransmitter or neuropeptide release from LNvs[56] and also the neuronal response of LNvs to neurotransmitters or neuropeptides acting on them (e.g., serotonin,[57] glutamate,[58] DH31[59] or GABA[26]), leading to a range of behavioural deficits previously reported, as also our observations with *Pdf-GAL4>UAS-Kir2.1* (Figure 4). We ablated the LNvs using *Dti* (*Pdf-GAL4>UAS-Dti*, Figure 5) which would not only result in the absence of the PDF neuropeptide, but also perhaps reduce levels of other neuropeptides like short-Neuropeptide F (sNPF) which is secreted in-part by the LNvs.[60] This manipulation will also result in absence of axonal projections from the LNvs, unlike in the case of mutants of Pdf^{01}, $Pdfr^{3369}$ and $Pdfr^{5304}$ (See Table 1). Axonal projections act as synaptic regions for other neurons like the above-mentioned DN1ps. Lack of these projections could influence behaviours that are modulated by LNv secretions other than PDF or also influence circuits and behaviours modulated by the neuronal projections of the LNvs, as discussed by Fernandez and colleagues.[16] Thus, it is possible that our genetic manipulations of the LNvs possibly affect other yet unknown aspects of this circuit which may result in abnormal allocation of activity across the night. Finally, we point out that in all our LD30 experiments, flies were reared under relatively cool LD25 and experienced LD30 conditions only as adults. Early work by Sayeed and Benzer showed no effect of rearing temperature on adult temperature preference- flies reared at 15, 20 or 25°C preferred 23–24.5°C as adults.[4] In another study, populations reared under 30°C for 10 generations preferred warmer temperatures than those reared at 25°C,[7] however, in this particular study, one cannot rule out evolved tendencies as opposed to plastic changes. Thus, it is possible that some of the behavioural changes we observe in adults are a consequence of a mismatch in temperature between rearing and experimentation and may not fully reflect behaviour of natural populations. This can be verified by rearing and testing flies at the same temperatures.

5 | CONCLUSION

To deal with varying environmental conditions, such as increases in global temperatures, animals in the wild, especially small poikilotherms, must adopt behavioural strategies to avoid harsh conditions. Modulation of activity levels via proper PDF signalling may be an important strategy employed by flies to beat the daily heat and not remain active at inappropriate times. Based on our observations with flies lacking proper PDF signalling, we present a role of PDF, PDFR and the LNvs in allocation of activity levels during the night under prolonged warm conditions and provide a neuronal basis to the role of the clock in such behaviour. Future studies will aim to delineate the roles of other neuronal subgroups, neuromodulators and thermal sensors in the fly that could potentially be part of the complex circuitry that brings about appropriate behaviour in flies under warm ambient conditions.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest to report.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding authors upon reasonable request.

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