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PROCEEDINGS B

Characterizing ontogeny of quantity discrimination in zebrafish

Eva Sheardown, Jose Vicente Torres-Perez, Sofia Anagianni, Scott E. Fraser, Giorgio Vallortigara, Brian Butterworth, Maria Elena Miletto-Petrazzini and Caroline H. Brennan

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Original submission:	4 March 2021
1st revised submission:	22 November 2021
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Final acceptance:	7 January 2022

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

Review History

RSPB-2021-0532.R0 (Original submission)

Review form: Reviewer 1

Recommendation
Accept with minor revision (please list in comments)

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

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

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

Is the length of the paper justified?
Yes

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

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

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

Is it accessible?
Yes

Is it clear?
Yes

Is it adequate?
Yes

Do you have any ethical concerns with this paper?
No

Comments to the Author
This clever set of experiments investigates the ontogeny of number discrimination in zebrafish from 21 days post fertilization. Original evidence is reported for the emergence of a reliable choice for the larger among two groups of conspecifics from day 27 post fertilization. New samples of fish from this age are thereafter tested in a series of numerical comparisons to assess the upper limits of this ability. Fish succeed in several comparisons, such as 1vs8, or whenever the ratio between groups was 1:2, but all experiments showed a consistent failure in the discrimination of 2vs.4 individuals. Such puzzling result is then elegantly discussed in terms of a processing limitation (of up to 5 items) of the attentional system allowing juvenile zebrafish to deal with comparisons among small numbers: the object tracking system. Alternative hypotheses are also discussed and convincingly discarded. Moreover, the approximate number system would be unable to take charge of 2vs.4 as this system would require a minimum numerical distance of 3 elements between the two sets. The idea that individual processing is involved in the discrimination of small quantities is not only sound, but it opens to intriguing manipulations and novel predictions, see for example some recent studies we carried out in few days old chicks (Rugani et al., 2017; 2020 Scientific Reports).
Overall I think this research is insightful and contributes novel evidence in the field which will be of interest to a wide audience, therefore I recommend publication. I would only point out to the authors some considerations on their control for the overall space. This condition contributes no evidence of a choice for the larger group and this null result seems difficult to be argumented. This is due to the fact that the use of individual and opaque compartments aligned one next to the other in a linear space does not offer, in my opinion, a satisfactory control for occupancy. Also it possibly provides a poor setting for the choice among what should be perceived as two groups: basically the physical and visual separation among individual fish may have altered the nature of this specific task.

Review form: Reviewer 2

Recommendation
Reject – article is scientifically unsound

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?
Marginal

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
This paper 'Characterising ontogeny of numerosity discrimination in zebrafish reveals use of multiple, numerical and non-numerical mechanisms' examines the question of how the ability to perform numerical (or rather 'quantity') discrimination develops in juvenile zebrafish. While there has exists multiple papers on numerical discrimination in both zebrafish and other fish species, few have looked at the behavior during the developmental process. The authors used a group size preference task in 4 experiments.

First, they used fish from 21-30 dpf split into 4 groups pooled across days matching for size. These groups were presented with 3 contrasts (1 vs 3, 2 vs 4, 2 vs 3). The only contrast that was successfully discriminated was 1 vs 3 for only the older two groups (26-28, 30). They then focus on older fish in the subsequent experiments. The second experiment involved fish from 27-33 dpf presented with a range of contrasts varying in difficulty using a modified version of the apparatus that could separate the stimuli fish. They found that the fish showed a significant preference only in the 1 v 8 contrast. The advantage of this modified apparatus is that the authors can control the overall space occupied by the shoal. For the third experiment, they had an uncontrolled and controlled presentation of 2 v 5, 2 v 3 and 2 v 4 contrasts to fish aged 30:33. In the uncontrolled portion, fish would discrimination 2 v 5 and 2 v 3 but there was no significant preference once overall space was made similar. Finally, the last experiment presented 30-33 dpf fish with ratios that involved slightly larger numbers (3 vs. 6) to examine how proximity to the limits of the subitizing range influences number discrimination. Fish were able to discriminate 1 v 2, 3 v 6, 2 v 5 but interestingly not 2 v 4.

As it stands, the research has several integral flaws that render it unsuitable for publication. The first major issue concerns the ability of the fish to do 'numerical discrimination' and in general, the suitability of the experimental paradigm to even study 'numerical discrimination'. Secondly, the major contribution of this paper would be the characterization of 'numerical discrimination' through development. However, this angle is not adequately explored with only 1 of 4 experiments actually containing a range of ages and there being no tracking of the use of 'multiple numerical and non-numerical mechanisms'.

MAJOR COMMENTS/CONCERNS

1. The paper is focused more on 'quantity discrimination' not 'numerical discrimination'. First, the argument for numerical discrimination presented is based on the failure of the fish to discriminate the 2 v 4 contrast even when other non-numerical information is present. Other 'easier' contrasts (2 v 3 and 2 v 5) were successfully discriminated between but only when overall space was not controlled for. There was no preference for the larger group when fish took up the same amount of space so they are unable to do the task of 'numerical discrimination'. Secondly, other non-numerical variables such as the overall size of the fish are not controlled for (such as 5 fish having the same total size as 2 fish) and are indeed very difficult to control for in these paradigms. Third, the fish don't have to distinguish a particular numerosity but are potentially making easier more than/less than judgments.

2. The major addition of this paper to the field of fish numerosity is characterizing 'ontogeny.' However, the only experiment that has a (still-limited) range of age groups is the first, non-controlled experiment. There is no hypothesized or evidenced time course for when certain 'multiple, numerical and non-numerical mechanisms' would develop and what behavioral patterns/discriminations they would be able to make with increased age due to these developments. More specific questions about the age of fish used are detailed in the below comments 4-6.

