SPECIAL ISSUE REVIEW

WILEY   EJN European Journal of Neuroscience   FENS

Flies as models for circadian clock adaptation to environmental challenges

Charlotte Helfrich-Förster [ID] | **Enrico Bertolini** [ID] | **Pamela Menegazzi**

Neurobiology and Genetics, Theodor-Boveri Institute, Biocentre, University of Würzburg, Würzburg, Germany

Correspondence
Charlotte Helfrich-Förster, Neurobiology and Genetics, Theodor-Boveri Institute, Biocentre, University of Würzburg, Würzburg, Germany.
Email: charlotte.foerster@biozentrum.uni-wuerzburg.de

Abstract

Life on earth is assumed to have developed in tropical regions that are characterized by regular 24 hr cycles in irradiance and temperature that remain the same throughout the seasons. All organisms developed circadian clocks that predict these environmental cycles and prepare the organisms in advance for them. A central question in chronobiology is how endogenous clocks changed in order to anticipate very different cyclical environmental conditions such as extremely short and long photoperiods existing close to the poles. Flies of the family Drosophilidae can be found all over the world—from the tropics to subarctic regions—making them unprecedented models for studying the evolutionary processes that underlie the adaptation of circadian clocks to different latitudes. This review summarizes our current understanding of these processes. We discuss evolutionary changes in the clock genes and in the clock network in the brain of different Drosophilids that may have caused behavioural adaptations to high latitudes.

KEYWORDS
circadian rhythm, cryptochrome, latitudinal adaptations, locomotor activity, pigment-dispersing factor

1 | INTRODUCTION

Circadian clocks enable animals to anticipate the regular daily changes in the environment and to time specific activities to specific times of the day (Daan, 2010). On a different timescale, circannual clocks evolved to anticipate yearly environmental changes that are most evident in the north and south of our planet (Helm & Lincoln, 2017). The circannual and circadian clocks may be linked/interlocked, as circadian clocks can provide the necessary time reference for measuring day length and prepare for the forthcoming winter or summer (Bünning, 1960; Denlinger, Hahn, Merlin, Holzapfel, & Bradshaw, 2017; Goldman, 2001; Pittendrigh & Minis, 1964; Saunders, 2013). Seasonal changes do not only induce photoperiodic responses such as overwintering or reproduction, they have also a strong impact on animal's daily activity pattern. This is most evident in small-sized animals, which are more subjected to rapid heat and energy loss than larger animals (Daan & Aschoff, 1975; Halle & Stemseth, 2000; Hoogenboom, Daan, Dallinga, & Schoenmakers, 1984; Ikeda et al., 2016).

Abbreviations: 5th LN$_v$, fifth ventrolateral neuron; Ca, calcium; *Clk*, *Clock* gene; CLK, Clock protein; CRY, Cryptochrome; CYC, CYC protein; *cyc*, *cycle* gene; DN, dorsal neurons; l-LN$_v$, large ventrolateral neurons; LN, lateral neurons; LN$_d$, dorsolateral neurons; *ls-tim*, long-short splice form of *timeless*; PDF, Pigment-Dispersing Factor; *Pdp1ε*, gene coding for the *PAR Domain Protein 1ε*; PDP1ε, PAR Domain Protein 1ε; *per*, *period* gene; PER, Period protein; POC, posterior optic commissure; SCN, suprachiasmatic nucleus; s-LN$_v$, small ventrolateral neurons; *s-tim*, short splice form of *timeless*; Thr-Gly, threonine-glycine; *tim*, *timeless* gene; TIM, Timeless protein; *vri*, *vrille* gene.

Edited by Rae Silver. Reviewed by Amita Sehgal.

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Insects highly depend on ambient temperature. On cold days, it is favourable for them to be active during the warmer parts of the day that occur during the photophase. In contrast, on hot days it is favourable for them to shift activity to the morning, the late evening, or even the night to avoid overheating (e.g., Fowler & Robinson, 1979). At least two mechanisms might help animals to be active at the best time of the day: (a) the quick response to acute ambient environment and (b) their circadian clocks. Here, we will refer to the latter.

For several small vertebrates it was observed that the clock has different waveforms under different day lengths (Aschoff, 1966; Daan & Aschoff, 1975). Birds, fishes, and several mammals show two activity bouts, one in the morning (M) and one in the evening (E). M and E activity bouts are close together under short days and separated by a pronounced siesta under long days. This pattern persists under subsequent constant conditions highlighting its endogenous nature. In hamsters, the bimodal activity is reflected by a bimodal rhythm of electrical firing in cultured slices of the brain master clock—the suprachiasmatic nuclei (SCN) (Jagota, de la Iglesia, & Schwartz, 2000). The two maxima in electrical firing are close together when the animals have previously been kept under a short photoperiod and far apart, when they have been kept under a long photoperiod (Jagota et al., 2000). Among insects, M and E activity bouts have been reported in fruit and house flies (*Drosophila melanogaster* and *Musca domestica;* Bazalova & Dolezel, 2017; Hamblen-Coyle, Wheeler, Rutila, Rosbash, & Hall, 1992; Helfrich-Förster, 2000; Prabhakaran & Sheeba, 2012, 2013; Wheeler, Hamblen-Coyle, Dushay, & Hall, 1993) as well as in some mosquitoes (Chiba, 1971; Chiba, Kubota, & Nakamura, 1982). Although, in fruit flies, M and E activity bouts usually merge to one major peak under constant conditions, the M bout remains visible as a shoulder on the E bout in most individuals and in some even as separate activity bout (Helfrich-Förster, 2000). Therefore, similar to vertebrates both activity bouts appear of endogenous nature. Comparable to vertebrates, the two fly activity bouts are closer together during cold and/or short days and further apart under hot and/or long days (Bazalova & Dolezel, 2017; Chiba, 1971; Majercak, Sidote, Hardin, & Edery, 1999; Rieger, Stanewsky, & Helfrich-Förster, 2003; Rieger, Peschel, Dusik, Glotz, & Helfrich-Förster, 2012). Thus, the adaptation of the daily activity patterns to different environmental conditions can be easily observed in flies, although individual animals are relatively short-lived and do usually not experience several seasons (only the individuals that overwinter experience autumn, winter, and spring). The great advantage of studying flies is that the molecular and neuronal mechanisms of the circadian clock are well-understood in *D. melanogaster* (see King & Sehgal, this issue) and start to emerge also in other Diptera (Bazalova & Dolezel, 2017; Bertolini et al., 2018; Codd et al., 2007; Gentile, Rivas, Meireles-Filho, Lima, & Peixoto, 2009; Gesto et al., 2015; Kaiser et al., 2016; Kyriacou, 2014; Meireles-Filho & Kyriacou, 2013; Meuti, Stone, Ikeno, & Denlinger, 2015; Noreen, Pegoraro, Nouroz, Tauber, & Kyriacou, 2018; Rivas et al., 2018; Rund, Hou, Ward, Collins, & Duffield, 2011). In *D. melanogaster*, recent studies have shown that M and E activity bouts are reflected by Ca^{2+} rhythms in the relevant clock neurons controlling M and E activity, respectively (Liang, Holy, & Taghert, 2016, 2017). As shown for the electrical activity in the SCN of hamsters, the Ca^{2+} peaks in the M and E neurons are close together in flies that have been kept under short-day conditions and far apart in flies that have been kept under long-day conditions (Liang et al., 2016). Thus, the different activity patterns of flies in short and long photoperiods have a neuronal basis (reviewed in Helfrich-Förster, 2017; Yoshii, Rieger, & Helfrich-Förster, 2012).

