Life at High Latitudes Does Not Require Circadian Behavioral Rhythmicity under Constant Darkness

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In Brief
Bertolini et al. find that drosophilids inhabiting high latitudes possess a circadian clock that is either not self-sustained or uncoupled from its output. This leads to behavioral arrhythmicity in constant darkness. The authors propose that this feature might be of adaptive value for animals to successfully colonize weakly rhythmic environments.

Highlights
- The Chymomyza genus carries the ancestral low-latitude D. melanogaster-like clock
- C. costata colonized high latitudes despite a low-latitude clock neuroarchitecture
- High-latitude drosophilids show arrhythmicity in constant darkness
- C. costata and D. ezoana achieve arrhythmicity via different strategies
Life at High Latitudes Does Not Require Circadian Behavioral Rhythmicity under Constant Darkness

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SUMMARY

Nearly all organisms evolved endogenous self-sustained timekeeping mechanisms to track and anticipate cyclic changes in the environment. Circadian clocks, with a periodicity of about 24 h, allow animals to adapt to day-night cycles. Biological clocks are highly adaptive, but strong behavioral rhythms might be a disadvantage for adaptation to weakly rhythmic environments such as polar areas [1, 2]. Several high-latitude species, including Drosophila species, were found to be highly arrhythmic under constant conditions [3–6]. Furthermore, Drosophila species from subarctic regions can extend evening activity until dusk under long days. These traits depend on the clock network neurochemistry, and we previously proposed that high-latitude Drosophila species evolved specific clock adaptations to colonize polar regions [5, 7, 8]. We broadened our analysis to 3 species of the Chymomyza genus, which diverged circa 5 million years before the Drosophila radiation [9] and colonized both low and high latitudes [10, 11]. C. costata, pararufithorax, and procnemis, independently of their latitude of origin, possess the clock neuronal network of low-latitude Drosophila species, and their locomotor activity does not track dusk under long photoperiods. Nevertheless, the high-latitude C. costata becomes arrhythmic under constant darkness (DD), whereas the two low-latitude species remain rhythmic. Different mechanisms are behind the arrhythmicity in DD of C. costata and the high-latitude Drosophila ezoana, suggesting that the ability to maintain behavioral rhythms has been lost more than once during drosophilids’ evolution and that it might indeed be an evolutionary adaptation for life at high latitudes.

RESULTS AND DISCUSSION

C. costata, pararufithorax, and procnemis Carry a D. melanogaster-like Clock, Regardless of Their Latitudinal Distribution

Cosmopolitan and low-latitude fly species (such as D. melanogaster, D. hydei, Z. indianus, and B. oleae) generally co-express the photopigment cryptochrome (CRY) and the neuropeptide pigment dispersing factor (PDF) in two clusters of ventrolateral clock neurons (i.e., small [s-LNvs] and large [l-LNvs]) [6, 7, 12, 13]. In contrast, all high-latitude Drosophila species so far investigated do not express CRY in the l-LNvs and PDF in the s-LNvs but express PDF in some cells located in the dorsal brain [4–6] (Figure 1A). To understand whether similar modifications occurred also in high-latitude species outside the Drosophila genus, we studied the clock neuronal network of Chymomyza costata from Finland and compared it to that of Chymomyza pararufithorax and procnemis from southern Japan (Figure 1B). To identify and characterize their clock neuron clusters, we used antibodies against D. melanogaster PAR domain protein 1 (PDP1), CRY, and PDF. All antibodies already proved to work reliably in several fly species [4–7, 12, 13].

