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## Review

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<sup>†</sup>We dedicate this article to the memory of Alexandre A. Peixoto (1963–2013), former PhD student of C.P.K. and dearest friend. Alex introduced rigorous population genetic analysis to latitudinal clock gene variation in *Drosophila*, and allied it to phenotypic analyses, thereby generating a powerful paradigm for identifying the selective forces acting on clock genes.

<sup>‡</sup>This work originated from the workshop 'The Diversity, Evolution and Mechanisms Controlling Activity Patterns' organized by Kronfeld-Schor, Bloch, Smale & Schwartz in 2012, Ein Gedi (Israel).

One contribution to a Special Feature 'Animal clocks: when science meets nature'.

# Latitudinal clines: an evolutionary view on biological rhythms<sup>†,‡</sup>

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Properties of the circadian and annual timing systems are expected to vary systematically with latitude on the basis of different annual light and temperature patterns at higher latitudes, creating specific selection pressures. We review literature with respect to latitudinal clines in circadian phenotypes as well as in polymorphisms of circadian clock genes and their possible association with annual timing. The use of latitudinal (and altitudinal) clines in identifying selective forces acting on biological rhythms is discussed, and we evaluate how these studies can reveal novel molecular and physiological components of these rhythms.

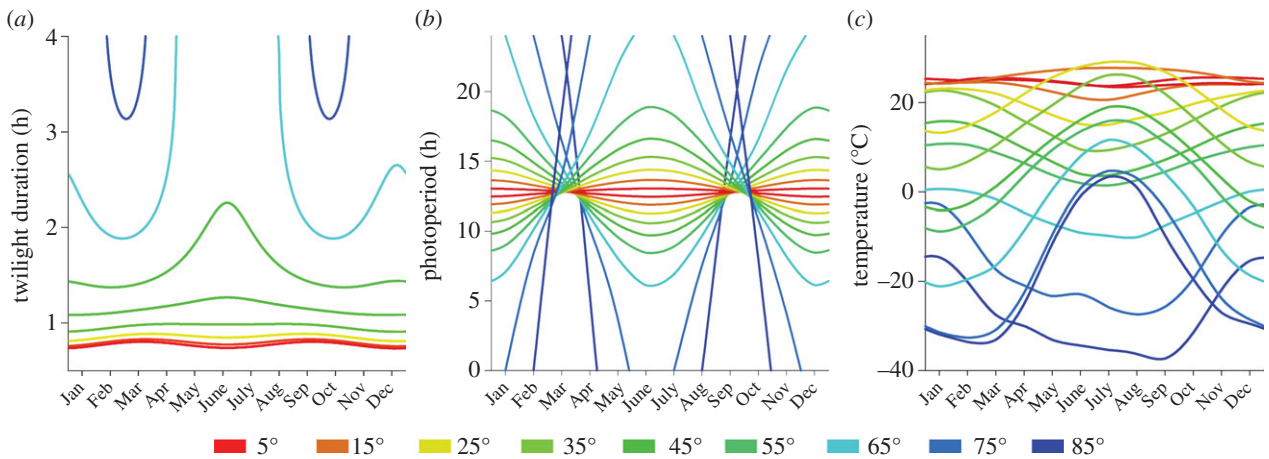
## 1. Introduction

Two celestial movements of the Earth have deeply impacted the evolution of life: its axial rotation and its elliptical orbit around the sun. They cause daily variation in solar radiation and temperature and, due to the tilt of the Earth's axis, annual variation in the angle of incidence of solar radiation leading to annual variation in light intensity, twilight duration, photoperiod and average daily temperature. Daily and annual variations in solar radiation determine primary production through synergistic effects of temperature and light availability on plant growth. Organisms depending directly or indirectly on primary production and temperature are under selection pressure to optimize annual and daily timing of physiology and behaviour [1]. Daily and annual variation in solar radiation depend on latitude, and this leads to latitude-specific selection pressures in many organisms. We expect that evolution has shaped daily (circadian) and annual (circannual and/or photoperiodic) timing systems as a function of latitude, resulting in phenotypic and genotypic clines yielding information on the underlying mechanisms [2].

