THE ROYAL SOCIETY PUBLISHING

PROCEEDINGS B

Microplastic exposure interacts with habitat degradation to affect behaviour and survival of juvenile fish in the field

Mark I. McCormick, Douglas P. Chivers, Maud C. O. Ferrari, Makeely I. Blandford, Gerrit B. Nanninga, Celia Richardson, Eric P. Fakan, George Vamvounis, Alexandra M. Gulizia and Bridie J. M. Allan

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Review timeline

Original submission:	17 April 2020
1st revised submission:	12 August 2020
2nd revised submission:	25 September 2020
Final acceptance:	6 October 2020

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

Review History

RSPB-2020-0876.R0 (Original submission)

Review form: Reviewer 1

Recommendation
Major revision is needed (please make suggestions in comments)

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

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

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

Is the length of the paper justified?
Yes

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

Do you have any concerns about statistical analyses in this paper? If so, please specify them explicitly in your report.
Yes

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

Is it accessible?
Yes

Is it clear?
Yes

Is it adequate?
Yes

Do you have any ethical concerns with this paper?
No

Comments to the Author
By considering both habitat degradation and microplastic exposure, the paper provides insights into the potential consequences of combined pressures on the behaviour and survival of juvenile reef fish. The researchers clearly identify the motivation for the study, test their research question using a reasonable experimental design and statistical analyses, and present strong evidence for an influence of the two stressors (and especially microplastic exposure) on fish behaviour and survival. We found the paper generally clear and easy to follow.

We felt a key weakness of this study was the lack of information on the microplastic concentrations these fish may be exposed to in the wild, which therefore makes it difficult to put the results into context. The authors did a good job of providing some information on what is known about microplastic concentrations in different parts of the ocean, but they also note microplastics have seldom been quantified in tropical waters, and never at their study site. It's unfortunate that even preliminary data on microplastic concentrations near where these fish were caught are not available. We appreciate that the authors seemed to use conservative amounts of microplastics in this study, but there is no way to know that these concentrations are actually conservative given the lack of data. This lack of data also means it is unknown whether microplastic exposure is even a problem for coral-reef fish at the end of their larval phase. Relatedly, the inclusion of supplementary feeding studies was useful to help address these concerns, but we were confused about some of those methods when reading the main text, including the sample sizes and the concentration of microplastics.

Please see below for additional specific comments, especially for places where the methods and results could be clarified, along with some suggestions to provide more explicit links between the stressors, observed behaviours and mortality to make the story even more compelling.

Line-by-line comments:

Introduction
Line 50-51: Whilst the combination of habitat degradation and microplastics may be novel, there has been a lot of recent research on the combined effects of multiple stressors more generally. As a starting point, there are two meta-analyses from 2008 in Ecology Letters focused purely on the effects of multiple stressors (Crain et al., Darling and Côté), plus many more studies since then (including some using microplastics or habitat degradation – some of which are cited in the Discussion). In addition, either here or elsewhere in the introduction, it could be beneficial to

include some information on previous fish behavioural research, addressing both habitat degradation and microplastic pollution, given that the focal point of the study seems to be behaviour as well as the combination of two environmental stressors.

Line 53-54: This line also seems a bit overstated. Experiments may not be the only way to determine the magnitude of multi-stressor interactions, and they may not truly inform us of the trajectory of future communities (because there are many more than just two stressors operating at a time, and species respond differently to different stressors, which will then affect their interactions). Please consider re-wording and/or providing references for these statements.
Lines 70-88: This paragraph could be condensed whilst still retaining the key points. For example, Lines 74-76 do not add any additional information to the prior sentence 'Plastic waste has been generated…' so could be removed.

Methods
Lines 101-110: This paragraph is perhaps too detailed for the main text, given that none of the microplastic concentrations provided are directly relevant to the study site, or even to coral reefs more generally. Consider either summarising this information more concisely, or placing a table in the supplementary materials reporting the previous study locations and concentrations/sizes of microplastics found. In addition, stating the concentration of microplastics used in this study would be beneficial, as currently it's difficult to determine how the reported concentrations align with what was used in the study.

Line 159-160: Could you tell if the food remaining was Artemia, microplastics, or both? Also, what do "beakers" and "jar" refer to – do these just mean tank, since there's been no previous mention of anything except tanks?

Line 163-167: The supplemental feeding studies are a nice addition, but as it's written it was not clear that these results are from different fish than those used in the main experiment (Line 313 is the first direct reference to supplementary studies, rather than just supplementary files). Please clarify whether the fish used in the supplemental studies were from the same collections as the ones used in the main experiment, and the sample sizes (number of individuals) for each.
Also, it appears that the supplemental studies used different concentrations of microplastics than used in the main experiment (or at least, the fish in the supplemental studies were only exposed to microplastics, and not microplastics + Artemia). This point should be made explicit in the main text, as the reported ingestions may not be representative of what the fish used in the main experiment ingested.

Line 175: Does placing the fish individually mean there was only one fish on each reef? Please be explicit whether there was only one individual fish per reef at a time.

Line 176-180: Some more details about the patch reefs would be helpful. For example - when were the patch reefs created (how old are they – which could influence any other chemical/visual cues from the live versus dead or degrading corals) and how complex are the patches (which could influence how susceptible the fish are to predators)? How many patch reefs were there, and how many in each treatment? For the dead-degraded corals – did they die naturally, how long had they been dead, and were they structurally intact (which may influence behaviour)? How were all fishes and invertebrates removed from the corals?

Line 194: Please clarify how boldness was assessed over the 3-min observation period prior to the pencil test.

Line 207-208: Did you ever find a tagged fish on a different reef?

Results & Figures
Lines 236-244: A reminder of what the behavioural index means could be helpful – so high

behavioural activity is associated with high bite rate, high movement, high boldness, etc.

Figure 1/S3: Whilst figure 1 clearly presents the behavioural index included in the PCA, Figure S3 gives a simple but clear overview of the individual behavioural trends, so maybe should be included in the main article (e.g., by making Figure 1 a 5-panel figure). Because the Discussion repeatedly refers to individual behavioural traits (e.g., lines 259-263, 269, 295-296, etc.), it would be useful to have the results for these individual behaviours in the main text rather than just the supplement.

Table S1: I apologise if this is naïve as I'm not familiar with the partial eta squares metric, but what does it mean that the effect size for the random effect of tank is larger than any other effect size? To me, this suggests that tank had a bigger influence on behaviour than plastic or habitat, which would be concerning.

Discussion
Line 266-275: This part was a bit unclear to me. For example, it makes sense that mortality is constrained to 100%, but is the behavioural index constrained too (relevant to the ceiling-truncation hypothesis)?

Line 277-280: Consider changing both uses of the word "will" to "may", given that 1) the amount of microplastics that these fish are naturally exposed to is unknown and 2) this study did not test the resilience of coral reef communities.

Line 287: Without any data on microplastic concentrations near coral reefs, it's still unclear that these are "realistic" concentrations.

Line 296-297: Some elaboration on the links between the behavioural metrics and survival would be useful (either here or elsewhere in the Discussion). From the results, there is not a clear mechanism linking these two. Is it possible to look at the correlation between high activity and hours survived (across the different treatments)? Showing that individuals that exhibited high activity died fastest (or died at all), for example, would provide convincing evidence that the observed behaviours were linked to survival.

