Peer Review File

Manuscript Title: Circadian plasticity evolves via regulatory changes in a neuropeptide gene

Reviewer Comments & Author Rebuttals

Reviewer Reports on the Initial Version:

Referee #1 (Remarks to the Author):

This work addresses the extremely important question of how the behavioral plasticity necessary for the global distribution of a species has evolved on a molecular genetic basis. Near the equator, species find the same environmental conditions throughout the year, whereas at higher latitudes they are subject to strong seasonal fluctuations. Therefore, species that have spread from the tropics to higher latitudes have developed a certain behavioral plasticity to survive. So far, very little is known about its molecular basis.

To decipher one of the underlying mechanisms, the authors used the circadian system of fruit flies, which is exceptionally well characterized in the cosmopolitan Drosophila melanogaster. Its close relative D. sechellia is endemic to islands near the equator. Genetic comparison of the two species thus promises answers to the burning question. Through a series of clever experiments, the authors identified the neuropeptide gene Pigment-dispersing factor (Pdf) as an important locus for evolution of circadian plasticity. The main results of the paper are the following:
1) D. sechellia cannot adapt their evening activity to long photoperiods demonstrating that the circadian system of D. sechellia is less plastic than that of D. melanogaster.
2) By crossing D. sechellia to different D. melanogaster circadian clock mutants, the authors identified the Pdf gene as the main gene necessary for circadian plasticity.
3) A 2.4 kb regulatory region immediately upstream of the Pdf gene 5' start codon is responsible for this plasticity (tested by reporter gene expression and downregulation of Pdf by Gal4-RNAi under control of the Pdf upstream regulatory region).
4) The Pdf upstream regulatory region is responsible for the expression level of Pdf mRNA: D. sechellia have lower Pdf expression levels than D. melanogaster.
5) The Pdf upstream regulatory region from D. sechellia appears to have lost transcription factor binding sites. Constructing a maximum likelihood phylogeny shows that the Pdf upstream regulatory sequences from D. sechellia form a monophyletic group that is different from D. melanogaster and D. simulans.
6) There is evidence for latitude-based selection the D. melanogaster Pdf 5'-regulatory region, causing circadian plasticity at higher latitudes.
7) Flies carrying the D. sechellia Pdf upstream regulatory region have a lower mating success under long photoperiods und consequently a lower fitness.

Strength of the study: The authors show for the first time that differences in Pdf expression caused by natural selection on the Pdf 5'-regulatory region increase behavioral plasticity and reproductive fitness in D. melanogaster under long photoperiods. Conversely, the loss of putative transcription factor binding sites in the Pdf 5'-regulatory region leads to a loss of circadian plasticity in D. sechellia and a lower fitness under long photoperiods.

Overall, the study is novel and of considerable significance. The taken approach is valid, the data are of high quality, and the story is logically presented. The abstract and the introduction are clear and appropriate. The conclusions are robust and well justified and appropriate credit is given to previous work. I could also not detect any flaws in the statistics.

Suggestions for improvement:
The study has a single weakness: the results of PDF-immunocytochemistry (and partly mRNA expression) do not completely match the results gained with the transcriptional reporters. Nevertheless, I agree with the authors that this might be explained by additional endogenous factors acting on PDF and think that this does not diminish the significance of this study (further explanations below).

The authors place great emphasis on determining Pdf mRNA and PDF peptide levels in the somata of the large lateral neurons (l-LNv), probably because they assume that these cells act as evening oscillators of the circadian clock (statement in line 277). However, this is a misunderstanding and needs to be corrected. The l-LNvs are neither morning nor evening oscillators, but rather function in the light input pathway to the clock. Some authors also call them "arousal neurons" (e.g. McCarthy et al., J Neurosci 22, 2011). They can be activated by light, which leads to increased PDF secretion (Sheeba et al. J Neurophysiol 99, 2008). The increased PDF secretion from the l-LNvs delays the oscillations of the evening oscillators, which encompass the dorsolateral clock neurons (LNds). The delay in LNd oscillations in turn leads to a delay in evening activity under long photoperiods (Menegazzi et al., Curr Biol 27, 2017; Schlichting et al., Curr Biol 29, 2019).

The problem is that one cannot measure increased PDF secretion by measuring PDF mRNA in the somata of the l-LNvs during the time of the evening peak (Figure 4 a, b). The latter measurements just give a rough estimate of the amount of Pdf mRNA during this time. The l-LNvs appear to produce high amounts of PDF peptide that is stored in their somata (Park et al., PNAS 97, 2000), thus one can assume that also Pdf mRNA is rather high and differences between the two species are hardly to detect. For me, showing Figure 4 a, b makes not much sense. In the s-LNvs, the situation is different, because they produce much less PDF, and indeed there are significant differences in Pdf mRNA levels between D. sechellia and D. melanogaster.

Specific minor comments:

Throughout the paper, please replace PDF protein with PDF peptide. PDF is only 18 aa long.

Line 263: The reference for Hr38 is missing.

Line 312: shouldn’t it read ‘morning peak’ instead of ‘evening peak’?

Line 335: in my opinion, it would be better to write: ‘dramatic advance in evening peak time’

Lines 430-431: I wonder why you did not include D. mauritiana in sequencing the Pdf 5’-regulatory region. It would be interesting to see where this species is placed in the maximum likelihood

phylogeny since is closer to D. sechellia than to D. melanogaster and also restricted to the tropics.

Lines 568-468: It might be useful to cite also Deppisch et al. (2022, J Biol Rhythms 37), because this paper shows that the cline in timeless polymorphism affects adaptation to long photoperiods.

Line 590: What about “constant photoperiods” instead of “constant conditions”?

Lines 788-798: the longevity assay needs to be explained in more detail. How many flies have been in each vial and how many vials have been used. Was there just one repetition of the longevity assay?

Figure 2: The molecular components of the circadian clock need some more explanation. The entire loop is rather small and hard to see. This is in particular true for Fig. 2g.

Figure 5a: the grey shade around the D. simulans strains is hard to see (at least on my print). I suggest making it a bit darker. The same is true for the grey lines in Fig. 5c.

Referee #2 (Remarks to the Author):

In this study Shahandeh and co-workers investigate differences in the photoperiodic adjustment of daily activity in two species of Drosophila and provide evidence these are explained by differences in the cis-regulatory region of a single neuropeptide, pigment dispersing factor. Data are presented to suggest that these differences influence the fecundity of flies under long summer-like days. Establishing that changes the regulatory region of a single identified neuropeptide underly these striking behavioral differences and fitness would be of significant and broad interest.

At the heart of the study is evidence that the equatorial D. sechellia fails to adjust its daily evening peak of activity in response to increases in daylength, instead maintaining a relatively stable phase relative to dawn. This contrasts with the well-established photoperiodic adjustment of the evening peak of activity in D. melanogaster, which delays the phase of its evening peak as daylength increases. Using a unique and impressive screen of hybrids between D. sechellia and clock mutants of D. melanogaster, the authors implicate the neuropeptide PDF as the factor driving these behavioral differences. In comparing pdf sequences in these two species the authors show the difference between them lies within the cis regulatory sequence rather than the coding sequence of pdf. Data are presented suggesting that this difference produces relatively low levels of pdf expression in D. sechellia.

Though the data presented are consistent with these conclusions, there are two major concerns that diminish the impact of the work. The first is that the authors have not tested their model as rigorously as they could have with the methods available to them. Second, the mechanism proposed for the differences in photoperiodic adjustment of daily activity does not reflect what is known about the relevance of PDF abundance in the regulation of evening peak phase in D. melanogaster.

Concern One:

The authors use a pdf-GFP element in melanogaster for each of the cis-coding regions and show that GFP levels are lower when the sechellia cis regulatory region is used to drive GFP. The authors also use GAL4 lines driven by the two pdf cis regulatory regions to drive PDF RNA interference constructs with different strengths and show that the resulting advance in the evening peak of activity under long day conditions tracks the strength of the GAL4 driver. However, there is an obvious alternative to this approach that would have tested the central model more directly. When Renn et al. (1999 Cell. 99:791) first characterized pdf in melanogaster, they showed that the pdf01 mutant's behavioral phenotypes were due to a loss of the pdf locus through genomic rescues of the mutant (that, is they introduced full length, including both the cis-regulatory and coding sequence, PDF into the genome of the pdf01 mutant) and showed that it rescued normal behavior. Repeating this approach and comparing sechellia and melanogaster cis regulatory regions would provide a strong test of the model. Comparing these two genomic rescues would be expected to show that sechellia cis-regulatory regions would fail to rescue the photoperiodic adjustment phenotypes displayed by pdf01 mutant and would result in lower levels of pdf expression in the brain. This would appear to be feasible and highly rigorous test of the central model that would carry much more weight than the relatively indirect approach used here.

Concern Two:

There is very little evidence that the changes in the levels of PDF peptide described here would be sufficient to produce the striking behavioral differences described in the study. As first reported by Renn et al. (1999), and replicated here, the loss of one copy of pdf has no effect on evening peak phase and the over-expression of pdf in the PDF neurons themselves produces no behavioral phenotypes (Helfrich-Forster 2000 J. Neurosci. 20:3339). Though the authors provide evidence that PDF peptide levels differ between the two species as specific times of the day, these differences do not appear to be severe enough to produce the phenotypes described if this previous work is considered. A much more severe reduction in PDF peptide would be expected to be required for significant behavioral effects (Shafer 2009 PLoS ONE 4:e8298). Finally, previous work has established that PDF from the large LNvs, but not the s-LNvs is responsible for photoperiodic adjustment of the evening peak of activity (Schlichting 2016 J. Neurosci. 36:9084). Remarkably, the authors present supplementary data (Fig. ED 7) that appear to show HIGHER levels of PDF in the large LNvs of sechellia. This is the opposite of what we would expect from the major conclusions of the study.

Additional Concerns:

The authors identify large LNv neurons as “evening cells.” This is not accurate. Though the l-LNvs adjust the relative phases of morning the evening peaks of activity, the field has long considered the dorsal lateral neurons and the and PDF negative 5th small LNv as the evening cells of the network.

The authors conclude from their behavioral experiments that sechellia lacks photoperiodic responses to increased daylength (i.e., to lack “behavioral plasticity”). However, it appears, from the data presented, that sechellia’s waveforms change as days become longer there is an increase in the breadth, amplitude, and amount of activity throughout the day. So, though sechellia lacks the coherent shift in phase, they do appear to respond behaviorally to increases in daylength.

The authors should acknowledge the significant body of existing work on photoperiodism and PDF in Drosophila species differences therein and evolution thereof. (see: Prabhakaran 2013 J. Exp. Biol. 216:4691; Prabhakaran 2012 J. Biol. Rhythm. 27:365; Abhilash 2020 J. Biol. Rhythm. 35: 145; Dani 2022 Front. Physiol. 13:954731).
The phyogenic analysis would benefit from the inclusion of a distant outgroup.

The authors use strains of wild-type flies that were collect a long time ago (Mid-1900s, I think for CS and Oregon R). These strains have been evolving in the lab for a very long time now, in contrast to the sechellia strains (I assume). One implication of this is that the former strains have been exposed to artificial light for many generations whereas sechellia has not (presumably). In other words, the differences between the species described here are not likely only a reflection of geographical source (equatorial versus temperate). It is acknowledges that the authors augment their work with additional melanogaster strains, but they should at the very least give more information about when the strains used were collected from the wild.

Additional tests should be employed to support the conclusion of natural selection (Tajimas’s D or Fu and Li; see Tauber 2007 Science 316:1895)

Referee #3 (Remarks to the Author):

Summary: Here the authors test the ability of D. sechellia, an equatorial species, to respond to different photoperiod lengths. Compared to D. melanogaster, D. sechellia have a reduced behavioral response to elongated day lengths. The authors screen candidate genes underlying the behavioral effects by taking advantage of the fact that D. melanogaster and D. sechellia can form hybrids, concluding that the neuropeptide PDF is a candidate gene. They examine the expression of PDF RNA and protein in clock cells. They hypothesize that the differences in phenotype between D. melanogaster and D. sechellia are due to changes differences in the promotor region of PDF. They create transgenic D. melanogaster flies having the D. melanogaster PDF promoter, and D. melanogaster flies having the D. sechellia PDF promoter. The transformed flies have altered responses to changes in photoperiod. The authors conduct some additional analyses and conclude that functionality was lost in the PDF promoter of D. sechellia, and that this loss affects fitness.

