Response to reviewer comments

Symbiotic nutrient cycling enables the long-term survival of Aiptasia in the absence of heterotrophic food sources

Comments by the reviewers are indicated in **black.**
Responses by the authors are indicated in **blue.**
New and revised manuscript sections are indicated in **green.**
Line numbers refer to the revised bioRxiv submission.

Recommender:
UC:
Thank you for submitting your preprint article "Symbiotic nutrient cycling enables the long-term survival of Aiptasia in the absence of heterotrophic food sources" to PCI Ecology.
Following careful assessment of your submission, two expert reviewers feel that it has potential for recommendation, so we would like to invite you to revise the paper following their comments.
Please see the attached reviewer comments for further details about necessary revisions.

A: Dear Ulisse,
we were delighted to see the interest expressed by the reviewers in our manuscript. As you will see from the responses below, we were able to incorporate all their suggestions. Most notably these changes include:

- reanalysis of NanoSIMS data to achieve a balanced experimental design
- correlative SEM + NanoSIMS imaging to inform on the nature of enrichment hotspots in the host tissue
- extended information on experimental design and replication

We believe that integrating the reviewers' positive and constructive feedback greatly enhanced the quality of our manuscript. We hope that our manuscript is now acceptable for recommendation in PCI Ecology and we look forward to your decision.

Sincerely,
Anders & Nils

Referee #1:

R1: The study by Rädecker and Meibom aims at understanding nutrient cycling in food deprived Cnidarian-Symbiodinium holobionts using the Aiptasia model. It combines stable isotope probing and NanoSIMS to provide direct evidence of enhanced C assimilation and similar N assimilation in symbionts between 1 year fed and unfed Aiptasia. Indirect evidences suggest more efficient C translocation from symbionts in artemia deprived holonbionts.
The paper is clear and easy to read.

A: We would like to thank the reviewer for their interest in our study and their constructive feedback. Please see below for a detailed point-by-point response.

R1: The title reflects the content of the article. The introduction provides a good background on the importance of trophic plasticity in corals with appropriate references. The objectives and general findings are clearly stated.

A: Thank you.

R1: The method used seems appropriate for the study however I do not have sufficient expertise in statistics to evaluate the *Statistical analyses* paragraph (lines 155 to 161). The Materials and methods section provides sufficient details for replication by others.

A: Thanks. Please note that in line with recommendations of Reviewer 2 we improved on the way NanoSIMS data are collected from the images to achieve a fully balanced study design. This did neither change the findings nor their interpretation, but it strengthened the analyses involved. To facilitate reproducibility of statistical results for the reader, the Zenodo repository submission further includes the R script with all analyses.

R1: *Animal husbandry & experimental design (line 84 to 98)* How many animals were kept in each batch and how many survived the one-year starvation?

A: Thanks for raising this important point. We started the experiment with 10 individuals in each of the treatments and no mortality was observed the experiment (with the exception of the loss of some individuals during the weekly cleaning due to human error).
This information is now included in the Material and Methods and Results sections, respectively.

L90: “However, while half of the animals (two culture containers with five animals each) were fed weekly with *Artemia* nauplii (regularly fed control), the other half (two culture containers with five animals each) was reared in the absence of any food sources (heterotrophically starved).”

L171: “After one year of husbandry in the absence of heterotrophic food sources, no mortality was observed and Aiptasia remained viable but had ceased any detectable asexual propagation via pedal lacerates.”

R1: Line 106; It mentioned that the centrifugation parameters are the same for both treatments. Is there a possibility that symbionts from unfed Aiptasia have different densities compare to symbionts from fed Aiptasia? If so, using the same centrifugation parameters in both treatments might not allow the author to capture all the symbiont fraction. Did the author check the host fraction to make sure it doesn’t content symbionts?

A: Indeed, given the differences in algal ultrastructure their density is likely different between treatments (note that we now describe these ultrastructural changes in the revised version of the manuscript). However, we assessed the host supernatant for algal symbiont contamination to ensure that algal symbionts were removed efficiently. Across samples, at least 95 % of algal symbionts were pelleted by centrifugation. Hence, even if changes in ultrastructure would have an effect on symbiont quantification, they cannot explain the pronounced differences reported here.
The manuscript now states, L102: "Host and algal symbiont fractions were immediately separated by centrifugation (3000 g, 3 min, sufficient to remove > 95 % of algal symbionts from the supernatant)…"

R1: Please clarify which controls were used for Stable Isotope probing and NanoSIMS.
A: We apologize that this important information was missing in the earlier version of the manuscript. To ensure the accuracy of NanoSIMS measurements and to normalize enrichment levels for labelled samples, we used Aiptasia that were kept under identical conditions but did not receive any heavy isotope label during the incubation. These unlabelled controls were processed in parallel to the labelled specimens using the same workflow.

