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Evolution of circadian behavioral plasticity through *cis*-regulatory divergence of a neuropeptide gene
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Abstract

Widely-distributed species experience substantial environmental variation, which they often accommodate through behavioral plasticity. Although this ability is integral to fitness, we have little understanding of the mechanistic basis by which plasticity evolves. One factor that varies seasonally and by latitude is photoperiod (day length). Many organisms, including the cosmopolitan *Drosophila melanogaster* display circadian plasticity, adjusting to fluctuating photoperiod by varying the timing of their activity to coincide with changing dawn/dusk intervals[1]. Here, we compare *D. melanogaster* with the closely-related ecological specialist *Drosophila sechellia*, an equatorial island endemic that experiences minimal photoperiod variation, to investigate the molecular-genetic basis of circadian plasticity evolution[2,3]. We discover that *D. sechellia* displays exceptionally little circadian plasticity compared to *D. melanogaster* and other non-equatorial drosophilids. Through a screen of circadian mutants in *D. melanogaster*/*D. sechellia* hybrids, we identify a role of the neuropeptide Pigment-dispersing factor (Pdf) in this loss. While the coding sequence of *Pdf* is conserved, we show that *Pdf* has undergone *cis*-regulatory divergence in these drosophilids. We document species-specific temporal dynamic properties of *Pdf* RNA and protein expression, as well as Pdf neuron morphological plasticity, and demonstrate that modulating Pdf expression in *D. melanogaster* can influence the degree of behavioral plasticity. Furthermore, we find that the *Pdf* regulatory region exhibits signals of selection across populations of *D. melanogaster* from different latitudes. Finally, we provide evidence that plasticity confers a selective advantage for *D. melanogaster* at higher latitudes, while *D. sechellia* likely suffers fitness costs through reduced copulation success outside its range. Our work defines *Pdf* as a locus of evolution for circadian plasticity, which might have contributed to both *D. melanogaster*'s global distribution and *D. sechellia*'s habitat specialization. Moreover, together with spatial changes in Pdf expression reported in high-latitude drosophilid species[4,5], our findings highlight this neuropeptide gene as a hotspot for circadian plasticity evolution.

Introduction

Nervous systems coordinate animals' behavioral responses to the external world to maximize survival and fitness. This task becomes more challenging when environments are not constant, a problem of substantial significance for broadly-distributed species. One way to face changing conditions is with behavioral plasticity, that is, the ability to adjust behavioral phenotypes to match fluctuations in the environment. There are many examples of plastic behaviors in nature: songbirds shift the frequency of their vocalizations in response to anthropogenic noise[6], ants alter their locomotor and foraging behaviors as a function of temperature[7], and lizards change their basking behavior based on altitude[8]. However, we have little understanding of whether and how behavioral plasticity is determined and evolves at the genetic and cellular level.

An important example of plastic behavior in animals is circadian activity, whereby species adjust their daily activity patterns in response to seasonal variation in day length[9]. This ability is critical because circadian activity in most animals coordinates specific behaviors with optimal activity periods throughout the day to, for example, avoid environmental stressors, maximize food availability, and

align with conspecifics for synchronized social and sexual behaviors[10,11]. As such, deviations from regular circadian patterns can negatively affect fitness and species persistence[12,13]. Drosophilids are a powerful system to study circadian behavioral plasticity. These flies display large bouts of activity surrounding dawn and dusk (termed morning and evening activity peaks), separated by a period of relative inactivity during the middle of the day[14]. The best-studied species, the cosmopolitan *Drosophila melanogaster*, plasticly adjusts its circadian rhythm depending upon seasonal variation in photoperiod[1]. Notably, the degree of photoperiod plasticity of different strains of this species correlates with the latitude of collection site[15]. Moreover, several distantly-related, high-latitude species have evolved divergent patterns of activity and extreme plasticity, allowing their daily activity to match long summer days[16].

A potentially interesting comparison species to *D. melanogaster* is *Drosophila sechellia*, a much closer relative that diverged 3-5 million years ago (Fig. 1a)[2,3]. *D. sechellia* is endemic to the equatorial islands of the Seychelles archipelago, where it experiences almost no seasonal photoperiod variation (Fig. 1a-b). Here, we discover striking differences in the circadian activity and plasticity of *D. sechellia* and *D. melanogaster*, notably an almost complete inability of *D. sechellia* to adapt to increased photoperiod. Taking advantage of the possibility to interbreed these species, we conducted a genetic screen of known circadian genes, offering unprecedented insights into the genetic and cellular underpinnings of circadian plasticity evolution, and a rare connection between genetic divergence and evolved differences in an ecologically-relevant behavior.

Results

D. sechellia displays reduced photoperiod plasticity compared to *D. melanogaster*

To test for species-specific differences in circadian plasticity, we first measured circadian behavior for *D. melanogaster* and *D. sechellia* under a standard 12 h light-dark cycle (12:12 h LD) as well as four extended photoperiod regimes ranging from mild (14:10 h LD) to extreme (20:4 h LD) (Fig. 1c). We used males of two strains each of *D. melanogaster* and *D. sechellia* (Supplementary Table 1), to distinguish interspecific from intraspecific phenotypic differences. The two *D. melanogaster* strains (*Dmel*CS and *Dmel*OR) were collected at ~41° N and ~44° N, respectively, while the *D. sechellia* strains (*Dsec*07 and *Dsec*28) are from the Seychelles archipelago, at ~4° S of the equator (Fig. 1a). The strains of each species therefore initially evolved in environments where they experienced large differences in annual photoperiod variation (Fig. 1b). Under each photoperiod, all strains displayed activity peaks during the morning and evening, although the timing of the evening peak varied by photoperiod (Fig. 1c). We quantified for each fly the average evening peak time of the last 4 of 7 days in a given photoperiod, allowing the first 3 days to serve as an acclimation period (Fig. 1c). For both *D. melanogaster* strains, we observed that as photoperiod increases, the timing of the evening activity peak is commensurately delayed (Fig. 1c). By contrast, for our *D. sechellia* strains, we observed strikingly little photoperiod plasticity, with a median delay in evening peak time of maximum ~1 h regardless of photoperiod length (Fig. 1c). Additionally, under all photoperiod regimes, *D. sechellia* ended its afternoon

150 siesta and began ramping up to evening peak activity a few hours earlier than *D.*
151 *melanogaster* (Fig. 1c).
152
153 ### *D. sechellia* displays reduced morning peak activity compared to *D.*
154 ### *melanogaster*
155
156 We noted that *D. sechellia* is far less active during the dark phase than either *D.*
157 *melanogaster* strain and displays little, if any, morning anticipation, i.e., increasing
158 activity in the hours leading up to lights-on (Fig. 1c-d). Quantification of pre-dawn
159 activity under 12:12 h LD in the 3 h preceding lights-on revealed prominent
160 differences between the species (Fig. 1d): *D. sechellia* is generally very inactive
161 during this time period, while the *D. melanogaster* strains display ample, albeit
162 strain-specific, activity. These observations led us to question whether the morning
163 peak of *D. sechellia* is a true activity peak or merely a startle response to lights-on.
164 To address this issue, we measured free-running activity by acclimating our strains
165 to 12:12 h LD before submitting them to constant dark conditions (DD). Both *D.*
166 *melanogaster* and *D. sechellia* remained rhythmic under DD (Fig. 1e), and each
167 strain displayed a period of ~24 h (Extended Data Fig. 1). By contrast, while *D.*
168 *melanogaster* displayed clear activity peaks at the subjective dawn, *D. sechellia*
169 exhibited very little activity at this timepoint, even during the first day of DD (Fig.
170 1e). This result supports the hypothesis that the morning peak of *D. sechellia*
171 observed under LD conditions (Fig. 1c) is predominantly a startle response to
172 lights-on. Consistently, when we quantify morning peak timing under 12:12 h LD,
173 we found that *D. melanogaster* reached peak activity before lights-on, as previously
174 described[17], while *D. sechellia* peaked only at or after lights-on (Fig. 1f).
175
176 ### Reduced circadian plasticity and morning activity represent an evolutionary
177 ### loss in *D. sechellia*
178
179 To determine if the species differences in circadian plasticity and morning activity
180 represent an evolutionary loss in *D. sechellia* or a trait gain in *D. melanogaster*, we
181 measured the activity of additional *D. melanogaster* strains collected from the
182 Lower Zambezi Valley (close to its ancestral range[18] (~16° S), as well as strains of
183 two species that have a more recent common ancestor with *D. sechellia* (Fig. 1a):
184 *D. simulans* (collected from Madagascar, its ancestral range[19], ~19° S) and *D.*
185 *mauritiana* (a species endemic to Mauritius, ~20° S) (Extended Data Fig. 2a).
186 Comparing 12:12 h and 16:8 h LD conditions, we observed a similar larger degree
187 of circadian plasticity (~2 h evening peak delay under the longer photoperiod) for
188 all of these strains compared to *D. sechellia* (~1 h evening peak delay). All of these
189 strains also exhibited significant morning anticipation (Extended Data Fig. 2b).
190 These results indicate that the lack of plasticity and reduction in morning peak
191 activity observed in *D. sechellia* likely represent evolutionary losses in this lineage,
192 and point to a potential connection between these two phenotypes.
193
194 ### A genetic screen for differences in circadian activity and plasticity
195
196 To identify the mechanistic basis of the species differences in circadian behaviors,
197 we employed a candidate genetic screening approach. Extensive research in *D.*
198 *melanogaster* has defined central brain circuitry of 150 circadian neurons, divided
199 into discrete groups with differing effects on circadian activity and network

dynamics[20]. Within each neuron, a gene regulatory feedback loop allows each cell to track a ~24 h period[21-23] and to control the rhythmic expression of downstream effector genes (Fig. 2a). The members of this network serve as excellent candidates for explaining species-specific circadian behaviors. To take as unbiased an approach as possible, we obtained loss-of-function mutations for the majority of the genes encoding proteins within this feedback loop in addition to several in the downstream network, including the neuropeptide Pigment-dispersing factor (Pdf), as well as other circadian neuropeptides, CCHa1 and ITP (Supplementary Table 1). Our cross-species behavioral analyses (Fig. 1c-d and Extended Data Fig. 2) indicated that reduced circadian plasticity and morning peak activity in *D. sechellia* represent evolutionary losses. We therefore reasoned that the causal *D. sechellia* alleles were more likely to be recessive to *D. melanogaster*, and designed a screen in *D. melanogaster-D. sechellia* hybrids (Fig. 2b). In brief, we generated hemizygous test hybrids containing *D. melanogaster* mutants for each individual candidate gene, to reveal the recessive phenotype of the *D. sechellia* allele at the same locus. We also generated heterozygous control hybrids, using the *D. melanogaster* w^{1118} strain (the most common genetic background of our mutant strains) or CS^W strain (in the case of *Pdf*; see Methods) and each of our *D. sechellia* strains. These control hybrids have one allele each from *D. melanogaster* and *D. sechellia*. Thus, any differences we observe between control and test hybrids is likely due to the loss of the *D. melanogaster* allele in the test hybrid background. To control for genetic background effects[24], we tested hybrids of both the *Dsec*07 and *Dsec*28 backgrounds. Finally, gene dosage effects were assessed by testing control hemizygotes in a (non-hybrid) *D. melanogaster* background, i.e., mutants crossed to w^{1118} or CS^W. Genes whose mutations displayed an effect in both test hybrid backgrounds compared to control hybrids, and no effect in hemizygous *D. melanogaster*, were considered the strongest candidates explaining interspecific phenotypic differences (Fig. 2c).

The *Pigment-dispersing factor* gene underlies evolved differences in circadian plasticity

To assess candidate genes for an effect on circadian plasticity, we observed test and control hybrids under a 16:8 h LD photoperiod. Control hybrids of either the w^{1118} (Extended Data Fig. 3a-d) or CS^W background (Fig. 2d-e) display a larger degree of phenotypic plasticity than their *D. sechellia* parental strain, confirming that the *D. melanogaster* genotype underlying plasticity is at least partially dominant to that of *D. sechellia*, though the degree of dominance depends on the *D. melanogaster* parental strain. We screened 14 genes covering the majority of the circadian transcriptional feedback loop and many of its modulator and effector genes. Mutations in only one reduced circadian plasticity in both test hybrid backgrounds but not in hemizygous *D. melanogaster*: *Pdf* (Fig. 2d-e, Extended Data Fig. 3e-f). This is a promising gene for explaining species differences because, in *D. melanogaster*, Pdf is essential for delaying the phase of the endogenous clock circadian neurons under long photoperiods[25], and flies lacking Pdf expression display reduced plasticity[26,27].

Potential broad-scale divergence of the circadian clock underlies *D. sechellia*'s reduced morning peak activity

We also screened these genotypes under 12:12 h LD and quantified pre-dawn activity (Extended Data Fig. 4a-d). In contrast to the dominance of the *D. melanogaster* phenotype for circadian plasticity (Extended Data Fig. 3a-d), *w[1118]* control hybrids display intermediate pre-dawn activity relative to either parental strain, suggesting a more complex genetic architecture of this species difference. Consistent with this idea, four genes displayed an effect in test hybrids of both backgrounds (Fig. 2f-g) and not in hemizygous *D. melanogaster* flies (Extended Data Fig. 4e-g). These encode the core transcriptional feedback loop protein CYC and the light-sensitive CRY, which is responsible for light-dependent synchronization of the molecular clock[28-30], as well as Hr38 and VRI, which are neural activity-dependent transcriptional or post-transcriptional regulators of *Pdf* expression, respectively[31].

Cis-regulatory evolution of *Pdf*

We subsequently focused our attention on *Pdf*, because of its unique significant effect on circadian plasticity and evidence that *trans*-regulation of *Pdf* expression influences morning peak activity. To understand how this gene differs between species, we first compared the *Pdf* coding sequence of 10 *D. melanogaster* and 6 *D. sechellia* isogenic strains (as well as 5 *D. simulans* lines). These sequences are predicted to encode peptides of near-perfect conservation, with no species-specific differences (Extended Data Fig. 5).

We therefore reasoned that divergence between species must be due to expression differences of Pdf. In *D. melanogaster*, this neuropeptide is expressed exclusively in 8 neurons in each central brain hemisphere: 4 large ventrolateral clock neurons (l-LNvs), which represent a subset of the "evening" cells, and 4 small ventrolateral clock neurons (s-LNvs), the "morning" cells (Fig. 3a). These neuronal subtypes have predominant roles in controlling evening and morning activity, respectively[32-34], although a functional clock is required in both for photoperiod plasticity[33,35]. Through qualitative Pdf immunofluorescence analysis, we observed a conserved spatial pattern of Pdf expression in *D. sechellia* (Fig. 3b), consistent with a survey of Pdf expression across a broader range of drosophilids[36]. This result suggested that species-specific differences instead exist in the temporal pattern and/or levels of expression.

