A natural *timeless* polymorphism allowing circadian clock synchronization in 'white nights'

Angelique Lamaze[1]*, Chenghao Chen[2]*, Solene Leleux[1], Min Xu[2], Rebekah George[1], and Ralf Stanewsky[1]**

[1]Institute of Neuro- and Behavioral Biology, Westfälische Wilhelms University, Münster, Germany

[2]Howard Hughes Medical Institute, Janelia Research Campus, Ashburn, Virginia, USA.

*equal contribution

**corresponding author

stanewsky@uni-muenster.de

Abstract

Daily temporal organisation of behavioural and physiological functions offers a fitness advantage for most animals. Optimized temporal niches are determined by an interplay between external environmental rhythms and internal circadian clocks. While daily light:dark cycles serve as a robust time cue (Zeitgeber) to synchronise circadian clocks, it is not clear how animals experiencing only weak environmental cues deal with this problem. Like humans, flies of the genus Drosophila originate in sub-Saharan Africa and spread North in Europe up to the polar circle where they experience extremely long days in the summer or even constant light (LL). LL is known to disrupt clock function, due to constant activation of the deep brain photoreceptor CRYPTOCHROME (CRY), which induces constant degradation of the clock protein TIMELESS (TIM). Temperature cycles are able to overcome these arrhythmia inducing effects of LL, reinstating clock protein oscillations and rhythmic behaviour. We show here that for this to occur a recently evolved natural allele (*ls-tim*) of the *timeless* gene is required, whereby the presence of this allele within the central clock neurons is sufficient. The *ls-tim* allele encodes a longer, less-light sensitive form of TIM (L-TIM) in addition to the shorter (S-TIM) form, the only form encoded by the ancient *s-tim* allele. Only after blocking light-input by removing functional CRY, *s-tim* flies are able to synchronise molecular and behavioural rhythms to temperature cycles in LL. Additional removal of light input from the visual system results in a phase advance of molecular and behavioural rhythms, showing that the visual system contributes to temperature synchronization in LL. We show that *ls-tim*, but not *s-tim* flies can synchronise their behavioural activity to semi-natural LL and temperature cycle conditions reflecting long Northern Europe summer days, the season when Drosophila populations massively expand. Our observations suggest that this functional gain associated with *ls-tim* is driving the Northern spread of this allele by directional selection.

Introduction

Like most organisms, *Drosophila melanogaster* rely on their endogenous circadian clock to regulate rhythmic physiological and behavioural outputs. This timer is equipped with two core clock proteins CLOCK(CLK) and CYCLE(CYC) to activate the transcription of the clock genes *period* (*per*) and *timeless* (*tim*). The translated PER and TIM proteins then terminate their own transcription through negative feedback [1]. This transcription/translation feedback loop, constitutes the molecular oscillator of the biological clock, which runs with a period of approximately 24 hours, even in absence of environment cues. On the other hand, this robust timing system interacts with the environment and resets itself by daily cues like fluctuating light and temperature (so called 'Zeitgeber'). CRY is an important blue light photoreceptor expressed in the *Drosophila* eye as well as in subsets of the clock neurons, which are composed of about 75 neurons expressing core clock genes in each brain hemisphere [2–4]. This central pacemaker contains seven anatomically well-defined clusters: three groups of dorsal neurons (DN1-3), the lateral posterior neurons (LPN), the dorsal lateral neurons (LNd) and the large and small ventral lateral neurons (l- and s-LNv). Together, they orchestrate timing of the locomotor activity patterns with external light and temperature fluctuations. When flies are exposed to light, CRY is activated and binds to TIM and the F-box protein JETLAG (JET), triggering TIM and CRY degradation in the proteasome to reset the clock network [5–8]. Therefore, exposure of flies to constant light (LL) leads to arrhythmicity, due to the constitutive degradation of TIM in clock neurons, mediated by CRY [9,10]. In addition, rhodopsin-mediated retinal photoreception contributes to circadian light input, and only if both CRY and the visual system function are ablated in parallel, circadian light synchronization is abolished [11]. Another important Zeitgeber to synchronise circadian rhythms is temperature. In mammals, temperature cycles (TC) with an amplitude of 1.5°C induce robust circadian gene

65 expression in cultured tissues [12]. Moreover, the daily fluctuation of body temperature (36°C -
66 38.5°C) generated by the suprachiasmatic nucleus (SCN) is employed to enhance internal
67 circadian synchronization [13]. In *Drosophila*, unlike cell autonomous light resetting by CRY,
68 clock neurons receive temperature signals from peripheral thermo sensory organs including
69 the aristae and mechanosensory chordotonal organs [14–17]. Interestingly, robust molecular and
70 behavioural entrainment to temperature cycles was observed under LL [18,19], suggesting that
71 cycling temperature can somehow rescue clock neurons from the effects of constant light,
72 but the underlying molecular mechanism is unknown.

73 This ability to synchronise circadian clocks to temperature cycles in constant light may have
74 ecological relevance. For instance, animals living above or near the Northern Arctic Circle
75 experience LL or near-LL conditions, while the temperature still varies between 'day' and
76 'night' (due to differences in light intensity). In Northern Finland summers (e.g., Oulu, 65°
77 North), the sun only sets just below the horizon and it never gets completely dark, so that
78 organisms experience so called 'white nights'. At the same time, average temperatures vary
79 by 10°C between day and night (www.timeanddate.com/sun/finland/oulu?month=7&year=2021), suggesting
80 that animals use this temperature difference to synchronise their circadian clock. *Drosophila*
81 *melanogaster* populate this region, with massive expansion of the population during the late
82 summer. It has been suggested that a recently evolved novel allele of the *tim* gene is
83 advantageous for Northern populations and that this allele is under directed natural selection
84 [20–22]. The novel *ls-tim* allele encodes a longer (by 23 N-terminal amino acids), less-light
85 sensitive form of Tim (L-Tim) in addition to the shorter (S-Tim) form, the only form encoded
86 by the ancient *s-tim* allele [7,8,20,23]. The reduced light-sensitivity of L-TIM is caused by a weaker
87 light-dependent interaction with CRY, thereby resulting in increased stability of L-TIM during
88 light, compared to S-TIM [7,8,20]. Indeed, *ls-tim* flies show reduced behavioural phase-responses

to light pulses [20] and are more prone to enter diapause during long summer days compared to *s-tim* flies [21]. It has been proposed that light-sensitivity of circadian clocks needs to be reduced in Northern latitudes, in order to compensate for the long summer days and presumably excessive light reaching the clock cells [24]. The *ls-tim* allele might therefore offer a selective advantage in Northern latitudes, which is indeed supported by the spread of this allele from its origin in Southern Italy 300-3000 years ago by directional selection [21,22].

Here we provide strong support for this idea, by showing that only *ls-tim* flies are able to synchronise their circadian clock and behavioural rhythms to temperature cycles in constant light (LLTC). The observation that wild type flies carrying the ancient *s-tim* allele are not able to synchronise to LLTC demonstrate the advantage of the *ls-tim* allele in Northern latitudes. Despite of their reduced light sensitivity, *ls-tim* flies can still synchronise their circadian clock because they can use temperature cycles as Zeitgeber. We show that *ls-tim* is also required for synchronisation under semi-natural conditions mimicking 'white nights' conditions as they occur in natural Northern latitude habitats of *Drosophila melanogaster*, supporting the adaptive advantage of this allele.

Results

ls-tim, but not *s-tim* flies are able to synchronise to temperature cycles in constant light

During our studies of how temperature cycles synchronise the circadian clock of *Drosophila melanogaster,* we noticed that some genetic control stocks did not, or only very poorly, synchronise their behavioural rhythms to temperature cycles in constant light (16°C : 25°C in LL). Further analysis revealed that the ability to synchronise to LLTC was correlated with the presence of the *ls-tim* allele, while flies that did not, or only poorly synchronise, carry the *s-*

tim allele. This was true for the two isogenic *w* strains *iso31* [25], and *iso* [26] (Figure 1A, B), as well as for common control stocks like *y w* (Figure S1A, B). As expected, regardless of *s-tim* or *ls-tim*, all flies showed normal synchronization to 12 hr : 12 hr light:dark (LD) cycles at constant temperature (Fig., 1A, S1A) [20]. Furthermore, as expected for wild type flies, independent of *s-tim* or *ls-tim*, all control stocks became arrhythmic in LL at constant temperatures (Fig. 1A, S1A) [27]. Finally, when exposed to temperature cycles in DD, both *s-tim* and *ls-tim* robustly synchronised their behavioural activity (Figure S1C), indicating that the *s-tim* allele specifically affects clock synchronization during LLTC.