This is a fascinating study and the authors should be commended on taking on this very challenging work. We recognize and appreciate that the hybrid screening was particularly difficult. However, the phenotypic measures presented and the statistical analysis leaves open some additional interpretations other than what the authors have concluded.

General note: our evaluation is based on the merged pdf, Extended Data Figs. 1-9, and the authors' Reporting Summary.

Major Comments:

1. General comment: Means and standard deviations are not presented for many of these measures within the text. It would be helpful for the reader to understand the magnitude and variability of the differences presented, i.e., Line 148, "~ 1 h" leaves the reader wondering.

2. A key result is "…an almost complete inability of D. sechellia to adapt to increased photoperiod" (Lines 117-119, Fig. 1c). The authors test the behavioral response of two strains of D. melanogaster and two strains of D. sechellia in four different photoperiod environments, and find that D. sechellia evening peak activity changes less than D. melanogaster as the daylength increases. There are several issues with this claim. First, the author's interpretation of the data as "almost complete inability to adapt" and "exceptionally little circadian plasticity" strikes us as an exaggeration. The D. sechellia flies under lengthened photoperiod become more variable in evening peak time, suggesting that some of them are responding to photoperiod changes. D. sechellia can successfully entrain to these extreme long photoperiods successfully, despite being an equatorial organism. One could argue that this demonstrates more circadian plasticity of their circadian clock. What is different between these two species is their phase of entrainment under long photoperiods. The authors' definition of 'circadian plasticity' should be defined in the Introduction. Also, the authors unfortunately use only two strains of D. sechellia to come to their conclusions. Enormous variability has been observed in for instance wildtype strains and populations of D. melanogaster for all sorts of behaviors, including circadian period. But this variability would not have been noticed if experimenters had only measured the behavior in two wildtype strains. Thus, it is not clear whether the lack of response to changes in photoperiod is strain- rather than species-dependent (and notice

that the differences between strains within species start to diverge as daylength increases, for example). There are more D.sechellia stocks published, e.g. (PMID: 14710171), and this study may benefit from using a few more stocks (or a wildtype population, if available) of D.sechellia as the conclusions about the species and evolution of certain traits will also gain more evidence. Third, the authors state in the Reporting Summary that all behavioral experiments were replicated, but, judging by the numbers of animals, the data in Fig. 1c do not appear to be replicated. In any case, no statistical test of the differences among replicates was made, so it is uncertain whether these results replicate across time.

3. A second key piece of data is the analysis of morning anticipation (Fig. 1d,e). The authors use the terms 'morning anticipation', 'morning peak activity', and 'pre-dawn activity' interchangeably when they are not the same thing. The authors do not use a standard method such as the Harrisingh Index to calculate morning anticipation, which compares the proportion of the activity in the 3 hours before dawn to all activity in the preceding 6 hours. Instead, the authors state in Lines 688-689 that "To quantify pre-dawn activity, the average normalized activity was calculated for each fly in 30 minute bins in the 3 h preceding dawn." Using the author's method, one would for example not realize that the Clk/w1118 flies in Extended Fig. 4e also lack morning anticipation as their pre-dawn activity is high. Nor is a startle response to "lights-on" the same as a failure to entrain. The DD data presented suggest that D. sechellia remains entrained.

4. Methods: The method used to normalize activity counts is unclear. Throughout the manuscript and figures authors mention "normalized activity" and "mean normalized activity", however the figures do not show any normalization, except that the highest possible activity for any bin can be 1, indicating that perhaps a maximum normalization was used. As this normalized activity forms the basis of mostly all other phenotypes (evening peak phase, pre-dawn activity) in this manuscript, a detailed description of its calculation needs to be provided.

5. The rolling triangular mean used to calculate the phase of evening peak may lead to shifted phases/timing relative to the original signal, and is very much dependent on the bin size. In addition, the activity profiles lack error bars, making it difficult to interpret the level of variability within a given strain.

6. Figure 2e: There does not appear to be a statistical test between Pdf01/07 and 07, or Pdf01/28 and 28. The first comparison looks non-significant, in which case the PDF allele of 07 drives the phenotype. But Pdf01/28 looks like it is halfway between 28/CSW and 28, more like what would be expected from additional background modifiers driving the phenotype.

7. Figure 3h, it is not clear from the figure whether there is a statistically significant difference between DmelPdf-GAL4 and DsecPdf-GAL4, which would indicate that behavioral differences may stem from the insertion of different transgenes, even though they are inserted into the same attP2 site. Also, there do not appear to be any statistical tests with the UAS-PdfRNAi genotype. The same is true for Figure 3j.

8. Figure 5c and d: The authors refer to an alternate 'allele' of the PDF promoter region, however, nowhere in the manuscript has this been defined. One would anticipate several polymorphic

variants within the promoter region. Thus the alternate 'allele' is really probably an alternate haplotype, and it seems likely that there would be more than two haplotypes segregating in the D. melanogaster promoter region.

9. Figure 3d-f and 5e: There might be a genotype X environment interaction here that the authors' analysis doesn't pick up. Why are the genotypes (CS and OR; 07 and 28) combined in 5e, f, and g? Additionally, the figure does not have error bars. A more rigorous survival analysis may shed some light on any genotype*photoperiod effect present.

10. Lines 495-511. Though females were not used for any other behavior, they are suddenly used for the copulation assay. The reasoning that circadian plasticity may impact copulation success would require inspection of locomotor activity data of both males and females and finding significant differences in activity phases.

11: Discussion, Lines 578-598: The authors tie the reduced fitness in D. sechellia with photoperiod plasticity, but there is little evidence presented demonstrating that the phenotypes are highly genetically correlated. As fitness is polygenic, a different gene could be contributing these effects or the D. sechellia strains may suffer from inbreeding depression, etc.

12. Extended Data Fig. 1: The authors test the circadian period in two strains of D. melanogaster and two of D. sechellia. They state in Lines 166-167 that each displayed a circadian period of ~24 hours; however, no data analysis was conducted to determine whether there were any significant differences in circadian period among these strains, which is relevant to their thesis. Is there a connection between the period differences and low PDF levels in D. sechellia? Shorter period leads to advanced phases; there can be a connection between D.sechellia having shorter period than D.melanogaster, and this could in turn explain some of the phase differences seen under longer photoperiods.

Minor Comments:

1. Results, Lines 139-140, the authors state that the strains of each species originally evolved in environments where they were exposed to large differences in annual photoperiod variation, implying that D. sechellia was exposed to large differences in photoperiod. Do the authors mean to say that the strains of D. melanogaster were exposed to large differences?

2. Results, Lines 191, 210: The authors state that the phenotypic differences represent an evolutionary loss for D. sechellia. This seems like a premature statement; to show evolutionary loss, one would need to know the precise genetic basis for the difference in behavior and the gain or loss of the element with respect to other species. At this point in the paper it makes more sense to formulate this sentence as a hypothesis.

3. Lines 286-288: It's not clear why the authors believe that the differences in expression "must" result from the divergence of cis-regulatory region. It would help the reader to know the details of the reasoning, because none of results until this point in the manuscript indicate any difference in expression; in fact the spatial patterns of Pdf expression are conserved between D.melanogaster and D.sechellia.

4. Fig. 2g: It is not clear what statistical comparisons the asterisks refer to.

5. Results, Lines 234-238, the authors state that the control hybrids have a larger degree of phenotypic plasticity. The differences among individual flies that they observe can be quantified.

6. Extended Data Fig. 2: The authors compare the time of evening peak activity and pre-dawn activity in two other strains of D. melanogaster, two strains of D. simulans, and 2 strains of D. mauritiana. No statistical differences among the strains are indicated, but there appear to be, leaving open the question of whether a statistical analysis was performed. The authors plot the mean D. sechellia results as an orange bar across the plots as they presumably did not measure behavior in the D. sechellia strains contemporaneously with the other species in the figure. The orange bar is not a valid comparison. A similar situation occurs in Extended Data Fig. 4.

Author Rebuttals to Initial Comments:

We thank the reviewers for their careful reading and constructive criticisms of our manuscript. Below, we provide responses to each of the raised issues.

Referee #1

This work addresses the extremely important question of how the behavioral plasticity necessary for the global distribution of a species has evolved on a molecular genetic basis. Near the equator, species find the same environmental conditions throughout the year, whereas at higher latitudes they are subject to strong seasonal fluctuations. Therefore, species that have spread from the tropics to higher latitudes have developed a certain behavioral plasticity to survive. So far, very little is known about its molecular basis.

To decipher one of the underlying mechanisms, the authors used the circadian system of fruit flies, which is exceptionally well characterized in the cosmopolitan Drosophila melanogaster. Its close relative D. sechellia is endemic to islands near the equator. Genetic comparison of the two species thus promises answers to the burning question. Through a series of clever experiments, the authors identified the neuropeptide gene Pigment-dispersing factor (Pdf) as an important locus for evolution of circadian plasticity. The main results of the paper are the following:
1) D. sechellia cannot adapt their evening activity to long photoperiods demonstrating that the circadian system of D. sechellia is less plastic than that of D. melanogaster.
2) By crossing D. sechellia to different D. melanogaster circadian clock mutants, the authors identified the Pdf gene as the main gene necessary for circadian plasticity.
3) A 2.4 kb regulatory region immediately upstream of the Pdf gene 5' start codon is responsible for this plasticity (tested by reporter gene expression and downregulation of Pdf by Gal4-RNAi under control of the Pdf upstream regulatory region).
4) The Pdf upstream regulatory region is responsible for the expression level of Pdf mRNA: D. sechellia have lower Pdf expression levels than D. melanogaster.
5) The Pdf upstream regulatory region from D. sechellia appears to have lost transcription factor binding sites. Constructing a maximum likelihood phylogeny shows that the Pdf upstream regulatory sequences from D. sechellia form a monophyletic group that is different from D. melanogaster and D. simulans.
6) There is evidence for latitude-based selection the D. melanogaster Pdf 5'-regulatory region, causing circadian plasticity at higher latitudes.
7) Flies carrying the D. sechellia Pdf upstream regulatory region have a lower mating success under long photoperiods und consequently a lower fitness.

Strength of the study: The authors show for the first time that differences in Pdf expression caused by natural selection on the Pdf 5'-regulatory region increase behavioral plasticity and reproductive fitness in D. melanogaster under long photoperiods. Conversely, the loss of putative transcription factor binding sites in the Pdf 5'-regulatory region leads to a loss of circadian plasticity in D. sechellia and a lower fitness under long photoperiods.

Overall, the study is novel and of considerable significance. The taken approach is valid, the data are

of high quality, and the story is logically presented. The abstract and the introduction are clear and appropriate. The conclusions are robust and well justified and appropriate credit is given to previous work. I could also not detect any flaws in the statistics.

Suggestions for improvement:

1. The study has a single weakness: the results of PDF-immunocytochemistry (and partly mRNA expression) do not completely match the results gained with the transcriptional reporters. Nevertheless, I agree with the authors that this might be explained by additional endogenous factors acting on PDF and think that this does not diminish the significance of this study (further explanations below).

RESPONSE: Please see the detailed response to comment #3.

2. The authors place great emphasis on determining Pdf mRNA and PDF peptide levels in the somata of the large lateral neurons (l-LNv), probably because they assume that these cells act as evening oscillators of the circadian clock (statement in line 277). However, this is a misunderstanding and needs to be corrected. The l-LNvs are neither morning nor evening oscillators, but rather function in the light input pathway to the clock. Some authors also call them "arousal neurons" (e.g. McCarthy et al., J Neurosci 22, 2011). They can be activated by light, which leads to increased PDF secretion (Sheeba et al. J Neurophysiol 99, 2008). The increased PDF secretion from the l-LNvs delays the oscillations of the evening oscillators, which encompass the dorsolateral clock neurons (LNds). The delay in LNd oscillations in turn leads to a delay in evening activity under long photoperiods (Menegazzi et al., Curr Biol 27, 2017; Schlichting et al., Curr Biol 29, 2019).

RESPONSE: We thank the reviewer for this detailed explanation and references. We have corrected our misrepresentation of the role of the l-LNvs in the text. Specifically, we have removed the term “evening cells” from the text and Figure 3a. Additionally, we have specified in the text that the s-LNv and l-LNvs contribute to the timing of morning and evening activity peaks (lines 294-296) and included the three indicated references.