Because our hybrid screen identified an effect of the *Pdf* locus itself, we hypothesized that differences in expression must result from divergence in the *cis*-regulatory region. To test this possibility, we cloned ~2.4 kb of genomic DNA immediately 5' of the start codon of *Pdf* from either *D. melanogaster* or *D. sechellia* – based upon a previous analysis in *D. melanogaster*[37] – upstream of a GFP reporter gene[38]. These transgenes were integrated in an identical genomic location in the same *D. melanogaster* genetic background, thereby permitting comparison of their activity in a common *trans* and genomic environment. As expected, both species' *Pdf* reporters exclusively labelled the l-LNvs and s-LNvs (Fig. 3c). We first measured reporter expression in the l-LNvs because, in *D. melanogaster*, Pdf expression in these cells is required to plasticly adjust the timing of the evening peak[4]. To process all samples in parallel for quantitative comparisons, we focused on behaviorally relevant time points under 12:12 h LD and 16:8 h LD conditions

299 (Fig. 3d-e). In the l-LNvs, throughout the evening activity peak under both
300 photoperiods, the *D. sechellia* 5'-regulatory region consistently drives lower and
301 more constant reporter expression relative to the *D. melanogaster* sequence (Fig.
302 3d-e). Notably, the *D. melanogaster Pdf* reporter displayed a sudden drop in
303 expression at 8 h (under 12:12 h LD) or at 12 h (under 16:8 h LD), prior to returning
304 to a higher level, which potentially reflects a new pulse in transcriptional activity
305 that appears sensitive to photoperiod.
306 　　　In *D. melanogaster*, the s-LNvs are essential for resetting the phase of the
307 circadian clock (likely through the cyclic release of Pdf[39,40]) and are necessary for
308 morning peak activity[32,34]. We therefore compared reporter expression in the s-LNv
309 axonal projections – where the largest cyclic Pdf expression is observed over a 24
310 h period[37] – for four time points spanning the morning activity peak (Fig. 3f). We
311 again observed that the *D. sechellia* sequence drives lower expression of the
312 reporter but, in contrast to the l-LNvs during the evening peak, with a similar
313 temporal fluctuation in expression. Together these results confirm functional
314 divergence of the 5' *cis*-regulatory region between *D. sechellia* and *D.*
315 *melanogaster*. This sequence is most likely to affect transcriptional activity but,
316 because it encompasses the 5'-UTR of *Pdf*, we cannot exclude that it (also)
317 influences transcript stability and/or translatability[41].
318

319 **Modifying *Pdf* expression reduces the magnitude of circadian plasticity and**
320 **morning anticipation**
321

322 Having identified species-specific properties in the *cis*-regulatory element of *Pdf*,
323 we tested whether these would be sufficient to impact behavior. Taking advantage
324 of the different reporter expression levels driven by the *D. melanogaster* and *D.*
325 *sechellia Pdf* 5' regions (Fig. 3d-f), we used these same sequences to generate
326 Pdf neuron Gal4 drivers to induce "strong" (*D. melanogaster Pdf-Gal4*) or "weak"
327 (*D. sechellia Pdf-Gal4*) knock-down of *Pdf* using a *UAS-Pdf^RNAi* effector[42]. We first
328 validated the anticipated distinct efficacy of RNAi with quantitative single molecule
329 RNA fluorescence *in situ* hybridization (smFISH). In *DmelPdf*-Gal4>*Pdf* RNAi flies,
330 *Pdf* transcripts were reduced to ~4% of the levels of control animals, while *DsecPdf*-
331 Gal4>*Pdf* RNAi flies expressed *Pdf* transcripts at ~20% of control levels (Extended
332 Data Fig. 6).
333 　　　We first observed these flies under 16:8 h LD conditions and quantified
334 evening peak timing (Fig. 3g-h). The *DmelPdf*-Gal4>*Pdf* RNAi flies displayed a
335 dramatic reduction in evening peak time relative to controls. By contrast, *DsecPdf*-
336 Gal4>*Pdf* RNAi flies display a small, and non-significant, decrease in evening peak
337 time relative to control animals, with a notable increase in variance. Importantly,
338 there is a significant difference between the RNAi-expressing genotypes despite
339 their otherwise identical genetic backgrounds. We also observed the behavior of
340 these flies under 12:12 h LD, and quantified pre-dawn activity (Fig. 3i-j). Both
341 *DmelPdf*-Gal4>*Pdf* RNAi and *DsecPdf*-Gal4>*Pdf* RNAi flies have reduced pre-
342 dawn activity relative to controls. However, the *DmelPdf*-Gal4>*Pdf* RNAi flies
343 displayed significantly less pre-dawn activity than the *DsecPdf*-Gal4>*Pdf* RNAi
344 flies. Together these results indicate that the level (and possibly temporal
345 dynamics) of *Pdf* expression – as determined by species-specific 5' *cis*-regulatory
346 regions – is sufficient to affect both circadian plasticity and morning anticipation in
347 an otherwise identical genetic background.
348

Species-specific, photoperiod-dependent differences in *Pdf* RNA expression

We next investigated how *cis*-regulatory divergence might influence endogenous *Pdf* RNA and protein expression. Using smFISH, we compared *Pdf* transcript levels between *D. melanogaster* and *D. sechellia* at the same fine temporal resolution as for the transgenic reporters. Under 12:12 h LD, quantification of transcript levels in l-LNvs throughout the evening peak revealed slightly higher initial *Pdf* expression in *D. sechellia* than *D. melanogaster* (Fig. 4a). The most striking difference, however, is a sudden drop in *Pdf* transcripts in *D. melanogaster*, but not *D. sechellia*, after lights-off (Fig. 4a). Under 16:8 h LD conditions, this reduction in *Pdf* RNA levels is no longer present (Fig. 4b). These observations indicate that transcriptional activity of *Pdf* is more dynamic in *D. melanogaster* than *D. sechellia*, resulting in a decrease of *Pdf* transcripts by the dark phase under 12:12 h LD. Such a pattern might additionally/alternatively result from differences in RNA stability between species. Regardless of the mechanism, this species-specific transcript depletion appears sensitive to photoperiod. Interestingly, such a pattern of *Pdf* transcription in the l-LNvs has not been previously described using bulk or single-cell RNA sequencing of these neurons in *D. melanogaster*[43,44] when sampling more broadly across a 24 h time period; however a decrease in *Pdf* RNA at 14 h (relative to the 2 h time point) was previously documented using smFISH[45], congruent with our results.

When we quantified *Pdf* RNA in the s-LNvs throughout the morning activity peak, we found that *Dmel*CS displayed overall more *Pdf* RNA than *Dsec*07 at each time point, particularly in the pre-dawn time points, with *Dsec*07 reaching near-similar levels only after lights-on (Fig. 4c). This difference in *Pdf* RNA levels is concordant with the differences in pre-dawn activity we observed between these species (Fig. 1c-d), and the implication of transcriptional and post-transcriptional regulators of *Pdf* expression in species-specific morning anticipation (Fig. 2f-g).

Species- and photoperiod-dependent differences in Pdf protein expression

We next used immunofluorescence to compare Pdf protein expression in *D. melanogaster* and *D. sechellia*. Similar to the transcript analyses, we quantified Pdf immunofluorescence in the l-LNvs in time points surrounding the evening activity peak under 12:12 h LD and 16:8 h LD conditions (Extended Data Fig. 7a-b). Under both photoperiods, we observed qualitative differences between these species in the overall pattern of staining intensity, but the much greater variability in signal intensity of these samples, particularly for *D. sechellia* under 16:8 h LD, made it difficult to connect back to our more quantitative measures of *Pdf* RNA levels (or to behavioral activity).

In the s-LNvs, we quantified Pdf fluorescence in the axonal projections for the same time points spanning the morning activity peak (Fig. 4d). In the relatively short time window analyzed, we observed a consistently high level of Pdf in *D. melanogaster*, including in the hours preceding lights-on. By contrast, in *D. sechellia*, Pdf signal is lower in the pre-dawn hours, and increases to an equivalent amount as *D. melanogaster* only by lights-on. This pattern corresponds well to that of the relative levels of *Pdf* transcripts, and to species differences in pre-dawn activity at these times (Fig. 1c-d). We also analyzed Pdf immunofluorescence in the s-LNv cell bodies, which display higher and less cyclic Pdf expression in *D. melanogaster*[37]. Consistently, we found Pdf signal remains high across the morning

399 peak times in the s-LNv soma of *D. melanogaster* (Fig. 4e). In *D. sechellia*, the Pdf
400 signal begins high and drops significantly only after lights-on (Fig. 4e). Together,
401 our observations of *Pdf* RNA levels and Pdf protein levels/distribution in the s-LNvs
402 of *D. sechellia* suggest that this species has a smaller and/or shorter pulse of *Pdf*
403 expression around the morning peak compared to *D. melanogaster*, such that once
404 this neuropeptide accumulates to high levels in the axon terminals at/after lights-
405 on (Fig. 4d), it becomes depleted in the soma (Fig. 4e). Species-specific dynamics
406 in Pdf spatial distribution might also reflect differences in intracellular transport
407 and/or secretion pathways.

409 **Species-specific circadian structural plasticity of Pdf neurons**

411 In *D. melanogaster*, the axonal projections of the s-LNvs to the dorsal circadian
412 neurons (Fig. 3a) display circadian structural plasticity, reaching peak branching
413 complexity during the day, and lower complexity during the night[46]. This
414 phenomenon depends, at least in part, on cyclic expression and release of Pdf from
415 both the s-LNvs and l-LNvs and the expression of the Pdf receptor[42]. To test if the
416 observed species-specific temporal patterns of Pdf expression are accompanied
417 by differences in the remodeling of these neurons, we quantified the branching
418 complexity of s-LNv projections in *D. melanogaster* and *D. sechellia* during the light
419 (2 h) and dark (14 h) phases (Fig. 4f). During the light phase, we found statistically
420 indistinguishable levels of complexity in the two species. However, in *D.
421 melanogaster*, branching complexity is significantly lower in the dark phase when
422 compared to *D. sechellia* (Fig. 4f). This apparent reduction of structural plasticity of
423 *D. sechellia* Pdf neurons corroborates the less dynamic changes in Pdf expression
424 in this species.

426 **Signals of *cis*-regulatory evolution and selection on the *Pdf* regulatory region**

428 Having characterized functional divergence of the *Pdf cis*-regulatory region
429 between species, we next asked whether the *D. sechellia* sequence exhibits any
430 evolutionary signature. We sequenced this region in all of our *D. melanogaster*, *D.
431 sechellia* and *D. simulans* strains. Overall, *D. simulans* and *D. melanogaster*
432 sequences share an average of ~97% pairwise sequence similarity, while *D.
433 sechellia* has an average pairwise sequence similarity of ~93% with *D.
434 melanogaster*. We used these sequences to construct a maximum likelihood
435 phylogeny (Fig. 5a). In contrast to the species tree, the *Pdf* 5'-regulatory
436 sequences from *D. sechellia* form a monophyletic group, while the *D. melanogaster*
437 and *D. simulans* sequences mostly cluster together (with the exception of a single
438 *D. simulans* sequence, which groups more basally with the *D. sechellia*
439 sequences). Motif enrichment analysis[47] identified putative regulatory sequences
440 in these species' 5' regions. While all such motifs were shared among the *D.
441 melanogaster* and *D. simulans* sequences, 8 of these sites are degenerated or
442 absent in *D. sechellia* (and one site is unique to this species) (Fig. 5b), indicating
443 that sequence divergence in the *D. sechellia Pdf* regulatory region is likely to affect
444 its function activity, potentially through the loss of transcription factor binding sites.
445   We next investigated whether the sequence divergence between *D.
446 melanogaster* and *D. sechellia* 5'-regulatory sequences might result, at least in part,
447 from natural selection on variants underlying circadian plasticity at higher latitudes.
448 We examined this possibility by determining whether variants within the *D.

449 *melanogaster Pdf* 5'-regulatory region are associated with higher degrees of
450 circadian plasticity observed with increasing latitudes[15]. The reverse analysis in *D.*
451 *sechellia* is not possible as this species is restricted to an equatorial latitude. Taking
452 advantage of a dataset of single nucleotide variant frequencies in the genomes of
453 globally-distributed populations of *D. melanogaster*[48], we chose 13 populations
454 representing a wide range of latitudes (Fig. 5c); all had a minimum read-depth of 5
455 to ensure confidence in variant frequencies. We calculated the average minor allele
456 frequency (MAF) across all variable sites detected within the *Pdf* 5'-regulatory
457 sequence and plotted these against the estimated latitude of the population
458 collection site. Because correlations could reflect the underlying population
459 structure as a result of demographic history as *D. melanogaster* emigrated from its
460 native range in Africa[49], as a control, we repeated this analysis for the putative
461 equivalent regulatory region (2.4 kb upstream from the start codon) of six other
462 neuropeptide genes. We found a strong positive correlation (Spearman's rho =
463 0.77) between population latitude and MAF for the *Pdf* 5'-regulatory region, but not
464 for any of the other neuropeptide genes (Fig. 5c, Extended Data Fig. 8). These
465 comparisons indicate that the effect of latitude on MAF of the *Pdf* 5'-regulatory
466 sequence is different than we would expect due to demography alone, suggesting
467 a potential role for selection on these sites in *D. melanogaster*. These results are
468 similar to recent reports of clinal variation in other circadian genes[50-53].
469   Lastly, we checked the MAFs of these variable sites in our laboratory strains.
470 These single nucleotide variants occur with a MAF of ~25% among our *D.*
471 *melanogaster* strains, but none were present in any of our *D. sechellia* strains (Fig.
472 5d). These results are consistent with a potential function of these variants in
473 increasing circadian plasticity. Together with the predicted motif differences
474 between *D. melanogaster* and *D. sechellia Pdf* 5'-regulatory sequences (Fig. 5b),
475 these data will help guide future analyses of the specific functional changes within
476 this region.
477
478 **Circadian plasticity is important for reproductive success**
479
480 To identify a mechanism by which natural selection might act, we asked if plasticity
481 in circadian activity impacts fitness. Photoperiod has been shown to affect lifespan
482 in many insects and other animals[54-56], leading us to reason that if flies have
483 reduced lifespans under extended photoperiod, then this might lead to reduced
484 total reproductive output. We recorded survivorship of individual *D. melanogaster*
485 and *D. sechellia* maintained at either 12:12 h or 16:8 h LD (Fig. 5e). Flies of both
486 species maintained under 16:8 h LD displayed a significant reduction in lifespan
487 relative to those under 12:12 h LD. This result is surprising in that it suggests that
488 *D. melanogaster*'s ability to plasticly adjust its activity to longer days does not
489 alleviate the cost of exposure to longer photoperiod. However, the detrimental
490 effect of longer photoperiod was not observed until after several weeks in both
491 species, in which time flies could certainly mate and produce offspring. It is
492 therefore unclear if the effect of extended photoperiod on lifespan would impact
493 fitness in nature, where lifespan is likely much shorter than under laboratory
494 conditions.
495   Circadian rhythms are important for synchronizing sexual behavior among
496 conspecifics[10,11]. We therefore reasoned that if circadian plasticity (or the lack
497 thereof) impacted copulation success, it would likely impact fitness. To test this
498 possibility, we acclimated male and female *D. melanogaster* and *D. sechellia*

499 virtins to 12:12 h LD and 16:8 h LD for 4 days. We then observed copulation rates
500 among male-female pairs in two assays: first, over a 2-hour period just after lights-
501 on (Fig. 5f), and second, over the course of one week (Fig. 5g). For *D.*
502 *melanogaster*, we observed no difference in copulation rates of flies between
503 treatments in either experiment. By contrast, there was a significant decrease in
504 copulation by *D. sechellia* acclimated to 16:8 h LD. Specifically, the decrease in
505 copulation rate in the short-term assay was largely maintained over the course of
506 several days in our long-term assay, indicating that flies that did not copulate within
507 2 h were unlikely to do so several days later. These results demonstrate that *D.*
508 *sechellia*'s reproductive output – and thus fitness – is highly likely to be impacted
509 by its lack of circadian plasticity under extended photoperiods that it would never
510 normally experience in nature. These data also suggest that by plasticly adjusting
511 its behavior, *D. melanogaster* is able to circumvent these negative effects.
512
513 **Discussion**
514
515 Identifying the genetic and neural mechanisms of behavioral plasticity is key to
516 understanding how organisms evolve(d) to inhabit variable environments, as well
517 as to projecting how they will persist in increasingly unstable ones[57]. However,
518 efforts to uncover the proximate causes of behavioral divergence are limited by a
519 lack of genetic access to multiple closely-related species, leaving remarkably few
520 cases where the molecular and/or cellular underpinnings of interspecific
521 differences in behaviors have been mapped[58], with the vast majority being in
522 (peripheral) sensory pathways (e.g.,[59-63]). Here, we have uncovered molecular and
523 cellular mechanisms of circadian plasticity differences in drosophilids, providing a
524 rare example linking differences in gene function, central neuron populations, and
525 behavioral differences between species.
526   By comparing the equatorial *D. sechellia* with closely-related, globally-
527 distributed species, we discovered a dramatic difference in the degree of
528 photoperiod plasticity and provided evidence for a key contribution of the *cis*-
529 regulatory region of the *Pdf* locus in this difference (Fig. 5h). In *D. melanogaster*,
530 Pdf expression is required in the l-LNvs for photoperiod plasticity[27], and previous
531 comparative work has described interspecific spatial differences in Pdf
532 expression[36], notably in high-latitude species where Pdf is restricted to the l-
533 LNvs[4,16]. Importantly, prior to our work, no functional connection between
534 divergence at the *Pdf* locus and species differences in behavior had been
535 established. Nevertheless, these observations, combined with our analyses in *D.*
536 *sechellia* and *D. melanogaster* – including the evidence for latitude-based selection
537 on the *D. melanogaster Pdf* 5'-regulatory region – point to the *Pdf* locus as a hotspot
538 of evolution. Given Pdf's terminal placement as an effector gene of the clock
539 network[31], its role in broadly synchronizing circadian clock neurons[64], and its strong
540 impact on circadian behaviors, changes in the *cis*-regulation of *Pdf* expression
541 might represent a minimally pleiotropic means of introducing plasticity into the clock
542 neuronal network, akin to regulatory changes of developmental genes that underlie
543 morphological evolution[65]. By contrast, divergence of core clock genes might
544 represent a more complex evolutionary trajectory, necessitating the coevolution of
545 multiple interacting loci.
546   *Pdf* evidently does not explain the entirety of the species differences in
547 plasticity: there are almost certainly contributions of additional loci that we have not
548 tested and/or more complex genetic interactions that we cannot identify with our

screen design. For example, the possibility of transvection (*trans*-regulation of alleles on homologous chromosomes)[24] in our hybrid screen might have masked the contributions of some divergent loci. Our observation of differences between *Pdf cis*-regulatory activity using transcriptional reporters, and endogenous *Pdf* RNA and Pdf protein levels in *D. sechellia* and *D. melanogaster* suggest that additional genes might nevertheless ultimately impact Pdf. Beyond species-specific *cis*-regulation characterized here, post-transcriptional regulation of *Pdf*[31], as well as control of the transport and secretion of this neuropeptide[66] are also potentially subject to divergent regulation. Indeed, in *D. melanogaster*, a *D. virilis* transcriptional reporter for *Pdf* labels the s-LNvs (in addition to several non-circadian cells) despite this species, like other high-latitude drosophilids[4,16], lacking endogenous Pdf expression in these neurons[5]; these observations suggest that mechanisms other than (or in addition to) *cis*-regulatory divergence underlie this spatial difference in expression. Finally, molecules functioning downstream of Pdf in controlling plasticity (e.g., Eyes absent[67]) are possible loci of evolutionary adaptations.