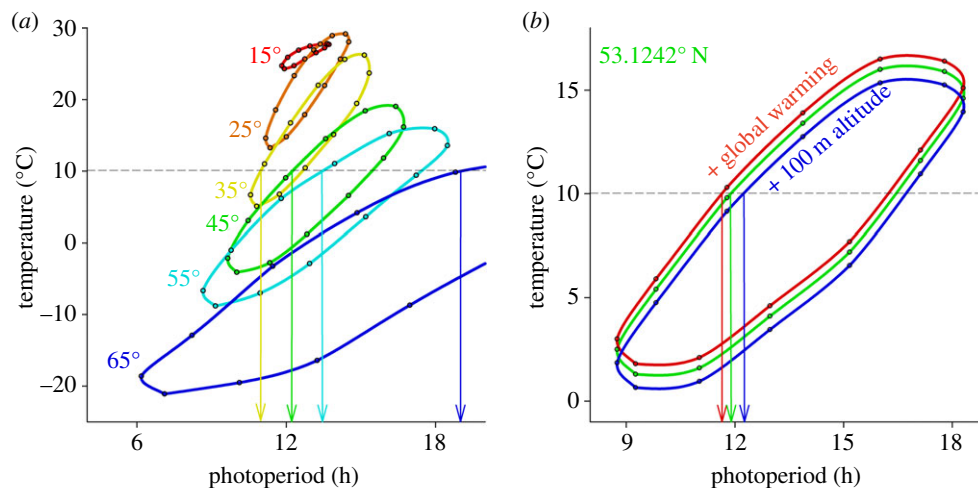
## 2. Latitudinal variation in annual light and temperature patterns: the use of ellipse-like photoperiod–temperature curves

Annual patterns in light and temperature vary with latitude (figure 1). More perpendicular solar transition across the horizon causes shorter twilights at the equator and around the equinoxes (figure 1a). Equatorial day length (civil twilight-based photoperiod) is nearly constant at approximately 13 h d<sup>-1</sup>. Away from the equator, photoperiod increases during summer and decreases during winter (figure 1b), eventually causing continuous light and continuous darkness near the poles.

Throughout the year, the incidence of solar radiation is more perpendicular at the equator than at the poles, causing higher temperatures around the equator. The Earth's axis is slanted relative to the annual orbital path around the sun and together they cause annual variation in solar radiation and temperature, with larger amplitude at the poles than in the tropics (figure 1c). The distribution of land mass and sea currents over the globe affect these patterns and cause



**Figure 1.** Annual patterns of (a) twilight duration, (b) photoperiod and (c) temperature, for different latitudinal ranges. (a) Twilight duration for Northern Hemisphere, defined as the time between  $-12^\circ$  (nautical twilight) and  $0^\circ$  solar altitude (approx. 20–80% of the log light intensity change between midnight and noon). (b) Photoperiod calculation based on civil twilight times at dawn and dusk. Civil twilight (solar altitude  $6^\circ$  below the horizon) is the moment when log light intensity changes most rapidly [3] and is often considered as the time of ‘lights on’ and ‘lights off’ for biological systems. (c) Monthly mean dry bulb air temperatures (mostly between 1960 and 1990) from 873 weather stations around the world obtained from the World Meteorological Organization (<http://www.wmo.int>), and globally averaged over  $10^\circ$  latitudinal bands. Colours indicate mid-latitude of each band (hemispheres plotted separately).



**Figure 2.** Ellipse-like annual PPT curves predict changing photoperiodic responses with changing latitude and altitude. (a) The annual temperature hysteresis leads to an ellipse-like relationship between temperature and photoperiod, with higher temperatures in autumn than in spring (dots indicate the mid-point of each month). The dotted lines (a,b) indicate a hypothetical threshold temperature at  $10^\circ\text{C}$  at which a certain species starts winter dormancy (e.g. diapause), resulting in a shift towards longer critical photoperiod (CPP) earlier in the year when this species moves north. This fundamental process forms the basis for the expectation that latitudinal clines in photoperiodic response mechanisms may exist in nature. (b) Global warming (by  $0.5^\circ\text{C}$ ) will shift PPT ellipses up, whereas altitude will shift PPT ellipses down (by  $0.649^\circ\text{C } 100\text{ m}^{-1}$ ). The photoperiod–diapause reaction norm (and accompanying CPP) is therefore expected to evolve under these environmental selection pressures (latitude, altitude and global warming).