Also, it seems that a major assumption is that any loss observed was due to predation-induced mortality. Elaborating on a few points related to this would be useful. 1) Were any predation events observed and/or any predators observed in the area of the reefs? When was loss rate highest (morning, afternoon, evening, or night), and does this correspond to higher predator activity levels? 2) How does the fact that there were no resident predators on the experimental reefs influence the interpretations of these results? For example, are the predators of these damselfish (listed on Line 125-127) resident or transient? It seems like some of the observed behaviours (e.g., staying farther from shelter) could increase exposure to transient predators, but may be useful in avoiding resident predators (see, for example, Hixon and Carr 1997 Science). So maybe on actual reefs the differences in mortality across treatments would not be as large?

Line 333: Some elaboration on why fish would exhibit higher activity levels after being exposed to microplastics, especially if the plastic is no longer in their system, would be useful.

Line 346: As the closing paragraph, a closing reference to the findings of the study could be included here.

Review form: Reviewer 2

Recommendation
Major revision is needed (please make suggestions in comments)

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

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

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

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No

Comments to the Author
I am happy for my 'additional comments to the editor' to be forwarded to the authors

Decision letter (RSPB-2020-0876.R0)

28-May-2020

Dear Dr McCormick:

I am writing to inform you that your manuscript RSPB-2020-0876 entitled "Microplastic exposure interacts with habitat degradation to affect behaviour and survival of juvenile fish in the field" has, in its current form, been rejected for publication in Proceedings B.

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

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

Please find below the comments made by the referees, not including confidential reports to the Editor, which I hope you will find useful. as you will see, there is recognition that your manuscript is both timely, and potentially advances our understanding in an important field. however, there are some fundamental issues requiring your considered attention. Details are provided below. Amongst these, are the demonstration of ecological relevance of the plastic beads, and in particular the extent to which weathered beads differ in their properties (which is highly unlikely), and also whether weathering was analagous with natural systems where microplastics will spend varying durations exposed to marine conditions. If you do choose to resubmit your manuscript, please upload the following:

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

To upload a resubmitted manuscript, log into http://mc.manuscriptcentral.com/prsb and enter your Author Centre, where you will find your manuscript title listed under "Manuscripts with Decisions." Under "Actions," click on "Create a Resubmission." Please be sure to indicate in your cover letter that it is a resubmission, and supply the previous reference number.

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

Reviewer(s)' Comments to Author:
Referee: 1
Comments to the Author(s)
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Referee: 2
Comments to the Author(s)
I am happy for my 'additional comments to the editor' to be forwarded to the authors

Author's Response to Decision Letter for (RSPB-2020-0876.R0)

See Appendix A.

RSPB-2020-1947.R0

Review form: Reviewer 1

Recommendation
Accept with minor revision (please list in comments)

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

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

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

Is the length of the paper justified?
Yes

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

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

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Is it accessible?
Yes

Is it clear?
Yes

Is it adequate?
Yes

Do you have any ethical concerns with this paper?
No

Comments to the Author
This paper describes the effects of microplastics and habitat degradation on the behaviour and survival of a coral-reef fish during a short-term field experiment. The experimental design seems sounds and the results of the study are very clear. Overall, we believe this manuscript has improved from the previous version and appreciate the authors' efforts in revising the paper based on our previous comments. In particular, the description of the supplemental feeding studies, behavioural assessments, and patch reefs are much clearer now. We have only a few places where the response to our previous comments may need a bit more explanation plus a few additional comments – please see below.
Abstract
Consider adding an opening background sentence explaining the rationale/context for the study.
Line 40: This study does not test the "resilience of coral-reef communities", so please delete/change this in the abstract.
Introduction
Line 70-71: It may be worth mentioning chemical cues directly here, since it seems like this is the mechanism being described.
Materials and methods
Lines 103- 107: The order of this paragraph was a bit hard to follow - it starts by describing the study species, then mentions the study site, then goes back to describing the study species. It may read more clearly if study location were moved to the start of the paragraph.
Lines 124-126: The response to reviewers and new supplemental material regarding microplastic concentrations were very thorough, and it is now clear that obtaining reliable information on the availability of microplastics to coral-reef fish is extremely difficult. Whilst there is reference to the supplementary material, a sentence in the main text stating that the exact concentrations of microplastics in the field are unknown and the difficulty of microplastic measurements in the field would be beneficial and more transparent.
Also, from reading the supplement it is not clear that these microplastic concentrations are "similar to an area of reef near an urban centre", especially given that the microplastic concentrations measured in the tropics were often <0.005 p/L, compared to the 167 p/L used in this study. A similar statement that the concentrations used "…will likely be analogous to microplastic levels…" found near tropical Indo-Pacific reefs is also in the Supplement (Line 87-90). However, if most of the text argues that there is a lot of uncertainty about microplastic availability, it then seems that one cannot also know the experimental concentrations are realistic. At the least, we suggest being fully transparent in the text about this uncertainty, therefore these results provide an example of what could happen (but not necessarily what will happen).
Line 130: Is there a reason for choosing this size of bead? For example, is it similar in size to food items for these fish, or is it a size that may be particularly common in plastic pollution now or in the future?
Results
Line 227-229: It seems that this line is arguing that microplastics are the dominant effect based on the difference in partial-ETA squared among the fixed effects. But based on Table S1, the tank effect is actually the largest, so this suggests that social systems (based on the response to reviewers) are more important than either stressor. At the least, this large tank effect and explanation should be acknowledged within the main text.

Discussion
Line 268: Same as for the abstract- this study does not test resilience, and we are unsure of a direct link between the behaviour of this damselfish species and the resilience of coral reefs. So, please consider changing/removing this phrasing.
Line 283-285: The response to reviewers provides a lot of details about predation-induced mortality being the likely cause of mortality. Explicitly including a statement to that effect with a reference could be useful (perhaps here, where risk-taking behaviour is mentioned, but predation is not).

Review form: Reviewer 2

Recommendation
Accept with minor revision (please list in comments)

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

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

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

Is the length of the paper justified?
Yes

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

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

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

Is it accessible?
N/A

Is it clear?
N/A

Is it adequate?
N/A

Do you have any ethical concerns with this paper?
No

Comments to the Author
Response by the authors to referee 1 (Para 4): Currently, what we can say of the concentrations available to fishes in tropical waters is that they are likely to be spatially variable and will pose an increasing threat in the future as the amount of microplastics within the environment increases.

Referee reply - The problem with this statement is that the paper is essentially crying-wolf about an impact that has not eventuated.
In the conclusions the following statement is made.

The predictions of increasing warming, storm frequency and severity [86] have led to a prognosis of a general decline in the quality of coral reefs globally [87]. Evidence from our study suggests that the consumption of microplastics can have a detrimental effect on juvenile fish that is of similar magnitude, at least over short time scales, to living in association with a degraded habitat.

Referee reply - The suggestion that that "the consumption of microplastics can have a detrimental effect on juvenile fish that is of similar magnitude to storm etc – is extrapolating in the extreme.

Referee 2 comment:
My fundamental concern about the use of fresh beads rather than weathered beads is indicated below.

Referee reply – From the addition of Supplementary Part 3 I am satisfied that the leachates from beads are minimal when compared for control water. It does, however, not address the issue of how the beads fare when subjected to digestive juices (ie fresh beads versus those weathered to microplastics in the ocean).

Decision letter (RSPB-2020-1947.R0)

16-Sep-2020

Dear Dr McCormick:

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

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

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

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

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

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

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

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

Data accessibility and data citation:
It is a condition of publication that you make available the data and research materials supporting the results in the article (https://royalsociety.org/journals/authors/author-guidelines/#data). Datasets should be deposited in an appropriate publicly available repository and details of the associated accession number, link or DOI to the datasets must be included in the Data Accessibility section of the article (https://royalsociety.org/journals/ethics-policies/data-sharing-mining/). Reference(s) to datasets should also be included in the reference list of the article with DOIs (where available).

