Feel the Energy
אוניברסיטת בן-גוריון בנגב
Ben-Gurion University of the Negev

Department of Geological and Environmental Sciences

March 16, 2020

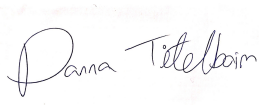
Dear Jack Middelburg,

Please find enclosed the revised version of our manuscript that I resubmit in the name of all co-authors. We believe that the reviewers raised some important issues that we have considered and changed the manuscript accordingly. The main issues raised by the reviewers were:

1) The use of our approach to separately examine the well-being of the foraminiferal host and the symbionts. We maintain that our approach is valid since calcification is a physiological trait performed only by the foraminifera and thus present a direct proxy of its wellbeing. The same is valid for photosynthesis, which is a physiological trait of the algal symbionts. Because of the exclusiveness of each parameter, we have selected them in order to get specific indications for the two components of the holobiont. Stress of the symbionts will indeed affect the host and vice versa. However, we are not examining the interaction between them but the specific response of foraminifera and algae. This is explained in more details including examples from previous publications in the responses to each reviewer and the explanation is also added to the manuscript. Additionally, to further clarify this issue in the manuscript, we have rephrased throughout the text to indicate "foraminiferal calcification rates and symbiotic net photosynthesis" as suggested by reviewer #1.
2) 2) Not enough strong evidence to support the discussion regarding the influence of pre-exposure on the thermal sensitivity of the holobionts. We accept the criticism that there are differences between the experiments other than temperature and that to make this case this will have to be done separately in a much more comprehensive manner. Therefore, we have excluded this part from the manuscript.

All other comments have been followed and are implemented in the current version of the MS. We believe that the result is a much-improved manuscript. Below is a point-by-point reply to all comments made by the reviewers (in blue).

Kind regards,



Dr Danna Titelboim

Response to comments by Reviewer 1:

This elegant study presents data of a laboratory experiment comparing, for 2 larger symbiont-bearing benthic foraminiferal species, their response to high temperature, in terms of the foraminiferal calcification rate and the photosynthetic rate of their symbionts.

These data are important, interesting, and deserve to be published. The study is very well conceived, and the high quality data are analysed with adequate statistical methods.

The text is rather short, and the information is quite dense. In such a case, the written text should be very precise, and all potential sources of ambiguity should be avoided. This is not always the case yet.

A main, recurrent problem in the discussion is that systematically, there is confusion between the holobiont (foraminiferal host + symbionts) and the host (the foraminifer without symbionts). Often, the authors speak about the host, when they mean the “whole foraminifer”, that is the holobiont. This is not surprising, because it is probably impossible to consider the foraminifer without its symbionts, which represent an essential part of it. For that reason, I think it’s impossible to compare the “well-being” of the foraminiferal host with that of the symbionts!

This becomes problematic when the calcification rate alone is supposed to represent perfectly well the general state of the “foraminiferal host”. Most times, when the authors compare “the host and the symbionts”, in reality, they compare the “calcification rate of the foraminifer” with the (photosynthesis rate of the) symbionts.

I agree that the photosynthetic rate probably describes the health of the symbionts very well, but I am not convinced that the same can be said about the foraminifer and its calcification rate. I would say that many other factors (together) determine (and can inform about) the wellbeing of the foraminiferal holobiont, the wellbeing of its symbionts being one of them!

Summarising, the authors should formulate things more carefully. They compare foraminiferal calcification rates with symbiotic photosynthetic rates. Then that’s what they should write!

Response: See response to main issue 1 in the first part of the letter. We have rephrased throughout the text to indicate "foraminiferal calcification rates and symbiotic net photosynthesis" as suggested by the reviewer.

Similarly, I think that some parts of the discussion go too far. The authors have only tested part of the response of the foraminiferal holobiont to high temperatures. Other indicators (locomotion, feeding, reproduction, etc.) may respond differently, and future climate change will probably lead to changes in other stressors (salinity, oxygenation, carbonate chemistry, etc.) as well. They should therefore be much less affirmative when they discuss the future evolution of larger BF communities.

Response: We agree with this point and changed it throughout the text. We specifically address the issues of other well-being indicators and of other stressors in the detailed comments below (comment 2 and 23).

Detailed comments:

1. Line 12: a “contribution to thermal tolerance” is somewhat strange. This suggest some pro rata contribution to the tolerance of several factors. In reality I would expect that the overall tolerance is determined by the element which is least tolerant, either the foraminifer or its symbionts.

Changed to: "In order to assess the holobiont thermal tolerance we separately evaluated foraminiferal calcification rates with symbiotic photosynthetic rates"

2. Line 13: a key question is to what point calcification (for foraminifera) and photosynthetic activity (for symbionts) can be considered representative for their tolerance. For the symbionts, since photosynthesis is a primary life process, this is probably the case. Concerning foraminiferal calcification, it is less evident that this is the best marker of tolerance. I would say overall activity (feeding, locomotion, pseudopod movements) and reproduction are more critical parameters. There are many observations of (active?!) foraminifera under stressed conditions without calcification (even of decalcified forams). Only on the long term, a lack of calcification may lead to disappearance. I think this point should be discussed.

Previous studies have demonstrated that calcification rates can be used as a direct parameter for comparing the temperature sensitivity of different calcifying organisms, this is due to the fact that calcification involves a profound consumption of energy. Therefore, calcification rates are directly linked to the range of optimal to suboptimal conditions of the organism (Lough & Barnes, 2000; Carricart-Ganivet et al., 2012). This was specifically demonstrated on different species of foraminifera (Schmidt et al., 2011, 2015, 2016a, 2016b; Vogel and Uthicke ,2012, Uthicke and Fabricius, 2012, Evans et al., 2015).

The Alkalinity anomaly method present an ideal experimental approach for detecting even subtle differences in performance under different treatments and while it is true that the overall activity and reproduction are critical parameters to indicate the well-being of foraminifera they will not identify as clearly and as quantitively the small differences between treatments. We added a short explanation of this in the method section.

3. Line 15: “sensitivity to 35°C”: what does that mean, if resilience is up to 32°C? The way it is formulated, the authors suggest that there is no sensitivity until 32°C which is evidently wrong. They probably mean something like “progressive loss of life functions between 32°C and 35°C”.

Changes as suggested.

4. Line 16: “future warming will change. . ..). The word “will” is definitely too affirmative. Replace by “may”.

Changes as suggested.

5. Line 17: “a synchronized response”: this suggest that there is some deliberate process behind it, like host and symbionts coordinating their activities. Since you don’t know this, it is better to use the more neutral term “synchronous”.

Changes as suggested.

6. Line 20: “pre-exposure to modest temperatures”. This is too imprecise. Should be “moderately high temperatures”.

The discussion regarding pre-exposure has been excluded from the manuscript (see response to the main issue #2 in the first part of the letter).

Introduction

7. Lines28-29: No, it is not this area, but the entire Mediterranean which can be considered as a miniature ocean.

Changes as suggested.

8. Line 41: “Some species live close to their thermal thresholds”: upper or lower? In fact, the further invasion of some LBF (*Amphistegina*) in the Western Med is hampered by the fact that they are limited by their LOWER temperature threshold (at present, it is too cold for them to go further west). I guess that you mean here that in the Eastern Med, at present, they live close to their UPPER threshold?! Please be more specific.

The text is corrected to indicate upper thresholds. It is true that species are limited by their lower temperature threshold, but it is still important to consider upper threshold as even small increase in temperature, predicted in the relatively near future will influence species that are close to their upper thresholds.

9. Lines43-44: “the relative contribution (positive or negative) of the host and symbiont algae to cope with rising temperatures”. As indicated before, this is really strange. The way it is written here, the sentence doesn’t make sense. You probably mean “the relative contribution to the tolerance of rising temperature”. But also that concept is very strange. This suggests that somehow you can quantify that when a LBF can still function at let’s say at 30˚C, what proportion of this resistance is due to the foram itself, and what proportion is due to (activities of) the symbionts. I think this is not possible! You simply want to investigate the “relative tolerance of host and symbiont algae to higher temperature”. With the underlying idea that the element with the lowest tolerance (host or symbionts) will probably be determinant for the tolerance of the holobiont.

Indeed, we mean that the component with lowest tolerance will limit the tolerance of the holobiont. We changed the wording to make it clearer.

10. Lines 45-46: “LBF species with different holobiont systems”: incorrect formulation: the LBF species IS the holobiont system (combination of host and symbionts)! The 2 species represent 2 different holobiont systems.

Changed.

Methods

11. Lines 67-68: “however, they were used to produce comparable data to that of related published papers (Schmidt et al., 2016b, 2016a, 2018; Titelboim et al., 2019).” This sentence is very unclear. “they” should be more specific, like “these light conditions”. But then, also the following part of the sentence doesn’t make sense to me. Who used these conditions to produce comparable data? You? Or the cited authors? But you say the data are comparable to data of these authors. So it is probably your data you talk about?! Then you should write: “however, while using these light conditions, we were able to produce data comparable to those presented in related published papers.”

Changed.

12. Finally, if I understand you right, I don’t see why the fact that you have comparable data as other others with the same conditions shows that these conditions are ok?! Maybe both your and other studies have unreliable results because by using insufficient light, you may have added an additional stress factor?! This eventuality should be discussed!

