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PROCEEDINGS B

Cellular bicarbonate accumulation and vesicular proton transport promote calcification in the sea urchin larva

Marian Y. Hu, Inga Petersen, William Chang, Christine Blurton and Meike Stumpp

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Review timeline

Original submission:	31 March 2020
1st revised submission:	25 June 2020
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Final acceptance:	7 August 2020

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

Review History

RSPB-2020-0552.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?
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

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
The study addresses a crucial but poorly understood aspect of biomineralisation, pH regulation, in the sea urchin embryo model. I highly recommend the paper for eventual publication as I think it contains novel and important information for understanding mechanisms of carbon and proton transport relevant to many different calcification models. The questions asked and the experiments used to tackle them are well-chosen and the data presentation is clear and informative. The interpretation is for the most part well-reasoned. There are some inconsistencies that the authors could address in revisions to the discussion and/or their diagrams.
Broadly speaking, the findings of the paper fall into two parts. A part that concerns pH regulation of the PMC cells and a part that concerns vesicles in the BFC cells. Both parts are well-worth publishing, but it is clear that understanding of the former is better than the latter. In the case of the vesicle part, many questions remain outstanding and their role in both acid-base regulation and calcification is far from clear. The authors are well aware of this, because they are cautious not to go far in their interpretations in the abstract or the discussion. This to be commended but it is rather inconsistent with their diagrams which show processes that are not discussed in the text. My main criticism of this exciting paper is that the authors need to align the text with the diagrams. That could mean being more explicit in the discussion that they are speculating about certain aspects and proposing future research into this speculation.
More specifically, figure 4 G clearly shows the possible transfer of protons from PMC to BFC. This is not really discussed explicitly and needs to be if it is included in a diagram. It is suggested that protons generated by calcification are somehow being taken up by the BFC cells, but if the authors go so far to put the arrow of proton flux at the meeting point of the two cell types in the diagram they need to discuss it in the text. Currently it is not even described properly in the figure legend. It can't be backed up by data but it can be proposed explicitly as a hypothetical mechanism. Furthermore, in the same diagram protons are expelled into the body cavity and the implication is that they are taken up by the BFC cells. Again this should be addressed in the discussion.
Additionally, there is a discrepancy between the diagram in Figure 2 J and Figure 4 G which complicates things a bit. In Figure 2J the authors show HCO3- entering the ACC vesicles in the PMCs. No proton export across the cell membrane is shown. This illustrates the argument that reducing NHE proton export from the PMCs helps bicarbonate accumulation by the NBC by not acidifying boundary layer of the PMCs. However, in figure 4G, CO2 enters the ACC vesicles and protons are expelled across the PMC membrane (possibly by a V-type H ATPase as the legend states). This would presumably acidify the PMC boundary layer, contrary to the strategy proposed in Figure 2 J. Could the authors deal with this contradiction between the figures and their overall proposed mechanism?

Another point to consider for the discussion is the eventual fate of the protons. The authors suggest that the BFC cells sequester protons generated from calcification in vesicles. This is a very elaborate mechanism compared to the alternative which is that protons are expelled from the PMCs into the primary body cavity which exchanges with seawater, thus dissipating the protons. In the BFC vesicle mechanism, what is the eventual fate of the protons? Why keep them in vesicles and where are they transported? While the authors don't have any data on this, the credibility of their proposed mechanism requires some discussion of the possibilities.
A final point concerns the pH regulatory capacity of the PMCs. If there is a reduction in NHE activity and thus capacity for pH regulation by the PMCs, why is not compensated for by the increase in activity of the bicarbonate transporters? Both transporters are involved in pH regulation. Do these types of transporters react on different time scales? pH regulation is assessed over short time scales here. Does NHE normally respond faster? Could that be part of the reason?
Minor typos:
Line 447. Weather should be whether.
Line 415. till here should be until here.

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?
Excellent

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

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Is it adequate?
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Do you have any ethical concerns with this paper?
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Comments to the Author
Overall assessment
How cells generate biominerals? is a fundamental question that intrigues biologist, chemists and material engineers for decades. The ability of the cells to condense minerals and keep them in amorphous phase and then control their crystallization direction and rate, is beyond the current state of the art in material sciences. Many marine organisms including corals and sea shells, use the mineral, CaCO3 to make their shells. In the process of CaCO3 calcification, the ion CO3(-2) needs to be accumulated, mostly through the reaction HCO3(-1)->CO3(-2) and the release of a proton. In this paper the authors use the sea urchin larval skeletogenesis as a model system to learn about cellular regulation of CO3 and proton concertation. The sea urchin is an excellent experimental system for both molecular and biochemical studies and therefore had been used extensively to decipher the biological control of biomineralization. This is a good system for this kind of studies and the authors have a unique expertise in studying the physiology of the sea urchin cells under different conditions and technically, the paper is sound. This would have made this work highly interesting and important to a broad audience, if it was presented properly. However, the current version of the paper lacks critical explanations about the broad context of the problem addressed, the rationale of the experimental design and the interpretation of the results. Hence, it should be significantly revised so it won't read like a detailed lab report but as a mature paper. I believe that after the required revisions, this paper will be of high significance and well received by the broad and interdisciplinary biomineralization community. My detailed comments to the authors are listed below.

General comment about the presentation
The way the paper is written now limits its readership to physiologists that study sea urchin skeletogenesis, which is a somewhat narrow audience. Yet, it has the potential of being interesting to biologists, chemists and material scientists that study biomineralization, as well as people that study climate change, if you write it thinking about them as your readers. You should start with a broader introduction of the importance of biomineralization, presenting the critical gaps in knowledge in the field, that you want to address in this paper. If you think that your main impact will be on climate change – explain the relevance in the first paragraphs of the introduction. The most important impact can't appear as the last line in the abstract or the last paragraphs of the discussion, you have to lead to the reader to that in order to convince them. After that you should explain why the sea urchin is such an excellent system for these studies and the specific mechanisms that you are investigating here. When referring to vertebrate it is important to note that vertebrates use a different mineral than CaCO3 (CaPO4), and clearly the cellular environment is completely different, but yet, the cells have similar challenges regarding the pH and proton exchange. You use the "regeneration" assay quite a lot in the paper, but you don't explain why you use it and what you expect to learn from it. Also, this is not a typical regeneration process where an organ is amputated and recovers, but a de-calcification process where the change in pH causes the mineral to dissolve. I wouldn't use the term "regeneration" in this context, but if you wish to use it – you have to explain what it is (also in the abstract) and why you refer to it as regeneration (in the text of the paper). I think you should mention this assay in the introduction – where it was used before, and what is the advantages/reasons to use this assay here. There is a lot of emphasize on the blastoceolar cells and their buffering capacity, but it is not clear why you think that they play a role in skeletogenesis, at least not in the results section. This needs to be explained. In general, a common rule is write an explanatory sentence at the beginning of each experimental paragraph that explains the goal in this experiments and a summary sentence at the end of each experimental paragraph that explains the essence of your results. I think that if you follow this rule throughout the paper it will increase its readability significantly. Also, please cite the recent papers in this subject (molecular and cellular control of sea urchin skeletogenesis) that are relevant to this work.