In this review, we focus on the circadian clocks of insects within the family Drosophilidae, which is part of the order Diptera (flies and mosquitoes; Figure 1a); they alone comprise many species that colonized almost all parts of the world. Currently, about 4,000 species are described (Brake & Bächli, 2008; O'Grady & DeSalle, 2018) but a number of surveys have projected the eventual number of Drosophilidae species to 5,200 (summarized in O'Grady & DeSalle, 2018). This is a huge number of species in comparison to the overall 6,399 known mammals (Burgin, Colella, Kahn, & Upham, 2018). Drosophilidae are thought to have originated in the tropics (as assumed for mammals) about 50 million years ago and have subsequently colonized virtually all environments including subarctic regions (Markow & O'Grady, 2007; Obbard et al., 2012; O'Grady & DeSalle, 2018; Russo, Mello, Frazão, & Voloch, 2013; Throckmorton, 1975). These elements make the Drosophilidae family an unprecedented model for studying the evolutionary processes that underlie the adaptation of circadian clocks to different environments. Here, we will review our current understanding of these processes.

2 | PHYLOGENY OF FLIES (DIPTERA) WITH A SPECIAL FOCUS ON THE DROSOPHILIDAE

The flies (Diptera) are historically divided into the Nematocera (lower Diptera, mosquitoes + others) and Brachycera (higher Diptera, "real" flies), but the Nematocera classification is controversial and this suborder was recently identified as paraphyletic to the Brachycera (Amorim & Yeates, 2006; Beckenbach, 2012). Here, we mention only few families of which some members have been studied in respect of rhythmic behaviour and/or clock genes (Figure 1a). Among these are mosquitoes (Culicidae) (Chiba, 1971; Chiba et al., 1982; Gentile et al., 2009; Meuti et al., 2015) and sand

(a)

(b)
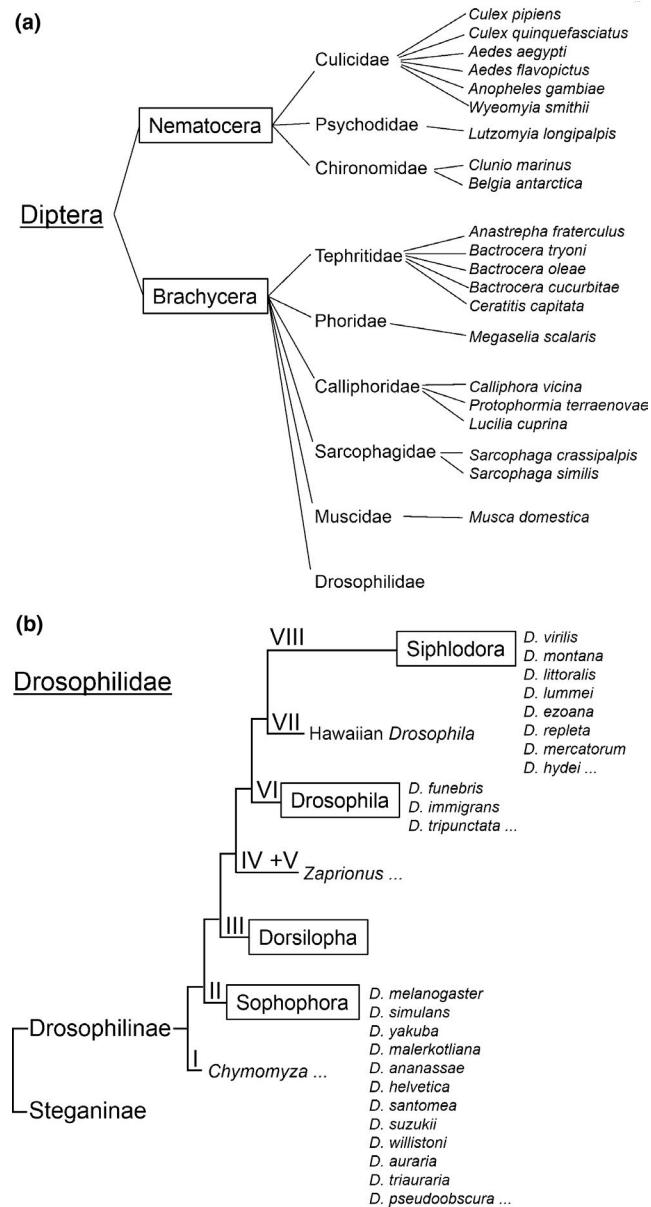
FIGURE 1 Rough classification of Diptera with a focus on the phylogeny of the family Drosophilidae. (a) The Diptera are historically divided in the Nematocera and Brachycera. The Nematocera contain ~34 and the Brachycera ~110 families. Here, only the families are shown, in which some species have been investigated in respect of their rhythms and/or clock genes. (b) Phylogeny of the family Drosophilidae (after Yassin, 2013 and O'Grady & DeSalle, 2018). They are divided into two sister groups, the Drosophilininae and Steganinae. The subfamily Drosophilinae is divided in eight clades (I–VIII) of which always the groups that are on the same "vertical" level are sister groups (e.g., *Chymomyza* is the sister group of the remaining Drosophilinae (clades II–VIII) and the group of Sophophora can be regarded as the sister group of clade III–VIII, etc.). As in a, emphasis is laid on those species that have been investigated from the chronobiological point of view. These stem predominantly from the Subgenera Sophophora and Siphlodora and are listed on the right. Furthermore, rhythms of the species from the genus Chymomyza (clade I) and Zaprionus (clade IV + V) have been studied. So far none of the species belonging to the subgenus Drosophila has been investigated for rhythms. The three species listed here are the key species of this group. Details see text