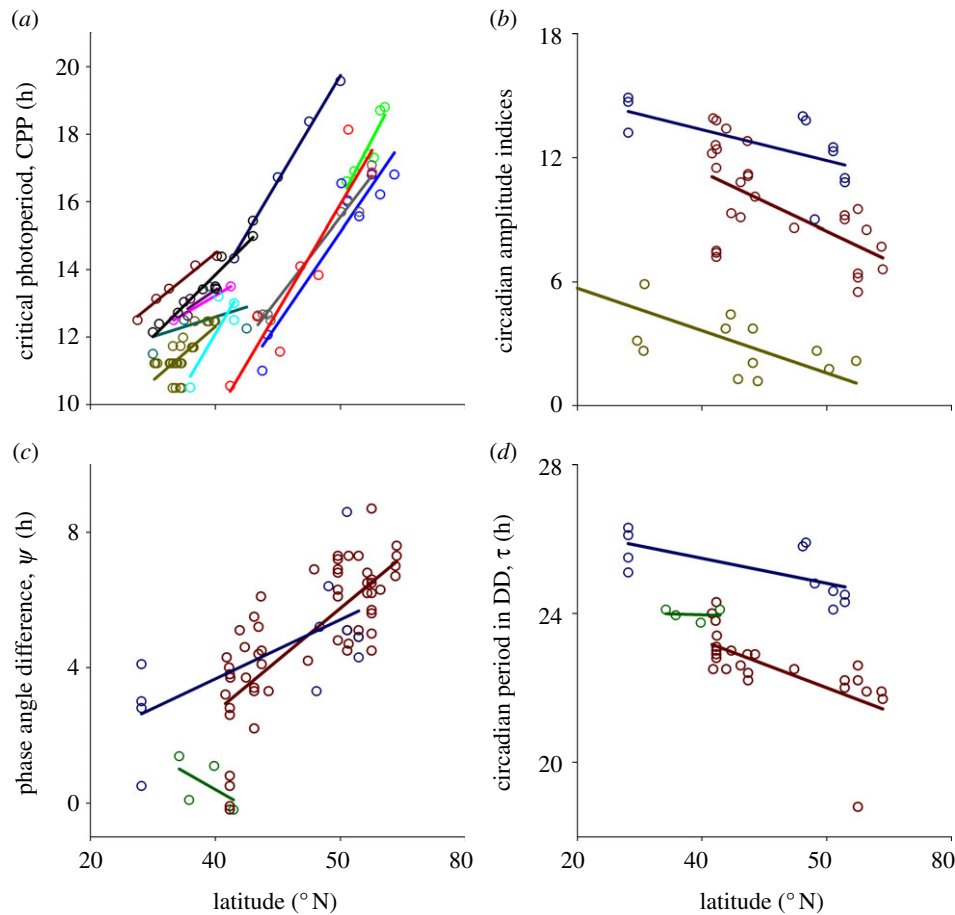
quantitative differences between the Southern and Northern Hemisphere, but the general patterns are largely similar.

For understanding latitudinal clines in timing, it is important to understand the correspondence between photoperiod and temperature (figure 1*b,c*). The temperature capacity of the Earth’s surface generates an annual hysteresis, with temperature lagging behind photoperiod. This causes a slanted ellipse-like annual relationship: the photoperiod–temperature (PPT) ellipse (figure 2*a*). This PPT ellipse changes position, angle and shape with latitude: when moving away from the equator, it shifts to lower temperatures and decompresses asymmetrically in photoperiod and temperature ranges (figure 2*a*), and eventually becomes more complex in shape approaching the (Ant-) Arctic circles.

Temperatures vary considerably between years, but photoperiod remains stable. Organisms therefore use photoperiod as

a proximate factor to tune the annual timing of physiology and behaviour to changes in ultimate factors such as temperature [4]. Thus, when many years of temperature data are used for constructing PPT ellipses, they can be useful tools to make evolutionary predictions on photoperiodism (figure 2). For instance, assume an insect at  $35^\circ\text{N}$  has optimized its physiology for photoperiodic diapause induction when temperatures drop below  $10^\circ\text{C}$ . The PPT ellipses can be used to illustrate that photoperiods below the critical photoperiod (CPP) should induce diapause (figure 2*a*, yellow PPT ellipse and arrow). They predict how the CPP will change when this species expands its distribution range north (figure 2*a*, green, turquoise and blue arrows). The expectation is that CPP will show a positive latitudinal cline, which may have a nonlinear complex shape (figure 2*a*).

Photoperiodic induction may change over years when global temperatures are changing [5]. This can be described



**Figure 3.** Latitudinal clines in photoperiodic and circadian timing mechanisms in insects. Latitude (in  $^{\circ}$  N) correlates with: (a) critical photoperiod for diapause induction (populations above 800 m altitude were excluded); (b) circadian amplitude of overt rhythms in light–dark (LD) using various indices (see text); (c) phase of entrainment in 3 L : 21 D; (d) circadian period in continuous darkness (DD). (a) Brown, *Sericanus montelus* (pupae) [13]; black, *Wyeomyia smithii* (larvae) [14]; purple, *Bruchidius dorsalis* (larvae) [15]; pink, *Chrysopa carnea* (adult) [16]; turquoise, *Homoeosoma electellum* (larvae) [17]; khaki, *Tetranychus pueraricola* (adult) [18]; cyan, *Orius sauteri* (adult) [19]; dark blue, *Acrionicta rumicis* (larvae) [11]; red, *Nasonia vitripennis* (larvae; maternally induced) [20]; green, *Drosophila montana* (adult) [21]; grey, *D. phalerata* (adult) [22]; blue, *D. transversa* (adult) [22]. (b)–(d) Khaki, *D. melanogaster* (oviposition) [23]; blue, *D. subobscura* (eclosion) [24]; red, *D. littoralis* (eclosion) [25]; green, *D. auraria* (eclosion) [26]. Three equatorial regression data points from Allemand & David [23] were not plotted in (b) for axis consistency.