Experimental approach and results:
The authors first studied the role of Na/H exchange in skeletogenesis. They studied the recovery of the skeletogenic pH from an ammonium pulse in the presence of H+ exchange inhibitors and observed a variable response to the inhibition. They used EIPA (please add reagent number to the methods section), that indeed inhibits Na+/H+-exchanger, but it also inhibits TRPP3 channels and macropinocytosis. They observed that the recovery of some of the skeletogenic cells to the ammonium pulse was impaired in EIPA (Fig. 1A-D). Additionally, the incorporation of calcium was reduced under EIPA treatment based on calcein pulse-chase experiments. They observed the expression of the Na+/H+-exchanger, SLC9a2 at the skeletogenic cells located near the body rods at this time, and suggest that this gene could be relevant to the effect of EIPA on calcification. However, the role of this gene or of other genes studied in this paper was not studied by genetic perturbation, and the fact that it is expressed in the skeletogenice cells at later stages of skeletogenesis is not a proof of the gene function – you should be more cautious about this in the abstract and discussion. Furthermore, I am not sure that I agree with the conclusion as macropinocytosis was shown to be the way that the skeletogenic cells uptake calcium and CO3 from the sea water (Vidavski et al 2014, 2015, 2016). EIPA blocks macropinocytosis and that could be the reason for the reduced calcium uptake– the authors should mention it and explain or prove that the main issue here is the NH-exchange blocking.

Then the authors present the regeneration assay (Fig. 2), without explaining why they use it and what they expect to learn from it that they can't learn from normal skeletogenesis – please add this reasoning to the introduction and/or the results section. Please explain how this relates to normal skeletogenesis as this is an extreme condition – what is your hypothesis. You have to explain what each inhibitor does in the text (DIDs, Bafilomycin A1) and instead or in addition to the detailed experimental concentrations and measured parameters, explain in words what processes you affected and the meaning of the results.

The authors compare the regulatory capacities of the skeletogenic cells with those of the blastoceolar cells present in the coelom of the larva (I wouldn't use the work "occur" for that). They don't explain why they do this comparison – the blastoceolar cells touch the skeletogenic cells but there is no cell fusion between these populations. The acidic vesicles detected in the blastocoelar cells are most likely lysosomes or autophagosomes and the effect of Bafilomycin on the acidification could be since it blocks the fusion between autopahgosomes and lysosomes and not directly related to skeletogenesis. They measure the levels of different isoforms of the V H+ ATPASE and observe divergent pattern during decalcification – some isoforms are upregulated and some are downregulated. However, without the knowledge about the specific spatial expression of the isoforms, it is hard to even guess what their role is. The human antibody for the ATPase show a stain in the sub-cellular vesicles of the blastoceolar cells, again, probably the autophagosomes and lysosomes. But it is not clear which of the isoforms it is actually detecting and what is the relevance to skeletogenesis. Also – the red stain in Fig. 4D is not explained in the text/figure caption. The model presented in Fig. 4G in not clear – how they got to it from the measurements and what it means. This part of the work need some more thinking and reasoning I believe.

Decision letter (RSPB-2020-0552.R0)

08-Jun-2020

Dear Dr Hu:

I am writing to inform you that your manuscript RSPB-2020-0552 entitled "Cellular bicarbonate accumulation and vesicular proton transport promote skeleton regeneration in the sea urchin larva" has, in its current form, been rejected for publication in Proceedings B.

This action has been taken on the advice of referees, who have recommended that substantial revisions are necessary. With this in mind we would be happy to consider a resubmission, provided the comments of the referees are fully addressed. However please note that this is not a provisional acceptance.

The resubmission will be treated as a new manuscript. However, we will approach the same reviewers if they are available and it is deemed appropriate to do so by the Editor. Please note that resubmissions must be submitted within six months of the date of this email. In exceptional circumstances, extensions may be possible if agreed with the Editorial Office. Manuscripts submitted after this date will be automatically rejected.

Please find below the comments made by the referees, not including confidential reports to the Editor, which I hope you will find useful. If you do choose to resubmit your manuscript, please upload the following:

1) A 'response to referees' document including details of how you have responded to the comments, and the adjustments you have made.
2) A clean copy of the manuscript and one with 'tracked changes' indicating your 'response to referees' comments document.
3) Line numbers in your main document.

To upload a resubmitted manuscript, 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 Resubmission." Please be sure to indicate in your cover letter that it is a resubmission, and supply the previous reference number.

Sincerely,
Dr Daniel Costa
mailto: proceedingsb@royalsociety.org

Associate Editor
Board Member: 1
Comments to Author:
Dear Marion Hu

Your manuscript has been assessed by two reviewers who found your manuscript very exciting scientifically. They both raise a number of concerns regarding presentation and interpretation that will necessitate a significant rewrite of the manuscript to address. They provide an extensive suggestions and comments to guide this process. I trust you will find the comments useful in your revision of the manuscript and I look forward to seeing the next version here or elsewhere.
Warm Regards,
Line K Bay

Reviewer(s)' Comments to Author:

Referee: 1
Comments to the Author(s)
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Referee: 2

Comments to the Author(s)

Overall assessment

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Author's Response to Decision Letter for (RSPB-2020-0552.R0)

See Appendix A.

RSPB-2020-1506.R0

Review form: Reviewer 2

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?
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

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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
The revised version of the manuscript addressed my major concerns: The replacement of the work "regeneration" with re-calcification and explanation about this assay and its' purpose, mentioning additional mechanisms that are affected by the inhibitors, etc. However, I still feel that some more work on the text is needed to improve the paper's readability. Below I list my suggestions for improving the paper clarity and significance, according to the order of their appearance:

Introduction:

Lines 64-67: I recommend you to revise to: "Thus, efficient trans-membrane transport systems that regulate pH and deliver calcification substrates to the calcification front, are a fundamental requisite of all calcifying systems with the underlying mechanisms not clearly understood."

Lines 77-78: I recommend you to revise to: "A range of matrix proteins that regulate crystal nucleation, ACC stabilization and recruitment of Ca2+ ions, is required for the proper development of the larval skeleton".

Lines 80-81: Missing "is": Carbon isotope studies demonstrated that approximately 60% of dissolved inorganic carbon (DIC) used to build the larval skeleton is derived

Line 85: Missing "of": to be critically involved in the cellular of accumulation HCO3 from the seawater

Line 92: Please repeat the meaning of the acronyms, NHE and VHA, that you defined in the abstract in the first time they appear in the text. Also – for HKA, it is first mentioned in line 93 – put the acronym there.

Line 95 and throughout the paper: Sp_SLC9a2 -> Sp-SLC9a2 or Sp-Slc9a2 – which ever you prefer but keep consistent.

Line 109: what do you mean by pH regulatory states? Maybe you mean regulatory mechanisms?

Results:

NHE exchange mechanisms

This paragraph still needs more explanation about the logic of the experiments to be clear. First you block NHE and you see an effect of the ability to regulate pH, and then you look for the responsible gene. Say it explicitly and separate into two paragraphs, the first about the inhibition and the second about the gene. Add one sentence to the beginning of the second paragraph, e.g.,: Our findings suggest that NHE proteins participate in the regulation of pH in the PMC and therefore we searched for the responsible gene". – or something along these lines. Summarize the findings in the end of the section – "we observed (NEH effect) and identified (SLC9) as a possible gene that mediates this function". Also - You have to mention the block of macro-pinocytosis by EIPA here, in the calcein experiment and not wait to the discussion, where it also damages the logical flow.

pHi regulatory capacities during skeleton re-calcification

1. Please change the first line of this paragraph to make it clearer to the reader: "To study pH regulatory dynamics in actively calcifying PMCs we used the re-calcification assay where the skeleton is dissolved and completely rebuild within few days. To conduct the re-calcification assay we expose pluteus larvae…". And then the rest of the paragraph. I think it is better to put this explanation here and not in the end of the introduction where it is a little too much information for this stage.

2. This section also needs to be divided into two paragraph, the first about the pH regulation during re-calcification (reporting the dynamic changes in different ion levels) and the second about the pharmacological experiments addressing the molecular mechanisms that underlie these dynamics. The second paragraph needs to start with: "to address the molecular mechanisms involved in these ionic changes we blocked this and that and observed the effect on ion levels". And then the rest of the paragraph follows. Summary Figure 2J has to be explained in the text, it is not self-explanatory.

