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Bivalve mollusk circadian clock genes can run at tidal frequency

Damien Tran, Mickael Perrigault, Pierre Ciret and Laura Payton

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Review timeline
Original submission: 18 October 2019
Revised submission: 25 November 2019
Final acceptance: 2 December 2019

Note: Reports are unedited and appear as submitted by the referee. The review history appears in chronological order.

Review History

RSPB-2019-2440.R0 (Original submission)

Review form: Reviewer 1

Recommendation
Accept with minor revision (please list in comments)

Scientific importance: Is the manuscript an original and important contribution to its field?
Good

General interest: Is the paper of sufficient general interest?
Good

Quality of the paper: Is the overall quality of the paper suitable?
Good

Is the length of the paper justified?
Yes

Should the paper be seen by a specialist statistical reviewer?
No

Do you have any concerns about statistical analyses in this paper? If so, please specify them explicitly in your report.
No

It is a condition of publication that authors make their supporting data, code and materials available - either as supplementary material or hosted in an external repository. Please rate, if applicable, the supporting data on the following criteria.

Is it accessible?
Yes

Is it clear?
Yes

Is it adequate?
Yes

Do you have any ethical concerns with this paper?
No

Comments to the Author
This manuscript by Tran et al. reports on an investigation of circadian and circatidal rhythms of both behavior (valve-opening) and expression of clock-associated genes in the oyster Crassostrea gigas. Experiments were done in natural conditions subject to exogenous light and tidal cues, as well as constant conditions to investigate endogenous rhythms. Both behavior and gene expression was seen to exhibit elements of both circadian and circatidal rhythms, even in constant conditions. The authors conclude that the bimodal (circadian and circatidal) expression of certain genes supports the presence of a single clock that regulates both rhythms.

Strengths
• This manuscript builds on previous work identifying circadian clock-associated genes in C. gigas, and adds elements of both behavior and circatidal analyses. This continues to build the knowledge base of an invertebrate in an under-represented clade (Lophotrochozoa) for circadian investigations.
• The experimental design and analyses for both the behavioral and gene expression components of the study were sound and well-conceived.
• While relatively far afield of the work in this study, Crassostrea is an important organism for aquaculture and the more we understand about this group of bivalves, the better for the associated fishery. Furthermore, circatidal rhythms have been observed for a long time but we still know very little about the mechanisms underlying these rhythms. Thus, this paper will be an integral component to our growing understanding of this poorly understood phenomenon.

Major Comments
• The authors analyzed valve activity and gene expression in natural and free-running conditions, which have been done many times before with many other animals. The paper claims to test the hypothesis that a single bimodal oscillator controls both circatidal and circadian rhythms, by looking at potential circatidal expression of genes that are also expressed with a circadian rhythm. But this is simply correlation. Testing of this hypothesis would need to involve what others have done, which is to knock down circadian genes, especially in the area of the circadian clock, and measure the effects on circatidal rhythms. While I agree that a single bimodal

oscillator for these two rhythms could explain the results from this current study, the experiments do not specifically test this, as the authors suggest. Furthermore, I suggest that the authors tone down their conclusions. In lines 305-310, the authors go well beyond their correlational study to suggest that they have "…demonstrated that the circadian clock could run at a tidal frequency…" They then go on to write, "Integrating the tidal cues with the daily cues likely occurs in a single clock…" Just because genes involved in the circadian clock are also seen to have a circatidal rhythm does not mean that the circadian clock can run at a tidal frequency. What if the circadian and circatidal clocks use similar genes but are located in different areas of the nervous system? It is important for the authors to remember that they have done a correlational investigation and they have not demonstrated evidence for any causative connection.

• Previous data were analyzed (reanalyzed?) in this study for constant darkness. This is fine, but it would be helpful to have the previous experiments and analyses of these investigations described in the Introduction. Currently, these experiments are only briefly mentioned in the last paragraph of the Introduction (references 21 and 22 are cited).

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Minor comments

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• Lines 125-126: There is missing text here, providing information about the primer sets. Later on in line 126, "method" is used twice.

• Lines 264-266: I have no idea what the authors are trying to say in these sentences. There might be some missing text.

Review form: Reviewer 2

Recommendation

Major revision is needed (please make suggestions in comments)

Scientific importance: Is the manuscript an original and important contribution to its field?
Good

General interest: Is the paper of sufficient general interest?
Good

Quality of the paper: Is the overall quality of the paper suitable?
Marginal

Is the length of the paper justified?
Yes

Should the paper be seen by a specialist statistical reviewer?
No

Do you have any concerns about statistical analyses in this paper? If so, please specify them explicitly in your report.
No

It is a condition of publication that authors make their supporting data, code and materials available - either as supplementary material or hosted in an external repository. Please rate, if applicable, the supporting data on the following criteria.

Is it accessible?
Yes

Is it clear?
Yes

Is it adequate?
Yes

Do you have any ethical concerns with this paper?
No

Comments to the Author
This manuscript is a study about the biological clock, which is very interesting topics of biology, especially for marine species, which may affected by both of solar and lunar reasons. The scientific question is sound and relevant but poorly presented. Major work of rephrasing and restructuring the manuscript is needed. Details are given below.
1: The authors should rephrase the MS, where some parts sentence should be put in Introduction section. Some of the discussion were enclosed in results section, which should be separate.
MATERIALS AND METHODS
Experimental model and field details
2: The authors state that oysters used were from natural recruitment and of comparable age (1.5 years of old, 65-75 mm shell length). If you used attachment to collect oysters in the nature, all of them is C.gigas ? No C.angulata in the Arcachon Bay? If you collected the oysters from intertidal rocks, it is difficult to distinguish the age of the wild oysters.
Valve activity behavior
3:15/14 oysters were used for Valve activity behavior detection, but I think the number of the oysters are not sufficient because of the diversity among individuals.
4: I think the VOA parameter itself is not enough to explain the activity of valve. Is the valve activity represent the activity of the oyster well? Maybe the filtration rate of the oyster is a

reasonable parameter to support each other.
Sampling for gene expression
5: Why gill instead of other tissues were used for gene expression study. What about the mantle tissue or adductor muscle?
6: Why the sample interval was 3.1h, instead of 1h or 2h, it seems that you know these genes have tidal rhythm before your experiment.
7: What's the basis for gene set chosen for expression detection like Cgclock, please give a reason in the material and method section.
8: I think the VOA and gene expression results could not demonstrated that the circadian clock could run at tidal frequency because of the insufficient oysters and parameters.