D. sechellia also displays greatly reduced morning activity compared to other drosophilids. As this phenotype is similar to that observed in *D. melanogaster Pdf* mutants[68], it is perhaps surprising that we did not find an effect of the *Pdf* locus itself in this difference. Rather, we identified several genes with the ability to regulate *Pdf* expression in *trans*, highlighting a different evolutionary trajectory to divergence of circadian plasticity that nevertheless converges upon this neuropeptide. The morning and evening oscillators partially overlap in function, sharing synaptic feedback[27,69], with both being required for long photoperiod adaptation[35]. The mechanistic and evolutionary connection (if any) between divergence of circadian plasticity and morning activity warrants further exploration. The reason, if any, for reduced morning peak activity in *D. sechellia* remains unclear; this issue might be illuminated by future analysis of the circadian pattern of other aspects of this species' behavior, such as courtship or feeding.

D. sechellia's loss of photoperiod plasticity is particularly intriguing in the context of this species' specialist ecology for the noni fruit of *Morinda citrifolia*, on which it exclusively feeds and breeds. Noni specialization has involved substantial evolution of its chemosensory behaviors[59,70-75]. Our work extends knowledge of this species' phenotypic divergence to non-host related behaviors. Why loss of circadian plasticity of *D. sechellia* likely leads to a severe fitness cost at high latitudes is unknown, but we speculate that longer photoperiods result in altered pheromone production, as observed in different seasonal morphs of *Drosophila suzukii*[76]. A more general consideration is why *D. sechellia* has lost circadian plasticity. One hypothesis is that in a constant environment, selection to maintain plasticity mechanisms is relaxed, leading them to degenerate over time. Alternatively, in stable environments, plasticity might come at a fitness cost, leading selection to favor its loss under constant conditions to enhance, for example, the robustness of this species' circadian activity. While we cannot currently discriminate between these two possibilities, if the latter is correct, our view of *D. sechellia*'s specialization must expand beyond host fruit preference evolution to restriction to an equatorial environment. Indeed, *D. sechellia*'s circadian phenotype might contribute to its restriction to the Seychelles archipelago, despite the much larger modern range of *M. citrifolia*[77]. Exploration of the impact of differences in circadian plasticity mechanisms to latitudinal constraint of other species seems warranted.

Author Contributions

M.P.S. and R.B. conceived the project. M.P.S. designed, performed and analyzed most experiments. L.A. performed the hybrid screening and sequencing of the *Pdf* gene region. L.L.D.D. contributed to the experiments in Fig. 1c. J.C. contributed to the experiments in Extended Data Fig. 3 and 4. R.K. assisted with preliminary behavioral experiments and, together with E.N. and R.B., provided input on experimental design, analysis and interpretation. M.P.S. and R.B. wrote the paper with feedback from all other authors.

Competing interests

The authors declare no competing interests.

Methods

***Drosophila* strains and rearing**

All flies were reared on a wheat flour-yeast-fruit juice media in non-overlapping 2-week cycles kept in 12:12 h LD at 25°C. For *D. sechellia* strains, we added an additional mixture of instant *Drosophila* medium (Formula 4-24 blue, Carolina bio-supply) mixed with juice of their host noni fruit (Raab Vitalfood).

For behavioral comparisons of *D. melanogaster*, *D. sechellia*, *D. simulans,* and *D. mauritiana* circadian behavior, at least two wild-type strains of each species were used (*Dmel*CS, *Dmel*OR, *Dmel*LZV L72 , *Dmel*LZV L76, *Dsec*07, *Dsec*28, *Dsim*MD221, *Dsim*MD242, *Dmau*90, and *Dmau*91). To screen candidate genes for effects on differences in circadian behavior between *D. melanogaster* and *D. sechellia*, we used *D. melanogaster* strains containing known loss-of-function mutations for genes previously associated with circadian behavior in *D. melanogaster*. A list of all fly strains and their hybridization success (when applicable), is provided in Supplementary Table 1. When strains were not available (*vrille*) or not hybridizable (*Jet* and *timeless*), we used *D. melanogaster* deficiency strains, containing engineered chromosomal deletions spanning the region of a candidate gene, in addition to many other loci[79]. In the case of *timeless*, a deficiency strain did not hybridize either. The *Pdf* strain we used is the *pdf[01]* allele[68] in the Canton-S genetic background (provided by Charlotte Förster), as we were unable to hybridize the original *pdf[01]* strain. To confirm that the effect we found (Extended Data Fig. 3) was not due to a difference in *D. melanogaster* genetic background, we additionally compared it to the same parental Canton-S (denoted here *Dmel*CS[W], where "W" = Würzburg), which displayed qualitatively similar pre-dawn activity and circadian plasticity to our own *Dmel*CS strain (Extended Data Fig. 9).

Hybrid crosses and circadian candidate gene screening

To screen available circadian candidate genes, we created *D. melanogaster/D. sechellia* hybrids as previously described[80]. In brief, very young virgin females were crossed to males that were collected as virgins and aged in high density for 5-7 days. To increase their interactions, we pushed a plug into the vial to leave 2-3 cm height above the food surface. These crosses yield only sterile but viable males. This method does not allow us to test sex-linked candidate genes, such as the core transcriptional feedback loop member *period*[81], and the Pdf receptor gene, *Pdfr*[64]. We aimed to phenotype at least 15 hybrids of each genotype, but due to the strong reproductive isolation between species, some genotypes were difficult to cross to *D. sechellia*, resulting in a low sample size.

***Drosophila* activity monitoring**

For all activity monitoring, we used 1-3 day old males in the *Drosophila* activity monitor (DAM) system[82] stored in small incubators that continuously monitor light and temperature conditions (TriTech Research DT2-CIRC-TK). In brief, this system uses an infrared beam that bisects a 5 mm glass tube, in which the fly is stored, to record activity as the number of beam crosses per minute. Flies are stored in tubes with a 5% sucrose 2% agar w/v solution at one end, and capped with a cotton plug

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676 at the other. Each monitor records the activity of up to 32 flies simultaneously, and
677 multiple monitors are stored in a single incubator. For each genotype, we recorded
678 flies over at least two technical replicates.

679 　　　　All flies were first exposed to 7 days of 12:12 h LD, and then shifted to one
680 of four extended photoperiod cycles for an additional 7 days: 14:10, 16:8, 18:6, or
681 20:4 h LD to allow us to measure 12:12 h LD-associated (i.e., morning anticipation)
682 and extended photoperiod-associated behaviors (i.e., evening peak plasticity) for
683 each fly. For assessment of free-running period, flies were exposed to 7 days of
684 DD following 7 days of 12:12 h LD . For each photoperiod regime, we took the
685 average activity of the final 4 days of each week-long period. The initial 3 days were
686 considered an acclimation period, and were discarded. All subsequent analyses
687 were performed in R using the Rethomics package[83].

688 　　　　To quantify pre-dawn activity, the average normalized activity was
689 calculated for each fly in 30 min bins in the 3 h preceding dawn. To quantify morning
690 and evening peak times, peak activity was identified from the average activity of
691 each fly in 10 min bins during the last 4 days of both the 12:12 h LD and extended
692 photoperiod using custom R scripts (available at:
693 github.com/mshahandeh/circ_plasticity). First, a rolling triangular mean was
694 applied to smooth the data. The data was split into two 12 h sections, the first
695 spanning the time around lights-on and the second spanning the time around lights-
696 off (at least 3 h preceding and 3 h after for both). The global peak was identified
697 within each data set and recorded as the timing of the morning peak and evening
698 peak, respectively.

699

700 **Construction of transgenic lines**

701

702 ~2.4 kb upstream of the *Pdf* start codon was PCR amplified from *D. melanogaster*
703 (*Dmel*CS) or *D. sechellia* (*Dsec*28) genomic DNA and Gateway cloned into the
704 pDONR221 vector, sequenced-verified, and subcloned into both pHemmarG
705 (Addgene #31221) for CD4:tdGFP reporters, and pBPGUw (Addgene #17575) for
706 Gal4 drivers. Constructs were injected and integrated into the attP2 landing site
707 (chromosome 3) in the *D. melanogaster* genome by BestGene Inc.
708 Oligonucleotides used for cloning and sequence verification are listed in
709 Supplementary Table 3.

710

711 **Single molecule mRNA FISH**

712

713 We performed single molecule mRNA fluorescent in-situ hybridization to quantify
714 *pdf* mRNA expression at various time points in the s-LNvs and l-LNvs. We used
715 the *Pdf* probe library described previously[45] bound to the Cy5 fluorophore
716 (LubioScience), and adapted a published protocol[84]. Brains were imaged using an
717 inverted confocal microscope (Zeiss LSM 710 or 880) equipped with a 40× or 63×
718 oil immersion objective using fixed settings to maximize comparability of images
719 within experiments. Images were processed in Fiji and RNA spots were counted
720 using the Fiji macro RS-FISH[85]. No signal was detected outside of the LNv cell
721 bodies. We compared RNA spot counts between strains within photoperiod
722 treatments using a Wilcoxon rank-sum test followed by post-hoc correction for
723 multiple tests[86]. We did not compare between experiments as these flies were
724 dissected, stained and imaged separately. We repeated smFISH throughout the
725 morning peak to ensure replicability of the overall pattern of expression. We did not

pool these data as they are from a separate staining/imaging and may not be as comparable.

Immunofluorescence

For immunofluorescence of whole-mount *Drosophila* brains, 1-2 day old males were collected and acclimated to a specific photoperiod for 4 additional days. To standardize sampling times, we fixed these flies in 4% paraformaldehyde for 2 h at room temperature with gentle agitation prior to dissection. Brains were dissected and stained essentially as described[87]. Primary and secondary antibodies and concentrations used are provided in Supplementary Table 2. Brains were imaged using an inverted confocal microscope (Zeiss LSM 710 or 880) equipped with a 20× or 40× objective using fixed settings to maximize comparability of images. To quantify fluorescence, images were processed in Fiji, by first creating a maximum intensity projection Z-stack which was then thresholded to remove background signal[88]. Relative fluorescence was measured for each set of neurons by structure (i.e., LNv soma or s-LNv dorsal axonal projections) as integrated density of pixel intensity, and the average of both hemispheres was recorded for each brain. We quantified all images blind to treatment (species, genotype, and timepoint). We compared Pdf immunofluorescence between strains within photoperiod treatments using a Wilcoxon rank-sum test followed by post-hoc correction for multiple tests[86]. We did not compare between experiments as these flies were dissected, stained, and imaged separately. We repeated immunostainings throughout the morning peak to ensure replicability of the overall pattern of expression. These data cannot be pooled, however, as they are from a separate staining/imaging and produce different fluorescence measurements (arbitrary units).

To compare structural plasticity of s-LNv axonal projections between *D. melanogaster* and *D. sechellia*, we imaged the most dorsal projections during two timepoints in the light and dark phase (2 h and 14 h, respectively) at 40× with a 2× digital zoom. We performed a Scholl analysis, counting the number of axonal crossings with concentric 10 μm arcs using the Neuroanatomy Fiji plugin[89]. The number of axonal crossings was averaged per hemisphere for each brain and compared using a Wilcoxon rank-sum test. We performed this experiment in two replicates and pooled replicates for analysis as fluorescence intensity was not measured.

***Pdf* gene region sequence comparisons**

We Sanger-sequenced the *Pdf* gene region using the oligonucleotides listed in Supplementary Table 3. The sequences were assembled and aligned in SnapGene software (www.snapgene.com) using MUSCLE v3.8.1551[90] and then visually inspected for errors. When appropriate, sequences were translated to an amino acid alignment and visualized using Jalview[91]. For 5'-regulatory sequences, we used the R package phangorn to generate maximum likelihood trees[92], using the modelTest function to identify the best fitting substitution model and performing standard bootstrapping to obtain support values. The MEME program was used to discover putative regulatory motifs common across all sequences[47]. We restricted this analysis to the top ten significant motifs identified.

Population genetic analysis of the *Pdf* 5' regulatory sequences in *D. melanogaster* and *D. sechellia*

To detect genomic patterns of clinal adaptation in the *Pdf* 5' sequences, we used a dataset of single nucleotide variants in globally distributed *D. melanogaster* populations[48]. We calculated the average MAF for each population in this region, and for the same-sized region upstream of the start codon of 6 control neuropeptide genes. Spearman's rho was used to correlate MAF with the latitude of the capital city in each country where the populations were sampled (precise latitudes were not available).

Longevity assay

To test for photoperiod-dependent differences in lifespan, we acclimated 1-day old *Dmel*CS, *Dmel*OR, *Dsec*07 and *Dsec*28 males to either 12:12 h LD or 16:8 h LD conditions. We held these flies in vials containing wheat flour-yeast-fruit juice media to which we added an additional mixture of instant *Drosophila* medium (Formula 4-24 blue, Carolina bio-supply) mixed with noni juice for *D. sechellia* and apple cider vinegar for *D. melanogaster*. Flies were transferred to fresh vials every 3 days to prevent the media from drying out. We recorded for each week-day the number of vials in which a fly died until all flies among one treatment per strain were dead. No significant differences were detected between strains within species (Fisher's exact test, all $p > 0.05$), which were therefore pooled to represent the species for analysis. We compared cumulative survival probability using the R package 'survival'[93].

Copulation rate assays

To test for photoperiod-dependent differences in copulation rate, we first acclimated 1-day old virgin *Dmel*CS, *Dmel*OR, *Dsec*07 and *Dsec*28 males and females to either 12:12 h LD or 16:8 h LD conditions for 4 days. For our short-term assay, we aspirated single females into 25 mm food vials containing wheat flour-yeast-fruit juice media, returned them to their respective photoperiods, and allowed them to recover for 24 h. The following day, 30 min after lights-on, we aspirated a single male of the same genotype into each tube, pushed the plug into the vial so that pairs had 2 cm above the food surface, forcing them to interact. We observed for copulation for 2 h, recording successfully and unsuccessfully copulating pairs. For our long-term assay, we similarly acclimated flies for 4 days, but aspirated male-female pairs of the same genotype into vials and returned them to their respective photoperiods. We transferred these pairs to new vials every 24 h, and scored copulation success per day based on the presence of offspring. Flies that produced no offspring over 7 days were considered to have never mated. We observed no differences between strains within species in either experiment (Fisher's exact test, all $p = 1$), so these data were pooled to represent the species for analysis. Copulation frequencies within species between treatments were compared using a Wilcoxon rank-sum test.

References

1. Rieger, D., Stanewsky, R. & Helfrich-Forster, C. Cryptochrome, compound eyes, Hofbauer-Buchner eyelets, and ocelli play different roles in the entrainment and masking pathway of the locomotor activity rhythm in the fruit fly *Drosophila melanogaster*. *Journal of biological rhythms* **18**, 377-391, doi:10.1177/0748730403256997 (2003).

2. Auer, T. O., Shahandeh, M. P. & Benton, R. *Drosophila sechellia*: A Genetic Model for Behavioral Evolution and Neuroecology. *Annu Rev Genet* **55**, 527-554, doi:10.1146/annurev-genet-071719-020719 (2021).