Ziegler and Uthicke, 2011 specifically indicate that photosymbionts of LBF acclimate very rapidly to different light leves in under 48 hours. This means that our 10 days acclimation is sufficient for them to adjust to the specific light level provided during the experiment. Furthermore, it is important to note that the light level is low in respect to the photosynthetic optimum and not other physiological functions of the holobiont. Based on our own experience of culturing *Amphistegina* in the lab for several years we can confirm that specimens look healthy (colorized, strong motility), and show substantial growth by producing new chambers in similar manner as field specimens.

2.2 Laboratory manipulative experiments

13. Line 77: “acclimated under constant conditions”: it is essential so say at what temperature!

This was all described in the supplementary but is now moved to the main text in the revised manuscript.

2.3. Results

14. Fig. 2b: no data for photosynthetic activity of *S. orbiculus* in winter. Why not? Explain in methods section! However, the caption of Suppl. Table S.3.2. mentions 3 groups!

This part has been excluded from the manuscript (see response to the main issue #2 in the first part of the letter).

15. Lines 115-119. “in the winter population, calcification decreases already after one week and is inhibited after three weeks”. This looks like an over-interpretation to me: in view of the overlapping error bars, I don’t think that the “week 1” values are statistically different for 25°C and 35°C! The supplementary table doesn’t inform us about this.

This part has been excluded from the manuscript (see response to the main issue #2 in the first part of the letter).

16. Fig. 3: I’m intrigued by the last line: “Abnormal measurement is marked as extreme and is not calculated as part of the average and error.” I would write “a single abnormal measurement, obtained after x weeks. . ...”. You have to add the info in which week this measurement was made!

Added to the main text and to the caption.

17. The regular text also describes this anomalous measurement and says “...as it is clearly damaged from sample handling”. I don’t see how inadequate sample handling can lead to such a value! I would simply not explain this single anomalous value.

This part is deleted.

Chapter 3.2. *Amphistegina*

18. Line 131 “Both calcification and photosynthesis responses remain synchronized throughout the experiment”. I don’t think you can say that. “Remain synchronized” means that there is an intrinsic interaction mechanism which explains why the responses of these two parameters are synchronous. “synchronised” is a wrong word. You should write: “are synchronous throughout the experiment”.

Changes as suggested.

19. Line 133: “calcification and photosynthesis were both inhibited”. However, calcification values are still slightly positive, so calcification doesn’t seem to be (entirely) inhibited!

Inhibited is replaced with "severely reduced"

20. Line 133 “and net photosynthesis was negative” That doesn’t add anything to “inhibited photosynthesis”. If you want to mention this, it should come BEFORE the conclusion of inhibited photosynthesis.

Since the first part of the sentence is now changed from "inhibited" to "severely reduced", it does add information about the result of this reduction.

Discussion

Lines 143-145: “Specifically, our results predict that with rising temperatures the relative contribution of *S. orbiculus* will increase since its calcification is not inhibited even at extreme temperatures, contrary to *A. lobifera*”.

I have three problems with this sentence:

21. (1) I would prefer when, before jumping to such a conclusion, you first briefly summarise the differences you found between the two species.

We moved the paragraph summarizing the differences between the species (this also refers to comment 24).

22. (2) Next, as said before, to me, the situation doesn't seem so "black-white" as you suggest (inhibition – no inhibition): for both species the calcification rate goes down at 35°C. It is true that the values go down much more for *A. lobifera*, but it doesn't become zero. If you think that a value of 10 µMol carbonate per individual per week means "no calcification", then you have to explain why!

Calcification rate values of 10 µMol carbonate is within the precision of the alkalinity measurements in this study. In fact, ±10 µMol is the maximum error of our results. However, we accept this comment since values might be slightly higher than zero and rephrased to - near inhibition.

23. (3) I think this conclusion goes much farther than you can go with your present results. I could imagine that a species no longer calcifies in the warmest month, but still survives these months without any major problems. Your observations only suggest that *A. lobifera* resists less well than *S. orbicularis* to high temperature. But that's not enough to go as far as you go, by concluding that in future, *Sorites* will progressively replace *Amphistegina*. In fact, temperature is one stress factor, but there may be others, which could covary with temperature, like salinity. Maybe the tolerance of the two species to raised salinity (or any other stress factor) is exactly the opposite?

We have adjusted the conclusions to better represent the scope and the significance of this study. Specifically, concerning the possible effect of salinity, our previous studies indicate that temperature is a much more prominent stressor than salinity (Titelboim et al., 2016, Kenigsberg et al., 2020). This is further supported by culturing experiment that is presently being carried out in our laboratory which is testing the separate and combined response of *A. lobifera* to elevated temperature and salinity. However, since this point is also true for other stressors and changes caused by future climate change, we have rephrased the conclusion.

24. Lines 159-166: there the authors summarise their main results. But this is way too late. This paragraph should already be inserted at line 143/144, before presenting the overly speculative final conclusion/suggestion.

We agree with this comment and moved this part to the beginning of the discussion.

25. Line 164 again mentions "inhibition of calcification", whereas the measure values are not zero. A more "nuanced" wording is absolutely necessary!

We agree, see response to comment 19 and 22 (inhibited is replaced with "severely reduced")

26. Line 166: "Moreover, the Symbiodinium symbionts clearly exhibit stress earlier than the host." True, that is to say, for the indicator you use, i.e., calcification rate. However, this may not be the best

indicator. Maybe the host would show stress just as early (or even earlier) if you would use another indicator (e.g., locomotion, feeding behaviour, reproduction, etc.). And finally, with symbionts showing signs of stress, it is hard to image the foram itself is not "feeling" signs of stress!

I simply want to underline that in my opinion you can't reduce the "well- being" of the foram to its calcification efficiency. This is only one element out of many others, which may not even be critical!

See response to comment 2.

27. Lines 166-67: "The different thermal sensitivity of the symbionts and host of *S. orbiculus*". Same remark here. You can't base your ideas on the "thermal sensitivity of the host" (= the whole holobiont) only on its calcification rate. I would say that the thermal sensitivity of *S. orbiculus* depends both on the thermal sensitivity of its calcification rate, on the thermal sensitivity of its symbionts and on the thermal sensitivity of many other of its life processes.

I think you should rather write: "The different thermal sensitivity of the calcification rate and of the symbionts of *S. orbiculus*".

Agree. We have changed this sentence as suggested.

28. Line 168-69: "Hallock et al., 2006b which (=who) suggested that the ectoplasm of bleached specimens is "preprogrammed" to continue calcification." → This sentence needs some more explanation!

This notion was given by Hallock et al., 2006b to explain their own observations on bleaching in *Amphistegina*.

29. Lines 169-70: "Our observation of *S. orbiculus* indicates that this discordance might be limited to a relatively short time after the bleaching". → I have no idea what you are talking about! What "discordance" do you mean? (probably the wrong word!). What can your observations on *S. orbicularis* tell us about bleaching? I'm lost! Please clarify!

We removed these sentences.

Conclusion

30. Lines 187-88 "Our study emphasizes the role of pre-exposure and acclimation processes in mitigating the effect of future warming." It is very strange to me that this point, which is only discussed very briefly at the end of the discussion, suddenly be- comes the main conclusion of your work!

The discussion regarding pre-exposure has been excluded from the manuscript (see response to the main issue #2 in the first part of the letter).

Response to comments by Reviewer 2:

General comments:

The study presented by Pinko et al. represents a comparative study of two different common LBF species, with different shell and symbiont types, exposed to elevated temperature over few weeks. The two main proxies assessed give insights into photosymbiont performance and holobiont health. Due to

subtle differences, the authors conclude that *Sorites orbiculus* will be less affected by climate change than *Amphistegina lobifera*. They also claim insights into distinct effects of preexposure to moderate temperatures regarding the LBFs thermal tolerance. Along the lines of former studies, the experiment shows that there are species-specific thresholds regarding temperature and duration of exposure, and that LBF from the Eastern Mediterranean, which are most likely Lessepsian invaders from the Red Sea, have a relatively high thermal tolerance. The further confirm that the photosymbionts seem to be the 'weaker' member of this symbiotic association, showing the earlier stress response. Hence, the study give further important proof of prior hypotheses on LBF thermal stress responses, and adds to the knowledge of species- dependent thresholds. Hence, I consider it important and valid to publish this data. However, the novel insights are limited, as I do not think that calcification can be considered as a host-specific response (as they suggest), and therefore this study does not assess the relative contribution of host and symbionts (see specific comments).

Specific comments:

1) Calcification cannot be considered as a host proxy, as it is largely influenced by photosynthesis. It is hence, as in many other studies, a holobiont proxy.

It is true that many key elements are involved in the biological machinery of the calcification process. Among these is the mutualistic partnership between the algal symbionts and the foraminiferal host. Nevertheless, calcification is a physiological trait preformed only by the foraminifera and thus present a direct proxy of its wellbeing (as in other calcifying organisms). This is based on a common observation that stress lowers the physiological activities that involves high consumption of energy. The same is true for photosynthesis, which is a physiological trait of the algal symbionts. Because of the exclusiveness of each parameter we have selected them to disentangle the complex relationship between the two components of the holobiont.

Since this issue was raised by both reviewers, we recognize the need for clarification in the paper and added an explanation of the rational to the text at the end of the introduction.

Although prior studies mostly used possibly less precise methods to assess growth (e.g. increase in surface area or buoyant weight in studies by Schmidt, Prazeres, Stuhr, Hallock and others), it basically gives similar information.