by shifting a PTT ellipse upwards by, for example,  $0.5^{\circ}\text{C}$  (figure 2b, green curve). This results in an approximately 10 min reduction in CPP (figure 2b, green and red curves and arrows). It is important for evolutionary studies to exploit altitude variation to predict effects of global warming [5,6]. Mean air temperature decreases on average  $6.49^{\circ}\text{C } 1000\text{ m}^{-1}$  altitude [7]. Thus, 100 m elevation will cause an opposite but comparable shift in PPT and CPP (figure 2b, blue curve and arrow). Sampling at different altitudes may generate a predictable altitudinal cline with a simple linear relationship between CPP and altitude. Indeed, the pitcher plant mosquito *Wyeomyia smithii* increases its CPP by 11 min for each degree north, which is similar to 100 m altitudinal increase causing 8 min increase in CPP [5,6]. Long-term changes in global air temperatures and altitudinal clines may be important tools to disentangle complex interacting traits in annual and daily timing in physiology and behaviour.

### 3. Latitudinal clines in photoperiodism

Latitudinal variation in photoperiodism has been investigated mainly in insects in which short photoperiods trigger the induction of diapause, a form of winter dormancy. Diapause can occur in all stages of the life cycle, from embryo to adult, and each species has a genetically determined

diapause stage [8]. Insects enter diapause in preparation for winter even when conditions are still favourable [9]. Typically, the developmental stage sensitive to environmental stimuli is earlier than the diapausing stage and sometimes it even occurs in the previous generation, as is the case for larval diapause with maternal induction [10].

Danilevskii [11] was the first to demonstrate a positive correlation between latitude and CPP for diapause induction. He showed that CPP increases by about 1 h every  $5\text{--}6^{\circ}$  latitude in the knot grass moth *Acrionicta rumicis* [11]. Positive correlations between CPP and latitude were later described in many other diapausing insects (figure 3a; reviewed by Tauber *et al.* [8] and Danks [12]). Latitudinal clines in CPP have further been demonstrated in adult reproductive diapause in the Japanese flower bug *Orius* [19] and in Finnish malt flies *Drosophila montana* [21], in pupal diapause in the butterfly *Sericanus montelus* [13] and in maternally induced larval diapause in the parasitoid wasp *Nasonia vitripennis* [20]. The combined data reveal a general positive correlation between CPP and latitude, with an increased slope above approximately  $40^{\circ}\text{N}$  (figure 3a).

The latitudinal cline in CPP for diapause induction has adaptive significance related to the seasonal cycles at different latitudes, since CPP is predicted to be longer at higher latitudes (figure 2a). Selection pressure on timing for photoperiodic diapause induction is also evident in studies on

alien species recently introduced in new environments. Alien insects can adapt to the seasonal cycles of a newly colonized geographical range by the rapid establishment of latitudinal clines in CPP [27–29]. Rapid change in CPP also occurs in response to global warming (figure 2b), as documented in northern populations of pitcher plant mosquito *W. smithii*, which shifted their photoperiodic response towards shorter CPP [30].

The latitudinal cline in CPP (figure 3a) leads to the prediction that the proportion of diapausing individuals increases with latitude. This general trend has been found in many species [8,12,31–34]. In some cases, populations at high latitudes evolved obligate diapause where all individuals enter diapause at a certain stage regardless of environmental conditions [35].

In the varied carpet beetle *Anthrenus verbasci*, natural photoperiodic conditions synchronize the endogenous, self-sustained circannual rhythm of the life cycle with the environment [36,37]. The fact that photoperiod acts as an entrainment factor suggests that properties of circannual rhythmicity might also change with latitude, but this remains to be investigated.

The power of photoperiod as an indicator of seasonal change diminishes towards lower latitudes. At more temperate latitudes, different cues can interact with day length and modify the photoperiodic response. Temperature plays an important role as modulating factor in southern populations of the mosquito *Aedes atropalpus* [38] and in the fly *Calliphora vicina* [39], where higher temperatures suppress diapause incidence and shorten the CPP. The larger temperature sensitivity of southern populations allows more flexibility of the photoperiodic response in locations where occasional warm conditions in late summer permit an extension of the reproductive season before winter. Saunders [40] showed that thermoperiod (daily temperature cycles) can alone induce diapause in *N. vitripennis*, but it is not known whether this thermoperiodic response varies across latitudinal gradients.

Photoperiodic induction of diapause sometimes behaves as a threshold accumulation counter, where individuals must experience a specific number of short days to enter diapause [41]. Beach [38] investigated variation in this trait in relation to latitude. Northern strains require fewer short photoperiods for diapause induction than southern populations, as was found in *N. vitripennis* [20]. The variation in required number of short days has similar adaptive significance: faster responses are beneficial in northern locations where seasonal change is abrupt (figure 1; [20]).

