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Rapid multi-generational acclimation of coralline algal reproductive structures to ocean acidification

B. Moore, S. Comeau, M. Bekaert, A. Cossais, A. Purdy, E. Larcombe, F. Puerzer, M.T. McCulloch and C.E. Cornwall

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Review timeline

Original submission:	18 January 2021
Revised submission:	8 April 2021
Final acceptance:	15 April 2021

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

Review History

RSPB-2021-0130.R0 (Original submission)

Review form: Reviewer 1 (J Fietzke)

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?
Yes

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 submitted manuscript of Moore et al. uses an interesting, meaningful approach to estimate the potential performance i.e. reproductive success of coralline algae under conditions of Ocean Acidification predicted for the future.
As such it is a continuation of the 2020 Cornwall et al. paper. Following a fairly complementary approach it is a nice continuation of the culturing study performed, which I consider as a plus, extracting more information from the existing sample material.
After revision I think this work will make a valuable contribution to RSPB.

I've got a couple of questions, though, which hopefully will help to improve the manuscript.

I think, it would be extremely helpful for the reader if the authors could provide information of the natural baselines of the parameters measured. Can you provide data for the conceptacle abundance and diameter for specimen of similar live-stage collected from their natural habitat? This would help to understand how of the generational trends observed compare to the "undisturbed" scenario.
It may be helpful to reconsider some of the structure of presentation. In my initial reading I've got a little distracted by the clear generational trends (e.g. Fig.2, Fig. S1-3). But this is perhaps not the major point here. One could argue, playing devil's advocate, those trends are indicative of the alga acclimatizing to the culturing environment, recovering from the shock of being transplanted from their natural habitat. So, those trends are not the main message.
One could even argue, that the stronger response in generation 2 (OA) is due to the fact, that this treatment represented the largest change from the natural situation.
Again, this is not the main point in my opinion.
What's key in my opinion is the observation, that there are NO significant pH-related differences in the performance after 3+ generations.
So, please, if you agree try to focus the presentation more into that direction, preventing misconception or unnecessary criticism.
Please, carefully revise the figures and its use. Try to find a better balance of what really needs to be presented in the main text, what can go to the supplements. E.g. Fig. 4 is not in particular helpful in the main text. It is confusing and I do not see the point of presenting particular trendlines for each generation/treatment. Also, not using common x axes is unnecessarily confusing.
Quite similar critique for Fig. S5 (? trendlines, axes ?). What is the underlying logic anyway? Conventionally, one would expect on the y axis the dependent parameter relating to parameter

on the x axis (total recruit area as a function of conceptacle abundances?).

It would also be helpful to be more clear from the very start about the key parameter of this study (mean pH). The additional details (e.g. origin of the samples, pH variability levels) are not used for detailed interpretation if I am not mistaken. So, either remove the hypotheses 2 and 3 from your introduction, or expand on those questions in the discussion.
Finally, maybe consider converting the conceptacle abundance, diameter and maybe also using the total growth into some kind of "total conceptacle volume" parameter. I'd played around with just abundance x diameter^3 ... looked interesting, if only I had the natural baseline ;)
I would consider such a parameter a fair representation of reproductive potential.

So, again, I think this is interesting material, which should be accepted for publication after some moderate revision.

Best wishes,
jf

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?
Marginal

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

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.
Yes

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 manuscript led by Moore et al. assess the transgenerational effects of seawater pH on coralline algae conceptacles. They describe an initial sensitivity to lower seawater pH via reduced conceptacle abundance, however, this sensitivity disappeared in later generations. This initial negative response to ocean acidification and later recovery was taken to suggest acclimation to ocean acidification conditions over only a few generations. This is an interesting paper that demonstrates the acclimation potential of an important marine calcifier and its reproductive output after multiple generations of reduced seawater pH exposure. The findings add nicely to the body of literature assessing ocean acidification on coralline algae growth/physiology. I do believe the manuscript would benefit from some edits and clarifications. (Please see attached comments)

Decision letter (RSPB-2021-0130.R0)

19-Mar-2021

Dear Mr Moore:

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:

Research ethics:
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.

Use of animals and field studies:
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. Please see our Data Sharing Policies (https://royalsociety.org/journals/authors/author-guidelines/#data). 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.

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

Associate Editor
Board Member: 1
Comments to Author:
Both reviewers have identified that the manuscript is of interest to the readers of this journal, however both identify that some further detail and clarification of content is needed and some refocusing of content would be useful. Both have provided constructive comments for consideration in revising your manuscript.