Previous studies have shown that S-TIM is more sensitive to light compared to L-TIM, due to stronger light-dependent binding of S-TIM to CRY [8,20]. It is therefore likely that the increased stability of L-TIM in the presence of light is responsible for the ability of *ls-tim* flies to synchronise to LLTC. Moreover, red eye pigments of wild type *Drosophila melanogaster* protect TIM and CRY proteins from light-dependent degradation in photoreceptor cells [8,28]. To test if eye pigmentation also influences synchronization of *s-tim* flies during LLTC, we analysed red-eyed wild type flies carrying either the *s-tim* or the *ls-tim* allele. While Canton S carries *ls-tim*, the wild-type strain collected in Tanzania [29] is homozygous for *s-tim*. In addition, a wild type strain collected in Houten (The Netherlands) was analysed in both *s-tim* and *ls-tim* background [21]. As expected, both *ls-tim* strains showed robust synchronization to LLTC (Figure S2A, B). In contrast to most of the white-eyed *s-tim* flies we tested, the two red-eyed *s-tim* strains showed synchronised behaviour (compare Figure 1A, B and Figure S2A, B). But while in *ls-tim* flies activity started to rise around the middle of the warm phase, activity of red-eyed *s-tim* flies increased several hours earlier with a persistent activity until the end of the thermophase (Figure S2 A, B). To test if the early activity increase observed in some of *s-tim* strains reflects proper synchronisation to LLTC, we compared behaviour of *s-tim* flies with

137 *tim*^{KO} mutant flies. *tim*^{KO} is a new *tim* null allele in the *iso31* background, in which the *tim* locus
138 has been replaced with a *mini-white* gene resulting in red eye colour [30]. Interestingly, although
139 *tim*^{KO} flies do not show clock-controlled behaviour in LD, their locomotor activity pattern in
140 LLTC is equivalent to what we observed in red eye *s-tim* flies (Figure 1C, S2C). We therefore
141 conclude that *s-tim* flies, regardless of their eye pigmentation, are not able to synchronise
142 their clock controlled behavioural activity rhythms to temperature cycles in constant light.
143 Next, we compared the behaviour of hemizygous *s-tim* and *ls-tim* flies. While *tim*^{KO}*/ls-tim* flies
144 showed normal LD and LLTC behaviour, *tim*^{KO}*/s-tim* flies only synchronised to LD (Figure 1C).
145 Interestingly, trans-heterozygous *s-tim/ls-tim* flies perfectly synchronise their behaviour to
146 LLTC, showing that *ls-tim* is dominant over *s-tim* for LLTC entrainment (Figure 1C).

147

148 **_s-tim_ flies fail to properly synchronise their clock protein oscillations to temperature cycles**
149 **in constant light**

150 To distinguish if the lack of behavioural synchronization is due to a defect within or
151 downstream of the circadian clock, we analysed PER and TIM oscillations during LLTC in clock
152 neurons of *s-tim* and *ls-tim* flies (Figure 2, S3). As expected for *s-tim* in LL, TIM levels were
153 lower compared to *ls-tim* flies, but detectable at all four time points we examined (ZT0, ZT6,
154 ZT12, ZT18). While the amplitude of TIM oscillations in *s-tim* was dramatically reduced
155 compared to *ls-tim* flies, we found that in the ventral and dorsal lateral clock neurons, TIM
156 oscillations in *s-tim* are phase advanced by 6 hr, reaching peak values at ZT6 compared to
157 ZT12 for *ls-tim* (Figure2A). In the three DN groups, S-Tim levels were constitutively low at all
158 four time points (Figure 2A). We also noticed that even in *ls-tim* flies, TIM peaks earlier
159 compared to LD and constant temperature conditions [31], correlated with the phase advance

of the behavioural evening peak in LLTC compared to LD (Figure 1, S1, S2) [32]. In addition, we found that S-TIM remains cytoplasmic at all time points studied, while in *ls-tim* flies TIM showed the typical nuclear accumulation at ZT0 (Figure S3A, C) [31]. Similarly, PER levels were drastically reduced in *s-tim* compared to *ls-tim* flies in the PDF-positive LNv and LNd, and PER was undetectable in the 5th s-LNv (Figure 2B, S3B, D). Due to the low levels it was impossible to clearly distinguish between cytoplasmic and nuclear localisation, but the results indicate constitutive nuclear and cytoplasmic PER distribution at all four time points examined (Figure S3 B, D). The only exception were the DN3, which showed significant PER oscillations in *s-tim* flies, indicating the existence of an alternative system to control PER oscillations at least in this group of neurons (Figure 2B). Overall, the results indicate that the drastic impairment of synchronised TIM and PER protein expression in clock neurons underlies the inability of *s-tim* behavioural synchronization to LLTC.

Cryptochrome depletion allows synchronisation of *s-tim* flies to temperature cycles in constant light

s-tim flies are more sensitive to light compared to *ls-tim* flies, presumably because the light-dependent interaction between CRY and S-TIM is stronger compared to that of CRY and L-TIM [8,20]. To test if the inability of *s-tim* flies to synchronise to LLTC is due to the increased S-TIM:CRY interaction and subsequent degradation of TIM [8], we compared the behaviour of *s-tim* and *ls-tim* flies in the absence of *cry* function using the same environmental protocol. As expected, both *cry^{02}* and *cry^{b}* mutant flies showed rhythmic behaviour in LL and constant temperature (Figure 3A, S4A) [9,26]. Strikingly, the *s-tim* flies lacking CRY were now able to synchronise to LLTC, similar to *cry^{02}* flies carrying the *ls-tim* allele (Figure 3A, B). Notably, upon release into LL and constant temperature, activity peaks of both genotypes were aligned with

184 those during the last few days in LLTC, indicating stable synchronisation of clock-driven
185 behavioural rhythms (Figure 3A, B, S4A).

186

187 **Cryptochrome depletion partially restores molecular synchronisation of *s-tim* flies to**
188 **temperature cycles in constant light**

189 The behavioural results of *s-tim* flies lacking CRY described above, suggest that PER and TIM
190 protein oscillations within clock neurons that underlie behavioural rhythms are also
191 synchronised in LLTC. To confirm that *s-tim* flies lacking functional CRY are able to synchronise
192 their molecular clock, we determined PER and TIM levels in different subsets of clock neurons
193 of *s-tim cry*^{02} flies at four different time points during LLTC. Overall, we observed robust PER
194 and TIM oscillations in s-LNv and LNd clock neurons of *s-tim cry*^{02} flies, demonstrating that
195 removal of CRY restores molecular synchronization in *s-tim* flies during LLTC (Figure 3C, D,
196 S4B, C). Nevertheless, PER and TIM oscillations were not identical to those observed in *ls-tim*
197 *cry*$^{+}$ flies under the same conditions (compare Figure 3C, D with Figure 2A, B). To our surprise,
198 we found desynchronization between and within groups. Notably, there was an obvious
199 discrepancy in terms of PER amplitude between the LNd/5th and the LNv PDF^{+} neurons, even
200 though these cells are positioned anteriorly (i.e., the reduction of amplitude is not caused by
201 brain tissue that could interfere with the confocal imaging) (Figure 3D, S4B, C). In contrast,
202 TIM showed clear oscillations in both groups of PDF^{+} LNv, with trough values during the first
203 half of the warm phase and increasing levels up to the middle of the cold phase (Figure 3C,
204 S4B, C). Furthermore, while the amplitude of PER and TIM oscillation is comparable within
205 the LNd/5th, there was a clear phase difference between LNd CRY^{+} and the 5th compared to
206 the LNd CRY^{-} (the LNd were distinguished based on the larger size of the CRY^{+} neurons), with

the trough of PER and TIM in the LNd CRY$^-$ phase-advanced by at least 6 hr compared to the CRY$^+$ neurons (Figure 3C, D, S4B). Moreover, the overall TIM phase is advanced by 6 hr compared to that of PER in these neurons. Apart from half of the ~15 DN1p neurons, the neurons belonging to the three DN groups do not express CRY [4]. Interestingly, in these neurons TIM peaks at ZT12 as in the LNd CRY$^-$, with the DN1p oscillating with the highest amplitude (Figure 3C). To summarize, in *s-tim cry^{02}* flies, the six LNd and the 5th sLNv are the only clock neurons showing high amplitude PER oscillations, and the CRY$^-$ LNd, and DN neurons show drastic phase advances of PER (LNd only) and TIM oscillations compared to the LNd CRY$^+$ and 5th LNv evening cells.

Rhodopsin photoreception contributes to circadian clock synchronization in constant light and temperature cycles.

The constitutive cytoplasmic localisation of TIM in *s-tim* flies during LLTC in both CRY$^+$ and CRY$^-$ cells (Figure S3A, C), suggests that the visual system also contributes to circadian temperature synchronisation in the presence of light. To test this hypothesis, we analysed *s-tim* flies lacking CRY, in which Rhodopsin-expressing photoreceptor cells are either absent (via cell ablation using GMR-hid), or in which the major phototransduction cascade is interrupted due to the absence of Phospholipase C-ß (PLC-ß, via loss-of-function mutation of *norpA*). Completely removing both the visual system and CRY renders the brain clock blind to light entrainment [11], which is exactly what we observed with the *s-tim GMR-hid cry^{01}* flies analysed here (Figure 4A). In contrast, due to *norpA*-independent Rhodopsin photoreception, *norpA^{P41} cry^b* double mutants can still be entrained to LD [33–35], consistent with what we here observe for the *norpA^{P41} s-tim cry^{02}* double mutants (Figure 4A). Strikingly, after switch to LLTC both

genotypes synchronise their behaviour, however with a clear phase advance compared to *s-tim* cry^{02} flies in the same condition (Figure 4B). Interestingly, $norpA^{P41}$ *s-tim* cry^{02} double mutants take longer to establish a similar early phase as the *s-tim GMR-hid* cry^{01} flies (Figure 4A). We attribute this difference to the initial synchronization of $norpA^{P41}$ *s-tim* cry^{02} flies to the LD cycle, and their maintained synchronised free running activity in LL and constant temperature (Figure 4A, B). In contrast, the *s-tim GMR-hid* cry^{01} flies are completely desynchronised at the beginning of the LLTC, presumably allowing for rapid synchronisation to the temperature cycle. In conclusion, the results indicate that a photoreceptors using a *norpA*-dependent signalling pathway play a role in phasing the behaviour in LLTC.

To see if the Rhodopsin contribution to phasing behaviour in LLTC has a molecular correlate, we analysed TIM expression in *s-tim* flies lacking PLC-ß and CRY ($norpA^{P41}$ cry^{b}). We observed a clear phase advance of TIM oscillations in LNd, the s-LNv and l-LNv, as well as DN1p clock neurons, with peak or close-to-peak levels occurring at ZT9 and being maintained at peak levels until ZT16 (Figure 4C, S5A). Compared to *ls-tim* and *s-tim* cry^{02} flies TIM cycles with a phase advance of about 3 hours (compare Figure 4C to Figures 2 and 3). The molecular phase advance of TIM cycling observed in several of the clock neuronal groups correlates with the behavioural phase advance we observe in *GMR-hid* cry^{01}, $norpA^{P41}$ cry^{02}, and $norpA^{P41}$ cry^{b} flies (Figure 4, S5D). Interestingly, single $norpA^{P41}$ *s-tim* flies completely abolished the low amplitude oscillations of cytoplasmic TIM abundance observed in *s-tim* flies (Figure 2A, Figure 4C, S5B, C). Not surprisingly, $norpA^{P41}$ *s-tim* flies also fail to synchronise their behavioural activity to LLTC (Figure S5D). Taken together, these results indicate that *norpA*-dependent visual photoreception contributes to synchronization of TIM oscillations in clock neurons during LLTC to influence the behavioural activity of wild type flies.