3. The problem is that one cannot measure increased PDF secretion by measuring PDF mRNA in the somata of the l-LNvs during the time of the evening peak (Figure 4 a, b). The latter measurements just give a rough estimate of the amount of Pdf mRNA during this time. The l-LNvs appear to produce high amounts of PDF peptide that is stored in their somata (Park et al., PNAS 97, 2000), thus one can assume that also Pdf mRNA is rather high and differences between the two species are hardly to detect. For me, showing Figure 4 a, b makes not much sense. In the s-LNvs, the situation is different, because they produce much less PDF, and indeed there are significant differences in Pdf mRNA levels between D. sechellia and D. melanogaster.

RESPONSE: We agree with the referee: Pdf secretion is the behaviourally relative cellular process but, to our knowledge, this has never been observed directly, even in *D. melanogaster*. Certainly this process cannot be measured by imaging the soma of the l-LNvs, which generally contain very high levels of Pdf throughout the day (lines 365-366). For this reason, we have re-focused this figure to discuss the numerous differences in Pdf expression in the s-LNvs (i.e., RNA levels, rhythmic accumulation in axon terminals), and Pdf-dependent circadian remodelling of s-LNv axons, which are

more likely to correlate with Pdf secretion. Accordingly, we have also revised the text to discuss primarily these species-specific differences. We have moved our measures of the l-LNvs to the supplement (Extended Data Figure 8) so that we still provide analysis for all Pdf cell types. We also limit our discussion of l-LNvs in the text, not least because the high variability in Pdf peptide levels in these neurons (Extended Data Figure 8c,d) – whether of biological or technical origin – constrains our ability to offer useful interpretations at this stage.

Specific minor comments:

Throughout the paper, please replace PDF protein with PDF peptide. PDF is only 18 aa long.

RESPONSE: We have replaced all instances of 'PDF protein' with the more accurate "Pdf peptide". (We follow the FlyBase conventions for gene/protein nomenclature in terms of capitalisation).

Line 263: The reference for Hr38 is missing.

RESPONSE: We have added the following reference:

Mezan, S., Feuz, J. D., Deplancke, B. & Kadener, S. PDF Signaling Is an Integral Part of the *Drosophila* Circadian Molecular Oscillator. *Cell Rep* **17**, 708-719, doi:10.1016/j.celrep.2016.09.048 (2016).

Line 312 (new line 329): shouldn't it read 'morning peak' instead of 'evening peak'?

RESPONSE: Here we intend to draw a comparison between our measures of reporter fluorescence in the s-LNv axonal projections during the morning peak and the data we discuss in the preceding paragraph examining reporter expression in the l-LNvs during the evening peak. We have re-worded this sentence to for clarity:

"*We again observed that the D. sechellia 5'-regulatory sequence drives lower expression of the reporter but, in contrast to reporter expression in the l-LNvs during the evening peak, with a similar temporal pattern.*"

Line 335 (new line 410): in my opinion, it would be better to write: 'dramatic advance in evening peak time'

RESPONSE: We have changed this sentence as suggested.

Lines 430-431 (new lines 457-458): I wonder why you did not include D. mauritiana in sequencing the Pdf 5'-regulatory region. It would be interesting to see where this species is placed in the maximum likelihood phylogeny since is closer to D. sechellia than to D. melanogaster and also restricted to the tropics.

RESPONSE: This is a very good idea, and we have updated the phylogeny in Figure 5a to include sequences from *D. mauritiana*, as well as *D. yakuba, D. santomea, D. tessieri* and *D. erecta* to root the tree (as per Referee 3's suggestion). Interestingly, *D. mauritiana*, an island endemic from the

tropics, is more similar to *D. sechellia* than cosmopolitan *D. melanogaster* and *D. simulans*, although the *D. sechellia* sequences still form a monophyletic group. We have further elaborated our discussion of these results accordingly in the text (lines 460-467).

Lines 568-468 (new lines 474-500): It might be useful to cite also Deppisch et al. (2022, J Biol Rhythms 37), because this paper shows that the cline in timeless polymorphism affects adaptation to long photoperiods.

RESPONSE: We have added this citation at the end of line 496, where we list previous work examining clinal variation in circadian genes.

Line 590 (new line 627): What about “constant photoperiods” instead of “constant conditions”?

RESPONSE: We have changed the phrasing as suggested.

Lines 788-798 (new lines 835-848): the longevity assay needs to be explained in more detail. How many flies have been in each vial and how many vials have been used. Was there just one repetition of the longevity assay?

RESPONSE: We have updated this section of the methods to include more detail. Specifically, that we observed 10 flies of each genotype (two *Dmel* strains and two *Dsec* strains) that we held individually in vials until their death. We also performed a second replicate of this experiment, verifying these results, and have provided these data in Extended Data Figure 11.

Figure 2: The molecular components of the circadian clock need some more explanation. The entire loop is rather small and hard to see. This is in particular true for Fig. 2g.

RESPONSE: We have provided a more detailed description of the diagram depicted in Figure 2a in the figure legend. We have increased the size of the loop in Figure 2a as far as possible and created a larger independent panel (Figure 2h) for the second.

Figure 5a: the grey shade around the D. simulans strains is hard to see (at least on my print). I suggest making it a bit darker. The same is true for the grey lines in Fig. 5c.

RESPONSE: We have made the grey slightly darker in Figure 5a, Figure 5c and Extended Data Figure 10.

Referee #2

In this study Shahandeh and co-workers investigate differences in the photoperiodic adjustment of daily activity in two species of Drosophila and provide evidence these are explained by differences in the cis-regulatory region of a single neuropeptide, pigment dispersing factor. Data are presented to suggest that these differences influence the fecundity of flies under long summer-like days. Establishing that changes the regulatory region of a single identified neuropeptide underly these striking behavioral differences and fitness would be of significant and broad interest.

At the heart of the study is evidence that the equatorial D. sechellia fails to adjust its daily evening peak of activity in response to increases in daylength, instead maintaining a relatively stable phase relative to dawn. This contrasts with the well-established photoperiodic adjustment of the evening peak of activity in D. melanogaster, which delays the phase of its evening peak as daylength increases. Using a unique and impressive screen of hybrids between D. sechellia and clock mutants of D. melanogaster, the authors implicate the neuropeptide PDF as the factor driving these behavioral differences. In comparing pdf sequences in these two species the authors show the difference between them lies within the cis regulatory sequence rather than the coding sequence of pdf. Data are presented suggesting that this difference produces relatively low levels of pdf expression in D. sechellia.

Though the data presented are consistent with these conclusions, there are two major concerns that diminish the impact of the work. The first is that the authors have not tested their model as rigorously as they could have with the methods available to them. Second, the mechanism proposed for the differences in photoperiodic adjustment of daily activity does not reflect what is known about the relevance of PDF abundance in the regulation of evening peak phase in D. melanogaster.

Concern One:

The authors use a pdf-GFP element in melanogaster for each of the cis-coding regions and show that GFP levels are lower when the sechellia cis regulatory region is used to drive GFP. The authors also use GAL4 lines driven by the two pdf cis regulatory regions to drive PDF RNA interference constructs with different strengths and show that the resulting advance in the evening peak of activity under long day conditions tracks the strength of the GAL4 driver. However, there is an obvious alternative to this approach that would have tested the central model more directly. When Renn et al. (1999 Cell. 99:791) first characterized pdf in melanogaster, they showed that the pdf01 mutant's behavioral phenotypes were due to a loss of the pdf locus through genomic rescues of the mutant (that, is they introduced full length, including both the cis-regulatory and coding sequence, PDF into the genome of the pdf01 mutant) and showed that it rescued normal behavior. Repeating this approach and comparing sechellia and melanogaster cis regulatory regions would provide a strong test of the model. Comparing these two genomic rescues would be expected to show that sechellia cis-regulatory regions would fail to rescue the photoperiodic adjustment phenotypes displayed by pdf01 mutant and would result in lower levels of pdf expression in the brain. This would appear to be feasible and highly rigorous test of the central model that would carry much more weight than the relatively indirect approach used here.

RESPONSE: This is an excellent suggestion, and we now have engineered allele-specific rescue strains, where the *D. melanogaster* or *D. sechellia* 5'-regulatory region is used to drive expression of Pdf in the *D. melanogaster Pdf* null background. In brief, we find that rescue strains with the *D. sechellia Pdf* 5'-regulatory sequence exhibit less plasticity (as well as lower morning activity) compared to those with the *D. melanogaster Pdf* 5'-regulatory sequence. These results are now provided in Figure 4e-h, and we have added text discussing these data to the Results and Discussion (lines 428-452 and lines 580-583, 596-602).

Concern Two:

There is very little evidence that the changes in the levels of PDF peptide described here would be sufficient to produce the striking behavioral differences described in the study. As first reported by Renn et al. (1999), and replicated here, the loss of one copy of pdf has no effect on evening peak phase and the over-expression of pdf in the PDF neurons themselves produces no behavioral phenotypes (Helfrich-Forster 2000 J. Neurosci. 20:3339). Though the authors provide evidence that PDF peptide levels differ between the two species as specific times of the day, these differences do not appear to be severe enough to produce the phenotypes described if this previous work is considered. A much more severe reduction in PDF peptide would be expected to be required for significant behavioral effects (Shafer 2009 PLoS ONE 4:e8298).

RESPONSE: The referee raises an important point regarding the contribution of Pdf expression differences to behavioural difference between species. Our genetic screen revealed that, under 16:8 h LD, the *D. sechellia Pdf* allele fails to complement the *D. melanogaster* allele when hemizygous in a hybrid background. It does not, however, imply that the *D. sechellia Pdf* allele is a complete loss-of-function (or even hypomorphic), as these hybrids display typical activity patterns under 12:12 h LD, unlike Pdf^{01} mutants. For this reason, our experiments are not directly comparable to previous hemizygous/overexpression manipulations in *D. melanogaster.*

The *Pdf* locus also does not explain the entirety of the species-specific differences: these hybrids still display greater evening peak plasticity than *D. sechellia* (lines 583-585). There are almost certainly multiple loci contributing to the large behavioural difference between species. While questions remain open about the mechanism, our new species-specific 5'-regulatory rescue experiment (described above) indicates that *Pdf* is a causal factor, albeit with a small effect alone. We note that species-specific differences in temporal expression of Pdf in s-LNvs, and the contribution of the 5'-regulatory region to these differences and to species-specific morning activity levels are clearer. It seems that evening peak plasticity is a more complex process; indeed, work in *D. melanogaster* has shown that it requires a functional clock in both the morning and evening oscillators (ref. 38).

We would like to emphasise that the motivation of our study was to understanding divergence in species' behaviours. Behavioural evolution is generally appreciated to be polygenic (e.g., doi:10.1534/genetics.118.300712; ref. 47)), which explains why it has been extremely hard to extract underlying molecular mechanisms of behavioural divergence – and relate to species' ecologies and fitness – as we have been able to do in our work.

Finally, previous work has established that PDF from the large LNvs, but not the s-LNvs is responsible for photoperiodic adjustment of the evening peak of activity (Schlichting 2016 J. Neurosci. 36:9084).

Remarkably, the authors present supplementary data (Fig. ED 7) that appear to show HIGHER levels of PDF in the large LNvs of sechellia. This is the opposite of what we would expect from the major conclusions of the study.

RESPONSE: Regarding differences in Pdf immunofluorescence in the l-LNvs (now in Extended Data Figure 8): expression in *D. sechellia,* particularly under extended photoperiods, is highly variable, and we do not detect any significant differences between the species so we cannot conclude that Pdf expression is higher in *D. sechellia* (as now mentioned explicitly in the text; lines 371-375).

As pointed out by Referee 1, expression in the l-LNv soma is generally high and hard to relate to rates of Pdf secretion, which is the process that is ultimately necessary to delay evening peak activity (see also our response at the top of page 3). For this reason, we have limited our discussion of these data and moved the figure panels to the Extended Data. In Figure 3 we now focus on the differences we observe in the s-LNvs, where expression and remodelling differences more likely reflect differences in secretion dynamics.

Additional Concerns:

The authors identify large LNv neurons as "evening cells." This is not accurate. Though the l-LNvs adjust the relative phases of morning the evening peaks of activity, the field has long considered the dorsal lateral neurons and the and PDF negative 5th small LNv as the evening cells of the network.