This is now clarified in the material and methods of the manuscript.
L120: "In addition, one additional animal per treatment was transferred into a vial filled with minimal artificial seawater medium without heavy isotope tracers to serve as unlabeled controls for NanoSIMS measurements and normalization."

R1: Please describe the number of ROI as well as ROI selection process for C data as it done in the following reference: Rädecker N, Raina J-B, Pernice M, Perna G, Guagliardo P, Kilburn MR, Aranda M, Voolstra CR (2018) Using Aiptasia as a model to study 345 metabolic interactions in cnidarian-Symbiodinium symbioses. Front Physiol 9:214
A: Thanks, we have now revised our ROI analysis to achieve a balanced statistical design as suggested by Reviewer 2. The materials and methods section now clearly describes sampling and replication of ROIs. Likewise, figure legends have been updated to include the level of replication for NanoSIMS data.
L152: "^{13}C and ^{15}N assimilation was quantified by drawing regions of interest (ROIs) of individual algal symbionts and surrounding host gastrodermis based on $^{12}C^{14}N^-$ maps. For this, algal symbiont ROIs were drawn by outlining individual algal cells and host ROIs were drawn in a circle with a 15 µm diameter around the centroid of the algal symbiont whilst excluding any algal symbionts and symbiosome content from the ROI. For unlabeled control Aiptasia, 80 host and 80 algal symbiont ROIs were analyzed across two animal replicates. For isotopically labeled Aiptasia, 120 host and 120 algal symbiont ROIs were analyzed across three animal replicates per treatment."

R1: The results are well-explained and are presented in appropriate format.
A: Thanks for the encouraging feedback.

R1: Please include data from the unlabeled controls in the raw data.
A: The raw data now include ROI data for unlabelled controls and Figure S2 further shows correlative SEM and NanoSIMS analysis of unlabelled samples.

R1: Line 186-187: The authors claimed that 13C assimilation within the host was primarily observed in lipid bodies however as there is no TEM correlation in this study, there is no evidence that structures labeled as lipid bodies on fig 2 A and B are lipid bodies. It would be useful if the author could provide the TEM correlated image or explain how they came to this labeling. In fact, those structures could just be symbionts appearing smaller because of the sectioning plane.

A: Valid point. We have now included correlative SEM and NanoSIMS images for fed and starved Aiptasia, clearly showing that enrichment hotspots correspond to host lipid bodies. Please refer to revised Fig. 2 and Fig. S2.

R1: Fig. 2 A and B: The scales are different (0.0105 to 0.0400 vs 0.105 to 0.400). I assume it is a mistake and it should read 0.0105 to 0.0400 on both scales. Please correct or explain otherwise.

A: Thanks for spotting this. Indeed, this was an error during figure formatting and has been corrected now.

R1: Fig 2 B, C and D: Please include number of replicates and ROI in the legend

A: Done.

L217: "120 regions of interest across three Aiptasia replicates were analyzed per symbiotic partner and treatment combination."

R1: Line 221-222: The author stated "translocation of photosynthates by algal symbionts remained sufficient to maintain the basal metabolic requirement of the host." Could the author specify number of Aiptasia in each batch at the end of the experiment? Knowing how many individuals survived the experiment would help support their claim. Also, in view basal metabolic requirement implies cell growth. Do the authors have any evidence of cell growth in the system that would support their claim?

A: Thanks, we agree that this is important information to include. The manuscript no clarifies that "two culture containers with five animals each" were used for each treatment and that "no mortality was observed" during the experiment. Unfortunately, we have no way to assess host cell growth in the present study. However, NanoSIMS images clearly show that starved hosts maintained active anabolic ammonium assimilation. Further, the revised manuscript now includes SEM images (Fig.S2) that reveal an increase in lipid bodies in the gastrodermis of starved Aiptasia. Taken together, we are confident that our findings support the notions that the host metabolism of starved Aiptasia was unlikely limited by photosynthate availability in the present study.

R1: Line 341-342: reference is incomplete :...118 (5)

A: The article number is now included.