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

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

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

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

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

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

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

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

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

Associate Editor Board Member
Comments to Author:
While both reviewers agree that the manuscript has improved from the previous version, they have highlighted a number of issues that would need to be addressed.

Reviewer(s)' Comments to Author:

Referee: 2

Comments to the Author(s).
Response by the authors to referee 1 (Para 4): Currently, what we can say of the concentrations available to fishes in tropical waters is that they are likely to be spatially variable and will pose an increasing threat in the future as the amount of microplastics within the environment increases.

Referee reply - The problem with this statement is that the paper is essentially crying-wolf about an impact that has not eventuated.
In the conclusions the following statement is made.

The predictions of increasing warming, storm frequency and severity [86] have led to a prognosis of a general decline in the quality of coral reefs globally [87]. Evidence from our study suggests that the consumption of microplastics can have a detrimental effect on juvenile fish that is of similar magnitude, at least over short time scales, to living in association with a degraded habitat.

Referee reply - The suggestion that that "the consumption of microplastics can have a detrimental effect on juvenile fish that is of similar magnitude to storm etc – is extrapolating in the extreme.

Referee 2 comment:
My fundamental concern about the use of fresh beads rather than weathered beads is indicated below.

Referee reply – From the addition of Supplementary Part 3 I am satisfied that the leachates from beads are minimal when compared for control water. It does, however, not address the issue of how the beads fare when subjected to digestive juices (ie fresh beads versus those weathered to microplastics in the ocean).

Referee: 1

Comments to the Author(s).
This paper describes the effects of microplastics and habitat degradation on the behaviour and survival of a coral-reef fish during a short-term field experiment. The experimental design seems sounds and the results of the study are very clear. Overall, we believe this manuscript has improved from the previous version and appreciate the authors' efforts in revising the paper based on our previous comments. In particular, the description of the supplemental feeding studies, behavioural assessments, and patch reefs are much clearer now. We have only a few places where the response to our previous comments may need a bit more explanation plus a few additional comments – please see below.
Abstract
Consider adding an opening background sentence explaining the rationale/context for the study.

Line 40: This study does not test the "resilience of coral-reef communities", so please delete/change this in the abstract.
Introduction
Line 70-71: It may be worth mentioning chemical cues directly here, since it seems like this is the mechanism being described.
Materials and methods
Lines 103- 107: The order of this paragraph was a bit hard to follow - it starts by describing the study species, then mentions the study site, then goes back to describing the study species. It may read more clearly if study location were moved to the start of the paragraph.
Lines 124-126: The response to reviewers and new supplemental material regarding microplastic concentrations were very thorough, and it is now clear that obtaining reliable information on the availability of microplastics to coral-reef fish is extremely difficult. Whilst there is reference to the supplementary material, a sentence in the main text stating that the exact concentrations of microplastics in the field are unknown and the difficulty of microplastic measurements in the field would be beneficial and more transparent.
Also, from reading the supplement it is not clear that these microplastic concentrations are "similar to an area of reef near an urban centre", especially given that the microplastic concentrations measured in the tropics were often <0.005 p/L, compared to the 167 p/L used in this study. A similar statement that the concentrations used "…will likely be analogous to microplastic levels…" found near tropical Indo-Pacific reefs is also in the Supplement (Line 87-90). However, if most of the text argues that there is a lot of uncertainty about microplastic availability, it then seems that one cannot also know the experimental concentrations are realistic. At the least, we suggest being fully transparent in the text about this uncertainty, therefore these results provide an example of what could happen (but not necessarily what will happen).
Line 130: Is there a reason for choosing this size of bead? For example, is it similar in size to food items for these fish, or is it a size that may be particularly common in plastic pollution now or in the future?
Results
Line 227-229: It seems that this line is arguing that microplastics are the dominant effect based on the difference in partial-ETA squared among the fixed effects. But based on Table S1, the tank effect is actually the largest, so this suggests that social systems (based on the response to reviewers) are more important than either stressor. At the least, this large tank effect and explanation should be acknowledged within the main text.
Discussion
Line 268: Same as for the abstract- this study does not test resilience, and we are unsure of a direct link between the behaviour of this damselfish species and the resilience of coral reefs. So, please consider changing/removing this phrasing.
Line 283-285: The response to reviewers provides a lot of details about predation-induced mortality being the likely cause of mortality. Explicitly including a statement to that effect with a reference could be useful (perhaps here, where risk-taking behaviour is mentioned, but predation is not).

Author's Response to Decision Letter for (RSPB-2020-1947.R0)

See Appendix B.

Decision letter (RSPB-2020-1947.R1)

06-Oct-2020

Dear Dr McCormick

I am pleased to inform you that your manuscript entitled "Microplastic exposure interacts with habitat degradation to affect behaviour and survival of juvenile fish in the field" has been accepted for publication in Proceedings B.

You can expect to receive a proof of your article from our Production office in due course, please check your spam filter if you do not receive it. PLEASE NOTE: you will be given the exact page length of your paper which may be different from the estimation from Editorial and you may be asked to reduce your paper if it goes over the 10 page limit.

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

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

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

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

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

Associate Editor:
Board Member
Comments to Author:
The comprehensive comments from both reviewers have been addressed in a thorough and constructive manner. I see no need for the paper to go out for further review. Congratulations to the authors for this nice paper.

Appendix A

Response to Reviewers' comments

Referee: 1

Comments to the Author(s)

By considering both habitat degradation and microplastic exposure, the paper provides insights into the potential consequences of combined pressures on the behaviour and survival of juvenile reef fish. The researchers clearly identify the motivation for the study, test their research question using a reasonable experimental design and statistical analyses, and present strong evidence for an influence of the two stressors (and especially microplastic exposure) on fish behaviour and survival. We found the paper generally clear and easy to follow.

We felt a key weakness of this study was the lack of information on the microplastic concentrations these fish may be exposed to in the wild, which therefore makes it difficult to put the results into context. The authors did a good job of providing some information on what is known about microplastic concentrations in different parts of the ocean, but they also note microplastics have seldom been quantified in tropical waters, and never at their study site. It's unfortunate that even preliminary data on microplastic concentrations near where these fish were caught are not available. We appreciate that the authors seemed to use conservative amounts of microplastics in this study, but there is no way to know that these concentrations are actually conservative given the lack of data. This lack of data also means it is unknown whether microplastic exposure is even a problem for coral-reef fish at the end of their larval phase. Relatedly, the inclusion of supplementary feeding studies was useful to help address these concerns, but we were confused about some of those methods when reading the main text, including the sample sizes and the concentration of microplastics.

Reply: Obtaining *reliable* data on the availability to animals of microplastics of this size (0.2mm dia) is very difficult. In coral reefs, as in other marine environments, there are large amounts of organic and inorganic (e.g. sand) matter that are around this size. Obtaining reliable data on the availability of microplastics to animals is very difficult because it not only requires good estimates of the concentrations of microplastics in the different habitats where the target animals may be present, but also requires information on the likelihood of the animal feeding on the particles when available. It is only recently that studies have tried to address this issue. Currently, estimates of concentrations come from surface water or sediment samples. Very few estimates of microplastic concentrations in the waters around tropical coral reefs are available and surprisingly few are available for inshore coastal areas near urban centres of the tropics (Purba et al. 2019; Li et al. 2020). An examination of the research on the Australian coastline found that concentrations of microplastics range up to 17.4 particles/L during high winds (Hitchcock 2020), with sediment concentrations in tropical waters of up to 38,790/kg dry sediment (Manalu et al. 2017). A brief literature summary has been included in Part 2 of the Supplementary file.