The Alkalinity anomaly method provides similar information to that produced by the measure of increase in surface area or buoyant weight. However, its main advantage is that it is much more accurate and thus presents an ideal experimental approach for detecting even subtle differences in performance under different treatments. This technique requires high level of analytical expertise and meticulous work measuring large number of samples (in order to replicate properly) but it can detect differences of even few single micromolars in carbonate production. Thus, this method is highly beneficial over the other common method.

Nearly all studies on LBF stress response assessed at least one holobiont parameter such as growth (often also others to get a better picture, as calcification / growth can be limited due to other factors that are independent of stress, hence, it is not a very good parameter anyways), and one or more photosymbiont parameters. The only study to my knowledge that actually managed to gain host-specific insights was Stuhr et al. 2018 (Scientific Reports) by differentiating between host and symbionts on the protein level. But even here, the influence of photosymbionts stress on host stress cannot be fully excluded.

It is true that stress of the symbiont will affect the host and vice versa. However, it is important to try and find indicators related to each of the component. Stuhr et al. 2018 identified differential expression in protein related specifically to host or symbiont. Under the same logic in this study we examine physiological activity only related to one of the components (calcification of foraminifera and photosynthesis of symbiont algae). The reviewer indicates that even in the data from Stuhr et al. 2018 the host is affected by symbionts stress but still doesn't exclude the fact that the stress is experienced by the foraminifera, we believe that the case is similar with our approach.

Due to the lack of novelty described above, I would suggest to the authors to focus more on the comparison between *A. lobifera* and *S. orbiculus*, and the detected differences in time-related responses (seasons and experimental duration), and emphasize these in more detail.

We changed the discussion to focus more on the comparison of the differences between the holobionts.

Furthermore, the methods section is very short and lacking a lot of details, descriptions etc., and many crucial information has unfortunately been moved to the supplementary materials. The same applies to some of the results, e.g. the statistics, which should be at least indicated in the text or the figures where significant. Even with the results provided in the supplementary materials, it is not possible to fully judge where statistically significant variation were detected due to the poor representation and lacking explanations.

We have added the information to the methods and results sections and the statistical indication of significance (test and p value) throughout the manuscript

I am also wondering why no further parameters were tested, and calcification and photosynthesis normalized by individual, which is very unusual and prohibits comparison with other studies.

Calcification rate and net oxygen production are quantitative very accurate parameters. As such, they were chosen for this study that aimed to recognize differences between treatments and between species. Normalizing these parameters by individual (as done by Evans et al., 2015, Titelboim et al., 2019) is meant to be more informative and more comparable than other ways of presenting this data that in many cases doesn't include the amount of foraminifera in the experiment or when normalized to mg is less specific because it can represent either more small specimens or less adult specimens that will have different growth rates.

It is also less precise if not all individual were of the same size (which they most likely were not).

Specimens were confined in size as mentioned in the manuscript in the method section. We have now added to the text the specific size fraction used for the experiments.

And I wonder, if you measured photosynthetic activity via oxygen production, why didn't you simply also measure respiration via oxygen consumption in darkness? This would have provided another valuable indicator for holobiont condition, and would have allowed to calculate gross oxygen production. So far, you only provide net values for photosynthesis, not considering that respiration is likely to be higher under high temperatures, which results in lower net photosynthesis, even though the actual production of oxygen may be constant.

Indeed. We have measured net photosynthesis. The use of this parameter clearly takes into account the fact that respiration lowers net photosynthesis. We understand from this comment that this was not clear and we adjusted the text to always say "net oxygen production/photosynthesis".

Lastly, I think this study could further be improved by discussing some interesting observational details such as bleaching (Did you observe it? In which species? Mottled or more gradual? Or mortality? Reproduction?) as well as by including time into the statistical evaluation of the results (e.g. two-factorial analysis of variance with time and temperature as factors).

There were no related observations of bleaching, mortality, reproduction etc (otherwise they would have been mentioned). This is in fact another indication that the parameters examined in this study are the most appropriate ones as their sensitivity can identify stress before a fatal response was reached. We added to the revised version additional statistical analyses, including two-factorial analysis of variance with time and temperature as factors.

Technical comments:

L2: "Roommate problems or successful collaboration?" this title sounds catchy, but the question is still as valid as before. . . I don't think the paper is resolving this question,

Following our response to comment 1 we believe that our title does describe the content of the paper and is not only valid but the formulation of it as a question actually emphasis the complexity of the relationship between the holobiont components.

-- and also the first part of the title is very broad (e.g. climate change includes more than just temperature stress) and should be a bit more specific

We except this part of the comment and changed the first part of the title to: "Foraminiferal holobiont thermal tolerance under future warming...."

L11: ". . . hyaline diatom-bearing *Amphistegina lob*ifera and the proceallaneaous dinoflagellate-bearing *Sorites* . . ."

Changed

L12-13: see discussion above

Changed according to the comments of reviewer 1

L16-L17: “future warming will significantly shift the relative contribution. . .” this is taking the implications way too far. You only see small differences in their response to 35◦C in respect of timing. Please be more specific (in general) and stick to what you actually show.

This was changed to "may" according to the comment by reviewer 1. Further, the word "significantly" was deleted to make the sentence less affirmative

L18-21: You mention pre-exposure for the first time here, and it is rarely described in the manuscript in general. What do you mean by this? The season? Or different acclimation temperatures? I also don’t understand why you suggest that it reduces thermal tolerance. Please reconsider these statements and adjust to your results and the discussion.

Following the review process, we have reconsidered this part of the manuscript and recognized that there are not enough evidences to make this case. Thus, we have decided to eliminate the results of the February experiment and the discussion regarding pre-exposure

L27-28: “. . . one of the regions most affected. . .”

Changed

L30: “Symbiont-bearing large benthic foraminifera . . .”

Changed

L34-35: To my knowledge, none of these studies really provided evidence for temperature control on symbionts composition. Some suggested that there may be a connection, but statements saying that they are “strongly controlled” would definitely require further proof, especially since other studies did show extremely flexible relationships (e.g. several Lee et al. studies, Pochon et al. 2007, Schmidt et al. 2016)

We have changed it to: "The symbiont composition of LBF was suggested to be controlled by temperatures"

L45: delete “calcifiers”

Deleted

L55-56: there are much earlier studies that describe these species in much more detail (such as Hansen & Burchard 1977 and Hottinger 1977) that deserve to be cited here

We added additional earlier references but we couldn’t find the ones specifically mentioned

L59: “. . . Israel, during. . .”

Changed

L60: the picked size fraction is crucial when it comes to assessing growth/calcification as it is strongly linked to ontogenetic phase. Hence, please provide this information in the manuscript and not in the supplemental materials.

Added

L62: the same accounts for the sample sizes. It is important to know in order to judge the power of the study.

Added

And ". . . 60-ml airtight. . ."

Changed

L 68: there is still a lot of detail missing regarding the culture conditions: was there water flow? What kind of water were they in? Were they fed? pH? . . .

There was no water flow, this is implicit from the description of airtight Erlenmeyer that are important for measurement of oxygen and alkalinity. We use natural sea water as indicated in line 88. We do not feed them because we use natural seawater filtered above 45 μm, that contain the foraminifera and symbiont algae nutrition. pH of water through the experiment was added to the results.

L65: Which temperatures? In the baths or the flasks? What means regularly? Once a week or once per hour would both be regularly but are very different.

The flasks are incubated in the water baths and thus the temperature of the water within the flasks is controlled by the water in the bath. These were monitored using HOBO data loggers that recorded temperature every 1 hour. In the manuscript, the word "regularly" is now replaced with this description.

L71: Why did you use calcification rate as the only parameter? Please explain.

February experiment is now eliminated from this manuscript because of the differences in methodologies.

L74: Same for: why did you only include *A. lobifera* for the spring experiment?

February experiment is now eliminated from this manuscript

L77: Which "constant conditions"?

These details were at the supplementary file but are now written in the manuscript itself

L79-80: How many samples didn't show oxygen production? And any suggestion why?

A total of three samples didn't show normal oxygen production (this was detailed in supplementary and now moved to the main text) and in any case the number of replicates per treatment didn't decrease below three. The word " apparent " was replaced with " similar values of net oxygen production as other samples" which describe the scenario better, we cannot speculate why this happened.

L81: How was temperature adjusted? What instruments did you use to control this?

The temperature is controlled by heating circulators with a thermostat and were adjusted manually.

L82: where are these temperatures expected? In the Med Sea? The Red Sea?

Added "in the Eastern Mediterranean"

L85: Was the water filtered? Pre-conditioned temperature-wise?

Water were filtered through 45μm to ensure nutrition for the holobionts but also reduce noise in the oxygen measurement. Other than that, the water was not treated.

L85-90: Please give more details on the method. Did you do this in the 60-ml flasks? What was the time frame? Were foraminifera pooled?

Water from all the Erlenmeyer flasks were replaced at the same time, immediately transferred to air-tight syringes, and then measured for their alkalinity and dissolved oxygen. Measurements of oxygen were conducted immediately and of alkalinity within the next two days. To ensure no changes in water properties accurse in this time frame standard material is measured before and after the first and last sample of the set, respectively. Foraminifera were kept inside the Erlenmeyer flasks throughout the experiment.

L91-93: Same details are missing here, as well as references. What instruments did you use? What light? Which temperatures? ". . . µg L-1 ..." and please define RDO.

This follows the same protocol as described in the last comment. We added the technical details of the sensor, fixed "µg L-1" and defined RDO in the text.

What was the accuracy value?