Interaction of vesicle-rich blastocoelar filopodial cells with PMCs and characterization of vesicular pH

The revision really improved the readability of this section. However, there are still a lot of technical details and not enough explanation of the biological meaning of the results. The proposed role of the blastocoelar cells is still not clear to me – is it to sequester the protons from the blastocoel? In Fig. 3C you write: "BFCs attach to PMCs and transport endocytosed vesicles across their filopodial network" It is not clear whether the vesicles are transported between BFCs or to the PMC from this statement and I don't think there is evidence for transport of vesicles between the blastocoelar cells and the PMCs. So, I don't understand what the vesicle with the question mark in Fig. 4G stands for.

Modulation of acid-base transporters during skeleton re-calcification

Add a sentence to the beginning of this section saying: "Our findings indicate that NBCs and NHEs might play a role in the regulation of the re-calcification process we therefore measured the change in the level of relevant genes during this process".

Increase in log scale is not fold: “1.18-fold (log2) and 0.84-fold (log2)” translate to actual changes in level.

Summarize the findings of this section in the last sentence.

Discussion

In general – the discussion provides very interesting references that give broader context to the paper’s findings, but it contains too much repeats and details of the results, which makes it hard to follow. All the explanation of the results should be in the result section and only briefly mentioned in the discussion. This would make the results and the discussion clearer.

pHi regulatory states of PMCs – again it is not clear to me what you mean by pH regulatory states. Either change the wording (to capacities or mechanisms) or explain. The point you make in this paragraph is extremely interesting – about the spatial difference along the body rods (not primary rods) that correlated with differential gene expression along these rods and also with the fact that the skeleton elongates at the tips (Sun and Ettensohn gene exp patterns 2014 – refer to, it is relevant). This paragraph should be written more clearly. The analogy to bone formation is interesting but the process is extremely different. Yet it helped me understand what you mean by different functional states. Why don’t you just way it clearly: “It seems that different PMCs have a different function that is related to their position along the body rods, the ones at the tips show… while the ones located away from the tips show…” This strongly correlates with the fact that they have different functions in the normal calcification process – calcification is promoted at the tips and inhibited along the rods (Sun and Ettensohn gene exp patterns 2014).

Lines 387-388: “However, unlike the situation in vertebrates where the..” and not “unlike the situation invertebrates”.

The discussion of the role of BFC doesn’t have a clear conclusion.

Decision letter (RSPB-2020-1506.R0)

21-Jul-2020

Dear Dr Hu

I am pleased to inform you that your manuscript RSPB-2020-1506 entitled "Cellular bicarbonate accumulation and vesicular proton transport promote calcification in the sea urchin larva" has been accepted for publication in Proceedings B.

The referee(s) have recommended publication, but also suggest some minor revisions to your manuscript. Therefore, I invite you to respond to the referee(s)' comments and revise your manuscript. Because the schedule for publication is very tight, it is a condition of publication that you submit the revised version of your manuscript within 7 days. If you do not think you will be able to meet this date please let us know.

To revise your manuscript, log into https://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. You will be unable to make your revisions on the originally submitted version of the manuscript. Instead, revise your manuscript and upload a new version through your Author Centre.

When submitting your revised manuscript, you will be able to respond to the comments made by the referee(s) and upload a file "Response to Referees". You can use this to document any changes you make to the original 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.

Before uploading your revised files please make sure that you have:

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2) A separate electronic file of each figure (tiff, EPS or print-quality PDF preferred). The format should be produced directly from original creation package, or original software format. PowerPoint files are not accepted.

3) Electronic supplementary material: this should be contained in a separate file and where possible, all ESM should be combined into a single file. 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.

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].

4) A media summary: a short non-technical summary (up to 100 words) of the key findings/importance of your manuscript.

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It is a condition of publication that data supporting your paper are made available either in the electronic supplementary material or through an appropriate repository (https://royalsociety.org/journals/authors/author-guidelines/#data).

In order to ensure effective and robust dissemination and appropriate credit to authors the dataset(s) used should be fully cited. To ensure archived data are available to readers, authors should include a 'data accessibility' section immediately after the acknowledgements section. This should list the database and accession number for all data from the article that has been made publicly available, for instance:
• DNA sequences: Genbank accessions F234391-F234402
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Once again, thank you for submitting your manuscript to Proceedings B and I look forward to receiving your revision. If you have any questions at all, please do not hesitate to get in touch.

Sincerely,
Dr Daniel Costa
mailto: proceedingsb@royalsociety.org

Associate Editor
Board Member
Comments to Author:
Dear Dr Hu,

A few remaining issues have been identified by a second review of the of the original reviewers. I recommend you address these remaining issues before the manuscript can be finally considered for publication in Proc B.

Best
Line K Bay

Reviewer(s)' Comments to Author:

Referee: 2

Comments to the Author(s).
The revised version of the manuscript addressed my major concerns: The replacement of the work "regeneration" with re-calcification and explanation about this assay and its' purpose, mentioning additional mechanisms that are affected by the inhibitors, etc. However, I still feel that some more work on the text is needed to improve the paper's readability. Below I list my suggestions for improving the paper clarity and significance, according to the order of their appearance:

Introduction:

Lines 64-67: I recommend you to revise to: "Thus, efficient trans-membrane transport systems that regulate pH and deliver calcification substrates to the calcification front, are a fundamental requisite of all calcifying systems with the underlying mechanisms not clearly understood."

Lines 77-78: I recommend you to revise to: "A range of matrix proteins that regulate crystal nucleation, ACC stabilization and recruitment of Ca2+ ions, is required for the proper development of the larval skeleton".

Lines 80-81: Missing "is": Carbon isotope studies demonstrated that approximately 60% of dissolved inorganic carbon (DIC) used to build the larval skeleton is derived

Line 85: Missing "of": to be critically involved in the cellular of accumulation HCO3 from the seawater

Line 92: Please repeat the meaning of the acronyms, NHE and VHA, that you defined in the abstract in the first time they appear in the text. Also – for HKA, it is first mentioned in line 93 – put the acronym there.

Line 95 and throughout the paper: Sp_SLC9a2 -> Sp-SLC9a2 or Sp-Slc9a2 – which ever you prefer but keep consistent.

Line 109: what do you mean by pH regulatory states? Maybe you mean regulatory mechanisms?

Results:

NHE exchange mechanisms

This paragraph still needs more explanation about the logic of the experiments to be clear. First you block NHE and you see an effect of the ability to regulate pH, and then you look for the responsible gene. Say it explicitly and separate into two paragraphs, the first about the inhibition and the second about the gene. Add one sentence to the beginning of the second paragraph, e.g.,: Our findings suggest that NHE proteins participate in the regulation of pH in the PMC and therefore we searched for the responsible gene". – or something along these lines. Summarize the findings in the end of the section – "we observed (NEH effect) and identified (SLC9) as a possible gene that mediates this function". Also - You have to mention the block of macro-pinocytosis by EIPA here, in the calcein experiment and not wait to the discussion, where it also damages the logical flow.

pHi regulatory capacities during skeleton re-calcification

1. Please change the first line of this paragraph to make it clearer to the reader: "To study pH regulatory dynamics in actively calcifying PMCs we used the re-calcification assay where the skeleton is dissolved and completely rebuild within few days. To conduct the re-calcification assay we expose pluteus larvae…". And then the rest of the paragraph. I think it is better to put this explanation here and not in the end of the introduction where it is a little too much information for this stage.