Reviewer(s)' Comments to Author:

Referee: 1

Comments to the Author(s)
The submitted manuscript of Moore et al. uses an interesting, meaningful approach to estimate the potential performance i.e. reproductive success of coralline algae under conditions of Ocean Acidification predicted for the future.
As such it is a continuation of the 2020 Cornwall et al. paper. Following a fairly complementary approach it is a nice continuation of the culturing study performed, which I consider as a plus, extracting more information from the existing sample material.
After revision I think this work will make a valuable contribution to RSPB.

I've got a couple of questions, though, which hopefully will help to improve the manuscript.

I think, it would be extremely helpful for the reader if the authors could provide information of the natural baselines of the parameters measured. Can you provide data for the conceptacle abundance and diameter for specimen of similar live-stage collected from their natural habitat? This would help to understand how of the generational trends observed compare to the "undisturbed" scenario.
It may be helpful to reconsider some of the structure of presentation. In my initial reading I've got a little distracted by the clear generational trends (e.g. Fig.2, Fig. S1-3). But this is perhaps not the major point here. One could argue, playing devil's advocate, those trends are indicative of the alga acclimatizing to the culturing environment, recovering from the shock of being transplanted from their natural habitat. So, those trends are not the main message.
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Please, carefully revise the figures and its use. Try to find a better balance of what really needs to be presented in the main text, what can go to the supplements. E.g. Fig. 4 is not in particular helpful in the main text. It is confusing and I do not see the point of presenting particular trendlines for each generation/treatment. Also, not using common x axes is unnecessarily confusing.
Quite similar critique for Fig. S5 (? trendlines, axes ?). What is the underlying logic anyway? Conventionally, one would expect on the y axis the dependent parameter relating to parameter on the x axis (total recruit area as a function of conceptacle abundances?).

It would also be helpful to be more clear from the very start about the key parameter of this study (mean pH). The additional details (e.g. origin of the samples, pH variability levels) are not used for detailed interpretation if I am not mistaken. So, either remove the hypotheses 2 and 3 from your introduction, or expand on those questions in the discussion.
Finally, maybe consider converting the conceptacle abundance, diameter and maybe also using the total growth into some kind of "total conceptacle volume" parameter. I'd played around with just abundance x diameter^3 ... looked interesting, if only I had the natural baseline ;)
I would consider such a parameter a fair representation of reproductive potential.

So, again, I think this is interesting material, which should be accepted for publication after some moderate revision.

Referee: 2

Comments to the Author(s)
The manuscript led by Moore et al. assess the transgenerational effects of seawater pH on coralline algae conceptacles. They describe an initial sensitivity to lower seawater pH via reduced conceptacle abundance, however, this sensitivity disappeared in later generations. This initial negative response to ocean acidification and later recovery was taken to suggest acclimation to ocean acidification conditions over only a few generations. This is an interesting paper that demonstrates the acclimation potential of an important marine calcifier and its reproductive output after multiple generations of reduced seawater pH exposure. The findings add nicely to the body of literature assessing ocean acidification on coralline algae growth/physiology. I do believe the manuscript would benefit from some edits and clarifications. (Please see attached comments)

Author's Response to Decision Letter for (RSPB-2021-0130.R0)

See Appendix A.

Decision letter (RSPB-2021-0130.R1)

15-Apr-2021

Dear Mr Moore

I am pleased to inform you that your manuscript entitled "Rapid multi-generational acclimation of coralline algal reproductive structures to ocean acidification" 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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Please remember to make any data sets live prior to publication, and update any links as needed when you receive a proof to check. It is good practice to also add data sets to your reference list.

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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.

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,
Dr Daniel Costa
Editor, Proceedings B
mailto: proceedingsb@royalsociety.org

Appendix A

Dear Dr Daniel Costa

Associate Editor

We wish to submit our revised manuscript RSPB-2021-0130 "Rapid multi-generational acclimation of coralline algal reproductive structures to ocean acidification" to Proceedings Royal Society B. The overall comments from Referees #1 and #2 are positive, as they suggest this manuscript would be of interest to Proceedings B readers and should be accepted after moderate revision. Therefore, after reading the referees comments we have made the suggested amendments in all but a few instances, and we now consider the manuscript to be much improved. For the majority of the referees' comments, please see our specific answers point-by-point below. We thank both referees and yourself for your time and effort.