flies (Psychodidae) (Meireles-Filho, Amoretty, De Souza, Kyriacou, & Peixoto, 2006; Meireles-Filho, da S. Rivas, et al., 2006). Mosquitoes and sand flies are very interesting because most of them are blood-sucking and potential disease vectors (Meireles-Filho & Kyriacou, 2013). Furthermore, they have undergone rapid speciation colonizing very different habitats all over the world. Their success may be partly due to rapidly evolving clock genes that allowed them to colonize different temporal niches (Kyriacou, 2014). Particularly interesting is also the pitcher plant mosquito, *Wyeomyia smithii*, which has undergone an evolutionary transition from blood feeding to obligate nonbiting and at the same time an expansion from the south to the north of North America (Bradshaw et al., 2018; Merz et al., 2013). This species has become a model species to investigate the genetic mechanisms underlying photoperiodic time measurement (Bradshaw, Emerson, Catchen, Cresko, & Holzapfel, 2012). In the family of Chironomidae, *Clunio marinus* and *Belgica antarctica,* both living in intriguing environments, are studied for their endogenous clocks. *Clunio marinus*, is the only fly that colonized sea water and it possesses circadian and circatidal clocks (Neumann, 1989; Kaiser et al., 2016). *Belgica Antarctica* the only insect endemic to Antarctica (Kobelková et al., 2015).

Thus far, the neuronal basis of the circadian clock has not been revealed in any of the "lower Diptera" species.

The Brachycera comprise about 120 families. Species of the following families have been investigated with respect to their rhythms: Tephritidae (An et al., 2002; An, Tebo, Song, Frommer, & Raphael, 2004; Bertolini et al., 2018; Chahad-Ehlers et al., 2017; Fuchikawa et al., 2010; Matsumoto et al., 2008; Mazzotta et al., 2005; Miyatake et al., 2002), Phoridae (Bostock, Green, Kyriacou, & Vanin, 2017), Calliphoridae (Muguruma, Goto, Numata, & Shiga, 2010; Saunders, 1997; Smith, 1987; Shiga & Numata, 2009; Warman, Newcomb, Lewis, & Evans, 2000; Yasuyama, Hase, & Shiga, 2015), Sarcophagidae (Goto & Denlinger, 2002; Koštál, Závodská, & Denlinger, 2009; Yamamoto, Nishimura, & Shiga, 2017; Yamamoto, Shiga, & Goto, 2017), Muscidae (Codd et al., 2007; Bazalova & Dolezel, 2017; Pyza & Meinertzhagen, 2003; Pyza, Siuta, & Tanimura, 2003), and Drosophilidae (see below) (Figure 1a). Among these, the genetic and neuronal basis of the circadian clock was revealed for the house fly *M. domestica* (Codd et al., 2007), the blow fly *Protophormia terraenovae* (Muguruma et al., 2010) and recently also for the olive fly, *Bactrocera oleae* (Bertolini et al., 2018). In all three fly species, it turned out to be rather similar to that of the fruit fly *D. melanogaster*, although the families of Tephritidae, Muscidae, Calliphoridae, and Drosophilidae have separated millions of years ago.

The phylogeny of the Drosophilidae is complex and still not completely resolved. A recent elaborate review can be found in O'Grady and DeSalle (2018). Most researchers agree to the idea that the Drosophilidae can be

divided into two sister subfamilies, the Steganinae and the Drosophilinae (discussed in O'Grady & DeSalle, 2018) (for the definition of sister groups, see Figure 1b). Since all of the species investigated for their endogenous clocks are within the Drosophilinae, we will only discuss this subfamily (Figure 1b). Yassin (2013) divided the Drosophilinae into eight major clades (see Figure 1b). Clade I can be regarded as sister clade of the other seven clades (II–VII; which cover the main *Drosophila* groups) and contains the genus *Chymomyza* (which is investigated from the chronobiological point of view (e.g., Koštál, 2011a,b; Stehlík, Závodská, Shimada, Sauman, & Kostál, 2008)) and several other genera that are not important for this review. Within the main *Drosophila* groups, clade II is again the sister group of the remaining six clades. Clade II coincides with the subgenus Sophophora and contains the "model" species *D. melanogaster* and other species that have been investigated from the chronobiological point of view such as *D. suzukii, D. yakuba, D. simulans, D. pseudoobscura, D. helvetica, D. ananassae, D. willistoni, D. auraria,* and *D. triauraria* (Gleason & Powell, 1997; Hamby, Kwok, Zalom, & Chiu, 2013; Hermann et al., 2013; Joshi, 1999; Low, Lim, Ko, & Edery, 2008; Nishinokubi et al., 2003; Nishinokubi, Shimoda, & Ishida, 2006; Ousley et al., 1998; Pittendrigh, 1967; Pittendrigh & Takamura, 1989; Prabhakaran, De, & Sheeba, 2013; Prabhakaran & Sheeba, 2012, 2013, 2014; Vanlalhriatpuia et al., 2007; Wheeler et al., 1991; Yamada & Yamamoto, 2011). Important for the present review is that the subgenus *Sophophora* has separated from the remaining groups (clades III–VIII), among which is the *Drosophila* subgenus (clade VI), about 30 million years ago. Clade III that contains the subgenus Dorsilopha is again the sister clade of the remaining clades (clades IV–VIII). Clades IV and V are heterogenous and the lineages within this groups are still under debate (O'Grady & DeSalle, 2018; Russo et al., 2013; Yassin, 2013). Therefore, we will treat them as one group (Figure 1b). This group includes the tropical genus *Zaprionus* that has been investigated from the chronobiological point of view (Beauchamp et al., 2018; Prabhakaran & Sheeba, 2013, 2014). The subgenus Drosophila (clade VI) has been redefined recently by Yassin (2013) and corresponds roughly to the immigrans-tripunctata radiation of Throckmorton (1975). Whereas the immigrans radiation consists of New World species, the tripunctata radiation contains Old World species. None of these species has been studied so far for their rhythmic behaviour. The last two clades are the Hawaiian *Drosophila* species and the subgenus Siphlodora. The subgenus Siphlodora was also recently redefined (Yassin, 2013) and corresponds roughly to the repleta-virilis radiation of Throckmorton (1975). Several species within the virilis radiation have colonized high-latitudes and were investigated for their rhythmic behaviour

and neuronal clock network (Bahn, Lee, & Park, 2009; Beauchamp et al., 2018; Hermann et al., 2013; Kauranen et al., 2012; Lankinen, 1986; Lankinen & Forsman, 2006; Menegazzi et al., 2017).