Review form: Reviewer 3

Recommendation
Accept with minor revision (please list in comments)

Scientific importance: Is the manuscript an original and important contribution to its field?
Excellent

General interest: Is the paper of sufficient general interest?
Excellent

Quality of the paper: Is the overall quality of the paper suitable?
Excellent

Is the length of the paper justified?
Yes

Should the paper be seen by a specialist statistical reviewer?
No

Do you have any concerns about statistical analyses in this paper? If so, please specify them explicitly in your report.
No

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Is it clear?
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Comments to the Author

Tran et al. study the transcript level changes of core circadian clock genes as well as valve opening behavior under natural and laboratory conditions. While the behavior exhibits both tidal, as well as diel/circadian periods under field and laboratory conditions (LD and DD), the transcript profiles of the core circadian clock genes exhibits remarkable differences between the field, lab LD and lab DD conditions. The most striking finding this paper presents is that several core "circadian" clock genes oscillate with tidal frequencies under DD conditions, but are diel under lab LD. The field data represent an interesting "combination" of both, maybe they are overall a bit closer to the lab DD, than the lab LD data (with per,cry1,cry2 being interesting exceptions).

This is an absolutely fascinating story and I am sure it will be much referred to in the future. It suggests that what we at present call "circadian" oscillator might not be strictly "circadian", but rather represent a flexible oscillator system that can be used to control inner time on the range of hrs.

Technically, the paper is solid. I have only few comments.

By convention: gene names should always be written in italics. Otherwise this will really cause confusion, as for molecular biologists non-italics means proteins are referred to.

The authors compare gene expression in gills, with valve opening behavior. Isn't the latter controlled by the muscle? In order to make a logical connect between the measured behavior and the gene expression, it would be nice if qPCR data from muscle would be available. If this is not possible, this aspect should be commented on and discussed.

Figure S1- light data are in lux. This is very a very unfortunate unit, as it is adjusted relative to the sensitivity of the human eye. Are there any measurements in photons/area/time or uW/area/time available? If not, comment in methods why not.

Supply of seawater- please also include a comment on what is exactly used. Artificial sea water or natural? In other words- could the water contain tidal chemosensory cues? (Even if, this wouldn't take away from the importance of the author's findings, but the interpretations would be a bit different, as it is not free-running, but still under possibly entraining conditions.)

Methods in S1 are really confusing about LD and temperature conditions 10:14 or 9:15 ? Two different temperatures are given. Please clarify.

As the authors do not really functionally show that the "circadian" clock genes are functionally required for the tidal rhythm to occur, it could still be possible that the observed transcript oscillation patterns are downstream of a still existing independent circatidal oscillator. This possibility needs to be included in the discussion and interpretation.

Suggestion for the heading: "Into the wild: …"

Line 70: "in" not "into"

Decision letter (RSPB-2019-2440.R0)

12-Nov-2019

Dear Dr Tran:

Your manuscript has now been peer reviewed and the reviews have been assessed by an Associate Editor. The reviewers' comments (not including confidential comments to the Editor) and the comments from the Associate Editor are included at the end of this email for your reference. As you will see, the reviewers and the Editors have raised some concerns with your manuscript and we would like to invite you to revise your manuscript to address them.

We do not allow multiple rounds of revision so we urge you to make every effort to fully address all of the comments at this stage. If deemed necessary by the Associate Editor, your manuscript will be sent back to one or more of the original reviewers for assessment. If the original reviewers are not available we may invite new reviewers. Please note that we cannot guarantee eventual acceptance of your manuscript at this stage.

To submit your revision please log into http://mc.manuscriptcentral.com/prsb and enter your Author Centre, where you will find your manuscript title listed under "Manuscripts with Decisions." Under "Actions", click on "Create a Revision". Your manuscript number has been appended to denote a revision.

When submitting your revision please upload a file under "Response to Referees" - in the "File Upload" section. This should document, point by point, how you have responded to the reviewers' and Editors' comments, and the adjustments you have made to the manuscript. We require a copy of the manuscript with revisions made since the previous version marked as 'tracked changes' to be included in the 'response to referees' document.

Your main manuscript should be submitted as a text file (doc, txt, rtf or tex), not a PDF. Your figures should be submitted as separate files and not included within the main manuscript file.

When revising your manuscript you should also ensure that it adheres to our editorial policies (https://royalsociety.org/journals/ethics-policies/). You should pay particular attention to the following:

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If your study contains research on humans please ensure that you detail in the methods section whether you obtained ethical approval from your local research ethics committee and gained informed consent to participate from each of the participants.

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If your study uses animals please include details in the methods section of any approval and licences given to carry out the study and include full details of how animal welfare standards were ensured. Field studies should be conducted in accordance with local legislation; please include details of the appropriate permission and licences that you obtained to carry out the field work.

Data accessibility and data citation:
It is a condition of publication that you make available the data and research materials supporting the results in the article. Datasets should be deposited in an appropriate publicly available repository and details of the associated accession number, link or DOI to the datasets must be included in the Data Accessibility section of the article

(https://royalsociety.org/journals/ethics-policies/data-sharing-mining/). Reference(s) to datasets should also be included in the reference list of the article with DOIs (where available).

In order to ensure effective and robust dissemination and appropriate credit to authors the dataset(s) used should also be fully cited and listed in the references.

If you wish to submit your data to Dryad (http://datadryad.org/) and have not already done so you can submit your data via this link
http://datadryad.org/submit?journalID=RSPB&manu=(Document not available), which will take you to your unique entry in the Dryad repository.

If you have already submitted your data to dryad you can make any necessary revisions to your dataset by following the above link.

For more information please see our open data policy http://royalsocietypublishing.org/data-sharing.

Electronic supplementary material:
All supplementary materials accompanying an accepted article will be treated as in their final form. They will be published alongside the paper on the journal website and posted on the online figshare repository. Files on figshare will be made available approximately one week before the accompanying article so that the supplementary material can be attributed a unique DOI. Please try to submit all supplementary material as a single file.