Currently, what we can say of the concentrations available to fishes in tropical waters is that they are likely to be spatially variable and will pose an increasing threat in the future as the amount of microplastics within the environment increases. Our current study was conducted at Lizard Island (14° 40' S, 145° 28' E), on the northern GBR, Australia. This study

site is ideal as it is in a remote part of the GBR with no urbanisation inland. To date, no information is available on the concentrations of microplastic particles in specific microhabitats foraged by the focal fish used for the study. Other studies have found that fish can preferentially select and consume microplastics within their environment (Nanninga et al. 2020). The experimental scenario used in the present study will likely be analogous to the microplastic levels currently found near inshore fringing reefs and inshore reefs near urban areas within the more populated parts of the tropical Indo-Pacific.

Please see below for additional specific comments, especially for places where the methods and results could be clarified, along with some suggestions to provide more explicit links between the stressors, observed behaviours and mortality to make the story even more compelling.

Line-by-line comments:

Introduction

Line 50-51: Whilst the combination of habitat degradation and microplastics may be novel, there has been a lot of recent research on the combined effects of multiple stressors more generally. As a starting point, there are two meta-analyses from 2008 in Ecology Letters focused purely on the effects of multiple stressors (Crain et al., Darling and Côté), plus many more studies since then (including some using microplastics or habitat degradation – some of which are cited in the Discussion). In addition, either here or elsewhere in the introduction, it could be beneficial to include some information on previous fish behavioural research, addressing both habitat degradation and microplastic pollution, given that the focal point of the study seems to be behaviour as well as the combination of two environmental stressors.

Reply: The text has been modified to note that recent research has started to examine the effects of co-occurring stressors.

"Historically, when researchers have explored the impact of stressors on the biology and ecology of organisms, they have looked at stressors in isolation (O'Brien et al. 2019). However, stressors are most likely to occur concurrently and this co-occurrence can lead to additive, antagonistic or synergistic effects (Crain et al. 2008; Darling and Cote 2008; Montoya José and Raffaelli 2010; Ormerod et al. 2010; Ferrari et al. 2015; Tekin et al. 2020). Accordingly, combined stressor effects have become an increasing focus in recent years (Côté et al. 2016; Jackson et al. 2016). Given the complexity of natural systems, manipulative experiments have proven key for characterising multi-stressor interactions, and these findings inform us of the potential trajectory of future communities."

Line 53-54: This line also seems a bit overstated. Experiments may not be the only way to determine the magnitude of multi-stressor interactions, and they may not truly inform us of the trajectory of future communities (because there are many more than just two stressors

operating at a time, and species respond differently to different stressors, which will then affect their interactions). Please consider re-wording and/or providing references for these statements.

Reply: This sentence has been deleted and the last sentence has been reworded.

"Given the complexity of natural systems, manipulative experiments have proven key for characterising multi-stressor interactions, and these findings will inform us of the potential trajectory of future communities."

Lines 70-88: This paragraph could be condensed whilst still retaining the key points. For example, Lines 74-76 do not add any additional information to the prior sentence 'Plastic waste has been generated…' so could be removed.

Reply: The sentence on lines 74-76 has been deleted.

Methods

Lines 101-110: This paragraph is perhaps too detailed for the main text, given that none of the microplastic concentrations provided are directly relevant to the study site, or even to coral reefs more generally. Consider either summarising this information more concisely, or placing a table in the supplementary materials reporting the previous study locations and concentrations/sizes of microplastics found. In addition, stating the concentration of microplastics used in this study would be beneficial, as currently it's difficult to determine how the reported concentrations align with what was used in the study.

Reply: This section has been reduced to a summary in the main manuscript (at the start of '*Laboratory conditioning*'), with a detailed examination of the concentrations found in the tropics and relevant considerations within Part 2 of the Supplementary file.

One of the problems with the discipline as a whole is the varying methods used to sample microplastics and the way these have been scaled-up to concentration estimates. When water samples have been taken they have usually been converted into particles/km^2, despite the common methodology sampling only the upper 30-40 cm of the water column. This means it will be mostly the positively buoyant types of microplastic particles. The quantities obtained will also be very weather dependent, with even light and moderate winds mixing the surface waters and potentially reducing the particles that have aggregated at the surface due to their buoyancy. It is unclear how particle concentrations for the upper part of the water column relate to concentrations within the water column near substrate that is occupied by most bottom-dwelling fishes. Why the field has decided to opt for a p/km^2 standardisation, when the collection methods used have sampled different depths of water is never discussed. We have opted to convert these water sample estimates to particles/L. Sediment samples have been standardised to particle/m^3 (though there is one instance where the authors have decided on using a dry weight standardisation that precludes our standardisation). Both water column and sediment plastic concentrations are relevant to bottom-dwelling fishes, particularly due to the large amount of resuspension

from the sediment that occurs in the shallow areas of the continental shelf occupied by most coral reef fishes. These issues have been explored in the Supplementary file.

Line 159-160: Could you tell if the food remaining was Artemia, microplastics, or both? Also, what do "beakers" and "jar" refer to – do these just mean tank, since there's been no previous mention of anything except tanks?

Reply: The 'beaker' has been replaced with 'tank'. Whether any plastic remained after feeding was not quantified in the current study.

Line 163-167: The supplemental feeding studies are a nice addition, but as it's written it was not clear that these results are from different fish than those used in the main experiment (Line 313 is the first direct reference to supplementary studies, rather than just supplementary files). Please clarify whether the fish used in the supplemental studies were from the same collections as the ones used in the main experiment, and the sample sizes (number of individuals) for each.

Reply: The results of the ingestion and egestion trials held within the Supplementary file are described in the "*Laboratory conditioning*" portion of the methods as they form a foundation to the current study. The species name for the egestion trial study (i.e., *Pomacentrus chrysurus*) is now emphasised in the main text. The details of the study (treatments, replicates etc) are documented in the Supplementary file.

Also, it appears that the supplemental studies used different concentrations of microplastics than used in the main experiment (or at least, the fish in the supplemental studies were only exposed to microplastics, and not microplastics + Artemia). This point should be made explicit in the main text, as the reported ingestions may not be representative of what the fish used in the main experiment ingested.

Reply: The goals of the two feeding trial studies in the Supplementary file (Parts 4 and 5) were quite different from the main study. One supplementary study was an ingestion study, to determine whether fish would actually feed on the particles and quantify any individual variability in ingestion. Ecological realism wasn't the main aim of either study, but rather whether they eat them when available, and if they did, whether they passed through their guts. In hindsight, it would have been better to keep the particle density and presence of *Artemia* consistent with the main study (the Sup study was an initially conducted pilot study). This methodological difference has been made plain in the text of the manuscript.

Line 175: Does placing the fish individually mean there was only one fish on each reef? Please be explicit whether there was only one individual fish per reef at a time.

Reply: You are correct. There was one fish per patch reef. This has been clarified in the text.

"Individual fish were placed by divers (MIM, MB) on one of ~ 50 small numbered patch reefs (~25 x 15 x 20 cm; one per patch) made of either live-healthy or dead-degraded *Pocillopora damicornis*, a bushy hard coral commonly used as a settlement habitat by *P. amboinensis* (McCormick et al. 2010; McCormick and Weaver 2012)."

Line 176-180: Some more details about the patch reefs would be helpful. For example - when were the patch reefs created (how old are they – which could influence any other chemical/visual cues from the live versus dead or degrading corals) and how complex are the patches (which could influence how susceptible the fish are to predators)? How many patch reefs were there, and how many in each treatment? For the dead-degraded corals – did they die naturally, how long had they been dead, and were they structurally intact (which may influence behaviour)? How were all fishes and invertebrates removed from the corals?