The accuracy of the optical dissolved oxygen sensor was better than ± 0.01 mg/L

And in general, you normalize both parameters by specimen. For better comparability, they are usually normalized by species size (given by surface area or weight etc.). Please consider doing so.

See response to specific comment 4

L95-97: Please give n for each parameter, treatment, time point, described which data got transformed, and which tests exactly got chosen "accordingly".

We added all of this information to the revised manuscript in 2.3 Statistical analysis as a summarizing table

L99: " . . .cases where normality . . . non-parametric Kruskal-Wallis test . . ."

Changed

L100: Please name the "proper" post-hoc tests.

This is also added to the summarizing table in section 2.3 statistical analysis in the revised manuscript

L106: you cannot say whether there would be differences in *A. lobifera* between winter and spring, but sounds as if you do. Please rephrase.

February (winter) experiment is now eliminated from this manuscript

L108: What is the "x" in your box plots? Please indicate significant differences by a letter report. And give n in captions. Also provide full species names and specify what the whiskers represent (SE?).

Figure and caption changed as suggested

L113f: Please avoid expressions like "much higher", "a decrease" or "substantially different". What does that mean? Please provide statistical test results and/or how big is the difference (twice as high, ~20 %...)

This sentence was deleted as part of the exclusion of the winter experiment however this comment was noted for the rest of the revised manuscript

L120: “The symbionts’ photosynthetic . . .”

The sentence is changed

and how is the “sensitivity pattern” different? Apart from one week in the 35◦C treatment, they look very similar to me.

This is what we mean. We rephrased it to: "indicates faster response than that presented by calcification rates"

L122: As mentioned before, please provide overview of statistical results here, ideally in figure.

Added

L123: If you mention this “abnormal value” please state in which way it was abnormal and why you suspect this to be related to handling. You say it’s not used for average and SE calculations. Does that also mean the further statistical analyses?

We added "abnormally high value" to indicate the way it is abnormal and considering the suggestion of reviewer 1 removed the statement about sample handling

Fig. 3: please jitter weeks more, so they are easier to recognize. In which week did you have the extreme value?

The graph is modified, and we also included the week of the extreme value in the legend and in the caption

L128: “significant negative response” I am not sure the word significant is used correctly here.

Removed the word "significant"

L134: “week” and what means “massive bleaching”? Please describe, give proportions etc.

This is added to the manuscript

L135-136: “... between 25◦C and 32◦C, and was thus clearly ...” As described in the general comments, please describe it more. What may be the reason for this bleaching? Were both species affected? *A. lobifera* could have a lower light tolerance than *Sorites* and could therefore bleach. Or since it only affected the lower temperature range, it could actually be related to reproduction. Why didn’t you exclude the bleached specimens from the analyses? I suggest to include the data anyways, e.g. only for comparison in the supplementary, or to conduct analyses on those samples that had no bleaching. Moreover, as the 35◦C had no bleaching, please provide at least these values to compare with former weeks.

As suggested, this is added and discussed in the revised manuscript

L142-143: Well, I think “clear differences in thermal tolerance” is a bit exaggerated. I would call them rather subtle.

We deleted this as part of the first reviewer comments

L144: This is not true, the calcification seems lower at 35◦C (significances missing), at least in the winter populations. Plus, the experimental exposure in spring was shorter, so the response may have just been delayed, as suggested by the reduced oxygen production.

As mentioned before, we excluded the winter experiment since it was done in slightly different conditions than the spring experiment. Furthermore, the difference between the species is not just in the magnitude but also in the timing. *Sorites orbuculus* reduced calcification only after the second week, which indicate it is more resilient than *A. lobifera*. It is true that this is a short-term experiment and therefore (and following the comment of reviewer 1) had changed the sentence to be less affirmative.

L147: please rephrase, something here doesn't make sense. And I again don't agree that you can state that there is a "strong dependence", as many other factors have been shown to be at least as important. Please also include some newer references here.

Rephrased and added a newer reference

L149&L161: "dinoflagellates" and change to "Symbiodinium" to Symbiodiniaceae (as you seem to be aware this taxonomic system has been revised)

Changed

L153: ". . . control a holobionts ..."

Changed to "... control the holobionts..."

L154-155: What "mechanism to cope with thermal stress was observed"? Please describe, I do not think this paper actually showed 'shuffling'.

Rephrased, observed was changed to suggested. We deleted 'shffuling' and better explained the finding of Schmidt

L156: ". . . explain the observation . . ."

Changed

L159: ". . . describes ..."

Changed

L164: "... 35◦C, whereas in ..."

Changed

L165: ". . . inhibited, indicating that it is. . ."

The sentence was completely changed following comment by reviewer 1

L166: That means you refer to *Sorites* only? When is "earlier"?

Does this refer to old line 162? If so, yes this refer to the symbionts of *Sorites*. Earlier, is from the first measurement after one week contrary to calcification that only decrease after the second week. This is now clarified in the text.

L168: An apparent higher sensitivity (earlier/stronger response of the symbionts than the holobiont) was also observed by other studies such as Prazeres et al. 2017, Stuhr et al. 2017, Schmidt et al. 2016

References added

L170: Here you mention bleaching again: so were these specimens that you measured calcification on already bleached? If there was, this may also indicate that there was another stress factor such as too high light intensity or the wrong light spectrum (e.g. Hallock once showed that blue light facilitated high growth rates but at the same time led to bleaching). Please discuss your observations.

No, these were not bleached. However, the decrease in photosynthesis is considered as a negative response of the symbiont algae and thus compared to the observation made by Hallock regarding bleaching. We added clarification of this in the text.

Light intensity in the experiments is not a factor causing bleaching since for *Amphistegina* this light was used before in published (Titelboim et al., 2019) and unpublished experiments. For *S. orbiculus*, this is not the case since through the 3 week experiments no bleaching was observed.

L172: The resolution is a very important point! One week is a long time for a foraminifera! Plus, so far I don't even know how long your calcification measurements or photosynthesis measurements took. They don't calcify continuously all day long, so the time frame may strongly influence the results. The same accounts for the photosynthesis, which varies over the time of the day.

Indeed, the weekly resolution limits our ability to identify changes in a shorter-term time interval. However, the aim of this study is not to quantify changes in the daily cycle of calcification and photosynthesis but to examine the response over longer time intervals (measurement per week).

The description of handling time and measurement is not relevant to this point and hopefully this was clarified in the response to comment on lines 85-90, and also in the text in the method section.

L174: Please specify the time of the onset.

This part was deleted with the discussion on pre exposure

L175: What do you mean by "very cold"? I think that is very relative... give a tem- perature range of what is usually encountered in the Med Sea in winter and spring, and ideally state what were the temperature measured during sampling in the methods section.

This part was deleted with the discussion on pre exposure

L178: ". . . symbionts, or both. However, while the *A. lobifera* spring . . . "

L180: In which way did they respond "negatively"? Please be a bit more specific so the reader does not have to go back to each of the studies you cite to find you what you mean, and "... that, while ..."

This part was deleted with the discussion on pre exposure

L181: ". . . temperatures benefits the . . ."

This part was deleted with the discussion on pre exposure

L186-187: There have been studies modeling the future changes in distribution and hence contribution, which should be cited here (e.g. Weinmann et al. 2013, 2017)

References added

L187: If you mention pre-exposure here (as in the abstract) please elaborate a bit more in which way they had different pre-exposures in the methods as well as the discussion parts.

Pre exposure is no longer discussed in this manuscript

L188-189: I don't understand this statement at all. Why? Where is you evidence for that?

This part was deleted with the discussion on pre exposure

L189: Again, I find "clearly shows" a bit exaggerated.

"Clearly" is deleted

Supplement 1: Is the number of replicates given the value before or after exclusion of same samples? Why are the numbers different for the different time points? How did you deal with this unbalanced design in your statistical analyses?

This has been moved to the main text of the revised manuscript. Regarding the unbalanced design: an unequal sample size is only a problem if it validates the homogeneity of variance assumption. Since ANOVA is considered robust to some departures from this assumption it is common to use it even if the number of samples is not similar in all treatments. Specifically, this is not an issue with our data since this assumption is valid with high significance in all cases.

You used once filtered and once unfiltered water. Why? And why did you pre- condition the spring *Sorites* to another temperature than the rest? This is very crucial information and must not be excluded from the actual manuscript! I am not sure if you can compare your data the way you do with all these differences.

Considering these differences, February experiment and the comparison between spring and winter populations was excluded from the manuscript. Each species was pre-conditioned to its specific optimal temperature.

Sometimes you give two numbers after the comma, sometimes six or other. . . please be consistent (and usually its three).

The supplement file was replaced, and this comment was followed in the new file.

Why do you give four stars (they are actually called asterisk)? Usually, these are used to indicate the level of significance, from one (lower end of significance) to three (highly significant).

Since this presentation of results is not clear we changed it to include p-values between all treatments

Why is some text red? Please explain in captions.

Significant results are marked in red. This is added to the captions of the new supplement

Table S2.2 and others: What are "1, 2 and 3" in your column headers? I cannot under- stand your statistical results if I don't know what is which group.

The number of the group is indexed in the left part of the table marked with { }. We believe and hope that this clarifies it.

Table S5.2: “Stars indicate homogenous groups and thus significant differences be- tween them”? This makes no sense to me, because if they are homogenous, they are similar, so no difference. . .

As mentioned in previous comments, since this presentation of results was not clear we changed it to include p-values between treatment

Foraminiferal holobiont thermal tolerance under future warming ~~climate change~~- Roommates problems or successful collaboration?