3 | THE MOLECULAR BASIS OF THE CIRCADIAN CLOCK IN *D. MELANOGASTER* AND ITS ADAPTATION TO DIFFERENT ENVIRONMENTAL CONDITIONS

In 2017, the Nobel Prize in Physiology/Medicine was awarded to Jeffrey Hall, Michael Young, and Michael Rosbash for their work that led to the understanding of the molecular basis of circadian rhythms in *D. melanogaster* (Callaway & Ledford, 2017). This work was initiated by Konopka and Benzer in the 1970s of the last century by the isolation of the *period* mutants (Konopka & Benzer, 1971). The *period* gene participates in interlocked molecular transcriptional/translational feedback loops (Hardin, 2011; Glossop, Lyons, & Hardin, 1999).

A first feedback loop involves the clock genes *period* (*per*), *timeless* (*tim*), *cycle* (*cyc*), and *Clock* (*Clk*), and their respective products. CLK and CYC form heterodimers and bind to E-box regulatory elements in the promoters of *per* and *tim*, activating their transcription. Consequently, *per* and *tim* mRNA levels rise and are translated in the cytoplasm, where their products PER and TIM are subjected to posttranslational modification, dimerize, and after a while enter the nucleus as a complex. In the nucleus, PER/TIM complexes bind to CLK/CYC and repress their transcriptional activity. Doing so, they negatively regulate their own expression. Subsequent PER and TIM destabilization and degradation stops the repression on CLK/CYC activity, and a new transcriptional-translational cycle restarts.

A second feedback loop involves the clock genes, *cycle* (*cyc*), *Clock* (*Clk*), *Vrille* (*Vri*), and *PAR Domain Protein 1ε* (*Pdp1ε*), and their respective products. *Vri* and *Pdp1ε* carry E-box regulatory elements in their promoters, therefore their expression is also activated by the active CLK/CYC complex. VRI accumulates earlier than PDP1ε and it represses the expression of *Clk*, acting at the level of VP-boxes (VRI/PDP regulatory elements (Emery & Reppert, 2004) present in its promoter region. PDP1ε accumulates later than VRI and finally promotes *Clk* expression. The synergistic activity of VRI and PDP1ε generates circadian transcription of *Clk*. Several additional clock factors, that are not focus of this review, fine-tune the transcriptional/translational feedback loops (for deeper insights see Glossop et al., 1999; Hardin, 2011; Hardin & Panda, 2013; Helfrich-Förster, 2017; Ozkaya & Rosato, 2012).

Environmental light-dark cycles synchronize the molecular oscillations via Rhodopsins and Cryptochrome (CRY) (reviewed in Helfrich-Förster, 2017; Yoshii, Hermann-Luibl,

& Helfrich-Förster, 2016). CRY makes the *Drosophila* extraordinary light sensitive (Vinayak et al., 2013), because it interacts directly with the core clock proteins: light-activated CRY binds to TIM and leads to its degradation in the proteasome (Ceriani et al., 1999; Naidoo, Song, Hunter-Ensor, & Sehgal, 1999). Without TIM, PER is also destabilized and degraded, which immediately resets the clock. When flies are exposed to constant light instead of light-dark cycles, TIM is permanently degraded, which makes the flies arrhythmic, whereas they remain rhythmic without functional CRY (Emery, So, Kaneko, Hall, & Rosbash, 1998; Emery, Stanewsky, Hall, & Rosbash, 2000a,b; Stanewsky et al., 1998). Due to the high light sensitivity of the flies, arrhythmicity of wild-type flies occurs already at rather low irradiances (Konopka, Pittendrigh, & Orr, 1989) and flies with CRY overexpressed show even a higher sensitivity towards light (Emery et al., 1998).

3.1 | Polymorphisms in TIM and PER affect clock sensitivity to light and temperature

The original *tim* gene gives rise to a short form of TIM (= S-TIM) that strongly interacts with CRY and is therefore sensitive to degradation by light. However, ~10,000 years ago, after *D. melanogaster* colonized Europe, a mutation occurred in the south eastern Italian populations, in which a single guanosine was inserted in the 5′ coding region of *tim* that resulted in the production of a long TIM isoform in addition to a short one (= LS-TIM). The *ls-tim* mutants are less light sensitive due to a reduced ability of LS-TIM to interact with CRY, and the *ls-tim* mutants gradually invaded Northern Europe (Sandrelli et al., 2007; Tauber et al., 2007). The lower light sensitivity of the flies prevents the flies from enhanced TIM degradation and from getting arrhythmic during the long exposure to light in northern summers. On the other hand, the *ls-tim* mutation causes females to enter diapause (reproductive arrest) earlier in autumn than the *s-tim* allele. Together with the reduced light-sensitivity of *ls-tim* flies, the earlier diapause induction is advantageous for a life in the north. Thus, the observed latitudinal cline in TIM polymorphism appears to be an evolutionary adaptation to the conditions at higher latitudes (Kyriacou, Peixoto, Sandrelli, Costa, & Tauber, 2008). Polymorphisms appear also present in CRY, but a clear latitudinal cline was so far not observed (Pegoraro et al., 2015).

Nevertheless, a latitudinal cline in PER polymorphism exists, but in contrast with the TIM polymorphism, the PER polymorphism does not affect light-sensitivity but has a role in temperature adaptation (reviewed in Costa & Kyriacou, 1998; Kyriacou et al., 2008). PER possesses an uninterrupted stretch of alternating threonine-glycine (Thr-Gly) repeats at its C-terminus (Yu, Colot, Kyriacou, Hall, & Rosbash, 1987). This unusual sequence is shorter in flies stemming from southern *D. melanogaster* populations and longer in flies stemming from northern populations in Europe. This latitudinal cline in the Thr-Gly length turned out to be important for temperature compensation of the clock. The northern populations with long Thr-Gly stretch had the most thermally stable periods that were close to 24 hr at all temperatures tested. In contrast, southern populations, with short Thr-Gly stretch, had free-running periods of 24 hr at 29°C, but the periods shortened significantly at cooler temperatures. Thus, the "temperature compensated" PER protein with long Thr-Gly stretch appears better adapted to the colder and thermally variable higher latitudes, whereas the PER protein with short Thr-Gly stretch is more suitable for the warmer Mediterranean region. However, this latitudinal cline of the PER Thr-Gly stretch was not observed in *D. melanogaster* populations collected along the coast of Australia, suggesting that there exist other adaptations to latitudinal clines in temperature (Weeks, McKechnie, & Hoffmann, 2006).

Nevertheless, the polymorphisms in PER and TIM offer a fine-tuning of daily rhythms to the environment and can be regarded as evolutionary adaptations of the clock to a life in warmer or colder regions.