In all three species, the spatial (Figures 1C and 1D) and temporal (Figure 4) expression patterns of PDP1, CRY, and PDF allowed us to identify as bona fide clock neurons the same main clusters known from D. melanogaster and other low-latitude species [5, 7, 12, 13, 17, 18]. These were dorsal neurons (DNs) located in the dorsal protocerebrum and lateral neurons (LNs) in the lateral cell body rind (Figures 1C and 1E). We could further subdivide the LNs into subgroups according to their size, anatomical location, and neurochemistry (Figures 1F–1K and S1). As in D. melanogaster, s- and l-LNvs co-express PDF (Figures 1F–1H and S1A) and CRY (Figures 1I–1K). The s-LNvs send PDF positive projections toward both the optic lobes, whereas the s-LNvs innervate the superior posterior protocerebrum (Figures 1C and 1D). One LN, located in close proximity to the s- and l-LNvs but intermediate in size between them (Figure S1A), is CRY positive but PDF negative and hereafter named PDF-LNvs (Figures 1I–1K). We propose that this neuron corresponds to the 5th s-LNv of D. melanogaster [17]. We could also identify one cluster of more dorsally located LNs, named
Figure 1. C. costata, C. pararufithorax, and C. procnemis Possess the Ancestral Low-Latitude Clock Network Neuroarchitecture

(A) Schematic representation of the CRY and PDF neurochemistry within the LNvs of low- (left) and high-latitude (right) Drosophila species.

(B) Collection sites of the fly strains considered in this work. C. costata (1) and D. ezoana (5) were collected at 65°57’N, near the Oulanka Research Station, Kuusamo, Finland. C. pararufithorax (2) and C. procnemis (3) were collected in Southern Japan, at 26°28’N (Okinawa) and 33°35’N (Fukuoka), respectively, and obtained by the National Drosophila Species Stock Center at Cornell University (Ithaca, New York). D. melanogaster flies (4) were obtained from a commonly used laboratory strain, derived from a wild-type line collected at 40°48’N [14]. The Chymomyza genus diverged circa 5 million years before the Drosophila radiation [9] and Chymomyza species are today found at both high and low latitudes [10, 11].

(C) PDP1 (green) and PDF (magenta) immunoreactive neurons in the brain of C. costata.

(D) PDP1 (green) and PDF (magenta) immunoreactive neurons in the brain of C. pararufithorax.

(E) PDP1 (green) and PDF (magenta) immunoreactive neurons in the brain of C. procnemis.

(F) PDP1 (green) and PDF (magenta) immunoreactive lateral neurons in C. costata.

(G) PDP1 (green) and PDF (magenta) immunoreactive lateral neurons in C. pararufithorax.

(legend continued on next page)
dorsolateral neurons (LNds), which, as in *D. melanogaster* [19], do not express PDF and are heterogeneous regarding CRY expression (Figures 1I–1K). In none of the *Chymomyza* species, we found PDF-expressing cells in the dorsal protocerebrum.

Based on these results, the architecture of the putative master clock of the three *Chymomyza* species showed conspicuous similarities with that of *D. melanogaster*. This was also true during development [17, 20–24] (Figures S1B–S1K). The parallelisms of the clock neuronal network among *D. melanogaster* and the *Chymomyza* species confirmed the idea that the *D. melanogaster*-like clock neuroarchitecture is the ancestral one [5, 6, 8] and was somewhat expected, given that we already described this pattern outside the Drosophilidae family (i.e., *Bactrocera oleae*) [12]. Based on CRY and PDF functions in *D. melanogaster* [5, 25–27], we proposed that the loss of PDF in the s-LNvs and CRY in the l-LNvs in the high-latitude species might allow them to better cope with the long days typical of polar summers [5, 7, 8]. In this scenario, *C. costata* becomes an exception: it colonized high latitudes but, unlike the high-latitude *Drosophila* species, it maintains the ancestral clock neuroarchitecture. This suggests *C. costata* might have evolved adaptations different from those observed within the *Drosophila* genus.