MINOR COMMENTS/CONCERNS

1. Since 'fish were pooled across days matching for size' (line 160), please write out the groups. The information is provided in the figure axis (21-22, 24-25, 26-28, 30) and would be nice provided in the procedural methods as well. Also would the last group be 29-30? Additionally, in a previous paragraph, the subjects are written as 24-33 days post fertilization (line 136).

2. Where is the supplementary figure that shows the time course of movement in the apparatus? Is there also a figure to show how the stimuli fish are mostly stationary since they were 'inserted into the stimulus compartments for 10 minutes prior to the test to control for movements?' How much movement is left and how does it differ with group size (especially of interest since discussion mentions how in motion is a cue to distinguish group size)? How close do the groups of fish end up being so that density can be used as a condition for discrimination?

3. For subjects that were removed who did 'not visit each choice area at least once (174)', did those fish stay in the middle of the compartment or only visit one side (e.g. the larger group and stayed there?). For example, would the results change if the output measure was the amount of time spent near larger group during the entire 5 minute test session (which would still be a measure of preference)? It also appears that some fish spent all of their time with the larger group (having a preference value of 1 in the figures)?

4. For the second experiment, why were only age 27-33 dpf fish used (Line 208)? Since only small numbers were used in the first experiment and the introduction talked about both small and large numerosities, it would be interesting here to see if different age groups would show different discriminations for different contrasts (e.g. 1 vs 8 vs 1 v 3). Similarly for the space control experiment, why were day 30-33 dpf fish used? Are individual fish only used once in each context or are they used multiple times over multiple days over experiments? How consistent are

they given the possibility of preference due to inter-individual differences and are there changes as they age (given that Fish ID is marked in the dataset)?

5. This also makes the following sentence in the abstract slightly misleading 'Fish showed group size preference from 26 days post fertilization (dpf) and from 27 dpf fish reliably chose the larger group when presented with discrimination ratios from 1:8 to 2:3' (Lines 72:74). It suggests that there is a behavioral change from 26 to 27 dpf while this sentence refers to separate experiments. Additionally, 'reliably choosing the larger group when presented with discimination ratios from 1:8 to 2:3' suggests that they can discriminate between ratios of that range but the data supports 1:8 in experiment 2 and 2:5, 2:3 in experiment 3.

6. Related to the above, the discussion paragraph on 'fish showed a strong preference for the larger group only in the easiest contrasts (1 vs. 8 and 1 vs.3), although we observed a tendency to join the larger group in 2 vs. 5 and 2 vs. 3. One possibility for the lack of significance in these later contrasts may be the relatively small sample size used as fish successfully discriminated 2 vs. 5 and 2 vs. 3 in subsequent experiments where a greater sample size was employed. (Lines 336:340).' However, the fish were also older in these subsequent experiments (experiment 2: (27:33) and experiment 3 (30:33)? If only the older fish 30:33 were plotted in experiment 2, would they show successful discrimination in these contrasts? Would there be a large enough sample size to do this?

7. How were the stimuli fish placed in the apparatus in Experiment 2, Figure 1c? Were they randomly placed in the compartments or lined up from one side or the middle? Dots were used to demonstrate positioning in Figure 1e and it would be useful to also have an example for Figure 1c. This is of interest since in Experiment 3, the contrast is between the fish preferences when space is controlled and non-controlled for. What is the positioning in the non-space controlled experiments (randomly, clustered, etc…)?

8. For Figure 2, the x axis is not contrast but age group? However, since the text mentions the other two contrasts and how there is no significant discrimation across age groups there, I am curious to see the data. Would it be possible to modify Figure 2 as a grouped bar plot to show all contrasts in the experiments?

9. Is there an overall effect of contrast in the controlled space experiment (Line 267)?
For consistently, please report Z and p values for 2 vs 4 (line 273).

10. For experiment 4, the procedural methods indicated that an additional contrast was presented (3 vs 9 to examine increases in numerical distance). However, this contrast appears to be missing from the statistics and figure? The 3 v 9 contrast would be interesting given the prediction that limits of the subitizing range influences number discrimation and the discussion on ratio and distance effects.

Decision letter (RSPB-2021-0532.R0)

18-Apr-2021

Dear Miss Sheardown:

I am writing to inform you that we have now obtained responses from referees on manuscript RSPB-2021-0532 entitled "Characterising ontogeny of numerosity discrimination in zebrafish reveals use of multiple, numerical and non- numerical mechanisms." which you submitted to Proceedings B. Unfortunately, on the advice of the Associate Editor and the referees, your manuscript has been rejected following full peer review. As you will see, the reviews are

somewhat different, however reviewer 2 points out a number of issues with the study that preclude me recommending it for publication. In particular, I share the reviewers' concerns about the control condition; reviewer 1 highlights some of the potentially challenges with the control, but as reviewer 2 emphasizes, the fact that the fish do not discriminate when you control for spatial distribution suggests that what you are really seeing is an ability to discriminate spatial area occupied by the fish, or quantity, but not numerosity per se. Competition for space in Proceedings B is currently extremely severe, as many more manuscripts are submitted to us than we have space to print. We are therefore only able to publish those that are exceptional, convincing and present significant advances of broad interest, and must reject many good manuscripts.