RESPONSE: We have corrected this error in the figures and throughout the text (see also the response to Referee 1, bottom of page 2).

The authors conclude from their behavioral experiments that sechellia lacks photoperiodic responses to increased daylength (i.e., to lack "behavioral plasticity"). However, it appears, from the data presented, that sechellia's waveforms change as days become longer there is an increase in the breadth, amplitude, and amount of activity throughout the day. So, though sechellia lacks the coherent shift in phase, they do appear to respond behaviorally to increases in daylength.

RESPONSE: We agree, and we have now replaced essentially all instances of "circadian plasticity" or "behavioural plasticity" with "evening peak plasticity" where necessary, and included a more specific definition in the Introduction of the evening peak plasticity we measure (lines 118-120).

The authors should acknowledge the significant body of existing work on photoperiodism and PDF in Drosophila species differences therein and evolution thereof. (see: Prabhakaran 2013 J. Exp. Biol. 216:4691; Prabhakaran 2012 J. Biol. Rhythm. 27:365; Abhilash 2020 J. Biol. Rhythm. 35: 145; Dani 2022 Front. Physiol. 13:954731).

RESPONSE: We acknowledge this prior interesting work and tried to cite as many of the relevant studies on other drosophilids as possible. For concision, we introduce this work only in the Results and Discussion section, following our implication of Pdf in *D. sechellia*'s behavioural divergence (rather than in the Introduction, where we feel it would provide too much detail too soon).

The phylogenetic analysis would benefit from the inclusion of a distant outgroup.

RESPONSE: We have included 4 additional drosophilid species to root the tree. We have further updated our discussion of these results accordingly (lines 460-467).

The authors use strains of wild-type flies that were collect a long time ago (Mid-1900s, I think for CS and Oregon R). These strains have been evolving in the lab for a very long time now, in contrast to the sechellia strains (I assume). One implication of this is that the former strains have been exposed to artificial light for many generations whereas sechellia has not (presumably). In other words, the differences between the species described here are not likely only a reflection of geographical source (equatorial versus temperate). It is acknowledged that the authors augment their work with additional melanogaster strains, but they should at the very least give more information about when the strains used were collected from the wild.

RESPONSE: We were aware of this difference, and for this reason chose to include the much more recently caught *D. melanogaster* and *D. simulans* strains (LZV and MD strains, as presented in Extended Data Figure 4), collected in ~2010 (ref. 56). These strains also display plasticity in their circadian phase, indicating that the difference we observe between *D. melanogaster* CS/OR and our *D. sechellia* strains is not simply a product of laboratory adaptation. While the stock center no longer maintains specific collection dates for its *D. sechellia* strains, these lines were likely collected in the 1990s (doi: 10.1534/genetics.113.154773), and the strains we use here have been in our lab since the early 2010s. Thus, they themselves are quite lab adapted.

It is an interesting general question as to whether long-term exposure to laboratory lighting conditions might impact circadian properties of animals. The only experiment we are aware of that has addressed this issue is the "Dark fly" project in *D. melanogaster*, where a strain has been maintained in constant darkness since 1954. Assessment of circadian rhythms of these flies after >1300 generations revealed no changes compared to control flies (doi:10.2108/zsj.28.195), suggesting that measurable alterations to circadian cycles do not occur over at least these relatively short timescales.

Additional tests should be employed to support the conclusion of natural selection (Tajimas's D or Fu and Li; see Tauber 2007 Science 316:1895)

RESPONSE: We have now included a Tajima's D and Fu and Li's F and D statistics for *D. sechellia* using 41 publicly available genomes recently sampled from the Seychelles. We additionally find evidence of selection acting at the *Pdf cis*-regulatory locus in this population, but not for two control neuropeptide genes (new Figure 5e). Unfortunately, such analyses are not possible for the *D. melanogaster* populations previously described, as haplotype data are not available.

Referee #3

Summary: Here the authors test the ability of D. sechellia, an equatorial species, to respond to different photoperiod lengths. Compared to D. melanogaster, D. sechellia have a reduced behavioral response to elongated day lengths. The authors screen candidate genes underlying the behavioral effects by taking advantage of the fact that D. melanogaster and D. sechellia can form hybrids, concluding that the neuropeptide PDF is a candidate gene. They examine the expression of PDF RNA and protein in clock cells. They hypothesize that the differences in phenotype between D. melanogaster and D. sechellia are due to changes differences in the promotor region of PDF. They create transgenic D. melanogaster flies having the D. melanogaster PDF promoter, and D. melanogaster flies having the D. sechellia PDF promoter. The transformed flies have altered responses to changes in photoperiod. The authors conduct some additional analyses and conclude that functionality was lost in the PDF promoter of D. sechellia, and that this loss affects fitness.

This is a fascinating study and the authors should be commended on taking on this very challenging work. We recognize and appreciate that the hybrid screening was particularly difficult. However, the phenotypic measures presented and the statistical analysis leaves open some additional interpretations other than what the authors have concluded.

General note: our evaluation is based on the merged pdf, Extended Data Figs. 1-9, and the authors' Reporting Summary.

Major Comments:

1. General comment: Means and standard deviations are not presented for many of these measures within the text. It would be helpful for the reader to understand the magnitude and variability of the differences presented, i.e., Line 148, "~ 1 h" leaves the reader wondering.

RESPONSE: We have added the specific medians mentioned by the referee (lines 151-153). To avoid cluttering the text, however, for most results we discuss general patterns rather than provide specific values, but all of this information is available both through the data visualisations in the figures. All raw data is also provided in the Source Data.

2. A key result is "…an almost complete inability of D. sechellia to adapt to increased photoperiod" (Lines 117-119, Fig. 1c). The authors test the behavioral response of two strains of D. melanogaster and two strains of D. sechellia in four different photoperiod environments, and find that D. sechellia evening peak activity changes less than D. melanogaster as the daylength increases. There are several issues with this claim. First, the author's interpretation of the data as "almost complete inability to adapt" and "exceptionally little circadian plasticity" strikes us as an exaggeration. The D. sechellia flies under lengthened photoperiod become more variable in evening peak time, suggesting that some of them are responding to photoperiod changes. D. sechellia can successfully entrain to these extreme long photoperiods successfully, despite being an equatorial organism. One could argue that this demonstrates more circadian plasticity of their circadian clock. What is different

between these two species is their phase of entrainment under long photoperiods. The authors' definition of 'circadian plasticity' should be defined in the Introduction.

RESPONSE: The referee raises several very good points. The increased variability in evening peak time observed in *D. sechellia* (particularly *Dsec*28) at longer photoperiods is due, in part, to a breakdown in their rhythmic activity combined with an overall reduction in activity levels, which affect our peak-picking algorithm's ability to identify obvious peaks at extreme photoperiods. To make this variability more apparent, we have now included non-normalised Extended Data Figure 1. Furthermore, we have also added pie charts to Figure 1c illustrating the percent of rhythmic/arrhythmic flies under each treatment and have elaborated on these results in the text (lines 153-162). Nonetheless, the referee is correct that the behaviour does not go entirely unchanged from 12:12 h LD into the extended photoperiod treatments, as *D. sechellia* do certainly lengthen the duration of their evening activity even though peak times do not vary. We have included in our introduction a more specific definition of the behavioural plasticity we measure here, evening peak delay (lines 118-120), and adopted the phrase "evening peak plasticity" rather than the more general "circadian plasticity" here and throughout.

Also, the authors unfortunately use only two strains of D. sechellia to come to their conclusions. Enormous variability has been observed in for instance wildtype strains and populations of D. melanogaster for all sorts of behaviors, including circadian period. But this variability would not have been noticed if experimenters had only measured the behavior in two wildtype strains. Thus, it is not clear whether the lack of response to changes in photoperiod is strain- rather than species-dependent (and notice that the differences between strains within species start to diverge as daylength increases, for example). There are more D.sechellia stocks published, e.g. (PMID: 14710171), and this study may benefit from using a few more stocks (or a wildtype population, if available) of D.sechellia as the conclusions about the species and evolution of certain traits will also gain more evidence.

RESPONSE: We have repeated the experiments at both 12:12 h LD and 16:8 h LD now using two additional *D. sechellia* strains (Extended Data Figure 4). For all strains, we find that *D. sechellia* displays reduced pre-dawn activity and reduced evening peak plasticity. These data, in combination with the four *D. melanogaster* strains we previously reported (as well as our analysis of several *D. simulans* and *D. mauritiana* strains; also in Extended Data Figure 4), strengthen our claims of species differences.

Third, the authors state in the Reporting Summary that all behavioral experiments were replicated, but, judging by the numbers of animals, the data in Fig. 1c do not appear to be replicated. In any case, no statistical test of the differences among replicates was made, so it is uncertain whether these results replicate across time.

RESPONSE: The Referee is correct in that the survey of circadian responses across multiple photoperiods in Figure 1c was not replicated, and we have amended the Reporting Summary accordingly. These experiments formed part of the exploratory stage of the project examining whether *D. melanogaster* and *D. sechellia* display differences in response to different photoperiods. For this reason we did not consider it necessary to replicate every photoperiod treatment,

particularly for the extreme (largely unnatural) photoperiod conditions (18:6 and 20:4 h LD), the latter of which led to arrhythmicity in both species. However, we acknowledge the referee's concern and we now have replicated the 12:12 h and 16:8 h LD treatments using two *D. melanogaster* and four *D. sechellia* strains (Extended Data Figure 4). The results – collected three years apart – clearly replicate. We also note that (in response to a comment below), we have also tested females of these species in an independent experiment, with very similar results (Extended Data Figure 2).

3. A second key piece of data is the analysis of morning anticipation (Fig. 1d,e). The authors use the terms 'morning anticipation', 'morning peak activity', and 'pre-dawn activity' interchangeably when they are not the same thing. The authors do not use a standard method such as the Harrisingh Index to calculate morning anticipation, which compares the proportion of the activity in the 3 hours before dawn to all activity in the preceding 6 hours. Instead, the authors state in Lines 688-689 that "To quantify pre-dawn activity, the average normalized activity was calculated for each fly in 30 minute bins in the 3 h preceding dawn." Using the author's method, one would for example not realize that the Clk/w1118 flies in Extended Fig. 4e also lack morning anticipation as their pre-dawn activity is high. Nor is a startle response to "lights-on" the same as a failure to entrain. The DD data presented suggest that D. sechellia remains entrained.

RESPONSE: We apologise for the confusing use of terminology. Although, in the course of our work, we initially interpreted the low pre-dawn activity of *D. sechellia* as a lack of morning anticipation, we subsequently realised from the analysis of activity under DD that *D. sechellia* simply has low activity in the mornings compared to *D. melanogaster*. We have strived to describe this phenotype of *D. sechellia* as "morning activity" in the revised manuscript. However, because lights-on causes a startle response, to measure morning activity in most experiments, we could only quantify activity before lights-on, and use the term "pre-dawn activity" when referring to how this activity was calculated. (Because we are not reporting on morning anticipation, we did not use the Harrisingh Index method).

We do not state in the text that the flies in DD are not entrained (in fact we state that they remain rhythmic under constant darkness (lines 177-178), we merely note that in the absence of a startle response to lights on, the free-running activity pattern of *D. sechellia* strains reveals significantly reduced activity around the subjective dawn when compared to *D. melanogaster* strains. We show that measuring pre-dawn activity during 12:12 h LD is a correlative measure for comparing differences in the magnitude of morning activity, which are revealed under constant darkness (DD). We have further elaborated this in the relevant results section (lines 181-184).

Concerning the *Clk/w*^{1118} flies, we previously noted that this abnormal phenotype was due to our use of the dominant-negative *Clk*^{JRK} allele. In this revision, we now replaced analysis of this atypical allele with the *Clk*^{out} loss-of-function allele, and we no longer see this effect. We have updated Extended Data Figure 6.

4. Methods: The method used to normalize activity counts is unclear. Throughout the manuscript and figures authors mention "normalized activity" and "mean normalized activity", however the figures do not show any normalization, except that the highest possible activity for any bin can be 1, indicating that perhaps a maximum normalization was used. As this normalized activity forms the basis of mostly all other phenotypes (evening peak phase, pre-dawn activity) in this manuscript, a detailed description of its calculation needs to be provided.

RESPONSE: We indeed performed a maximum normalisation and now added a description of this in the Methods.

