Revision 1

Dear Dr. Schwartz,
I am writing to resubmit our manuscript, “LimoRhyde: a flexible approach for differential analysis of rhythmic transcriptome data,” for consideration as an original article in JBR. We were encouraged by the reviewers’ thoughtful comments, and have thoroughly revised the manuscript accordingly. In particular, we have now used simulations to validate and explore LimoRhyde’s performance. Our point-by-point responses are below.

Thank you for your consideration. I look forward to hearing from you.

Sincerely,

Jacob Hughey

Reviewer: 1

Comments to the Author
In the manuscript titled ‘LimoRhyde: a flexible approach for differential analysis of rhythmic transcriptome data’, the author presented an approach for detecting statistical significant variation in rhythmicity. Through decomposing the time variable into multiple components by LimoRhyde, time-series data could fit linear model, which makes it easier to apply differential expression tools in identifying differential rhythmicity. Comparing with DODR, LimoRhyde gives similar results in analyzing circadian data under two experimental conditions. In addition, LimoRhyde can handle complex experimental designs that are not suitable for DODR. With more time-series datasets being generated, LimoRhyde will provide a new aspect in mining these datasets.

Major issues:
1. The pipelines in Fig. 1A (DR first then DE) and Fig. 3A (DE first then DR) are different. Even RAIN and JTK_CYCLE can not analyze randomly spaced time-series data used in Fig. 3, other available method like Lomb-Scargle (Glynn E.F., Bioinformatics, 2006) or consinor regression could analyze this kind of dataset. Is it possible to analyze human brain data with the same pipeline shown in Fig.1A? If so, will the author get quite different results?

We have now revised the analysis pipeline for the human brain data to be consistent with the pipeline for the mouse data (i.e., rhythmicity -> differential rhythmicity -> differential expression). The results are virtually unchanged, because the number of differentially rhythmic genes is relatively small and the number of differentially expressed genes is massive. In fact, our use of LimoRhyde for identifying rhythmic genes is equivalent to cosinor regression. We have now also

attempted to identify rhythmic genes in the human brain dataset using Lomb-Scargle, which yielded only 30 rhythmic genes ($q \leq 0.1$; Suppl. Fig. S4B), so we have not pursued it further.

2. As evaluated by Decard A. et al.(Bioinformatics, 2013), each rhythmic detection method had different strengths. Has the author evaluated the influence on DR and DE analysis caused by applying different rhythmic detection methods before running LimoRhyde? Simulated datasets used in DODR may be helpful for this evaluation.

With the exception of now trying Lomb-Scargle for the human brain dataset, we have not tried the various methods for identifying rhythmic genes. The differences between these methods are orthogonal to the main point of our paper, which is to describe an approach for identifying differential rhythmicity and differential expression.

We have used simulations, however, to validate LimoRhyde's ability to detect differential rhythmicity (Fig. 2 and Suppl. Fig. S3) and to show the high concordance between LimoRhyde and DODR (Suppl Fig. S4).

3. Will the significant cut-off used in defining rhythmic or not rhythmic affect the DR and DE analysis? If so, what is the influence of setting a more strict or less strict cut-off in the DR and DE analysis?

We have added supplemental plots to address this question (Suppl. Fig. S2 and S5). In short, the number of differentially rhythmic genes increases as the cutoffs for rhythmicity and differential rhythmicity become less stringent. The number of differentially expressed genes, on the other hand (which tends to be large), depends only modestly on the cutoffs for rhythmicity and differential rhythmicity, but increases as the cutoff for differential expression becomes less stringent.

4. How about applying LimoRhyde in analyzing other time-series transcriptome datasets not studying circadian rhythms (e.g. cell cycle)? A case study using cell cycle dataset will extend the application of LimoRhyde. Other than transcriptome datasets, could LimoRhyde be used to analyze time-series omics data (e.g. proteomics and metabolomics) with missing values?

Thank you for the suggestions. Although we considered analyzing cell cycle data, we found no datasets that included samples from both a full time-course (post synchronization in vitro) and from multiple conditions that it would make sense to compare. The advantage of circadian datasets is that most of them are based on tissues in vivo.

We are eager to try LimoRhyde on other data types. In our experience, though, proteomics and metabolomics data often differ from transcriptome data in terms of signal-to-noise and number of features. In addition, the two largest circadian proteomics datasets we know of collected samples from only a single condition (Robles et al. 2016 and Wang et al. 2016). For these reasons, we wish to keep the current manuscript focused on gene expression.

5. A vignette or shinny app along with the LimoRhyde package showing the whole pipeline from time-series data to the DR and DE results will be helpful for users of LimoRhyde.

Thank you for the suggestion. We have now added a vignette to the LimoRhyde R package, which walks through each step of a typical analysis.

6. Those detected DR genes show difference in amplitude or phase in this study. However, it will be great if LimoRhyde could do a statistical test on the amplitude and phase difference between different conditions. In addition, other possible DR genes, with period length variation under difference conditions, may be ignored by DODR and LimoRhyde. These may be out of the range of this paper, but could be mentioned in the discussion part.

We agree these are interesting problems to address in future work, and have added a couple sentences to the Discussion.

Minor points:
1. The sub-figure order in Fig. 1 needs to be re-organized.

We have revised the layout of Fig. 1.

Reviewer: 2

Comments to the Author
This paper uses a linear model to quantify how rhythms in gene expression that are measured by cosinor regression vary across a series of conditions. In this sense, the approach, named LimoRhyde, can be viewed as a straightforward generalization of the existing DODR approach, which exploits differences in sinusoidal fits to compare two conditions. While overall the method looks like a useful tool that would be of interest to readers of JBR, the manuscript would be improved by addressing the following comments.