On a more positive note, based on my reading of the paper and the advice that I have received, I would like to offer you the opportunity to transfer your manuscript file to another Royal Society journal, Royal Society Open Science. Royal Society Open Science is a fast, open journal publishing high-quality research across all of science and mathematics. The journal operates objective peer review, optional open peer review, and will publish any article deemed to sufficiently advance the field by the reviewers and editors, leaving judgement of potential impact of the work to the reader. The journal publishes Registered Reports and encourages the submission of negative results. You can find out more about the scope of the journal and the benefits of publication here https://royalsocietypublishing.org/journal/rsos

If you wish to have your manuscript transferred to Royal Society Open Science please ensure that you revise your text to address all of the reviewers' comments relating to scientific soundness. Please particularly ensure that your conclusions do not overstate the results of your study. Once submitted to Royal Society Open Science your manuscript will be assessed by an Associate Editor who will decide whether further reviewer advice is required. If no further advice is needed and all of your revisions are satisfactory your manuscript will be immediately accepted for publication.

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or by clicking 'approve' or 'reject' in your Author Center.

Once you have approved the transfer you will be prompted to complete the transfer of your article via the Royal Society Open Science submission system.

Please find below the comments received from referees concerning your manuscript, not including confidential reports to the Editor. If you approve transfer to Royal Society Open Science, these reviews will accompany your paper.

Thank you for your interest in Proceedings B.

Sincerely,
Dr. Sarah Brosnan
Editor, Proceedings B
mailto: proceedingsb@royalsociety.org

Associate Editor
Board Member: 1
Comments to Author:
The manuscript has now been evaluated by two experts in the field. After careful evaluation of the reviews and referee's comments, I believe reviews raised serious concerns about the methodology and the support for the conclusions drawn.

I was particularly concerned by the major comments brought up by referee 2, explaining why he/she does not believe the claims made by the authors about number discrimination are correct. I think the concerns raised by reviewer 2 are quite concerning and significant. I also agree that the novel aspect, of studying the development of number discrimination, is only explored in a limited part of the study.

Reviewer(s)' Comments to Author:
Referee: 1
Comments to the Author(s)
This clever set of experiments investigates the ontogeny of number discrimination in zebrafish from 21 days post fertilization. Original evidence is reported for the emergence of a reliable choice for the larger among two groups of conspecifics from day 27 post fertilization. New samples of fish from this age are thereafter tested in a series of numerical comparisons to assess the upper limits of this ability. Fish succeed in several comparisons, such as 1vs8, or whenever the ratio between groups was 1:2, but all experiments showed a consistent failure in the discrimination of 2vs.4 individuals. Such puzzling result is then elegantly discussed in terms of a processing limitation (of up to 5 items) of the attentional system allowing juvenile zebrafish to deal with comparisons among small numbers: the object tracking system. Alternative hypotheses are also discussed and convincingly discarded. Moreover, the approximate number system would be unable to take charge of 2vs.4 as this system would require a minimum numerical distance of 3 elements between the two sets. The idea that individual processing is involved in the discrimination of small quantities is not only sound, but it opens to intriguing manipulations and novel predictions, see for example some recent studies we carried out in few days old chicks (Rugani et al., 2017; 2020 Scientific Reports).
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Referee: 2
Comments to the Author(s)
This paper 'Characterising ontogeny of numerosity discrimination in zebrafish reveals use of multiple, numerical and non-numerical mechanisms' examines the question of how the ability to

perform numerical (or rather 'quantity') discrimination develops in juvenile zebrafish. While there has exists multiple papers on numerical discrimination in both zebrafish and other fish species, few have looked at the behavior during the developmental process. The authors used a group size preference task in 4 experiments.

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MAJOR COMMENTS/CONCERNS

1. The paper is focused more on 'quantity discrimination' not 'numerical discrimination'. First, the argument for numerical discrimination presented is based on the failure of the fish to discriminate the 2 v 4 contrast even when other non-numerical information is present. Other 'easier' contrasts (2 v 3 and 2 v 5) were successfully discriminated between but only when overall space was not controlled for. There was no preference for the larger group when fish took up the same amount of space so they are unable to do the task of 'numerical discrimination'. Secondly, other non-numerical variables such as the overall size of the fish are not controlled for (such as 5 fish having the same total size as 2 fish) and are indeed very difficult to control for in these paradigms. Third, the fish don't have to distinguish a particular numerosity but are potentially making easier more than/less than judgments.

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MINOR COMMENTS/CONCERNS

1. Since 'fish were pooled across days matching for size' (line 160), please write out the groups. The information is provided in the figure axis (21-22, 24-25, 26-28, 30) and would be nice provided in the procedural methods as well. Also would the last group be 29-30? Additionally, in a previous paragraph, the subjects are written as 24-33 days post fertilization (line 136).

2. Where is the supplementary figure that shows the time course of movement in the apparatus? Is there also a figure to show how the stimuli fish are mostly stationary since they were 'inserted into the stimulus compartments for 10 minutes prior to the test to control for movements?' How much movement is left and how does it differ with group size (especially of interest since discussion mentions how in motion is a cue to distinguish group size)? How close do the groups of fish end up being so that density can be used as a condition for discrimination?

3. For subjects that were removed who did 'not visit each choice area at least once (174)', did those fish stay in the middle of the compartment or only visit one side (e.g. the larger group and stayed there?). For example, would the results change if the output measure was the amount of time spent near larger group during the entire 5 minute test session (which would still be a measure of preference)? It also appears that some fish spent all of their time with the larger group (having a preference value of 1 in the figures)?

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5. This also makes the following sentence in the abstract slightly misleading 'Fish showed group size preference from 26 days post fertilization (dpf) and from 27 dpf fish reliably chose the larger group when presented with discrimination ratios from 1:8 to 2:3' (Lines 72:74). It suggests that there is a behavioral change from 26 to 27 dpf while this sentence refers to separate experiments. Additionally, 'reliably choosing the larger group when presented with discimination ratios from 1:8 to 2:3' suggests that they can discriminate between ratios of that range but the data supports 1:8 in experiment 2 and 2:5, 2:3 in experiment 3.