One of Referee #1's primary concerns is that the significant effect of ocean acidification observed in generation two may have been driven by lab or transplant effects, and thus they suggest focusing more on generation three plus. Firstly, we find it highly unlikely that these potential effects would interact only with our ocean acidification treatments (and not present-day treatments), in such a way that it would underpin the significant effect of OA in generation two. Furthermore, interpreting the results in such a way would cast doubt on the majority of aquaria-based ocean acidification studies, as historically these studies have focused on first generation wild organisms that have recently been transplanted to aquaria. Secondly, the results of our reciprocal transplant experiment indicate that lab/transportation effects did not drive the acclimation reported here. Thirdly, such an alteration in interpreting the results would require a vast restructuring of this publication, as the second-generation trends underlie our primary conclusion that coralline algae acclimate after multiple generations of exposure. Therefore, considering the positive response of both referees to our initial submission, it would seem that such a restructuring is not desired.

Both reviewers requested that trends relating to the coralline algae's site of origin (Shell vs Tallon) and the aquaria treatments level of pH variability (high vs low), be given more discussion. To address this, we have added two new paragraphs to the discussion and additional information related to these variables in the results. Furthermore, we have now moved Figure S1 that displays the effect of all variables on conceptacle abundance to the primary manuscript. Thus, placing further emphasis upon site of origin and pH variability effects within the primary manuscript.

An additional concern related to the presentation of Figure's 4 and S5. As these figures' present the multi-generational relationship of coralline algal conceptacle abundance with growth and recruitment, they highlight an interesting and novel aspect of this study. Therefore, we believe they warrant a place within this publication, but have now moved Figure 4 to the supplementary as per Referee #1's suggestion. We have also included a new "Growth and Recruitment Measurements" section within the methods, that clarifies the presentation and purpose of these figures. Moreover, additional methodological details and Figures S1-S4 have been added to the manuscript in order to reduce the reliance on previous publications for information on the approach used here. However, note that methodological information has been moved to a supplementary methods document, in order to ensure the allowed page limit is not exceeded.

We believe this publication shows novel results from a complex experimental set-up that cannot be easily replicated in the future, and therefore hope that upon considering the revisions made here, you will accept this manuscript for publication.

Regards

Billy Moore, Drs Steeve Comeau, Christopher E. Cornwall, and co-authors

Referee 1
(*Comments to Author*)
(Response)

"I think, it would be extremely helpful for the reader if the authors could provide information of the natural baselines of the parameters measured. Can you provide data for the conceptacle abundance and diameter for specimen of similar live-stage collected from their natural habitat? This would help to understand how of the generational trends observed compare to the "undisturbed" scenario."

Baseline measurements of conceptacle abundances and size from natural populations were not conducted during this study. Moreover, as far as we are aware such baseline data does not exist for this species, with much of the previous work on conceptacles focusing on their structure, rather than abundance or size. Also as far as we are aware, there is no data regarding the frequency of reproductive mode, ploidy, etc for this species – apart from brief mentions that sexual organisms had not been encountered by morph-anatomical taxonomists in textbooks (Townsend and Huisman, 2018).

Additionally, the coralline algae assessed in our study were juveniles of a known age. Therefore, even if baseline data from wild coralline algae could be obtained, these would be collected from individuals that are of an unknown age and life-stage. Thus, we believe that use of these measurements as "undisturbed" baselines for the juvenile coralline algae used in our study, would likely be less accurate than those generated from our present-day treatments.

No changes made.

"It may be helpful to reconsider some of the structure of presentation. In my initial reading I've got a little distracted by the clear generational trends (e.g. Fig.2, Fig. S1-3). But this is perhaps not the major point here. One could argue, playing devil's advocate, those trends are indicative of the alga acclimatizing to the culturing environment, recovering from the shock of being transplanted from their natural habitat. So, those trends are not the main message.

One could even argue that the stronger response in generation 2 (OA) is due to the fact, that this treatment represented the largest change from the natural situation. Again, this is not the main point in my opinion. What's key in my opinion is the observation, that there are NO significant pH-related differences in the performance after 3+ generations. So, please, if you agree try to focus the presentation more into that direction, preventing misconception or unnecessary criticism."

As we agree that no significant pH differences in generation 3+ are important we have added lines 348 and 468 to draw greater attention to this. However, we respectfully disagree that the significant effect of OA observed in generation 2 is not important, as such a result underlies our overall conclusion that "acclimation" has occurred.