Online supplementary material will also carry the title and description provided during submission, so please ensure these are accurate and informative. Note that the Royal Society will not edit or typeset supplementary material and it will be hosted as provided. Please ensure that the supplementary material includes the paper details (authors, title, journal name, article DOI). Your article DOI will be 10.1098/rspb.[paper ID in form xxxx.xxxx e.g. 10.1098/rspb.2016.0049].

Please submit a copy of your revised paper within three weeks. If we do not hear from you within this time your manuscript will be rejected. If you are unable to meet this deadline please let us know as soon as possible, as we may be able to grant a short extension.

Thank you for submitting your manuscript to Proceedings B; we look forward to receiving your revision. If you have any questions at all, please do not hesitate to get in touch.

Best wishes,
Professor Gary Carvalho
mailto: proceedingsb@royalsociety.org

Associate Editor
Board Member: 1
Comments to Author:
Three reviewers reviewed the manuscript submitted by Tran et al.

Overall, the reviewers found the paper to be of importance with well executed experiments and of a broad interest to the journal's readership. However, a few minor to semi-major issues were raised that require revision before a final decision may be made. In particular I highlight the following examples; however, please address all of the reviewers' comments before returning your manuscript.

1. Occasionally the phrasing used is unclear and where appropriately highlighted by the reviewer/s, these should be address. I would perhaps recommend a careful re-reading of the manuscript by an independent researcher for assistance on language clarity. Also, please ensure that UK spelling is used and not American English.

2. Be careful not to over-interpret the data as presented. Edit as suggested by the reviewers.

3. Please update the Introduction and Discussion as suggested by Rev 1.

4. The reviewers have questioned the title, I suggest removing the "Into the wild" tagline.

5. Please comment on the sample size used vrs the intrinsic diversity amongst individuals.

6. Gene expression: consider including qPCR data from muscle or sufficiently justify why this is not possible.

7. Change the lux unit to photons/area/time as suggested.

8. Please include a more detailed Ethics Statement in the main text, including any local ethical approvals from relevant universities.

9. Some of the labels on the graphs (both main figs. and supp. figs) are very small, please consider enlarging the font size so that they are clearer to the reader if the paper is accepted.

Kind regards
Wayne Davies

Reviewer(s)' Comments to Author:

Referee: 1

Comments to the Author(s)
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Referee: 3

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Figure S1- light data are in lux. This is very a very unfortunate unit, as it is adjusted relative to the sensitivity of the human eye. Are there any measurements in photons/area/time or uW/area/time available? If not, comment in methods why not.

Supply of seawater- please also include a comment on what is exactly used. Artificial sea water or natural? In other words- could the water contain tidal chemosensory cues? (Even if, this wouldn't take away from the importance of the author's findings, but the interpretations would be a bit different, as it is not free-running, but still under possibly entraining conditions.)

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Suggestion for the heading: "Into the wild: …"

Line 70: "in" not "into"

Author's Response to Decision Letter for (RSPB-2019-2440.R0)

See Appendix A.

Decision letter (RSPB-2019-2440.R1)

02-Dec-2019

Dear Dr Tran

I am pleased to inform you that your manuscript entitled "Bivalve mollusk circadian clock genes can run at tidal frequency" has been accepted for publication in Proceedings B.

You can expect to receive a proof of your article from our Production office in due course, please check your spam filter if you do not receive it. PLEASE NOTE: you will be given the exact page

length of your paper which may be different from the estimation from Editorial and you may be asked to reduce your paper if it goes over the 10 page limit.

If you are likely to be away from e-mail contact please let us know. Due to rapid publication and an extremely tight schedule, if comments are not received, we may publish the paper as it stands.

If you have any queries regarding the production of your final article or the publication date please contact procb_proofs@royalsociety.org

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All supplementary materials accompanying an accepted article will be treated as in their final form. They will be published alongside the paper on the journal website and posted on the online figshare repository. Files on figshare will be made available approximately one week before the accompanying article so that the supplementary material can be attributed a unique DOI.

Thank you for your fine contribution. On behalf of the Editors of the Proceedings B, we look forward to your continued contributions to the Journal.

Sincerely,
Professor Gary Carvalho
Editor, Proceedings B
mailto: proceedingsb@royalsociety.org

Appendix A

Ms. No. RSPB-2019-2440

Title : Into the wild, bivalve mollusk circadian clock genes run at tidal frequency

Response to Associate Editor and Referees.

Associate Editor

Board Member: 1

Comments to Author:

Three reviewers reviewed the manuscript submitted by Tran et al.

Overall, the reviewers found the paper to be of importance with well executed experiments and of a broad interest to the journal's readership. However, a few minor to semi-major issues were raised that require revision before a final decision may be made. In particular I highlight the following examples; however, please address all of the reviewers' comments before returning your manuscript.

We thank the Associate Editor to let us the opportunity to revise the manuscript. We have carefully read the comments of the referees. We hope that our answers and revisions will convinced the associate editor and the referees.

Please, find our responses (in blue) to the associate editor and reviewers' comments. Line numbers refer to the "tracked changes" revised manuscript (this is not the same line numbers in the revised version without tracked change). Changes in the manuscript are highlighted in blue font in "the tracked changes" manuscript included in this file 'response to referees'.

1. Occasionally the phrasing used is unclear and where appropriately highlighted by the reviewer/s, these should be address. I would perhaps recommend a careful re-reading of the manuscript by an independent researcher for assistance on language clarity. Also, please ensure that UK spelling is used and not American English.

As mentioned to the referee 2, we previously corrected and edited by Nature Research Editing Service (English Language Editing, Gold version) for this manuscript. The certificate is in attached file.

Moreover, the referee 2 said we have to rephrase or to change some sentences to a paragraph to another one, but he never cite the paragraphs or the sentences concerned. So it is difficult to modify in a good way. We did our best.

2. Be careful not to over-interpret the data as presented. Edit as suggested by the reviewers.

Ok, we have moderated our conclusion in different sentences. See the comment in the response to the referee 1.

3. Please update the Introduction and Discussion as suggested by Rev 1.

See the comment in the response to the referee 1

4. The reviewers have questioned the title, I suggest removing the "Into the wild" tagline.

Ok, this is done.