2. This section also needs to be divided into two paragraph, the first about the pH regulation during re-calcification (reporting the dynamic changes in different ion levels) and the second about the pharmacological experiments addressing the molecular mechanisms that underlie these dynamics. The second paragraph needs to start with: "to address the molecular mechanisms involved in these ionic changes we blocked this and that and observed the effect on ion levels". And then the rest of the paragraph follows. Summary Figure 2J has to be explained in the text, it is not self-explanatory.

Interaction of vesicle-rich blastocoelar filopodial cells with PMCs and characterization of vesicular pH

The revision really improved the readability of this section. However, there are still a lot of technical details and not enough explanation of the biological meaning of the results. The proposed role of the blastocoelar cells is still not clear to me – is it to sequester the protons from the blastocoel? In Fig. 3C you write: "BFCs attach to PMCs and transport endocytosed vesicles across their filopodial network" It is not clear whether the vesicles are transported between BFCs or to the PMC from this statement and I don't think there is evidence for transport of vesicles between the blastocoelar cells and the PMCs. So, I don't understand what the vesicle with the question mark in Fig. 4G stands for.

Modulation of acid-base transporters during skeleton re-calcification

Add a sentence to the beginning of this section saying: "Our findings indicate that NBCs and NHEs might play a role in the regulation of the re-calcification process we therefore measured the change in the level of relevant genes during this process".

Increase in log scale is not fold: “1.18-fold (log2) and 0.84-fold (log2)” translate to actual changes in level.

Summarize the findings of this section in the last sentence.

Discussion

In general – the discussion provides very interesting references that give broader context to the paper’s findings, but it contains too much repeats and details of the results, which makes it hard to follow. All the explanation of the results should be in the result section and only briefly mentioned in the discussion. This would make the results and the discussion clearer.

pHi regulatory states of PMCs – again it is not clear to me what you mean by pH regulatory states. Either change the wording (to capacities or mechanisms) or explain. The point you make in this paragraph is extremely interesting – about the spatial difference along the body rods (not primary rods) that correlated with differential gene expression along these rods and also with the fact that the skeleton elongates at the tips (Sun and Ettensohn gene exp patterns 2014 – refer to, it is relevant). This paragraph should be written more clearly. The analogy to bone formation is interesting but the process is extremely different. Yet it helped me understand what you mean by different functional states. Why don’t you just way it clearly: “It seems that different PMCs have a different function that is related to their position along the body rods, the ones at the tips show… while the ones located away from the tips show…” This strongly correlates with the fact that they have different functions in the normal calcification process – calcification is promoted at the tips and inhibited along the rods (Sun and Ettensohn gene exp patterns 2014).

Lines 387-388: “However, unlike the situation in vertebrates where the..” and not “unlike the situation invertebrates”.

The discussion of the role of BFC doesn’t have a clear conclusion.

Author's Response to Decision Letter for (RSPB-2020-1506.R0)

See Appendix B.

Decision letter (RSPB-2020-1506.R1)

07-Aug-2020

Dear Dr Hu

I am pleased to inform you that your manuscript entitled "Cellular bicarbonate accumulation and vesicular proton transport promote calcification in the sea urchin larva" 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.

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Your article has been estimated as being 10 pages long. Our Production Office will be able to confirm the exact length at proof stage.

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Electronic supplementary material:
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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,
Editor, Proceedings B
mailto: proceedingsb@royalsociety.org

Appendix A

Referee: 1

Comments to the Author(s)
The study addresses a crucial but poorly understood aspect of biomineralisation, pH regulation, in the sea urchin embryo model. I highly recommend the paper for eventual publication as I think it contains novel and important information for understanding mechanisms of carbon and proton transport relevant to many different calcification models. The questions asked and the experiments used to tackle them are well-chosen and the data presentation is clear and informative. The interpretation is for the most part well-reasoned. There are some inconsistencies that the authors could address in revisions to the discussion and/or their diagrams.
Broadly speaking, the findings of the paper fall into two parts. A part that concerns pH regulation of the PMC cells and a part that concerns vesicles in the BFC cells. Both parts are well-worth publishing, but it is clear that understanding of the former is better than the latter. In the case of the vesicle part, many questions remain outstanding and their role in both acid-base regulation and calcification is far from clear. The authors are well aware of this, because they are cautious not to go far in their interpretations in the abstract or the discussion. This to be commended but it is rather inconsistent with their diagrams which show processes that are not discussed in the text. My main criticism of this exciting paper is that the authors need to align the text with the diagrams. That could mean being more explicit in the discussion that they are speculating about certain aspects and proposing future research into this speculation.

Author´s reply:

We are grateful for the positive and constructive feedback of reviwer#2 on our work. We fully agree that there were inconsistencies and conflicts in our proposed models and that figure were insufficiently explained in the text. We carefully revised our manuscript by addressing and accepting all comments raised by reviewer#1 including a stronger speculation regarding the fate of the protons liberated by the calcification process. We feel that this has largely improved the clarity of this relatively complex work. To better address the comments raised by reviewer#1 we divided the original review into separate points and addressed them in a point to point manner. All changes made in the text are marked in red in the “track-changes” version. We hope reviewer#1 is happy with this revised version of our manuscript.

Specific comments

Reviewer#1

More specifically, figure 4 G clearly shows the possible transfer of protons from PMC to BFC. This is not really discussed explicitly and needs to be if it is included in a diagram. It is suggested that protons generated by calcification are somehow being taken up by the BFC cells, but if the authors go so far to put the arrow of proton flux at the meeting point of the two cell types in the diagram they need to discuss it in the text. Currently it is not even described properly in the figure legend. It can’t be backed up by data but it can be proposed explicitly as a hypothetical mechanism. Furthermore, in the same diagram protons are expelled into the body cavity and the implication is that they are taken up by the BFC cells. Again this should be addressed in the discussion.

Author´s reply: We fully agree to the comment of reviewer#1 that our models were insufficiently integrated into the text. We revisited the diagrams with slight modifications and now explicitly explain our finding and hypotheses in the text:

L451-460: "Here it remains unresolved which of the VHA isoforms is responsible for the massive sequestration of protons in BFCs and what the biological function may be. It can be speculated that this massive transport of protons into vesicular structures of BFCs is associated with a removal of protons liberated by the calcification process. Acidic vesicles may then be exocytosed into the primary body cavity or transported through the filopodial network to be released into the seawater at the highly permeable ectoderm. Furthermore, besides the possibility of direct secretion, vesicular sequestration of protons may also serve other cellular functions like enhanced protein degradation and processing in acidic lysosomes or autophagosomes during skeleton re-calcification [41]."

Conclusion L476-486: "In contrast to the situation in vertebrate osteoblasts, mineralizing cells of the sea urchin embryo reduce NHE based pH_i regulatory capacities during extensive calcification events. However, the necessity to remove protons liberated by the calcification process suggests an alternative route of proton removal from the calcification front in PMCs. Here our vesicular pH measurements demonstrate a sub-cellular sequestration of protons in PMCs and BFCs potentially supporting pH_i regulation during the clacification process (Figure 4 G). It remains a matter of future investigations whether acidic vesicles in PMCs are exocytosed into the primary body cavity of if they can be transferred from PMCs to BFCs to be secreted or used for other cellular processes during skeleton repair (depicted by question marks in Figure 4G). Despite these open questions regarding the removal of protons from the calcification front our results clearly demonstrate efficient cellular mechanisms of DIC accumulation during the mineralization event (Figure 4G)."

Reviewer#1

Additionally, there is a discrepancy between the diagram in Figure 2 J and Figure 4 G which complicates things a bit. In Figure 2J the authors show HCO3- entering the ACC vesicles in the PMCs. No proton export across the cell membrane is shown. This illustrates the argument that reducing NHE proton export from the PMCs helps bicarbonate accumulation by the NBC by not acidifying boundary layer of the PMCs. However, in figure 4G, CO2 enters the ACC vesicles and protons are expelled across the PMC membrane (possibly by a V-type H ATPase as the legend states). This would presumably acidify the PMC boundary layer, contrary to the strategy proposed in Figure 2 J. Could the authors deal with this contradiction between the figures and their overall proposed mechanism?