3. Jones, C. D. The genetics of adaptation in *Drosophila sechellia*. *Genetica* **123**, 137-145 (2005).

4. Menegazzi, P. *et al.* Adaptation of Circadian Neuronal Network to Photoperiod in High-Latitude European Drosophilids. *Curr Biol* **27**, 833-839, doi:10.1016/j.cub.2017.01.036 (2017).

5. Bahn, J. H., Lee, G. & Park, J. H. Comparative analysis of Pdf-mediated circadian behaviors between *Drosophila melanogaster* and *D. virilis*. *Genetics* **181**, 965-975, doi:10.1534/genetics.108.099069 (2009).

6. Roca, I. T. *et al.* Shifting song frequencies in response to anthropogenic noise: a meta-analysis on birds and anurans. *Behavioral Ecology* **27**, 1269-1274, doi:10.1093/beheco/arw060 (2016).

7. Andrew, N. R., Hart, R. A., Jung, M. P., Hemmings, Z. & Terblanche, J. S. Can temperate insects take the heat? A case study of the physiological and behavioural responses in a common ant, *Iridomyrmex purpureus* (Formicidae), with potential climate change. *J Insect Physiol* **59**, 870-880, doi:10.1016/j.jinsphys.2013.06.003 (2013).

8. Caldwell, A. J., While, G. M. & Wapstra, E. Plasticity of thermoregulatory behaviour in response to the thermal environment by widespread and alpine reptile species. *Animal Behaviour* **132**, 217-227, doi:10.1016/j.anbehav.2017.07.025 (2017).

9. Muraro, N. I., Pirez, N. & Ceriani, M. F. The circadian system: plasticity at many levels. *Neuroscience* **247**, 280-293, doi:10.1016/j.neuroscience.2013.05.036 (2013).

10. Emerson, K. J., Bradshaw, W. E. & Holzapfel, C. M. Concordance of the circadian clock with the environment is necessary to maximize fitness in natural populations. *Evolution* **62**, 979-983, doi:10.1111/j.1558-5646.2008.00324.x (2008).

11. Wang, G. *et al.* Clock genes and environmental cues coordinate *Anopheles* pheromone synthesis, swarming, and mating. *Science* **371**, 411-415, doi:10.1126/science.abd4359 (2021).

12. Beaver, L. M. *et al.* Loss of circadian clock function decreases reproductive fitness in males of *Drosophila melanogaster*. *Proceedings of the National Academy of Sciences of the United States of America* **99**, 2134-2139, doi:10.1073/pnas.032426699 (2002).

13. Horn, M. *et al.* The Circadian Clock Improves Fitness in the Fruit Fly, *Drosophila melanogaster*. *Frontiers in physiology* **10**, 1374, doi:10.3389/fphys.2019.01374 (2019).

14. Hardeland, R. & Stange, G. Comparative studies on the circadian rhythms of locomotor activity of 40 *Drosophila* species. *Journal of Interdisciplinary Cycle Research* **4**, 353-359, doi:10.1080/09291017309359398 (1973).

15   Rieger, D., Peschel, N., Dusik, V., Glotz, S. & Helfrich-Forster, C. The ability to entrain to long photoperiods differs between 3 *Drosophila melanogaster* wild-type strains and is modified by twilight simulation. *Journal of biological rhythms* **27**, 37-47, doi:10.1177/0748730411420246 (2012).

16   Beauchamp, M. *et al.* Closely Related Fruit Fly Species Living at Different Latitudes Diverge in Their Circadian Clock Anatomy and Rhythmic Behavior. *Journal of biological rhythms* **33**, 602-613, doi:10.1177/0748730418798096 (2018).

17   Bywalez, W. *et al.* The dual-oscillator system of *Drosophila melanogaster* under natural-like temperature cycles. *Chronobiol Int* **29**, 395-407, doi:10.3109/07420528.2012.668505 (2012).

18   Lachaise, D. *et al. Historical biogeography of the Drosophila melanogaster species subgroup.* 159-225 (Plenun Press, 1988).

19   Dean, M. D. & Ballard, J. W. Linking phylogenetics with population genetics to reconstruct the geographic origin of a species. *Mol Phylogenet Evol* **32**, 998-1009, doi:10.1016/j.ympev.2004.03.013 (2004).

20   Nitabach, M. N. & Taghert, P. H. Organization of the *Drosophila* circadian control circuit. *Curr Biol* **18**, R84-93, doi:10.1016/j.cub.2007.11.061 (2008).

21   Hardin, P. E. Molecular genetic analysis of circadian timekeeping in *Drosophila. Adv Genet* **74**, 141-173, doi:10.1016/B978-0-12-387690-4.00005-2 (2011).

22   Hermann-Luibl, C. & Helfrich-Forster, C. Clock network in *Drosophila. Curr Opin Insect Sci* **7**, 65-70, doi:10.1016/j.cois.2014.11.003 (2015).

23   Dubowy, C. & Sehgal, A. Circadian Rhythms and Sleep in *Drosophila melanogaster. Genetics* **205**, 1373-1397, doi:10.1534/genetics.115.185157 (2017).

24   Stern, D. L. Identification of loci that cause phenotypic variation in diverse species with the reciprocal hemizygosity test. *Trends Genet* **30**, 547-554, doi:10.1016/j.tig.2014.09.006 (2014).

25   Vaze, K. M. & Helfrich-Forster, C. The Neuropeptide PDF Is Crucial for Delaying the Phase of *Drosophila*'s Evening Neurons Under Long Zeitgeber Periods. *Journal of biological rhythms* **36**, 442-460, doi:10.1177/07487304211032336 (2021).

26   Yoshii, T. *et al.* The neuropeptide pigment-dispersing factor adjusts period and phase of *Drosophila*'s clock. *J Neurosci* **29**, 2597-2610, doi:10.1523/JNEUROSCI.5439-08.2009 (2009).

27   Schlichting, M. *et al.* A Neural Network Underlying Circadian Entrainment and Photoperiodic Adjustment of Sleep and Activity in *Drosophila. J Neurosci* **36**, 9084-9096, doi:10.1523/JNEUROSCI.0992-16.2016 (2016).

28   Yoshii, T., Todo, T., Wulbeck, C., Stanewsky, R. & Helfrich-Forster, C. Cryptochrome is present in the compound eyes and a subset of *Drosophila*'s clock neurons. *J Comp Neurol* **508**, 952-966, doi:10.1002/cne.21702 (2008).

29   Koh, K., Zheng, X. & Sehgal, A. JETLAG resets the *Drosophila* circadian clock by promoting light-induced degradation of TIMELESS. *Science* **312**, 1809-1812, doi:10.1126/science.1124951 (2006).

30   Peschel, N., Chen, K. F., Szabo, G. & Stanewsky, R. Light-dependent interactions between the *Drosophila* circadian clock factors cryptochrome,

922   jetlag, and timeless. *Curr Biol* **19**, 241-247, doi:10.1016/j.cub.2008.12.042
923   (2009).

924   31   Gunawardhana, K. L. & Hardin, P. E. VRILLE Controls PDF Neuropeptide
925   Accumulation and Arborization Rhythms in Small Ventrolateral Neurons to
926   Drive Rhythmic Behavior in *Drosophila*. *Curr Biol* **27**, 3442-3453,
927   doi:10.1016/j.cub.2017.10.010 (2017).

928   32   Grima, B., Chelot, E., Xia, R. & Rouyer, F. Morning and evening peaks of
929   activity rely on different clock neurons of the *Drosophila* brain. *Nature* **431**,
930   869-873, doi:10.1038/nature02935 (2004).

931   33   Delventhal, R. *et al.* Dissection of central clock function in *Drosophila*
932   through cell-specific CRISPR-mediated clock gene disruption. *Elife* **8**,
933   e48308, doi:10.7554/eLife.48308 (2019).

934   34   Stoleru, D., Peng, Y., Agosto, J. & Rosbash, M. Coupled oscillators control
935   morning and evening locomotor behaviour of *Drosophila*. *Nature* **431**, 862-
936   868, doi:10.1038/nature02926 (2004).

937   35   Menegazzi, P. *et al.* A Functional Clock Within the Main Morning and
938   Evening Neurons of *D. melanogaster* Is Not Sufficient for Wild-Type
939   Locomotor Activity Under Changing Day Length. *Frontiers in physiology*
940   **11**, 229, doi:10.3389/fphys.2020.00229 (2020).

941   36   Hermann, C. *et al.* The circadian clock network in the brain of different
942   *Drosophila* species. *J Comp Neurol* **521**, 367-388, doi:10.1002/cne.23178
943   (2013).

944   37   Park, J. H. *et al.* Differential regulation of circadian pacemaker output by
945   separate clock genes in *Drosophila*. *Proceedings of the National Academy*
946   *of Sciences of the United States of America* **97**, 3608-3613,
947   doi:10.1073/pnas.97.7.3608 (2000).

948   38   Han, C., Jan, L. Y. & Jan, Y. N. Enhancer-driven membrane markers for
949   analysis of nonautonomous mechanisms reveal neuron-glia interactions in
950   *Drosophila*. *Proceedings of the National Academy of Sciences of the*
951   *United States of America* **108**, 9673-9678, doi:10.1073/pnas.1106386108
952   (2011).

953   39   Zhang, L. *et al.* DN1(p) circadian neurons coordinate acute light and PDF
954   inputs to produce robust daily behavior in *Drosophila*. *Curr Biol* **20**, 591-
955   599, doi:10.1016/j.cub.2010.02.056 (2010).

956   40   Liang, X., Holy, T. E. & Taghert, P. H. A Series of Suppressive Signals
957   within the Drosophila Circadian Neural Circuit Generates Sequential Daily
958   Outputs. *Neuron* **94**, 1173-1189 e1174, doi:10.1016/j.neuron.2017.05.007
959   (2017).

960   41   Ryczek, N., Lys, A. & Makalowska, I. The Functional Meaning of 5'UTR in
961   Protein-Coding Genes. *International journal of molecular sciences* **24**,
962   doi:10.3390/ijms24032976 (2023).

963   42   Herrero, A. *et al.* Coupling Neuropeptide Levels to Structural Plasticity in
964   *Drosophila* Clock Neurons. *Curr Biol* **30**, 3154-3166,
965   doi:10.1016/j.cub.2020.06.009 (2020).

966   43   Ma, D. *et al.* A transcriptomic taxonomy of *Drosophila* circadian neurons
967   around the clock. *Elife* **10**, doi:10.7554/eLife.63056 (2021).

968   44   Kula-Eversole, E. *et al.* Surprising gene expression patterns within and
969   between PDF-containing circadian neurons in *Drosophila*. *Proceedings of*
970   *the National Academy of Sciences of the United States of America* **107**,
971   13497-13502, doi:10.1073/pnas.1002081107 (2010).

45 Long, X., Colonell, J., Wong, A. M., Singer, R. H. & Lionnet, T. Quantitative mRNA imaging throughout the entire *Drosophila* brain. *Nat Methods* **14**, 703-706, doi:10.1038/nmeth.4309 (2017).

46 Fernandez, M. P., Berni, J. & Ceriani, M. F. Circadian remodeling of neuronal circuits involved in rhythmic behavior. *PLOS Biol.* **6**, e69, doi:10.1371/journal.pbio.0060069 (2008).

47 Bailey, T. L. & Elkan, C. Fitting a mixture model by expectation maximization to discover motifs in biopolymers. *Proc Int Conf Intell Syst Mol Biol* **2**, 28-36 (1994).

48 Bergland, A. O., Tobler, R., Gonzalez, J., Schmidt, P. & Petrov, D. Secondary contact and local adaptation contribute to genome-wide patterns of clinal variation in *Drosophila melanogaster. Mol. Ecol.* **25**, 1157-1174, doi:10.1111/mec.13455 (2016).

49 Arguello, J. R., Laurent, S. & Clark, A. G. Demographic History of the Human Commensal *Drosophila melanogaster. Genome Biol. Evol.* **11**, 844-854, doi:10.1093/gbe/evz022 (2019).

50 Khatib, L., Subasi, B. S., Fishman, B., Kapun, M. & Tauber, E. Unveiling Subtle Geographical Clines: Phenotypic Effects and Dynamics of Circadian Clock Gene Polymorphisms. *Biology* **12** (2023).

51 Lamaze, A. *et al.* A natural timeless polymorphism allowing circadian clock synchronization in "white nights". *Nat Commun* **13**, 1724, doi:10.1038/s41467-022-29293-6 (2022).

52 Tauber, E. *et al.* Natural selection favors a newly derived timeless allele in *Drosophila melanogaster. Science* **316**, 1895-1898, doi:10.1126/science.1138412 (2007).

53 Sawyer, L. A. *et al.* Natural variation in a *Drosophila* clock gene and temperature compensation. *Science* **278**, 2117-2120, doi:10.1126/science.278.5346.2117 (1997).

54 Costanzo, K. S., Schelble, S., Jerz, K. & Keenan, M. The effect of photoperiod on life history and blood-feeding activity in *Aedes albopictus* and *Aedes aegypti* (Diptera: Culicidae). *J Vector Ecol* **40**, 164-171, doi:10.1111/jvec.12146 (2015).

55 Li, J. C. & Xu, F. Influences of light-dark shifting on the immune system, tumor growth and life span of rats, mice and fruit flies as well as on the counteraction of melatonin. *Biol Signals* **6**, 77-89, doi:10.1159/000109112 (1997).

56 Amin, M. R., Suh, S. J. & Kwon, Y. J. Effects of photoperiod and hibernation duration on the lifespan of *Bombus terrestris. Entomological Research* **37**, 89-94, doi:10.1111/j.1748-5967.2007.00058.x (2007).

57 Ducatez, S., Sol, D., Sayol, F. & Lefebvre, L. Behavioural plasticity is associated with reduced extinction risk in birds. *Nature ecology & evolution* **4**, 788-793, doi:10.1038/s41559-020-1168-8 (2020).

58 Courtier-Orgogozo, V., Arnoult, L., Prigent, S. R., Wiltgen, S. & Martin, A. Gephebase, a database of genotype-phenotype relationships for natural and domesticated variation in Eukaryotes. *Nucleic Acids Res.* **48**, D696-D703, doi:10.1093/nar/gkz796 (2020).

59 Auer, T. O. *et al.* Olfactory receptor and circuit evolution promote host specialization. *Nature* **579**, 402-408, doi:10.1038/s41586-020-2073-7 (2020).

60   Leary, G. P. *et al.* Single mutation to a sex pheromone receptor provides adaptive specificity between closely related moth species. *Proc. Natl. Acad. Sci. USA* **109**, 14081-14086, doi:10.1073/pnas.1204661109 (2012).

61   Wisotsky, Z., Medina, A., Freeman, E. & Dahanukar, A. Evolutionary differences in food preference rely on Gr64e, a receptor for glycerol. *Nature Neuroscience* **14**, 1534-1541, doi:10.1038/nn.2944 (2011).

62   Baldwin, M. W. *et al.* Sensory biology. Evolution of sweet taste perception in hummingbirds by transformation of the ancestral umami receptor. *Science* **345**, 929-933, doi:10.1126/science.1255097 (2014).

63   Seeholzer, L. F., Seppo, M., Stern, D. L. & Ruta, V. Evolution of a central neural circuit underlies *Drosophila* mate preferences. *Nature* **559**, 564-569, doi:10.1038/s41586-018-0322-9 (2018).

64   Shafer, O. T. *et al.* Widespread receptivity to neuropeptide PDF throughout the neuronal circadian clock network of *Drosophila* revealed by real-time cyclic AMP imaging. *Neuron* **58**, 223-237, doi:10.1016/j.neuron.2008.02.018 (2008).

65   Carroll, S. B. Evo-devo and an expanding evolutionary synthesis: a genetic theory of morphological evolution. *Cell* **134**, 25-36, doi:S0092-8674(08)00817-9 [pii]10.1016/j.cell.2008.06.030 (2008).

66   Ding, K. *et al.* Imaging neuropeptide release at synapses with a genetically engineered reporter. *Elife* **8**, e46421, doi:10.7554/eLife.46421 (2019).

67   Hidalgo, S., Anguiano, M., Tabuloc, C. A. & Chiu, J. C. Seasonal cues act through the circadian clock and pigment-dispersing factor to control EYES ABSENT and downstream physiological changes. *Curr Biol* **33**, 1006-1008, doi:10.1016/j.cub.2023.02.028 (2023).