5. The rolling triangular mean used to calculate the phase of evening peak may lead to shifted phases/timing relative to the original signal, and is very much dependent on the bin size. In addition, the activity profiles lack error bars, making it difficult to interpret the level of variability within a given strain.

RESPONSE: We shared concerns about this possible effect, and therefore used 10-min bins (as detailed in the Methods) to minimise it as much as possible. We note that the same effect would be present in each strain, and thus differences between strains would not be affected even if each value is slightly inflated. We have added error bars to the activity plots to make the increased variance at higher photoperiods, particularly in *D. sechellia*, more apparent.

6. Figure 2e: There does not appear to be a statistical test between Pdf01/07 and 07, or Pdf01/28 and 28. The first comparison looks non-significant, in which case the PDF allele of 07 drives the phenotype. But Pdf01/28 looks like it is halfway between 28/CSW and 28, more like what would be expected from additional background modifiers driving the phenotype.

RESPONSE: We performed all pairwise comparisons (within the *D. sechellia* 28 and 07 genotypes separately) with post-hoc correction for multiple comparisons. Both comparisons between the *D. sechellia* strains and the *DmelPdf*^{01}*/Dsec* hybrids were not significant after correcting for multiple corrections (and marginally significant prior). The full list of P-values is provided in the Source Data. As there are a total of 20 comparisons, to maintain visual clarity, we chose to only indicate those we discuss directly in the text. Additionally, although these specific comparisons were not significant following post-hoc correction, we do acknowledge in the text that the *Pdf* locus is unlikely to explain the entirety of the effect (lines 448-452, 580-588).

7. Figure 3h, it is not clear from the figure whether there is a statistically significant difference between DmelPdf-GAL4 and DsecPdf-GAL4, which would indicate that behavioral differences may stem from the insertion of different transgenes, even though they are inserted into the same attP2 site. Also, there do not appear to be any statistical tests with the UAS-PdfRNAi genotype. The same is true for Figure 3j.

RESPONSE: We confirm and now indicate on the plot (in new Figure 4c) that the evening peak timing of the two Gal4 insertions is not significantly different. We do not show comparisons to the *UAS-Pdf*^{RNAi} strain, as this strain was crossed to the *w*^{1118} strain (so flies had only one copy of the *UAS* transgene), and we suspect the greater evening peak delay than any other strain reflects this outcrossing. We stress that that most pertinent comparison is between flies of the same genetic background and identical set of transgenes (differing only in the species origin of the *Pdf* 5'-regulatory region in the *Gal4* line).

8. Figure 5c and d: The authors refer to an alternate 'allele' of the PDF promoter region, however, nowhere in the manuscript has this been defined. One would anticipate several polymorphic

variants within the promoter region. Thus the alternate 'allele' is really probably an alternate haplotype, and it seems likely that there would be more than two haplotypes segregating in the D. melanogaster promoter region.

RESPONSE: The data that we use here (from doi:10.1111/mec.13455; ref. 49), unfortunately does not include haplotype data, but simply a table of SNV frequencies in different populations across the genome. We averaged the frequencies for all minor alleles (the less common allele at each variable site) within our neuropeptide regulatory regions to calculate the average minor allele frequency. We exclusively refer to this value as minor allele frequency in the text, as well as replacing the term "alternative allele" with "minor allele" in the Figure 5c-d (we agree our mix of terminology was confusing). We have added the following to the Methods section to make the approach clearer:

"*For each population, we calculated the average minor allele frequency (MAF) across all variable sites in this region, and for the same-sized region upstream of the start codon of 6 control neuropeptide genes.*"

9. Figure 3d-f and 5e: There might be a genotype X environment interaction here that the authors' analysis doesn't pick up. Why are the genotypes (CS and OR; 07 and 28) combined in 5e, f, and g? Additionally, the figure does not have error bars. A more rigorous survival analysis may shed some light on any genotype*photoperiod effect present.

RESPONSE: For Figure 3d-f, we are showing the results comparing two transcriptional reporter strains created using the *D. melanogaster* CS and *D. sechellia* 07 *Pdf cis*-regulatory regions. There are only two strains compared here, there is no pooling of genotypes that could mask a genotype-by-environment interaction.

For the longevity analysis in the original Figure 5e (now Figure 5f), we collected data on 10 flies of each strain (two strains per species) under two environmental conditions. In all cases we found the same result: flies held under a 16:8 h LD cycle displayed a reduced life-span. We chose to pool the strains here for visual simplicity (4 lines rather than 8) because there was no significant difference between strains of the same species (i.e., no G x E effect). This simplicity seemed warranted for what is, essentially, a negative result. In response to a comment from Referee 1, we have replicated this longevity experiment, verifying these results, now presented in Extended Data Figure 11. Here, we present the strain-specific results of both replicates (including those from Figure 5f).

Because the results of our copulation assay are integral to the main results regarding the potential for selection to act, we have expanded our sample size and number of *D. sechellia* strains. We have updated Figure 5f-g to include these more detailed results, as well as our description of them in the text. In the 2 h copulation experiment, we consistently find a reduction in copulation success across four *D. sechellia* strains; we have additionally bootstrapped error bars on these proportions (new Figure 5g).

We note that for the 3-day copulation experiment (original Figure 5g), with the addition of more strains and increased N, we no longer observe a consistent reduction in copulation success for all strains: only in *Dsec*07 and marginally in *Dsec*13. However, 3 days is an exceptionally long time for two flies to interact (especially unencumbered with other animals in a culture vial), so the lack of a consistent effect at this timescale is unsurprising. Detection of consistent reduction over 2 h would

be sufficient to affect fitness in the field, where individual flies likely interact only for much shorter periods of time.

10. Lines 495-511. Though females were not used for any other behavior, they are suddenly used for the copulation assay. The reasoning that circadian plasticity may impact copulation success would require inspection of locomotor activity data of both males and females and finding significant differences in activity phases.

RESPONSE: We have now measured circadian activity for females of *D. melanogaster* and *D. sechellia* under both 12:12 h LD and 16:8 h LD (new Extended Data 2). We observed reduced evening peak plasticity among our *D. sechellia* strains when compared to *D. melanogaster* (as well as reduced pre-dawn activity). The similar reduction in plasticity of both male and female *D. sechellia* suggest that decreased copulation success under longer photoperiod is not due to de-synchronisation of activity phases; we suggest in the Discussion that it might instead reflect altered pheromone production (as reported in *D. suzukii*; doi:10.1038/s41598-023-32652-y; ref. 84).

11: Discussion, Lines 578-598: The authors tie the reduced fitness in D. sechellia with photoperiod plasticity, but there is little evidence presented demonstrating that the phenotypes are highly genetically correlated. As fitness is polygenic, a different gene could be contributing these effects or the D. sechellia strains may suffer from inbreeding depression, etc.

RESPONSE: We have changed the wording “fitness cost” to “reproductive cost”, as we are specifically referring to the copulation experiments (Figure 5g) where we demonstrate reduced reproductive ability of *D. sechellia* under increased photoperiods. While this is likely to impact fitness at higher latitudes, we acknowledge that we did not measure fitness directly.

12. Extended Data Fig. 1: The authors test the circadian period in two strains of D. melanogaster and two of D. sechellia. They state in Lines 166-167 that each displayed a circadian period of ~24 hours; however, no data analysis was conducted to determine whether there were any significant differences in circadian period among these strains, which is relevant to their thesis. Is there a connection between the period differences and low PDF levels in D. sechellia? Shorter period leads to advanced phases; there can be a connection between D.sechellia having shorter period than D.melanogaster, and this could in turn explain some of the phase differences seen under longer photoperiods.

RESPONSE: We have now included a statistical analysis in Extended Data Figure 3 (previously Extended Data Figure 1) comparing circadian period between the focal *D. melanogaster* and *D. sechellia* strains. Essentially, there are several significant differences, but not in a species-specific manner. For example, *Dmel*OR displays a shorter period than *Dsec*07, but a similar period to *Dsec*28. *Dmel*CS displays a longer period than all strains. Because no significant conclusions can be made from these data, we have restricted these results to the Extended Data figure and legend.

Minor Comments:

1. Results, Lines 139-140, the authors state that the strains of each species originally evolved in environments where they were exposed to large differences in annual photoperiod variation, implying that D. sechellia was exposed to large differences in photoperiod. Do the authors mean to say that the strains of D. melanogaster were exposed to large differences?

RESPONSE: Yes, we realise our phrasing was ambiguous and reworded it to make it more concise and hopefully clearer (line 139-142):

"The D. melanogaster strains thus initially evolved in environments with annual photoperiod variation on the scale of several hours, while the D. sechellia strains only ever experienced variation on a scale of minutes (Fig. 1b)."

2. Results, Lines 191, 210: The authors state that the phenotypic differences represent an evolutionary loss for D. sechellia. This seems like a premature statement; to show evolutionary loss, one would need to know the precise genetic basis for the difference in behavior and the gain or loss of the element with respect to other species. At this point in the paper it makes more sense to formulate this sentence as a hypothesis.

RESPONSE: Here we are specifically discussing the transitions in phenotypic plasticity across the phylogeny, irrespective of the mechanism. We acknowledge the referee's point and have now replaced "evolutionary loss" with "phenotypic loss" to clarify this claim, also tempering our language by stating is it a *likely* loss. Because all three sister species display some degree of evening peak plasticity (even when collected from the tropics, Extended Data Figure 2), while *D. sechellia* does not, a loss of phenotypic plasticity in the *D. sechellia* lineage represents the most parsimonious scenario, rather than a gain of plasticity in multiple other lineages. This proposition is subsequently given some support by our phylogenetic and motif analyses. We discuss this further in lines 468-473.

3. Lines 286-288: It's not clear why the authors believe that the differences in expression "must" result from the divergence of cis-regulatory region. It would help the reader to know the details of the reasoning, because none of results until this point in the manuscript indicate any difference in expression; in fact the spatial patterns of Pdf expression are conserved between D.melanogaster and D.sechellia.

RESPONSE: The screen results point to the *Pdf* locus itself (Figure 2d-e). However, the peptide sequence is perfectly conserved between species. Thus, the differences must be attributable to differences in expression (spatial and/or temporal (including levels)), and not peptide function. Differences in *trans*-acting factors influencing Pdf function would have resulted in identification of *trans*-acting genes in our screen, which was not the case. Thus, we conclude *cis*-regulation. We have clarified this logic in the text (lines 290-294). Of course, this logic provides motivation for all of the subsequent experiments in the paper where we investigate (and find) *cis*-regulatory difference in *Pdf*.

4. Fig. 2g: It is not clear what statistical comparisons the asterisks refer to.

RESPONSE: These comparisons are explained in the figure caption:

"*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).*"

5. Results, Lines 234-238, the authors state that the control hybrids have a larger degree of phenotypic plasticity. The differences among individual flies that they observe can be quantified.

RESPONSE: At these lines we state that the control hybrids have a larger degree of evening peak plasticity than their *D. sechellia* parent strains. These are indeed quantified and represented by the boxplots in Figure 2e. Statistical differences between them are indicated by the asterisks above. We hope this clarifies the presentation for the referee.

6. Extended Data Fig. 2: The authors compare the time of evening peak activity and pre-dawn activity in two other strains of D. melanogaster, two strains of D. simulans, and 2 strains of D. mauritiana. No statistical differences among the strains are indicated, but there appear to be, leaving open the question of whether a statistical analysis was performed. The authors plot the mean D. sechellia results as an orange bar across the plots as they presumably did not measure behavior in the D. sechellia strains contemporaneously with the other species in the figure. The orange bar is not a valid comparison. A similar situation occurs in Extended Data Fig. 4.

RESPONSE: We have removed the orange line from Extended Data Figure 4 (previously Extended Data Figure 2). We have now included additional data –collected in parallel – and indicated on the figure all significant differences that were detected between strains. All pairwise comparisons prior to, and following Bonferroni correction are provided in the Source Data.

In Extended Data Figures 5 and 6 (previously Extended Data Figures 3 and 4), the *D. sechellia* parental control data were collected in parallel with the control and test hybrids (full spread of the data already shown in Figure 2), so the orange line here does represent a valid comparison.

Reviewer Reports on the First Revision:

Referee #1 (Remarks to the Author):

The authors addressed my concerns well. I also checked the statistical analysis and found no problems.

There only one issue that needs to be addressed in the text (line 277):
The l-LNv are NOT a subset of the "evening" cells. They are required to plasticly adjust the timing of the evening peak as the authors correctly wrote in lines 294-297.
I ask the authors to correct their statement.