#### 4. Latitudinal variation in properties of circadian clocks

Endogenous circadian rhythm characteristics may also associate with latitude, because of their possible role in photoperiodism as well as in the timing of daily behaviours [42]. Such variation might reveal important selection pressures on circadian function. Yet, latitudinal variation in circadian rhythms has not been as extensively studied as photoperiodism.

In plants [43], observed free-running periods in circadian leaf movement rhythms of various species were generally shorter further north. For two dandelion (*Taraxacum*) species, this holds also within the genus. No analyses of latitudinal strains within the same species are known to us apart from a study on leaf movements in *Arabidopsis thaliana* that,

which reports that both the period ( $\tau$ ) in constant conditions and phase angle difference ( $\psi$ ) in a light–dark (LD) cycle are positively associated with latitude [44].

In animals, nearly all latitudinal studies deal with *Drosophila* (figure 3b–d). Allemand & David [23] established a latitudinal cline in daily rhythms of oviposition in 12 L : 12 D in *Drosophila melanogaster* from equatorial Africa (approx. 0° N) to Scandinavia (approx. 62° N). The entrained state however precluded assessment of whether the variation is endogenous or due to masking. Lankinen contributed extensively on circadian pupal eclosion rhythms performed in LD and continuous darkness (DD), in *Drosophila littoralis* [25] from 41.6° N (Caucasus) to 69° N (northern Finland) and in *Drosophila subobscura* [24] from the Canarian Islands (28° N) into Scandinavia (62° N). Two further studies deal with the eclosion rhythm of *Drosophila auraria* from Japan [26,45], but these cover only an 8° latitude range (figure 3c,d).

Latitudinal clines in the amplitude of circadian rhythms are summarized for three fruitfly species (figure 3b). The measures of amplitude vary and cannot directly be compared among species. Allemand & David [23] used peak-to-width ratio of the daily peak (in % h<sup>-1</sup>) of eggs laid. Lankinen [24,25,46] used mean % h<sup>-1</sup> in the peak 5 h of daily eclosion in DD. Within species, there is a steep decline in the circadian amplitude with increasing latitude. Pittendrigh & Takamura [26] have argued that the amplitude of the overt rhythm is not the same as that of the circadian pacemaker. They concluded from the northward decrease in amplitude of the phase response curve that the amplitude of the circadian pacemaker increases towards the north. This would also explain the more rapid damping of the eclosion rhythm—slave to the pacemaker—in DD found further north by Lankinen [24] and Pittendrigh *et al.* [45].

In stable entrainment, the phase angle difference ( $\psi$ , defined as time between rhythm and zeitgeber phase markers) increases (i.e. the rhythm advances) as one goes north in two species [23,24]. The data shown (figure 3c) are for 3 L : 21 D. Pittendrigh & Takamura [26] showed, however, that the latitudinal differences in *D. auraria* vary strongly with photoperiod, and that the variations do not necessarily reflect those of the pacemaker's  $\psi$ , where the latitudinal difference is reversed between 14 L : 10 D and 1 L : 23 D. The period  $\tau$  of the free-running circadian rhythm (figure 3d) decreased from equator to pole in *D. littoralis* and *D. subobscura*, but was not associated with latitude—over a smaller range—in *D. auraria* [26].

It is remarkable that no studies have directly addressed latitudinal variation of circadian organization in vertebrates. Some studies indicate a latitudinal cline in aspects of retinal photoreception, which possibly indicates an adaptation to lower daylight intensities and longer twilight durations at higher latitudes. In fish, the degree of retinal cone contraction is under stronger endogenous control in tropical fish than in polar species [47]. In humans, the frequency of colour deficiency increases from 2 to 10 per cent from the equator to the polar circle [48], a feature that might relate to increasing scotopic light sensitivity [49]. Birds and mammals have been studied at different latitudes to assess the effects of natural zeitgeber properties [3], but not the evolutionary adaptation of their circadian systems to latitude. One study aimed at finding directional selection on circadian clock genes in humans concluded that the genetic variation among world populations was due to genetic drift rather than latitudinal selection [50]. However, another revealed that genetic

variation in the *hPer2* gene appeared to be under directional selection in non-African populations [51].