68   Renn, S. C., Park, J. H., Rosbash, M., Hall, J. C. & Taghert, P. H. A pdf neuropeptide gene mutation and ablation of PDF neurons each cause severe abnormalities of behavioral circadian rhythms in *Drosophila*. *Cell* **99**, 791-802, doi:10.1016/s0092-8674(00)81676-1 (1999).

69   Duhart, J. M. *et al.* Circadian Structural Plasticity Drives Remodeling of E Cell Output. *Curr Biol* **30**, 5040-5048, doi:10.1016/j.cub.2020.09.057 (2020).

70   Prieto-Godino, L. L., Schmidt, H. R. & Benton, R. Molecular reconstruction of recurrent evolutionary switching in olfactory receptor specificity. *Elife* **10**, e69732, doi:10.7554/eLife.69732 (2021).

71   Prieto-Godino, L. L. *et al.* Evolution of Acid-Sensing Olfactory Circuits in Drosophilids. *Neuron* **93**, 661-676, doi:10.1016/j.neuron.2016.12.024 (2017).

72   Alvarez-Ocana, R. *et al.* Odor-regulated oviposition behavior in an ecological specialist. *Nat Commun* **14**, 3041, doi:10.1038/s41467-023-38722-z (2023).

73   Ibba, I., Angioy, A. M., Hansson, B. S. & Dekker, T. Macroglomeruli for fruit odors change blend preference in *Drosophila*. *Die Naturwissenschaften* **97**, 1059-1066, doi:10.1007/s00114-010-0727-2 (2010).

74   Dekker, T., Ibba, I., Siju, K. P., Stensmyr, M. C. & Hansson, B. S. Olfactory shifts parallel superspecialism for toxic fruit in *Drosophila melanogaster* sibling, *D. sechellia*. *Curr Biol* **16**, 101-109 (2006).

75   Reisenman, C. E., Wong, J., Vedagarbha, N., Livelo, C. & Scott, K. Taste adaptations associated with host specialization in the specialist *Drosophila sechellia*. *J Exp Biol* **226**, jeb244641, doi:10.1242/jeb.244641 (2023).

76 Karpati, Z., Deutsch, F., Kiss, B. & Schmitt, T. Seasonal changes in photoperiod and temperature lead to changes in cuticular hydrocarbon profiles and affect mating success in *Drosophila suzukii*. *Sci Rep* **13**, 5649, doi:10.1038/s41598-023-32652-y (2023).

77 Razafimandimbison, S. G., McDowell, T. D., Halford, D. A. & Bremer, B. Origin of the pantropical and nutriceutical *Morinda citrifolia* L. (Rubiaceae): comments on its distribution range and circumscription *Journal of Biogeography* **37**, 520-529 (2010).

78 Nair, S., Bahn, J. H., Lee, G., Yoo, S. & Park, J. H. A Homeobox Transcription Factor Scarecrow (SCRO) Negatively Regulates Pdf Neuropeptide Expression through Binding an Identified cis-Acting Element in *Drosophila melanogaster*. *Mol Neurobiol* **57**, 2115-2130, doi:10.1007/s12035-020-01874-w (2020).

79 Cook, R. K. *et al.* The generation of chromosomal deletions to provide extensive coverage and subdivision of the *Drosophila melanogaster* genome. *Genome Biol* **13**, R21, doi:10.1186/gb-2012-13-3-r21 (2012).

80 Shahandeh, M. P. & Turner, T. L. The complex genetic architecture of male mate choice evolution between *Drosophila* species. *Heredity (Edinb)* **124**, 737-750, doi:10.1038/s41437-020-0309-9 (2020).

81 Zhou, J., Yu, W. & Hardin, P. E. CLOCKWORK ORANGE Enhances PERIOD Mediated Rhythms in Transcriptional Repression by Antagonizing E-box Binding by CLOCK-CYCLE. *PLOS Genet.* **12**, e1006430, doi:10.1371/journal.pgen.1006430 (2016).

82 Chiu, J. C., Low, K. H., Pike, D. H., Yildirim, E. & Edery, I. Assaying locomotor activity to study circadian rhythms and sleep parameters in *Drosophila*. *J Vis Exp*, e2157, doi:10.3791/2157 (2010).

83 Geissmann, Q., Garcia Rodriguez, L., Beckwith, E. J. & Gilestro, G. F. Rethomics: An R framework to analyse high-throughput behavioural data. *PLOS ONE* **14**, e0209331, doi:10.1371/journal.pone.0209331 (2019).

84 Yuan, Y., Padilla, M. A., Clark, D. & Yadlapalli, S. Streamlined single-molecule RNA-FISH of core clock mRNAs in clock neurons in whole mount *Drosophila* brains. *Frontiers in physiology* **13**, 1051544, doi:10.3389/fphys.2022.1051544 (2022).

85 Bahry, E. *et al.* RS-FISH: precise, interactive, fast, and scalable FISH spot detection. *Nat Methods* **19**, 1563-1567, doi:10.1038/s41592-022-01669-y (2022).

86 Holm, S. A Simple Sequentially Rejective Multiple Test Procedure. *Scandinavian Journal of Statistics* **6**, 65-70 (1979).

87 Ostrovsky, A., Cachero, S. & Jefferis, G. Clonal analysis of olfaction in *Drosophila*: immunochemistry and imaging of fly brains. *Cold Spring Harb Protoc* **2013**, 342-346, doi:10.1101/pdb.prot071720 (2013).

88 Schindelin, J. *et al.* Fiji: an open-source platform for biological-image analysis. *Nat Methods* **9**, 676-682, doi:10.1038/nmeth.2019 (2012).

89 Ferreira, T. A. *et al.* Neuronal morphometry directly from bitmap images. *Nat Methods* **11**, 982-984, doi:10.1038/nmeth.3125 (2014).

90 Edgar, R. C. MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Res.* **32**, 1792-1797, doi:10.1093/nar/gkh34032/5/1792 [pii] (2004).

91 Procter, J. B. *et al.* Alignment of Biological Sequences with Jalview. *Methods Mol Biol* **2231**, 203-224, doi:10.1007/978-1-0716-1036-7_13 (2021).

92 Schliep, K. P. phangorn: phylogenetic analysis in R. *Bioinformatics* **27**, 592-593, doi:10.1093/bioinformatics/btq706 (2011).

93 Therneau, T. M. & Grambsch, P. M. *Modeling survival data : extending the Cox model.* (Springer, 2000).

94 Matute, D. R., Gavin-Smyth, J. & Liu, G. Variable post-zygotic isolation in *Drosophila melanogaster*/*D. simulans* hybrids. *J Evol Biol* **27**, 1691-1705, doi:10.1111/jeb.12422 (2014).

Figure legends

Fig. 1. *D. sechellia* displays reduced circadian plasticity and lower morning activity than *D. melanogaster*.

a, Left: phylogeny of the *Drosophila melanogaster* sub-group. Right: modern ranges of the focal species of this study, *D. melanogaster* (*Dmel*) and *D. sechellia* (*Dsec*), are indicated by the shaded regions (blue and orange, respectively) on the map, with the approximate collection sites of the wild-type strains used.

b, Approximate seasonal photoperiod variation at the collection sites of the *D. melanogaster* (left) and *D. sechellia* strains (right).

c, Top: mean normalized activity of two *D. melanogaster* (CS and OR, blue) and two *D. sechellia* (07 and 28, orange) strains under the indicated photoperiods. Plots depict normalized average activity of the last 4 days of a 7-day photoperiod, for extended photoperiods, following 7 days of 12:12 h LD. Vertical dashed lines indicate the average timing of the evening peak for each strain. Here and elsewhere, yellow and grey bars indicate timing of lights-on and lights-off, respectively. Overall, *D. sechellia* strains were slightly less active than *D. melanogaster* strains. Bottom: box plots depict evening peak time quantifications for individual flies under each photoperiod. Here and elsewhere, box plots show the median (bold line), interquartile range (box), and whiskers represent the final quartiles. All data points are shown overlaid on box plots. Outliers are points that fall beyond the box plot whiskers. Letters indicate significant differences: $p < 0.05$ (pairwise Wilcoxon test with Bonferroni correction). Sample sizes (numbers of individual flies) are as follows: 12:12 h LD: CS (18), OR (21), 07 (24), 28 (19); 14:10 h LD: CS (22), OR (22), 07 (19), 28 (13); 16:8 h LD: CS (18), OR (21), 07 (24), 28 (19); 18:10 h LD: CS (22), OR (23), 07 (21), 28 (11); 20:4 h LD: CS (21), OR (22), 07 (19), 28 (18).

d, Mean normalized activity of *D. melanogaster* and *D. sechellia* strains under a 12:12 h LD cycle during the morning activity peak (same data from **c.** restricted to -6 to 6 h). Left: plots depict average activity of the last 4 days of a 7-day recording period. Dashed boxes highlight the pre-dawn period, 3 h before lights-on. Right: mean normalized activity of individual flies within this pre-dawn period. Letters indicate significant differences: $p < 0.001$ (pairwise Wilcoxon test with Bonferroni correction). Sample sizes as follows: CS (89), OR (93), 07 (95), 28 (91).

e, Double plotted actograms depicting the transition from the last 2 days of 12:12 h LD to the first 2 days of constant darkness (DD) for each strain. Dashed boxes highlight morning activity peak period during DD, 3 h before and after subjective lights-on. Sample sizes as follows: CS (29), OR (32), 07 (29), 28 (22). Grey bars indicate timing of subjective lights-on during DD.

f, Morning peak time, in hours from lights-on, for individual flies from **d**. Letters indicate significant differences: $p < 0.001$ (pairwise Wilcoxon test with Bonferroni correction).

Fig. 2. A screen of circadian clock genes reveals distinct genetic architectures underlying interspecific differences in plasticity and morning activity.

a, Molecular components of the circadian clock in *D. melanogaster*.

b, Crossing schemes used to generate hemizygous test hybrids, heterozygous control hybrids, and hemizygous *D. melanogaster* flies in a controlled genetic background. The fourth ("dot") chromosome is not shown.

c, Schematics illustrating the sought-after behavioural phenotypes of test hybrids, and anticipated phenotypes of control hybrids and hemizygous *D. melanogaster* controls: positive candidate gene test hybrids, but not corresponding controls, will display reduced circadian plasticity and/or reduced morning activity.

d, Mean normalized activity of the indicated control and hybrid genotypes under a 16:8 h LD cycle. Plots depict average activity of the last 4 days of a 7-day extended photoperiod, following 7 days of 12:12 h LD. Vertical dashed lines indicate the average timing of the evening peak for each strain. Sample sizes as follows: CS^W (16), Pdf^{01}/CS^W (47), 07/CS^W (25), 28/CS^W (23), 07/Pdf^{01} (37), 28/Pdf^{01}(40), 07 (24), 28 (19). Full screen results are shown in Extended Data Fig. 3.

e, Evening peak time for the flies depicted in **d**. Asterisks indicate significant differences: * = $p < 0.05$ and *** = $p < 0.001$ (Wilcoxon tests with Bonferroni correction). Comparisons were made only between control and test hybrids of the same genetic backgrounds.

f, Mean normalized activity of the indicated genotypes under a 12:12 h LD cycle, illustrating the screened mutations displaying reduced morning anticipation in test hybrids. Dashed boxes highlight the pre-dawn area used to quantify morning anticipation. Full screen results are shown in Extended Data Fig. 4.

g, Mean normalized pre-dawn activity for the genotypes in **f.** Asterisks indicate significant differences: ** = $p < 0.01$ and *** = $p < 0.001$ (Wilcoxon tests comparing each test hybrid to the control hybrid strain (07/w^{1118}) with Bonferroni correction). Top right: the circadian molecular network in which screen hits for morning anticipating are highlighted in green; genes in light grey were unable to be tested (see Methods).

Fig. 3. Species-specific *cis*-regulatory elements impact *Pdf* expression and have the potential to impact behaviour.

a, Schematic of the circadian clock network in *D. melanogaster*, which is composed of ~75 neurons in each brain hemisphere that are divided into distinct groups. The groups comprising the morning and evening cells are indicated on the left and right hemispheres, respectively. Pdf-positive small and large ventrolateral neurons (s-LNvs and l-LNvs) are highlighted in purple.

b, Immunofluorescence for Pdf (green) and Cadherin-N (magenta) on whole-mount brains of the indicated strains at 2 h under 12:12 h LD conditions.

c, Representative images of reporter expression visualized by Pdf (left) and GFP immunofluorescence (middle) showing faithful labelling (merge, right) of s-LNvs and l-LNvs for both the *D. melanogaster* (top) and *D. sechellia* (bottom) *Pdf* 5'-regulatory regions. For **b** and **c**, scale bars, 100 μm.

d, Top: Schematic illustrating the average activity patterns of *D. melanogaster* and *D. sechellia* during behaviourally relevant time points (labelled with

arrowheads) within the evening peaks under 12:12 h LD conditions where we analyzed *Pdf* expression. These summaries were derived from the data in Fig. 1c-d. Bottom-left: representative images of GFP immunofluorescence in the l-LNvs for the *D. melanogaster* and *D. sechellia Pdf* 5'-regulatory sequence-GFP reporter strains under 12:12 h LD at one time point (10 h). Bottom-right: GFP fluorescence quantifications at 5 time points spanning the evening activity peak period.

e, Top: Schematic illustrating the average activity patterns of *D. melanogaster* and *D. sechellia* during behaviourally relevant time points (labelled with arrowheads) within the evening peaks under 16:8 h LD conditions where we analyzed *Pdf* expression. Bottom-left: representative images of GFP reporter immunofluorescence in the l-LNvs for the *D. melanogaster* and *D. sechellia* strains under 16:8 h LD at one time point (10 h). Bottom-right: GFP fluorescence quantifications at 5 time points spanning the evening activity peak period.

f, Top: Schematic illustrating the average activity patterns of *D. melanogaster* and *D. sechellia* during behaviourally relevant time points (labelled with arrowheads) within the morning peaks under 12:12 h LD conditions where we analyzed *Pdf* expression. Bottom-left: representative images of GFP immunofluorescence in the s-LNv axon terminals for the *D. melanogaster* and *D. sechellia Pdf* reporter strains at one time point (-2 h). We observed the same general pattern when measuring fluorescence in the s-LNv soma. Bottom-right: GFP fluorescence quantifications at 4 time points spanning the morning activity peak period. For **d-f**, N = 5 for each strain and time point. Despite weak signal in some *D. sechellia* images, the projections were easily identified in threshholded images.

g, Mean normalized activity of the indicated *D. melanogaster* genotypes under a 16:8 h LD cycle. Plots depict average activity of the last 4 days of a 7-day extended photoperiod, following 7 days of 12:12 h LD. Vertical dashed lines indicate the average timing of the evening peak for each strain. Sample sizes as follows: *DmelPdf-Gal4/+* (28), *DsecPdf-Gal4/+* (28), *UAS-Pdf^{RNAi}/+* (16), *DmelPdf-Gal4/UAS-Pdf^{RNAi}* (31), *DsecPdf-Gal4/UAS-Pdf^{RNAi}* (24).

h, Evening peak time for the flies shown in **g**.

i, Mean normalized activity of the indicated *D. melanogaster* genotypes under a 12:12 h LD cycle. Plots depict average activity of the last 4 days of a 7-day recording period. Dashed boxes highlight the pre-dawn period, 3 h before lights-on. Sample sizes as follows: *DmelPdf-Gal4/+* (31), *DsecPdf-Gal4/+* (29), UAS-*Pdf^{RNAi}/+* (47), *DmelPdf-Gal4/UAS-Pdf^{RNAi}* (34), *DsecPdf-Gal4/UAS-Pdf^{RNAi}* (30).

j, Mean normalized pre-dawn activity for the genotypes in **i**.

For **d-f**, Lines connect medians of each time point within genotypes. Scale bars, 10 μm.

For **d-f, h** and **j**, Asterisks indicate significant differences: * = $p < 0.05$ and *** = $p < 0.001$ (Wilcoxon tests with Bonferroni correction).