6. Related to the above, the discussion paragraph on 'fish showed a strong preference for the larger group only in the easiest contrasts (1 vs. 8 and 1 vs.3), although we observed a tendency to join the larger group in 2 vs. 5 and 2 vs. 3. One possibility for the lack of significance in these later contrasts may be the relatively small sample size used as fish successfully discriminated 2 vs. 5 and 2 vs. 3 in subsequent experiments where a greater sample size was employed. (Lines 336:340).' However, the fish were also older in these subsequent experiments (experiment 2: (27:33) and experiment 3 (30:33)? If only the older fish 30:33 were plotted in experiment 2, would they show successful discrimination in these contrasts? Would there be a large enough sample size to do this?

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9. Is there an overall effect of contrast in the controlled space experiment (Line 267)?
For consistently, please report Z and p values for 2 vs 4 (line 273).

10. For experiment 4, the procedural methods indicated that an additional contrast was presented (3 vs 9 to examine increases in numerical distance). However, this contrast appears to be missing from the statistics and figure? The 3 v 9 contrast would be interesting given the prediction that limits of the subitizing range influences number discrimation and the discussion on ratio and distance effects.

Author's Response to Decision Letter for (RSPB-2021-0532.R0)

See Appendix A.

RSPB-2021-2544.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?
Good

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

Is the length of the paper justified?
Yes

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

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

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?
Yes

Comments to the Author
"The reviewer mentions the possibility that our setup did not allow for satisfactory control of occupancy."

I meant that the null result obtained may be due to the specificities of the apparatus employed in which the individual fish in the two sets compared could not see one another. I asked you to take this into account in the discussion.

"According to the occupancy model, each element in a set is hypothesised to have a region of influence around it and perceived numerosity is based on overlapping regions of influence of neighbouring elements. To date, clustering (total area of the regions of influence) has been found to decrease perceived numerosity in humans (Allik & Tuulmets, 1991, Percept Psychophys ; Beltramini et al., 2016, Atten. Percept. Psychophys) but its impact on quantitative abilities in animals has been poorly investigated (Beltramini et al., 2018, J. Comp. Psychol.)."

I guess you meant Bertamini et al., not Beltramini.

"Despite the present study did not control for occupancy, we believe it is unlikely that fish relied on this cue as it cannot explain the result obtained in 2 vs 3 contrast when the overall space was controlled. In fact, according to the model, the clustering would have caused stronger underestimation of the perceived numerosity in the group with three fish than in the group with 2 spatially distant fish. In this scenario, it would have been almost impossible to identify the shoal with more fish as the total area of the regions of influence in the larger shoal would have been perceived similar to the total area on the other side thus leading to a random choice. However, the significant preference we observed for the 3-fish group indicates that the subjects clearly perceived that shoal as the larger one."

I am not sure such effect would be effective for very small numerosities such as 2 vs. 3. In that case your line of reasoning would not hold.

"One more remark: if fish used other non-numerical cues (e.g. contour length) they should have been able to distinguish 2 vs 4 as well as 2 vs 3 and 2 vs 5. The consistent failure in discriminating only 2 vs 4 thus excludes that fish were only using non-numerical information, but rather suggests that fish relied on some other kind information, namely, numerical information."

Neither am I very convinced by this argumentation.

In all I simply feel the authors have missed the point in my previous review and consequently have failed to take it into account in their revised manuscript.

Review form: Reviewer 3

Recommendation
Accept with minor revision (please list in comments)

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

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

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

Is it clear?
Yes

Is it adequate?
Yes

Do you have any ethical concerns with this paper?
Yes

Comments to the Author
The Authors replied to the comments almost, but not entirely, satisfactory. I also wonder why their considerations were not inserted in the revised manuscript. Thus, I will revise the manuscript again once we will receive the revised document.
The main aspects that should be better integrated in the current version are the following:

- the use of individual and opaque compartments aligned one next to the other in a linear space does not offer, in my opinion, a satisfactory control for quantitative information. Even if the amount of movement in each compartment is limited and possibly similar, it preserves the fact that the movement of three fish is larger than the movement of two fish, additionally three fish occupy three slots, thus occupying a larger space than two fish.
Moreover, the volume surface and other physical quantitative variables that co-vary with number, are still larger in the larger group.
Thus, I find correct the sentence "Here we investigated quantitative skills of juvenile zebrafish using a 'group size preference" (lines 255 and 256). These are quantitative skills and they should be described as such in the whole manuscript, including the title.

- Although fascinating, these results are puzzling. The lack of discrimination in the 2 vs. 4 comparison while they discriminate not only between two small quantities 1 vs. 2 but also for sets which comprise a small and a large quantity 3 vs. 6. A deeper discussion though is needed (please see the previous revision).
"One more remark: if fish used other non-numerical cues (e.g. contour length) they should have been able to distinguish 2 vs 4 as well as 2 vs 3 and 2 vs 5. The consistent failure in discriminating only 2 vs 4 thus excludes that fish were only using non-numerical information, but rather suggests that fish relied on some other kind information, namely, numerical information."

I miss the logic behind this affirmation, in fact an alternative explanation can be that in all cases they are using quantitative information (but these experiments, lacking for control for possible physical quantitative information do not allow to conclude that). Moreover, using a frequentist statistical analysis does not allow to conclude anything about null results. If the Authors are interested in discussing "non-significant" results a Bayesian approach is more appropriate.