Reply: The patches have now been described in more detail with reference to a previously published figure. There were ~ 50 small patches (~25 of each). Dead degraded coral was sourced from the ref edge (as noted in the methods) and had naturally died. Resident fishes were removed from the habitat patches by use of a hand net and then transported onto the main reef. Crabs and shrimps were induced to move onto habitat fragments with the aid of a piece of wire and they were then also transported onto the main reef when possible.

"Individual fish were placed by divers (MIM, MB) on one of ~ 50 small numbered patch reefs (~25 x 15 x 20 cm; one per patch) made of either live-healthy or dead-degraded *Pocillopora damicornis*, a bushy hard coral commonly used as a settlement habitat by *P. amboinensis* (McCormick et al. 2010; McCormick and Weaver 2012). The live coral and degraded corals used had similar structural complexity. For our purpose, we define a degraded habitat as *Poc. damicornis* that had been dead for approximately 3 months to 1 year, had a similar structural complexity to live coral, but was covered with a variety of sessile invertebrates and algae (e.g., see Fig. 1 in (McCormick and Lönnstedt 2016)). Patches were placed on 20 x 20-cm paving tiles buried up to their top in the sand to prevent the sand from damaging the live coral. Substrata were sourced from the base of the main reef edge and were vacant of all fishes and mobile invertebrates."

Line 194: Please clarify how boldness was assessed over the 3-min observation period prior to the pencil test.

Reply: The boldness description has been rewritten to improve clarity. This part has been moved to the Supplementary file due to space restrictions of the main manuscript.

"The boldness score follow methodology used in previous studies on small fishes (McCormick 2009,2012) and was categorized as: 0 if the fish was positioned within a small hole and seldom emerged; 1 if it retreated to a hole when approached by the pencil tip and took more than 5 sec to re-emerge, with weak or tentative strikes at food; 2 if fish shied to shelter of the patch when approached by the pencil tip, but emerged quickly and purposefully struck at food; and 3 if the fish did not retreat to shelter when approached, but rather explored around the coral patch, and struck aggressively at food (McCormick 2009). Previous research showed that this boldness measure is repeatable across different time scales (e.g., repeatability values of ~ 0.5 over a 2 h period;(White et al. 2013; White et al. 2015)). Three-minute behavioural assessments have previously been found to

be sufficiently long to obtain a representative estimate of an individual's behaviour (McCormick and Weaver 2012; White et al. 2015). Video cameras could not be used to create a reliable record of these assessments because fish move around their topographically complex habitat patches."

Line 207-208: Did you ever find a tagged fish on a different reef?

Reply: In this study, no tagged fish were found to migrate from the reef on which they were released. This has now been noted.

Results & Figures

Lines 236-244: A reminder of what the behavioural index means could be helpful – so high behavioural activity is associated with high bite rate, high movement, high boldness, etc.

Reply: A sentence has been added to the legend of Figure 1 to remind the reader that there is a positive relationship between the behavioural index (PC1) and all variables (listed in the legend) that are used in the analysis.

Figure 1/S3: Whilst figure 1 clearly presents the behavioural index included in the PCA, Figure S3 gives a simple but clear overview of the individual behavioural trends, so maybe should be included in the main article (e.g., by making Figure 1 a 5-panel figure). Because the Discussion repeatedly refers to individual behavioural traits (e.g., lines 259-263, 269, 295-296, etc.), it would be useful to have the results for these individual behaviours in the main text rather than just the supplement.

Reply: We agree with the reviewer that this would be a good idea, and initially did undertake the suggestion, but space restrictions of the main manuscript have meant that we must leave the figure in the Supplementary file.

Table S1: I apologise if this is naïve as I'm not familiar with the partial eta squares metric, but what does it mean that the effect size for the random effect of tank is larger than any other effect size? To me, this suggests that tank had a bigger influence on behaviour than plastic or habitat, which would be concerning.

Reply: A reference to partial eta squared and its interpretation is now given in the methods. The effect size for tanks is quite large. This is neither unusual nor surprising. Fish within tanks often develop social systems (as they do in nature) and that promotes variability in phenotypic expression. This variability among groups cannot be attributed to either the main effects or interactions. Of interest is that, despite the variance at the tank level, there are strong patterns of difference between and within treatments.

Discussion

Line 266-275: This part was a bit unclear to me. For example, it makes sense that mortality is constrained to 100%, but is the behavioural index constrained too (relevant to the ceiling-truncation hypothesis)?

Reply: The behavioural index will be somewhat constrained by the behaviour it represents. The species' behaviour will sit within a behavioural envelope that is specific to the species, life-stage and context. For instance, there is only so far that one of our study animals will stray from shelter, and there is a maximum rate at which they will forage.

Line 277-280: Consider changing both uses of the word "will" to "may", given that 1) the amount of microplastics that these fish are naturally exposed to is unknown and 2) this study did not test the resilience of coral reef communities.

Reply: Good point. "will" has been changed to "may" as suggested.

Line 287: Without any data on microplastic concentrations near coral reefs, it's still unclear that these are "realistic" concentrations.

Reply: "Realistic" has been deleted.

Line 296-297: Some elaboration on the links between the behavioural metrics and survival would be useful (either here or elsewhere in the Discussion). From the results, there is not a clear mechanism linking these two. Is it possible to look at the correlation between high activity and hours survived (across the different treatments)? Showing that individuals that exhibited high activity died fastest (or died at all), for example, would provide convincing evidence that the observed behaviours were linked to survival.

Reply: A Spearman's rank order correlation has been used to examine the correlation between the behavioural index and survival (in hours) ($\rho = 0.33$, $p = 0001$, $n = 135$). This suggests that survival increases as fish become more conservative in their behaviour (because there are strong negative relationships between PC1 and behavioural measures, as stated in the *Statistical analyses* section). The link between risk taking behaviour and death through predation has been emphasised by edits to the first paragraph of the discussion.

Also, it seems that a major assumption is that any loss observed was due to predation-induced mortality. Elaborating on a few points related to this would be useful. 1) Were any predation events observed and/or any predators observed in the area of the reefs? When was loss rate highest (morning, afternoon, evening, or night), and does this correspond to higher predator activity levels? 2) How does the fact that there were no resident predators on the experimental reefs influence the interpretations of these results? For example, are the predators of these damselfish (listed on Line 125-127) resident or transient? It seems like some of the observed behaviours (e.g., staying farther from shelter) could increase exposure to transient predators, but may be useful in avoiding resident predators (see, for example,

Hixon and Carr 1997 Science). So maybe on actual reefs the differences in mortality across treatments would not be as large?

Reply: We have now included additional details of the study system and the likelihood that loss of small damselfish equates to their mortality.

We have conducted over 50 published predator-prey experiments using the methodology similar to that described in our study so we have a pretty good understanding of the idiosyncrasies of the system. During the 2000's we conducted a major series of experiments that quantified the trajectories of mortality and the traits of prey that influence their probability of survival (Hoey and McCormick 2004; McCormick and Hoey 2004; McCormick and Meekan 2007; McCormick 2009; Fuiman et al. 2010; Meekan et al. 2010; McCormick 2012; Poulos and McCormick 2014; Poulos and McCormick 2015; McCormick et al. 2018). We have also done many field experiments where we have looked at how prey learn about the identity of predators (Lönnstedt et al. 2012; Lönnstedt et al. 2014; Lönnstedt and McCormick 2015; McCormick and Lönnstedt 2016; McCormick et al. 2017) and how this is affected by various environmental stressors (Munday et al. 2010; McCormick 2012; McCormick et al. 2013; McCormick et al. 2019). Along with these studies we have examined the ecology of some of the most significant predators on the reef edge (McCormick and Meekan 2007; Feeney et al. 2012; Bosiger and McCormick 2014).