We understand the referee's view that the overall generational increase in conceptacle abundances could be due to the alga acclimatizing to the culturing environment. However, we find it unlikely that our OA treatments would interact with this artefact in such a way that it would underpin the significant effect of OA in generation 2 (across all site/pH variability treatment combinations). Moreover, if the reviewers proposed interaction between transport/culturing stress and OA treatments does exist, it would question the validity of most aquaria-based OA studies, as many of these only focus on first- or second-generation organisms.

Additionally, our reciprocal transplant experiment was employed to test whether acclimation to the culturing environment underpinned our trends. Our observation that conceptacle abundances were 25% lower (significant pairwise difference) in coralline algae transplanted from present-day to OA treatments in generation 7, compared to those kept in OA treatments for 7 generations, suggests that lab effects did not drive the acclimation reported here.

"Please, carefully revise the figures and its use. Try to find a better balance of what really needs to be presented in the main text, what can go to the supplements. E.g. Fig. 4 is not in particular helpful in the main text. It is confusing and I do not see the point of presenting particular trendlines for each generation/treatment. Also, not using common x axes is unnecessarily confusing."

Following the recommendation of the reviewer, we have moved Figure 4 to the supplementary. We also present our reasoning below for the inclusion of this figure in the supplementary and highlight the changes we have made to increase the clarity and relevance of this figure.

We believe that by displaying the relationship between conceptacle abundance and growth for each generation/treatment we are able to show that these two physiological processes do not acclimate to OA at the cost of the other. For example, if such a trade-off was driving this acclimation you would expect to see a negative relationship between growth and conceptacle abundance within the later "acclimated" generations under OA. However, this does not occur as a positive relationship between these two variables is observed for each generation/treatment combination. This is important as demonstrating that two physiological responses (growth and conceptacle abundance) both acclimate to OA across multiple generations, is one of the novel aspects of this study.

We have now added an additional paragraph to the supplementary methods labelled "Growth and Recruitment Measurements" that clearly states the reasoning behind displaying these generational/treatment trends. Furthermore, we have added two sentences to the discussion (lines 367-372) that specifically link the trends observed in Figure 4 (now Figure S6) to our conclusion that trade-offs between growth and conceptacle abundance are not occurring.

Common x axes are now used in Figure S6.

"Quite similar critique for Fig. S5 (? trendlines, axes?). What is the underlying logic anyway? Conventionally, one would expect on the y axis the dependent parameter relating to parameter on the x axis (total recruit area as a function of conceptacle abundances?)."

The primary aim of figure S5 (now Figure S7) was to demonstrate the relationship between a population's conceptacle abundance and the area of recruitment produced from such conceptacles (e.g., total area of next-generation recruitment within the tank). Figure S7 and the positive trends within this figure show this because, as the number of conceptacles increase, the total area of next-generation recruits produced from those conceptacles also increases. This result emphasises the value of our conceptacle measurements, as it highlights a clear link between conceptacles and fecundity, which is a of vital ecological importance.

We choose to present conceptacle abundance as the independent parameter and recruit area as the dependent parameter, as the aim was to show that higher conceptacle abundances (in one generation) lead to higher areas of recruitment (of the next generation). Therefore, making area of recruitment from the next generation dependent upon conceptacle abundance of the prior generation. We have now clarified this and have added an additional paragraph to the supplementary methods labelled "Growth and Recruitment Measurements" that details how recruitment was measured. This paragraph also makes it clear that the recruitment measurements represent next-generation recruitment and are therefore dependent upon conceptacle abundances of the prior generation. We also highlight the underlying logic of this figure at the end of the paragraph.

As for above, common x axes for each generational figure have now been used.

"It would also be helpful to be more clear from the very start about the key parameter of this study (mean pH). The additional details (e.g. origin of the samples, pH variability levels) are not used for detailed interpretation if I am not mistaken. So, either remove the hypotheses 2 and 3 from your introduction or expand on those questions in the discussion."

Although we believe that the trends relating to mean pH and generation are the most interesting result of this study, we also believe that comparisons made between different pH variability treatments and the origin of the samples are important. Therefore, more focus has been placed on these parameters in the results and discussion. See lines 260-264, 438-463.

We have also moved Figure 2 to the supplementary (now Figure S6) and replaced this with Figure S1 (now Figure 2), which shows conceptacle abundance data split by mean treatment pH, treatment pH variability and site of origin. Thus, placing further emphasis on these parameters within the primary manuscript.

"Finally, maybe consider converting the conceptacle abundance, diameter and maybe also using the total growth into some kind of "total conceptacle volume" parameter. I'd played around with just abundance x diameter^3 ... looked interesting, if only I had the natural baseline. I would consider such a parameter a fair representation of reproductive potential."