Fig. 4. *Pdf* expression differences observed between *D. melanogaster* and *D. sechellia*.

a, Left: representative images of *Pdf* smFISH in the l-LNv soma in the CS and 07 strains under 12:12 h LD at one time point (14 h), with RNA spots (green)

1278   identified by RS-FISH. Right: quantifications of RNA spots at 5 time points
1279   spanning the evening activity peak period.
1280 **b**, Left: representative images of *Pdf* smFISH in the l-LNv soma in the CS and
1281   07 strains under 16:8 h LD at one time point (14 h), with RNA spots (green)
1282   identified by RS-FISH. Left: quantifications of RNA spots in the CS and 07
1283   strains under 16:8 h LD at 5 time points spanning the evening activity peak
1284   period.
1285 **c**, Left: representative images of smFISH in the s-LNv soma for the CS and 07
1286   strains under 12:12 h LD at one time point (-2 h) with RNA spots identified
1287   by RS-FISH. Right: quantifications of RNA spots in the 07 and CS strains at
1288   4 time points spanning the pre-dawn period.
1289 **d**, Left: representative images of Pdf immunofluorescence in the s-LNv axon
1290   terminals for the CS and 07 strains at one time point (-4 h). Right:
1291   quantifications of Pdf signals at 4 time points spanning the morning activity
1292   peak period.
1293 **e**, Left: representative images of Pdf immunofluorescence in the s-LNv cell
1294   bodies for the CS and 07 strains at one time point (2 h). Right: quantifications
1295   of Pdf signals at 4 time points spanning the morning activity peak period.
1296 **f**, Left: representative images of Pdf immunofluorescence in the s-LNv axon
1297   terminals for the CS and 07 strains during the day (2 h) and night (14 h).
1298   Right: quantifications of axonal branching complexity quantifications.
1299 For **a**-**f**, N = 5 brains per strain per time point. Plotted values are the average of
1300   left and right hemispheres. Lines connect medians of each time point within
1301   genotypes. Asterisks indicate significant differences: * = $p < 0.05$ and *** =
1302 $p < 0.001$ (Wilcoxon tests with Bonferroni correction). All scale bars, 10 μm.
1303
1304 **Fig. 5. Evidence for selection on the *Pdf* 5'-regulatory sequence and fitness**
1305 **effects of circadian plasticity loss.**
1306 **a**, A midpoint rooted maximum likelihood phylogeny of *Pdf* 5'-regulatory
1307   sequences from 10 *D. melanogaster* (blue), 6 *D. sechellia* (orange), and 5
1308   *D. simulans* (grey) strains. Bootstrap support values are shown for key
1309   internal nodes (100 bootstraps). The species tree is depicted at the bottom
1310   left for comparison.
1311 **b**, A motif analysis of the *Pdf* 5'-regulatory sequences from **a**. The *Pdf* start
1312   codon is on the right, and the different types of predicted regulatory motifs
1313   for each species are shown as distinct colored boxes on the + or – strand.
1314   Species-specific diagrams depict all motifs found for each species. Motifs
1315   observed in *D. melanogaster* and *D. simulans* but absent in all *D. sechellia*
1316   sequences are marked with downward facing arrows; one motif unique to all
1317   sequences of *D. sechellia* is marked with an upward facing arrow. No
1318   variation in motif location was observed among the 6 *D. sechellia* strains.
1319 **c**, Left: the 13 *D. melanogaster* populations selected from Ref.[48] and the
1320   approximate latitude of their collection sites. Right: plot of minor allele
1321   frequency in the *Pdf* 5'-regulatory sequences (blue) of these 13 populations
1322   against latitude, revealing a significant positive correlation (Spearman's rho
1323   = 0.77). No such correlation is observed for the putative 5'-regulatory
1324   sequences of 6 control neuropeptide genes (grey); detailed data points are
1325   shown in Extended Data Fig. 8.
1326 **d**, Average minor allele frequency of variable sites from the analysis in **c** in the
1327   laboratory *D. sechellia* and *D. melanogaster* lines from **a**. Variable sites are

significantly underrepresented in *D. sechellia* relative to *D. melanogaster* strains (p <0.05, Fisher's exact test).

e, Cumulative survival probability for *D. melanogaster* (*Dmel*CS + *Dmel*OR, blue) and *D. sechellia* (*Dsec*07 + *Dsec* 28, orange) maintained at 12:12 h LD (circles) or 16:8 h LD (squares) for 52 days. No significant differences were observed between strains of the same species by photoperiod, and were thus pooled (Fisher's exact test, all p > 0.05). Pooled data were compared between photoperiods within species using a log-rank test.

f, Percent of copulating pairs observed for *D. melanogaster* (*Dmel*CS + *Dmel*OR, blue) and *D. sechellia* (07 + 28, orange) after 2 h for flies acclimated to 12:12 h LD (left) compared to 16:8 h LD (right, Wilcoxon test). No significant differences were observed between strains of the same species by photoperiod, and were thus pooled (Fisher's exact test, all p = 1). Sample sizes as follows: *D. melanogaster* 12:12 h LD (22), *D. melanogaster* 16:8 h LD (26), *D. sechellia* 12:12 h LD (29), and *D. sechellia* 16:8 h LD (32).

g, Same as **f**, except after 3 days (left) or 7 days (right). Sample sizes as follows: *D. melanogaster* 12:12 h LD (26), *D. melanogaster* 16:8 h LD (31), *D. sechellia* 12:12 h LD (36), and *D. sechellia* 16:8 h LD (38).

h, Schematic of the main findings of this work: the equatorial species *D. sechellia* has lost circadian plasticity, in part through *cis*-regulatory changes in the *Pdf* 5' region, which lead to less dynamic expression.

For **c, e-g**, Asterisks indicate significant differences: * = p < 0.05, ** = p < 0.01, and *** = p < 0.001.

Extended Data Fig. 1. *D. melanogaster* and *D. sechellia* strains exhibit ~24 h periods. Periodogram analysis from 5 days of constant darkness (DD) for *D. melanogaster* (CS and OR) and *D. sechellia* (07 and 28) strains. Period estimates: CS (24.36 h), OR (23.45 h), 07 (23.16 h), 28 (23.57 h). Sample sizes as in Fig. 1e

Extended Data Fig. 2. Tropical *D. melanogaster*, *D. simulans* and *D. mauritiana* strains display prominent circadian plasticity and morning anticipation.

a, Top: mean normalized activity of two recently-collected strains of *D. melanogaster* (LZV L72 and LZV L76, from the Lower Zambezi Valley), *D. simulans* (MD221 and MD242, from Madagascar) and *D. mauritiana* (*Dmau*90 and *Dmau*91, from Mauritius) under the indicated photoperiods. Plots depict average activity of the last 4 days of a 7-day recording period. Dashed lines highlight the average evening peak time. Bottom: evening peak time for these flies. The orange line depicts the median evening peak time of individuals of both *D. sechellia* strains (from Fig. 1c). Sample sizes as follows: LZV L72 (29), LZV L74 (46), MD221 (27), MD242(34), *Dmau*90 (19), *Dmau*91 (28).

b, Top: mean normalized activity of the same strains as in **a** under a 12:12 h LD cycle (same as in **a**). Plots depict average activity of the last 4 days of a 7-day recording period. Dashed boxes highlight the pre-dawn period, 3 h before lights-on. Bottom: Mean normalized activity of individual flies within this pre-dawn period. Sample sizes as follows: LZV L72 (41), LZV L74 (61), MD221 (57), MD242 (52), *Dmau*90 (33), *Dmau*91 (34). The orange line

depicts the median pre-dawn activity of individuals of both *D. sechellia* strains (from Fig. 1d).

Extended Data Fig. 3. Screen results for the genetic basis of interspecific differences in circadian plasticity.

a, Mean normalized activity of the indicated control and hybrid genotypes under a 16:8 h LD cycle. Plots depict average activity of the last 4 days of a 7-day extended photoperiod, following 7 days of 12:12 h LD. Vertical dashed lines indicate the average timing of the evening peak for each strain. Sample sizes as follows: w^{1118} (22), 07/w^{1118} (53), 07/*CCHa1* (34), 07/*Clk* (20), 07/*cwo* (16), 07/*cyc* (33), 07/*Cry* (21), 07/*Fer2* (17), 07/*Hr38* (50), 07/*ITP* (23), 07/*Jet* (22), 07/*Pdf* (37), 07/*PDP1* (4), 07/*Rh7* (16), 07/*scro* (22), 07/*vri* (23).

b, Evening peak time for the flies depicted in **a**. Asterisks indicate significant differences: ** $p < 0.01$ and *** = $p < 0.001$ (Wilcoxon tests comparing each test hybrid to the control hybrid strain (07/w^{1118}) with Bonferroni correction). n.s. = not significantly different. The orange line marks the median evening peak delay of the *D. sechellia* parental strain (07).

c, Mean normalized activity of the indicated control and hybrid genotypes under a 16:8 h LD cycle. Plots depict average activity of the last 4 days of a 7-day extended photoperiod, following 7 days of 12:12 h LD. Vertical dashed lines indicate the average timing of the evening peak for each strain. Sample sizes as follows: w^{1118} (22), 28/w^{1118} (31), 28/*CCHa1* (4), 28/*Clk* (17), 28/*cwo* (27), 28/*cyc* (52), 28/*Cry* (28), 28/*Fer* (8), 28/*Hr38* (23), 28/*Itp* (25), 28/*Jet* (16), 28/*Pdf* (40), 28/*scro* (31), 28/*vri* (29), 28 (19).

d, Evening peak time for the flies depicted in **c**. Asterisks indicate significant differences: * = $p < 0.05$ (Wilcoxon tests comparing each test hybrid to the control hybrid strain (28/w^{1118}) with Bonferroni correction). Red asterisks denote a significant increase in circadian plasticity. n.s. = not significantly different. The orange line marks the median evening peak delay of the *D. sechellia* parental strain (28).

e, Mean normalized activity of the indicated hemizygous *D. melanogaster* genotypes that displayed an effect in both hybrid backgrounds under a 16:8 h LD cycle. Plots depict average activity of the last 4 days of a 7-day extended photoperiod, following 7 days of 12:12 h LD. Vertical dashed lines indicate the average timing of the evening peak for each strain. Sample sizes as follows: w^{1118} (22), *Clk*/w^{1118} (6), *Pdf*/w^{1118} (37).

f, Evening peak time for the flies depicted in **e**. Asterisks indicate significant differences: *** = $p < 0.001$ and ** = $p < 0.01$ (Wilcoxon tests comparing each test hemizygote to the control strain (w^{1118}) with Bonferroni correction). Red asterisks denote a significant increase in circadian plasticity.

g, Summary of the overlapping hits. *A priori*, we considered the strongest candidates would display a reduction in circadian plasticity in both *Dsec*07 and *Dsec*28 hybrids, but not in w^{1118} hemizygotes; only *Pdf* fulfilled these criteria. Note: the *Clk* mutant used in this screen is a dominant negative allele, and thus we expect the behaviour of *Clk*/w^{1118} mutants to display a total *Clk* loss-of-function phenotype. Interestingly, we do not observe this phenotype in either test hybrid genotype, indicating divergence of the *Clk* locus between species. If and how this divergence affects behaviour requires subsequent investigation.

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1429 **Extended Data Fig. 4. Screen results for the genetic basis of interspecific**
1430 **differences in morning anticipation.**

1431 **a**, Mean normalized activity of the indicated genotypes under a 12:12 h LD
1432 cycle. Dashed boxes highlight the pre-dawn area used to quantify morning
1433 anticipation. Sample sizes as follows: w^{1118} (78), 07/w^{1118} (69), 07/*CCHa1*
1434 (21), 07/*Clk* (19), 07/*cwo* (43), 07/*cyc* (43), 07/*Cry* (26), 07/*Fer* (18), 07/*Hr38*
1435 (87), 07/*ITP* (23), 07/*Jet* (49), 07/*Pdf* (42), 07/*PDP1* (23), 07/*Rh7* (33),
1436 07/*scro* (58), 07/*vri* (28), 07 (40).

1437 **b**, Mean normalized pre-dawn activity for the genotypes in A. Asterisks indicate
1438 significant differences: ** = $p < 0.01$ and *** = $p < 0.001$ (Wilcoxon tests
1439 comparing each test hybrid to the control hybrid strain (07/w^{1118}) with
1440 Bonferroni correction). Red asterisks denote a significant increase in
1441 circadian plasticity. The orange line marks the median pre-dawn activity of
1442 the *D. sechellia* parental strain (07).

1443 **c**, Mean normalized activity of the indicated genotypes under a 12:12 h LD
1444 cycle. Dashed boxes highlight the pre-dawn area used to quantify morning
1445 anticipation. Sample sizes as follows: w^{1118} (78), 28/w^{1118} (22), 28/*CCHa1*
1446 (4), 28/*Clk* (64), 28/*cwo* (20), 28/*cyc* (56), 28/*Cry* (66), 28/*Fer* (5), 28/*Hr38*
1447 (43), 28/*ITP* (25), 28/*Jet* (22), 28/*Pdf* (21), 28/*PDP1* (14), 28/*scro* (38), 28/*vri*
1448 (33), 28 (36).

1449 **d**, Mean normalized pre-dawn activity for the genotypes in C. Asterisks indicate
1450 significant differences: * = $p < 0.05$ and *** = $p < 0.001$ (Wilcoxon tests
1451 comparing each test hybrid to the control hybrid strain (28/w^{1118}) with
1452 Bonferroni correction). The orange line marks the median pre-dawn activity
1453 of the *D. sechellia* parental strain (28).

1454 **e**, Mean normalized activity of the indicated hemizygous *D. melanogaster*
1455 genotypes that displayed an effect in a hybrid background under a 12:12 h
1456 LD cycle. Dashed boxes highlight the pre-dawn area used to quantify
1457 morning anticipation. (07 or 28) under a 12h LD cycle. Plots depict average
1458 activity of the last 4 days of a 7 day recording period. Dashed boxes highlight
1459 the pre-dawn period, 3 h before lights-on. Sample sizes as follows: w^{1118}
1460 (78), *Clk*/w^{1118} (25), *cyc*/w^{1118} (31), *Cry*/w^{1118} (32), *Hr38*/w^{1118} (25), *vri*/w^{1118}
1461 (46).

1462 **f**, Mean normalized pre-dawn activity for the genotypes in **e**. Asterisks indicate
1463 significant differences: * = $p < 0.05$ (Wilcoxon tests comparing each test
1464 hemizygote to the control strain (w^{1118}) with Bonferroni correction). Red
1465 asterisks denote a significant increase in pre-dawn activity.

1466 **g**, Summary of the overlapping hits from each of the above genotypes. *A priori*,
1467 we considered the strongest candidates to display a reduction in morning
1468 anticipation in *Dsec*07 and *Dsec*28 hybrids, but not in w^{1118} hemizygotes.
1469 See Extended Data Fig. 3g legend for notes on the *Clk/w^{1118}* mutant
1470 phenotype.

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1472 **Extended Data Fig. 5. The predicted Pdf protein sequence is highly**
1473 **conserved between *D. melanogaster*, *D. sechellia* and *D. simulans*.**
1474 Alignment of the predicted Pdf protein sequence of 10 *D. melanogaster*, 6 *D.*
1475 *sechellia* and 5 *D. simulans* strains. Amino acid residues are coloured by similarity,
1476 periods indicate conserved amino acid residues and letters indicate variable

residues. No fixed differences are observed between species. The consensus sequence is displayed at the bottom.

Extended Data Fig. 6. Validation of differential *Pdf* transcript depletion by smFISH.
Left: representative smFISH images for one genetic control (*UAS-Pdf^RNAi^/+*), *DmelPdf-Gal4/UAS-Pdf^RNAi^* and *DsecPdf-Gal4/UAS-Pdf^RNAi^* strains with RNA spots identified by RS-FISH. Right: RNA spot quantifications. N = 5 for each genotype.

Extended Data Fig. 7. Pdf immunofluorescence in the l-LNvs of *D. melanogaster* and *D. sechellia* during the evening peak.
    a, Left: representative images of Pdf immunofluorescence in the l-LNv soma for the CS and 07 strains under 12:12 h LD at one time point (6 h). Right: quantifications of Pdf signals at 5 time points spanning the evening activity peak period.
    b, Quantifications of Pdf signals at 5 time points spanning the evening activity peak period in the l-LNv soma for the CS and 07 strains under 16:8 h LD.

Extended Data Fig. 8. Correlations of minor allele frequency and latitude in *D. melanogaster* populations for control neuropeptide genes.
Each plot depicts the correlation between minor allele frequency and latitude for the putative 5'-regulatory region (~2.4 kb upstream of the start codon) for the indicated neuropeptide genes. For reference, the analysis for the *Pdf* 5'-regulatory region (from Fig. 5c) is shown on the first plot. Values for Spearman's rho and Bonferroni corrected p-values are listed above each plot.