**Chymomyza costata from the Very North Maintains Southern Behavior under Long Summer Days**

*Drosophila* species carrying the high-latitude clock show a rather unimodal daily locomotor activity profile and adjust to very long days by delaying their evening activity bout [4–6]. Such a locomotor activity profile depends, at least partially, on the PDF-negative s-LNvs and CRY-negative l-LNvs that characterize the clock network of these species [5]. Indeed, in *D. melanogaster*, flies with no PDF (especially in the s-LNvs) lack the morning activity bout becoming unimodal [25, 26], whereas flies with no CRY (especially in the l-LNvs) delay their evening activity more than wild-types [5, 27]. The *Chymomyza* species considered here, despite their latitude of origin, carry a low-latitude *D. melanogaster*-like clock neuronal network and should therefore show a *D. melanogaster*-like locomotor activity under LD, i.e., strong bimodality and inability to track dusk under long photoperiods. The locomotor activity of *C. pararufithorax* and *C. procnemis* was never analyzed before, whereas few studies reported on the locomotor activity of *C. costata*. In 1970, Nuorteva and Hackman inferred information based on malt fruit bait catches in the field (Inari, Finland, 68° N [28]; *C. costata* showed one bout of activity concomitant with the time of maximum environmental temperature (i.e., circa 25°C). On the day of collection, the authors recorded a minimum temperature of circa 2°C, making it hard to determine whether the activity peak observed was the result of a unimodal activity profile or merely a consequence of the low temperature. Based on later studies [29–32], we can assert that the most likely explanation is the latter. It is well known that cold exposure suppresses locomotor activity in insects (see [33] for a review on the topic) and cool temperatures can induce a unimodal activity profile even in the strongly bimodal *D. melanogaster* [29–32].

To verify whether *C. costata* shows a high-latitude behavior despite carrying a low-latitude clock network, we recorded its locomotor activity, as well as that of *C. pararufithorax* and *C. procnemis*, under light:dark (LD) cycles with 12, 16, and 20 h of light per day (LD12:12, LD16:8, and LD20:4, respectively; Figures 2 and S1). For comparison, we used a laboratory strain of *D. melanogaster*, with a low-latitude clock and locomotor activity, and a Finnish strain of *D. ezoana*, with high-latitude clock and locomotor activity (Figure 1B). As previously reported, under long days (i.e., LD20:4), *D. ezoana* showed a later evening activity than *D. melanogaster* (Figures 2A and 2B; Table S1) and delayed its evening activity peak to a greater extent under increasing day length (Figure S1; Table S2). The locomotor activity of *C. pararufithorax* and *C. procnemis* from southern Japan closely resembled that of *D. melanogaster*, with rather sharp activity peaks at lights on and evening activity bouts at lights off under LD12:12 but advanced compared to lights-off in LD16:8 and LD20:4 (i.e., 3 and 5 h before lights off, respectively; Figure 2A; Table S1). The timing of *C. costata*’s locomotor activity was very similar to that of the low-latitude *Chymomyza* species (Figure 2A); it did not show a later evening activity (Figure 2B; Table S1), and it was not able to delay more under increasing day length (Figure S2; Table S2).

Animals living in subarctic regions are not only exposed to extremely long photoperiods but also to days with continuous light (LL), which, most likely via persistent activation of the photopigment CRY [35], drives behavioral arrhythmicity in all low-latitude fly species so far investigated [5, 6, 12]. In contrast, we found that high-latitude *Drosophila* species retain some rhythmicity in LL and showed that this is due to the lack of CRY expression within their l-LNvs [5]. In agreement with this, the three *Chymomyza* species considered here express CRY in their l-LNvs (Figure 1) and are unable to maintain rhythmicity in LL (Figure S2).