253

254 **_ls-tim_ expression in clock neurons is sufficient for temperature synchronisation in constant**
255 **light.**

256 Because *norpA*-dependent visual system function contributes to synchronization of TIM
257 oscillations in clock neurons during LLTC (Figure 4C), we wondered if expressing the *ls-tim*
258 allele specifically in clock neurons or photoreceptors in otherwise *s-tim* flies, would also
259 restore synchronization. For this we first recombined a *UAS-ls-tim* [36] transgene with the tim^{KO}
260 allele (Methods) and crossed the recombinant flies to *s-tim* flies and tim^{KO} stocks. As
261 expected, *UAS-ls-tim,* tim^{KO} */ s-tim* and *UAS-ls-tim,* tim^{KO} */* tim^{KO} flies did not synchronise to
262 LLTC (Figure 5A, B, S6A). Next, we crossed *UAS-ls-tim,* tim^{KO} flies to *Clk856-Gal4* (expressed in
263 all clock neurons and not in photoreceptor cells: [37]), and to *Rh1-Gal4* (expressed in
264 photoreceptorcells R1 to R6, but not in clock neurons: [38]). Strikingly, expression of *ls-tim* in
265 clock neurons was sufficient to restore robust synchronization to LLTC, while expression in R1
266 to R6 had no effect (Figure 5, S6A-C). While we cannot rule out a role for *ls-tim* in the R7 and
267 R8 cells, the results unequivocally show that presence of the less-light-sensitive L-TIM form in
268 clock neurons is sufficient for allowing temperature synchronisation in LL.

269

270 **The _ls-tim_ allele enables flies to synchronise in white nights under semi natural conditions**

271 In order to determine if the *ls-tim* allele can be advantageous in natural conditions, we
272 analysed behaviour under LLTC conditions experienced in the summer in Northern Europe.
273 We decided to mimic the conditions of a typical summer day in Oulu, Finland (65° North) for
274 two reasons. First, *Drosophila melanogaster* populate Northern Scandinavian regions in this

latitude and overwinter here (e.g., [39]). Second, from mid-May to the end of June day length varies from 19-22 hours, and the rest of the 'night' corresponds to civil twilight, where the sun does not set more than 6° below the horizon and general activities can be performed without artificial light ('white nights', maximum darkness between 1-3 lux). At the same time average temperatures vary by 10°C between day and night, reaching an average maximum of 20°C in July. To mimic these conditions, we programmed a 2.5 hr period with 1 lux light intensity (civil twilight) and 12 hr of 200 lux interspersed by ramps with linear increases (morning) or decreases (evening) of light intensity (Figure 6A). Temperature cycled over 24 hr with linear ramps between 12°C and 19°C, reaching its minimum towards the end of the civic twilight period and its maximum in the middle of the 200 lux phase (Figure 6A). Using these conditions, we analysed two w^+ and one w^- *s-tim* strains. Interestingly, all of these strains showed the same broad activity phase covering a large part of the 200 lux day period (Figure 6B, C, S7). In addition, all *s-tim* strains showed a pronounced 2nd activity peak during the 3.5 hours of down-ramping the light intensity from 200 lux to 1 lux (Figure 6B, C, S7). In contrast, both *ls-tim* strains we tested (w^+ and w^-) showed only one defined activity peak during the 2nd half of the 200 lux phase and activity increase coincided precisely with the onset of the temperature decrease (Figure 6B, C, S7). The results indicate that the synchronised circadian clock in *ls-tim* flies is responsible for a suppression of behavioural activity during the phase of increasing temperature. Interestingly, a similar repression of behavioural activity during ramped temperature cycles in DD depends on the gene *nocte,* which is required for temperature synchronisation during DD and LL [15,16]. *nocte* mutants steadily increase their activity with rising temperature[15], similar to what we observe here for *s-tim* flies (Figure 6B, S7A), indicating a failure to synchronize their clock to the temperature cycle. To test this, we also analysed clock-less flies (tim^{KO}), which showed essentially the same behaviour as *s-tim*

299 flies (Figure 6B, C), indicating that *s-tim* flies are not able to synchronise their clock in Northern
300 summer conditions as for example experienced in Oulu. Finally, to test if the same mechanism
301 responsible for the lack of *s-tim* synchronization to rectangular laboratory LLTC conditions
302 operates under semi-natural conditions, we also analysed *s-tim cry*^{02} flies under Oulu summer
303 conditions. Strikingly, without CRY, *s-tim* flies showed essentially the same behaviour as *ls-*
304 *tim* flies (Figure 6B, C), indicating that the reduced light-sensitivity of the L-TIM:CRY
305 interaction enables *ls-tim* flies to synchronise their clock in Northern summers.

306

307 **Discussion**

308 Light and temperature serve as two universal Zeitgebers to time the circadian clock in
309 *Drosophila* and many other organisms. In *Drosophila,* exposure to constant light breaks down
310 the clock machinery leading to arrhythmic locomotor activity [10,27]. This is likely due to
311 constitutively low TIM levels in clock neurons caused by constant activation of the circadian
312 photoreceptor CRY [5,8,9]. Cycling temperature, on the other hand, serves as another potent
313 Zeitgeber to synchronise the circadian clock independent of light, suggesting the circadian
314 thermo input is distinct from the light input at the circuit level. Interestingly, temperature
315 cycles can 'override' the effects of constant light and restore rhythmicity, both at the
316 molecular and behavioural level [18,19]. Core clock proteins such as TIM and PER abolish their
317 oscillation when exposed to constant light, but the rhythmic expression of those proteins is
318 restored by temperature cycles in both peripheral tissues and central pacemakers, suggesting
319 the existence of a functional clock in these conditions. But the mechanisms that protect TIM
320 from constant degradation by light during temperature cycles was so far an unresolved
321 question.

We show here that only flies that carry the novel *ls-tim* allele can be synchronised to temperature cycles in LL, whereas flies carrying the ancient *s-tim* allele cannot. *ls-tim* is derived from *s-tim* by the insertion of single G nucleotide, which enables the usage of an additional upstream Methionine. As a result, *ls-tim* flies generate two TIM proteins: the original S-TIM (1398 amino acids) and L-TIM, carrying 23 additional N-terminal amino acids. In contrast, *s-tim* flies can only produce S-TIM [23]. L-TIM is less sensitive to light (more stable) compared to S-TIM, due to a weaker light-dependent interaction with the photoreceptor CRY [8,20]. This explains why *ls-tim* flies show reduced behavioural phase shifts in response to brief light pulses and why they are more prone to enter diapause in long photoperiods compared to *s-tim* flies [20,21]. The impaired L-TIM:CRY interaction is also the reason why *ls-tim* flies can synchronise to LLTC, because removal of CRY enables *s-tim* flies to synchronise as well (Figure 3, S3). Nevertheless, both *s-tim* and *ls-tim* flies do become arrhythmic in LL at constant temperature, meaning that in *ls-tim* flies temperature cycles still somehow overcome the arrhythmia inducing effects of constant light. Presumably L-TIM levels in LL are below a threshold to support rhythmicity at constant temperatures, while above a threshold enabling the response to rhythmic temperature changes.

Our observation that removal of visual system function in the context of a *cry* mutant background leads to a behavioural phase advance, supports a role for visual system light input in phasing behaviour during temperature entrainment. Interestingly, in constant darkness and temperature cycles, wild type flies show the same early activity phase at the beginning of the thermo period as visual system impaired *cry* mutants in LLTC (Figure 4A, B, S5D) [32]. Moreover, restricting clock function to the the 5th s-LNv, and the majority of the LNd and DN neurons in *cry* mutant flies, resulted in an activity peak late in the thermo phase, both in LLTC and DDTC conditions, similar to that of wild type flies in LLTC (Figure 1A) [32]. The drastic phase

346 difference in DDTC between wild type and *cry* mutant flies with a functional clock restricted
347 to dorsal clock neurons, indicates that these dorsally located neurons (including at least some
348 of the E-cells) are sufficient to drive behaviour in 16°C : 25°C temperature cycles, but that
349 other clock neurons contribute to setting the behavioural activity phase in the absence of
350 light or impaired light-input to the circadian clock neurons (Figures 1, 3, 5) [32,40].

351

352 The *ls-tim* allele arose approximately 300-3000 years ago in southern Europe from where it is
353 currently spreading northward by seasonal directional selection [21,22]. *ls-tim* enhances
354 diapause, which presumably serves as driving force for this natural selection, by providing
355 advantages in coping with the shorter day-length and earlier winter onset in higher latitudes.
356 [21,22]. In addition to an earlier onset of winter, northern latitudes are also characterized by
357 extremely long photoperiods in the summer, and north of the Arctic Circle even constant light.
358 Our finding that *ls-tim* enables flies to synchronise to temperature cycles in constant light and
359 particularly to semi-natural conditions mimicking white nights in Finland, indicates that this
360 allele provides an additional fitness advantage during long photoperiods. Considering the
361 massive population expansion of *Drosophila* during the summer and that daily timing of
362 activity offers a fitness advantage (e.g., [41]), we propose that the ability to synchronise to
363 temperature cycles in long summer days constitutes the main positive selection drive for this
364 allele. This positive drive is further boosted by the dominance of *ls-tim* over *s-tim,* i.e.,
365 heterozygous *ls-tim/s-tim* flies are able to synchronise as efficiently to LLTC as homozygous
366 *ls-tim/ls-tim* flies do (Figure 1, S1C).