Referee #2 (Remarks to the Author):

In my review of the original submission I expressed two major concerns. The first was that the authors had failed to conduct a direct test of the central model by rescuing a loss of function Pdf01 mutation in D. melanogaster with full length D. sechellia Pdf. The second was that the explanation for how changes in the cis regulatory sequence of Pdf result in a loss of photoperiodic adjustment was not consistent with previous work on PDF regulation of locomotor rhythms and photoperiodic adjustment.

In response to my first concern the authors have, laudably, conducted the definitive experiment and state in their rebuttal that "…rescue strains with the D. d Pdf 5'-regulatory sequence exhibit less plasticity (as well as lower morning activity) compared to those with the D. melanogaster Pdf 5'-regulatory sequence," and citing new data in Figure 4e-h. However, examination of the data reveal striking similarity between the two rescue strains under long days (4e) and a failure to produce a sechellia-like reduction in photoperiodic adjustment to long days (comparing 1C 12:12 and 16:8 for sechellia with the data in 4d). Thus, the definitive experiment failed to support the author's model of Pdf's cis-regulatory region being responsible for differences in plasticity. As the authors themselves state, the evolutionary change that produced differences in photoperiodic adjustment is probably mediated by multiple genetic loci, which is not the provocative model that the original manuscript put forth.

In response to my second major concern, the authors have stated that Pdf expression in D. sechellia is highly variable, and that consistent effects of l-LNv Pdf expression were not detectable when sechellia regulatory elements are used to gauge expression. This is troubling, given the fact that Pdf's role in photoperiodic adjustment of locomotor rhythms is mediated by the l-LNv neurons (Schlichting 2016 J. Neurosci. 36:9084) and that the model presented in the original manuscript concluded that decreases in PDF was the mechanism underlying reduced photoperiodic adjustment of daily activity peaks. The authors have moved l-LNv expression data to extended data and have shifted their focus to the s-LNvs and their Pdf expression and structural remodeling. However, the s-LNvs are not thought to be strong drivers of the evening peak phase under long photoperiods. This weakens the central model of the study. The authors pivot from an examination of Pdf expression to the structural plasticity of Pdf neurons, presenting evidence that there is reduced dynamic remodeling of sLNvs when sechellia regulatory regions are used to drive Pdf expression in melanogaster. However, this is only shown for flies reared under LD12:12 and there is no compelling evidence presented here or in the literature that structural plasticity has any bearing on the timing of the evening peak or in its adjustment to photoperiod. To the contrary, there is strong evidence that the sites of plasticity are not required for the PDF mediated functions of the s-LNv, including the setting of evening peak phase (Fernandez et al. 2020 Curr. Biol. 30:2225). These new findings therefore do not provide a compelling explanation for the behavioral differences observed between species.

The authors have addressed all of the minor concerns listed in my original review.

Referee #3 (Remarks to the Author):

The authors have satisfactorily addressed most of our points in their revision. They tested two additional strains of D. sechellia, and they tested females of the original two D. sechellia and D. melanogaster strains and see the same result as they did with males. They acknowledge the issue with replication of these data, providing a new Figure, Extended Data Fig. 4 that replicates their original findings.

The authors have also clearly defined their terms. They adopt the phrase 'evening peak plasticity' instead of 'circadian plasticity' to clarify their claims. Further, they clarify their morning phenotype as either 'morning activity' or 'pre-dawn activity'.

They also addressed some of the statistical and data analysis issues we mentioned. They clarified the normalization method they used for the activity counts. They acknowledged the effect of the rolling triangular mean. They acknowledge the possibility of additional background modifiers contributing to the effects they observe, in addition to Pdf. The p-values, corrected and uncorrected, are provided in the Source Data. In addition, they added the comparison of evening peak timing of the two Gal4 insertions, and they are not significantly different.

There are a few lingering issues with the revision:

1. There is still an issue concerning our original Comment 8 under Major Comments. The authors state that they calculate an average minor allele frequency for the Pdf promoter region. Calculating an average minor allele frequency is problematic. As the authors note, they cannot determine haplotypes as they only have allele frequency data. However, by averaging across polymorphisms, the authors assume that there must be high linkage disequilibrium among the polymorphisms in the Pdf promoter region. This is contrary to what is known about wild populations of D. melanogaster: that linkage disequilibrium decays over 10-30 bp on average (PMID: 22318601). These prior data suggest that each polymorphism should be examined separately.

2. Related to our original Comment 9: Lines 146-154 and Fig. 1c: The authors' thesis rests on the data presented in Fig. 1c. In the figure they compare the response of different strains under different photoperiods. To look at the figure, one would conclude, for example, that under a 16:8 photoperiod that Canton-S and Oregon-R are responding differently than sec07 and sec28. But the author's thesis is that D. sechellia is less responsive to changes in photoperiod. However, this hypothesis is not tested explicitly. The five plots in 1c could be combined as one figure showing the reaction norms of each genotype/species across increasing photoperiod. There should be a strong species X environment interaction.

3. Comment on new Table 5e: How did the authors determine significance for Tajima's D and Fu and Li's D* and F*? There are asterisks next to the numbers in the table in Fig. 5e, but no description of how this significance was determined has been provided. Also, in the materials and methods there is no explanation of how these numbers were calculated, or how the statistical significance was determined. In the Science Tauber paper mentioned by one of the reviewers, 1000 coalescent

simulations were done to determine the significance of the Tajima's D and Fu and Li's D* and F*. Was the same strategy applied here?

Minor Comments:

1. In Fig 2g, It is still not clear what statistical comparisons the asterisks refer to, and this typo persists after revision. The authors stated in response to this comment that "Asterisks indicate significant differences: ** = P < 0.01 and *** = P < 0.001 (Wilcoxon tests comparing each test hybrid to the control hybrid strain (07/w1118) with Bonferroni correction)." However, the control hybrid strain 28/w1118 is part of this figure also. This should be clarified.

2. Results, Line 503, the authors state that they used 82 Pdf 5'-regulatory sequences from individuals recently sampled from the Seychelles archipelago[56] to calculate Tajima's D, Fu and Li's D* and Fu and Li's F* statistics. However, in the Methods, Line 825, the number of sequences used is 41.

3. In reply to Comment 9, authors respond: "Detection of consistent reduction over 2 h would be sufficient to affect fitness in the field, where individual flies likely interact only for much shorter periods of time." Please provide references supporting this assumption, as this forms the basis of one of the major conclusions of the paper.

Author Rebuttals to First Revision:

We thank the reviewers for their additional positive and critical feedback on our manuscript. Below, we provide responses to each of the raised issues.

Referee #1

The authors addressed my concerns well. I also checked the statistical analysis and found no problems.

There only one issue that needs to be addressed in the text (line 277):
The l-LNv are NOT a subset of the "evening" cells. They are required to plasticly adjust the timing of the evening peak as the authors correctly wrote in lines 294-297.
I ask the authors to correct their statement.

RESPONSE: We have now resolved this issue.

Referee #2

In my review of the original submission I expressed two major concerns. The first was that the authors had failed to conduct a direct test of the central model by rescuing a loss of function Pdf01 mutation in D. melanogaster with full length D. sechellia Pdf. The second was that the explanation for how changes in the cis regulatory sequence of Pdf result in a loss of photoperiodic adjustment was not consistent with previous work on PDF regulation of locomotor rhythms and photoperiodic adjustment.

In response to my first concern the authors have, laudably, conducted the definitive experiment and state in their rebuttal that "…rescue strains with the D. d Pdf 5'-regulatory sequence exhibit less plasticity (as well as lower morning activity) compared to those with the D. melanogaster Pdf 5'-regulatory sequence," and citing new data in Figure 4e-h. However, examination of the data reveal striking similarity between the two rescue strains under long days (4e) and a failure to produce a sechellia-like reduction in photoperiodic adjustment to long days (comparing 1C 12:12 and 16:8 for sechellia with the data in 4d). Thus, the definitive experiment failed to support the author's model of Pdf's cis-regulatory region being responsible for differences in plasticity. As the authors themselves state, the evolutionary change that produced differences in photoperiodic adjustment is probably mediated by multiple genetic loci, which is not the provocative model that the original manuscript put forth.

RESPONSE: We thank the reviewer for appreciating our substantial efforts in executing the species-specific rescue approach that they recommended. In contrast to this reviewer, we consider that these data agree with – and strengthen – the data and in-text arguments we originally presented. Based on the results of our hybrid screen, it would have been extremely surprising if the rescue with the transgene containing the *D. sechellia Pdf* 5'-regulatory sequence (in an otherwise *D. melanogaster* genetic background) produced a fully *D. sechellia*-like phenotype. We find a subtler, but still statistically-significant difference, indicating that the *Pdf* regulatory region does have an effect (~30 min difference in evening peak delay), while not explaining the entirety of the species difference (~2 h). We respectfully consider that the reviewer's description of the "*striking similarity*" of the rescue genotypes is misleading, as it does not accurately reflect the consistent difference detected between the rescue strains for the evening peak plasticity (as well as the pre-dawn activity) phenotypes.

We also emphasise that we never claimed that the *Pdf* locus explains the entirety of the species difference in circadian plasticity. In the original submission, we already recognised the implication that additional loci contribute to this effect, as *Pdf* test hybrids do not completely resemble their corresponding *D. sechellia* parental strains in terms of evening peak plasticity, and instead represent an intermediate phenotype to these strains and the control hybrids (Fig. 2e). These data are consistent with a polygenic model, as emphasised in the text:

Lines 435-436: *"Pdf clearly does not explain the entirety of the species differences in plasticity, as is true of most behaviours[47]."*

Neurogenetic studies often seek large phenotypic effects of loss-of-function mutations in individual genes, an undoubtedly powerful approach to identify key molecular players in nervous system development and function (including the various screens that identified the circadian molecular feedback loop). By contrast, the relatively nascent field of evolutionary neurogenetics necessarily expects to relate interspecific differences in single genes to smaller behavioural effects. Pinpointing these is hard, which is why there are only very few documented examples demonstrating causal relationships. Importantly, such phenotypic effects can still impact organismal fitness – and so the evolutionary process – in nature. We have carefully revised the text to eliminate any over-claims or ambiguity about the degree of contribution of *Pdf* to the circadian plasticity phenotype.

In response to my second major concern, the authors have stated that Pdf expression in D. sechellia is highly variable, and that consistent effects of l-LNv Pdf expression were not detectable when sechellia regulatory elements are used to gauge expression. This is troubling, given the fact that Pdf's role in photoperiodic adjustment of locomotor rhythms is mediated by the l-LNv neurons (Schlichting 2016 J. Neurosci. 36:9084) and that the model presented in the original manuscript concluded that decreases in PDF was the mechanism underlying reduced photoperiodic adjustment of daily activity peaks. The authors have moved l-LNv expression data to extended data and have shifted their focus to the s-LNvs and their Pdf expression and structural remodeling. However, the s-LNvs are not thought to be strong drivers of the evening peak phase under long photoperiods. This weakens the central model of the study. The authors pivot from an examination of Pdf expression to the structural plasticity of Pdf neurons, presenting evidence that there is reduced dynamic remodelling of sLNvs when sechellia regulatory regions are used to drive Pdf expression in melanogaster. However, this is only shown for flies reared under LD12:12 and there is no compelling evidence presented here or in the literature that structural plasticity has any bearing on the timing of the evening peak or in its adjustment to photoperiod. To the contrary, there is strong evidence that the sites of plasticity are not required for the PDF mediated functions of the s-LNv, including the setting of evening peak phase (Fernandez et al. 2020 Curr. Biol. 30:2225). These new findings therefore do not provide a compelling explanation for the behavioral differences observed between species.

RESPONSE: For clarity, we subdivide our response into three parts, which we hope collectively address all of the inter-related points of this reviewer:

1. Expression data. First, we wish to clarify that our expression analysis is composed of two types of approaches. Approach (i) comparing *Pdf* RNA, Pdf peptide (and Pdf neuron morphology, which is an indirect read-out of Pdf signalling levels) in *D. melanogaster* and *D. sechellia* (new Fig. 3b-e, ED Fig. 8). Approach (ii) comparing the transcriptional activity of species-specific *Pdf* 5'-regulatory region in transgenic constructs in a common *D.*

melanogaster background (new Fig. 3f-i). Approach (ii) demonstrates clear functional differences in the *Pdf* 5'-regulatory region in terms of levels and dynamics of reporter expression in both l-LNvs and s-LNvs. We go on in Fig. 4 to show such functional differences are behavioural relevant, notably through the rescue experiments discussed above (Fig. 4e-h), as well as showing signs of diversification between species and selection within species (Fig. 5a-e).

