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

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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

expression in cultured tissues¹². Moreover, the daily fluctuation of body temperature (36°C - 38.5°C) generated by the suprachiasmatic nucleus (SCN) is employed to enhance internal circadian synchronization¹³. In *Drosophila*, unlike cell autonomous light resetting by CRY, clock neurons receive temperature signals from peripheral thermo sensory organs including the aristae and mechanosensory chordotonal organs^{14–17}. Interestingly, robust molecular and behavioural entrainment to temperature cycles was observed under LL^{18,19}, suggesting that cycling temperature can somehow rescue clock neurons from the effects of constant light, but the underlying molecular mechanism is unknown.

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

to light pulses²⁰ and are more prone to enter diapause during long summer days compared to *s-tim* flies²¹. 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 cells²⁴. 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 Tanzania²⁹ 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* background²¹. 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

tim^{KO} mutant flies. *tim*^{KO} is a new *tim* null allele in the *iso31* background, in which the *tim* locus has been replaced with a *mini-white* gene resulting in red eye colour³⁰. Interestingly, although *tim*^{KO} flies do not show clock-controlled behaviour in LD, their locomotor activity pattern in LLTC is equivalent to what we observed in red eye *s-tim* flies (Figure 1C, S2C). We therefore conclude that *s-tim* flies, regardless of their eye pigmentation, are not able to synchronise their clock controlled behavioural activity rhythms to temperature cycles in constant light. Next, we compared the behaviour of hemizygous *s-tim* and *ls-tim* flies. While *tim*^{KO}/*ls-tim* flies showed normal LD and LLTC behaviour, *tim*^{KO}/*s-tim* flies only synchronised to LD (Figure 1C). Interestingly, trans-heterozygous *s-tim*/*ls-tim* flies perfectly synchronise their behaviour to LLTC, showing that *ls-tim* is dominant over *s-tim* for LLTC entrainment (Figure 1C).

***s-tim* flies fail to properly synchronise their clock protein oscillations to temperature cycles in constant light**

To distinguish if the lack of behavioural synchronization is due to a defect within or downstream of the circadian clock, we analysed PER and TIM oscillations during LLTC in clock neurons of *s-tim* and *ls-tim* flies (Figure 2, S3). As expected for *s-tim* in LL, TIM levels were lower compared to *ls-tim* flies, but detectable at all four time points we examined (ZT0, ZT6, ZT12, ZT18). While the amplitude of TIM oscillations in *s-tim* was dramatically reduced compared to *ls-tim* flies, we found that in the ventral and dorsal lateral clock neurons, TIM oscillations in *s-tim* are phase advanced by 6 hr, reaching peak values at ZT6 compared to ZT12 for *ls-tim* (Figure 2A). In the three DN groups, S-Tim levels were constitutively low at all four time points (Figure 2A). We also noticed that even in *ls-tim* flies, TIM peaks earlier compared to LD and constant temperature conditions³¹, 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 TIM⁸, 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

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

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

The behavioural results of *s-tim* flies lacking CRY described above, suggest that PER and TIM protein oscillations within clock neurons that underlie behavioural rhythms are also synchronised in LLTC. To confirm that *s-tim* flies lacking functional CRY are able to synchronise their molecular clock, we determined PER and TIM levels in different subsets of clock neurons of *s-tim cry⁰²* flies at four different time points during LLTC. Overall, we observed robust PER and TIM oscillations in s-LNv and LNd clock neurons of *s-tim cry⁰²* flies, demonstrating that removal of CRY restores molecular synchronization in *s-tim* flies during LLTC (Figure 3C, D, S4B, C). Nevertheless, PER and TIM oscillations were not identical to those observed in *Is-tim cry⁺* flies under the same conditions (compare Figure 3C, D with Figure 2A, B). To our surprise, we found desynchronization between and within groups. Notably, there was an obvious discrepancy in terms of PER amplitude between the LNd/5th and the LNv PDF⁺ neurons, even though these cells are positioned anteriorly (i.e., the reduction of amplitude is not caused by brain tissue that could interfere with the confocal imaging) (Figure 3D, S4B, C). In contrast, TIM showed clear oscillations in both groups of PDF⁺ LNv, with trough values during the first half of the warm phase and increasing levels up to the middle of the cold phase (Figure 3C, S4B, C). Furthermore, while the amplitude of PER and TIM oscillation is comparable within the LNd/5th, there was a clear phase difference between LNd CRY⁺ and the 5th compared to 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 CRY⁴. 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 cry⁰²* 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-β (PLC-β, via loss-of-function mutation of *norpA*). Completely removing both the visual system and CRY renders the brain clock blind to light entrainment¹¹, which is exactly what we observed with the *s-tim GMR-hid cry⁰¹* 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 cry⁰²* double mutants (Figure 4A). Strikingly, after switch to LLTC both