We feel that the comparative study of circadian systems adapted to different latitudes is in its infancy. The best studies are based on fruitflies (figure 3). There are clear latitudinal trends in amplitude (negatively), phase ( $\psi$ , positively) and free-running period ( $\tau$ , negatively). Opposite trends in  $\psi$  and  $\tau$  are to be expected, as a longer  $\tau$  would lead to a smaller positive or more negative  $\psi$ . We should, however, be aware of the fact that the observed clines refer to a slave oscillator rather than the pacemaker [26]. Indeed, these authors derive some evidence from their data, in spite of the narrow latitude range, that both amplitude and period of the pacemaker may increase towards the north. Pittendrigh *et al.* [45] provided a theory on selection pressure leading to increased amplitude and reduced phase response curve (PRC) amplitude as one goes north. As the entraining photoperiod increases, pacemaker amplitude diminishes. Increased pacemaker amplitude towards the poles would help offset the negative—damping—effect of the longer days of summer. The higher pacemaker amplitude might suppress the amplitude of the slave oscillation, which may be responsible for the reversed latitudinal cline observed in overt rhythms. The suggestion is that proper timing is preserved in arctic regions by such adjustments in the pacemaking system, no matter whether the ‘goal’ is photoperiodic time measurement or daily behavioural tuning. These fascinating propositions can be tested in other species with greater north–south spread than *D. auraria*.

## 5. Latitudinal clines in clock gene polymorphisms

The first clock gene identified, *period* (*per*) in *D. melanogaster*, encodes a variable length tract of Thr-Gly residues in which the two major variants, (*Thr-Gly*)<sub>17</sub> and (*Thr-Gly*)<sub>20</sub> accounted for 90 per cent of the length variation, and were distributed as a latitudinal cline stretching from the Mediterranean to Scandinavia [52]. A similar weaker cline was also detected in Australia, an observation consistent with the view that natural selection was maintaining the polymorphism [53]. A statistical analysis of linkage disequilibria around the Thr-Gly region confirmed that balancing selection was maintaining the European polymorphism [54]. Further phenotypic studies revealed that the two major European variants showed different patterns of temperature compensation that adapted them to warmer or cooler regions, which paralleled each variant’s latitudinal distribution [55]. These studies suggested that the closer the free-running period was to 24 h, the fitter the variant appeared to be, a conclusion that was dramatically demonstrated in competition experiments between pairs of Cyanobacterial clock mutants exposed to different length LD cycles [56].

A second gene, *timeless* (*tim*), encodes the major light-sensitive circadian regulator in *Drosophila* [57,58] and a natural 5’ polymorphism generates two *tim* alleles through the use of alternative start codons [59]. *ls-tim* produces a long and short TIM isoform, whereas *s-tim* generates the shorter isoform only [60]. A latitudinal cline in the frequencies of these allelic variants is observed, so that in the Mediterranean, *ls-tim* is at levels of 80 per cent, but falls to 20 per cent in Scandinavia [60]. *ls-tim* flies have lower circadian light-sensitivity than *s-tim*, and *ls-tim* females enter reproductive

diapause prematurely compared with *s-tim*, even at long day-lengths (diapause is gently photoperiodic in *D. melanogaster*) [60,61]. Indeed, one could envision this *ls-tim* ovarian response to reflect reduced photoperiodic light sensitivity, in that these females appear to misinterpret a long day as a short day, mirroring their circadian response to light.

Both of these *ls-tim* responses thus appear to be adaptive in seasonal environments, particularly in higher latitudes where winter comes earlier when photoperiods are still long. An earlier diapause could be put to good use, and reduced circadian light sensitivity could mitigate the damaging effects on rhythmicity of long summer days. Thus, it was puzzling that *ls-tim* females were found predominantly in the lower latitudes. The solution to this apparent contradiction was revealed when phylogenetic analysis of *tim* sequences revealed that *ls-tim* was relatively new, being, at most, a few thousand years old, and at least, a few hundred years old ([60], V. Zonato and C. P. Kyriacou 2013, unpublished data). Subsequent spatial analyses incorporating more southern European populations revealed that *ls-tim* population frequencies accurately reflected their distance from southern Italy, suggesting the mutation originated from this region [60]. This was confirmed by neutrality tests that revealed directional, not balancing selection acting on *ls-tim*. Thus, *ls-tim* frequencies have increased throughout Europe because of the seasonal nature of the photoperiodic environment, something that is absent in the ancestral homelands of sub-Saharan Africa, where *D. melanogaster* evolved before migrating to Europe 14 000 years ago.

A higher amplitude oscillator to diminish the effects of light at high latitudes has been proposed by Pittendrigh *et al.* [45]. An alternative mechanism may be to simply diminish the input of light to the clock. In flies, this appears to be resolved by generating a less light-sensitive TIM molecule because the amplitude of the *tim* mRNA rhythm, which may be a reflection of the oscillator, does not differ between the two *tim* alleles [61]. Secondly, the *tim* studies raise the possibility that in insects, circadian clock molecules are also involved in photoperiodism, first suggested by Bünning [62] and confirmed at the molecular level in plants [63]. However, the different isoforms of *tim* in *D. melanogaster* affect the levels of diapause, but *not* the photoperiodic slope of diapause induction. In a number of other insect species, the pitcher plant mosquito *W. smithii* [64], *Chymomyza costata* [65], *Drosophila triauraria* [66], the flesh fly *Sarcophaga bullata* [67,68] and the bean bug *Riptortus pedestris* [69–71], clock genes have been directly or indirectly implicated in diapause. This relationship between circadian clock genes and diapause could indicate direct involvement of the circadian clock with diapause, but does not exclude pleiotropic effects of these genes on the diapause mechanism. Taken together, the evidence seems to indicate an involvement of the circadian system in diapause induction, but the mechanisms seem to vary considerably between species [72,73].