Author´s reply: We would like to thank reviewer#1 for pointing out this discrepancy in our models. We revisited the concept of boundary layer acidification and think that this point is rather speculative and does not directly contribute conclusions of the present work. Thus we decided to omit the aspect of reduced boundary layer acidification increased Na+ driving force for HCO3- uptake by reduced NHE activity from the manuscript. We revised Fig 2J and the text accordingly:

L400-404:" In contrast, PMCs reduce pH_i regulatory capacities and become insensitive to the Na^+/H^+ exchange inhibitor EIPA, suggesting a reduction in NHE-based pH regulatory capacities during

skeleton re-calcification. Although decreased proton extrusion capacities of PMCs during active mineralization seem counter intuitive,……"

Reviewer#1

Another point to consider for the discussion is the eventual fate of the protons. The authors suggest that the BFC cells sequester protons generated from calcification in vesicles. This is a very elaborate mechanism compared to the alternative which is that protons are expelled from the PMCs into the primary body cavity which exchanges with seawater, thus dissipating the protons. In the BFC vesicle mechanism, what is the eventual fate of the protons? Why keep them in vesicles and where are they transported? While the authors don't have any data on this, the credibility of their proposed mechanism requires some discussion of the possibilities.

Author´s reply: We agree to the reviewer´s comment that the fate of protons in the vesicular sequestration model is a very interesting question, though it remains highly speculative. We observed that during skeleton regeneration the skeleton is initially formed with irregularities (branching) that gradually disappear over time suggesting a shaping of the skeleton after the major re-mineralization event. Accordingly, one of our hypotheses is, that maybe, sequestration and storage of protons can be used to shape the skeleton by controlled release of protons similar to the situation in vertebrate osteoclasts. Another possibility would be a trafficking of acidic vesicles along filopodial networks of PMS and maybe also BFCs and subsequent exocytosis across the larval ectoderm. This would protect the extracellular space from any acid-load generated by calcification. Finally, organelles like lysosomes require acidic conditions to degrade and recycle proteins that may represent an important aspect during skeleton repair. Thus, massive sequestration of protons may be relevant for protein turnover, but at the same time has the potential to support removal of protons liberated by the calcification process. We added some of these speculations to the discussion part of our manuscript.

L451-460:" Here it remains unresolved which of the VHA isoforms is responsible for the massive sequestration of protons in BFCs and what the biological function may be. It can be speculated that this massive transport of protons into vesicular structures of BFCs is associated with a removal of protons liberated by the calcification process. Acidic vesicles may then be exocytosed into the primary body cavity or transported through the filopodial network to be released into the seawater at the highly permeable ectoderm. Furthermore, besides the possibility of direct secretion, vesicular sequestration of protons may also serve other cellular functions like enhanced protein degradation and processing in acidic lysosomes or autophagosomes during skeleton re-calcification [41]."

Reviewer#1

A final point concerns the pH regulatory capacity of the PMCs. If there is a reduction in NHE activity and thus capacity for pH regulation by the PMCs, why is not compensated for by the increase in activity of the bicarbonate transporters? Both transporters are involved in pH regulation. Do these types of transporters react on different time scales? pH regulation is assessed over short time scales here. Does NHE normally respond faster? Could that be part of the reason?

Author´s reply: We understand the reviewer´s point. Although increased HCO3- accumulation can help to buffer protons, this is not a long-term solution to regulate pHi during continuous release of protons by the calcification process. This would require a continuous increase in buffer capacity as well. Accordingly, there must be an alternative route of proton removal from the cell. Given the overall decreased pH_i regulatory capacity of PMCs it can be hypothesized that protons are locally removed from the calcification vesicles by vesicular proton sequestration mechanisms. Another possibility for reduced NHE activity may involve an enhanced expression of proton channels that allow protons to leave the call along an electrochemical gradient similar to what has been proposed for the calcification process in coccolithophores.

Minor typos:
Reviewer#1
Line 447. Weather should be whether.
Author´s reply: This sentence has been reworded and corrected

Reviewer#1
Line 415. till here should be until here.

Author´s reply: We corrected this: "…unresolved until here how…"

Referee: 2

Comments to the Author(s)
Overall assessment
How cells generate biominerals? is a fundamental question that intrigues biologist, chemists and material engineers for decades. The ability of the cells to condense minerals and keep them in amorphous phase and then control their crystallization direction and rate, is beyond the current state of the art in material sciences. Many marine organisms including corals and sea shells, use the mineral, CaCO3 to make their shells. In the process of CaCO3 calcification, the ion CO3(-2) needs to be accumulated, mostly through the reaction HCO3(-1)->CO3(-2) and the release of a proton. In this paper the authors use the sea urchin larval skeletogenesis as a model system to learn about cellular regulation of CO3 and proton concertation. The sea urchin is an excellent experimental system for both molecular and biochemical studies and therefore had been used extensively to decipher the biological control of biomineralization. This is a good system for this kind of studies and the authors have a unique expertise in studying the physiology of the sea urchin cells under different conditions and technically, the paper is sound. This would have made this work highly interesting and important to a broad audience, if it was presented properly. However, the current version of the paper lacks critical explanations about the broad context of the problem addressed, the rationale of the experimental design and the interpretation of the results. Hence, it should be significantly revised so it won't read like a detailed lab report but as a mature paper. I believe that after the required revisions, this paper will be of high significance and well received by the broad and interdisciplinary biomineralization community. My detailed comments to the authors are listed below.

Author´s reply:

We would like to thank reviewer#2 for the positive and constructive criticism on our work. We carefully revised our manuscript by addressing and accepting all comments raised by reviewer#2. In particular, we added information about the scientific rationale at the beginning and a brief conclusion of the main findings at the end of each experimental section in the discussion. We feel that this has largely improved the clarity of this relatively complex work.

We restructured and revised the introduction to provide a better overview on the topic and to clearly point out the research questions addressed in this work. To better address the comments raised by reviewer#1 we divided the original review into separate points and addressed them in a point to point manner. All changes made in the text are marked in red in the "track-changes" version. We once again thank reviewer#2 for the constructive criticism and hope that he/she is happy with this revised version of our manuscript.

General comment about the presentation

Reviewer#2

The way the paper is written now limits its readership to physiologists that study sea urchin skeletogenesis, which is a somewhat narrow audience. Yet, it has the potential of being interesting to biologists, chemists and material scientists that study biomineralization, as well as people that study climate change, if you write it thinking about them as your readers.

Author´s reply: We are grateful for these general and constructive comments on our work We carefully revised the manuscript according to these suggestions (see following point-to point comments) and think that it has been largely improved.

Reviewer#2

You should start with a broader introduction of the importance of biomineralization, presenting the critical gaps in knowledge in the field, that you want to address in this paper. If you think that your main impact will be on climate change – explain the relevance in the first paragraphs of the introduction. The most important impact can't appear as the last line in the abstract or the last paragraphs of the discussion, you have to lead to the reader to that in order to convince them.

Author´s reply: We now added a broader introductory paragraph at the beginning of the introduction: "To generate $CaCO_3$ shells and skeletons, calcifying organisms must accumulate Ca^{2+} ions and dissolved inorganic carbon (e.g. HCO_3^- and CO_3^{2-}) by cellular transport mechanisms [1-3]. During this mineralization process, protons are liberated that need to be removed from the calcification front to allow further mineral precipitation [1]. Thus, efficient trans-membrane transport systems to regulate pH and to deliver calcification substrates to the calcification front are a fundamental requisite of all calcifying systems with the underlying mechanisms being little understood. This mechanistic knowledge will have important implications for our understanding of the mineralization process in marine calcifiers, and their ability to cope with rapid changes in the seawater carbonate system due to the phenomenon of ocean acidification [4]."