genotypes synchronise their behaviour, however with a clear phase advance compared to *s-tim cry⁰²* flies in the same condition (Figure 4B). Interestingly, *norpA^{P41} s-tim cry⁰²* double mutants take longer to establish a similar early phase as the *s-tim GMR-hid cry⁰¹* flies (Figure 4A). We attribute this difference to the initial synchronization of *norpA^{P41} s-tim cry⁰²* 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 cry⁰¹* 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- β 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 *Is-tim* and *s-tim cry⁰²* 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 cry⁰¹*, *norpA^{P41} cry⁰²*, 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.

***ls-tim* expression in clock neurons is sufficient for temperature synchronisation in constant light.**

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

The *ls-tim* allele enables flies to synchronise in white nights under semi natural conditions

In order to determine if the *ls-tim* allele can be advantageous in natural conditions, we analysed behaviour under LLTC conditions experienced in the summer in Northern Europe. We decided to mimic the conditions of a typical summer day in Oulu, Finland (65° North) for 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 temperature¹⁵, 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*

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

Discussion

Light and temperature serve as two universal Zeitgebers to time the circadian clock in *Drosophila* and many other organisms. In *Drosophila*, exposure to constant light breaks down the clock machinery leading to arrhythmic locomotor activity^{10,27}. This is likely due to constitutively low TIM levels in clock neurons caused by constant activation of the circadian photoreceptor CRY^{5,8,9}. Cycling temperature, on the other hand, serves as another potent Zeitgeber to synchronise the circadian clock independent of light, suggesting the circadian thermo input is distinct from the light input at the circuit level. Interestingly, temperature cycles can ‘override’ the effects of constant light and restore rhythmicity, both at the molecular and behavioural level^{18,19}. Core clock proteins such as TIM and PER abolish their oscillation when exposed to constant light, but the rhythmic expression of those proteins is restored by temperature cycles in both peripheral tissues and central pacemakers, suggesting the existence of a functional clock in these conditions. But the mechanisms that protect TIM from constant degradation by light during temperature cycles was so far an unresolved 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-TIM²³. 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 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

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

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

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

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

Methods

Fly stains

Flies were reared on cornmeal-sucrose food at 18 C or 25°C under 12 hr : 12 hr LD cycles and 60% humidity until used in experiments. The following strains were used in this study: *norpA^{P41}* and *norpA^{P41}*, *cry^b 35*, *norpA^{P41}*, *cry⁰² 34*; *cry^b 3*, *cry⁰¹*, *cry⁰²* and *w*; *iso s-tim*²⁶, *Clk856-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-tim2.5* transgene encodes L-TIM and S-TIM³⁶ and is inserted on a *s-tim* chromosome. It was combined with *tim^{KO} 30* using standard meiotic recombination. Wild type stocks used were 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 PCR²³.

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 histograms⁴⁶. Behavior was quantified using a custom Excel macro³⁰. 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 strain³⁰. 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 described in¹⁵ 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 AlexaFluor-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

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

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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).