These findings confirm the idea that flies carrying the ancestral low-latitude clock can neither delay their evening activity peak under long photoperiods nor maintain some rhythmic locomotor activity in LL, implying that these traits might not be essential for life in the North. While the clock of *D. ezoana* was driving a rather flexible locomotor activity, adjusting to a broad range of day lengths, the clock of *C. costata* seemed less plastic, with a morning and an evening activity peak tightly coupled to each other in LD. This difference under entrained conditions might be important in light of the circannual behavior of each species: *D. ezoana* overwinters as adult [36], whereas *C. costata* undergoes a developmental arrest at the larval stage [37, 38]. It was proposed that a clock driving robust rhythms, or in other words less sensitive to day length changes, might be a disadvantage in animals with strong circannual cycles [2, 39], like *D. ezoana* but unlike *C. costata* adults.
Several high-latitude species lack rhythmicity in behavior and physiology at least under certain environmental conditions [3, 40–43]. High-latitude Drosophila species, such as D. ezoana, show arrhythmic behaviors when released under constant darkness in the laboratory [4–6, 44]. It is known from D. melanogaster that PDF expression, especially within the s-LNvs, is required for rhythmic locomotor activity in DD [5, 25]. The behavioral arrhythmity observed in DD in high-latitude Drosophila species is therefore most likely due to the lack of PDF expression within their s-LNvs [5]. In C. costata kept in DD, Lankinen and Riihima described weak eclosion rhythm and arrhythmic locomotor activity [45]. We verified whether C. costata, C. pararufithorax, and C. procnemis are able of maintaining behavioral rhythms by recording their locomotor activity in DD to compare it to that of D. melanogaster and D. ezoana. All analyzed D. melanogaster, but only 11% of D. ezoana (3 out of 27), were found rhythmic (Figure 3; Table S3). In accordance with the hypothesis that PDF-positive s-LNvs would lead to a rhythmic locomotor activity in DD, all C. pararufithorax and C. procnemis proved to be rhythmic (Figure 3; Table S3).
contrast, despite its PDF-positive s-LNvs, C. costata was more similar to D. ezoana rather than D. melanogaster: only 2 flies out of 29 (7%) showed some clear circadian rhythmicity (Figure 3; Table S3). In a more permissive analysis, flies that showed complex rhythm components were scored as rhythmic (Figures S3A and S3B). This analysis increased the percentage of the individuals considered rhythmic (i.e., to 59% in D. ezoana and 28% in C. costata) but did not alter the comparison among species, with the low-latitude ones being markedly more rhythmic (Figure S3; Table S3).

A clock unable to sustain rigid rhythms, in addition to a pronounced sensitivity to temperature stimuli, might be of adaptive significance for northern species that might need to respond to unpredictable changes in the environment independently of their endogenous timing [1, 45]. The absence of behavioral rhythmicity in DD in C. costata and D. ezoana might be the output of a quickly dampening clock or of a clock that is not self-sustained. To investigate the two possibilities, we looked at the oscillation of PDP1 within the clock neurons (Figure 4). For comparison, we conducted this experiment also in D. melanogaster, where we expected rhythmic PDP1 expression [46]. PDP1 oscillated in all three species when flies were exposed to LD cycles (Figure 4A). PDP1 oscillation persisted in DD in the rhythmic D. melanogaster but was lost in the arrhythmic D. ezoana (Figure 4B). The loss of behavioral rhythms in D. ezoana might therefore be explained by a loss of core clock protein cycling. This is likely a consequence of the absence of PDF in the s-LNvs. It is known from D. melanogaster that the clock neurons, and especially the s-LNvs, which function as a master pacemaker in DD driving locomotor activity rhythms [5], rely on PDF signaling to maintain a strong oscillation in their molecular clock [47]. In C. costata, despite the arrhythmic locomotor activity, the s-LNvs are expressing PDF, and we could expect PDP1 cycling in DD. This is indeed the case: PDP1 cycles in a circadian manner under constant conditions (Table S4). We wondered whether, in C. costata, the reason behind the arrhythmic locomotor activity might rather be in a loss of coupling between the master clock and its output. To address this issue, we quantified PDF staining intensity at the s-LNv terminals. The role of PDF as an output factor of the circadian clock is conserved among several insect species [25, 48–53]. Moreover, in D. melanogaster, the neuropeptide is known to be rhythmically released at the s-LNv terminals of wild-type, but not of arrhythmic clock mutants [54, 55]. In C. costata, we found PDF cycling in LD, where flies showed strong locomotor activity rhythms, but not in DD, where they became mostly arrhythmic (Figures 4C and S4). For comparison, we analyzed PDF staining intensity also in the rhythmic C. procnemis. In accordance with their locomotor activity, C. procnemis showed cycling in PDF intensity both in LD and DD (Figure S4). In D. melanogaster, daily changes in PDF intensity correlate with daily reorganization of the s-LNv axonal morphology [56]. Furthermore, disruption of either PDF accumulation at the s-LNv terminals or s-LNv arborization rhythms leads to arrhythmic locomotor activity [5, 57, 58]. Based on these
isms maintaining behavioral rhythms when exposed to their environmental conditions. In some cases, as in D. melanogaster, it occurred later during the day, i.e., 23.75 h ± 0.86 (Z = 29; p < 0.01). This observation fits with the free-running period for this wild-type strain of D. melanogaster, which is slightly lower than 24 h. PDP1 expression levels (±SEM) over time are represented as solid lines (s-LNvs in red, PDF LNvs in blue, and LNds in green). The light regime is represented by the environmental bars on top of each panel (white for day and black for night). Zeitgeber time is plotted starting from the lights-on transition (ZT0), and night hours are represented by the shaded area in the background. The boxplots in each panel represent the peak time of PDP1 within the clock neuron clusters. Among the LNvs, the s-LNvs were not considered because immunocytochemical assays fail to show clock protein cycling within these cells in flies kept in DD [15, 16]. We could not analyze data for the PDF LNvs in D. ezoana because, in this species, PDF is expressed only within the i-LNvs.