Reviewer#2

After that you should explain why the sea urchin is such an excellent system for these studies and the specific mechanisms that you are investigating here.

Author´s reply: The broader introductory paragraph on biomineralization is now followed by the current knowledge on skeleton formation in the sea urchin larva. In the end of this paragraph specific research questions that were addressed in this work are pointed out.

L73-93: For more than a century, the sea urchin larva ……This requires substantial cellular proton buffering and export capacities with the underlying mechanisms being largely unknown. Thus, the present work aims at identifying ion transporters in PMCs that are critically involved in the calcification process of the sea urchin larva.

Reviewer#2

When referring to vertebrate it is important to note that vertebrates use a different mineral than CaCO3 (CaPO4), and clearly the cellular environment is completely different, but yet, the cells have similar challenges regarding the pH and proton exchange.

Author`s reply: We agree to the point. Due to space limitations we decided to omit this paragraph and now focus on calcifying invertebrates in the introduction.

Reviewer#2

You use the “regeneration” assay quite a lot in the paper, but you don’t explain why you use it and what you expect to learn from it. Also, this is not a typical regeneration process where an organ is amputated and recovers, but a de-calcification process where the change in pH causes the mineral to dissolve. I wouldn’t use the term “regeneration” in this context, but if you wish to use it – you have to explain what it is (also in the abstract) and why you refer to it as regeneration (in the text of the paper). I think you should mention this assay in the introduction – where it was used before, and what is the advantages/reasons to use this assay here.

Author`s reply: We agree to this point raised by reviewer’2 and now refer to “re-calcification” assay. We also added information to the last paragraph of the introduction, explaining why we apply this assay and what we expect from it.

L113-120: “To study pH regulatory processes in actively calcifying PMCs we then used a re-calcification assay where the skeleton is dissolved and completely rebuild within few days [29]. This approach allowed us to study pH regulatory mechanisms in actively calcifying PMCs in a stage where organogenesis and development are largely completed. Intra-cellular as well as intra-vesicular pH measurements were performed to characterize pH conditions in these compartments during skeleton re-calcification suggesting an important contribution of vesicular pH homeostasis in the calcification process.”

Reviewer#2

There is a lot of emphasize on the blastoceolar cells and their buffering capacity, but it is not clear why you think that they play a role in skeletogenesis, at least not in the results section. This needs to be explained.

Author`s reply: We added this information to the respective paragraph in the results section:

L254-264: “During the phase of skeleton re-calcification the number of large blastocoelar filopodial cells (BFCs) highly increase in the extracellular space of the primary body cavity and interact with PMCs and their syncycium (Figure 3A)…….. Since BFCs are strongly associated with PMCs, pH regulatory capacities as well as vesicular pH determinations of these two cell types were compared to see if their physiology shows similar responses during skeleton re-calcification or not.”

Reviewer#2

In general, a common rule is write an explanatory sentence at the beginning of each experimental paragraph that explains the goal in this experiments and a summary sentence at the end of each experimental paragraph that explains the essence of your results. I think that if you follow this rule throughout the paper it will increase its readability significantly.

Author`s reply: We followed the advice of reviewer#2 to explain the experimental rationale at the beginning of each experimental part and to provide a short conclusion about the main findings at the end of the respective paragraph. We are very grateful for this suggestion since we agree that this significantly improved the clarity and readability of this relatively complex manuscript.

For example: L366-369: "A first set of experiments addressed the presence and role of proton transporting enzymes, including Na^+/H^+-exchangers (NHEs) and V-type H^+-ATPases (VHA) in pH_i regulatory capacities of PMCs. Determination of pH_i regulatory capacities …"

L402-406: "…NHE inhibitor EIPA. Based on pharmacological and biochemical evidences, we conclude that NHEs are part of the pH regulatory machinery of PMCs. However, our results also demonstrate that Na^+/H^+ exchange activity mainly serves pH_i regulatory processes of PMCs during maintenance of the skeleton with only a minor contribution to the calcification process."

L407-412: "Decalcification experiments of the present work confirmed earlier studies demonstrating the ability of sea urchin larvae to fully re-calcify their calcitic endoskeleton after dissolution by acidic conditions [33]. This assay was used to stimulate calcification rates of PMCs allowing us to study pH regulatory capacities and mechanisms of PMCs during active calcification…."

L438-442: "Based on these observations and findings of the present work it can be concluded that cellular DIC accumulation is strongly stimulated during skeleton re-calcification. However, unlike the situation invertebrates where the acid-load generated by the mineralization process is compensated by increased NHE activity, PMCs seem to utilize another route to remove protons from the calcification front."

L443-446: "In a next step we investigated the potential role of vesicular acid sequestration as an alternative route to locally remove protons from the cytosol. Intra-vesicular pH measurements demonstrated…"

L475-481: "Here it remains unresolved which of the VHA isoforms is responsible for the massive sequestration of protons in BFCs and what the biological function may be. It can be hypothesized that this massive transport of protons into vesicular structures of BFCs is associated with a removal of protons liberated by the calcification process and/or potentially also serves other cellular functions like enhanced protein degradation and processing in acidic lysosomes or autophagosomes during skeleton re-calcification [39]."

Reviewer#2

Also, please cite the recent papers in this subject (molecular and cellular control of sea urchin skeletogenesis) that are relevant to this work.

Author`s reply:

We added the following references

Shashikant T, Khor JM, Ettensohn CA. 2018 From Genome to Anatomy: The Architecture and Evolution of the Skeletogenic Gene Regulatory Network of Sea Urchins and Other Echinoderms. Genesis. Sep 27. doi: 10.1002/dvg.23253

Morgulis et al. 2019 Possible cooption of a VEGF-driven tubulogenesis program for biomineralization in echinoderms. PNAS. 116 (25) 12353-12362

Reviewer#2

Experimental approach and results:
The authors first studied the role of Na/H exchange in skeletogenesis. They studied the recovery of the skeletogenic pH from an ammonium pulse in the presence of H+ exchange inhibitors and observed a variable response to the inhibition.

They used EIPA (please add reagent number to the methods section), that indeed inhibits Na+/H+-exchanger, but it also inhibits TRPP3 channels and macropinocytosis. They observed that the recovery of some of the skeletogenic cells to the ammonium pulse was impaired in EIPA (Fig. 1A-D). Additionally, the incorporation of calcium was reduced under EIPA treatment based on calcein pulse-chase experiments. They observed the expression of the Na+/H+-exchanger, SLC9a2 at the skeletogenic cells located near the body rods at this time, and suggest that this gene could be relevant to the effect of EIPA on calcification.

However, the role of this gene or of other genes studied in this paper was not studied by genetic perturbation, and the fact that it is expressed in the skeletogenice cells at later stages of skeletogenesis is not a proof of the gene function – you should be more cautious about this in the abstract and discussion.

Author`s reply:

We agree to the reviewers´ point that our studies did not use genetic perturbations to link protein function to the respective encoding gene and we carefully revised our manuscript by toning down any statement linking functionality to a specific gene (e.g. SLC9a2 or different ATP6V6 isoforms). We left the function of the HCO3- cotransporter (Sp_Slc4a10) in the manuscript since our previous study (Hu et al. 2018 elife: e36600) demonstrated that knock-down of this transporter leads to reductions in HCO_3^- accumulation in PMCs. However, our pharmacological and functional characterizations allow us to narrow down transport pathways to a group of transporters like Na+/H+-exchangers, V-type H+-ATPases or HCO_3- transporters. Thus, we think that our conclusions for the involvement of NHEs in pHi regulation, Na^+/HCO_3^- transporters in HCO3- accumulation and the role of the V-Type H+-ATPase in vesicular acidification are justified. We specifically mention that the identification of the responsible gene encoding the respective transporters remains a question for further research.

e.g. L406: "Although the genetic basis remains unresolved, our pharmacological and biochemical studies demonstrated that NHEs are part of the pH regulatory machinery of PMCs.