3.2 | Differential splicing of the clock genes contributes to the adaptation to different temperatures

In addition to polymorphisms in the clock genes, *D. melanogaster* flies can adapt their activity pattern to changes in the environment by splicing *per* and *tim* differently. The mRNAs of both genes are differentially spliced in their 3′ untranslated regions, and in both cases the degree of splicing is dependent on the environmental temperature. *Per* shows an enhanced splicing at low and an attenuated splicing at higher environmental temperatures and long days (Majercak et al., 1999; Majercak, Chen, & Edery, 2004). For *tim* it is the opposite: it is spliced to a larger degree at high temperatures than at low ones (Montelli et al., 2015). Enhanced *per* splicing at low temperatures leads to a quicker accumulation of *per* mRNA and PER protein, which accelerates the molecular cycle and advances the evening activity of the flies, whereas reduced *per*-splicing at high temperatures slows down the molecular cycle and delays the evening activity. This explains activity timing of the flies under cold and short autumn days and long and hot summer days (Majercak et al., 2004; Montelli et al., 2015): under autumn condition the flies start evening activity early in the day and lack the midday siesta almost completely, whereas under summer conditions they have a pronounced siesta and start evening activity late. Most interestingly, differential *tim* splicing enhances these seasonal adaptations. The unspliced TIM isoforms at low temperatures have a higher affinity for CRY what leads to an

earlier degradation of TIM and an advance of evening activity under cold days (Montelli et al., 2015). Under warm summer days the spliced TIM isoforms have less affinity to CRY, TIM is degraded later and evening activity is also later. Thus, *per-* and *tim-*splicing work in the same direction—advancing evening activity under short winter days and delaying it under long summer days.

The thermal regulation of *per-*splicing can be regarded as a mechanism that facilitated the radiation of *D. melanogaster* from the tropics to temperate climates (Figure 2a). Consistent with this hypothesis, Low et al. (2008), Low, Chen, Yildirim, and Edery (2012) discovered several single nucleotide polymorphisms (SNPs) in *per's* 3′ untranslated region of *D. melanogaster* that modulate its splicing efficiency. Most significantly, there was a latitudinal cline in these SNPs in wild-caught populations of flies originating along the east coast of the United States with the least efficiently spliced versions associated with a longer midday siesta in regions where temperatures can reach high levels. Similarly, an altitudinal cline in *per* splicing was associated in *D. melanogaster* that influences midday siesta (Cao & Edery, 2017). This demonstrates that natural selection can work at the level of splicing signals and that differential splicing plays an important role in the thermal adaptation of life forms. Without doubt there are selection mechanisms that are independent of the *per* gene that adapted the rhythms of *D. melanogaster* flies to different temperatures as was shown by Maguire, Schmidt, and Sehgal (2014) for natural populations living at different altitudes in Africa. Nonetheless, we will continue to focus our review on the clock genes and latitudinal adaptations.

4 | EVOLUTIONARY ADAPTATIONS OF THE MOLECULAR CLOCK IN SPECIES OTHER THAN *D. MELANOGASTER*
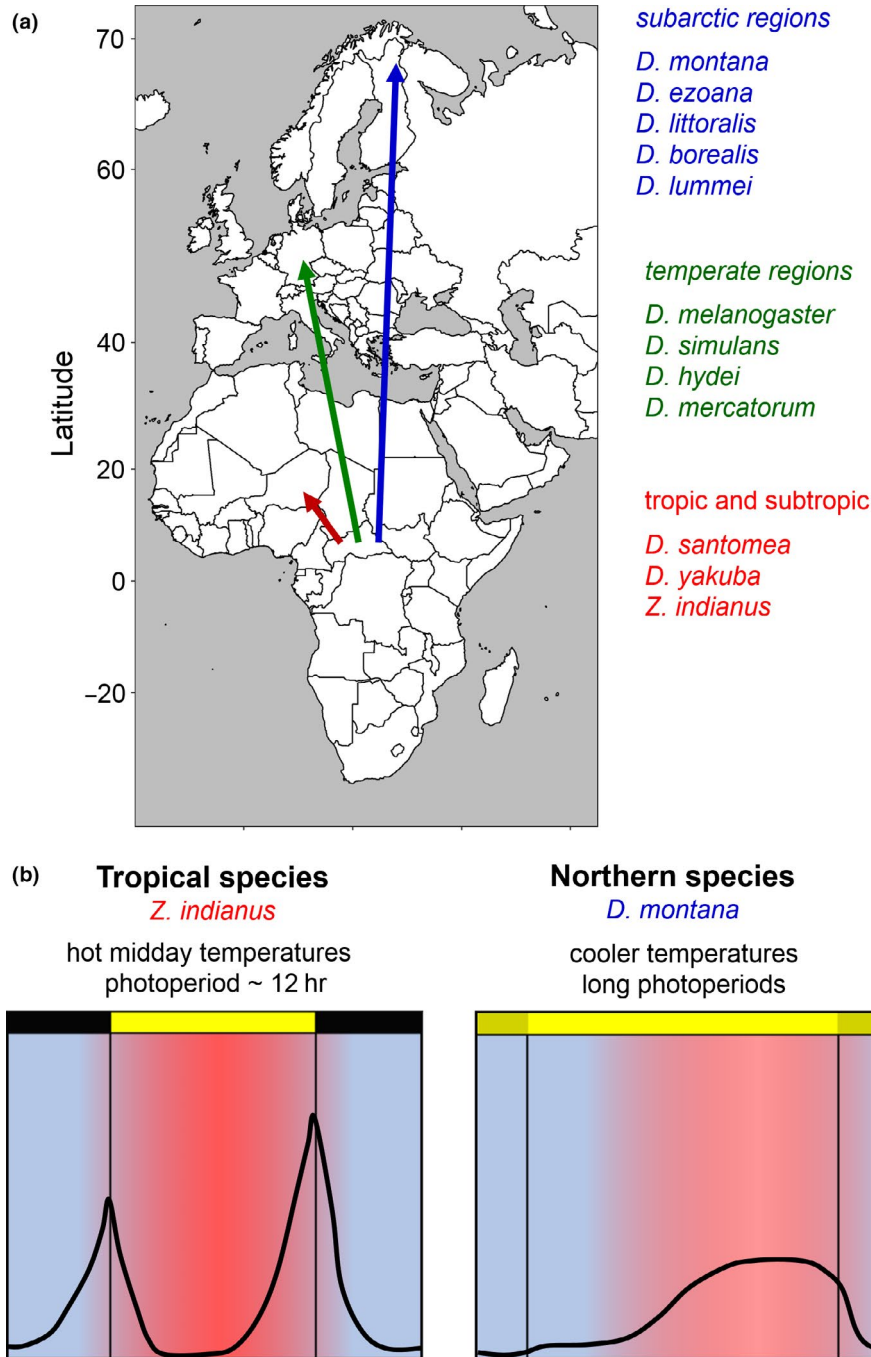
Like in *D. melanogaster*, there is thermal regulation of *per-*splicing in *D. simulans* that also invaded temperate regions, but not in *D. yakuba* and *D. santomea*, which have a more ancestral distribution in equatorial regions of Africa (Low et al., 2008), where day length and temperature exhibit little fluctuation throughout the year (Figure 2). *D. simulans* flies also show similar PER polymorphisms in the Thr-Gly repeats (Rosato, Peixoto, Barbujani, Costa, & Kyriacou, 1994). Thus, the thermal adaptations in form of per-splicing and PER polymorphisms have most likely facilitated colonization of temperate northern zones on earth. The repetitive threonine-glycine (Thr-Gly) region at the PER C-terminus has been investigated in several flies and found to be the longest in *D. pseudoobscura* (Colot, Hall, & Rosbash, 1988; Costa, Peixoto, Thackeray, Dalgleish, & Kyriacou, 1991; Nielsen et al., 1994; Peixoto, Costa, Wheeler, Hall,