10. Marrus, S., Zeng, H. & Rosbash, M. Effect of constant light and circadian entrainment of flies: evidence for light-mediated delay of the negative feedback loop in *Drosophila*. *Embo J* **15**, 6877–6886 (1996).
11. Helfrich-Förster, C., Winter, C., Hofbauer, A., Hall, J. C. & Stanewsky, R. The circadian clock of fruit flies is blind after elimination of all known photoreceptors. *Neuron* **30**, 249–61 (2001).
12. Brown, S., Zimbrun, G., Fabienne, F.-O., Preitner, N. & Schibler, U. Rhythms of Mammalian Body Temperature Can Sustain Peripheral Circadian Clocks. *Curr Biol* **12**, 1574–1583 (2002).
13. Buhr, E. D., Yoo, S.-H. & Takahashi, J. S. Temperature as a Universal Resetting Cue for Mammalian Circadian Oscillators. *Science* **330**, 379–385 (2010).
14. Chen, C. *et al.* *Drosophila* Ionotropic Receptor 25a mediates circadian clock resetting by temperature. *Nature* **527**, 516–520 (2015).
15. Chen, C., Xu, M., Anantaparakorn, Y., Rosing, M. & Stanewsky, R. *nocte* Is Required for Integrating Light and Temperature Inputs in Circadian Clock Neurons of *Drosophila*. *Curr Biol* **28**, 1595–1605.e3 (2018).
16. Sehadova, H. *et al.* Temperature entrainment of *Drosophila*'s circadian clock involves the gene *nocte* and signaling from peripheral sensory tissues to the brain. *Neuron* **64**, 251–66 (2009).
17. Yadlapalli, S. *et al.* Circadian clock neurons constantly monitor environmental temperature to set sleep timing. *Nature* **555**, 98–102 (2018).
18. Glaser, F. & Stanewsky, R. Temperature Synchronization of the *Drosophila* Circadian Clock. *Curr Biol* **15**, 1352–1363 (2005).
19. Yoshii, T. *et al.* Temperature cycles drive *Drosophila* circadian oscillation in constant light that otherwise induces behavioural arrhythmicity. *Eur J Neurosci* **22**, 1176–1184 (2005).
20. Sandrelli, F. *et al.* A Molecular Basis for Natural Selection at the timeless Locus in *Drosophila melanogaster*. *Science* **316**, 1898–1900 (2007).
21. Tauber, E. *et al.* Natural selection favors a newly derived timeless allele in *Drosophila 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 *Is-tim*. *J Biol Rhythms* **33**, 15–23 (2018).
23. Rosato, E. *et al.* Conceptual translation of timeless reveals alternative initiating methionines in *Drosophila*. *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 of *Drosophila* clock 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).

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

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

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 5th 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 (*Is-*
 587 *tim; cry⁰²*): 17, (*s-tim; cry⁰²*):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: (*Is-tim; cry⁰²*): 76; (*s-tim;*
 590 *cry⁰²*): 96. N for right panel: (*Is-tim; cry⁰²*): 36; (*s-tim;cry⁰²*): 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; cry⁰²* 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; cry⁰¹*): 17, (*norpA^{P41}; s-tim; cry⁰²*): 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, cry⁰²*): 56/45, (*norpA^{P41}; s-tim; cry⁰²*): 49/40, (*GMR-hid, s-tim; cry⁰¹*): 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

Figure 2**A** **B**

Figure 3**A** **B** **C** **D**

Figure 4

Figure 5

A

B

— *Clk856-Gal4, s-tim/tim^{KO}* — *Clk856>ls-tim, s-tim/tim^{KO}* — *UAS-ls-tim, s-tim/tim^{KO}*

Figure 6

A

B

Canton S

w⁺;s-tim [Hu]

s-tim;cry⁰²

tim^{KO}

C

— Canton S — *s-tim;cry⁰²*

— *s-tim* [Hu] — *tim^{KO}*