367 Interestingly, other high-latitude *Drosophila* species also show reduced light sensitivity of
368 their circadian clock, although via a different mechanism. These species (for example *D.*

369 *ezoana* and *D. littoralis*) reduce light-sensitivity of the circadian clock by omitting CRY
370 expression from the l-LNv clock neurons, thereby enabling their adaptation to long
371 photoperiods [29,42]. Furthermore, several Northern latitude fly species have lost the ability to
372 maintain free-running rhythms in constant darkness, implying that a circadian clock is not
373 required in long summer day conditions [29,42,43]. It is not known however, if these species are
374 able to synchronise to white nights or to temperature cycles in LL. Our results indicate that
375 under Northern latitude summer conditions, the lack of a robust clock can be compensated
376 by the ability to synchronise molecular behavioural and rhythms to temperature cycles.
377 Nevertheless, it seems clear that independent strategies have evolved allowing insects to
378 cope with light and temperature conditions in high-latitudes.

379

380

381 **Methods**

382

383 **Fly stains**

384 Flies were reared on cornmeal-sucrose food at 18 C or 25°C under 12 hr : 12 hr LD cycles and
385 60% humidity until used in experiments. The following strains were used in this study:
386 $norpA^{P41}$ and $norpA^{P41}$, cry^{b} [35], $norpA^{P41}$, cry^{02} [34]; cry^{b} [3], cry^{01}, cry^{02} and *w; iso s-tim* [26], *Clk856-*
387 *gal4* [37], *y w; s-tim* and *y w; ls-tim* [44], *w; iso31 ls-tim* [25], *gmr-hid* [45]; *Rh1-gal4* (BL8688). The *UAS-*
388 *tim2.5* transgene encodes L-TIM and S-TIM [36] and is inserted on a *s-tim* chromosome. It was
389 combined with tim^{KO} [30] using standard meiotic recombination. Wild type stocks used were
390 Canton S (*ls-tim,* Jeffrey Hall lab), Tanzania (*s-tim*) [29], and Houten (Hu) (*s-tim* and *ls-tim*

versions) [21]. If necessary *ls-tim* and *s-tim* chromosomes were exchanged using standard genetic crosses. *ls-tim/s-tim* genotypes were confirmed by PCR [23].

Behavioral assays

3-5 days old male flies were used for locomotor activity tests with the *Drosophila* Activity Monitor System (DAM,Trikinetics Inc). Fly activity was recorded in light- and temperature-controlled incubators (Percival,USA) every minute. Environmental protocols are indicated on next to the first actogram in every figure. LLTC was phase delayed with respect to the original LD cycle by 5h. Light intensity was between 400 and 800 lux (white fluorescence light). DDTC was advanced by 8h with respect to the initial LD cycle. A fly tool box implemented in MATLAB (Math Works) was employed for plotting actograms and histograms [46]. Behavior was quantified using a custom Excel macro [30]. 30min bin activity was normalized to the maximum level of activity for each fly. The median of this normalized activity was plotted, allowing to visualize the level of synchronization within a strain [30]. The same macro was used for plotting the light and temperature in Figure 6A, C, and Figure S7B).

Immunohistochemistry and quantification

Immunostaining of whole-mount brains was performed as descried in [15] for Figure 4C. In brief, flies for LLTC experiments were reared in LL and 25°C for 3 days, followed by 6 days of 25°C: 16°C LLTC. Brains were fixed and dissected on the day 7 of the temperature cycles at the indicated time points. Primary rat anti-TIM (1:1,000; [47]), and secondary rabbit AlexaFlour-488 (Invitrogen, 1:500) were applied for 12 hours at 4°C before the brains were mounted in Vectashield mounting medium. Brains were observed with a Leica TCS SP8 confocal

414 microscope with a 20x objective. To quantify the staining signals, pixel intensity of stained
415 neurons and background for each neural groups were measured using ImageJ (NIH), the signal
416 intensities were determined by subtracting average background signals from neuronal signals
417 from pixel values of two surrounding regions. Average intensities for each time point and
418 neuronal group represent at least 8 hemispheres for each genotype. Data were normalized
419 by setting the peak value to 1 and the ratio from each time point was then divided by the
420 peak value. For all the other immunostaining experiments, the protocol used was the same
421 as previously described [30]. Flies were placed in LL for 2 days and then entrained with a LLTC
422 cycle and dissected on the 6th cycle. Brains were dissected in PBST 0.1% and fixed for 20 min
423 at room temperature in PFA 4%. After 3 washes brains were blocked for one hour at room
424 temperature in PBST 0.1% + 5% goat serum. Primary antibodies were incubated for 48 h (in
425 PBST 0.1% + 5% goat serum) at 4°C, while secondary incubation was done overnight at 4°C.
426 Brains were mounted using Vectashield. Rat anti-TIM generated against TIM-fragment 222-
427 577 [48] (kind gift of Isaac Edery) was used at 1/2000. Monoclonal anti-PDF (DSHB) was used at
428 1/1000, and pre-absorbed Rabbit anti-PER [49] was used at 1/15000. Secondary antibodies
429 used: goat cross absorbed anti-mice 488 ++ 1/2000 (Invitrogen), goat anti-rabbit 555 1/2000
430 (Invitrogen) and anti-rat 647 1/1000 (Invitrogen). Brains were imaged with a Leica TCS SP8
431 confocal microscope with a 63x objective. Average intensity was measured using ImageJ and
432 quantification was normalized to the background: (signal-background)/background [50].

433

434 **Acknowledgements:**

435 We thank Isaac Edery for anti-TIM antibodies and Francois Rouyer and Charalambos Kyriacou
436 for fly stocks. This work was supported by a BBSRC research grant given to RS (BB/H001204/1)

and by the European Union's Horizon 2020 Research and Innovation Programme under the Marie Skłodowska-Curie grant agreement no. 765937 (CINCHRON).

References

1. Tataroglu, O. & Emery, P. The molecular ticks of the Drosophila circadian clock. *Curr Opin Insect Sci* **7**, 51–57 (2015).
2. Peschel, N. & Helfrich-Förster, C. Setting the clock - by nature: Circadian rhythm in the fruitfly *Drosophila melanogaster*. *FEBS Letters* **585**, 1435–1442 (2011).
3. Stanewsky, R. *et al.* The cry^b mutation identifies *cryptochrome* as a circadian photoreceptor in *Drosophila*. *Cell* **95**, 681–92 (1998).
4. Yoshii, T., Todo, T., Wülbeck, C., Stanewsky, R. & Helfrich-Förster, C. Cryptochrome is present in the compound eyes and a subset of *Drosophila*'s clock neurons. *J Comp Neurol* **508**, 952–66 (2008).
5. Busza, A., Myai, E.-L., Rosbash, M. & Emery, P. Roles of the Two Drosophila CRYPTOCHROME Structural Domains in Circadian Photoreception. *Science* **304**, 1503–1506 (2004).
6. Koh, K., Zheng, X. & Sehgal, A. JETLAG Resets the Drosophila Circadian Clock by Promoting Light-Induced Degradation of TIMELESS. *Science* **312**, 1809–1812 (2006).
7. Peschel, N., Veleri, S. & Stanewsky, R. Veela defines a molecular link between Cryptochrome and Timeless in the light-input pathway to Drosophila's circadian clock. *Proc Natl Acad Sci U S A* **103**, 17313–17318 (2006).
8. Peschel, N., Chen, K. F., Szabo, G. & Stanewsky, R. Light-dependent interactions between the Drosophila circadian clock factors cryptochrome, jetlag, and timeless. *Curr. Biol.* **19**, 241–7 (2009).
9. Emery, P., Stanewsky, R., Hall, J. & Rosbash, M. A unique circadian-rhythm photoreceptor. *Nature* **404**, 456–457 (2000).

462 10. Marrus, S., Zeng, H. & Rosbash, M. Effect of constant light and circadian entrainment ofperSflies:
463 evidence for light-mediated delay of the negative feedback loop inDrosophila. *Embo J* **15**, 6877–
464 6886 (1996).
465 11. Helfrich-Förster, C., Winter, C., Hofbauer, A., Hall, J. C. & Stanewsky, R. The circadian clock of
466 fruit flies is blind after elimination of all known photoreceptors. *Neuron* **30**, 249–61 (2001).
467 12. Brown, S., Zumbrunn, G., Fabienne, F.-O., Preitner, N. & Schibler, U. Rhythms of Mammalian
468 Body Temperature Can Sustain Peripheral Circadian Clocks. *Curr Biol* **12**, 1574–1583 (2002).
469 13. Buhr, E. D., Yoo, S.-H. & Takahashi, J. S. Temperature as a Universal Resetting Cue for
470 Mammalian Circadian Oscillators. *Science* **330**, 379–385 (2010).
471 14. Chen, C. *et al.* Drosophila Ionotropic Receptor 25a mediates circadian clock resetting by
472 temperature. *Nature* **527**, 516–520 (2015).
473 15. Chen, C., Xu, M., Anantaprakorn, Y., Rosing, M. & Stanewsky, R. nocte Is Required for Integrating
474 Light and Temperature Inputs in Circadian Clock Neurons of Drosophila. *Curr Biol* **28**, 1595-
475 1605.e3 (2018).
476 16. Sehadova, H. *et al.* Temperature entrainment of Drosophila's circadian clock involves the gene
477 nocte and signaling from peripheral sensory tissues to the brain. *Neuron* **64**, 251–66 (2009).
478 17. Yadlapalli, S. *et al.* Circadian clock neurons constantly monitor environmental temperature to
479 set sleep timing. *Nature* **555**, 98–102 (2018).
480 18. Glaser, F. & Stanewsky, R. Temperature Synchronization of the Drosophila Circadian Clock. *Curr*
481 *Biol* **15**, 1352–1363 (2005).
482 19. Yoshii, T. *et al.* Temperature cycles drive Drosophila circadian oscillation in constant light that
483 otherwise induces behavioural arrhythmicity. *Eur J Neurosci* **22**, 1176–1184 (2005).
484 20. Sandrelli, F. *et al.* A Molecular Basis for Natural Selection at the timeless Locus in Drosophila
485 melanogaster. *Science* **316**, 1898–1900 (2007).
486 21. Tauber, E. *et al.* Natural selection favors a newly derived timeless allele in Drosophila
487 melanogaster. *Science* **316**, 1895–1898 (2007).