- Lines 77-78 "These findings suggest that the systems involved in numerosity representation in fish do not operate separately from other cognitive mechanisms."
This sentence is vague, please explain how this data show that the numerosity representation in fish do not operate separately from them. (If it is unfeasible in the abstract this concept need to be considered in the discussion).

Decision letter (RSPB-2021-2544.R0)

06-Dec-2021

Dear Miss Sheardown:

Your manuscript has now been peer reviewed and the reviews have been assessed by an Associate Editor. As you will see, the reviewers and the AE have raised some concerns with your manuscript and we would like to invite you to revise your manuscript to address them. In particular, both Reviewers, and in particular Reviewer 1 (the second one listed below), highlight some points that they raised earlier that were not addressed in the revision, so please be sure to carefully address these as you revise. 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.

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 this is not an acceptance pending revision, and 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,
Dr Sarah Brosnan

Editor, Proceedings B
mailto: proceedingsb@royalsociety.org

Associate Editor
Comments to Author:
I believe this is an interesting paper studying numerosity discrimination in zebrafish. The questions behind this study are important one and I believe this study could be a valuable contribution to cognitive science.

We have received reviews for the resubmitted version of this manuscript, which were in general positive. I am concerned about the points raised by reviewer 2 (again) that have not been addressed by authors. I thus encourage them to carefully review outstanding comments, adjust the manuscript and respond to referees during manuscript revision.

Reviewer(s)' Comments to Author:
Referee: 3
Comments to the Author(s).
The Authors replied to the comments almost, but not entirely, satisfactory. I also wonder why their considerations were not inserted in the revised manuscript. Thus, I will revise the manuscript again once we will receive the revised document.
The main aspects that should be better integrated in the current version are the following:

- the use of individual and opaque compartments aligned one next to the other in a linear space does not offer, in my opinion, a satisfactory control for quantitative information. Even if the amount of movement in each compartment is limited and possibly similar, it preserves the fact that the movement of three fish is larger than the movement of two fish, additionally three fish occupy three slots, thus occupying a larger space than two fish.
Moreover, the volume surface and other physical quantitative variables that co-vary with number, are still larger in the larger group.
Thus, I find correct the sentence "Here we investigated quantitative skills of juvenile zebrafish using a 'group size preference" (lines 255 and 256). These are quantitative skills and they should be described as such in the whole manuscript, including the title.

- Although fascinating, these results are puzzling. The lack of discrimination in the 2 vs. 4 comparison while they discriminate not only between two small quantities 1 vs. 2 but also for sets which comprise a small and a large quantity 3 vs. 6. A deeper discussion though is needed (please see the previous revision).
"One more remark: if fish used other non-numerical cues (e.g. contour length) they should have been able to distinguish 2 vs 4 as well as 2 vs 3 and 2 vs 5. The consistent failure in discriminating only 2 vs 4 thus excludes that fish were only using non-numerical information, but rather suggests that fish relied on some other kind information, namely, numerical information."
I miss the logic behind this affirmation, in fact an alternative explanation can be that in all cases they are using quantitative information (but these experiments, lacking for control for possible physical quantitative information do not allow to conclude that). Moreover, using a frequentist statistical analysis does not allow to conclude anything about null results. If the Authors are interested in discussing "non-significant" results a Bayesian approach is more appropriate.

- Lines 77-78 "These findings suggest that the systems involved in numerosity representation in fish do not operate separately from other cognitive mechanisms."
This sentence is vague, please explain how this data show that the numerosity representation in fish do not operate separately from them. (If it is unfeasible in the abstract this concept need to be considered in the discussion).

Referee: 1
Comments to the Author(s).
"The reviewer mentions the possibility that our setup did not allow for satisfactory control of occupancy."

I meant that the null result obtained may be due to the specificities of the apparatus employed in which the individual fish in the two sets compared could not see one another. I asked you to take this into account in the discussion.

"According to the occupancy model, each element in a set is hypothesised to have a region of influence around it and perceived numerosity is based on overlapping regions of influence of neighbouring elements. To date, clustering (total area of the regions of influence) has been found to decrease perceived numerosity in humans (Allik & Tuulmets, 1991, Percept Psychophys ; Beltramini et al., 2016, Atten. Percept. Psychophys) but its impact on quantitative abilities in animals has been poorly investigated (Beltramini et al., 2018, J. Comp. Psychol.)."

I guess you meant Bertamini et al., not Beltramini.

"Despite the present study did not control for occupancy, we believe it is unlikely that fish relied on this cue as it cannot explain the result obtained in 2 vs 3 contrast when the overall space was controlled. In fact, according to the model, the clustering would have caused stronger underestimation of the perceived numerosity in the group with three fish than in the group with 2 spatially distant fish. In this scenario, it would have been almost impossible to identify the shoal with more fish as the total area of the regions of influence in the larger shoal would have been perceived similar to the total area on the other side thus leading to a random choice. However, the significant preference we observed for the 3-fish group indicates that the subjects clearly perceived that shoal as the larger one."

I am not sure such effect would be effective for very small numerosities such as 2 vs. 3. In that case your line of reasoning would not hold.

"One more remark: if fish used other non-numerical cues (e.g. contour length) they should have been able to distinguish 2 vs 4 as well as 2 vs 3 and 2 vs 5. The consistent failure in discriminating only 2 vs 4 thus excludes that fish were only using non-numerical information, but rather suggests that fish relied on some other kind information, namely, numerical information."

Neither am I very convinced by this argumentation.

In all I simply feel the authors have missed the point in my previous review and consequently have failed to take it into account in their revised manuscript.

Author's Response to Decision Letter for (RSPB-2021-2544.R0)

See Appendix B.