5. Please comment on the sample size used vrs the intrinsic diversity amongst individuals.

See the comment in the response to the referee 2

6. Gene expression: consider including qPCR data from muscle or sufficiently justify why this is not possible.

See the comment in the response to the referee 2 and 3

7. Change the lux unit to photons/area/time as suggested.

See the comment in the response to the referee 3

8. Please include a more detailed Ethics Statement in the main text, including any local ethical approvals from relevant universities.

We added a new sentence in this way in the Materials and Methods section. Now ln 607-609.

9. Some of the labels on the graphs (both main figs. and supp. figs) are very small, please consider enlarging the font size so that they are clearer to the reader if the paper is accepted.

Ok, we have enlarged when it was possible the font size of figures 2, 3 & 4 and SI figures (2 & 3).

Kind regards

Wayne Davies

We hope it will be correct now.

Sincerely,

Damien Tran

Referee 1

Comments to the Author(s)

This manuscript by Tran et al. reports on an investigation of circadian and circatidal rhythms of both behavior (valve-opening) and expression of clock-associated genes in the oyster Crassostrea gigas. Experiments were done in natural conditions subject to exogenous light and tidal cues, as well as constant conditions to investigate endogenous rhythms. Both behavior and gene expression was seen to exhibit elements of both circadian and circatidal rhythms, even in constant conditions. The authors conclude that the bimodal (circadian and circatidal) expression of certain genes supports the presence of a single clock that regulates both rhythms.

Strengths

- This manuscript builds on previous work identifying circadian clock-associated genes in C. gigas, and adds elements of both behavior and circatidal analyses. This continues to build the knowledge base of an invertebrate in an under-represented clade (Lophotrochozoa) for circadian investigations.
- The experimental design and analyses for both the behavioral and gene expression components of the study were sound and well-conceived.
- While relatively far afield of the work in this study, Crassostrea is an important organism for aquaculture and the more we understand about this group of bivalves, the better for the associated fishery. Furthermore, circatidal rhythms have been observed for a long time but we still know very little about the mechanisms underlying these rhythms. Thus, this paper will be an integral component to our growing understanding of this poorly understood phenomenon.

Major Comments

- The authors analyzed valve activity and gene expression in natural and free-running conditions, which have been done many times before with many other animals. The paper claims to test the hypothesis that a single bimodal oscillator controls both circatidal and circadian rhythms, by looking at potential circatidal expression of genes that are also expressed with a circadian rhythm. But this is simply correlation. Testing of this hypothesis would need to involve what others have done, which is to knock down circadian genes, especially in the area of the circadian clock, and measure the effects on circatidal rhythms.

The referee is right. We agree, to absolutely prove the hypothesis, functional approaches should be used, such as knock-out technique on circadian gene expressions and then see what happen at the behavioral level. That's why we already mentioned this issue in our manuscript, in the discussion section (see below). We explained why knock-down technique (RNAi) is not sufficient to prove the hypothesis and we also mentioned that unfortunately other functional approaches are not available on bivalves to date.

Ln 770-777: "*To validate the transcriptional regulatory actions of each clock gene, functional approaches are necessary. Unfortunately, the use of the Drosophila S2 cell transcriptional assay [14] is adapted for light entrainment experiments but not for investigations of the effects of tidal zeitgeber. The knockout gene technology, using CRISPR-Cas9 [45] gene-editing, is not currently operational for bivalves. Finally, gene interference that allows the knockdown of clock gene expression [46] does not give a clear and definitive response, as gene transcripts that might be sufficient to maintain the transcriptional feedback loops of the clock machinery always remain."*

Ln 813-825: "*Despite their merits, the limits of these studies are that RNAi applications lead to a decreased-but-not-abolished expression of the targeted genes [14,16,17]. These knockdown studies do not preclude the possibility that interference in clock gene expression was not sufficient to affect tidal oscillation. Depending on the species and the ecological niches they occupy, circadian rhythm is likely more labile than circatidal rhythm. Thus, the partial knockdown of the clock gene would at least partially disrupt the circadian pattern but would have no effect or less of an effect on the tidal pattern, which would be more robust in the littoral species. To validate or refute the unimodal tidal clock hypothesis (dissociated from the circadian one), the use of a complete knockout technique by mutagenesis, whose availability is still limited in marine organisms, might be a promising approach*."

While I agree that a single bimodal oscillator for these two rhythms could explain the results from this current study, the experiments do not specifically test this, as the authors suggest. Furthermore, I suggest that the authors tone down their conclusions. In lines 305-310, the authors go well beyond their correlational study to suggest that they have "…demonstrated that the circadian clock could run at a tidal frequency…" They then go on to write, "Integrating the tidal cues with the daily cues likely occurs in a single clock…" Just because genes involved in the circadian clock are also seen to have a circatidal rhythm does not mean that the circadian clock can run at a tidal frequency. What if the circadian and circatidal clocks use similar genes but are located in different areas of the nervous system? It is important for the authors to remember that they have done a correlational investigation and they have not demonstrated evidence for any causative connection.

We understand the referee. As suggested we tone down our conclusion.

We modify the sentences:

Ln 829-830: "*Our results demonstrated that the circadian clock could run at tidal frequency.*" by

"Our results **showed** that the circadian clock **genes** could run at tidal frequency."

Ln 832-834: "*Integrating the tidal cues with the daily cues likely occurs in a single clock that would give bimodal or unimodal oscillation outputs according the balance between tidal and daily cues in each specific location inhabited by oysters*." By

"*Integrating the tidal cues with the daily cues **might** occurs in a single clock that would give bimodal or unimodal oscillation outputs according the balance between tidal and daily cues in each specific location inhabited by oysters*."

The referee said that possibly a circadian clock and a circatidal clock can share the same genes. But in our comprehension, the canonical clock genes such as clock, bmal, per… are the core clock.

Basically, the circadian clock is the interconnected expression of the canonical circadian core clock genes that generate a rhythmic output and not something else or something more (except of course the genes involved in the peripheral loops of regulation).

- Previous data were analyzed (reanalyzed?) in this study for constant darkness. This is fine, but it would be helpful to have the previous experiments and analyses of these investigations described in the Introduction. Currently, these experiments are only briefly mentioned in the last paragraph of the Introduction (references 21 and 22 are cited).