(B) PDP1 oscillation within the lateral clock neurons (s-LNvs, PDF LNvs, and LNds) of C. costata, D. melanogaster, and D. ezoana under the first day of constant darkness. We found time-dependent PDP1 fluctuation in all clock neuron clusters of D. melanogaster (s-LNvs [H (6) = 38.04; p < 0.001], PDF LNvs [H (6) = 28.58; p < 0.001], LNds [H (6) = 43.7; p < 0.001]) and in the LNds of C. costata s-LNvs (H (7) = 43.29; p < 0.001). Fluctuations of PDP1 in C. costata s-LNvs and PDF LNvs were not significantly time dependent (s-LNvs [H (6) = 11.29; p = 0.08], PDF LNvs [H (6) = 9.84; p = 0.13]). PDP1 cycling was lost in D. ezoana (s-LNvs [H (6) = 6.12; p = 0.41], LNds [H (6) = 4.09; p = 0.66]). In C. costata, PDP1 maximum occurred 20.6 ± 0.82 h after the beginning of DD, which is comparable to the peak time in LD that occurred 19.05 ± 0.79 h after lights-on (U = 191; p > 0.05). In D. melanogaster, PDP1 maximum occurred 20.8 ± 0.2 h after the beginning of DD, whereas in LD, it occurred later during the day, i.e., 23.75 ± 0.86 (Z = 29; p < 0.01). This observation fits with the free-running period for this wild-type strain of D. melanogaster, which is slightly lower than 24 h. PDP1 expression levels (±SEM) over time are represented as solid lines (s-LNvs in red, PDF LNvs in blue, and LNds in green). Night and day (i.e., subjective night and subjective day) of the previous LD cycle are represented by the environmental bars on top of each panel (dark gray for night and light gray for day). Circadian time is plotted starting from the beginning of constant conditions (CT0); subjective day and subjective night are represented by the light- and dark-gray-shaded areas, respectively. The peak time of PDP1 within the clock neuron clusters is represented as mean ± SEM.

Observations and our findings in C. costata and C. procnemis, we speculate that loss of PDF cycling at the s-LNvs terminals could be the cause of C. costata’s arrhythmicity. We can hypothesize that absence of rhythmic PDF release would eventually translate into a stopped clock [47, 59] or into a lack of coupling between the clock and its output. This idea would fit with previous observations that found dampening rhythms in C. costata (i.e., eclosion rhythm and per mRNA cycling) [45, 60].