22. Zonato, V., Vanin, S., Costa, R., Tauber, E. & Kyriacou, C. P. Inverse European Latitudinal Cline at the timeless Locus of Drosophila melanogaster Reveals Selection on a Clock Gene: Population Genetics of ls-tim. *J Biol Rhythms* **33**, 15–23 (2018).

23. Rosato, E. *et al.* Conceptual translation oftimelessreveals alternative initiating methionines inDrosophila. *Nucleic Acids Res* **25**, 455–458 (1997).

24. Pittendrigh, C., Kyner, W. & Takamura, T. The Amplitude of Circadian Oscillations: Temperature Dependence, Latitudinal Clines, and the Photoperiodic Time Measurement. *J Biol Rhythms* **6**, 299–313 (1991).

25. Koh, K. *et al.* Identification of SLEEPLESS, a sleep-promoting factor. *Science* **321**, 372–376 (2008).

26. Dolezelova, E., Dolezel, D. & Hall, J. Rhythm Defects Caused by Newly Engineered Null Mutations in Drosophila's cryptochrome Gene. *Genetics* **177**, 329–345 (2007).

27. Konopka, R., Pittendrigh, C. & Orr, D. Reciprocal behaviour associated with altered homeostasis and photosensitivity ofDrosophilaclock mutants. *J Neurogenet* **6**, 1–10 (1989).

28. Suri, V., Qian, Z., Hall, J. & Rosbash, M. Evidence that the TIM Light Response Is Relevant to Light-Induced Phase Shifts in Drosophila melanogaster. *Neuron* **21**, 225–234 (1998).

29. Menegazzi, P. *et al.* Adaptation of Circadian Neuronal Network to Photoperiod in High-Latitude European Drosophilids. *Curr Biol* **27**, 833–839 (2017).

30. Lamaze, A. *et al.* Regulation of sleep plasticity by a thermo-sensitive circuit in Drosophila. *Sci Rep* **7**, 40304 (2017).

31. Shafer, O., Rosbash, M. & Truman, J. Sequential nuclear accumulation of the clock proteins Period and Timeless in the pacemaker neurons of Drosophila melanogaster. *J Neuroscience* **22**, 5946–5954 (2002).

32. Gentile, C., Sehadova, H., Simoni, A., Chen, C. & Stanewsky, R. Cryptochrome antagonizes synchronization of Drosophila's circadian clock to temperature cycles. *Curr Biol* **23**, 185–95 (2013).

513 33. Ogueta, M., Hardie, R. C. & Stanewsky, R. Non-canonical Phototransduction Mediates
514 Synchronization of the Drosophila melanogaster Circadian Clock and Retinal Light Responses.
515 *Curr Biol* **28**, 1725-1735.e3 (2018).
516 34. Ogueta, M., Hardie, R. C. & Stanewsky, R. Light Sampling via Throttled Visual Phototransduction
517 Robustly Synchronizes the Drosophila Circadian Clock. *Curr Biol* **30**, 2551-2563.e3 (2020).
518 35. Szular, J. *et al.* Rhodopsin 5- and Rhodopsin 6-mediated clock synchronization in Drosophila
519 melanogaster is independent of retinal phospholipase C-β signaling. *J Biol Rhythms* **27**, 25–36
520 (2012).
521 36. Yang, Z. & Sehgal, A. Role of molecular oscillations in generating behavioral rhythms in
522 Drosophila. *Neuron* **29**, 453–467 (2001).
523 37. Gummadova, J., Coutts, G. & Glossop, N. Analysis of the Drosophila Clock Promoter Reveals
524 Heterogeneity in Expression between Subgroups of Central Oscillator Cells and Identifies a Novel
525 Enhancer Region. *J Biol Rhythms* **24**, 353–367 (2009).
526 38. Zeng, H., Hardin, P. & Rosbash, M. Constitutive overexpression of the Drosophila period protein
527 inhibits period mRNA cycling. *Embo J* **13**, 3590–3598 (1994).
528 39. Kapopoulou, A. *et al.* Demographic analyses of a new sample of haploid genomes from a
529 Swedish population of Drosophila melanogaster. *Sci Rep* **10**, 22415 (2020).
530 40. Lorber, C., Stanewsky, R. & Lamaze, A. *Circadian clock neurons maintain phase control over daily*
531 *behavioral activity patterns under diverse environmental conditions*.
532 http://biorxiv.org/lookup/doi/10.1101/2020.06.02.128918 (2020)
533 doi:10.1101/2020.06.02.128918.
534 41. van der Vinne, V. *et al.* Maximising survival by shifting the daily timing of activity. *Ecol Lett* **22**,
535 2097–2102 (2019).
536 42. Beauchamp, M. *et al.* Closely Related Fruit Fly Species Living at Different Latitudes Diverge in
537 Their Circadian Clock Anatomy and Rhythmic Behavior. *J Biol Rhythms* **33**, 602–613 (2018).

538 43. Bertolini, E. *et al.* Life at High Latitudes Does Not Require Circadian Behavioral Rhythmicity
539 under Constant Darkness. *Curr Biol* **29**, 3928-3936.e3 (2019).
540 44. Chen, K. F., Peschel, N., Zavodska, R., Sehadova, H. & Stanewsky, R. QUASIMODO, a Novel GPI-
541 anchored zona pellucida protein involved in light input to the Drosophila circadian clock. *Curr*
542 *Biol* **21**, 719–29 (2011).
543 45. Klarsfeld, A. *et al.* Novel features of cryptochrome-mediated photoreception in the brain
544 circadian clock of Drosophila. *J Neuroscience* **24**, 1468–1477 (2004).
545 46. Levine, J., Funes, P., Dowse, H. & Hall, J. Signal analysis of behavioral and molecular cycles. *BMC*
546 *Neurosci* **3**, 1 (2002).
547 47. Rush, B., Murad, A., Emery, P. & Giebultowicz, J. Ectopic CRYPTOCHROME renders TIM light
548 sensitive in theDrosophilaovary. *J Biol Rhythms* **21**, 272–278 (2006).
549 48. Sidote, D., Majercak, J., Parikh, V. & Edery, I. Differential effects of light and heat on
550 theDrosophilacircadian clock proteins PER and TIM. *Mol Cell Biol* **18**, 2004–2013 (1998).
551 49. Stanewsky, R. *et al.* Temporal and spatial expression patterns of transgenes containing
552 increasing amounts of theDrosophilaclock geneperiodand alacZreporter: mapping elements of
553 the PER protein involved in circadian cycling. *J Neuroscience* **17**, 676–696 (1997).
554 50. Lamaze, A. *et al.* The E3 ubiquitin ligase CTRIP controls CLOCK levels and PERIOD oscillations in
555 Drosophila. *Embo Rep* **12**, 549–557 (2011).
556
557
558
559

Figure legends

Figure 1: *s-tim* flies cannot synchronise their behaviour to temperature cycles in constant light. A) Group actograms of the genotypes indicated representing average activity of one experiment. Environmental conditions are indicated next to the actogram on the upper left. LD: 12h-12h Light-Dark constant 25°C, LL: constant light and 25°C, LLTC: LL and 25°C-16°C temperature cycles. White areas: lights-on, and 25°C, grey areas: lights-off and 25°C during LD and lights-on and 16°C during LLTC. N (*w, iso31 ls-tim*): 20, (*w, iso s-tim*): 20. **B)** Median of normalised activity during day 6 of LLTC of independent experiments combined. Yellow bar: thermophase, blue bar cryophase (12h each). N (*w, iso31 ls-tim*): 74, (*w, iso s-tim*): 65. **C)** Left: Group actograms of the genotypes indicated representing average activity of one experiment as in (A). Right: median of the normalised activity during LLTC6. N: (tim^{KO}): 9, (*w;s-tim/ls-tim*): 15, (tim^{KO}/*ls-tim*): 5, (tim^{KO}/*s-tim*): 12.

Figure 2: During constant light and temperature cycles TIM and PER oscillations are strongly dampened in clock neurons of *s-tim* flies. Averages of normalised TIM **(A)** and PER **(B)** levels (see Methods) in the different groups of clock neurons during day six of LLTC in *y w; ls-tim* (blue lines) and *y w; s-tim* flies (orange lines). Number of brain hemispheres/time points = 4 to 10. Error bars = *sem* (standard error of the mean). PER was not detectable in the 5^{th} s-LNv of *s-tim* flies. A Mann Whitney test was performed (with a Bonferroni correction) to compare *s-tim* ZT6 with the other time points to determine the significance of potential oscillations. For TIM, no significant oscillations were observed in any of the DN groups. For PER, only the DN3 showed significant oscillations: * $p<0.05$, ** $p<0.01$, *** $p<0.0001$.

583

584 **Figure 3: Cryptochrome depletion partially restores behavioural and molecular**
585 **synchronisation during constant light and temperature cycles in *s-tim* flies. A)** Group
586 actograms of one representative experiment as described in the legend for Figure 1A. N *(ls-*
587 *tim; cry^{02}*): 17, (*s-tim; cry^{02}*):20. **B)** Median of normalised activity of independent experiments
588 combined during day 6 (left panel) of LLTC and day two of LL after LLTC (right panel). Yellow
589 bar: thermophase, blue bar: cryophase (12h each). N for left panel: (*ls-tim; cry^{02}*): 76; (*s-tim;*
590 *cry^{02}*): 96. N for right panel: (*ls-tim; cry^{02}*): 36; (*s-tim;cry^{02}*): 54. **C-D)** Averages of normalised
591 TIM (C) and PER (D) levels in the different groups of clock neurons during day six of LLTC in *s-*
592 *tim; cry^{02}* flies. Number of brain hemispheres/time point = 4 - 10. Error bars = *sem* (standard
593 error of the mean).