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Abstract

Understanding the response of marine organisms to expected future warming is essential. Large Benthic Foraminifera (LBF) are symbiont bearing protists considered to be major carbonate producers and ecosystems engineers. We examined the thermal tolerance of two main types of LBF holobionts characterized by different algal symbionts and shell types (resulted from alternative biomineralization mechanisms): The hyaline diatom-bearing *Amphistegina lobifera* and the porcelaneous dinoflagellate-bearing *Sorites* ~~*hyaline diatom bearing, Amphistegina lobifera, and the porcelaneous dinoflagellate bearing, Sorites*~~ *orbiculus*. In order to assess the holobiont thermal tolerance we separately evaluated foraminiferal calcification rates and symbionts net photosynthesis ~~To assess the relative contribution of host and symbiont algae to the holobiont thermal tolerance we separately evaluated their response by measuring calcification rates and photosynthetic activity~~ under present-day and future warming scenarios. Our results show that both holobionts exhibit progressive loss of life functions between 32ºC and 35ºC.~~thermal resilience up to 32ºC and sensitivity to 35ºC.~~ This sensitivity differs in the magnitude of their response: calcification of *A. lobifera* was drastically reduced ~~completely inhibited~~ compared with *S. orbiculus*. Thus, future warming may ~~will~~ significantly shift the relative contribution of the two species as carbonate producers. Moreover, *A. lobifera* exhibited a synchronous ~~synchronized~~ response of calcification and net photosynthesis~~the host and symbionts~~. In contrast, in *S. orbiculus* the symbionts decreased net photosynthesis prior to calcification. This implies that algal symbionts are limiting the resilience of the halobiont. ~~possibly limiting its resilience. Our results also demonstrate the role of pre-exposure and acclimation processes of host, symbionts or both in mitigating future warming. It highlights the possibility that while pre-exposure to moderate temperatures benefits the holobiont, in cases of extreme temperature it might reduce its thermal tolerance.~~

1 Introduction

Since the beginning of the industrial revolution anthropogenic activity has been leading to rapid ocean warming. This negatively affects marine ecosystems and specifically symbiont bearing calcifiers (Kawahata et al., 2019). The observed rate of global Sea Surface Temperature (SST) rise stands on 0.11°C per decade and future scenario predicts a similar rate until the end of the century (IPCC, 2014). Therefore, the Mediterranean can be presented in biogeographic models as a "miniature ocean" providing indications on global patterns in marine ecosystems in a warmer world (Lejeusne et al., 2010). Warming in the Eastern Mediterranean is expected to rise almost four times more rapidly than global forecast (Macias et al., 2013). Thus, the Eastern Mediterranean is expected to be one of the regions most affected ~~one of the most affected regions~~ by global warming. ~~Therefore, the Mediterranean this area can be presented in biogeographic models as a "miniature ocean" providing indications on global patterns in marine ecosystems in a warmer world (Lejeusne et al., 2010).~~

Symbiont-bearing Large Benthic Foraminifera ~~Symbiont bearing Large Benthic foraminifera~~ (LBF) are single-celled ecosystems engineers. Their carbonate production is estimated as at least 5% of the annual production in reef and carbonate shelf environments (Langer, 2008; Langer et al., 1997). Temperature is a major factor in the distribution of LBF that exhibit distinct thresholds for reproduction, survival, bleaching, and calcification (Evans et al., 2015; Hallock et al., 2006a; Langer et al., 2012; Langer and Hottinger, 2000; Schmidt et al., 2011; Titelboim et al., 2019; Weinmann et al., 2013). The symbiont composition of LBF was suggested ~~appears~~ to be ~~strongly~~ controlled by temperatures (Momigliano and Uthicke, 2013; Prazeres, 2018; Prazeres et al., 2017; Prazeres and Renema, 2019; Schmidt et al., 2018) which explains the observation that species-specific thermal tolerance is associated with more diverse algal symbionts (Stuhr et al., 2018).

Many LBF species are Lessepsian invaders, which often comprise over 90% of the foraminiferal population in the Eastern Mediterranean (Hyams-Kaphzan et al., 2014; Titelboim et al., 2016). Their invasion and successful establishment are facilitated by rising temperatures, as in the case of other Lessepsian organisms (Por, 1978, 2010; Zenetos et al., 2010, 2012). However, some of these species currently live very close to their upper thermal thresholds and consequently, their presence will be impeded in the relatively near future (Titelboim et al., 2016). The thermal sensitivity of some LBF species has already been investigated (Schmidt et al., 2011, 2016b; Stuhr et al., 2018; Titelboim et al., 2019). Yet, the relative contribution (positive or negative) of the holobiont components ~~of the host and symbiont algae~~ to cope with rising temperatures has not been fully constrained.

In this study, we present the thermal sensitivity of two very dominant and prominent LBF holobiont systems (Fig. 1). Specifically, our study separately assesses the thermal sensitivity of the foraminiferal host calcification rate and algal symbionts net photosynthesis ~~by tracking their calcification rate and photosynthetic activity~~ as an indication of their well-being under different warming scenarios ~~(Fig. 1)~~. This approach was chosen since calcification is a physiological activity done only by the foraminifera and thus presents a proxy to its wellbeing (like many organisms, when stressed lowering physiological activities that involve~~s~~ high consumption of energy). The same is true for photosynthesis ~~and algae, this is a physiological activity only possible by the symbiont algae. Photosynthesis is~~which is a primary life process and thus present an efficient

indicator for the tolerance of the symbiont algae. Because of the exclusiveness of each parameter we ~~use them to~~could use them ~~try and~~to disentangle the complex relationship between the two components of the holobiont.

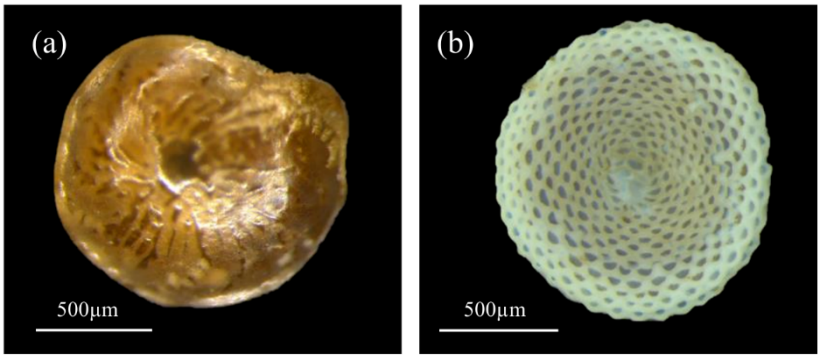

Figure 1: The holobionts examined in this study. a) *Amphistegina lobifera* and b) *Sorties orbiculus*. Note the green-brownish color of the symbiont algae.

2 Materials and methods

2.1 Specimens collection and handling

In this study, we targeted two LBF species that represent different types of holobiont systems, which differ in their shell construction mechanism and algal symbionts: *Amphistegina lobifera* (diatom bearing hyaline, Larsen, 1976, Prazeres et al., 2017; Schmidt et al., 2015, 2016b) and *S. orbiculus* (dinoflagellate bearing porcelaneous, Merkado et al., 2013; Pawlowski et al., 2001; Pochon et al., 2014). Both species have cosmopolitan distributions, are very common in warm shallow marine environments (Langer and Hottinger, 2000) and display different thermal tolerances (Titelboim et al., 2016). Specimens were picked from macro-algal samples that were scraped from beach rocks at Shikmona, northern Mediterranean coast of Israel ~~during at~~ February and May 2019. To reduce variance in growth derived from ontogenetic variability, the specimens were picked between the specific size fractions of 750-1000 µm~~(see details in Supplement 1 Table S1)~~. Live specimens (indicated by their symbiont color and motility) were cleaned by brushing, divided into groups with an equal number of of specimens (40 *S. orbiculus* and 30 *A. lobifera*)~~individuals (details in Supplement 1 Table S1)~~, and transferred into 60-ml~~60 ml~~ airtight Erlenmeyer flasks filled with natural seawater filtered to 0.45 µm, from here on referred to as 'samples'.

~~During the experiments, the samples were placed in temperature-controlled water baths, which maintained constant temperatures of ± 0.5°C, temperatures were monitored using HOBO data loggers that recorded temperature every one hourregularly. During the cultivating period, the samples were kept under a daily cycle of 12 hours light / 12 hours dark using fluorescent light of ~ 30 µmol photons m^{-2} s^{-1}. These are lower than the photosynthetic optimum for *A. lobifera* (Ziegler and Uthicke, 2011), However, using these light conditions, we were able to produce data comparable to those presented in related~~

~~published papers (Schmidt et al., 2016b, 2016a, 2018; Titelboim et al., 2019). These light levels should not cause stress since LBF acclimate rapidly to different light leves (in under 48 hours, Ziegler and Uthicke, 2011) and thus the 10 days acclimation is sufficient for them to adjust to the specific light level provided during the experiment.however, they were used to produce comparable data to that of related published papers (Schmidt et al., 2016b, 2016a, 2018; Titelboim et al., 2019).~~

2.2 Laboratory manipulative experiments

We conducted temperature manipulative experiments on *S. orbiculus* and *A. lobifera*. In these experiments, the well-being of both holobionts was examined by separately determining the responses of the foraminiferal calcification rate and symbiont algae net photosynthesis to elevated temperatures. These are both very accurate quantitative parameters. As such, they were chosen for this study that aimed to recognize even subtle differences between treatments and between species.