Our study shows that arrhythmicity in high-latitude environments can be achieved via either loss of molecular oscillation within the master clock or lack of coupling between the master clock and its output. These results fit well with previous findings in other organisms and can be generalized to weakly rhythmic environments all over the world. Lu and coworkers [39] showed that the molecular clock stops cycling in reindeer fibroblasts in DD, whereas Beale and coauthors [61] reported that the circadian clock of the Mexican blind cavefish Astyanax mexicanus keeps ticking under constant conditions but fails to drive rhythmic behavior.

There have been previous reports of arctic and subarctic organisms maintaining behavioral rhythms when exposed to their natural environment [62–64]. Nevertheless, most studies conducted on high-latitude animals revealed loss of circadian phenotypes, especially under exposure to constant conditions in the laboratory [3–6, 39, 40, 45, 65]. Recently, Hazlerigg and Tyler [66] proposed that high-latitude large mammals might have not been under the selective pressure of maintaining a circadian endogenous clock, being continuously exposed to daily temporal information. This is quite likely since reindeers, given that their lack of circadian rhythmicity depends on a loss of molecular clock function [43]. We argue that, if organisms living in weakly rhythmic environment would not need a circadian clock, they would as well not show circadian phenotypes under entrained conditions. Several studies [62, 64], including this one, reported relatively strong molecular and behavioral rhythms in animals exposed to cycling environmental conditions. In some cases, as in C. costata or A. mexicanus, the circadian molecular clock keeps ticking also under constant conditions but seems to be uncoupled from its output. The fact that so many species phylogenetically distant from each other possess a circadian clock, which, under constant conditions, is not self-sustained or is uncoupled from its output, suggests that this is a feature that might be of some advantage.
in weakly rhythmic environments (see also [1]). For instance, in those moments of the year in which external time cues would not suffice to reliably entrain the clock, animals would be able to quickly respond to favorable or unfavorable changes in the environment, independently of their internal timing.

**STAR METHODS**

Detailed methods are provided in the online version of this paper and include the following:

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**SUPPLEMENTAL INFORMATION**

Supplemental Information can be found online at https://doi.org/10.1016/j.cub.2019.09.032.

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**AUTHOR CONTRIBUTIONS**

Conceptualization, P.M., E.B., and C.H.-F.; Methodology, E.B., P.M., and F.K.S.; Validation, E.B. and P.M.; Formal Analysis, E.B.; Investigation, E.B., P.M., F.K.S., C.H.-F., D.Z., and H.S.; Resources, C.H.-F.; Data Curation, E.B. and P.M.; Writing – Original Draft, P.M. and E.B.; Writing – Review and Editing, F.K.S., D.Z., C.H.-F., and H.S.; Visualization, E.B., F.K.S., and P.M.; Supervision, P.M.; Project Administration, P.M.; Funding Acquisition, C.H.-F. and P.M.

**DECLARATION OF INTERESTS**

The authors declare no competing interests.