594

595 **Figure 4: Visual system function delays behavioural activity and TIMELESS oscillations of**
596 **Cryptochrome-depleted *s-tim* flies in constant light and temperature cycles. A)** Group
597 actograms of one representative experiment as described in the legend for Figure 1A. N
598 *(GMR-hid, s-tim; cry^{01}*): 17, (*norpA^{P41}; s-tim; cry^{02}*): 19. **B)** Median of normalised activity of
599 independent experiments combined during day 6 (left panel) of LLTC and day two of LL after
600 LLTC (right panel). Yellow bar: thermophase, blue bar: cryophase (12h each). N for left/right
601 panel (*s-tim, cry^{02}*): 56/45, (*norpA^{P41}; s-tim; cry^{02}*): 49/40, (*GMR-hid, s-tim; cry^{01}*): 32/30. **C)**
602 Quantification of TIM levels in different clusters of clock neurons of *y w; s-tim*, *norpA^{P41}; s-tim*
603 and *norpA^{P41}; s-tim ; cry^{b}* during day 5 of LLTC. Orange and blue bars indicate 25°C and 16°C,
604 respectively. 8 – 10 brain hemispheres were analysed for each genotype. Error bars indicate

sem (standard error of the mean). Two-way ANOVA with Bonferroni multiple comparisons was applied (* p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001).

Figure 5: Expression of *ls-tim* within clock neurons is sufficient for synchronisation to temperature cycles in constant light. A) Group actograms of one representative experiment of the indicated genotypes as described in the legend for Figure 1A . N *(UAS-ls-tim, tim^{KO}/s-tim)*: 9, (*Clk856-Gal4; s-tim/UAS-ls-tim, tim^{KO}*): 20, (*Clk856-Gal4; s-tim/tim^{KO}*): 17. **B)** Median of normalised activity during day 6 of LLT from the flies shown in A. Yellow bar: thermophase, blue bar cryophase (12h each).

Figure 6: Only *ls-tim* flies are able to synchronise to Northern latitude summer conditions. A) Changes in daily light intensity (left graph) and temperature (right graph) recorded within the incubator programmed to reflect summer conditions in Oulu, Finland (65° North). **B)** Group actograms and corresponding histograms of the last three days of one representative experiment in semi-natural Oulu conditions. White areas and bars: periods of light, and light ramping. Grey areas and bars, periods of 0.1 lux, reflecting civil twilight. Triangles under the histograms indicate the initiation of the light ramping (increase and decrease between 0.1 lux and 200 lux). The blue line above the histograms indicate the temperature ramping (between 12°C and 19°C). N (Canton S): 19; (*w^+; s-tim [Hu]*): 20; (*s-tim; cry^{02}*): 21; (*tim^{KO}*): 14. C) Median of normalised activity of independent experiments combined during the 6th day of Oulu condition. Yellow and blue lines indicate light and temperature ramping, respectively. N (Canton S): 38; (*w^+; s-tim [Hu]*): 39; (*s-tim; cry^{02}*): 41; (*tim^{KO}*): 33.

Figure 1

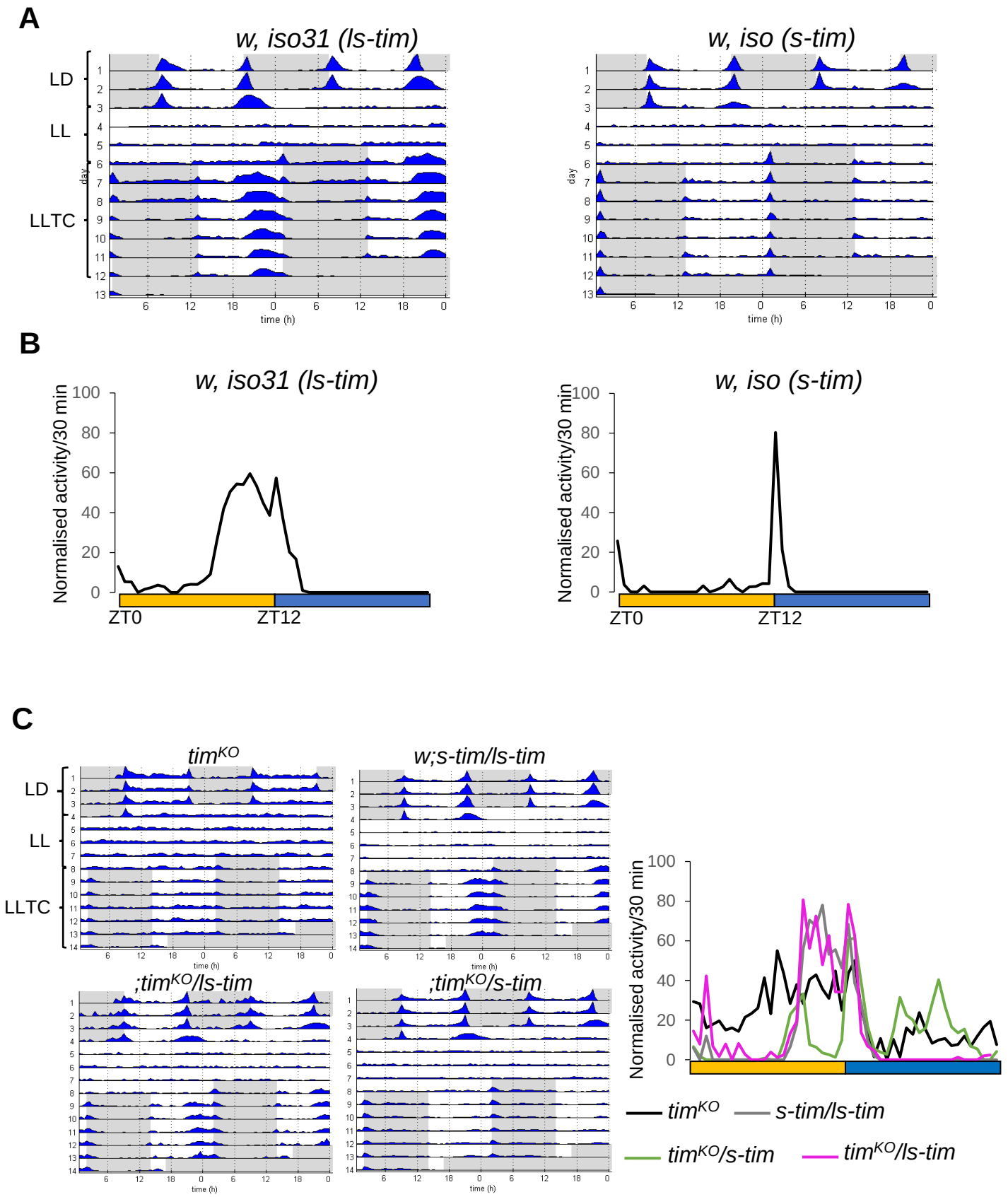

A
w, iso31 (ls-tim)
w, iso (s-tim)
LD
LL
LLTC
day
time (h)
B
w, iso31 (ls-tim)
w, iso (s-tim)
Normalised activity/30 min
ZT0
ZT12
C
timKO
w;s-tim/ls-tim
;timKO/ls-tim
;timKO/s-tim
timKO
s-tim/ls-tim
timKO/s-tim
timKO/ls-tim