During the experiments, the samples were placed in temperature-controlled water baths, which maintained constant temperatures of ± 0.5ºC, temperatures were monitored using HOBO data loggers that recorded temperature every one hour. During the cultivating period, the samples were kept under a daily cycle of 12 hours light / 12 hours dark using fluorescent light of ~30 µmol photons m^{-2} s^{-1}. These are lower than the photosynthetic optimum for *A. lobifera* (Ziegler and Uthicke, 2011), However, using these light conditions, we were able to produce data comparable to those presented in related published papers (Schmidt et al., 2016b, 2016a, 2018; Titelboim et al., 2019). These light levels should not cause stress since LBF acclimate rapidly to different light levels (in under 48 hours, Ziegler and Uthicke, 2011) and thus the 10 days acclimation is sufficient for them to adjust to the specific light level provided during the experiment.

~~We conducted temperature manipulative experiments on *S. orbiculus* and *A. lobifera*. The temperature manipulative experiments were conducted separately on specimens collected during February and May 2019 (i.e. winter and spring populations). The February 2019 experiment examined only the calcification rate of *S. orbiculus*. Next, we conducted another experiment on *S. orbiculus* in May 2019 to examine possible variation in thermal tolerance between winter and spring populations (following Schmidt et al., 2016a). The temperature manipulative experiments on *A. lobifera* were conducted only on the spring population that was sampled in May 2019. In this these experiments, the well-being of both holobionts was examined by separately determining the responses of the foraminiferal calcification rate and symbiont algae net photosynthesisforaminiferal hosts (calcification rate) and their symbiont algae (photosynthetic activity) to elevated temperatures. These are both very accurate quantitative parameters. As such, they were chosen for this study that aimed to recognize even subtle differences between treatments and between species.~~

All samples were acclimated under constant conditions for at least ten days. ~~(~~Acclimation ~~T~~temperatures were ~~the~~ optimal ~~temperature~~ for each species: 27ºC for *S. orbiculus* and 25ºC for *A. lobifera* and other conditions are as described in 2.1~~)~~. Then, the calcification rate ~~(February and May populations)~~ and net photosynthesis~~photosynthetic activity (May populations)~~ were measured to establish the performance baselines of the different species and the natural variability between samples, under equal conditions. Two samples (one *A. lobifera* replicate from 25ºC treatment and one *S. orbiculus* replicate from 30ºC) did not exhibit similar values of net oxygen production as the majority of samples and were excluded from the rest of the study

to avoid bias. ~~Samples that did not exhibit apparent photosynthetic activity (i.e. oxygen production) were excluded from the rest of the study.~~ At the end of the acclimation period, seawater was replaced in all samples and the temperature of each bath was slowly adjusted (1°C/hour). The examined treatments (25°C, 30°C, 32°C, 35°C) represent current and future temperatures expected in the Eastern Mediterranean until the end of the century (Macias et al., 2013). Each temperature treatment included four replicates unless reduced to three following low performance of the symbionts (*A. lobifera* 25°C and *S. orbiculus* 30°C). ~~Each temperature treatment included three to five replicates (Supplement 1 Table S1).~~

After acclimation, ~~the samples were exposed to the designated temperatures for a total duration of three (May experiment) or four (February experiment) weeks. After~~ following each week, the water was replaced with fresh natural seawater with verified pH of 8.0-8.1 and salinity of 38.4-39.2. ~~-~~The replaced water from all the samples was transferred to air-tight syringes and then all oxygen samples were immediately measured~~.~~ ~~alkalinity~~Alkalinity ~~samples~~measurements ~~-~~were conducted over the next two days. To ensure no changes accurse in this time frame standard material was measured before and after the first and last sample of the set, respectively. Calcification rates (µmol $CaCO_3$ $week^{-1}$ $specimen^{-1}$) were calculated using the Alkalinity ~~Anomaly a~~Anomaly ~~Method m~~Method (Smith and Key, 1975). In this method, the calcification rate is determined from the change in total alkalinity of the seawater caused by the precipitation of $CaCO_3$. These are determined by comparison to a control sample containing no foraminifera. Accuracy was assessed by analyses of the Scripps Institute of Oceanography reference seawater (~~Batch 154, February and~~ Batch 180~~, May~~) and an internal standard. Calcification rate involves high energetic consumption and as such is drastically influenced by stress levels of a calcifying organism and was specially shown to be related to thermal stress in benthic foraminifera (Evans et al., 2015, Schmidt et al., 2016b, Titelboim et al., 2019).

~~Photosynthetic~~ Net photosynthesis ~~activity~~ (ΔO_2 µg L^{-1} ~~-~~$specimen^{-1}$) was measured as net oxygen production compared with a control sample containing no foraminifera. Dissolved oxygen was measured using Eutech DO 450 connected to a Rugged Dissolved Oxygen (RDO) sensor. ~~RDO optical dissolved oxygen sensor.~~ Accuracy was assessed by calibration of the sensor against Winkler titration. ~~Photosynthesis is a primary life process and thus present an efficient indicator for the tolerance of the symbiont algae.~~

2.3 Statistical analysis

To examine whether differences in calcification rates and net ~~photosynthetic~~ photosynthesis ~~activity~~ are significant between temperature treatments and between weeks, statistical analyses were performed using STATISTICA10 software. For each set of data, we tested assumptions of normality of the residuals and homogeneity of variances and a statistical test was chosen accordingly. If both assumptions were valid ANOVA was performed, in cases where ~~were~~ normality was valid and homogeneity was violated Welch's ANOVA test was applied. In cases where ~~were~~ normality was violated the non-parametric test Kruskal-Wallis was applied. Each was followed by the proper post-hoc test. All statistical analysed are summarized in table 1.

Table 1: description of all statistical analyses conducted in this study including which statistical test was preformed, if data was transformed, and the number of samples in each data set.

Data		Description	Number of samples	Statistical analysis
Baseline	Calcification rate	Comparison between *S. orbiculus* and *A. lobifera* after acclimation period	*S. orbiculus*: 15 *A. lobifera*: 14	1-way ANOVA on log-transformed data
	Net photosynthesis	Comparison between *S. orbiculus* and *A. lobifera* after acclimation period	*S. orbiculus*: 15 *A. lobifera*: 15	1-way ANOVA on log-transformed data
Sorites orbiculus	Calcification rate	Comparision between 4 temperatures and 3 weeks	15	2-way ANOVA and Tukey HSD test
	Net photosynthesis	Comparison between 4 temperatures	Weeks 1&2: 15 Week 3: 14	Kruskal Wallis test and Multiple comparisons
		Comparison between the 3 weeks		1-way ANOVA
Amphistegina lobifera	Calcification rate	Comparison between 4 temperatures and 2 weeks	15	2-way ANOVA on log-transformed data and Tukey HSD test.
	Net photosynthesis	Comparison between 4 temperatures and 2 weeks	15	2-way ANOVA and Tukey HSD test
	Third week bleaching of *A. lobifera*	Comparing the number of bleached specimens between 4 temperatures	15	1-way ANOVA on log-transformed data and Tukey HSD test.

3 Results

Our experimental design takes into consideration biological variability in calcification rates and net photosynthesis ~~photosynthetic activity~~ between different species ~~and populations~~. This notion is based on previous observations that different species even from the same genus, and different populations of the same species display different calcification rates under the same conditions (i.e. baseline, Titelboim et al., 2019). Specifically, ~~Indeed,~~ among our experiments the activity baseline of both calcification and net photosynthesis ~~the hosts and the symbionts~~ are significantly different between *A. lobifera* and *S. orbiculus* ~~and between winter and spring populations~~ (One-way ANOVA: p value < 0.001, Fig. 2, Supplementary Tables ~~Supplement 1 Tables S2~~ S1 and ~~S3~~S2). Hence, the thermal tolerance of the two holobionts was separately evaluated for each experiment.