Decision letter (RSPB-2021-2544.R1)

05-Jan-2022

Dear Miss Sheardown

I am pleased to inform you that your Review manuscript RSPB-2021-2544.R1 entitled "Characterising ontogeny of quantity discrimination in zebrafish" has been accepted for publication in Proceedings B.

The referee(s) do not recommend any further changes but please can you upload your data to Dryad and include the Dryad DOI in the Data Accessibility section of the submission system.

Please proof-read your manuscript carefully and upload your final files for publication. Because the schedule for publication is very tight, it is a condition of publication that you submit the revised version of your manuscript within 7 days. If you do not think you will be able to meet this date please let me know.

To upload your 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 Revision." Your manuscript number has been appended to denote a revision.

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

1) A text file of the manuscript (doc, txt, rtf or tex), including the references, tables (including captions) and figure captions. Please remove any tracked changes from the text before submission. PDF files are not an accepted format for the "Main Document".

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

3) Electronic supplementary material: this should be contained in a separate file from the main text and the file name should contain the author's name and journal name, e.g authorname_procb_ESM_figures.pdf
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 see: https://royalsociety.org/journals/authors/author-guidelines/

4) Data-Sharing and data citation
It is a condition of publication that data supporting your paper are made available. Data should be made available either in the electronic supplementary material or through an appropriate repository. Details of how to access data should be included in your paper. Please see https://royalsociety.org/journals/ethics-policies/data-sharing-mining/ for more details.

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=RSPB-2021-2544.R1 which will take you to your unique entry in the Dryad repository.

5) For more information on our Licence to Publish, Open Access, Cover images and Media summaries, please visit https://royalsociety.org/journals/authors/author-guidelines/.

Once again, thank you for submitting your manuscript to Proceedings B and I look forward to receiving your final version. If you have any questions at all, please do not hesitate to get in touch.

Sincerely,
Dr Sarah Brosnan
Editor, Proceedings B
mailto:proceedingsb@royalsociety.org

Decision letter (RSPB-2021-2544.R2)

07-Jan-2022

Dear Miss Sheardown

I am pleased to inform you that your manuscript entitled "Characterising ontogeny of quantity discrimination in zebrafish." 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.

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Your article has been estimated as being 9 pages long. Our Production Office will be able to confirm the exact length at proof stage.

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Please remember to make any data sets live prior to publication, and update any links as needed when you receive a proof to check. It is good practice to also add data sets to your reference list.

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

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

Sincerely,
Proceedings B
mailto: proceedingsb@royalsociety.org

Appendix A

Reviewer 1.

- **Comments to the Author(s)**
  This clever set of experiments investigates the ontogeny of number discrimination in zebrafish from 21 days post fertilization. Original evidence is reported for the emergence of a reliable choice for the larger among two groups of conspecifics from day 27 post fertilization. New samples of fish from this age are thereafter tested in a series of numerical comparisons to assess the upper limits of this ability. Fish succeed in several comparisons, such as 1vs8, or whenever the ratio between groups was 1:2, but all experiments showed a consistent failure in the discrimination of 2vs.4 individuals. Such puzzling result is then elegantly discussed in terms of a processing limitation (of up to 5 items) of the attentional system allowing juvenile zebrafish to deal with comparisons among small numbers: the object tracking system. Alternative hypotheses are also discussed and convincingly discarded. Moreover, the approximate number system would be unable to take charge of 2vs.4 as this system would require a minimum numerical distance of 3 elements between the two sets. The idea that individual processing is involved in the discrimination of small quantities is not only sound, but it opens to intriguing manipulations and novel predictions, see for example some recent studies we carried out in few days old chicks (Rugani et al., 2017; 2020 Scientific Reports).
- **Overall I think this research is insightful and contributes novel evidence in the field which will be of interest to a wide audience, therefore I recommend publication. I would only point out to the authors some considerations on their control for the overall space. This condition contributes no evidence of a choice for the larger group and this null result seems difficult to be argumented. This is due to the fact that the use of individual and opaque compartments aligned one next to the other in a linear space does not offer, in my opinion, a satisfactory control for occupancy. Also it possibly provides a poor setting for the choice among what should be perceived as two groups: basically the physical and visual separation among individual fish may have altered the nature of this specific task.**

We thank the reviewer for the positive comments on our manuscript. We agree that the experimental set up used does not fully mirror a condition naturally encountered in nature as zebrafish is a shoaling species that form tight swimming groups. However, a limitation of the classical setting used to measure shoal choice preference, in which stimuli are free to swim in a compartment with no internal partitions, is that multiple non-numerical variables that covary with the number of shoal members are available, thus preventing the possibility to draw any possible conclusion on the information (discrete or continuous) used for discrimination.

For this reason, here we adopted the same experimental setup and procedure that have successfully been used in literature to investigate the influence of continuous variables, such us the overall space occupied by the shoals, their densities and swimming activity on quantitative abilities in different fish species (guppies: Bisazza et al., 2010, *PLoS ONE*; mosquitofish: Dadda et al., 2009, *Cognition*; angelfish: Gómez-Laplaza and Gerlai, 2012, *Front. Comp. Psychol.*; Gómez-Laplaza and Gerlai, 2013, *Anim Cogn*).