The previous data were used here to be tested with an algorithm not used in the previous articles to see rhythmic activities. In previous use of these data, the aim was not the same. Thus, this was not helpful to mention the results using these data (especially according to the limit of the words for the manus length).

However, these papers where the data were already used are mentioned several times:

In introduction section: ln 596 (mentioned by the referee)

In M&M section: ln 632, 643

In results section: 702, 715, 717, 719

In discussion section: 740, 760

In SI methods: the reference 21 and 22 are mentioned as reference 3 and 4 in SI.

- There are many strange differences in gene expression, based on the RAIN analysis, between the field experiments and controlled laboratory conditions. For example, cry1, cry2, and per do not express circatidal rhythms in the field, when they are exposed to tides (Fig. 3C). Yet they do express circatidal rhythms in DD in the laboratory, when they are not exposed to tides (Fig. 4C). There are numerous other differences in statistically-supported rhythms between Fig. 3C and 4C, but these differences are never explained.

As pointed out by the referee, it still remains numerous questioning about the circadian clock genes expression. But this is the first report of clock gene expression of bivalves in both field and lab. We are aware that the story is not achieved and other experiments are necessary to understand mechanistically the clockwork. However, an explanation could be done about the reason why cry1, cry2, and Per express daily rhythms in the field but express circatidal rhythms in DD. Indeed, these genes could be more sensitive to the daily zeitgeber than to the tidal zeitgeber, although they can be synchronized by both. And so, in field and lab LD conditions, cry1, cry2, and Per expressions run at daily frequency, but in free-running the intrinsic rhythm could be circatidal. As such explanations are speculative, it was not added in the discussion to not to burden the text.

• In the first paragraph of the Discussion, the case is made that this is the first example of bimodal (circatidal and circadian) activity in a subtidal organism. However, there has been extensive research over the last decade on circatidal and circadian rhythms in the horseshoe crab (Limulus polyphemus), that lives in subtidal areas and enters the intertidal zone only to breed. Reference to this extensive body of research is missing from this paper.

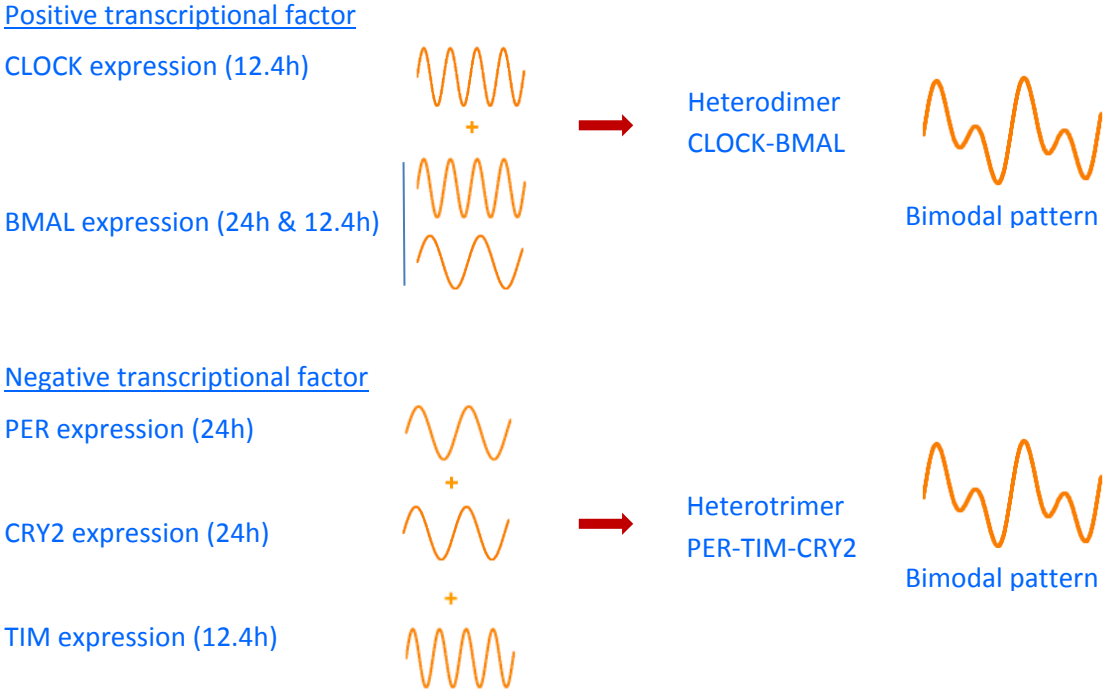
The referee is right. Many studies have been done last decade with the horseshoe crab (*Limulus polyphemus*), especially by Pr. C. Chabot and his team. In fact, specifically (and it was unclear in our manus) we mentioned studies done at behavioral and also at molecular levels. Which, to our knowledge, wasn't done for the horseshoe crab until now. However, as specified by the referee we will mention now the research done on the behavior of the horseshoe crab.

We modified the sentence ln 732-736 and we added a new reference ([34] *Anderson, R.L., Watson, III, W.H. and Chabot, C.C. 2017. Local tidal regime dictates plasticity of expression of locomotor activity rhythms of American horseshoe crabs, Limulus Polyphemus. Mar. Biol. 164:63. doi: 10.1007/s00227-017-3098-9*).

Consequently, we modified the numbering of the references after the reference 34.

• Lines 235-238: I fail to see how tidal expression of CLOCK and bimodal expression of BMAL1 could result in bimodal rhythms. The previous sentence states that CLOCK and BMAL1 operate as heterodimers. If CLOCK is not expressed with a circadian rhythm, then how could it form a heterodimer with the circadian component of BMAL1 expression, when circatidal and circadian rhythms are not always in synchrony? The same issue arises in lines 241-244, when the authors mention that CRY2 and PER have circadian rhythms of expression, whereas TIM has a circatidal rhythm. They then make the case that a heterotrimer of these proteins could result in bimodal repression of the clock.

To explain to the referee, we show with a schema:



To obtain a bimodal pattern of transcriptional factors, it could be done an analogy with the behavioral bimodal pattern, which have been explained by Enright in 1976 (Resetting a tidal clock: a

phase-response curve for *Excirolana*). A bimodal pattern that takes into account daily and tidal cues, has a pattern that can evolve from an unimodal circadian pattern to an unimodal circatidal pattern, including different bimodal patterns, accordingly to the different biotopes. See the figure below.