While these latter studies do not include latitudinal perspectives, some recent studies have suggested that other non-clock genes may contribute to diapause and are spatially patterned along the latitudinal axis. For example, molecular variation in the *couch-potato* (*cpo*) gene in *D. melanogaster* covaries with diapause levels and shows clinal variation along the East Coast of the USA [74]. In Australia, a similar situation occurs, but the clinal frequencies of the relevant *cpo* variant is driven by strong linkage disequilibrium with the nearby *Payne* inversion, which varies significantly with

latitude on this continent [74]. A cline for variation in the gene encoding phosphatidylinositol-3-kinase (*Dp110*), which is involved in insulin signalling, has also been reported in the USA and Canada [75], as has a cline observed for variation in the insulin receptor gene, *InR*, in both America and Australia [76]. While variation in *Dp110* appears to change diapause levels [75], the natural variation in *InR* has not been studied in terms of its effects on the phenotype. However, *InR* mutant females do have immature ovaries [77]. Consequently, it appears that the insulin pathway, which is important for lifespan [78], may provide a substrate for natural selection along a latitudinal cline that influences life-extending seasonal phenotypes such as diapause [79]. How clock genes interact, genetically and neuroanatomically, with the insulin pathway to generate the overwintering response remains to be established.

In vertebrates, progress at the molecular level has been slower than in *Drosophila*. Clock gene polymorphisms in birds have first been described by Fidler & Gwinner [80] in an effort to identify genetic differences between diurnal and nocturnal species. They examined sequences of three components of the core circadian oscillator: *BMAL1*, *CLOCK*, and *PER2* and found the *Clock* poly-glutamine (*Clock*-polyQ) region to be polymorphic within- and among-species. [80]. Populations of both the non-migratory Blue Tit *Cyanistes caeruleus* and the migratory Bluethroat *Luscinia svecica* showed considerable polymorphism in *Clock*-polyQ (nine and seven *Clock*-polyQ alleles, respectively) and a positive association between *Clock*-polyQ allele length and breeding latitude (30° range) was found in the blue tit [81]. Corresponding analyses for Bluethroat populations failed to show such a pattern between *Clock*-polyQ and breeding latitude [81]. Also the swallow genus *Tachycineta* that breeds across North, South and Central America showed no evidence for an association between *Clock*-polyQ allele size and breeding latitude across populations from five species [82]. This parallels the lack of latitude *Clock*-polyQ association in the barn swallow *Hirundo rustica* [83]. Similarly, studies in Pacific salmon species (genus *Oncorhynchus*) that overlap in their geographical breeding also generated similar inconsistent patterns of latitudinal clines in *Clock*-polyQ frequency [84,85]. Thus, the association between breeding latitude and *Clock*-polyQ length polymorphism is certainly not a general pattern in vertebrates.

## 6. Discussion

Variation in timing is typically measured in experiments where different strains are exposed to the same controlled environmental conditions. The observed phenotypic differences are based on genetic differences likely to result from selection caused by environmental conditions along latitudinal gradients. Taken together, there is evidence that predicted latitudinal clines indeed exist in circadian and photoperiodic properties. For photoperiodic responses this relationship can be well understood. All insect species investigated show increasing CPP for winter diapause induction in more northern populations (figure 3a), confirming the pioneering work of Danilevskii [11]. This trend is fully consistent with the theoretical prediction derivable from adaptive day length responses (figure 2a), where low autumn temperatures occur earlier in the year and at longer photoperiods when moving away from the equator (figure 2a). Although these clines have

almost exclusively been studied in insects, there is no reason why they could not also exist in other organisms. The molecular machinery for photoperiodism in mammals has been well described in laboratory studies [86–88], and it seems timely to take this knowledge to the field [89] where evidence for natural selection acting upon this mechanism can be found by using latitudinal cline approaches.

Few studies address latitudinal variation in circadian properties. The available data in *Drosophila* suggest three general patterns in latitudinally distributed species: circadian rhythm amplitude of overt rhythms in standard artificial LD cycles decreases in more polar populations (figure 3b), while phase angle difference increases (figure 3c) and, correspondingly, free-running period decreases (figure 3d). The amplitude decrease at higher latitudes might be related to more extreme daily light and temperature cycles. The increase in phase angle difference (i.e. advanced phase in entrainment) may reflect the cline in period (figure 3c,d). We are further aware of the fact that rhythm amplitude does not necessarily reflect pacemaker amplitude, which has been suggested to increase towards the north, as a way to offset the suppressing effect of long photoperiods [26].