As suggested by the reviewer a volume parameter was created by abundance x diameter^3. After plotting this new parameter in the same way as conceptacle abundance in Figure 2, it is clear that the same trends persist for conceptacle volume. Additionally, models of volume showed a significant effect of generation and a significant interaction between mean treatment pH and generation, as was observed for conceptacle abundance. However, the significant effect of other factors such as mean treatment pH and site of origin was not present when assessing volume, and the significance of the mean treatment pH and generation interaction was much reduced. This is likely due to the influence of diameter measurements on volume, as diameter was only affected by generation when assessed individually.

After completing this analysis, we believe that if we were to combine these two variables, we would lose some of the detailed information that our study provides, as the differing responses of conceptacle abundance and size to OA would be hidden by the overall effect of OA on volume.

We thank the reviewer for this suggestion and do believe that volume would also represent a good measure of reproductive potential. No changes made.

Referee 2
(*Comments to Author*)
(Response)

"Throughout the manuscript, readers are directed to various other publications for details on many aspects of the study design because this is a follow up on a previously published study, however, this takes away from the strength of this paper. I strongly recommend these details should be included in this manuscript so that readers are able to clearly understand all components of the study."

Additional details have now been added throughout the methods to ensure the experimental approach used here can be understood, without the need to refer to previous publications. Moreover, supplementary figures S1-S4 have been added, in order to provide information on the study sites, study site pH regimes, aquaria treatment pH regimes and multi-generational methods used in this study. However, in order to adhere to the 10-page limit set by the journal a large portion of the methods has been moved to a new document titled supplementary methods therefore additions are not highlighted through tracked changes.

"While I really appreciate the assessment of differing levels of pH variability, the way this component of your study is presented makes it sound like you are comparing static versus variable pH effects. I suggest you rephrase this component in your study to clearly differentiate your treatments as 'low variability pH' and 'high variability pH' treatments."

We thank the reviewer for highlighting this inconsistency.

This has now been adjusted throughout the manuscript so that different levels of pH variability are always referred to as “low variability pH” and “high variability pH” treatments, rather than constant and variable pH treatments. The addition of Figure S3 also makes this clear.

“This study does a nice job comparing the presented results to those of similar studies, however, I wish the importance of these observations was made more clearly. For example, in the discussion on acclimation to ocean acidification of the conceptacles, I would have appreciated a bit more detailed discussion of the benefits of this acclimation of this specific metric.”

A discussion as to how maintaining conceptacle abundance/size under future ocean acidification will affect coralline algae and the wider coral reef community has been added to the start of the discussion (lines 349-360).

“Similarly, discussing the importance of coralline algae for the future of reefs would strengthen the importance of this research to the community before those just interested in coralline algae or marine calcifiers.”

See response to comment above and lines 354-360 in discussion. Also see lines 57-62 in the introduction.

Abstract

Line 33: Replace ‘as’ with ‘because’

Amended.

Line 37: remove ‘using four pH treatments’

Removed.

Line 38: By ‘organismal pH’ do you mean the pH history of the algae? I suggest clarifying this.

“Organismal pH” is replaced with “the pH regime experienced in their natural habitat”.

Line 39: Remove comma

Amended.

Lines 39-42: Suggest rewording the sentence for clarity to state: "We show that second generation coralline algae exposed to ocean acidification treatments had conceptacle abundances 60% lower than those kept in present day conditions, suggesting that conceptacle development is initially highly sensitive to ocean acidification."

Reworded.

Line 42: Remove "we also demonstrate that"

Removed.

Introduction

"I think the introduction could use a bit more work to introduce the topics being assessed in your study. A broader connection to the ecological concerns associated with ocean acidification or coralline algae in general would help convince the reader of the importance of this work."

Lines 50-55 have been added to the start of the introduction in order to introduce some of the concerns relating to future ocean acidification. Line 57-58 has also been added to emphasise the importance of coralline algae to coral reefs.

"Additionally, I think introducing your assessment of conceptacles should be shifted to follow your paragraphs on generational response to ocean acidification and pH variability. The other paragraphs do a better job discussing some generic patters in coralline algae responses to acidification that I think will assist in your story before introducing conceptacles. Then your introduction will finish off with a stronger discussion of conceptacles, which is the novel component of your study."

The order of the introduction has been amended as suggested.

Line 50: Remove comma and replace 'as' with 'because'

Removed and amended.