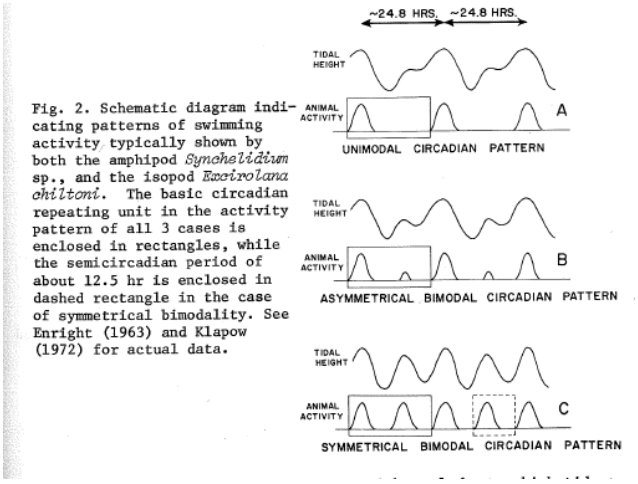


Fig. 2. Schematic diagram indicating patterns of swimming activity typically shown by both the amphipod *Synchelidium* sp., and the isopod *Excirolana chiltoni*. The basic circadian repeating unit in the activity pattern of all 3 cases is enclosed in rectangles, while the semicircadian period of about 12.5 hr is enclosed in dashed rectangle in the case of symmetrical bimodality. See Enright (1963) and Klapow (1972) for actual data.

To have more detailed explanations of the possible mechanism proposed, see the work of J. Enright (see the 2 papers below), with a transposition of the behavioral pattern to the gene/protein expressions pattern:

Zeitschrift für vergleichende Physiologie 46, 276—313 (1963)

From the Scripps Institution of Oceanography, of the University of California, La Jolla, California

THE TIDAL RHYTHM OF ACTIVITY OF A SAND-BEACH AMPHIPOD

By

JAMES T. ENRIGHT* **

Resetting a tidal clock: a phase-response curve for *Excirolana*

J. T. Enright

But this makes no sense when TIM is only expressed every 24 hours and only a couple of times a month would that expression maxima be synchronized with the tidal rhythms.

Sorry, we do not understand the referee. Why the referee is speaking about TIM expression only a couple of times a month? This is not a conclusion of our work.

Minor comments

- I'm not a big fan of "Into the wild" in the title. Compared to the scope of biological literature out there, there is little that is "wild" about this investigation.

Ok, we modify the title. Ln 517-518
Now: "Bivalve mollusk circadian clock genes **can** run at tidal frequency"

- Lines 125-126: There is missing text here, providing information about the primer sets. Later on in line 126, "method" is used twice.

We thank the referee. The end of the sentence was missing. Now, the sentence is complete. Ln 428-429.

And, we removed the word “method” in excess. More detailed explanations of the method are done in Methods SI.

- Lines 264-266: I have no idea what the authors are trying to say in these sentences. There might be some missing text.

The hypothesis exposed here was to say: as the rhodopsin-likes genes might be under control of the clockwork that run tidally, their expression are also tidally expressed. These genes are putatively involved in the light transduction. The shells are opened according to the tides, making the light more available to the gills inside the palleal cavity accordingly to the tidal rhythm. This could justify an expression of the rhodopsin-like genes based on the tidal rhythm.

As mentioned by the referee, the explanations were not very clear. To clarify, we modified the sentences ln 784-787.

Now: « *Similarly, a tidal rhythmicity of the CgRhodopsin-likes 1-3, that are homologs of the rhodopsin involved in light signal transduction [32], is observed. One can be argued that this tidal rhythmicity is explained by a tidal periodicity of light availability to the gills inside the palleal cavity, as a result of the valve opening tidal rhythm*.”

Referee 2

Comments to the Author(s)

This manuscript is a study about the biological clock, which is very interesting topics of biology, especially for marine species, which may affected by both of solar and lunar reasons. The scientific question is sound and relevant but poorly presented. Major work of rephrasing and restructuring the manuscript is needed. Details are given below.

1: The authors should rephrase the MS, where some parts sentence should be put in Introduction section.

We know that we are not native English speaking people. We are sorry about that. So, to be understood, we were previously corrected and edited by Nature Research Editing Service (English Language Editing, Gold version), see below and the attached file of the certificate. We don’t know how to do more for this manuscript.

SPRINGER NATURE | Author Services

Nature Research Editing Service Certification

This is to certify that the manuscript titled Into the wild, mollusk bivalve's canonical circadian clock genes run at tidal frequency was edited for English language usage, grammar, spelling and punctuation by one or more native English-speaking editors at Nature Research Editing Service. The editors focused on correcting improper language and rephrasing awkward sentences, using their scientific training to point out passages that were confusing or vague. Every effort has been made to ensure that neither the research content nor the authors' intentions were altered in any way during the editing process.

Documents receiving this certification should be English-ready for publication; however, please note that the author has the ability to accept or reject our suggestions and changes. To verify the final edited version, please visit our verification page. If you have any questions or concerns over this edited document, please contact Nature Research Editing Service at support@as.springernature.com.

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Authors: TRAN DAMIEN

Key: 92E7-622E-90DE-7365-CA0P

This certificate may be verified at **secure.authorservices.springernature.com/certificate/verify**.

Some of the discussion were enclosed in results section, which should be separate.

We are not sure to see what sentences of the results section should be in the discussion section, because the referee didn't gave us the information. After reading carefully the results section, we might see two sentences that should be putted in the discussion. We hope that it is the sentences that the referee is speaking about.

The first sentence in the Results previously in the sub-paragraph *Bimodal valve activity behavior in the field* "These results that oyster behavior exhibits both tidal and daily rhythms were validated by previous one-year studies at the same field location." is now in the discussion ln 734-736.

The second sentence in the Results previously in the sub-paragraph *Bimodal and circatidal behavior in free-running conditions* "This result for *C. gigas* is confirmed by previous results by Perrigault and Tran." is now in the discussion ln 739-740.