By following tagged newly settled fish on the main reef we have obtained realistic mortality trajectories for the study species used in the current study and found these to be exponential decay curves (e.g. McCormick and Meekan 2007), similar to those that we find on patch reefs close to the reef edge. Many of our previous studies have placed elastomer tagged fish on patch reefs (as did the current study) and this has allowed us to identify individuals that may have moved. One study on patch reefs similar in size and position to those in the current study for a closely related newly settled damselfish (*Pomacentrus wardi*) found no movement (McCormick and Meekan 2010). In an earlier study, we pushed the numbers of newly settled fish (*Pomacentrus amboinensis*) on patch reefs slightly larger than the ones used in the current study to 20 fish/patch (the upper end of recruitment levels). These fish were batch tagged, with different colours being used on neighbouring reefs within a grid of reefs. We found that movement, even under these somewhat extreme densities was very low (2 out of 300 fish; Hoey and McCormick 2004). Observations during behavioural trials also reveal that juveniles who stray too far from a shelter patch were quickly lost to predation, by predators such as lizardfish. The loss rate of fish from these experimental patches is always between the last census of the day (about an hour before dusk) and the first census of the day (about an hour after dawn) (e.g., McCormick 2009, 2012, McCormick and Meekan 2007). The loss of fish over this dusk to dawn period is in keeping with the crepuscular activity patterns often found for piscivores (e.g., *Cephalopholis cyanostigma*, Bosiger and McCormick 2014).

Line 333: Some elaboration on why fish would exhibit higher activity levels after being exposed to microplastics, especially if the plastic is no longer in their system, would be useful.

Reply: The third to last paragraph of the discussion has been elaborated to discuss these points.

Line 346: As the closing paragraph, a closing reference to the findings of the study could be included here.

Reply: A sentence has been added that further summarises the findings in the concluding paragraph of the discussion.

Reviewer 2.

My fundamental concern about the use of fresh beads rather than weathered beads is indicated below.

General comments

Although there have been multiple publication on the effects of microplastics, the effects have not been considered in an interactive sense with other factor such as habitat degradation.

The goals were sensible and the experimental design is largely sound, but a concern I have is the use of fresh plastic bed that have not been weathered as you would expect for microplastics that are current affecting our oceans.

Fresh beads for example are known to release pentane isomers that can may cause headache, dizziness and nausea in humans; there is also a cocktail of metal oxides (e.g., Zn and Ni) that presumably leech with time. The experiments in this paper therefore may have simply drugged/poisoned the fish rather than truly testing the weathered plastics (that would be chemically benign), as would be in our oceans. Coral reefs are rarely near the source of plastic pollution, impacts therefore would be largely restricted to well weathered particles from distant sources.

Reply: We agree with Reviewer 2 that the effects of additives could have a significant effect on the behaviour of fishes. In fact, we are currently working on this very topic using controlled microplastic samples with known toxins, such as plasticisers, in collaboration with a polymer chemist (Dr. George Vamvounis, JCU and Alexandra Gulizia, who are now authors on the current paper).

As mentioned in the methods of the manuscript, we used Polystyrene microbeads (200-300µm) from Polysciences. These are manufactured to be composed of pure polystyrene as mentioned in the Safety Data Sheet provided by the company. However, the reviewer's comment is very pertinent so we have addressed whether our beads release any chemicals using three chemical analytical methods detailed below. These analyses have now been added to the manuscript as Part 3 of the Supplementary file.

1. **Thermal Gravimetric Analysis (TGA)**: This analysis looked at the weight of the beads as a function of temperature. This analysis is relevant because it will show if low boiling point materials (such as pentane, ~35 ˚C) are emitted from the bead (hence lose weight at a lower temperature). The TGA analysis revealed a small decrease at around 100 ˚C (attributed to water moisture) and a decomposition at 400 ˚C. No decrease in weight was observed around 35 ˚C, indicating no pentane is present in the sample. The TGA of the beads were also compared to commercially available polystyrene and the decomposition temperature was chemically similar to these beads.
2. **Proton - Nuclear Magnetic Resonance (NMR)**: This technique is an analytical method that is commonly used to analyse/identify organic molecules such as pentane. Therefore, we simulated the experimental conditions, where we analysed if any leaching of organic molecules occurred in the microplastics in D_2O (heavy water) for 24 hours (which was much longer than the 15 minute immersion in the current experiment) at room temperature. The resultant heavy water was analysed using NMR to determine if pentane (or any other organic molecule) was present due to leaching. It can be seen in the supplementary data that no peaks were observed after exposing the beads in heavy water for 24 hours, meaning organic molecules were not leached.
3. **Fourier Transform Infrared analysis (FT-IR)**: This method helps identify the chemical structure of the material. The FT-IR spectrum of the beads matches perfectly with pure polystyrene. In addition, no associated peaks were attributed to the metal oxides (ZnO): a broad peak at 3400 cm^{-1} was not present as illustrated by NIST(https://webbook.nist.gov/cgi/cbook.cgi?ID=B6004648&Mask=80).

In summary, the TGA, NMR and FT-IR analysis showed no evidence that pentane and metal oxides were present/leaching from the beads. We are therefore confident that the beads themselves are the only contributing factor for the behaviour observed in the manuscript. Upon further investigation, we believe that the reviewer could be confusing these beads with thermally expandable microbeads (or more commonly called Expancel), which have a hydrocarbon in their core (such as pentane) and metal oxides on the surface. Our experiments demonstrate that these chemicals (and others) do not exist within the microbeads that we used and that they have a representative toxicity level as weathered pure polystyrene. All of this data (and the source of the beads) has been added to the Supplementary file and are referenced in the main text.

Accordingly for the paper to be accepted the authors need to demonstrate some or all of the following (i) fresh beads are no different to weathered bead in their properties (which is highly unlikely); (iii) the beads that were used weathered for a period of time that equates with the microplastics that have spent weeks to years in the ocean.

Reply: An examination of the leaching properties of the beds has now been undertaken and it was found that no detectable plastic compounds were released from the microbeads over

a 24h period at a normal summer ambient temperature for the reef at the study location (28C). This is not surprising as other studies have found that polystyrene is inert.

Other comments

Materials and methods

The justification for amount of plastic fed to the fish was weak. No data were available on the abundance of microplastics at Lizard Island.

Reply: True, no data is currently available for any natural system that is relevant to these demersal (bottom dwelling) fishes. The problem is that plastic particles that are ~200microns dia are very difficult to collect and identify. However, as plastics degrade they break up into smaller and smaller particles. The smaller they are, the more plentiful they should be with an exponential increase with decreasing size (e.g. Hitchcock 2020). Our study and other unpublished data we have shows that these small damselfish can pass the microbeads through their guts, which also means that even if we sample a fish and find no plastic within its gut, this does not mean it has not eaten any, but rather it has eaten any within the last 14 hours. Gut sampling from wild fish would lead to an underrepresentation of the magnitude of the problem. We have undertaken a brief literature review of the microplastic concentrations found in tropical waters and included this as Part 1 of the Supplementary file. We have also included a brief paragraph on the choice of concentration in the methods of the main document.

Some data are mentioned on the abundance of microplastics from temperate regions – line 104; diff references mixed m^2, km^2 m^3, depending on how the data (in some cases it is clear that surface hauls were made) were collected 2D metrics would include depth of the water column? No information is provided on the particle sizes collected to calculate those numbers?