The reviewer mentions the possibility that our setup did not allow for satisfactory control of occupancy. According to the occupancy model, each element in a set is hypothesised to have a region of influence around it and perceived numerosity is based on overlapping regions of influence of neighbouring elements. To date, clustering (total area of the regions of influence) has been found to decrease perceived numerosity in humans (Allik & Tuulmets, 1991, *Percept Psychophys* ; Beltramini et al., 2016, *Atten. Percept. Psychophys*) but its impact on quantitative abilities in animals has been poorly investigated (Beltramini et al., 2018, *J. Comp. Psychol.*).
Despite the present study did not control for occupancy, we believe it is unlikely that fish relied on this cue as it cannot explain the result obtained in 2 vs 3 contrast when the overall space was controlled. In fact, according to the model, the clustering would have caused stronger underestimation of the perceived numerosity in the group with three fish than in the group with 2 spatially distant fish. In this scenario, it would have been almost impossible to identify the shoal with more fish as the total area of the regions of influence in the larger shoal would have been perceived similar to the total area on the other side thus leading to a random choice. However, the significant preference we observed for the 3-fish group indicates that the subjects clearly perceived that shoal as the larger one. As occupancy cannot explain the result in 2 vs 3, it is implausible to think that this non-numerical cue affected performance only in some contrasts but not in others: if fish were using occupancy as a proxy for the discrimination, they should have used it in every comparison.
One more remark: if fish used other non-numerical cues (e.g. contour length) they should have been able to distinguish 2 vs 4 as well as 2 vs 3 and 2 vs 5. The consistent failure in discriminating only 2 vs 4 thus excludes that fish were only using non-numerical information, but rather suggests that fish relied on some other kind information, namely, numerical information.

Reviewer 2.

- **The paper is focused more on 'quantity discrimination' not 'numerical discrimination'. First, the argument for numerical discrimination presented is based on the failure of the fish to discriminate the 2 v 4 contrast even when other non-numerical information is present. Other 'easier' contrasts (2 v 3 and 2 v 5) were successfully discriminated between but only when overall space was not controlled for. There was no preference for the larger group when fish took up the same amount of space so they are unable to do the task of 'numerical discrimination'. Secondly, other non-numerical variables such as the overall size of the fish are not controlled for (such as 5 fish having the same total size as 2 fish) and are indeed very difficult to control for in these paradigms. Third, the fish don't have to distinguish a particular numerosity but are potentially making easier more than/less than judgments.**

We thank the reviewer for their comments on our manuscript, the points raised will help us improve the delivery of our research. Fish have been shown to discriminate number, and this is the terminology used in literature when subject has to choose between number of items (Agrillo & Bisazza, 2018, *Philos. Trans. R. Soc. B*). We agree with reviewer two on his first point that the results of experiment 3 where space is controlled for show that this task is not purely numerical, as when there is a control for overall space the discrimination of the contrasts 2 v

5 and 2 v 3 is lost. However, although the failure of the fish to discriminate 2 v 4 is not evidence for the task being purely numerical discrimination, it is evidence that numerical information is one of the cues being used to discriminate, along with other information such as overall space (line 355-364). A theory of magnitude (ATOM) proposes there is a common magnitude system for space, time and number (Walsh, 2003, *TiCS*). This theory is supported by Webers' law, which is seen in space, time and number across a range of species highlighting the similarity in cognitive processes for the three concepts with journals citing this previously. (Gibbon, 1977, *Psychol. Rev*; Cheng, 1990, *J. Comp. Physiol*; Perdue *et al.*, 2012, *Anim. Cogn*). ATOM seems to suggest that if the zebrafish are using overall space as a quantitative cue, in theory they will be using the same system as for numerical discrimination therefore they can be using both modalities, space and number. ATOM, along with our evidence that the zebrafish perform 5 vs 2 and 3 v 2 but fail with 4 vs 2 confirms that they are not using only spatial cues, as if that was the case, they should successfully discriminate 4 vs 2. We also agree that other controls of non-numerical variables could allow pinning down of exactly what information may be being used by the zebrafish to discriminate, however, as stated by the reviewer controlling overall size when using conspecifics as stimuli is difficult in this paradigm and would introduce other confounding variables such as affinity/aversion for smaller or larger conspecifics, this is known as the oddity effect. Although we have not run extensive continuous controls none of the other non-numerical explanations can account for this data with the failure of 2 v 4, as any explanation should apply to all contrasts. We have amended the discussion in the manuscript in line 295-296.

- **The major addition of this paper to the field of fish numerosity is characterizing 'ontogeny.' However, the only experiment that has a (still-limited) range of age groups is the first, non-controlled experiment. There is no hypothesized or evidenced time course for when certain 'multiple, numerical and non-numerical mechanisms' would develop and what behavioural patterns/discriminations they would be able to make with increased age due to these developments.**

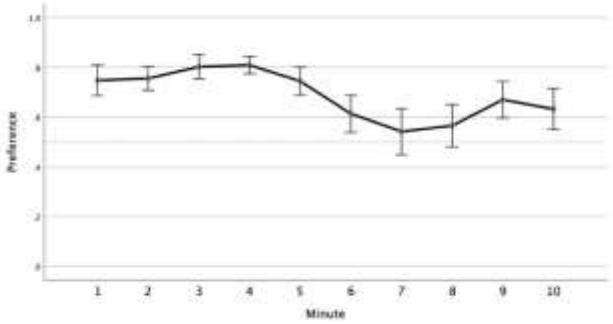
Reviewer two raises some important points about the lack of exploration of the ontogeny especially in relation to the two systems we hypothesise are at work when zebrafish are presented with small and large sets to discriminate. We thank them for this suggestion and to address this we will be repeating experiment 1 in the set up used for experiment 2-4, with age groups 21-23 dpf, 24-26 dpf, 27-29 dpf and 30-33 dpf. We will use contrasts of varying difficulty and use contrasts from the small and large numerosities (1 v 3, 2 v 5, 2 v 4, 2 v 3). This will allow us to investigate the time course of development of these different mechanisms of discrimination and if there is an ontogeny of not only ability to discriminate but also an ontogeny in the acuity of these systems. These experiments will be completed by June 4th 2021.