MATERIALS AND METHODS

Experimental model and field details

2: The authors state that oysters used were from natural recruitment and of comparable age (1.5 years of old, 65-75 mm shell length). If you used attachment to collect oysters in the nature, all of them is C.gigas ? No C.angulata in the Arcachon Bay? If you collected the oysters from intertidal rocks, it is difficult to distinguish the age of the wild oysters.

We understand the referee. We measured the length of the shells but the age was approximate, given by the oyster farm that collected the oysters in nature in subtidal conditions. As the study was not focused on the different stages of age of the oysters, we gave this approximate information.

Now, we suggest to modify the sentence ln 602 "All investigations were performed on Pacific oysters, *C. gigas*, of comparable age (**approximatively** 1.5 years old, 65-75 mm shell length).

To answer to the issue about *C. angulata*, since the beginning of the seventies in France, this specie has disappear. Indeed, a viral disease (iridolike viruses) has caused the definitive disappearance of this specie that have been replaced by *C. gigas* specie.

See references:

Grizel, H., Heral, M. 1991.Introduction into France of the Japanese oyster (*Crassostrea gigas*). ICES Journal of Marine Science, Volume 47, Issue 3, 1991, Pages 399–403, https://doi.org/10.1093/icesjms/47.3.399.

Renault, T. 2016. Iridolike Viruses of Mollusks. Aquaculture Virology. Pp 507-512. https://doi.org/10.1016/B978-0-12-801573-5.00036-X

Valve activity behavior

3:15/14 oysters were used for Valve activity behavior detection, but I think the number of the oysters are not sufficient because of the diversity among individuals.

We disagree with the referee. First, 29 oysters were used for valve activity: 15 oysters in the field and 14 oyster in the lab. We studied all these oysters in parallel during the whole experiment, recorded continuously at 10 Hz.
In the 18-days field experiment we have a total of 86 400 data (measure at 10Hz during a day) x 18 days, i.e. 1 555200 data analyzed.
In the 7-days lab experiment we have a total of 86 400 data x 7 days, i.e. 604800 data analyzed.
To our knowledge, we have never seen such amount of data to analyze behavioral studies.
Moreover, the statistical significance of the rhythms using Cosinor is very strong. The inter-individual variability is very low, see SI figure 2 &3.
Finally, the results showed in this study are already validated by two 1-year previous studies we did in the field (Tran et al. 2011 and Payton et al. 2017) and one study done in the lab (Perrigault et al. 2017).

4: I think the VOA parameter itself is not enough to explain the activity of valve. Is the valve activity represent the activity of the oyster well? Maybe the filtration rate of the oyster is a reasonable parameter to support each other.

VOA, which is the valve opening amplitude, is the best parameter to study the rhythmic behavior of the shell. We can measure with our technique (HFNI valvometer) very sensitive valve movement and spacing, at nanometer level.

We agree with the referee to say that filtration rate of the gills is as well a very good parameter to see the activity of the bivalve, but it is not a behavioral parameter but rather a physiological parameter. However, VOA has a good correlation with the gills filtration rate. The increase of VOA is correlated with the increase of filtration rate (Personal data in Lab experiment)

Technically, and until now, it is impossible to record in the field filtration rate for days/weeks, with 15 bivalves in parallel. Even the use of benthic bell that might be use in the field is not really appropriate, because the mollusk is isolated by the bell (necessary for the measure of a clearance) from the surrounding environment, such as for example the tidal water current, food, water pressure... On contrary, the HFNI valvometer biosensor allow to study without any behavioral disturbances for months the bivalves in their environment (see for the biosensor: Andrade et al. 2016. High frequency non-invasive (HFNI) bio-sensors as a potential tool for marine monitoring and assessments. Front. Mar. Sci. 3. (doi:10.3389/fmars.2016.00187))

Sampling for gene expression

5: Why gill instead of other tissues were used for gene expression study. What about the mantle tissue or adductor muscle?

We agree, the adductor muscle could have been a good candidate as well, as it is directly involved in the mechanic of the valve movement. The gills were chosen because it is a more integrative tissue. The gills filtration (see the above comment of the referee) necessary for the respiration and the feeding is directly correlated with the VOA, and a good parameter of the physiological status of the bivalve. Moreover, the decision to use the gills was also motivated by the fact we have published previous studies on gills gene expression, thus we had a background of knowledge with the clock gene expression in the gills that can support our work.

6: Why the sample interval was 3.1h, instead of 1h or 2h, it seems that you know these genes have tidal rhythm before

Yes, as we wanted to test our hypothesis that clock genes can run at tidal frequency, we precisely chosen a sampling time based on the tidal cycle, i.e. every 3.1h. This sampling time was chosen as well to fit perfectly with the RAIN algorithm procedure, used to detect rhythmic activity in gene expression.

7: What's the basis for gene set chosen for expression detection like Cgclock, please give a reason in the material and method section.

The aim of this study was to test how the canonical circadian clock genes cycle in the natural environment. These core clock genes are clock, bmal, per, tim, cry1 &2, rev-erb, ror. We sequenced these genes in *C. gigas* (Perrigault et al. 2017), and called them Cgclock, Cgbmal …ect.

To explicit, we modified the sentence ln 643-646.

Now : "*Primer sets of circadian clock genes (Cgclock, Cgbaml, Cgper, Cgtim, Cgcry2, Cgcry1, Cgrev-erb, Cgror), clock-associated genes (Cghiomt, CgRhodopsin 1, 2 and 3) and housekeeping genes applied in this study are listed in table S3*."

8: I think the VOA and gene expression results could not demonstrated that the circadian clock could run at tidal frequency because of the insufficient oysters and parameters.

We disagree with the referee. We think on the contrary that we have a lot of data and oysters used in our work.

For the valve behavior, see the comment above.

And for clock gene expression, we have used 226 oysters (112 oysters for field experiment, 42 for LD experiment and 72 for DD experiment), which is a lot, even if it is of course possible to do more. We think we are statistically robust. If we compare with papers published in the same field of studies, usually we can see that most the time, there are only 3 replicates or individuals by sampling time.

Referee 3

Comments to the Author(s)

Tran et al. study the transcript level changes of core circadian clock genes as well as valve opening behavior under natural and laboratory conditions. While the behavior exhibits both tidal, as well as diel/circadian periods under field and laboratory conditions (LD and DD), the transcript profiles of the core circadian clock genes exhibits remarkable differences between the field, lab LD and lab DD conditions. The most striking finding this paper presents is that several core "circadian" clock genes oscillate with tidal frequencies under DD conditions, but are diel under lab LD. The field data

represent an interesting “combination” of both, maybe they are overall a bit closer to the lab DD, than the lab LD data (with per,cry1,cry2 being interesting exceptions).