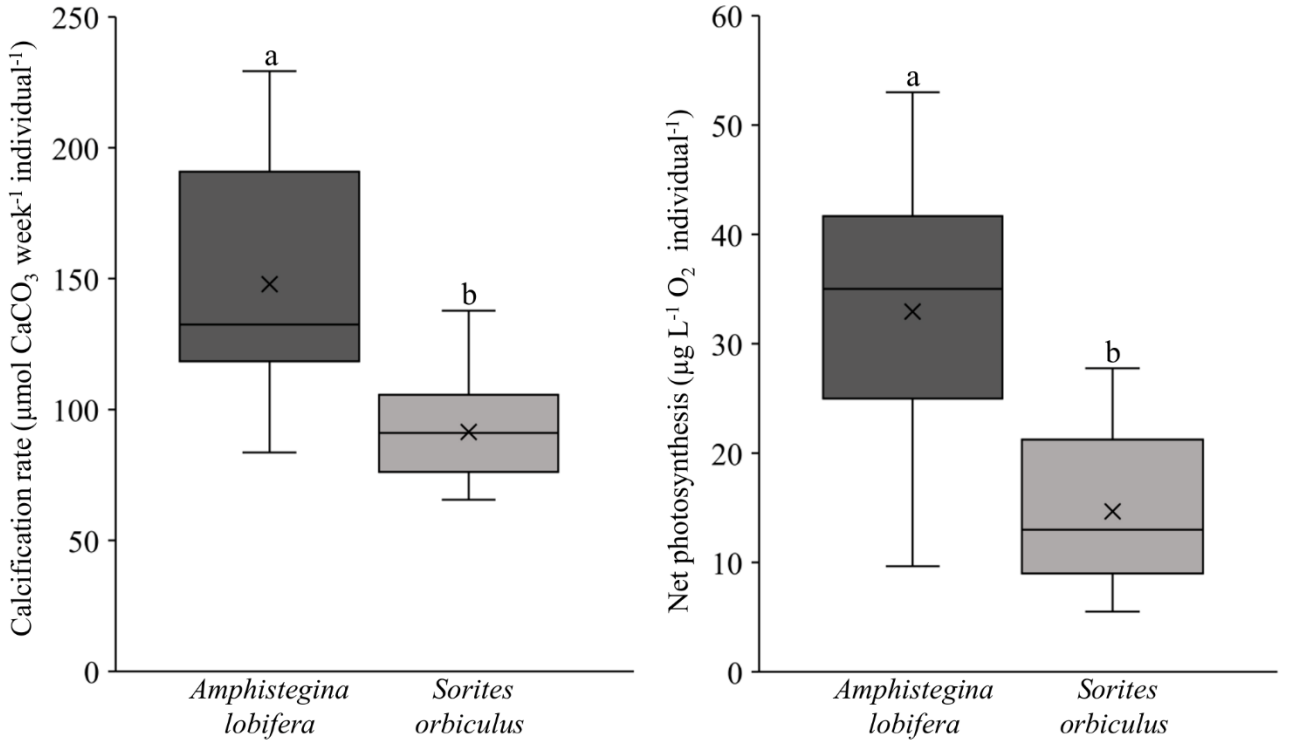

Figure 2: Activity baseline of the ~~hosts~~ foraminiferal calcification rate (left) and symbionts net photosynthesis (right) of *A. lobifera* (n = 14, 15) and *S. orbiculus* (n = 15, 15) ~~indicated by calcification rates (left) and photosynthesis activity (right), respectively~~. Note, the ~~distinct~~ significant differences in baseline values of both calcification rates (p <0.001) and photosynthetic activity (p <0.001) between the two holobionts ~~and between winter and spring populations of *S. orbiculus*~~. Error Bars represent minimum and maximum values.

3.1 *Sorites orbiculus* (porcelaneous- dinoflagellate holobiont system)

Calcification rates of ~~the~~ *S. orbiculus* ~~spring population are much higher than those of the winter population indicated both in the baseline measurements (Fig. 2) and in the experiments (Fig. 3). Comparison between their calcification responses~~ under the different temperature treatments exhibited ~~reveals overall similar trends of~~ highest values at 25°C, 30°C, and 32°C. A mild not statistically significant ~~a~~ decrease was observed at 35°C ~~. The negative response at 35°C is substantially different between the populations: in the winter population, calcification decreases already after one week and is inhibited after three weeks (Fig. 3). Whereas in the spring population the calcification rate is reduced only , and then remains low, but is not inhibited~~ (Fig. 3, ~~for statistical analyses,~~ Supplementary Tables ~~see Supplement 1 Tables S4~~ S3.1 and S3.2). ~~The symbionts' n~~Net photosynthesis ~~indicates a~~show ~~faster and clearer response than that presented by calcification rates. Symbionts photosynthetic activity of the spring population indicates different thermal sensitivity patterns than that of the host. Throughout the experiment~~ already from ~~week 1 and with no differences between the weeks (one-way ANOVA, p-value = 0.66, Supplementary Table S4.1) ,~~ p show positive values ~~were observed~~ under 25°C, 30°C, and 32°C. At 35°C, net photosynthesis was negative ~~and gradually decreased~~

during the experiment (Fig. 3, for statistical analyses, see Supplementary Table S4.1 and 4.2Supplement 1 Table S6). Unaccountably, in week 3 one sample exhibited an abnormal high value (i.e. extreme in Fig. 3) with respect to previous weeks as well as to other replicates and thus was not included in the average and error calculations -nor in the statistical analysisas it is clearly damaged from sample handling.

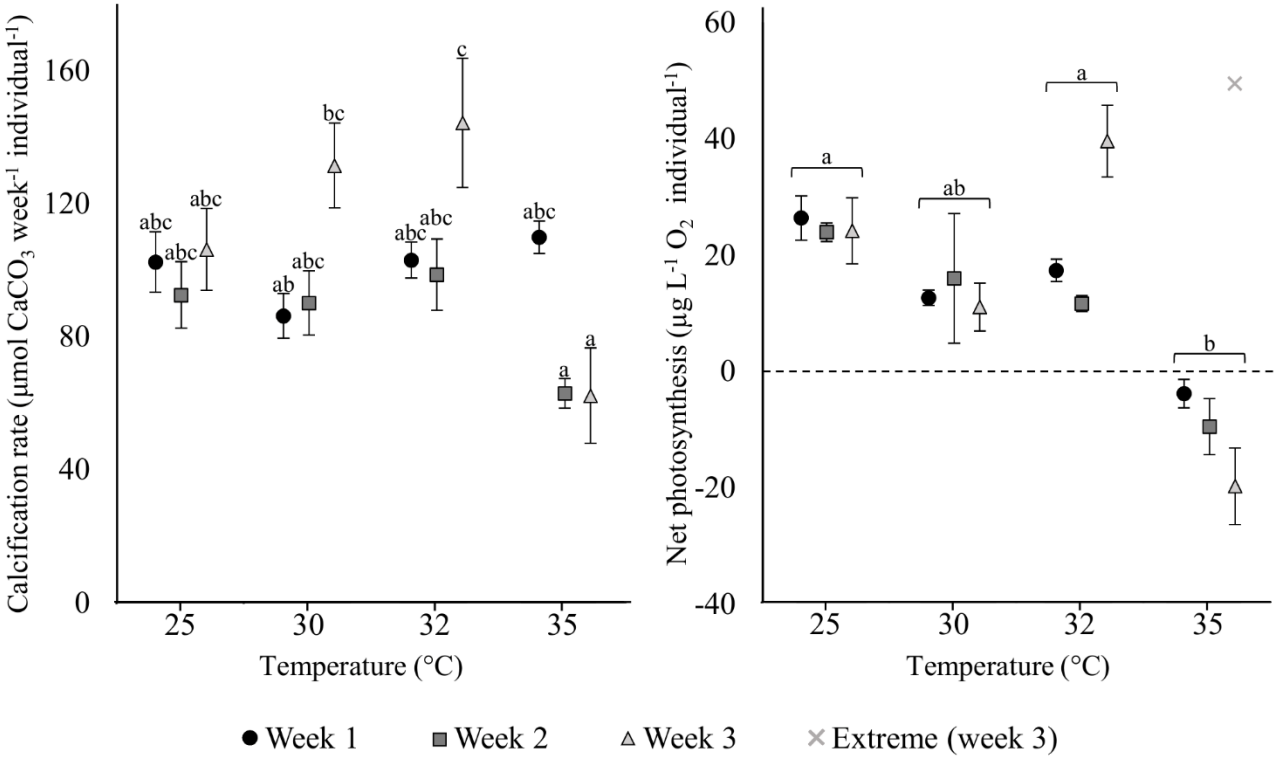

Figure 3: Calcification rates (left) (a, b) and net photosynthesis photosynthetic activity (eright) of *S. orbiculus* winter population (a) and spring population (b, c). Letters represent the results of athe post hoc tests (Supplementary Tables S3.2 and S4.2). Error bars are SE. Note, the significant negative response of host and symbionts at 35°C (a-c) with the exception of the spring population calcification rate at week 1 (b). A single abnormal measurement, obtained at week 3 Abnormal measurement is marked as extreme and is not calculated as part of the average and erroraverage, error, and statistical analysis.

3.2 *Amphistegina lobifera* (hyaline diatom holobiont system)

Both calcification and net photosynthesis are synchronous responses remain synchronized throughout the experiment. After the first and second weeks, calcification rates and net photosynthetic photosynthesis activity exhibited the highest values under 25°C, 30°C, and 32°C. At 35°C calcification and net photosynthesisphotosynthesis were both severely reduced inhibited and

net photosynthesis was negative (Fig. 4, Supplementary Tables S5 and S6~~Supplement 1 Tables S7 S5and 8S6~~). Between the second and third weeks, many specimens exhibit massive bleaching that occurred in different treatments between 25°C-32°C in similar proportions (Tukey HSD post-hoc test, p values> 0.1, Supplementary Table S7.2) and thus was clearly not related to the different temperature. Bleaching in the 35°C treatment did not exceed 2 specimens per replicate (Supplementary Table S11~~1~~). For this reason, measurements of the third week are excluded from the results.