The reviewer comments that "*consistent effects of l-LNv Pdf expression were not detectable when sechellia regulatory elements are used to gauge expression*" and that "*there is reduced dynamic remodeling of sLNvs when sechellia regulatory regions are used to drive Pdf expression in melanogaster*", but we stress that we only compared these phenotypes between species (Approach (i)), and so cannot ascribe them exclusively to differences in *Pdf* 5'-regulatory sequences. Indeed, our data from the Approach (i) expression analysis reveals that the 5'-regulatory sequence differences do not always match differences in *Pdf* RNA and peptide levels between species. Such a mismatch, while not simple to understand mechanistically currently, is unsurprising, as we note in lines 438-440:

"Beyond cis-regulatory differences in Pdf characterised here, the translation[20], transport and secretion of this neuropeptide[48] are all potentially subject to divergent regulation."

The "*highly variable*" expression referred to by the reviewer is limited to the peptide expression in the l-LNvs (ED Fig. 8), in particular in *D. sechellia*, and thus no significant differences were detected with *D. melanogaster*. We note that immunofluorescence in l-LNv soma is only semi-quantitative, affected by the spatial arrangement of cells (which is quite variable across brains), and we suspect we could only ever detect large differences in protein levels with this method. Consequently, we acknowledge the difficult of interpreting these data, as we state in lines 252-257:

"Pdf immunofluorescence intensity in the l-LNv soma was not significantly different between these species under both photoperiods, but displayed substantial variability, particularly for D. sechellia under 16:8 h LD (Extended Data Fig. 8c-d). If and how Pdf secretion rate in the l-LNv axon termini differs between these species under distinct photoperiods remains unclear."

2. Data organisation. In response to the reviewer's comments about the reorganisation of data in the revision, we refocused our data presentation on the s-LNvs in the main figure (largely based on the recommendations of the other two reviewers), because the differences we observed here have a better understood relationship to Pdf secretion, at least in *D. melanogaster*. We did not present new data or remove data regarding Pdf expression. However, we realise from the comments of this reviewer that confusion over the nature of the expression analyses (i.e. Approach (i) vs (ii) mentioned above) likely arose from the embedding of the Approach (i) data between the expression analysis of the *Pdf* 5'-regulatory

reporters and the causal contribution of these 5'-regulatory regions to behavioural differences. To help clarify the message – and also to comply with length limitations – we have now re-ordered the expression data to present all the interspecific difference in *Pdf* RNA, peptide (and Pdf neuron morphology) first (i.e. new Fig. 3a-e), following by focused analysis on activity of the 5'-regulatory sequence (i.e. new Fig. 3f-i), which then more naturally flows into Fig. 4-5.

3. Roles of l-LNvs and s-LNvs in evening plasticity. We fully appreciate that there is a body of literature in *D. melanogaster* emphasising the role of Pdf in l-LNvs in determining timing of the evening peak under extended photoperiods, which makes it difficult to relate to the variable Pdf expression we observed in the soma of these cells in *D. sechellia*, as described above. However, there is some nuance in this dogma. For example, in Schlichting J Neurosci 2016, while loss of Pdf expression in the l-LNvs alone, but not in the s-LNvs alone, reduces evening peak plasticity, loss of Pdf in *both* l- and s-LNvs has an even greater effect, hinting that there is a contribution of Pdf in the s-LNvs to this facet of circadian behaviour (in the model in Fig. 6 of that paper, Pdf from both l- and s-LNvs are shown to signal to evening cells). In addition, in Menegazzi Curr Biol 2017, evening peak delay under long photoperiod could be restored by provision of Pdf only in central neurons (i.e. in *neither* l- nor s-LNvs), suggesting that the l-LNv population does not need to be the sole source of this neuropeptide. More generally, timing of evening peak activity under different photoperiod regimes is also not exclusively under the control of Pdf-expressing LNvs, but rather a complex and dynamic neural network (e.g. Stoleru Cell 2007) that is not yet fully understood, even in *D. melanogaster*. Whether such core control mechanisms are identical in *D. sechellia* is unknown, and this would require many new genetic/transgenic reagents and several years of investigation.

In our work, we do not intend to challenge or explain models of circadian control in *D. melanogaster*, as our experiments do not explicitly address these issues. Rather, we report behavioural and cellular level phenotypic differences between these drosophilid species and provide evidence of one genetic cause – the *Pdf* 5'-regulatory region – that contributes to these differences. We believe this work is important, as it provides a rare example linking changes in gene function, central neuron populations, and ecologically-relevant behavioural differences between species.

In the Discussion text, we deliberately did not posit a model in which lower *Pdf* expression in *D. sechellia* results in reduced circadian plasticity but recognise that the cartoon we provide in Fig. 5h might have conveyed this oversimplified idea. We have now removed the schematic displaying differences in *Pdf* expression dynamics to avoid any misunderstanding.

The authors have addressed all of the minor concerns listed in my original review.

Referee #3

The authors have satisfactorily addressed most of our points in their revision. They tested two additional strains of D. sechellia, and they tested females of the original two D. sechellia and D. melanogaster strains and see the same result as they did with males. They acknowledge the issue with replication of these data, providing a new Figure, Extended Data Fig. 4 that replicates their original findings.

The authors have also clearly defined their terms. They adopt the phrase 'evening peak plasticity' instead of 'circadian plasticity' to clarify their claims. Further, they clarify their morning phenotype as either 'morning activity' or 'pre-dawn activity'.

They also addressed some of the statistical and data analysis issues we mentioned. They clarified the normalization method they used for the activity counts. They acknowledged the effect of the rolling triangular mean. They acknowledge the possibility of additional background modifiers contributing to the effects they observe, in addition to Pdf. The p-values, corrected and uncorrected, are provided in the Source Data. In addition, they added the comparison of evening peak timing of the two Gal4 insertions, and they are not significantly different.

There are a few lingering issues with the revision:

1. There is still an issue concerning our original Comment 8 under Major Comments. The authors state that they calculate an average minor allele frequency for the Pdf promoter region. Calculating an average minor allele frequency is problematic. As the authors note, they cannot determine haplotypes as they only have allele frequency data. However, by averaging across polymorphisms, the authors assume that there must be high linkage disequilibrium among the polymorphisms in the Pdf promoter region. This is contrary to what is known about wild populations of D. melanogaster: that linkage disequilibrium decays over 10-30 bp on average (PMID: 22318601). These prior data suggest that each polymorphism should be examined separately.

RESPONSE: We appreciate the reviewer's point and have reanalysed these data taking the approach that they recommend. We performed individual correlations between the frequency of the minor allele and latitude for every variable site within the putative regulatory regions of *Pdf* and the same two control genes we used in our analysis of *D. sechellia* sequences, *sNPF* and *AstC*. In Fig. 5c, we now plot the correlation coefficients for each of these single nucleotide variant (SNV) frequencies. We find that all of the SNV frequencies within the *Pdf* regulatory region display a positive correlation with latitude, with an average correlation coefficient of 0.514. Contrastingly, the SNV frequencies within each of our control genes display a wide range of values, with average coefficients much closer to 0. Importantly, we find significant differences between the distributions of coefficients for *Pdf* and both control genes, but not between the control genes themselves. We have modified our descriptions of these data in both the Results (lines 367-378) and Methods (lines 668-671).

2. Related to our original Comment 9: Lines 146-154 and Fig. 1c: The authors' thesis rests on the data presented in Fig. 1c. In the figure they compare the response of different strains under different photoperiods. To look at the figure, one would conclude, for example, that under a 16:8 photoperiod that Canton-S and Oregon-R are responding differently than sec07 and sec28. But the author's thesis is that D. sechellia is less responsive to changes in photoperiod. However, this hypothesis is not tested explicitly. The five plots in 1c could be combined as one figure showing the reaction norms of each genotype/species across increasing photoperiod. There should be a strong species X environment interaction.

RESPONSE: We performed a nested two-way ANOVA, with strains nested within species, to explicitly test for a G × E interaction. There is a highly significant interaction ($p < 2e\text{-}16$). In new ED Fig. 1a, we present the reaction norm, as well as the results of the ANOVA, and we discuss the results of this test in the text (lines 123-124).

3. Comment on new Table 5e: How did the authors determine significance for Tajima's D and Fu and Li's D* and F*? There are asterisks next to the numbers in the table in Fig. 5e, but no description of how this significance was determined has been provided. Also, in the materials and methods there is no explanation of how these numbers were calculated, or how the statistical significance was determined. In the Science Tauber paper mentioned by one of the reviewers, 1000 coalescent simulations were done to determine the significance of the Tajima's D and Fu and Li's D* and F*. Was the same strategy applied here?

RESPONSE: Yes, we replicated the methods of the Tauber Science paper, and provide these details in the Methods (lines 676-678).

Minor Comments:

1. In Fig 2g, It is still not clear what statistical comparisons the asterisks refer to, and this typo persists after revision. The authors stated in response to this comment that "Asterisks indicate significant differences: ** = P < 0.01 and *** = P < 0.001 (Wilcoxon tests comparing each test hybrid to the control hybrid strain (07/w1118) with Bonferroni correction)." However, the control hybrid strain 28/w1118 is part of this figure also. This should be clarified.

RESPONSE: We apologise for the confusion; for full clarity, we have now added bars indicating each comparison to Fig. 2g and updated the legend.

2. Results, Line 503, the authors state that they used 82 Pdf 5'-regulatory sequences from individuals recently sampled from the Seychelles archipelago[56] to calculate Tajima's D, Fu and Li's D* and Fu and Li's F* statistics. However, in the Methods, Line 825, the number of sequences used is 41.

RESPONSE: We obtained 41 genomes and phased these by chromosome to generate 82 haplotypes.

3. In reply to Comment 9, authors respond: "Detection of consistent reduction over 2 h would be sufficient to affect fitness in the field, where individual flies likely interact only for much shorter periods of time." Please provide references supporting this assumption, as this forms the basis of one of the major conclusions of the paper.

RESPONSE: During condensing of main text and necessary substantial trimming references within this section, we have moved this sentence to justify our experimental design in the Methods. There, we have provided the following two citations, showing that pairwise interactions in social groups of flies last on average for seconds (PMID 34924963), and that willing females will accept mating in naturalistic settings within seconds of being approached by courting males (PMID 33318613) (Line 709).

Reviewer Reports on the Second Revision:

Referee #2 (Remarks to the Author):

The study makes a strong case that melanogaster and sechellia display significantly different photoperiodic responses to increasing daylength, with the latter displaying little or none of the evening peak delay displayed by melanogaster under long day conditions. It also clearly establishes that these two species differ in cis-regulatory sequences of the neuropeptide encoding gene Pigment dispersing factor (Pdf), suggesting that changes in the regulation of Pdf expression might underlie, at least in part, the behavioral differences seen between these two species under long day conditions. The authors also make a nice case that the two species are differentially adapted to day-length changes with the equatorial sechellia displaying an apparently lower reproduction under long day conditions. I agree with the authors that this is of significant interest.

However, I remain unconvinced by the authors'assessment that their "… results indicate that the level (and possibly temporal dynamics) of Pdf expression is sufficient to affect evening peak plasticity." I understand that one should not expect changes at one gene's regulatory sequence to explain all the of the difference in photoperiodic adjustment, however, the authors spend a significant proportion of the study present Pdf expression data that fail to provide a compelling explanation for why the difference in Pdf's cis regulatory region contribute to the stark difference in photoperiodic adjustment, particularly given the potent role the PDF plays in this process.

My main concern remains the results of the work done to examine how differences in Pdf expression between the two species might explain, at least partially, the behavioral differences under long day conditions. Much of this comes down to 1.) a disagreement with the authors regarding the results shown in Figure 4e and 2.) the limitation of the analysis of Pdf expression, particularly in the l-LNvs.