Line 53: Replace semicolon with colon

Amended.

Lines 56-58: Suggest rewording as, "Previous research has demonstrated reduced calcification and growth in mature coralline algae under ocean acidification"

Reworded.

Lines 58-60: Suggest rewording as, "Moreover, coralline algal recruitment and calcification during early life-stages appear to be highly susceptible to reduced seawater pH lower than 7.91 as demonstrated through decreasing calcification rate and ~90% reduced recruitment rate (or success)."

Reworded.

Line 87: Missing the author associated with your reference here.

Author information added.

Line 104: Remove the comma and 'or' and make your sentence more of a list: "…[10,27], or by selecting…"

Amended as recommended.

Line 105: For your use of 'acclimation' in this paper, I am not sure that this is always the correct term to use instead of adaptation. Wouldn't your demonstration of tolerance of ocean acidification conditions across generations be better described as adaptation? I would ensure you are assigning this correctly. Either way, you would benefit by clearly defining what you mean by 'acclimation' early on in your manuscript.

We consider it more likely that acclimation over multiple generations was occurring here, though we could not rule out adaptation. Adaptation is commonly associated with genetic changes across generations (as in Lohbeck, Riebesell and Reusch, 2012; Sunday *et al.*, 2014), whereas acclimation is used to describe non-genetic plasticity in responses (as in (Donelson *et al.*, 2012; Goncalves *et al.*, 2016; Zhao *et al.*, 2018)). Therefore, as the coralline algae here were most likely reproducing asexually (for reasons described in lines 171-174), and as the change in performance occurred within less than two years, the chances of selective genetic adaptation driving the reduced effect of OA we see across generations is minimal. Therefore, the term acclimation is used as it better describes the more likely non-genetic processes facilitating the reduced effect across generations observed here.

A clear definition of the term acclimation has been added to the introduction (lines 77-81). Additionally, a sentence (similar to that in this response) has been added to the discussion (lines 362-365) stating why we believe the multi-generational change in performance in these coralline algae fits this description of acclimation.

Line 107: Do you mean pre-exposure to pH variability here? This is unclear.

Adjusted to state that exposure to low pH because of pH variability does not facilitate acclimation.

Line 109: Replace 'calcifiers' with 'coralline algae' since your discussion at this point is only covering the algae.

Replaced.

Lines 113: Again, I am not clear what you mean by 'organismal pH history' here. I think it will benefit from a clear explanation to guide your reader.

Replaced organismal pH history with "the pH regime experienced by coralline algae in their natural habitat" in order to give a clear definition of what is meant.

Lines 116-118: Add a comma or semicolon before each numbered hypothesis in your list.

Commas added before each number.

Methods

Lines 124-134: Your discussion of your study design would benefit including map demonstrating locations of sample collection as well as data on the pH variability at the different sites.

A map displaying the location of the two islands from which generation 1 coralline algae were collected has now been added to the supplementary (Figure S1). A figure displaying the average pH over 24 hours at the Tallon, and Shell island sites has been added to the supplementary (Figure S2).

Line 134: Separate your discussion of your experimental setup into a different paragraph from your sample collection sites descriptions.

Paragraphs separated as recommended.

Line 134: Again, your use of "mean pH" and "pH variability" suggests that one treatment was static, while the other was not, but looking at your pH mean/range suggests that they both varied. I understand that biological processes make a static pH difficult to control, but I am not sure this treatment difference is clear here. This is also another part where your reader is directed to another study for important details on the methods/design that needs to be clearly addressed in this current manuscript. This treatment difference may also benefit from a figure depicting how treatment pH was variable in these treatment (comparison of pH 8 low and pH 8 high variability over a 24 hour period).

Adjusted throughout the manuscript to state "differing levels of pH variability" and we have now consistently referred to the treatments with low pH variability as "low variability pH treatments" as opposed to constant or static.

Additional methodology information has now been added in the supplementary methods document as described above.

A figure displaying the pH regime of each of the four treatments has been added to the supplementary (Figure S3).

Line 140: Here, I like that you used high and low pH variability! I suggest you do this throughout just like this and remove reference to mean versus variable pH.

Amended as described above.

Line 156: This light level seems a bit low, where did you get this level from? I would recommend referencing work that has quantified ecologically relevant light levels.

The coralline algae rhodoliths used in this study were collected from under seaweed/corals on the reef, where light levels are much lower than open reef habitats. See Anthony and Hoegh-Guldberg, (2003) for a discussion of light microhabitats on coral reefs.