& Kyriacou, 1992; Peixoto et al., 1998). *D. pseudoobscura* flies have a wide geographic distribution and extend from Mexico along the western third of the North American continent to British Columbia (Wang & Hey, 1996). Thus, this species is exposed to very different climatic zones and needs to have a well temperature compensated clock, which was demonstrated by generating hybrid *per* transgenes between *Drosophila pseudoobscura* and *D. melanogaster* (Noreen et al., 2018; Peixoto et al., 1998). Nevertheless, not all species with wide geographic distribution have a long Thr-Gly region in PER. For example, the Thr-Gly region is short in the *D. virilis* group (subgenus Siphlodora; Figure 1b) and seems to encompass just two pairs of Thr-Gly (Hilton & Hey, 1996; Lankinen & Forsman, 2006). *Drosophila littoralis* flies, which are latitudinally exceptionally widespread from Mediterranean regions to the northern side of the Arctic Circle, do not even show a latitudinal cline in the length of the Thr-Gly stretch (Lankinen & Forsman, 2006). This shows that this region is not always included in the adaptive clock variability; obviously, these flies found other solutions to adapt their clocks to northern zones on earth.

Evolutionary modifications in TIM or CRY have so far not been demonstrated for fly species other than *D. melanogaster*, but they are most likely. At least a reduction in circadian photosensitivity of northern fly populations has been documented in Japanese *D. auraria* flies and was interpreted as being an adaptation of the circadian system to the long summer day lengths (Pittendrigh & Takamura, 1989). It is important to mention that all so far reported results are gained in species that belong to the Sophophora subgenus and are consequently relatively close to *D. melanogaster* (Figure 1b).

5 | EVOLUTIONARY ADAPTATIONS OF THE NEURONAL CLOCK NETWORK ACROSS SPECIES

Only a few fruit fly species have colonized subarctic regions (Figure 2a). Among these are *D. littoralis*, *D. montana*, *D. ezoana*, and *D. lummei*, all belonging to the *virilis-repleta* radiation that corresponds to the newly introduced subgenus *Siphlodora* (Figure 1b; O'Grady & DeSalle, 2018). In subarctic regions, the flies are not only exposed to cold winters that require effective overwintering strategies (e.g., a strictly photoperiodically controlled diapause), but also to very long photoperiods up to constant light in spring/summer (Figure 2b). The activity pattern of the high latitude species is quite different from the bimodal activity pattern of flies that inhabit lower latitudes (Beauchamp et al., 2018; Kauranen et al., 2012; Menegazzi et al., 2017): they show reduced activity in the morning, no siesta, and broad activity in the afternoon that extends until dusk (Figure 2b). Most interestingly, this

(a)

subarctic regions

D. montana
D. ezoana
D. littoralis
D. borealis
D. lummei

temperate regions

D. melanogaster
D. simulans
D. hydei
D. mercatorum

tropic and subtropic

D. santomea
D. yakuba
Z. indianus

(b)

Tropical species
Z. indianus

hot midday temperatures
photoperiod ~ 12 hr

Northern species
D. montana

cooler temperatures
long photoperiods

FIGURE 2 Colonization of northern latitudes by Drosophilidae and simplified activity patterns of tropical and subarctic flies. (a) Drosophilids are thought to have their origin in the tropics. Certain species still have a tropical or subtropical habitat (red). Others have colonized temperate regions (green) and still others colonized subarctic regions (blue). (b) The tropics are characterized by photoperiods that remain close to 12 hr throughout the year and hot midday temperatures. Flies living in this region (e.g., *Zaprionus indianus*) avoid the midday heat by taking a siesta and being active during the morning and evening. The summers in subarctic regions are characterized by very long photoperiods with moderate temperatures that are most pleasant during the afternoon. Flies living in this region (e.g., *Drosophila montana*) show a broad activity band in the afternoon that extends until dusk

activity pattern even persists to a certain extent under laboratory conditions with constant pleasant temperatures, demonstrating that the activity pattern is genetically determined; most probably, an altered circadian clock in the brain causes it. We do not know yet, to what degree alterations in the clock proteins contribute to the altered activity patterns, but most likely, they significantly do so. A recent study has conducted a phylogenetic analysis of the clock genes *period*, *timeless*, *Clock*, *cycle,* and the *cryptochrome* gene in 12 *Drosophila* species, among which was *D. virilis* that belongs to the subgenus

Siphlodora (Noreen et al., 2018). As may be expected from the phylogenetic tree (Figure 1b), *period*, *Clock*, and *Cycle* of *D. virilis* showed the largest genetic distance to the respective genes in *D. melanogaster*. Nevertheless, this was slightly different for *timeless*, where the largest distance was present in *D. pseudoobscura* that belongs to the Sophophora subgenus (Figure 1b). For *cryptochrome* the largest genetic difference was revealed for *D. willistoni*, another species of the Sophophora subgenus that is assumed to be closely related to *D. melanogaster* (Figure 1b). Altogether, this indicates that the clock genes have differentially evolved, most probably due to different environmental challenges as already nicely revealed in mosquitoes (see above).

In addition to a putatively altered molecular clock, high latitude flies possess an altered neuronal clock network in comparison to *Drosophila* species that remained in tropic regions or colonized temperate regions on earth (Bahn et al., 2009; Hermann et al., 2013; Kauranen et al., 2012; Kauranen, Ala-Honkola, Kankare, & Hoikkala, 2016; Menegazzi et al., 2017; Beauchamp et al., 2018). In the following, we will summarize the most important aspects of the clock network in the brain of *D. melanogaster* and then describe the observed alterations in the brains of high latitude species.