1.) I respectfully disagree with the authors' interpretation of the critical data shown in Fig. 4e-g. If the cis-regulatory region of sechellia were a significant determinant this species' lack of photoperiodic adjustment to long days we would expect the genomic rescue of Pdf null melanogaster mutants with sechellia Pdf sequences to result in sechellia-like photoperiodic responses, or, at the very least, an intermediate phenotype. But this is not what the data are telling us. These rescues look very much like melanogaster with regard to their behavioral timing under 16:8 LD. In fact, one of the two replicates revealed no significant difference in the evening peak phases between the two rescues. I acknowledge that the sechellia Pdf rescue is slightly phase-advanced relative to the melanogaster Pdf rescue, but photoperiodic delay is clearly intact in the sechellia Pdf rescue flies.

2.) I also persist in my concern that the study still fails to offer a compelling explanation of how differences in Pdf expression. The authors describe difference between species in Pdf mRNA and PDF peptide expression in the s-LNvs (higher in melanogaster cell bodies, but lower in dorsal termini at certain timepoints) and in the expression of GFP reporter of Pdf expression and Pdf mRNA in l-LNvs (more complex than the data reported for s-LNvs). I am not convinced that these differences provide a clear explanation for the differences in behavior, especially given what we know about how PDF functions to adjust behavior photoperiodically. I also do not understand the rationale for why the

“semi-quantitative” nature of PDF IHC, which the authors state is “affected by the spatial arrangement of cells (which is quite variable across brains)” should apply only the l-LNvs (s-LNvs are deeper smaller, and traditionally more difficult to image) and only to PDF but not to GFP reporter. Given the established importance of PDF released from the l-LNvs in photoperiodic adjustment, the failure to see a clear and compelling difference in PDF peptide expression between species that accounts for behavioral differences remains a weakness of the study. However, I acknowledge that I appear to be out of step with my fellow reviewers and am happy to defer to their judgment here.

Other concerns with the new revision:

Unless I am mistaken (a distinct possibility), there appear to be issues with figures. For example, Fig. 3j is cited at the bottom of page 6 but Figure 3’s panels only go to panel “i.” Supplemental figure 8 is said in the text to have four panels (a-d), but there are only two in the figure provided. (e.g., line 255 cites Extended Data Fig. 8c-d).

Furthermore, at the bottom of page 5, the authors state that “Pdf signal remains high across the morning peak times in the s-LNv soma of D. melanogaster,” citing Fig. 3e, but this panel shows dorsal termini and only two timepoints spread across the diurnal cycle.

In general, the description of results in the text on page 5, lines 225-246, don’t always clearly match the data in Figure 3. For example, the authors state that “In D. sechellia, the Pdf signal begins high and drops significantly only after lights on,” directing the reader to Fig. 3i, but the figure panel clearly shows Pdf levels dropping at the same times and starting before lights-on.

I should have noticed in the earlier submissions, but in figure 4, the statistical comparisons don’t appear include UAS controls. Including these parental controls would be critical for conclusions the authors are making here.

Referee #3 (Remarks to the Author):

The authors have sufficiently addressed our remaining questions. Further, we agree with the author's rebuttal comments to Reviewer #2; in particular, we agree that natural interspecific variants are more likely to have more subtle effects on behavioral phenotypes than engineered mutations.

We did notice that in the main text, the callouts for Figure 3 no longer line up with the Figure or its legend:
a. Line 222 states that "the spatial distribution of this neuropeptide in D sechellia (Fig. 3b)" but Fig. 3b is the smFISH data. Shouldn't this be 3c and/or 3d?
b. Line 231, shouldn't the reference to Figure 3c be 3b as it is referring to the smFISH data?
c. Lines 231-233 state that the immunofluorescence of Pdf in axonal terminals of the s-LNvs are shown in Fig. 3d, but the figure legend states that this is immunofluorescence in the soma.
d. Lines 241 and 246 refer to Fig. 3e; shouldn't this be 3d instead?
e. Line 268 refers to Fig. 3f, shouldn't this be 3e?
e. Line 272 refers to Fig. 3k, which is no longer in the figure or legend.

Author Rebuttals to Second Revision:

We thank the reviewers for their re-reading and additional comments on our manuscript. Below, we provide responses to each of the raised issues.

Referee #2

The study makes a strong case that melanogaster and sechellia display significantly different photoperiodic responses to increasing daylength, with the latter displaying little or none of the evening peak delay displayed by melanogaster under long day conditions. It also clearly establishes that these two species differ in cis-regulatory sequences of the neuropeptide encoding gene Pigment dispersing factor (Pdf), suggesting that changes in the regulation of Pdf expression might underlie, at least in part, the behavioral differences seen between these two species under long day conditions. The authors also make a nice case that the two species are differentially adapted to day-length changes with the equatorial sechellia displaying an apparently lower reproduction under long day conditions. I agree with the authors that this is of significant interest.

However, I remain unconvinced by the authors' assessment that their "… results indicate that the level (and possibly temporal dynamics) of Pdf expression is sufficient to affect evening peak plasticity." I understand that one should not expect changes at one gene's regulatory sequence to explain all the of the difference in photoperiodic adjustment, however, the authors spend a significant proportion of the study present Pdf expression data that fail to provide a compelling explanation for why the difference in Pdf's cis regulatory region contribute to the stark difference in photoperiodic adjustment, particularly given the potent role the PDF plays in this process.

My main concern remains the results of the work done to examine how differences in Pdf expression between the two species might explain, at least partially, the behavioral differences under long day conditions. Much of this comes down to 1.) a disagreement with the authors regarding the results shown in Figure 4e and 2.) the limitation of the analysis of Pdf expression, particularly in the l-LNvs.

RESPONSE: We have responded in detail to the reviewer's concerns as they have itemised them below. Here we note that our results text quoted above by the reviewer ("… results indicate that …. evening peak plasticity") refers specifically to the results of our RNAi experiment in *D. melanogaster*, and is not referring to the difference in behavior between species. At this point in the manuscript, we do not claim to have connected differences in Pdf expression and/or temporal dynamics to behavioural differences *between species*. Instead, our RNAi experiment in *D. melanogaster* demonstrates that differences in *Pdf* expression *can* impact behavior, at least in *D. melanogaster*. We acknowledge that this RNAi experiment comes first within the results section originally-titled "***Pdf* regulatory regions contribute to**

species differences", which perhaps led to unintended emphasis of the conclusions that can be drawn from this experiment alone. In reformatting the manuscript, this section title is now shortened to "***Pdf* regulatory regions affect plasticity**", which we believe is a more straightforward description of the results subsequently presented.

1.) I respectfully disagree with the authors' interpretation of the critical data shown in Fig. 4e-g. If the cis-regulatory region of sechellia were a significant determinant this species' lack of photoperiodic adjustment to long days we would expect the genomic rescue of Pdf null melanogaster mutants with sechellia Pdf sequences to result in sechellia-like photoperiodic responses, or, at the very least, an intermediate phenotype. But this is not what the data are telling us. These rescues look very much like melanogaster with regard to their behavioral timing under 16:8 LD. In fact, one of the two replicates revealed no significant difference in the evening peak phases between the two rescues. I acknowledge that the sechellia Pdf rescue is slightly phase-advanced relative to the melanogaster Pdf rescue, but photoperiodic delay is clearly intact in the sechellia Pdf rescue flies.

RESPONSE: We respectfully still disagree with the reviewer on this point. The reviewer appears to consider photoperiodic adjustment as a qualitative trait, rather than a quantitative one. We measure a small but significant reduction in photoperiod delay between species-specific Pdf rescue strains, in an otherwise identical genetic background. Here, we believe we are aligned with Reviewer 3's view that "natural interspecific variants are more likely to have more subtle effects on behavioral phenotypes than engineered mutations".

2.) I also persist in my concern that the study still fails to offer a compelling explanation of how differences in Pdf expression. The authors describe difference between species in Pdf mRNA and PDF peptide expression in the s-LNvs (higher in melanogaster cell bodies, but lower in dorsal termini at certain timepoints) and in the expression of GFP reporter of Pdf expression and Pdf mRNA in l-LNvs (more complex than the data reported for s-LNvs). I am not convinced that these differences provide a clear explanation for the differences in behavior, especially given what we know about how PDF functions to adjust behavior photoperiodically. I also do not understand the rationale for why the "semi-quantitative" nature of PDF IHC, which the authors state is "affected by the spatial arrangement of cells (which is quite variable across brains)" should apply only the l-LNvs (s-LNvs are deeper smaller, and traditionally more difficult to image) and only to PDF but not to GFP reporter. Given the established importance of PDF released from the l-LNvs in photoperiodic adjustment, the failure to see a clear and compelling difference in PDF peptide expression between species that accounts for behavioral differences remains a weakness of the study. However, I acknowledge that I appear to be out of step with my fellow reviewers and am happy to defer to their judgment here.

RESPONSE: We agree with the reviewer that we do not find an obvious species-specific differences in endogenous Pdf peptide levels that can easily account for the difference in behaviour. We have been careful not to make any such claims, nor hypothesise at this stage about a mechanistic basis of Pdf's contribution to circadian differences. The measures of

expression we present are merely descriptive, and future tools in *D. sechellia* to assess, for example, secretion dynamics of this peptide will be required.

Other concerns with the new revision:

Unless I am mistaken (a distinct possibility), there appear to be issues with figures. For example, Fig. 3j is cited at the bottom of page 6 but Figure 3's panels only go to panel "i." Supplemental figure 8 is said in the text to have four panels (a-d), but there are only two in the figure provided. (e.g., line 255 cites Extended Data Fig. 8c-d).

Furthermore, at the bottom of page 5, the authors state that "Pdf signal remains high across the morning peak times in the s-LNv soma of D. melanogaster," citing Fig. 3e, but this panel shows dorsal termini and only two timepoints spread across the diurnal cycle.

In general, the description of results in the text on page 5, lines 225-246, don't always clearly match the data in Figure 3. For example, the authors state that "In D. sechellia, the Pdf signal begins high and drops significantly only after lights on," directing the reader to Fig. 3i, but the figure panel clearly shows Pdf levels dropping at the same times and starting before lights-on.

RESPONSE: A last-minute merging of panels a and b in the previous version of this figure before submission resulted in the text references all being one panel off. We have corrected this in the revised version of the manuscript and thank the reviewer for catching the error.

I should have noticed in the earlier submissions, but in figure 4, the statistical comparisons don't appear include UAS controls. Including these parental controls would be critical for conclusions the authors are making here.

RESPONSE: We previously addressed this point in our initial response to reviewer 3, included below for convenience:

"We do not show comparisons to the UAS-Pdf^{RNAi} strain, as this strain was crossed to the w^{1118} strain (so flies had only one copy of the UAS transgene), and we suspect the greater evening peak delay than any other strain reflects this outcrossing. We stress that that most pertinent comparison is between flies of the same genetic background and identical set of transgenes (differing only in the species origin of the Pdf 5'-regulatory region in the Gal4 line)."

Nonetheless, we note that the full list of all Bonferroni-corrected pairwise comparisons is provided in the Source Data. In the case of evening peak delay, the *UAS-Pdf^{RNAi}* strain (crossed to *w^{1118}*) displays significantly greater evening peak delay than any other strain.

Referee #3

The authors have sufficiently addressed our remaining questions. Further, we agree with the author's rebuttal comments to Reviewer #2; in particular, we agree that natural interspecific variants are more likely to have more subtle effects on behavioral phenotypes than engineered mutations.

RESPONSE: We thank the reviewer for their positive feedback and, in particular, their assistance with our population genetic analysis.

We did notice that in the main text, the callouts for Figure 3 no longer line up with the Figure or its legend:

a. Line 222 states that "the spatial distribution of this neuropeptide in D sechellia (Fig. 3b)" but Fig. 3b is the smFISH data. Shouldn't this be 3c and/or 3d?
b. Line 231, shouldn't the reference to Figure 3c be 3b as it is referring to the smFISH data?
c. Lines 231-233 state that the immunofluorescence of Pdf in axonal terminals of the s-LNvs are shown in Fig. 3d, but the figure legend states that this is immunofluorescence in the soma.
d. Lines 241 and 246 refer to Fig. 3e; shouldn't this be 3d instead?
e. Line 268 refers to Fig. 3f, shouldn't this be 3e?
e. Line 272 refers to Fig. 3k, which is no longer in the figure or legend.

RESPONSE: A last-minute merging of panels a and b in the previous version of this figure before submission resulted in the text references all being one panel off. We have corrected this in the revised version of the manuscript and thank the reviewer for catching the error.