Referee #2:

R2: The manuscript by Rädecker and Meibom reports on a long-term starvation experiment of the sea anemone Aiptasia, an emerging model system for the study of cnidarian-Symbiodiniaceae symbioses. Aiptasia is a promising symbiosis model system. Aiptasia are small mixotrophic anemones which feed on free-living prey (and maybe particulate or dissolved organic matter??) and on photosynthate transferred from their algal endosymbionts. It can be reared and manipulated in the laboratory and can be used to improve our understanding of Cnidarian-Symbiodiniaceae symbioses. Among other reasons, studying this model is relevant because ultimately it may be used to tackle important basic biology questions about economically, ecologically and evolutionary relevant ecosystems, such as coral reefs which are based on similar symbiotic interaction with Symbiodiniaceae. With their long-term experiment, the authors aimed at studying the relative contributions to nutrition from the symbionts and from external sources.
The mains conclusions of the manuscript are:
1. The contribution of symbiont derived nutrients to the host metabolism remained unaffected by long-term starvation due to an increase in algal photosynthesis and more efficient carbon translocation.
2. Feeding on external prey "is not essential to fulfilling the energy requirements of the holobiont on a one-year timescale"
3. Feeding on external prey "is a critical source of nitrogen required for holobiont growth under oligotrophic conditions".

Overall, I found the manuscript relatively clear, the model and the research questions interesting, however, I think the authors may be overinterpreting their data, some essential controls are not provided, and the number of replicates is very low. The conclusions are based on a very small number of replicates, only three anemones were analyzed in each batch. Although I understand that NanoSIMS is not high throughput, for dry weight, symbiont cell count and protein content, however more than three individuals could have been analyzed easily.

A: We would like to thank the Reviewer for this encouraging and constructive feedback. We agree that more replicated for the physiology would have been desirable. However, the cultures used for the experiment here were initially intended for a different experimental purpose, which is why we only started with 10 animals per treatment to begin with. Only once we noticed the pronounced phenotypic changes in starved animals, we decided to turn these cultures into their dedicated experimental comparison as presented here. Due to the low starting number of animals (and the loss of some individuals during weekly cleaning), we were limited with the number of replicates available for the measurements presented here.

The initial number of animals in each treatment is now included in the materials and methods section of the manuscript (L90).

R2: A small terminology comment: It would be okay to talk about the autotrophic versus the heterotrophic nutrition for the symbiosis but throughout the manuscript, the authors refer to symbiont derived photosynthate as *autotrophic nutrients* for the host and to external food sources (i.e. Artemia) as *heterotrophic nutrients*. In my opinion, that this is inadequate and confusing. How do you define an autotrophic or heterotrophic nutrient? In both cases the nutrients are organic compounds and the Aiptasia host feeds on them heterotrophically.

Some external prey in the environment may be autotrophs, heterotrophs, particulate organic matter, or dissolved compounds therefore *heterotrophic/autotrophic nutrient* is, I think, not a good way to go.

A: Valid point. We agree that this terminology, while common in the coral field, lacks precision. We have thus revised the manuscript and now refer to the incorporation of "algal-derived nutrients" or "photosynthates" when referring to the host metabolism.

R2: A concern I have is the technique used to quantify the symbiont density. The authors homogeneized the host, then centrifuged the homogenate and considered the supernatant as the "host fraction" and the pellet as the "symbiont fraction". Is this an established and validated method in Aiptasia? Without any reference for this method or any form of control provided by the author, it is hard to believe that this is a reliable way to separate host and symbiont. Was the supernatant (considered "host fraction") also analyzed by the CellDrop cell count? This would be an easy and essential control to check whether the host fraction was really symbiont free. Similarly, was the pellet observed under light microscopy to confirm that it is comped of symbiont cells only?

The reason I'm pointing this out is that these data are essential to conclusion #1 and the starvation may be inducing some changes in the symbiont cells as shown in some papers (Bedgood et al 2020; Ladriere et al 2008). The 80% symbiont density could be in part due to a technical bias. If the symbionts in starved host have smaller size and/or density they may not pellet as much. Another easy way to better support the drastic reduction of symbiont density would be to add micrographs of histological staining (Toluidin blue) of semi-thin section from both treatments. A 80% reduction in symbiont density would be quite obvious.

A: Thanks, this aligns with a comment by Reviewer 1 above. Please note that only animal tentacles were processed for resin embedding. Hence, we cannot corroborate any conclusions on whole-body symbiont densities based on semi-thin sections. However, all host supernatants were inspected for algal contamination using the CellDrop system. In all samples at least 95% of algal symbionts were successfully pelleted by centrifugation. While this does not completely rule out treatment-related biases, the effects cannot explain he pronounced differences observed here. Combined with the visual paling of starved animals (Fig. 1). We thus have no doubt that starvation reduced the density of algal symbionts.

The manuscript now clarifies, L102: "Host and algal symbiont fractions were immediately separated by centrifugation (3000 g, 3 min, sufficient to remove > 95 % of algal symbionts from the supernatant)…"

R2: Regarding the NanoSIMS ROI analysis, why is the number of ROI so different between host (Fed: n=25, Starved n=22) and symbiont (Fed n=144, starved n=140)? This can affect the outcome of the statistical tests.