Figure 2

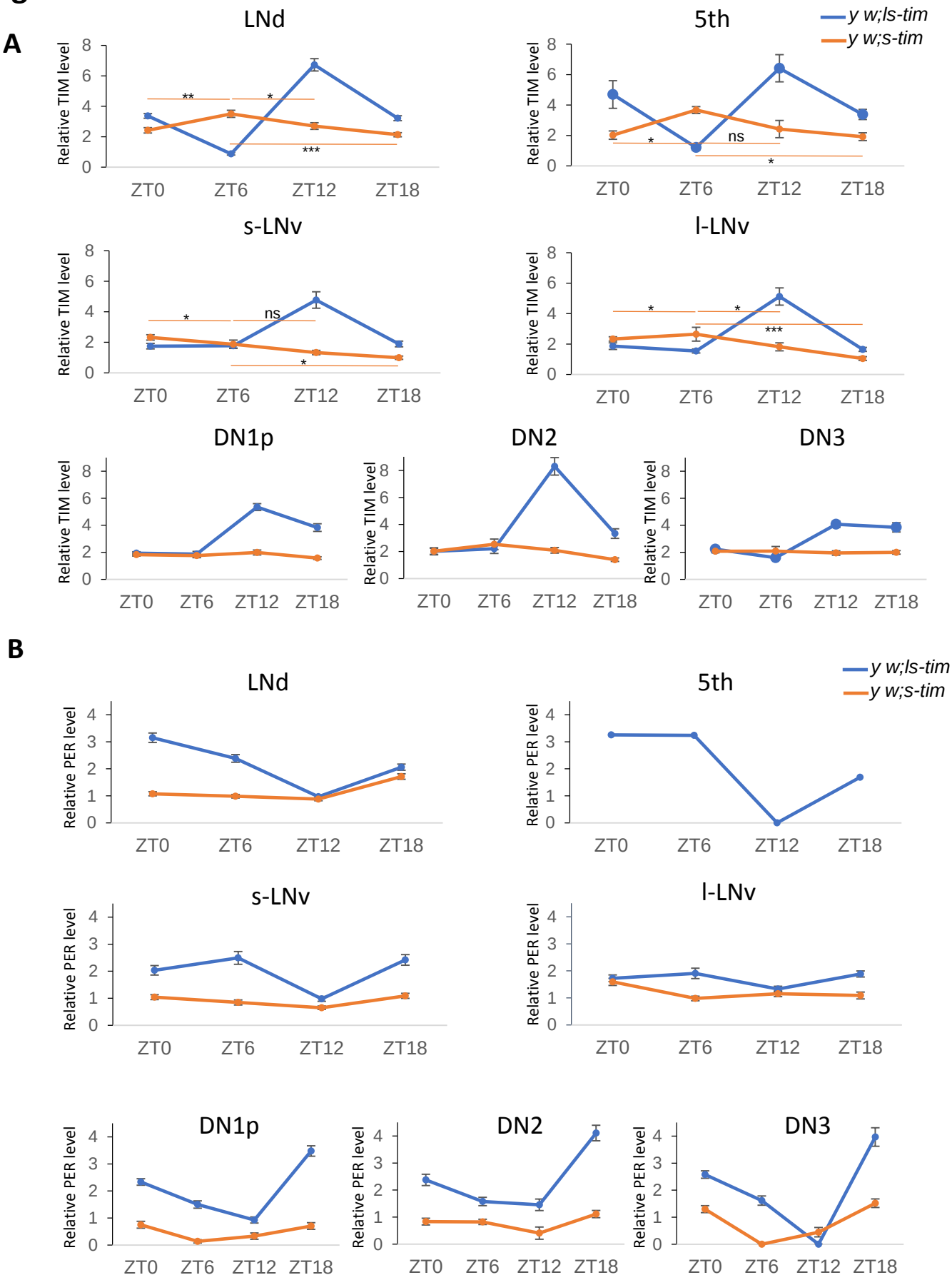

A
LNd
5th
y w;ls-tim
y w;s-tim
Relative TIM level
ZT0
ZT6
ZT12
ZT18
**
*
***
ns
s-LNv
l-LNv
DN1p
DN2
DN3
B
Relative PER level

Figure 3

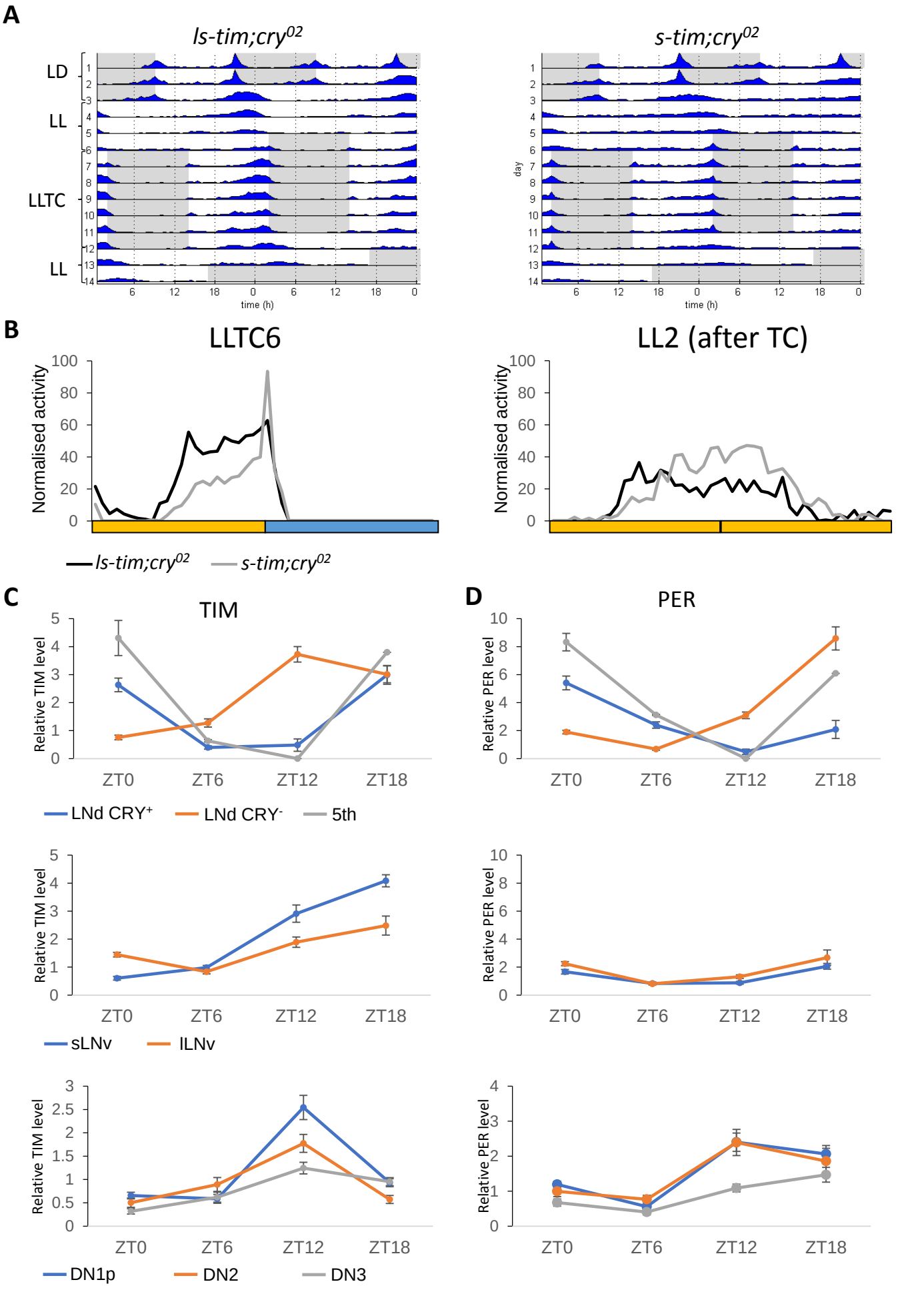

A
ls-tim;cry02
s-tim;cry02
LD
LL
LLTC
LL
day
time (h)
B
LLTC6
LL2 (after TC)
Normalised activity
ls-tim;cry02
s-tim;cry02
C
TIM
Relative TIM level
ZT0
ZT6
ZT12
ZT18
LNd CRY+
LNd CRY-
5th
sLNv
lLNv
DN1p
DN2
DN3
D
PER
Relative PER level

Figure 4

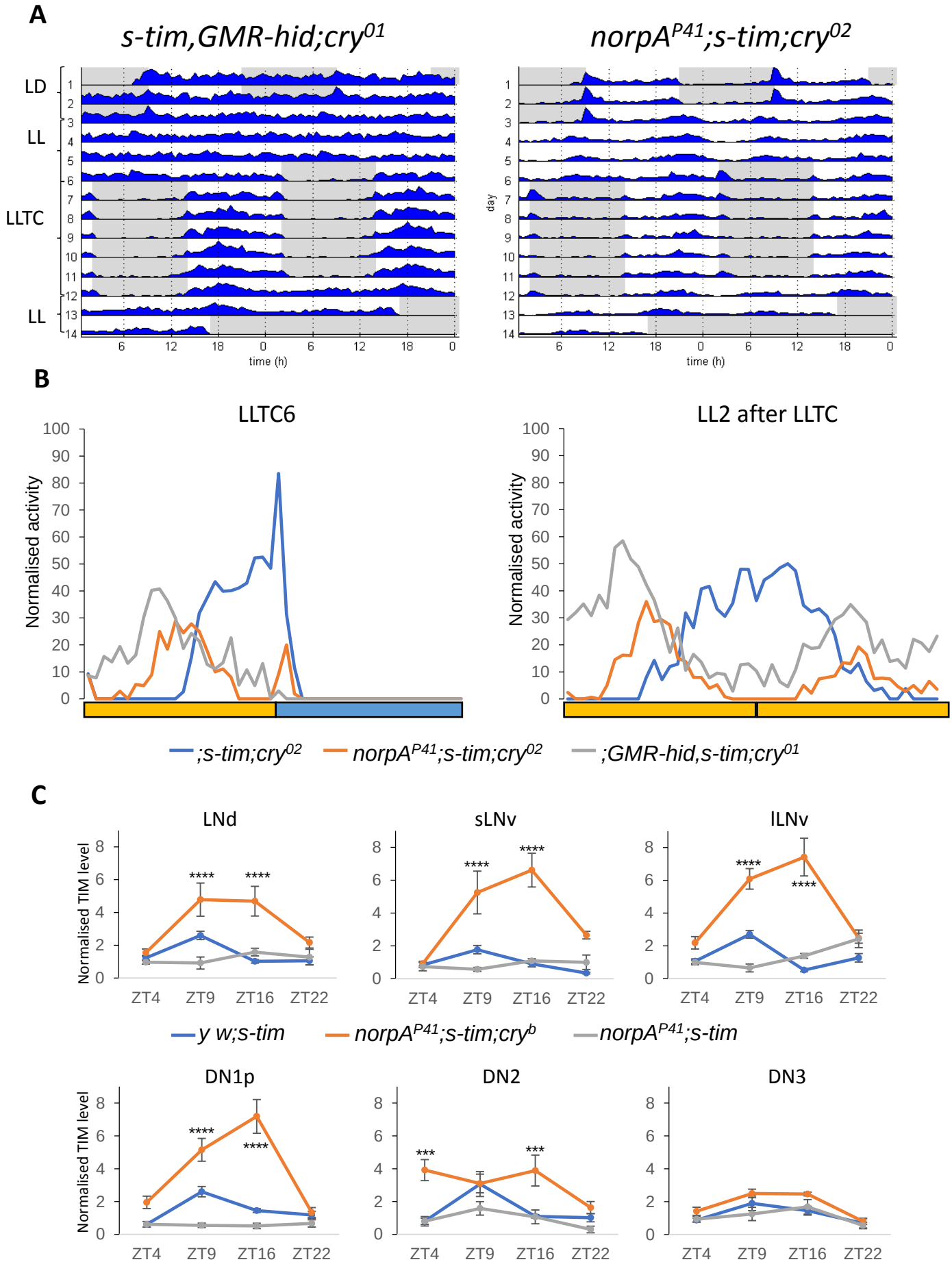
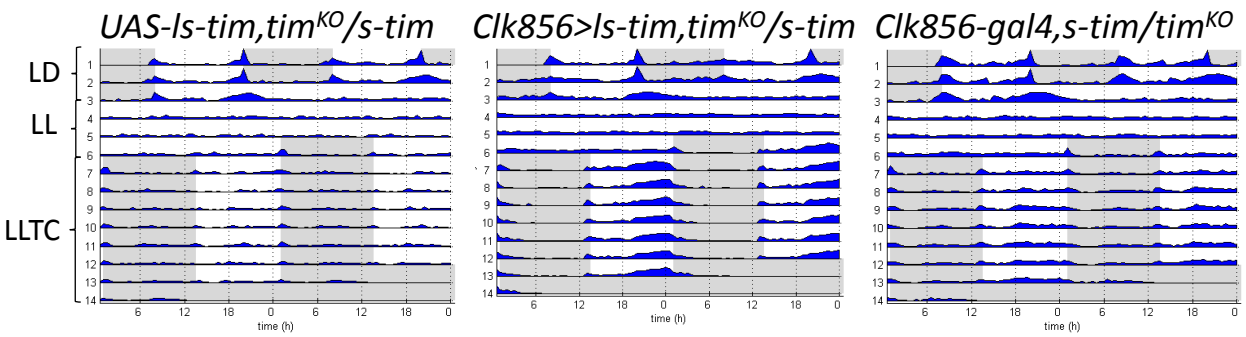