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STAR METHODS

KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Antibodies          |        |            |
| mouse anti-PDF      | Developmental Studies Hybridoma Bank | RRID: AB_760350, PDF C7 antibody |
| rabbit anti-CRY     | Takeshi Todo (Osaka University) | [19]; RRID: AB_2570229 |
| rabbit anti-PDP1    | Justin Blau (New York University) | [67]; RRID: AB_2569283 |
| goat anti-rabbit Alexa Fluor-488 | Thermo Fisher Scientific | # A-11008; RRID: AB_143165 |
| goat anti-mouse Alexa Fluor-555 | Thermo Fisher Scientific | # A28180; RRID: AB_2536164 |
| goat anti-mouse Alexa Fluor-647 | Thermo Fisher Scientific | # A-21240; RRID: AB_141658 |
| Chemicals, Peptides, and Recombinant Proteins |        |            |
| Agar-Agar, danish   | Carl Roth | #4508.1 |
| D-Sucrose           | Carl Roth | #9286.1 |
| Paraformaldehyde (PFA) | Alfa Aesar | #J19943 |
| Phosphate-Buffered Saline (PBS) | Sigma-Aldrich | #P5493 |
| Triton X-100        | Carl Roth | #3051 |
| Normal Goat Serum   | Sigma-Aldrich | #G9023 |
| NaN3                | Morphisto | #13741 |
| Vectashield         | Vector Laboratories | #101098-042 |
| Experimental Models: Organisms/Strains |        |            |
| Drosophila melanogaster | Laboratory strain (Neurobiology and Genetics, University of Wurzburg, Germany). | [14, 68] |
| Drosophila ezoana   | Strain collected by Hannele Kauranen, (Division of Ecology and Evolutionary Biology, University of Jyvaskyla, Finland) and Lill Bahr (Department of Neurobiology and Genetics, University of Würzburg, Germany), and maintained at the Department of Neurobiology and Genetics, University of Würzburg, Germany. | N/A |
| Chymomyza costata   | Dr. Kondo Shu (National Institute of Genetics, Shizuoka, Japan); Dr. Ari Riihimaa (Department of Biology, University of Oulu, Finland). | N/A |
| Chymomyza procnemis | The Cornell University Drosophila Species Stock Center | 20000-2640.00 |
| Chymomyza pararufithorax | The Cornell University Drosophila Species Stock Center | 20000-2650.00 |
| Software and Algorithms |        |            |
| DAM system          | https://trikinetics.com | N/A |
| ImageJ/Fiji         | [69] |            |
| CLEAN               | [70] |            |
| R                   | - | [71] |
| ggplot2             | - | [72] |
| Inkscape            | https://inkscape.org | N/A |
| CorelDraw           | https://www.coreldraw.com/en/ | N/A |

LEAD CONTACT AND MATERIALS AVAILABILITY

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Pamela Menegazzi (pamela.menegazzi@uni-wuerzburg.de). This study did not generate new unique reagents.
EXPERIMENTAL MODEL AND SUBJECT DETAILS

Chymomyza costata and Drosophila ezoana were collected in Oulu (Finland) in 2012 and 2013, respectively. Chymomyza procnemis and Chymomyza pararufithorax were purchased from the Drosophila Species Stock Center (Cornell University), and they were originally collected in Southern Japan, Fukuoka (1981) and Okinawa (1996), respectively. Canton-S is a common D. melanogaster laboratory strain [14, 68].

All flies were reared on standard Drosophila medium (8% malt extract, 8% corn flour, 2.2% sugar beet molasses, 1.8% yeast, 1% soy flour, 0.8% agar, 0.3% hydroxybenzoic acid).

D. melanogaster, C. pararufithorax, and C. procnemis were kept at constant 18°C under LD12:12, whereas D. ezoana and C. costata were reared at 18°C under constant illumination in order to prevent the induction of photoperiodic diapause.

METHOD DETAILS

Immunocytochemistry

Immunocytochemistry was performed as previously described [32]. The duration of PFA fixation was optimized for each species, because of difference in size: 2.5 hours for D. melanogaster, C. procnemis, and C. pararufithorax; 3 hours for C. costata and D. ezoana; at room temperature. Larval and pupal brains were fixed 2 hours at room temperature. Primary antibody concentrations were as follow: anti-PDF (1:1000), anti-PDP1 [67], anti-CRY (1:1000) [19], diluted in PBS (0.3% Triton X-100 in PBS), 5% NGS and 0.002% NaN3. Secondary antibodies were used at 1:400 in PBS with 5% NGS. Whole brains were mounted in Vectashield. For CRY- PDF double stainings, C. pararufithorax and C. procnemis larva were kept 24 hours in constant darkness before collection. Stainings in C. costata pupae as well as CRY-PDF stainings in C. costata larvae were performed in Czech Republic. For the latter, larvae were kept in DD for 72 hours before collection.