A: We agree that a balanced experimental design is desirable. We have thus revised the sampling strategy of NanoSIMS ROIs to achieve an even number of host and algal symbiont ROIs across treatments. Notably, while this enhanced the power of the statistical analysis, the results and their implications remain identical to the previous version of the manuscript.

L152: "^{13}C and ^{15}N assimilation was quantified by drawing regions of interest (ROIs) of individual algal symbionts and surrounding host gastrodermis based on $^{12}C^{14}N^-$ maps. For this, algal symbiont ROIs were drawn by outlining individual algal cells and host ROIs were drawn in a circle with a 15 µm diameter around the centroid of the algal symbiont whilst excluding any algal symbionts and symbiosome content from the ROI. For unlabeled control Aiptasia,

80 host and 80 algal symbiont ROIs were analyzed across two animal replicates. For isotopically labeled Aiptasia, 120 host and 120 algal symbiont ROIs were analyzed across three animal replicates per treatment."

R2: Figure 2B has a different scale (10 folds higher in starved host) than 2A, I suppose this is an error because the box plot data do not show a higher enrichement in the starved host. Please, correct or comment on this.
A: Apologies, indeed this was an error during figure formatting that has been corrected now.

R2: Line 150 the authors mention a unlabeled control but no data are provided, could the author add these data?
A: Absolutely, ROI data of unlabelled controls have been included in the Zenodo submission and Fig. S2 now includes a correlative SEM+NanoSIMS analysis of unlabelled control animals.

R2: Line 222: "Indeed, patterns of host 13C enrichment (Fig. 2A-C) were not affected by heterotrophic starvation indicating that photosynthate availability for the host was not impaired". The current NanoSIMS data provided in the manuscript do not show a clear 13C enrichment in the host above that of the natural abundance of 13C. Tu support this, the authors need to provide the ROI data of the unlabeled control, and ideally an aposymbiotic host control incubated with 13C-bicarbonate as well (to control for host incorporation of inorganic C via anaplerosis, see recent papers from Harald R. Gruber-Vodicka here and here). At the very least, the unlabeled control should be added to the Fig.2.
A: We agree that resin infiltration contaminated the host tissue with 'unlabelled' carbon. Yet, individual ^{13}C hotspots are clearly evident in the host tissue. As such, Figure 2 shows that all host ROIs have a positive ^{13}C atom % excess and are thus more enriched that unlabelled animal (which are used for the data normalization). Hence, there is no doubt that host tissues are enriched in ^{13}C in both treatments compared to unlabelled animals. To further corroborate this, we have now included Fig. S2 to facilitate a direct comparison of NanoSIMS images and data between labelled and unlabelled Aiptasia.

R2: Line 184-186: "13C enrichment from 13C-bicarbonate assimilation/translocation was highest in the algal symbionts with host 13C enrichment primarily observed in lipid bodies". What makes you think that these are lipid bodies? Lipids are not preserved by glutaradehyde or paraformaldehyde fixation (OsO4 fix lipids) and therefore they are washed away during samples dehydration and result in "empty vacuoles" on resin section. In absence of TEM correlation, how can you make confirm that these enriched area are not tangential sections of enriched symbiont cells?
A: Apologies, this was an error on our side. Of course, the samples underwent secondary fixation in OsO_4 (which is now clarified in the materials and methods). As such, lipid bodies were preserved in the sample and could be easily distinguished from algal cells based on their high carbon and low nitrogen counts.
To further verify this notion, we have now included an additional figure correlating NanoSIMS images with SEM images of the same region to show that enrichment hotspots correspond to electron-dense lipid bodies in the host tissue (see Fig. 2).
L195: "…host ^{13}C enrichment primarily observed in localized hotspots corresponding to lipid bodies in the correlative SEM images…"

R2: The authors have kept 2 batches of Aiptasia anemone in the lab for one year. One batch was regularly fed with Artemia while a second batch relied solely on the nutritional contribution of their photosynthetic symbionts for nutrition. The study clearly shows that at least some Aptasia survived and maintained their association with their nutritional symbionts even after one year of diet restriction. This seems to be relevant information on its own and would benefit from being discussed against similar starvation experiments from the literature. However, the authors should provide the total number of Aptasia individuals at the beginning and at the end of the experiment in the two batches. How many anemones survived after 1 year starvation, how many died? How does that compare to the batch fed with Artemia? This essential to know in order to draw the conclusion #2

A: We agree that the survival rate is important to evaluate the effects of starvation here. In the present study, we did not observe any mortality in either treatment (except for accidentally flushing animals away during the weekly cleaning). The absence of treatment-related mortality is now highlighted in the manuscript:

L171: "After one year of husbandry in the absence of heterotrophic food sources, no mortality was observed…"