A first evolutionary hypothesis is that these latitudinal clines have evolved primarily to support photoperiodic time measurement at different latitudes [90]. Circadian systems are assumed to be involved in photoperiodic time measurement [62,91] and such involvement has been demonstrated in many taxa, including birds, mammals, insects, etc. [1], although there are exceptions [92]. Even if a circadian system is involved, it does not necessarily mean that variation between populations in CPP is reflected in variation in circadian parameters, as shown clearly both in phenotypic analyses in the pitcher plant mosquito [14] and in crossing studies in *D. littoralis* [46]. Describing geographical, genetic or phenotypic correlations may not clarify the precise mechanism of circadian involvement in photoperiodism, if it exists. The classical debate between *external coincidence timing* (single circadian oscillator interacting with the LD cycle) and *internal coincidence timing* (two interacting circadian oscillators) models for circadian involvement has indeed not been settled. Hut & Beersma [2] suggested that predictions for latitudinal clines in circadian period are radically different when assuming an endogenous representation of day length generated by either external or internal coincidence. External coincidence would produce a monotone cline potentially crossing 24 h. Internal coincidence timing would produce a cline that deviates more from 24 h towards the poles, but does not cross 24 h towards the equator. So far, no cline in circadian period was reliably found to cross 24 h, but the cline in *D. subobscura* approaches 24 h at higher latitudes favouring an external coincidence timing model in *Drosophila* (figure 3d).

Recent molecular studies on mammalian photoperiodism [86–89] have clearly shown an involvement of the circadian system at the level of melatonin regulation and at the hypothalamic induction pathway. This pathway is well conserved across various species studied. Mammals therefore represent an excellent (and virtually unexploited) system to study selection pressures on photoperiodism and circadian organization by using latitudinal clines.

A second, very general, hypothesis is that the systematically shorter circadian periods in *Drosophila* at higher latitudes are required to enable circadian entrainment under long

summer photoperiods. This can be theoretically understood from phase response curves where very long light pulses only generate phase delays, as was demonstrated in mice [93]. This phenomenon would nearly preclude long photoperiod entrainment unless the endogenous circadian period in DD is shorter than 24 h. The long summer photoperiods in the north may select for shorter endogenous periods than the more modest photoperiod lengths in temperate regions. Assessments of clines in other species, specifically small mammals, would be needed to test the generality of this prediction.

Additional selection pressure may be formed by effects of latitude on the phase of circadian entrainment. Twilight duration can be expected to affect phase of circadian entrainment [94] either through zeitgeber strength [94–96], or through species-specific effects of twilight on circadian period under short photoperiods [97,98].

Examination of latitudinal clines in clock gene polymorphisms in free-living vertebrate populations is in its early stages, and does not allow general conclusions. These molecular studies have focused solely on *Clock* gene polymorphisms, without yielding convincing general patterns. Compensatory mechanisms in the circadian molecular network can readily explain why focusing on a single gene may not be a fruitful approach [99]. The types of neutrality tests that also detect the signatures of natural selection cannot generally be performed on repeated sequences like *Clock*-polyQ, so alternative methods need to be used [54,100]. Consequently, any connection between clock gene variation, latitude, phenotypes and selection in vertebrates so far remains speculative. Further studies examining multiple

gene polymorphisms in more species will be essential to get to grips with the molecular basis of latitudinal clines and their implications for selection. However, the study by Cruciani *et al.* [51] mentioned earlier is particularly intriguing because these authors observed that in non-African human populations *hPER2* showed evidence for positive selection. *hPER2*, like *TIM* in *Drosophila*, is important for circadian light resetting [101]. As humans and flies both emerged out of sub-Saharan Africa, could it be that they were challenged with changing photoperiods at higher latitudes, which led to natural selection on the clock genes underlying circadian photosensitivity?

Despite these caveats, we conclude that establishing latitudinal variation is a powerful tool to study mechanisms underlying circadian organization and photoperiodic responses and to reveal selective forces responsible for daily and seasonal adaptation [2,102]. Further exploitation of latitudinal clines will lead to novel insights into both the mechanism and evolutionary significance of daily and annual biological rhythms. To understand evolution and adaptive significance of daily and annual timing systems, direct selection on circadian entrainment should be disentangled from selection through circadian involvement in photoperiodism. This task asks for studies that combine available knowledge of the underlying molecular mechanisms in daily and annual timing with detailed phenotypic and genotypic description of populations at different latitudes and altitudes.

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