Reply: As with all disciplines in their early days, there are problems of methods standardization. Plastics research is no different. Most studies use different methods to sample for microplastics, different logic to scale up the estimates, and different spatial standardisations. This lack of standardisation is a general issue with the field and a number of recent reviews have suggested methods standardisation as an important way forward (e.g. Elkhatib and Oyanedel-Craver 2020). We have done our best to standardise estimates of microplastic concentrations in the water column to per litre, while estimates of microplastics in sediment are per cubic metre.

In the experiment 200 microplastics were added per 35 L added line 154. How does this relate to the literature?

Reply: Microplastic-exposed fish (10 fish per 1.2L tank) were given access to 200 beads + 1000 *Artemia* for two 15 min feeding episodes each day for 6 consecutive feedings. The logic of the choice in microplastic concentrations is now given within Supplementary Part 2.

I note that the consumption of beads was highly variable. See line 165 –1-33 particles were taken per fish, mean 4.5. 60% of fish ate 3 or fewer particles. Given this high variation the variation in results (ie Figs. 1 and 2 were surprizingly low.

Reply: Exactly, that is the point discussed in paragraph 5 of the discussion. Our feeling is that it is likely to be due to consumption and a rapid throughput of particles through the gut. Fish of this small size and developmental stage have very high metabolisms (Nilsson et al. 2007) and eating plastic may promote a small energy deficit. This is likely to promote feeding over vigilance.

Line 165 – if 200 particles were added, how does this result in a calculation of 40 particles available to each of four fish?
The acclimation of fish in the field (in cages) was sensible.

Reply: This refers to one of the feeding trials in the Supplementary file where 4 fish are given access to 40 beads within an 0.8L container. Details have now been added to the main text to clarify this point.

3 min observation period line 189 seems very short? .. or have I missed something? Boldness measured indicated is repeatable based on the literature, but not in this study.

Reply: Correct. Studies on fish of the same developmental stage and species from Lizard Island have found that behaviour is very repeatable in these young fish and can be assessed within a 3 minute assay (e.g., McCormick and Meekan 2010, White et al. 2015).

Statistical procedures:
Line 223 – Why type 1 SS, Type 3 generally considered more robust?

Reply: Type I sums of squares are used for designs that involve nested components otherwise the variance estimates are incorrectly apportioned (SAS-Institute-Inc. 2019).

Line 227 why unequal samples sizes?

Reply: We tried to obtain a balanced design, but the complexity of getting fish through the various steps in the experiments, and the availability of fish from light traps, meant that the treatments ended up being slightly unbalanced.

Effect sizes – line 229 procedure? Reference the source?
Reply: A citation for partial eta squared as a measure of effect size is now given (Richardson 2011).

Results
Fig 2 very clear result – microplastics a problems and dead coral – although trend that later

less mort NS!
Reply: Yes, the results are quite clear.

Discussion
There is not even a brief discussion on how microplastic could affect fish – be that chemical or through abrasion of the alimentary canal.

Reply: Additional discussion has been added to the mechanisms what promotes risk-taking behaviour after microplastic exposure in these fish. The findings of our new chemical analyses suggest that the polystyrene spheres are inert and so it seems more likely that they lead to a nutritional deficit, leading to the promotion of foraging over vigilance.

Line 274 – re: 100% mortality – this may have been due to toxic fresh beads rather than increased predation through greater risk taking by experimental fish.

Reply: Given the findings of the chemical analyses we have now included in the Supplementary file, it seems unlikely that the beads are toxic. Pure polystyrene should be pretty much inert, and this is the findings of the weathering study, where beads were placed in water for 24hours (considerably longer than the 15min used in the experiment). The most parsimonious hypothesis is that the mortality associated the consumption of microplastics is that it results in a nutritional deficit that leads to greater risk taking, and higher risk leads to higher mortality rates.

Line 286 re; relatively conservative concentrations of microplastics. For this to be stated, estimates of microplastics per unit volume need to use the same units. No data were available on actual concentrations of microplastics in the field at Lizard Is.

Reply: "relatively conservative" is relative to previous studies. This has now been clarified. Many studies have tended to have microplastics continuously present for many days prior to assessments of their impact. In contrast, our study used pulses of microplastics over short periods of time. For instance, Weber et al. (2020) used concentrations of up to 100 million particles/L for 2 weeks in a study of the effect of microplastics on mussels.

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Appendix B

Referee: 2

Comments to the Author(s).
Response by the authors to referee 1 (Para 4): Currently, what we can say of the concentrations available to fishes in tropical waters is that they are likely to be spatially variable and will pose an increasing threat in the future as the amount of microplastics within the environment increases.

Referee reply - The problem with this statement is that the paper is essentially crying-wolf about an impact that has not eventuated.
In the conclusions the following statement is made.

The predictions of increasing warming, storm frequency and severity [86] have led to a prognosis of a general decline in the quality of coral reefs globally [87]. Evidence from our study suggests that the consumption of microplastics can have a detrimental effect on juvenile fish that is of similar magnitude, at least over short time scales, to living in association with a degraded habitat.

Referee reply - The suggestion that that "the consumption of microplastics can have a detrimental effect on juvenile fish that is of similar magnitude to storm etc – is extrapolating in the extreme.

Reply: Our goal was not to in any way imply that the effects of microplastic consumption are similar in magnitude to the effects of an increasing frequency of storms, bleaching etc associated with a changing climate. The comments concerns the small scale effects on fish of the degraded coral habitat rather than the larger scale drivers of the change from live-coral dominated to an alternative community composition. The text has been modified to clarify this point.

"Evidence from our study suggests that the consumption of microplastics may have a detrimental effect on juvenile fish that is of similar magnitude, at least over small spatial and temporal scales, to living in association with a degraded habitat."

Referee 2 comment:
My fundamental concern about the use of fresh beads rather than weathered beads is indicated below.

Referee reply – From the addition of Supplementary Part 3 I am satisfied that the leachates from beads are minimal when compared for control water. It does, however, not address the issue of how the beads fare when subjected to digestive juices (ie fresh beads versus those weathered to microplastics in the ocean).

Reply: We are happy to hear that the referee agrees that the effect of leachates would be minimal in this study. In the chemical analyses we present in the Supplementary file we have shown that the fresh beads studied were composed of only polystyrene, so the polymer is the only component in the fresh plastic bead. Under harsh acidic or basic conditions, the plastics could possibly degrade, however we believe this is not the case under gastric digestion conditions. Co-authors Vamvounis and Gulizia have performed a number of additional experiments to understand the effects of digestion methods (to remove biological matter from plastics) on polystyrene microplastics. Vamvounis and Gulizia found no effect on the molecular weight distribution of polystyrene microplastics after exposing them to nitric acid (69% nitric acid (pH <1) for up to 12 hours at 30 °C), meaning the polystyrene did not break down under those conditions (the co-authors have a manuscript in preparation). The conditions studied by Vamvounis and Gulizia was more extreme

than the acidity of gastric digestion in fishes (pH ~4) at sea surface temperatures of roughly 28 °C for 6-8h, so the beads are unlikely to degrade under the milder gastric conditions.

Weathering of microplastics can change the shape of particles and biofilms develop rapidly on particles, which are known to change their sorption of other molecules. The maximum 15 minute tank residency of the beads used in the current study is unlikely to be long enough for the biofilms that may have developed to have any significant effect on fish. We have cited Liu et al. (2020) which is an in depth review of weathering in microplastics and noted in the manuscript that understanding the role that weather plays will be a key component of understanding the effects of microplastics on marine organisms (bottom of the third-to-last paragraph of the discussion).