Therefore, the 150 μmol quanta m^{-2} s^{-1} utilized in this study was selected to reflect the understory light environment from which the algae were collected. Moreover, as the light levels of the understory habitats were unknown, and as too high light levels can inhibit the growth of coralline algae (Leukart, 1994; Gao and Zheng, 2010), it was deemed appropriate to select a light level that would definitely not inhibit growth.

Information has now been added to the supplementary methods that justifies (as in the response above) the selection of these light levels.

Line 182: Missing parenthesis after reference 13.

Removed.

Line 215: Remove '-' from 'mm-2' it is redundant

Removed from all figures.

Line 221: Replace 'less' with 'fewer'

Replaced.

Line 227: Replace 'as for this generation of' with 'because'

Replaced "as for this generation of recruitment" with because

Line 246: For your header tanks suppling seawater, where all 12 used at the same time? If so, I would recommend using a random effect of header tank as well.

Header tank has now been incorporated into the model as a random effect, as suggested by the reviewer. The primary differing result of including this factor in the model is that the previously observed significant interactive effect of mean treatment pH and pH variability on conceptacle abundance has been lost. Therefore, results and discussion have been amended to reflect this.

Line 248: Since conceptacle diameter is not count data, I do not believe that a Poisson distribution would be appropriate here. You should utilize a gamma distribution for these data

A gamma distribution has now been used for analyses of diameter data. However, no difference in the significant effects were observed. New model results are now reported in Table S7.

Results

Lines 297-301: Because you are seeing pH variability treatment effects here, it is appropriate to group your abundance data by low versus high pH only. You should supplement figure 2 with a figure that demonstrates the effects of pH variability since you determined it resulted in a significant difference. Additionally, you have not discussed differences in your two different populations, if any. I would recommend at least mentioning if there were no population differences, so you assessed them together.

To avoid grouping, we have now moved Figure S1 (now Figure 2) which displays the separate effects of mean treatment pH, level of pH variability and site of origin (population) on conceptacle abundances to the primary manuscript.

As the updated model (including header tank) no longer shows an interactive effect between mean treatment pH and pH variability we have added line 262 stating this. The results state that site of origin did have a significant effect on conceptacle abundances, however as no interactive effects with other factors were observed this result has little meaning within our study. We have added lines 262 to the results that state no interactive effects with mean treatment pH or pH variability were observed. We have also included line 263, stating that the lack of interactive effects warrant abundance data analyses with site of origin and level of pH variability grouped together.

We have now added lines 438-463 to the discussion, to discuss the lack of effect of site of origin and level of pH variability in more detail.

Discussion

Line 398: Again, I suggest you review your use of acclimation versus adaptation here and throughout your discussion.

See response to previous acclimation vs adaptation comment.

Lines 400-401: Replace "Our observation that multi-generational acclimation is observed for conceptacle abundance and growth [13]…" with "This observation…"

Replaced.

Line 403-405: This sentence is unclear as to where you are drawing this conclusion from.

Now clearly state that this conclusion was reported in Cornwall *et al.,* 2020.

Line 407-409: Here, is the reproductive success improved compared to the first generation under ocean acidification or compared to control conditions? This is an important distinction to make in this sentence.

Sentence has been rewritten to make this clearer:
"For example, the reproductive output of marine invertebrates such as polychaetes [41] and copepods [42] is initially impaired under ocean acidification, however this effect disappears by the third generation, as the reproductive output of populations exposed to ocean acidification does not differ from controls"

Line 408: Replace "generation two" with "second generation" and I would do this throughout the discussion for an easier read

Replaced throughout discussion.

Line 410: Replace '≤' with 'fewer than' (or something similar)

Replaced with "within"

Lines 411-414: Suggest modifying these lines like this: "…clams [44], suggesting various biological processes are capable of rapid acclimation to ocean acidification. However, our results and those of Cornwall et al [13] suggest that different processes may exhibit contrasting acclimation times."

Amended as recommended.

Line 418: 'Linear' should not be capitalised

Amended.

Line 441, 461: Reference author missing here

Added author information.

Lines 445-450: This comparison of studies could benefit from being fleshed out a bit more. What are the morphological differences you are referring to? Also, I think emphasizing different species/population responses here is important.

A more detailed explanation of the morphological differences has now been added in lines 416-424.

Lines 462-463: You tested this with two different population from different pH variability environments, correct? Did you see any differences between populations? It would be really interesting to see if the more variable population outperformed the other (or vice versa). I recommend a discussion of this here (and mention of it in your results).

