Response to Reviewers' comments
Hughes et al. "Guidelines for genome scale analysis of biological rhythms"

We thank the editor and reviewers for their helpful comments regarding our manuscript, and we're delighted there was enthusiasm for publishing this article. Please find below our responses to specific comments.

Editor's Comments to Author:

1. Please format citations in the text and reference list in JBR style (author, year in text and alphabetical order in bibliography).

Corrected.

Reviewer(s)' Comments to Author:

Reviewer: 1

Comments to the Author
This thoughtful and well-written paper provides highly useful recommendations for the execution and interpretation of metabolomics and genome-wide transcriptomics and proteomics experiments around the circadian cycle. I very much like the undogmatic style of the piece. The authors correctly point out that different experimental rigor has to be applied for evaluating approaches aimed at describing large number of rhythmic components and experiments serving as a "treasure troughs" for finding components whose cyclic abundance will subsequently be reexamined in depth by additional techniques. I only have two suggestions that the authors may want to consider.

In circadian biology the term "amplitude" is frequently misused for "fold change". In physics the amplitude of an oscillation is defined as (maximal value – minimal value)/2. For a wave in the ocean the amplitude is highly relevant for boats and swimmers, irrespective of the absolute depth of the water. For example, a wave with an amplitude of five meters exerts the same effect on a floating object, regardless of whether the depth of the water is 20 meters or 2,000 meters. In contrast, in circadian gene expression (or metabolomics) the term "amplitude" is nearly irrelevant. For example, the amplitude of mRNAs fluctuating between 1 and 101 molecules per cell or 950 and 1050 molecules is 50 for both. While the former oscillation is likely to be physiologically relevant, the latter is probably not. What counts is the "fold change". This reviewer is embarrassed to admit that he is also guilty of having confounded the terms "amplitude" and "fold change", but this is no good reason for continuing to confuse the two terms.

We agree and now include a section paraphrasing the distinction drawn by the reviewer between amplitude and fold change.

The normalization of data sets is unsatisfactory in most publications. In RNA-seq experiments the data sets are usually normalized to the total number of mappable reads/experiment. In circadian studies this is only acceptable if the total amount of RNA per cell remains constant throughout the day. However, this is not necessarily the case. A recent study demonstrated that total liver RNA/cell oscillates during the day (Sinturel et al., 2017, Cell 169:651-663). Hence, DNA, representing cell number, is probably the only reliable biochemical denominator for the normalization of gene expression data. This also applies to comparisons between cell/tissue types, as the RNA/DNA ratio differs dramatically between cell/tissue types (Schmidt & Schibler, 1995, J Cell Biol 128:467-483). Ideally, the RNA/DNA ratio should be determined for every time point and/or cell type/tissue type. A simple method on how to accomplish this is provided in Sinturel et al. (2017).

We agree and now include this suggestion and these citations in the updated manuscript.

Reviewer: 2

Comments to the Author
This is a very useful article and will be a great resource for the field. It is written in an understandable language and makes a complicated topic approachable. I only have a few minor suggestions for revision:

P. 7 – For the recommendation of sampling of 48 hours, authors should clarify that this only holds true in constant conditions with no environmental zeitgebers or if the data are serially collected on the same subjects. For example, if data are collected in an LD cycle using population sampling, there is no reason to suspect that collecting 8 samples (from 8 subjects) at two hours after lights on would be any different from collecting another 8 samples from a different set of 8 subjects 24 hours later, but still 2 hours after lights on. In other words, when population sampling, there is no "repeat."

We now emphasize that the recommended 48 h sampling applies under constant conditions.

P. 8 The issue above is addressed in the second paragraph, but this paragraph includes multiple concepts including, period length, as well as consecutive cycles in LD. Maybe LD cycles should be discussed first, followed by the constant conditions paragraph. The issue of period length should be described in a separate paragraph. One could argue that period should not even be measured in the case of a zeitgeber (LD cycle) since tau = T in this case.

We have reorganized this section at the reviewer's suggestion.

General comments:
Please be more specific about what is meant by duplication or concatenation. Are the terms meant synonymously?

We now include a definition of 'duplication and concatenation' in the text and the Figure Legends.

It would be great if the authors could address how to handle repeated measures on the same individuals, which can substantially increase statistical power. This is mentioned as Key Area #1 but not enough attention is given to it in the manuscript.

We agree that statistical power changes when sampling from a single individual rather than from a population. We direct readers to two recently developed methods for addressing inter-individual variation. In response to the reviewers comment, we now elaborate on the rationale for including this in our "Key Areas" text box.