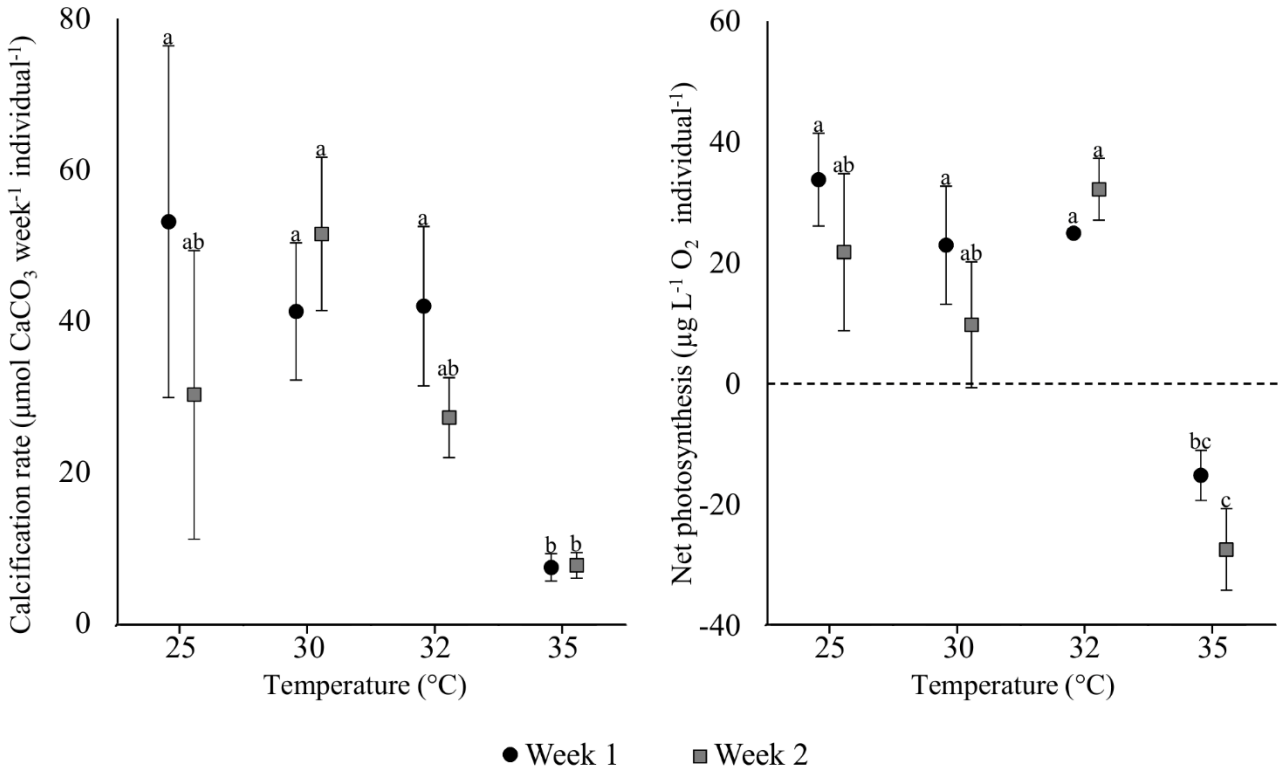

Figure 4: Calcification rates (~~a~~left) and net photosynthesis ~~photosynthetic activity~~ (~~b~~right) of *A. lobifera* ~~spring population~~. Note the synchronous ~~synchronized significant~~ negative response ~~of both host and symbiont~~ at 35°C. Error bars are SE, and letters represent the results of the post hoc tests between temperatures and weeks (Supplementary Tables S~~7.2~~5.2 and ~~8.2~~S6.2).

4 Discussion

~~*Amphistegina lobifera* and *S. orbiculus* are both considered as prominent calcifiers based on their massive occurrences and widespread distribution (Langer and Hottinger, 2000). Our study reveals clear differences in their thermal tolerance as expressed by both calcification rates and their algal performance. Specifically, our results predict that with rising temperatures the relative contribution of *S. orbiculus* will increase since its calcification is not inhibited even at extreme temperatures,~~

~~contrary to *A. lobifera*. This highlights the need for species-specific considerations for more accurate predictions on the fate of LBF and their future contribution to global carbonate production.~~

Our study separately describes the thermal sensitivity of the foraminifera and the algal symbionts in two types of holobiont systems: *A. lobifera* hosting diatoms mostly from the order Fragilariales (Barnes, 2016; Prazeres et al., 2017; Schmidt et al., 2016b, 2018) and *S. orbiculus* hosting dinoflagellates, Symbiondiniaceae (Merkado et al., 2013; Pawlowski et al., 2001; Pochon et al., 2007). Both species are considered as prominent calcifiers based on their massive occurrences and widespread distribution (Langer and Hottinger, 2000) and both record a graduate decline in physiological performance between 32ºC to 35ºC (Figs. 3-4). Both holobionts show thermal resilience up to 32ºC and a negative response at 35ºC (Figs. 3 and 4). Yet, they differ in respect to the magnitude of their responses: *A. lobifera* and its diatom symbionts share similar thermal sensitivity with near inhibition of calcification and negative net photosynthesis at 35ºC, whereas in *S. orbiculus* calcification is less dramatically reduced at 35ºC, indicating it is more resilient to extreme SST than *A. lobifera*. Moreover, the Symbiondiniaceae symbionts exhibit stress earlier (already after the first week) then calcification that was not reduced at the first week and only slightly reduced after. The different thermal sensitivity exhibited by calcification rate and by symbionts of *S. orbiculus* imply that they might be a limiting factor for the host to cope with future warming. A similar apparent discordance was previously observed in *Amphistegina* (Prazeres et al. 2017, Stuhr et al. 2017, Schmidt et al. 2016 and Hallock et al., 2006b). Hallock et al., 2006 suggested that the ectoplasm of bleached specimens is "preprogrammed" to continue calcification. Possible explanations for the synchronized response of the *A. lobifera* holobiont in this study are either 1) similar thermal sensitivity of the symbiont and the host or 2) the weekly resolution of measurements may not capture a short discordance time between the responses of the symbiont and host.

It was previously shown that corals ability to cope with elevated temperatures is ~~strongly dependent on~~ related to their partnering with functionally diverse symbionts (Baker et al., 2004; Howells et al., 2012; Jones et al., 2008; Poquita-Du et al., 2020; Rowan, 2004)~~,~~ although their symbiosis is limited to dinoflagellate from the *Symbiondiniaceae* ~~*Symbiodinium*~~ *"*Clades*"* (LaJeunesse et al., 2018; Silverstein et al., 2015). LBF are known to host different kinds of symbionts (Pochon et al., 2007), which include dinoflagellates, diatoms, unicellular chlorophytes, unicellular rhodophytes and/or cyanobacteria (reviewed in Lee, 2006). Whereas the general types of the symbiont (algal genus) seem to be phylogenetically fixed, there appears to be considerable flexibility in symbiont infestation, even within one individual (Lee, 2006). This versatile symbiont partnership may control the holobionts thermal tolerance and provide one of the key factors in their response to future warming. For example, a mechanism to cope with thermal stress was suggested ~~observed~~ in *Pararotalia calcariformata*, an extremely heat tolerant symbiont bearing foraminifera, that host a diverse ~~by 'shuffling' of~~ symbiont community of diatoms. In case of thermal stress, functionally relevant members of the symbiont community can become more dominant and magnify the ability

of the holobiont to tolerate elevated temperatures~~communities~~ (Schmidt et al., 2018). This might also explain ~~an~~ the observation that species-specific differences in the thermal tolerance of *Amphistegina* species are ~~related~~ correlated to different symbiont assemblages. Specifically, a larger diversity of algal symbionts was associated with the more tolerant species (Stuhr et al., 2018).

~~Our results also reveal different thermal tolerance of the two *S. orbiculus* populations demonstrated by the onset of their response to 35°C. The main difference between the two populations is the pre-exposure to the very cold winter in the Eastern Mediterranean compared with much more moderate spring temperatures (Schmidt et al., 2016b; Titelboim et al., 2016). The substantial effect of seasonal pre-exposure on the thermal tolerance of a population demonstrates the existence of acclimation processes of the host, the symbiont or both. However, while *A. lobifera* spring population exhibited sensitivity only to 35°C, a previous study that examined the thermal tolerance of a summer population indicates that the latter negatively responded already to 30°C (Titelboim et al., 2019). These observations highlight the notion that while pre-exposure to moderate temperature benefit the holobiont, in cases of extreme temperatures (cold or warm) it might reduce its thermal tolerance. In the context of ocean warming, this implies that while acclimation may mitigate some increase in SST, pre-exposure beyond a certain threshold will most likely reduce the thermal tolerance of LBF.~~

Conclusions

Considering the role of LBF in the carbon cycle and as ecosystem engineers, their future with expected warming is a major concern. Previous study modelled the predicted changes in the distribution of LBF and their contribution to carbonate production (e.g. Langer, 2008; Langer et al., 2013; Weinmann et al., 2013; Weinmann and Langer, 2017). ~~Moreover,~~ However, our results highlight~~s~~ the need for species-specific considerations for more accurate predictions on the fate of LBF.

~~the relative carbonate production of different LBF species is presently not equal and rising temperatures will most likely change their relative contribution. Our study emphasizes the role of pre-exposure and acclimation processes in mitigating the effect of future warming. It implies that with expected rising SST exceeding certain thresholds, pre-exposure to extreme temperatures will have a negative influence on thermal tolerance.~~ Our study ~~clearly~~ shows that LBF have different thermal tolerances that are limited by the sensitivity of their eukaryotic algal symbionts. Considering recent findings on the significant role of the prokaryotic microbiome on the physiological performance of LBF (Prazeres, 2018; Prazeres et al., 2017), it will be highly valuable also to explore in future studies their specific contribution to the thermal tolerance of the holobiont~~.~~.

Data availability

All data related to the manuscript is available in the Supplement ~~2~~.

Author contribution

The study was designed by D.T. and D.P. Sampling and culturing experiments were carried out by D.P and D.T. using facilities provided by S.A.; Interpretation of data and writing of the manuscript were done by all authors: D.P., D.T., and S.A.

Competing interests

The authors declare that they have no conflict of interest.

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