In *D. melanogaster,* the central clock consists of dorsal and lateral neurons that express the core clock genes and form an extensive neuropeptidergic network in the brain (Figure 3a; reviewed in Helfrich-Förster et al., 2007; Hermann-Luibl & Helfrich-Förster, 2015; Helfrich-Förster, 2017; Schubert, Hagedorn, Yoshii, Helfrich-Förster, & Rieger, 2018). One of the best conserved and most important neuropeptide in the insect circadian clock is the pigment-dispersing factor (PDF) (e.g., Beer et al., 2018; Helfrich-Förster et al., 2000; Helfrich-Förster, 2014; Ikeno, Numata, Goto, & Shiga, 2014; Renn, Park, Rosbash, Hall, & Taghert, 1999; Shafer & Yao, 2014; Wei et al., 2014). In *D. melanogaster*, PDF is expressed in four small ventro-lateral neurons (s-LN$_v$) and in four large ventro-lateral neurons (l-LN$_v$) (Figure 3a), which have different roles in the clock network. The s-LN$_v$ are major pacemaker neurons that are essential for robust rhythmic activity under constant darkness and for pronounced morning activity under cycling environmental conditions. Furthermore, they are connected in a light-dependent manner to the dorsal lateral neurons (LN$_d$) and to one group of the dorsal neurons, the DN$_{1p}$, and by this way shape the activity pattern under different light conditions (Chatterjee et al., 2018). The l-LN$_v$ are dispensable for rhythmic activity, but they are part of the light-input pathway to the clock and they control the phase of evening activity, especially under long photoperiods (Schlichting et al., 2016; Menegazzi et al., 2017). PDF is released in a rhythmic manner from the s-LN$_v$ to the dorsal brain, whereas such a rhythm has not been demonstrated in the l-LN$_v$ (Park et al., 2000; Fernández, Berni, & Ceriani, 2008). The l-LN$_v$ produce amidated PDF that is more stable

than the PDF from the s-LN$_v$ (Park et al., 2008) and that may have long-lasting effects on other clock neurons expressing the PDF-receptor (Im, Li, & Taghert, 2011; reviewed in Helfrich-Förster, 2014). Most importantly, PDF from the l-LN$_v$ (or amidated synthetic PDF) phase-delays the clock neurons that control evening activity (LN$_d$ and 5th LN$_v$) (Yoshii et al., 2009; Liang et al., 2017; Menegazzi et al., 2017). Flies without functional PDF signalling cannot phase-delay the Ca^{2+} oscillations in the evening neurons (Liang et al., 2016) and consequently cannot phase-delay evening activity (Renn et al., 1999; Yoshii et al., 2009). Thus, their evening activity occurs in the middle of the day instead of in the late afternoon, and this is most evident under long photoperiods (Menegazzi et al., 2017; Schlichting et al., 2016; Yoshii et al., 2009). In contrast, flies in which the l-LN$_v$ run and secrete PDF into the central brain instead of into the optic lobes exhibit a long siesta and late evening activity already under 12:12 hr light-dark cycles (Wülbeck, Grieshaber, & Helfrich-Förster, 2008). All this fits into the picture that PDF from the l-LN$_v$ phase-delays evening activity, although we cannot exclude that also PDF from the s-LN$_v$ contribute to this effect.

As mentioned above, CRY is a similarly important molecule in the circadian clock of *D. melanogaster*, because it interacts directly with TIM and synchronizes the molecular clock to environmental light-dark cycles. CRY is not the only fly circadian photoreceptor. The compound eyes and the extraretinal eyelets contribute essential aspects of entrainment and suffice to synchronize flies that lack functional CRY (reviewed in Yoshii et al., 2016). Nevertheless, CRY is responsible for the extraordinary light sensitivity of the circadian clock. Flies without functional CRY are not able to phase-shift their activity rhythms in response to short light-pulses and they do not become arrhythmic under constant light (Emery et al., 1998, 2000a,b; Kistenpfennig, Hirsh, Yoshii, & Helfrich-Förster, 2012). CRY is expressed in about half of the clock neurons, among which are the PDF-positive neurons (Figure 3a).

High latitude *Drosophila* species show evident differences in the expression of PDF and CRY in the clock neurons in comparison to *D. melanogaster*: They lack PDF in the s-LN$_v$ and CRY in the l-LN$_v$ (Bahn et al., 2009; Beauchamp et al., 2018; Hermann et al., 2013; Kauranen et al., 2012; Menegazzi et al., 2017). Furthermore, their l-LN$_v$ invade the central brain in addition to the optic lobes (Figure 3b). The lack of PDF in the s-LN$_v$ explains the reduced morning activity of the flies, and the PDF arborizations of the l-LN$_v$ in the central brain may explain their broad late evening activity. In addition, they do not become completely arrhythmic under constant light that might be caused by the absence of CRY in the l-LN$_v$. Most interestingly, we could partly mimic the activity patterns of the high-latitude *Drosophila* species in *D. melanogaster* by expressing PDF only in the l-LN$_v$ and the central brain and by additionally downregulating CRY in
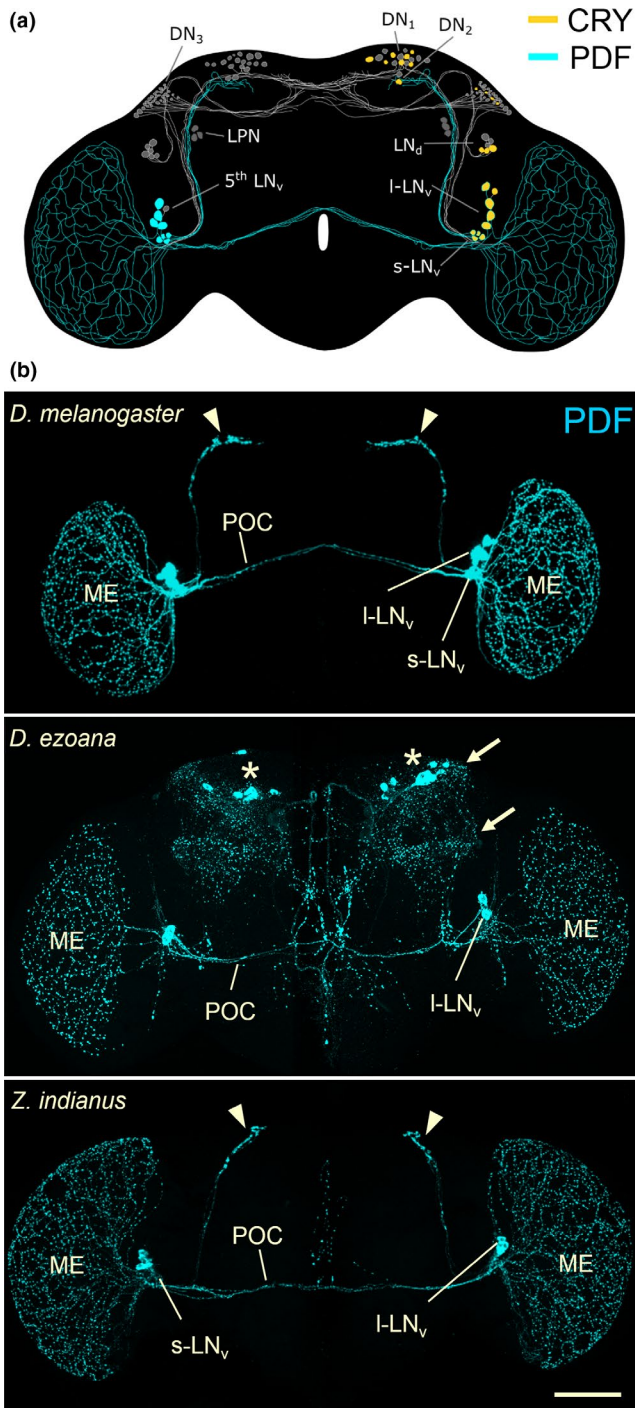
FIGURE 3 Clock network in the fruit fly brain. (a) Clock neurons and their neurites in *Drosophila melanogaster*. The neurons consist of lateral neurons (s-LN$_v$, l-LN$_v$, LN$_d$, 5th LN$_v$, LPN) and dorsal neurons (DN$_1$, DN$_2$, DN$_3$) that are heterogeneous in respect of neuropeptide and Cryptochrome (CRY) expression. Here, we focus on the s-LN$_v$ and l-LN$_v$ that express the Pigment-Dispersing Factor (PDF; cyan, left hemisphere) and CRY (yellow, right hemisphere). (b) Confocal pictures showing the PDF neurons and their arborizations in the brain and the medulla of the optic lobe for *D. melanogaster*, the high-latitude species *D. ezoana* and the tropic species *Zaprionus indianus*. *D. melanogaster* expresses PDF in the s-LN$_v$ and in the l-LN$_v$. The l-LN$_v$ send arborizations to the medulla (ME) and via the posterior optic commissure (POC) to the other brain hemisphere. The s-LN$_v$ project into the central brain and terminate close to the dorsal neurons (arrowheads in b, compare with a). *D. ezoana* expresses PDF only in the l-LN$_v$. These also project via the POC to the other brain hemisphere, but many fibres leave the POC and invade the central brain. Here, the latter come close to the LN$_d$ and dorsal neurons (arrows in b, compare with a). In the dorsal central brain PDF is additionally expressed in non-clock neurons (asterisks). *Z. indianus* expresses PDF in the s-LN$_v$ and the l-LN$_v$ as does *D. melanogaster* and the overall arborization pattern of these two groups of neurons also largely resembles *D. melanogaster*. The cell bodies of the s-LN$_v$ are only weakly stained in this individual, but the terminals of the s-LN$_v$ in the dorsal brain are nicely visible (arrow heads). Scale bar: 100 μm