Referee: 1

Comments to the Author(s).
This paper describes the effects of microplastics and habitat degradation on the behaviour and survival of a coral-reef fish during a short-term field experiment. The experimental design seems sounds and the results of the study are very clear. Overall, we believe this manuscript has improved from the previous version and appreciate the authors' efforts in revising the paper based on our previous comments. In particular, the description of the supplemental feeding studies, behavioural assessments, and patch reefs are much clearer now. We have only a few places where the response to our previous comments may need a bit more explanation plus a few additional comments – please see below.
Abstract
Consider adding an opening background sentence explaining the rationale/context for the study.

Reply: Two sentences have been added to the start of the abstract. "Coral reefs are degrading globally due to increased environmental stressors including warming and elevated levels of pollutants. These stressors affect not only habitat forming organisms, such as corals, but they may also directly affect the organisms that inhabit these ecosystems."

Line 40: This study does not test the "resilience of coral-reef communities", so please delete/change this in the abstract.

Reply: the word 'community' has been exchanged with 'populations'. From a management perspective, if we reduce the number of stressors a local population is under, then it will maximise the ability of that population to respond to stressors that cannot be controlled; basically it's reducing its burden of physiological stress. We believe 'resilience' is the correct term.

Introduction
Line 70-71: It may be worth mentioning chemical cues directly here, since it seems like this is the mechanism being described.

Reply: 'alarm cue mediated…' has been added to the sentence.

Materials and methods
Lines 103- 107: The order of this paragraph was a bit hard to follow - it starts by describing the study species, then mentions the study site, then goes back to describing the study species. It may read more clearly if study location were moved to the start of the paragraph.

Reply: The paragraph has been reorganised as suggested.

Lines 124-126: The response to reviewers and new supplemental material regarding microplastic concentrations were very thorough, and it is now clear that obtaining reliable information on the availability of microplastics to coral-reef fish is extremely difficult. Whilst there is reference to the supplementary material, a sentence in the main text stating that the exact concentrations of microplastics in the field are unknown and the difficulty of microplastic measurements in the field would be beneficial and more transparent.
Also, from reading the supplement it is not clear that these microplastic concentrations are "similar to an area of reef near an urban centre", especially given that the microplastic concentrations measured in the tropics were often <0.005 p/L, compared to the 167 p/L used in this study. A similar statement that the concentrations used "…will likely be analogous to microplastic levels…" found near tropical Indo-Pacific reefs is also in the Supplement (Line 87-90). However, if most of the text argues that there is a lot of uncertainty about microplastic availability, it then seems that one cannot also know the experimental concentrations are realistic. At the least, we suggest being fully transparent in the text about this uncertainty, therefore these results provide an example of what could happen (but not necessarily what will happen).

Reply: The reviewer makes a very good point that the concentrations in nature and the availability to organisms is unknown. The concentrations in the upper water column are generally low, whilst concentrations within the sediment can be very high. Given the importance of sediment resuspension in shallow tropical waters where most of the biodiversity exists, and the high abundance of heterotrophs that feed on suspended matter in marine environments (Shurin et al. 2006), it is likely that the amount of plastic available to and locked into marine food webs is more than upper water column plankton samples would suggest. There is some suggestions that organism may also preferentially target plastics, suggesting that plastics may be ingested even at relatively low concentrations. These arguments are given in the supplementary text. However, as the reviewer points out, it is currently unknown how realistic the concentrations of plastics used in our study compare to their availability to our target fish. We have modified the last paragraph of section 2 of the Supplementary file, which provides the logic for the choice of microplastic particle concentration used in the study.

"The experimental scenario used in the present study may be analogous to the microplastic levels found near inshore fringing reefs and inshore reefs near urban areas within the tropical Indo-Pacific. However, given the current uncertainty of the availability of microplastics to specific organisms in nature, caution must be exercised in the interpretation of the results of our experiments and what they mean for fishes on reefs."

In the methods section of the main document we have added a cautionary 'may' to the statement regarding the microplastic concentration used. This same sentence points the reader to Part 2 of the Supplementary file that gives a detailed account of the logic. Discussion of this point within the main document is brief due to space constraints.

In the abstract we have removed 'reduction' from: 'Our results highlight that the reduction of microplastics in the environment…' and instead put 'Our results highlight that attaining low concentrations of microplastic in the environment…'. The latter does not assume anything about the concentrations of microplastics in the environment.

Line 130: Is there a reason for choosing this size of bead? For example, is it similar in size to food

items for these fish, or is it a size that may be particularly common in plastic pollution now or in the future?

Reply: The beads are of a size that represents a good sized food item for a small recruit fish and are of a similar size to an *Artemia* shrimp. Shrimps and copepods of this size represent an important component of their diet. Beads of this size are also commercially used in cream cleansers and facial scrubs and so enter sediments via storm water and treated effluent. This information has now been added to Supplementary Part 2.

"Microbeads of 200-300 µm were used in the current study for a number of reasons. These beads are of a similar size to a newly hatch brine shrimp (*Artemia* spp.), which was also to be used in the experiment. This size also represents the mean size of beads used in many commercial face cleansers (60 - 800 µm, mean 264 µm) [20]."

Results

Line 227-229: It seems that this line is arguing that microplastics are the dominant effect based on the difference in partial-ETA squared among the fixed effects. But based on Table S1, the tank effect is actually the largest, so this suggests that social systems (based on the response to reviewers) are more important than either stressor. At the least, this large tank effect and explanation should be acknowledged within the main text.

Reply: The large effect size has now been noted in the results. We do not find this high effect size surprising given the aggressive nature of this species to similar sized individuals (e.g., Meekan et al. 2010, McCormick 2012). We believe that the large effect size is likely an artefact of the unavoidable experimental conditions, which kept fish in a confined space with limited opportunity to avoid behavioural interaction. The analysis however, takes these experimental effects (i.e., tank effects), which are well known in ecology, into account by nesting Tank within Plastic treatment.

Sentences within the Results have been rewritten "There was a significant difference among tanks in the behavior of fish within the plastic treatments ($F_{19,14} = 2.78$, $p = 0.03$) likely due to the social hierarchies established over the confinement period and this term also had a large effect size ($\eta_p^2 = 0.8$). However, these differences were consistent among habitats (i.e., interaction: $p = 0.67$)."

Discussion

Line 268: Same as for the abstract- this study does not test resilience, and we are unsure of a direct link between the behaviour of this damselfish species and the resilience of coral reefs. So, please consider changing/removing this phrasing.

Reply: In the first sentence 'will' has been changed to 'may'. The last sentence of the first paragraph 'population' has been substituted for 'community'. We believe that the findings of the study have implications for population resilience because our data suggests that both the loss of coral and the consumption of plastics detrimentally affect these fish. If we reduce the stressful effects of plastic by reducing consumption then the fish should be better able (more resilient) to cope with natural stressors.

Line 283-285: The response to reviewers provides a lot of details about predation-induced mortality being the likely cause of mortality. Explicitly including a statement to that effect with a reference could be useful (perhaps here, where risk-taking behaviour is mentioned, but predation is not).

Reply: We have added to the sentence that the enhanced risk exposes the juveniles to predators that have high feeding rates and selective for risk-taking fish. “Fish exposed to microplastics moved further from shelter and took more risks, exposing themselves to the predators that have high feeding rates and are highly selective for juvenile fish that stray from shelter [44, 46]. This high risk behaviour dramatically reduced survival compared to fish not exposed to plastic and living on live coral.”

References used in the Response

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