Extended Data Fig. 9. Qualitatively similar circadian plasticity and pre-dawn activity in two Canton-S strains.
    a, Mean normalized activity of two *Canton-S* (CS and CS^W^) strains collected under a 16:8 h LD cycle. Plots depict average activity of the last 4 days of a 7-day extended photoperiod, following 7 days of 12:12 h LD. Vertical dashed lines indicate the average timing of the evening peak for each strain. Sample sizes as follows: CS (18),CS^W^ (16).
    b, Evening peak time for the flies depicted in **a** is shown for each strain. No significant difference was observed between strains (Wilcoxon test).
    c, Mean normalized activity of two Canton-S strains (CS and CS^W^) under a 12:12 h LD cycle. Plots depict average activity of the last 4 days of a 7-day recording period. Dashed boxes highlight the pre-dawn period, 3 h before lights-on. Sample sizes as follows: CS (29), CS^W^ (42).
    d, The average activity for flies shown in **c** within the previously indicated pre-dawn period, mean normalized pre-dawn activity, is shown for each strain. Asterisks indicate significant differences: ** = $p < 0.01$ (Wilcoxon test).

Supplementary Tables

Supplementary Table 1. *Drosophila* strains.

Stock Name	Genotype	Species	Reference	Hybridized
Canton-S (CS)	*wt*	*D. melanogaster*	RRID:BDSC_64349	N/A
Oregon-R (OR)	*wt*	*D. melanogaster*	RRID:BDSC_2376	N/A
LZV L72	*wt*	*D. melanogaster*	[94]	N/A
LZV L76	*wt*	*D. melanogaster*	[94]	N/A
LZV A10	*wt*	*D. melanogaster*	[94]	N/A
MD221	*wt*	*D. simulans*	[94]	N/A
MD242	*wt*	*D. simulans*	[94]	N/A
LZV L47	*wt*	*D. simulans*	[94]	N/A
*Dsim*04	*wt*	*D. simulans*	DSSC 14021-0251.004	N/A
*Dsim*196	*wt*	*D. simulans*	DSSC 14021-0251.196	N/A
*Dsec*07	*wt*	*D. sechellia*	DSSC 14021-0248.07	N/A
*Dsec*28	*wt*	*D. sechellia*	DSSC 14021-0248.28	N/A
*Dsec*19	*wt*	*D. sechellia*	DSSC 14021-0248.19	N/A
*Dsec*21	*wt*	*D. sechellia*	DSSC 14021-0248.21	N/A
*Dsec*30	*wt*	*D. sechellia*	DSSC 14021-0248.30	N/A
*Dsec*31	*wt*	*D. sechellia*	DSSC 14021-0248.31	N/A
*Dmau*90	*wt*	*D. mauritiana*	DSSC 14021-0241.90	N/A
*Dmau*91	*wt*	*D. mauritiana*	DSSC 14021-0241.91	N/A
w^{1118}	w^{1118}	*D. melanogaster*	RRID:BDSC_3605	07 and 28
Würzburg Canton-S (CS^W)	*wt*	*D. melanogaster*	Gift of C. Förster	07 and 28
Pdf^{01}	Pdf^{01} (in CS^W background)	*D. melanogaster*	Gift of C. Förster	07 and 28
Hr38	*w*; dpy ov1 bw1 Hr3856/CyO, P{GAL4-twi.G}2.2, P{UAS-2xEGFP}AH2.2*	*D. melanogaster*	RRID:BDSC_76590	07 and 28
Clk	Clk^{Jrk}	*D. melanogaster*	RRID:BDSC_80927	07 and 28
PDP1	w^{1118}; *Pdp13135/TM3, Sb1*	*D. melanogaster*	RRID:BDSC_80925	07 and 28
cyc	cyc^{01}	*D. melanogaster*	RRID:BDSC_80929	07 and 28
scro	$scro^{Z211}$	*D. melanogaster*	RRID:BDSC_81875	07 and 28
cwo	w^{1118}; *PBac{RB}cwoe04207/TM6B, Tb1*	*D. melanogaster*	RRID:BDSC_85593	07 and 28
Cry	w^{1118}; *ss cryb*	*D. melanogaster*	RRID:BDSC_80921	07 and 28
Df(vri)	w^{1118}; *Df(2L)Exel6011, P{XP-U}Exel6011/CyO*	*D. melanogaster*	RRID:BDSC_7497	07 and 28
Rh7	*y1; Rh70*	*D. melanogaster*	RRID:BDSC_83716	07, not 28
Jet^c	*y1 w*; jetc*	*D. melanogaster*	RRID:BDSC_27641	No
Jer	*y1 w*; jetr*	*D. melanogaster*	RRID:BDSC_27641	No

Df(Jet)	w[1118]; Df(2L)ED7853, P{3'.RS5+3.3'}ED7853/SM6a	D. melanogaster	RRID:BDSC_24124	07 and 28
CCHa1	y[1] w[*]; Mi{y[+mDint2]=MIC}CCHa11[MI09190]	D. melanogaster	RRID:BDSC_51261	07, poorly with 28
ITP	w[1118]; PBac{w[+mC]=RB}ITP[e02889]/CyO	D. melanogaster	RRID:BDSC_85570	07 and 28
tim	y[1] w[*]; tim[01]	D. melanogaster	RRID:BDSC_80922	No
Df(tim)	y1 w*; Df(2L)drm-P2, P{lacW}ND-PDSWk10101/SM6b	D. melanogaster	RRID:BDSC_6507	No
sr	y[1]w[*]; P{neoFRT}82Bsr[155]/TM3, Sb[1]	D. melanogaster	RRID:BDSC_36535	No
Fer2	w[1118]; PBac{RB}Fer2e03248	D. melanogaster	RRID:BDSC_86028	07, poorly with 28
DmelPdf-Gal4	y1 w67c23;;P{Dmel-Pdf-Gal4}attP2	D. melanogaster	This work	N/A
DsecPdf-Gal4	y1 w67c23;;P{Dsec-Pdf-Gal4}attP2	D. melanogaster	This work	N/A
DmelPdf-CD4:tdGFP	y1 w67c23;;P{DmelPdf-CD4:tdGFP}attP2	D. melanogaster	This work	N/A
DsecPdf-CD4:tdGFP	y1 w67c23;;P{DsecPdf-CD4:tdGFP}attP2	D. melanogaster	This work	N/A
UAS-Pdf[RNAi]	y[1] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.JF01820}attP2	D. melanogaster	RRID:BDSC_25802	N/A

1523

1524 **Supplementary Table 2. Antibodies.**

Antibody	Dilution	Reference/source	Identifier
Mouse anti-Pdf C7	1:400	Developmental Studies Hybridoma Bank	AB_760350 AB_2315084
Rabbit anti-GFP	1:1000	Invitrogen	Cat #A-11122
Rat anti-DNCadherin (DN-Ex#8) (Cadherin-N)	1:25	Developmental Studies Hybridoma Bank	AB_528121
Goat Alexa488 anti-mouse	1:100	Molecular Probes, Jackson Immunoresearch	AB_2338840
Goat Alexa488 anti-rabbit	1:100	Molecular Probes, Jackson Immunoresearch	AB_2338049
Donkey Cy5 anti-rat	1:200	Molecular Probes, Jackson Immunoresearch	AB_2340672

1525

1526 **Supplementary Table 3. Oligonucleotides.**

Purpose	PCR product	Sequences (5'-3') sense / antisense
Pdf gene sequencing	CDS + 5'-regulatory region I	cattttccttcgacgcacca / ccaactgccgagctagctat
	5'-regulatory region II	aaacttaatagctagctcggcag / aatgtggctgcatggaaagt
	5'-regulatory region III	aaacattgacccaactccgc / gtttcatccttaccagcgcc
Entry clone for Pdf 5'-regulatory region Gateway cloning	5' regulatory region (~2.4 kb upstream of start codon)	ggggacaactttgtacaaaaaagttggcaccggtccacatagtgcccagta / ggggacaactttgtacaagaaagttggcaatagtccgaggagctggaagg

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34
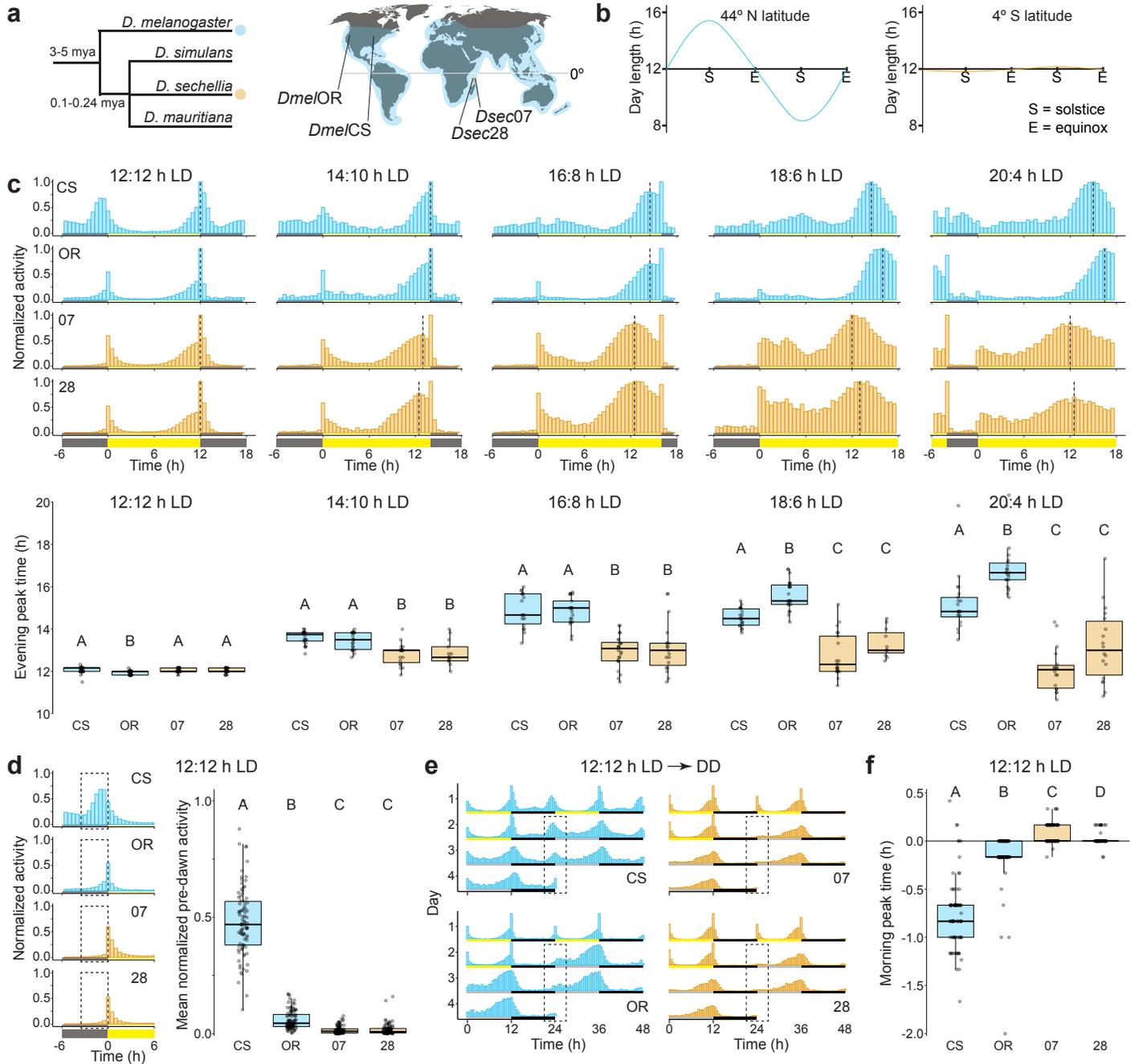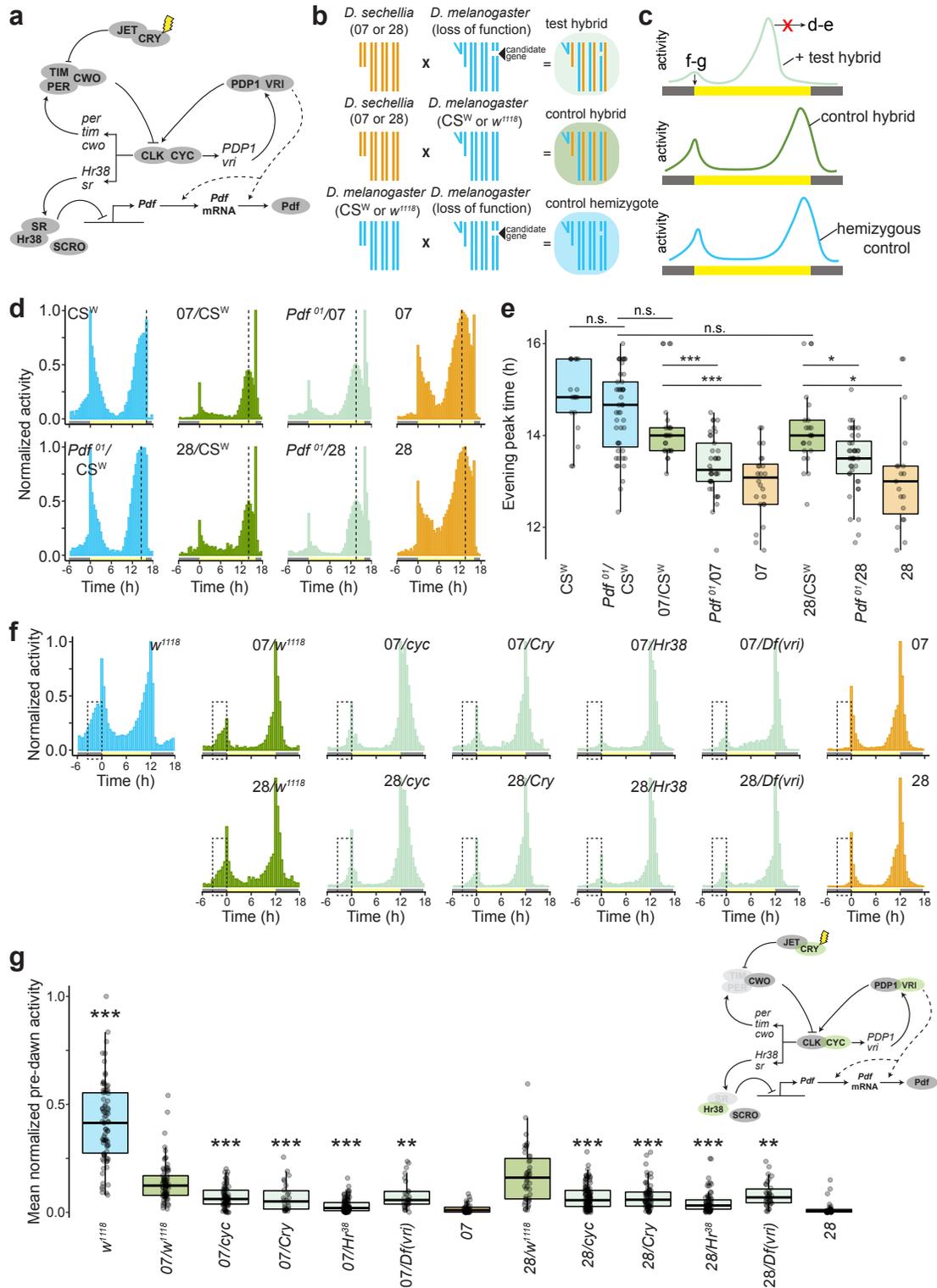
Figure 1