1. No explicit mathematical details are given. While I understand that the audience is biologically oriented, a few equations making explicit the form of the sinusoidal fit and how its output is then used in the linear models would make it easier to understand the method. Related, a few mathematical expressions are embedded in the figures, but I find it confusing that they do not include the coefficients that are the outputs of the fits.

We have now added mathematical details to the Methods section, to clarify how the sinusoidal fit is used in the linear model and what differential rhythmicity and differential expression are testing for. We have also switched the description of the linear models in the figures from R's model formula notation to standard vector notation.

2. The paper notes that it incorporates an empirical Bayes procedure to improve the statistical analysis. In this regard, the author should cite Hutchison et al. bioRxiv:118521, which investigates the impact of empirical Bayes on analysis of circadian transcriptomic data.

Thanks for bringing this work to our attention. We have now cited it in the Introduction.

3. On p. 4, the author makes an aside that one could test for differential variability. Even looking at the cited reference, it is hard to understand what this means---I guess it is the variability of expression within sets of genes. More explanation is needed, or this remark is just a distraction that should be removed.

This remark was getting too far into the weeds, so we have removed it.

4. While the biological examples presented are nice, the author should apply the method with simulated data, which would allow computing the performance against known answers and verification that the statistics are correct. This would also allow for investigating the origins of the differences in performance compared with DODR.

Thank you for this suggestion. We have now applied LimoRhyde to simulated data and validated its performance against the known labels (Fig. 2 and Suppl Fig. S3). In the simulations, the statistics from LimoRhyde and DODR are highly concordant (Suppl. Fig. S4). Overall, the small differences between DODR (specifically, robustDODR) and LimoRhyde (followed by limma) seem to be the differences between a rank-based test and a parametric test.

5. On p. 8 (and again on p. 9 in the Discussion), the author says that the random timing of samples precludes the use of RAIN and JTK_CYCLE. This is not true---one can sample the reference waveforms in these methods at arbitrary times and thus make them match the data.

Thank you for correcting us on this point. As far as we can tell, however, the current APIs of the two methods make it impractical to analyze data with a large number of randomly spaced time-points. We have revised the relevant text on page 8 to say “... making the use of RAIN or JTK_CYCLE infeasible”. We have revised the relevant text on page 9 to say “... scenarios for which the current implementations of methods such as JTK_CYCLE and RAIN are ill-suited”.

6. In Fig. 3C, the author groups the samples into two age ranges, but the advantage of the method is that it could treat larger numbers of age ranges. The author should look at the performance as a function of the number of groups into which the samples are binned.

For the LimoRhyde analysis in Fig. 3, we did not bin the data at all. We actually used the continuous values of age to identify differentially rhythmic and differentially expressed genes. We only binned the data into two groups to use ZeitZeiger to estimate the age-dependent

changes in amplitude and phase of differentially rhythmic genes. We have revised the text and figure caption to make this clearer.

7. On p. 10, the author discusses potential issues with analyzing free-running organisms. It was not clear to me why one could not also account for period differences by extension of the method.

The issue with period differences is not a technical one. Our point is that the results will depend on how one aligns the two time-courses, since ultimately, all times become equivalent to something between 0 and 2π. We have revised that part of the Discussion accordingly.

Revision 2

Referee 1
The manuscript titled 'LimoRhyde: a flexible approach for differential analysis of rhythmic transcriptome data' was comprehensively revised. I recommend it to be published on Journal of Biological Rhythms after fixing two minor issues.

1. The numbers mentioned in the manuscript and in the Fig. 4C are in-consistent. E.g. the manuscript mentioned '196 genes were differentially rhythmic', but it is 195 in the Fig. 4C.

Thanks for catching this inconsistency. The figure was correct, so we have changed the number in the manuscript to 195.

2. Workflows were shown with Table1 and Table 2, which were not as intuitive as figures (e.g. Fig. 1A in the primary submission). Is it possible to replace them with supplemental figures?

While the schematics may have been more intuitive to some, they were not capable of concisely conveying all the relevant information. We have now tweaked the tables to make them more understandable.

Referee 2
Overall, the authors have done a good job at responding to the reviewer comments. Below, I provide a few additional comments the authors can consider.

p. 9, line 12: Pearson correlation coefficients are reported. However, because the q-values vary over orders of magnitude, the Spearman correlation coefficient would be more informative.

We have now changed the calculation to the Spearman correlation coefficient. The value to two significant digits is unchanged (0.91).

The authors mentioned in the response to Reviewer 2 Point 6 that they revised the text and caption for Figure 4 to make clearer that they used the continuous values of age in the analysis for Figure 4. However, I could not find these changes in the highlighted manuscript.

The highlighting was done post hoc, so we missed a few changes. We have reproduced the relevant revisions to the manuscript in italics below. The figure caption was not changed.

- "... we used an additive model in LimoRhyde, including terms for age *(as a continuous variable in years)*, zeitgeber time, and brain region"
- "... we altered the linear model to include an interaction between age and zeitgeber time, *maintaining age as a continuous variable*"

Indeed, given that the ability to handle continuous data is the main advantage of the method, it would be nice to provide visualizations in Figure 4 that made clear the continuous variations. The authors should consider plotting the model coefficients as functions of the age and using a heatmap to show the variation of the data with age.

Thanks for the suggestion. We have now used the model coefficients to plot the expected expression of a given gene as a function of zeitgeber time and age (Fig. 4F). These plots turned out to be more understandable than heatmaps.

Figure S3B would be clearer if the authors plotted the p-values against the expected p-values under the null distribution, so that a uniform distribution of results would fall on the diagonal.

As suggested, we have now revised Fig. S3B to show quantile-quantile plots of the p-values of differential rhythmicity.