Confocal imaging and image analysis

Preparations were scanned by a Leica TCS SPE (Leica, Wetzlar, Germany) using 20x objective (ACS APO 20.0x0.60 IMM). Imaging data were visualized and analyzed in Fiji [69].

The PDP1 staining intensity was measured within manually selected ROIs corresponding to the nuclei of the clock neurons (s-LNv, PDF~LNv, LNd) as mean gray level. The measurement was subtracted with the background intensity, and averaged between the cells, hemispheres, and flies (See also [5, 32]). The PDP1 staining index was normalized to the maximum for each condition.

PDP1 peak timing was calculated averaging the time points of the 10% highest values among all time points. To determine cycling of PDP1 under the first day of DD we removed CT0 and CT3 from the comparison because here PDP1 levels are still dropping from the peak of the previous LD cycle. PDF levels were measured as described in [54], whereas cell size diameters were measured as in [18].

Locomotor activity recording and analyses

Locomotor activity of male flies (7 to 14 days old) was recorded using the Drosophila Activity Monitors (DAM, Trikinetics), isolating single flies in glass tubes of the diameter of 5 mm (for D. melanogaster, C. pararufithorax, and C. procnemis) or 7 mm (for D. ezoana and C. costata). Food was provided within the tube as 2% sucrose and 4% agar. Monitors were placed in light boxes where light:dark cycles were generated with a light intensity of 100 lux, at constant 20°C.

For the recording of locomotor activity under LD cycles: flies were either exposed to each photoperiod (LD12:12, or LD16:8, or LD20:4; See Figure 2 and Table S2) for seven days or exposed to increasing photoperiods for 7 days each (from LD12:12 to LD16:8 to LD20:4; see Figure S1, related to Figure 2, and Table S2, related to Figure 2). For recordings under DD, flies were released into constant conditions (DD, 20°C) after 7 days of entrainment under LD16:8. Data were collected in 1 minute intervals using the DAM system 2.1.3 software.

The activity profiles under LD cycles were calculated over the last three days of the entrainment, averaging the daily activity profile of single flies; average activity plots were normalized to the maximum for each condition. The timing of the E-peak was determined as described in [73]. To calculate the delay in evening activity peak under increasing photoperiods (Figure S2) we subtracted the peak time in the shorter photoperiod (LD12:12 or LD16:8) to that recorded in the longer photoperiod (LD16:8 or LD20:4, respectively).

For recordings in DD, individual fly activity is shown in actograms where days are double-plotted on the x axis and the activity (infrared beam crossings/15 minutes) is shown as a black bars. Rhythmicity under DD was analyzed using the periodograms Lomb-Scargle (implemented in Fiji, ActogramJ [74]) and CLEAN [70]. In addition, circadian rhythms of individual fly activity were scored blindly by five observers as “rhythmic,” “complex,” or “arrhythmic.” Complex rhythmicity was scored when multiple rhythm components were identified (e.g., [75]). Rhythmicity was summarized and plotted in percentages.

The free-running period of the rhythmic flies was calculated and reported with power (PN, Lomb-Scargle). Flies that died during of the experiment were excluded from the analyses.

QUANTIFICATION AND STATISTICAL ANALYSIS

Statistics was performed in R [71] and figures were generated using ggplot2 [72]. The normal distribution of samples was tested using the Shapiro-Wilk test, and homogeneity of variances among levels was tested using Levene’s test. According to the outcome, 1-way
ANOVA or Kruskal-Wallis H tests were applied. Pairwise comparisons between group levels (using Student’s t test or Wilcoxon rank-sum test) were corrected for multiple testing using the Benjamini-Hochberg method. Chi-Square test was used to compare proportions of “rhythmic,” “complex” and “arrhythmic” flies. All statistical details can be found in the figures and figure legends.

**DATA AND CODE AVAILABILITY**

The published article includes all datasets analyzed during this study. Requests for further information or raw data should be directed to and will be fulfilled by the Lead Contact, Pamela Menegazzi (pamela.menegazzi@uni-wuerzburg.de).