A
s-tim,GMR-hid;cry01
norpAP41;s-tim;cry02
LD
LL
LLTC
LL
time (h)
day
B
LLTC6
LL2 after LLTC
Normalised activity
;s-tim;cry02
norpAP41;s-tim;cry02
;GMR-hid,s-tim;cry01
C
LNd
sLNv
lLNv
Normalised TIM level
ZT4
ZT9
ZT16
ZT22
y w;s-tim
norpAP41;s-tim;cryb
norpAP41;s-tim
DN1p
DN2
DN3

Figure 5

A


UAS-ls-tim,tim^KO/s-tim
Clk856>ls-tim,tim^KO/s-tim
Clk856-gal4,s-tim/tim^KO
LD
LL
LLTC
time (h)

B

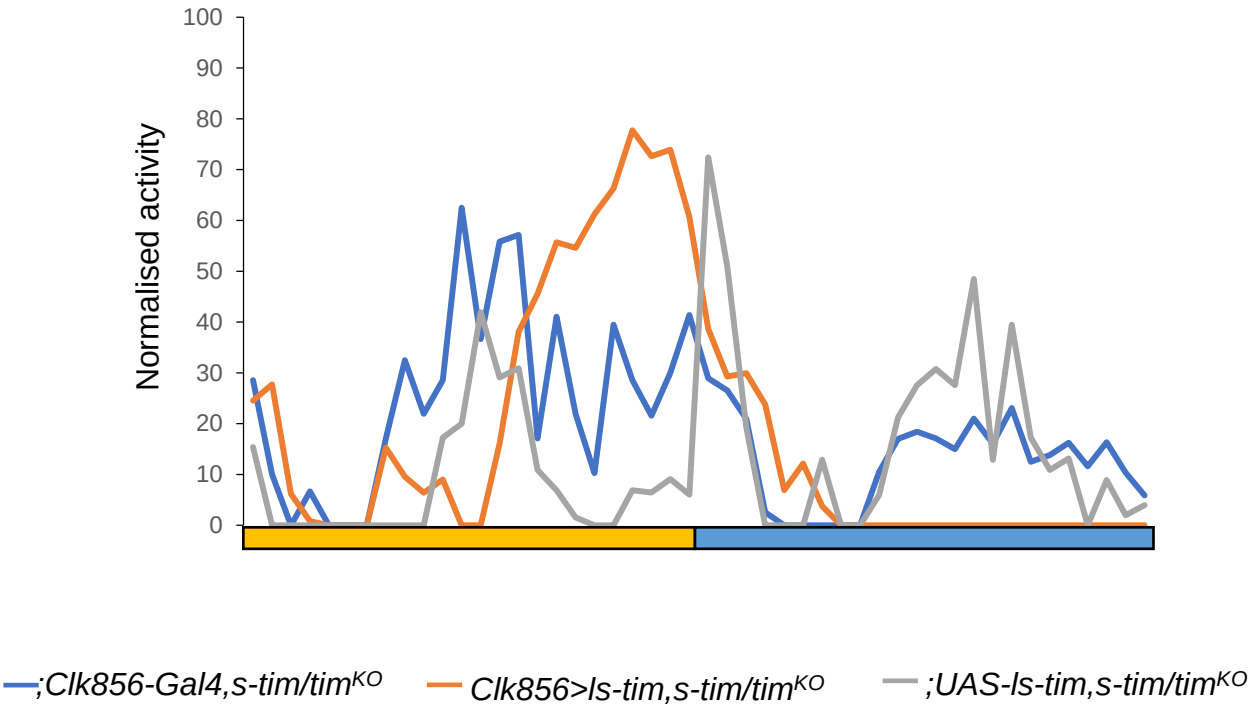
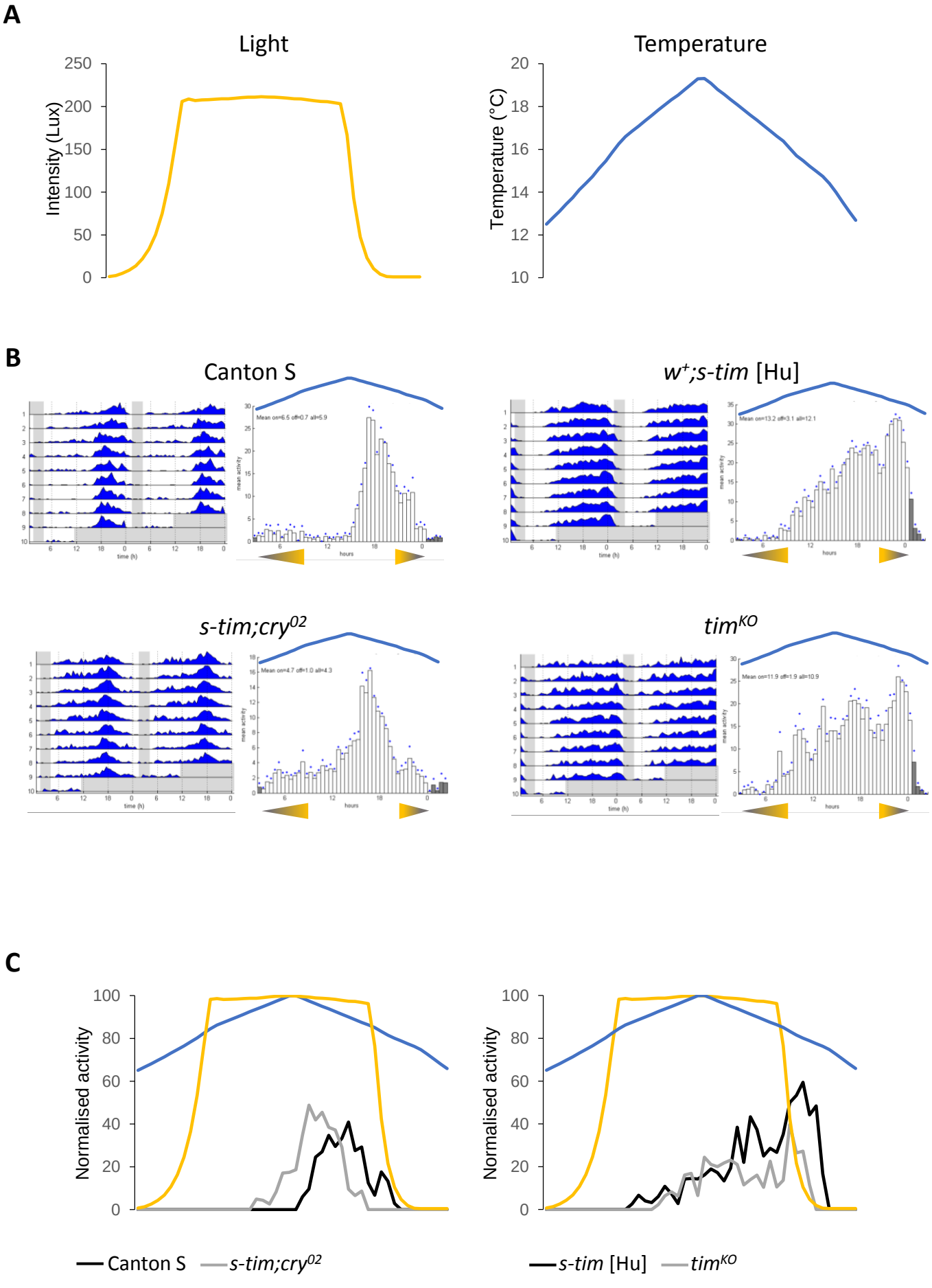

Normalised activity
100
90
80
70
60
50
40
30
20
10
0
;Clk856-Gal4,s-tim/tim^KO
Clk856>ls-tim,s-tim/tim^KO
;UAS-ls-tim,s-tim/tim^KO

Figure 6


A
Light
Intensity (Lux)
250
200
150
100
50
0
Temperature
Temperature (°C)
20
18
16
14
12
10
B
Canton S
w+;s-tim [Hu]
s-tim;cry02
timKO
time (h)
hours
mean activity
C
Normalised activity
100
80
60
40
20
0
Canton S
s-tim;cry02
s-tim [Hu]
timKO