L 486: "Here it remains unresolved which of the VHA isoforms is responsible for the massive sequestration of protons in BFCs and what the biological function may be."

Information about the inhibitors has been added to the material and methods section in the supplemental part:

"….For pharmacological treatments the inhibitors 5-(N-Ethyl-N-isopropyl)amiloride (EIPA; Sigma-Aldrich A3085), Bafilomycin A1 (Sigma-Aldrich 19-148) and Disodium 4,4'-diisothiocyanatostilbene-2,2'-disulfonate; (DIDS, Sigma-Aldrich D3514) targeting Na^+/H^+-exchangers, V-Type H^+-ATPases and HCO_3^- transporters were added to the Tris-buffered FSW at a 2 x concentration."

Reviewer#2

Furthermore, I am not sure that I agree with the conclusion as macropinocytosis was shown to be the way that the skeletogenic cells uptake calcium and CO3 from the sea water (Vidavski et al 2014, 2015, 2016). EIPA blocks macropinocytosis and that could be the reason for the reduced calcium uptake– the authors should mention it and explain or prove that the main issue here is the NH-exchange blocking.

Author`s reply:

We understand and agree to the point raised by reviewer#2: We agree that the slight reduction in calcification may be due to a direct inhibition of macropinocytosis and added this information to the discussion: L367-374: "Here it should be noted that EIPA has been demonstrated to inhibit macropinocytosis [35], and thus reductions in calcification rates under the inhibitor treatment may be a result of reduced Ca^{2+} uptake by vesicular pathways. Although the genetic basis remains unresolved, our pharmacological and biochemical studies demonstrated that NHEs are part of the pH regulatory machinery of PMCs. However, our results also demonstrate that Na^+/H^+ exchange activity mainly serves pH_i regulatory processes of PMCs during maintenance of the skeleton with only a minor contribution to the calcification process."

Reviewer#2

Then the authors present the regeneration assay (Fig. 2), without explaining why they use it and what they expect to learn from it that they can't learn from normal skeletogenesis – please add this reasoning to the introduction and/or the results section. Please explain how this relates to normal skeletogenesis as this is an extreme condition – what is your hypothesis.

Author`s reply:

We added an appropriate paragraph to the end of the introduction addressing these points. See previous comment.

Reviewer#2

You have to explain what each inhibitor does in the text (DIDs, Bafilomycin A1) and instead or in addition to the detailed experimental concentrations and measured parameters, explain in words what processes you affected and the meaning of the results.

Author`s reply:

We carefully revised our manuscript by better explaining the action of the respective inhibitors and the interpretations of these results.

e.g. L402: "Based on pharmacological and biochemical evidences, we conclude that NHEs are part of the pH regulatory machinery of PMCs."

L420: "Pharmacological inhibition of cellular HCO_3^- transport by DIDS led to a further decrease in pH_i regulatory capacities of PMCs supporting the concept of increased HCO_3^- accumulation during skeleton re-mineralization."

Reviewer#2

The authors compare the regulatory capacities of the skeletogenic cells with those of the blastoceolar cells present in the coelom of the larva (I wouldn't use the work "occur" for that). They don't explain why they do this comparison – the blastoceolar cells touch the skeletogenic cells but there is no cell fusion between these populations.

Author`s reply:

We added two sentences explaining the rationale and conclusion of this comparison: L468-472: "A comparison of PMCs and BFCs demonstrated substantially reduced pH regulatory capacities during skeleton re-calcification while those of BFCs resemble those of PMCs in a non-remineralizing mode. This difference in pH_i regulation of PMCs and BFCs further underline different functions of these two cell types during skeleton re-mineralization."

The sentence using "occur" has been rephrased: "skeleton the number of large blastocoelar filopodial cells (BFCs) largely increase in…"

Reviewer#2

The acidic vesicles detected in the blastocoelar cells are most likely lysosomes or autophagosomes and the effect of Bafilomycin on the acidification could be since it blocks the fusion between autopahgosomes and lysosomes and not directly related to skeletogenesis. They measure the levels of different isoforms of the V H+ ATPASE and observe divergent pattern during decalcification – some isoforms are upregulated and some are downregulated. However, without the knowledge about the specific spatial expression of the isoforms, it is hard to even guess what their role is. The human antibody for the ATPase show a stain in the sub-cellular vesicles of the blastoceolar cells, again, probably the autophagosomes and lysosomes. But it is not clear which of the isoforms it is actually detecting and what is the relevance to skeletogenesis.

Author`s reply:

Although proton transport by V-type-H+ATPases seem to be critically involved in the calcification process we agree to the criticism of Reviewer#2 that the exact mechanism how and which of the V-type H+-ATPases contributes to the re-mineralization event remains unresolved. To avoid any over interpretation, we toned down our conclusion on this paragraph and clearly state the remaining knowledge gaps:

L 464-469: " Here it remains unresolved which of the VHA isoforms is responsible for the massive sequestration of protons in BFCs and what the biological function may be. It can be hypothesized that this massive transport of protons into vesicular structures of BFCs is associated with a removal of protons liberated by the calcification process and/or potentially also serves other cellular functions like protein degradation and processing in acidic lysosomes or autophagosomes [39]."

Reviewer#2

Also – the red stain in Fig. 4D is not explained in the text/figure caption.

Author`s reply: We added this information to the figure legend." ……..re-calcification (D2) (green) and DraQ5 as a counterstain for nuclei (red)"

Reviewer#2

The model presented in Fig. 4G in not clear – how they got to it from the measurements and what it means. This part of the work need some more thinking and reasoning I believe.

Author`s reply:

We agree that the model depicted in figure 4 was insufficiently explained. We revised parts of our discussion and conclusion by better explaining the findings and open questions of the present work summarized in figure 4G.

L446-454: "Here it remains unresolved which of the VHA isoforms is responsible for the massive sequestration of protons in BFCs and what the biological function may be. It can be speculated that this massive transport of protons into vesicular structures of BFCs is associated with a removal of protons liberated by the calcification process. Acidic vesicles may then be exocytosed into the primary body cavity or transported through the filopodial network to be released into the seawater at the highly permeable ectoderm. Furthermore, besides the possibility of direct secretion, vesicular sequestration of protons may also serve other cellular functions like enhanced protein degradation and processing in acidic lysosomes or autophagosomes during skeleton re-calcification [41]."

L470-481:" In contrast to the situation in vertebrate osteoblasts, mineralizing cells of the sea urchin embryo reduce NHE based pH_i regulatory capacities during extensive calcification events. However, the necessity to remove protons liberated by the calcification process suggests an alternative route of proton removal from the calcification front in PMCs. Here our vesicular pH measurements demonstrate a sub-cellular sequestration of protons in PMCs and BFCs potentially supporting pH_i regulation during the calcification process (Figure 4 G). It remains a matter of future investigations whether acidic vesicles in PMCs are exocytosed into the primary body cavity of if they can be transferred from PMCs to BFCs to be secreted or used for other cellular processes during skeleton repair (depicted by question marks in Figure 4G). Despite these open questions regarding the removal of protons from the calcification front our results clearly demonstrate efficient cellular mechanisms of DIC accumulation during the mineralization event (Figure 4G)."

