Reviewer: 1

Comments to the Author
The submitted manuscript offers a computational study of circadian gene expression across different data sets coming from several species (including human) and tissues. I found the manuscript nicely written and scientifically sound, having said that I'm not a native speaker of English. The quality of figures is good.

The manuscript presents quite exciting findings about the temporal patterns of gene expression.

Thank you for your comments.

General remarks:
- Please comment in the manuscript on how the study was conceived - was it the availability of the data that motivated the particular design of the study?
We have now commented briefly on this point in the Introduction. In short, it was a combination of recently published datasets and our recent development of ZeitZeiger.

- I believe that both abstract and the introductory sections could be substantially improved. Your results don't merely "highlight the value of multi-tissue analysis", but rather question the consensus role of the SCN as a central circadian pacemaker in mammals.
We appreciate your enthusiasm for our findings! We do allude to “implications” in the abstract and elaborate on them in the Discussion. In light of decades of work on the role of the SCN and the fact that our study is descriptive in nature, however, we are reluctant to overstate our results and would prefer to let readers draw their own conclusions.

- Introduction: I don't see why conservation of clock genes should automatically imply the conservation of clock dynamics.
We apologize for not explaining that statement. Our reasoning was that if the genes are conserved, then the protein-protein and regulatory interactions are likely also conserved. Since it is primarily the interactions between clock genes and proteins that control the dynamics, this implies that the dynamics of the clock may also be conserved.

- An important question to the interpretation of your results: if genes A and B are both rhythmic and 8 hours apart, what makes you think that it is gene B that is 8 hours time-delayed relative to gene A and not gene A that is 24-8=16 hours time delayed relative to gene B?
We have clarified this point in the text. As we originally wrote in the Results, “peak time in brain was 6-8 h later (alternatively, 16-18 h earlier) than peak time in blood”. Indeed, these interpretations are equivalent and indistinguishable. We consistently referred to it as a delay simply because 8 is less than 16.

- An explanation how you classified your genes into rhythmic/non-rhythmic would make the manuscript convincing.

As explained below, we have now clarified how we decided which genes to keep in the analysis.

- In relation to the previous one: I think there are two *independent* components of circadian rhythmicity: (1) a "nice" (whatever particular meaning that might have) waveform of the signal during one day and (2) the periodicity itself, i.e. the property of being periodically repeated over day after day. You can have a most beautiful sine-looking time trace over one day, but if it's not repeated during the second day, you cannot speak of circadian rhythmicity. Next, most data that you show here is on 0-24 CT scale. Whereas you can assess the first property, for the second you need longer time courses.
We might call the first component “periodicity” and the second component “stability”. Although stability may be useful on longer timescales in constant conditions, we would argue that when the system is near the limit cycle (e.g., fully entrained), there is no difference between one day and the next; there is only the average behavior throughout the limit cycle, i.e., periodicity. This is what we are quantifying.

More technical questions:
- In Material & Methods, Selecting the Samples, it would be a good idea to start with at least a sentence containing a short overview of what data you have used, i.e. "we analyzed N datasets, including those from A, B and C under Alpha Beta and Gamma conditions".
As requested, we have added an overview sentence in the Materials and Methods.

- Please specify on the total number of data points in the analysed data, the number of data points per day and the total length of the experiments in days, ideally each time you discuss a new data set.
We have added all this information to the supplementary table. Given the large number of datasets analyzed in this study, in the interest of readability, we would prefer to not mention all these details in the Results section.

- In Processing the Expression Data: please explain your conversion of counts to transcripts per million in a bit more detail. What counts? Transcripts per million of what? Why did you decide to go with the given log formula?
We have revised our processing of the RNA-seq data and expanded our explanation of it. Transcripts per million (transcripts) is related to the proportion of transcripts in a sample that map to a given feature (https://haroldpimentel.wordpress.com/2014/05/08/what-the-fpkm-a-review-rna-seq-expression-units). The purpose of the log-transformation was to make the data comparable to the log-transformed microarray data. Because the minimum tpm is zero, we used log(tpm + 1).

- How do you control the order of the fitting spline against over-fitting? Did you cross-validate your fits?
We apologize for omitting this information. To prevent overfitting, the periodic spline fits were based on only three knots. We did not run the full cross-validation, but did observe some overfitting at higher numbers of knots.

- How do you enforce the periodicity of the fitting spline and its derivative? What happens to the fit if the underlying data is not periodic/rhythmic?
The periodicity is enforced within the bigspline function, of which we are not the authors. In our experience, however, the function works very well. If the data is not rhythmic, then the spline fit will be flat (but still technically periodic) and the signal-to-noise ratio will be zero, which is the case for many genes (Suppl. Fig. S1).

- What is your criterion for a "clear rhythmicity" p. 5, lines 30-31?
The criterion was post hoc, but the clock genes we examined seemed to fall into one of two groups, with a convenient cutoff being SNR>1 for at least one dataset each in blood and brain. Admittedly, ARNTL only meets this criterion in one of three blood datasets.