This is an absolutely fascinating story and I am sure it will be much referred to in the future. It suggests that what we at present call “circadian” oscillator might not be strictly “circadian”, but rather represent a flexible oscillator system that can be used to control inner time on the range of hrs.

Technically, the paper is solid. I have only few comments.

By convention: gene names should always be written in italics. Otherwise this will really cause confusion, as for molecular biologists non-italics means proteins are referred to.

Ok, we modified in the whole manuscript and figures. All the genes are in italics now.

The authors compare gene expression in gills, with valve opening behavior. Isn't the latter controlled by the muscle? In order to make a logical connect between the measured behavior and the gene expression, it would be nice if qPCR data from muscle would be available. If this is not possible, this aspect should be commented on and discussed.

We understand the referee. The question was raised as well by the referee 2.

See our comment:

“We agree, the adductor muscle could have been a good candidate as well, as it is directly involved in the mechanic of the valve movement. The gills were chosen because it is a more integrative tissue. The gills filtration (see the above comment of the referee) necessary for the respiration and the feeding is directly correlated with the VOA, and a good parameter of the physiological status of the bivalve. Moreover, the decision to use the gills was also motivated by the fact we have published previous studies on gills gene expression, thus we had a background of knowledge with the clock gene expression in the gills that can support our work.”

Moreover, one can be answered that if the muscle is the effector of the valve behavior, the gills are more likely to be the “controlling organ”. Indeed, for the gills filtration at the origin the nutrition and respiration processes in bivalves, the valve behavior should be driven by the gills need.

Figure S1- light data are in lux. This is very a very unfortunate unit, as it is adjusted relative to the sensitivity of the human eye. Are there any measurements in photons/area/time or uW/area/time available? If not, comment in methods why not.

The referee is right. Unfortunately in the field, the measure of light was in lux and not in irradiance. We had only HOBO datalogger (HOBO Pendant® Temperature/Light Data Logger, Onset Computer Corporation, Bourne, MA, USA) available at that time that measured only in lux. It is not possible to directly do a conversion from lux to µmol m^{-2} s^{-1}. It is only possible for a specific wavelength.

So, to mention it, we modified the sentence in Methods S1 in the sub-paragraph *Site characteristics of the field study.*

Now : “*Temperature (°C) and light intensity* ***in Lux (n.b. the conversion to light irradiance in µmol*** m^{-2} s^{-1} ***was not possible)*** *were measured all along the field study every 10 minutes by a data logger (HOBO Pendant® Temperature/Light Data Logger, Onset Computer Corporation, Bourne, MA, USA) fixed to the bag with oysters equipped for valve activity study and were provided figure S1.*”

Supply of seawater- please also include a comment on what is exactly used. Artificial sea water or natural? In other words- could the water contain tidal chemosensory cues? (Even if, this wouldn't take away from the importance of the author's findings, but the interpretations would be a bit different, as it is not free-running, but still under possibly entraining conditions.)

the referee has a very good question. We are totally agree with him. To explain, we supply with natural seawater but we did a procedure to remove any putative cycling environmental cues. Indeed, first, the water in pumped every 2 weeks approximatively in the sea, mixed and stored in a very tank (50 m3), then after decantation, the water is filtrated at 5 µm to remove phytoplankton. Then, the water is stored in another transient tank in the experimental room. In this tank, before filling the experimental units, the water is homogenized in terms of temperature, oxygen (normoxia), pH…

Especially to remove any cycles of putative tidal cues, the whole amount of water is mixed with pumps and air bubbling. Consequently, the water used for the experiment has the same composition and without any tidal cue cycles possible.

To clarify, we modified the sentence ln 619:

"Then, the oysters were placed in the laboratory under constant darkness (dark-dark, DD) **and homogenized filtrated seawater**."

Moreover, in SI methods we explained more.

See in the paragraph "Experiments in controlled environments":

Now added:

"*To prevent from any putative tidal chemosensory cues in the seawater supply, this natural seawater was, first, stored and mixed, in one time, in a 50 m^3 tank. Then, this water was stored in transient tank in the experimental room. In this 150-L tank, the seawater is homogenized with pumps and air-bubbling to break any putative cycles of environmental cues, related to tides for example*."

Methods in S1 are really confusing about LD and temperature conditions 10:14 or 9:15 ? Two different temperatures are given. Please clarify.

Both exist. There are two different experiments done in different times. These different light regimes were chosen according to the natural photoperiod when the experiment were done, to avoid a putative effect of photoperiod changes in the results.

First experiment (lab DD condition). The oysters were acclimated to 10:14 light dark regime and then in constant darkness.

Second experiment (Lab LD condition): The oysters were studied in 9:15 light dark regime.

As the authors do not really functionally show that the "circadian" clock genes are functionally required for the tidal rhythm to occur, it could still be possible that the observed transcript oscillation patterns are downstream of a still existing independent circatidal oscillator. This possibility needs to be included in the discussion and interpretation.

This question is very interesting. The referee is right, we didn't proven functionally our hypothesis, and other hypothesis could be proposed. According to the idea of the referee, it can be argue that a "master tidal oscillator" could constrain a "slave circadian clock" to run at tidal frequency. But, this hypothesis seems not the more efficient (especially in terms of energy cost). Why going through the circadian clock to run at tidal rhythmicity. In that case, it would be more efficient to have a tidal clock that directly give the tidal periodicity to the genes under control of the clock.

However, to take into account the hypothesis of the referee, we added a new sentence about that in the discussion, ln 820-822.

"*Finally, although the strong arguments of this study in favor of a bimodal clock, the possibility to have a "master tidal clock" that drives a "slave circadian clock" to run at tidal frequency might be raised*."

Suggestion for the heading: "Into the wild: …"

According to the referee 1 and the associate editor, we have already modified the title. Ln 517-518.

Now: "Bivalve mollusk circadian clock genes **can** run at tidal frequency"

Line 70: "in" not "into"

Agree. We modified. Now ln 586.