Appendix B

Reviewer(s)' Comments to Author:

Referee: 2

Comments to the Author(s).
Reviewer#2: The revised version of the manuscript addressed my major concerns: The replacement of the work “regeneration” with re-calcification and explanation about this assay and its’ purpose, mentioning additional mechanisms that are affected by the inhibitors, etc. However, I still feel that some more work on the text is needed to improve the paper’s readability. Below I list my suggestions for improving the paper clarity and significance, according to the order of their appearance:

Authors: We are grateful for the constructive criticism of reviewer#2 on our manuscript, that significantly improved the clarity of the text. We accepted and included all suggestions made by reviwer#2 into our manuscript and replied in a point by point manner in the following response letter.

Introduction:

Reviewer#2: Lines 64-67: I recommend you to revise to: “Thus, efficient trans-membrane transport systems that regulate pH and deliver calcification substrates to the calcification front, are a fundamental requisite of all calcifying systems with the underlying mechanisms not clearly understood.”

Authors: Accepted.

Reviewer#2: Lines 77-78: I recommend you to revise to: “A range of matrix proteins that regulate crystal nucleation, ACC stabilization and recruitment of Ca2+ ions, is required for the proper development of the larval skeleton”.

Authors: Accepted and rephrased.

Reviewer#2: Lines 80-81: Missing “is”: Carbon isotope studies demonstrated that approximately 60% of dissolved inorganic carbon (DIC) used to build the larval skeleton is derived

Authors: Corrected

Reviewer#2: Line 85: Missing “of”: to be critically involved in the cellular of accumulation HCO3 from the seawater

Authors: Corrected

Reviewer#2: Line 92: Please repeat the meaning of the acronyms, NHE and VHA, that you defined in the abstract in the first time they appear in the text. Also – for HKA, it is first mentioned in line 93 – put the acronym there.

Authors: We added the acronyms.

Reviewer#2: Line 95 and throughout the paper: Sp_SLC9a2 -> Sp-SLC9a2 or Sp-Slc9a2 – which ever you prefer but keep consistent.

Authors: We now use Sp_XX consistently throughout the manuscript.

Reviewer#2: Line 109: what do you mean by pH regulatory states? Maybe you mean regulatory mechanisms?

Authors: We now refer to pH regulatory mechanisms here.

Results:

NHE exchange mechanisms

Reviewer#2: This paragraph still needs more explanation about the logic of the experiments to be clear. First you block NHE and you see an effect of the ability to regulate pH, and then you look for the responsible gene. Say it explicitly and separate into two paragraphs, the first about the inhibition and the second about the gene. Add one sentence to the beginning of the second paragraph, e.g.,: Our findings suggest that NHE proteins participate in the regulation of pH in the PMC and therefore we searched for the responsible gene". – or something along these lines. Summarize the findings in the end of the section – "we observed (NEH effect) and identified (SLC9) as a possible gene that mediates this function". Also - You have to mention the block of macro-pinocytosis by EIPA here, in the calcein experiment and not wait to the discussion, where it also damages the logical flow.

Authors: We agree to the suggestions of reviewer#2 and made all recommended changes to the text.

pHi regulatory capacities during skeleton re-calcification

Reviewer#2: 1. Please change the first line of this paragraph to make it clearer to the reader: "To study pH regulatory dynamics in actively calcifying PMCs we used the re-calcification assay where the skeleton is dissolved and completely rebuild within few days. To conduct the re-calcification assay we expose pluteus larvae…". And then the rest of the paragraph. I think it is better to put this explanation here and not in the end of the introduction where it is a little too much information for this stage.

Authors: We agree and moved this information from the introduction to the beginning of this section.

Reviewer#2: 2. This section also needs to be divided into two paragraph, the first about the pH regulation during re-calcification (reporting the dynamic changes in different ion levels) and the second about the pharmacological experiments addressing the molecular mechanisms that underlie these dynamics. The second paragraph needs to start with: "to address the molecular mechanisms involved in these ionic changes we blocked this and that and observed the effect on ion levels". And then the rest of the paragraph follows. Summary Figure 2J has to be explained in the text, it is not self-explanatory.

Authors: We agree and divided this paragraph. We now explain figure 2J at the end of this paragraph.

Interaction of vesicle-rich blastocoelar filopodial cells with PMCs and characterization of vesicular pH

Reviewer#2: The revision really improved the readability of this section. However, there are still a lot of technical details and not enough explanation of the biological meaning of the results. The proposed role of the blastocoelar cells is still not clear to me – is it to sequester the protons from the blastocoel? In Fig. 3C you write: "BFCs attach to PMCs and transport endocytosed vesicles across their filopodial network" It is not clear whether the vesicles are transported between BFCs or to the PMC from this statement and I don't think there is evidence for transport of vesicles between the blastocoelar cells and the PMCs. So, I don't understand what the vesicle with the question mark in Fig. 4G stands for.

Authors: We added further explanations to this section regarding the potential role of vesicular proton sequestration in BFCs. The arrow with the question mark does not depict a vesicle but a potential membrane transporter or channel.

Modulation of acid-base transporters during skeleton re-calcification

Reviewer#2: Add a sentence to the beginning of this section saying: "Our findings indicate that NBCs and NHEs might play a role in the regulation of the re-calcification process we therefore measured the change in the level of relevant genes during this process".

Authors: We added this sentence to the beginning of this paragraph.

Reviewer#2: Increase in log scale is not fold: "1.18-fold (log2) and 0.84-fold (log2)" translate to actual changes in level.

Authors: We now provide real fold-change values. i.e. "…2.27-fold and 1.79-fold…"

Reviewer#2: Summarize the findings of this section in the last sentence.

Authors: We added summarizing sentence to the end of this paragraph

Discussion

Reviewer#2: In general – the discussion provides very interesting references that give broader context to the paper's findings, but it contains too much repeats and details of the results, which makes it hard to follow. All the explanation of the results should be in the result section and only briefly mentioned in the discussion. This would make the results and the discussion clearer.

Authors: We carefully revised our discussion and avoided unnecessary description of results in this paragraph.

Reviewer#2: pHi regulatory states of PMCs – again it is not clear to me what you mean by pH regulatory states. Either change the wording (to capacities or mechanisms) or explain. The point you make in this paragraph is extremely interesting – about the spatial difference along the body rods (not primary rods) that correlated with differential gene expression along these rods and also with the fact that the skeleton elongates at the tips (Sun and Ettensohn gene exp patterns 2014 – refer to, it is relevant). This paragraph should be written more clearly. The analogy to bone formation is interesting but the process is extremely different. Yet it helped me understand what you mean by different functional states. Why don't you just way it clearly: "It seems that different PMCs have a different function that is related to their position along the body rods, the ones at the tips show… while the ones located away from the tips show…" This strongly correlates with the fact that they have different functions in the normal calcification process – calcification is promoted at the tips and inhibited along the rods (Sun and Ettensohn gene exp patterns 2014).

Authors: We see the reviewers point and now stress the importance of this observation including suggested references: "…..body rods. The ones at the tip use EIPA insensitive mechanisms for calcification while the ones located away from the tips show EIPA sensitive incorporation of Ca^{2+}. This strongly correlates with the observation that PMCs have different functions in the normal calcification process where calcification is promoted at the tips and inhibited along the rods (Sun and Ettensohn 2014). Further evidence for different functional states of PMCs is provided by *in situ* hybridization analyses in combination with calcein pulse chase experiments [28]."

Reviewer#2: Lines 387-388: "However, unlike the situation in vertebrates where the.." and not "unlike the situation invertebrates".

Authors: Corrected

Reviewer#2: The discussion of the role of BFC doesn't have a clear conclusion.

Authors: This is true, but we feel that this observation is important for the calcification process that opens new venues for future research.