- What is the sensitivity of your phase detection algorithm towards noise? Can you provide a measure for it, for example, a confidence interval?
This is an excellent point. We have now performed simulations to determine how accurately ZeitZeiger can detect peak and trough times, and have added these results to the text and the supplement. For 200 observations randomly spaced in time and SNR=2 (assuming sinusoidal periodicity and iid additive Gaussian noise), our algorithm can detect the peak or trough time with a median absolute error of 0.3 h and a 95% confidence interval of 1.1 h (Suppl. Fig. S3).

- Computing mean, std for cyclic variables (i.e. phases) is a bit more subtle than for linear quantities, and median is downright impossible (you don't have a unique notion of "between" with cyclic quantities like phases or angles, consider what would be "between" 12am and 12pm). Please comment on using those statistics for your analysis.
Thank you for making this point. In this study, we used the circular difference and the circular mean. We mentioned these in “Analyzing circadian gene expression” of the Methods, and we have now clarified the caption for Figure 3. In Figure 2C and Suppl. Fig. S5B, we used the absolute phase difference, so we believe the non-circular versions of mean, median, and standard deviation are valid. We have clarified these points in the text.

- How do you define "highly conserved"? You've got examples for both differences and similarities between expression phases in different species.
This is an interesting question. Here we have not tried to rigorously define conservation in the context of dynamics. We have clarified our usage of “conserved” in the Discussion, namely, similar relative phasing of clock gene expression (Suppl. Fig. S10).

Reviewer: 2

Comments to the Author
This manuscript presents a meta-analysis of phase distribution among different mammals and multiple tissues. The authors first compared the phase distribution of core clock genes between

human blood and brain, and then expanded the analysis to a few other mammals and their SCNs. This addresses an important issue, and I expect the authors' conclusions will be of interest to the circadian field.

Thanks for your feedback.

Major issues:
1. In the supplementary table S1, the authors mention that samples from GSE48133 followed a LD 16:8 light-dark regimen. However, it seems as if from the original paper, the volunteers experienced a dark–dim light cycle which was scheduled to a 28-h day. It is important for the authors to clarify this point.
Thank you for catching this error. The samples we used were from the first day of the forced-desynchrony protocol, for which the onset of darkness was 24 hours after the onset of darkness from the previous (baseline) day. Therefore, the subjects perceived that day as dim-dark 14.7:9.3. We have revised the supplementary table accordingly.

2. The authors controlled the age for samples from brain datasets by splitting them into >40 or <=40, However, they did not mention the overall age distribution in each subgroup after splitting. Including this piece of information will be worthwhile for estimating the degree of phase shifting between brain and blood based on the fact of age related phase-shifting. If a different age threshold were used, would their results change?
We apologize for omitting this information. The age distribution of donors in the younger group was 29.7 ± 8.2 years (compared to 27 ± 5 years for blood donors). This has now been added to the Materials and Methods. Using a cutoff of 35 years gave very similar results.

3. Processing the metadata to infer CT0 is problematic. In the absence of better computational methods, the authors are subject to the whims of human biological rhythms… i.e. social or professional jetlag, pathological states, caffeine, fluorescent lighting, etc. One wonders whether the unique phasing of the human brain is an artifact of these well-documented perturbations. This manuscript could be improved with a much more detailed discussion of these ambiguities.
We have now expanded our discussion of this possibility from only "... the unnatural environment in which many humans live (especially with respect to light)". We should note, however, that all brain donors died rapidly and were carefully screened for psychiatric disorders. We have also added analysis of another possible variable, namely the post-mortem interval (Suppl. Fig. S6 and S7).

4. Related to this, the authors in the Discussion speculate that the unique phasing of the human brain may be due to "evolutionary adaptation". Even with their cautious, diplomatic language ("it seems reasonable to wonder…") this is a bridge too far. Wikipedia tells me that the conventional date for widespread use of fire dates to ~125-250k years ago. This is an awfully short amount of time to evolve a phase-shifted clock. I recommend the authors look at GWAS studies of clock gene polymorphisms as humans left Africa (closer to 1,000k years ago) in order to flesh out this argument in more satisfying detail.

Thank you for calling us out on this. Because data from non-human diurnal mammals are so limited, and to keep the manuscript focused, we have expunged the speculation about evolutionary adaptation.

5. "…the periodic nature of circadian time (e.g. CT4 is 6 h ahead of CT20". Please correct this calculation.
This rookie mistake has been corrected.

6. Is the rhesus macaque data really necessary? I would stipulate that one sample at each of six time points is junk and unusable. Including weak data in a meta-analysis is very dangerous without independent checks.
We don't disagree. To be honest, we were desperate to include data from non-human diurnal mammals. The rhesus macaque data has now been removed.

7. Regarding the SCN data from mice; there's an unpublished data set on CircaDB (the eventual citation will be "Ballance et al.). Since these data are already publically available, I recommend including them.
If that dataset is the same as GSE70384, then it is already included. We have added the citation information to the supplementary table.

8. Figure 3B is a confusing jumble of colors. I recommend the authors rethink this figure for color-blind readers.
Thanks for the suggestion. We have removed the mapping of species to colors and shifted the mapping of circadian time to colors.

Minor issues:
1. There are two places where the font changes. I assume this is a relic of the drafting and editing process. In any case, it can be eliminated.
This turned out to be a mysterious artifact of converting from a Google Doc to a docx file. It should be fixed now.