Figure 2

Figure 3
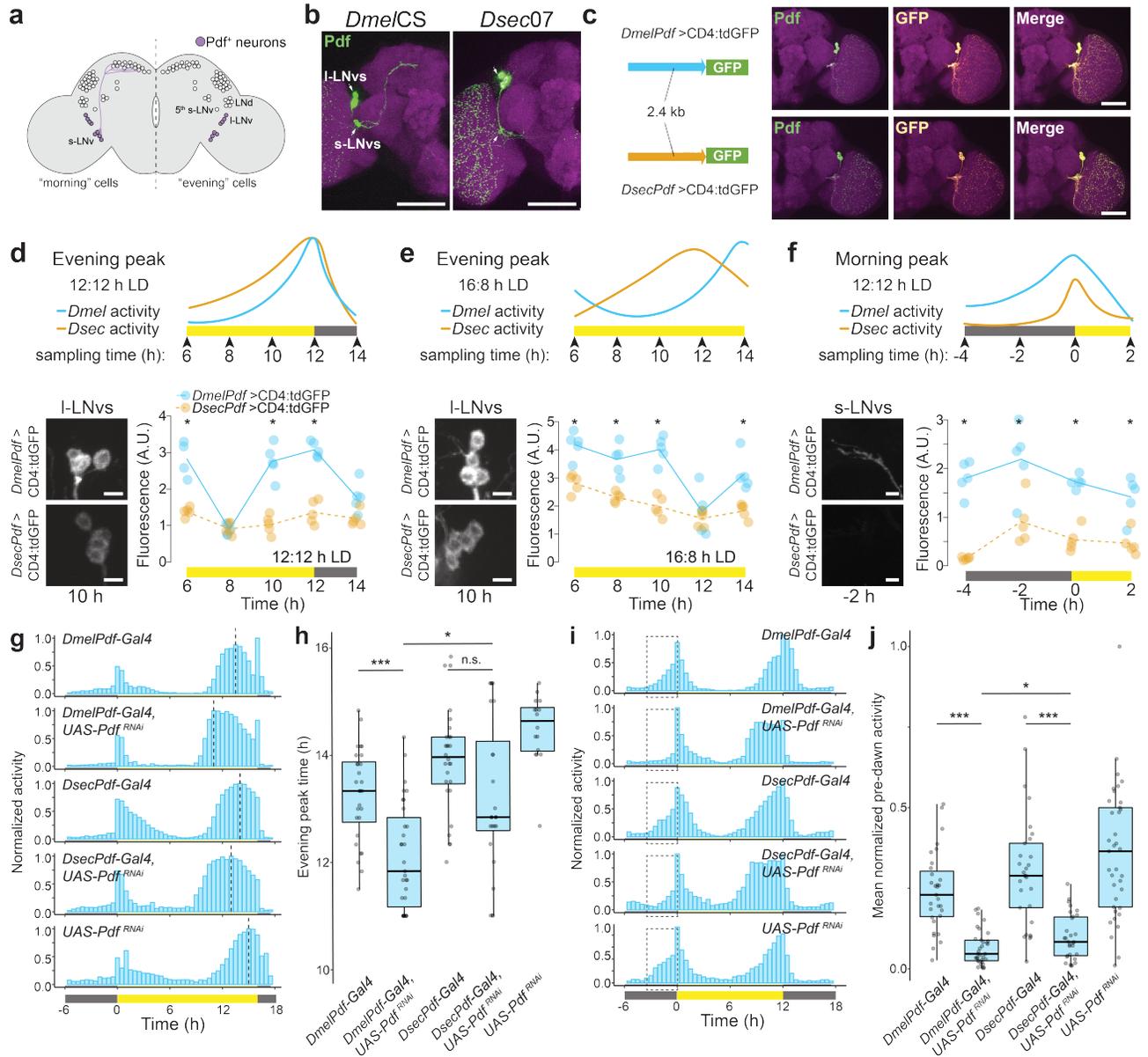
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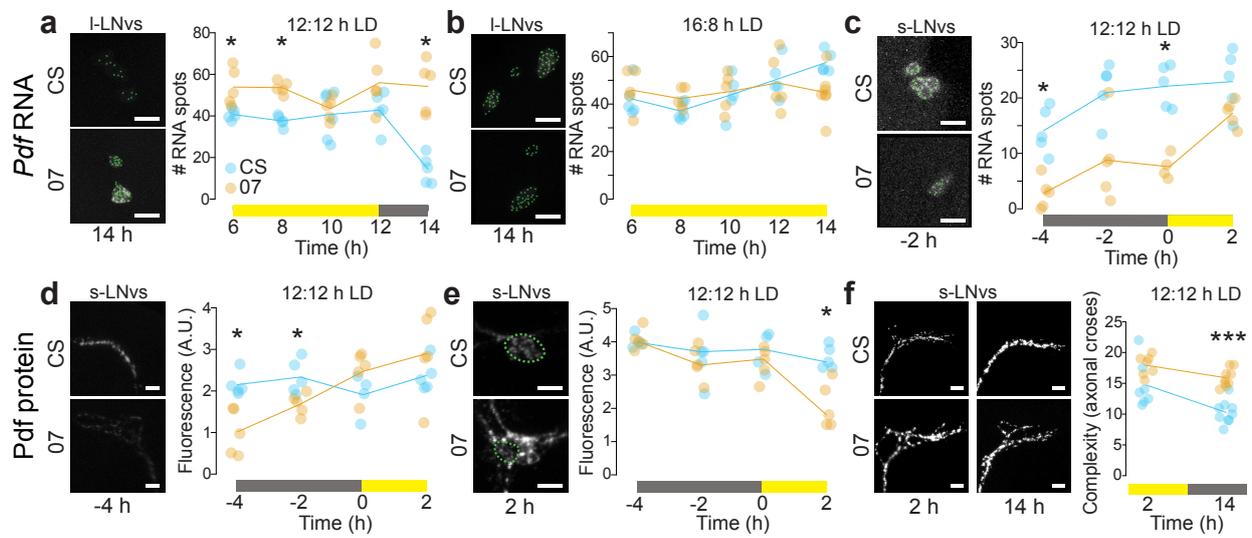
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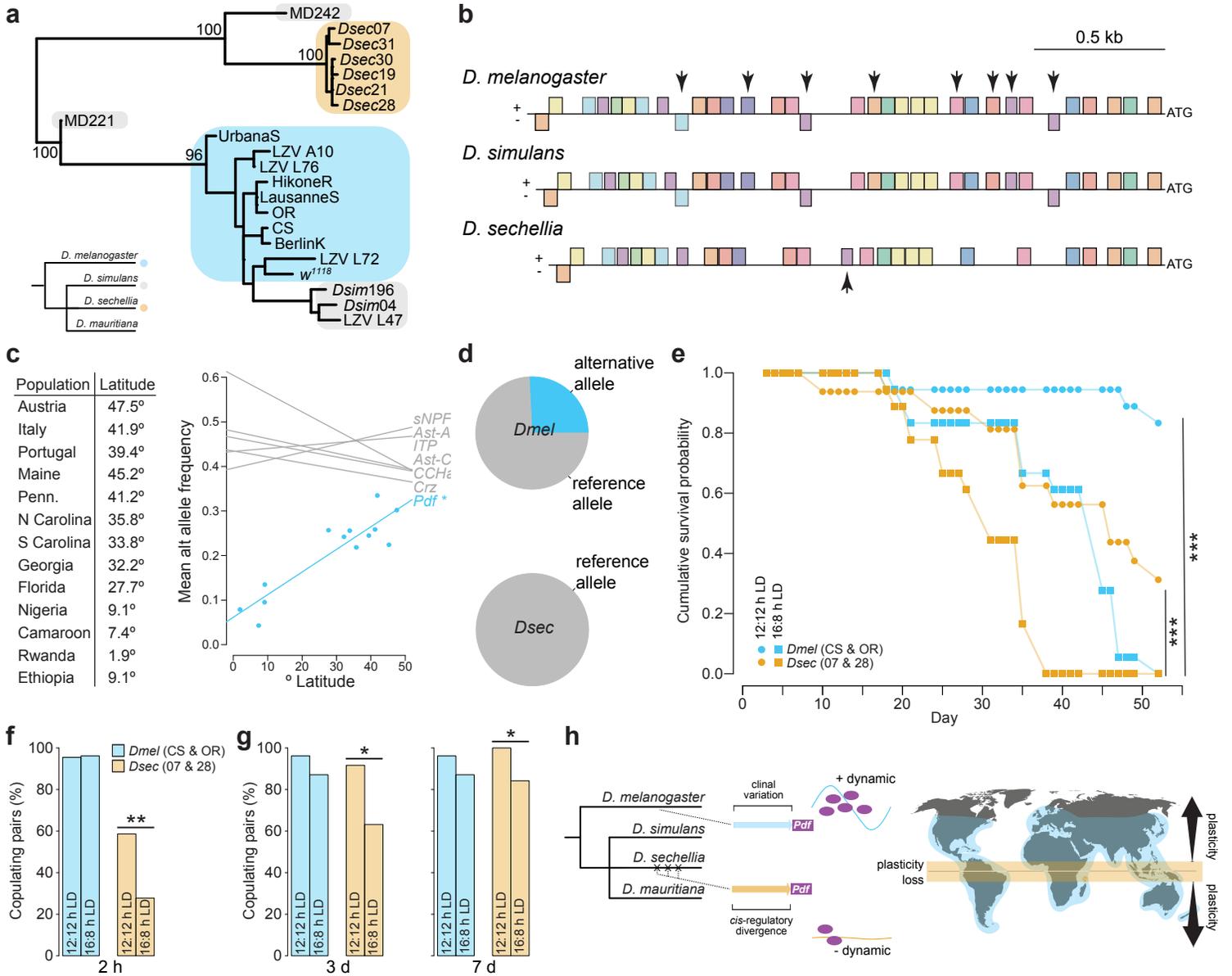
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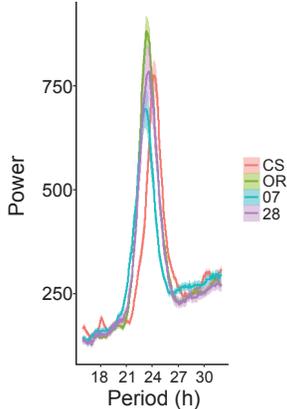
ED Figure 2
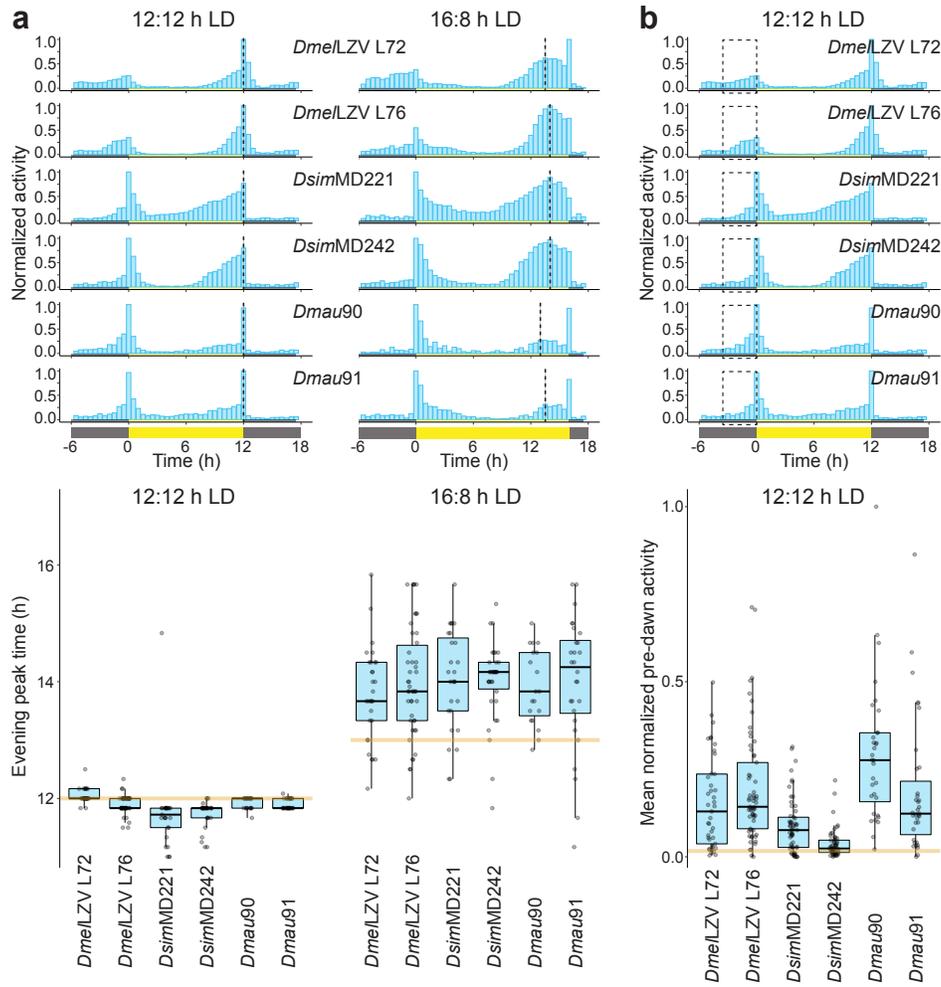
ED Figure 3
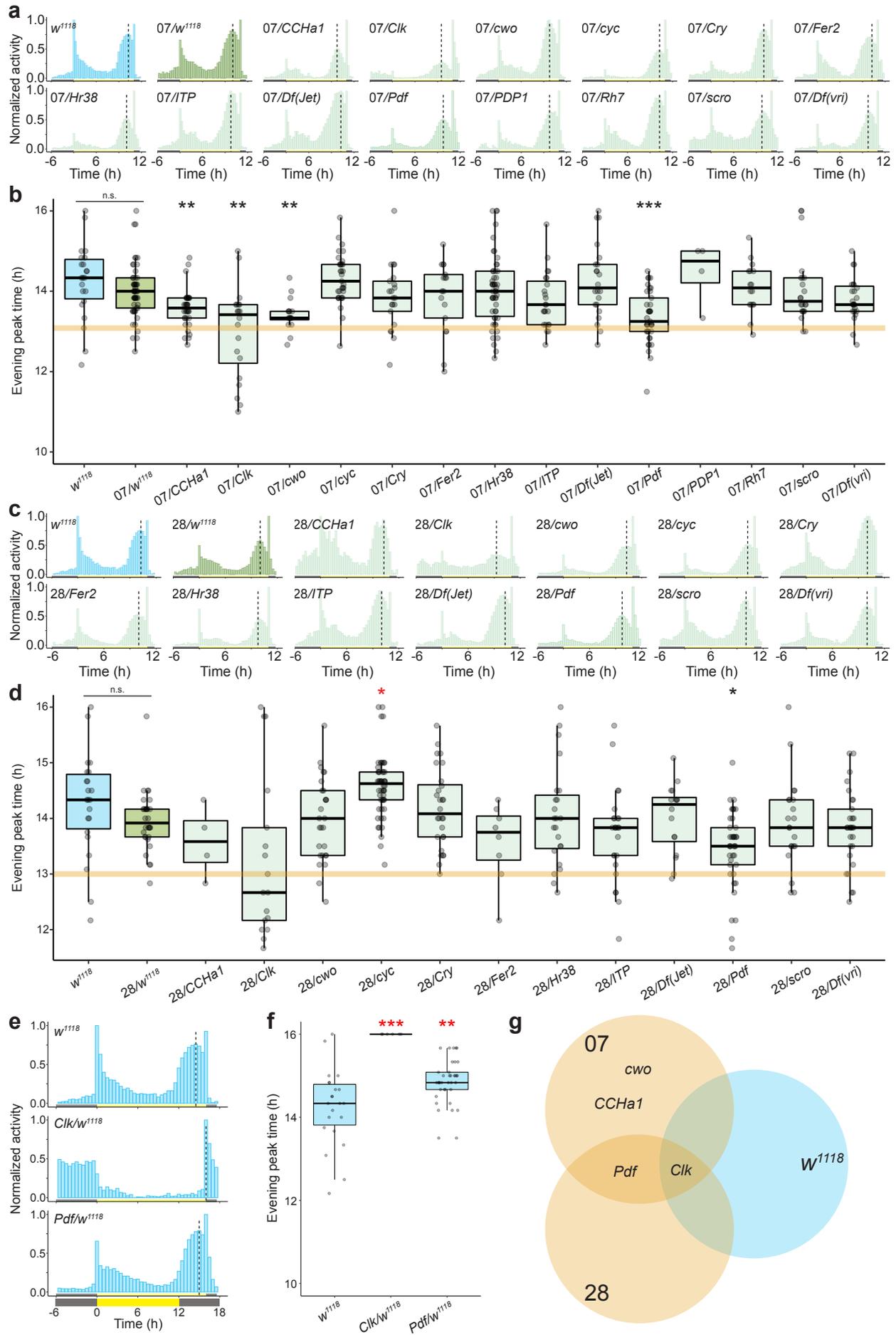
ED Figure 4

ED Figure 5

ED Figure 6

l-LNvs

DsecPdf-Gal4, *DsecPdf-Gal4,* *DmelPdf-Gal4,* *UAS-Pdf* RNAi *UAS-Pdf* RNAi *UAS-Pdf* RNAi

2 h

RNA spots

UAS-Pdf RNAi

DmelPdf-Gal4,
UAS-Pdf RNAi

DsecPdf-Gal4,
UAS-Pdf RNAi

ED Figure 7

a Pdf protein

l-LNvs

CS

07

6 h

a 12:12 h LD

Fluorescence (A.U.)

CS
07

Time (h)

b 16:8 h LD

Fluorescence (A.U.)

Time (h)

ED Figure 8

ED Figure 9