Yes, two populations from environments with different pH variability were investigated. As mentioned above, the results now state that population (site of origin) did have an overall independent effect on conceptacle abundance, however this did not interact with other fixed factors of interest (such as mean treatment pH or level of pH variability), therefore the overall conclusion was that site of origin did not influence the response of coralline algae to OA.

We have added a discussion of this lack of effect in lines 452-463.

Line 469: Missing author reference here.

Reference removed.

Lines 477-479: Suggest rewording "...under ocean acidification in generation two algae. Yet, our results show that coralline algal reproductive structures can rapidly acclimate to ocean acidification after just three generations of exposure." to "...in second generation coralline algae reared under simulated ocean acidification conditions. However, in just three generations of exposure to these same ocean acidification conditions the coralline algal reproductive structures can rapidly acclimate."

Reworded as recommended.

Line 479: When you mention the comparison to growth rates here, I would provide that time scale for reference.

Added "as growth took six generations to acclimate".

Figures

Figure 1: Suggest adding a scale bar on both images.

Added.

Figure 2: Why are your different pH treatments combined here? If you are going to combine treatments, you need to provide statistical evidence to do that. This should be clearly detailed in your methods/results.

As mentioned above, lines 262-264 now state: “No interactive effects between site of origin, mean treatment pH and level of pH variability were observed, therefore abundance and size data from different populations and pH variability treatments were grouped for subsequent analyses of mean treatment pH and generational effects.”

The replacement of Figure 2, with Figure S1 in the primary manuscript also reduces the emphasis on grouping different populations and levels of pH variability together.

Supplemental Materials

Figure S1/S3: It would be helpful to label your different populations with the normal pH variability that they were accustomed to so that readers can better assess how these coralline algae performed under ‘normal’ variability for each population.

Figure 2 (formerly Figure S1) and Figure S9 include labels that state which site the coralline algae are from (“Shell Island” or “Tallon Island”). Therefore, from the methods, the reader is able to distinguish the natural pH variability that each population is used to. Labelling the figures with Shell/Tallon island as opposed to the actual pH range or high/low variability site, was used to avoid confusion with the “low variability pH treatment” and “high variability pH treatment” labels used to detail the variability of aquaria pH treatments.

No changes made.

Tables S6-11: These tables need to also include your model estimates and standard error.

Added to S6-S11.

References used here:

Anthony, K. R. N. and Hoegh-Guldberg, O. (2003) ‘Variation in coral photosynthesis, respiration and growth characteristics in contrasting light microhabitats: an analogue to plants in forest gaps and understoreys?’, *Functional Ecology*. 17(2), pp. 246–259.

Donelson, J. M. *et al.* (2012) ‘Rapid transgenerational acclimation of a tropical reef fish to climate change’, *Nature Climate Change*. 2(1), pp. 30–32. doi: 10.1038/nclimate1323.

Gao, K. and Zheng, Y. (2010) ‘Combined effects of ocean acidification and solar UV radiation on photosynthesis, growth, pigmentation and calcification of the coralline alga *Corallina sessilis* (Rhodophyta)’, *Global Change Biology*. 16(8), pp. 2388–2398.

Goncalves, P. *et al.* (2016) ‘Rapid transcriptional acclimation following transgenerational exposure of oysters to ocean acidification’, *Molecular ecology*, 25(19), pp. 4836–4849. doi: 10.1111/mec.13808.
Leukart, P. (1994) ‘Field and laboratory studies on depth dependence, seasonality and light requirement of growth in three species of crustose coralline algae (*Corallinales, Rhodophyta*)’, *Phycologia*. Taylor & Francis, 33(4), pp. 281–290.

Lohbeck, K. T., Riebesell, U. and Reusch, T. B. H. (2012) ‘Adaptive evolution of a key phytoplankton species to ocean acidification’, *Nature Geoscience*. 5(5), pp. 346–351.

Sunday, J. M. *et al.* (2014) ‘Evolution in an acidifying ocean’, *Trends in ecology & evolution*. 29(2), pp. 117–125.

Townsend, R. A. and Huisman, J. M. (2018) *Algae of Australia: Marine Benthic Algae of North-Western Australia*.

Zhao, L. *et al.* (2018) ‘Transgenerational acclimation to seawater acidification in the Manila clam *Ruditapes philippinarum*: Preferential uptake of metabolic carbon’, *Science of the Total Environment*. 627, pp. 95–103. doi: 10.1016/j.scitotenv.2018.01.225.