the l-LN$_v$ (Menegazzi et al., 2017). This indicates that there is indeed a causal relation between PDF/CRY expression and activity patterns.

Further evidence comes from the comparison of the PDF network and CRY expression in fly species that are also from the Siphlodora subgenus, but did not invade subarctic regions, such as *D. hydei* and *D. mercatorum* (Figure 1b). These showed a very similar clock network as *D. melanogaster* in which CRY and PDF was present in the s-LN$_v$ and l-LN$_v$ and no or very little PDF in the

central brain (Beauchamp et al., 2018). Very similarly, *Zaprionus indianus* and *Z. camerounensis* that are also distantly related to the Sophophora subgenus, but restricted to tropic regions, exhibit a CRY/PDF expression pattern that is undistinguishable from *D. melanogaster* (Figure 3b) (Beauchamp et al., 2018). Even subtropic species outside the Drosophilidae such as olive flies, *B. oleae* (Diptera: Tephritidae), have a clock network that strongly resembled that of *D. melanogaster* (Bertolini et al., 2018). Together, these results strongly suggest that the *D. melanogaster*-like clock network is the ancestral one that is preserved in all fly species that did not invade high latitudes. Only flies that colonized the very north, may have lost PDF in the s-LN$_v$, gained PDF fibres from the l-LN$_v$ running into the central brain, and have lost CRY in the l-LN$_v$. If our hypothesis is true, there might even exist fly species in the Sophophora group that colonized the north and developed the clock network typical for high latitude species. Indeed, the latter appears to apply for *D. pseudoobscura* that have the typical high latitude clock network (Hermann et al., 2013). *D. pseudoobscura* did not invade subarctic regions, but they colonized regions in Canada up to 60°N.

Nevertheless, as already discussed for the Thr-Gly repeat in PER of the Siphlodora subgenus, there are different ways how the circadian clock can adapt to high latitude habitats. The species *Chymomyza costata*, which is at the base of the Drosophilinae (Figure 1b) is well-known for its extreme cold resistance (Koštál, 2011a,b). *C. costata* flies are distributed

in Eastern Siberia, Northern Lapland, Iceland, and from northern Japan to the Artic Cycle (Hackman, Lakovaara, Saura, Sorsa, & Vepsalainen, 1970). Nevertheless, they possess a *D. melanogaster*-like clock network and their rhythmic behaviour under artificial long photoperiods in the lab is also *D. melanogaster*-like (Bertolini and Menegazzi, unpublished observations). This confirms that the clock network determines rhythmic behaviour, but it also shows that the circadian clock of *C. costata* flies has found other ways to adapt to high-latitudes. Future studies have to reveal these mechanisms.

6 | CONCLUSION

The dissection of the circadian clock into its molecular components propelled the understanding of circadian timing mechanisms. Clock genes evolve rapidly (Colot et al., 1988) permitting latitude-specific modifications that enable organisms to maintain an optimal timing while colonizing higher latitudes (Hut, Paolucci, Dor, Kyriacou, & Daan, 2013; Kyriacou et al., 2008). Here, we discussed additional modifications of the brain clock network evolved in *Drosophila* species that colonized very high latitudes. These modifications concern the clock factors CRY and PDF that are involved in circadian photoreception and the timing of activity. It will be most promising to investigate, whether similar modifications are also present in other insect species that colonized very high latitudes and if not, which solutions these found to cope with the environmental challenges at these regions from a clock watcher's perspective.

ACKNOWLEDGEMENTS

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

DATA ACCESSIBILITY

This review does not report unpublished primary data.

AUTHOR CONTRIBUTIONS

C.H.F., E.B., and P.M. planned and discussed the outline of the review, E.B. designed Figure 3a and the graphical abstract, C.H.F. wrote and E.B. and P.M. corrected the manuscript.

ORCID

Charlotte Helfrich-Förster http://orcid.org/0000-0002-0859-9092

Enrico Bertolini http://orcid.org/0000-0002-7573-7467

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