- **Since 'fish were pooled across days matching for size' (line 160), please write out the groups. The information is provided in the figure axis (21-22, 24-25, 26-28, 30) and would be nice provided in the procedural methods as well. Also would the last group be 29-30? Additionally, in a previous paragraph, the subjects are written as 24-33 days post fertilization (line 136).**

This experiment is no longer in the manuscript, as we repeated the ontogeny experiment using the group size preference assay. However we took the reviewers suggestion and referred to the age details of the groups in the new experiment as 'we pooled across days matching for size (21-22, 24-25, 26-28, 30 dpf).' See lines 154-155.

- **Where is the supplementary figure that shows the time course of movement in the apparatus? Is there also a figure to show how the stimuli fish are mostly stationary since they were 'inserted into the stimulus compartments for 10 minutes prior to the test to control for movements?' How much movement is left and how does it differ with group size (especially of interest since discussion mentions how in motion is a cue to distinguish group size)? How close do the groups of fish end up being so that density can be used as a condition for discrimination?**

We are sorry the reviewer did not receive the supplementary figure of the time course of movement in the apparatus as it was submitted to proceedings b but see supplementary figure below. It is not time course that is measured however but is preference for the larger group, which we found was greater in the first 5 minutes of behavioral analysis therefore we could optimize the assay and record for just 5 minutes.



We did not record the amount of motion between shoals of different sizes in experiment 1 to see that they decreased over time, this was purely an observation and a protocol that has been implemented in other open shoal assays (Gómez-Laplaza and Gerlai, 2012, *Front. Comp. Psychol*). This practice of leaving stimuli for extended time before the trial was only used in experiment 1 and as we will repeat the ontogeny experiment using the procedure in experiments 2-4 this measure of movement in the open shoal assay should no longer be pertinent, and movement in the preference procedure has been shown in similar set ups with different fish (Gómez-Laplaza and Gerlai, 2012, *Front. Comp. Psychol.;* Gómez-Laplaza and Gerlai, 2013, *Anim Cogn*)

- **For subjects that were removed who did 'not visit each choice area at least once (174)', did those fish stay in the middle of the compartment or only visit one side (e.g. the larger group and stayed there?). For example, would the results change if the output measure was the amount of time spent near larger group during the entire 5 minute test session (which would still be a measure of preference)? It also appears that some fish spent all of their time with the larger group (having a preference value of 1 in the figures)?**

This experiment is no longer in the manuscript. The subjects that were removed only visited one side of the arena so we could not conclude that they had made a choice between the two groups as they only interacted with one. This removal was only done in the open shoal set up for experiment 1, the group size preference set up had the added procedure of the holding cylinder to ensure the subject has opportunity to view both groups before making a choice. When we generated the output measure as time spent near the group during the entire 5 minutes the results did not change. If you check the dryad data set there is no individuals in experiment 1 (Fig. 2) that had a preference of 1, some have 0.98 which may appear as 1 in the graph, but the absolute value is less than 1. As we don't have this exclusion criteria in the further experiments, due to the holding cylinder, there will be preference values of both 1 and 0.

- **For the second experiment, why were only age 27-33 dpf fish used (Line 208)? Since only small numbers were used in the first experiment and the introduction talked about both small and large numerosities, it would be interesting here to see if different age groups would show different discriminations for different contrasts (e.g. 1 vs 8 vs 1 v 3). Similarly for the space control experiment, why were day 30-33 dpf fish used? Are individual fish only used once in each context or are they used multiple times over multiple days over experiments? How consistent are they given the possibility of preference due to inter-individual differences and are there changes as they age (given that Fish ID is marked in the dataset)?**

We used 27-33dpf fish in experiment 2 as the ontogeny work from experiment 1 informed us they showed preference from at least 26 dpf so any age above this suitable for our set up was used (26-33dpf). The questions posed regarding use of small and large numerosities with ontogeny are again interesting and should be addressed with the ontogeny experiment we have outlined. The testing equipment was only free on the weekend when performing our space control experiment which led to the use of fish only in the 30-33dpf age range.

Since a free-choice test is based on the spontaneous tendency to join the larger shoal when a single fish is placed in an unfamiliar environment, the fish should not be re-tested otherwise, they might perceive the experimental tank as a partially familiar environment and might be less motivated to join the social companions. For this reason, individual fish were only used once, as stated in line 137-138, consistent with a plethora of studies on numerical abilities in fish that used the spontaneous choice test (Agrillo et al., 2012, *PLoS ONE*; Gómez-Laplaza and Gerlai 2013*, Anim Cogn*. Mehlis et al., 2015, *Anim. Cogn*).

Fish ID is marked in the data set only to track trial number not to track individuals, it's just the name assigned by ethovision. We discuss how individual differences can cause variation in but we don't know how consistent they are and if this changes with age. Others have found social preference (Dresoti et al., 2015, *Front. Neural Circuits*) and doesn't change with age but other fish species ability to discriminate numerosity changes with age.

- **This also makes the following sentence in the abstract slightly misleading 'Fish showed group size preference from 26 days post fertilization (dpf) and from 27 dpf fish reliably chose the larger group when presented with discrimination ratios from**

1:8 to 2:3' (Lines 72:74). It suggests that there is a behavioral change from 26 to 27 dpf while this sentence refers to separate experiments. Additionally, 'reliably choosing the larger group when presented with discimination ratios from 1:8 to 2:3' suggests that they can discriminate between ratios of that range but the data supports 1:8 in experiment 2 and 2:5, 2:3 in experiment 3.

We thank the reviewer for pointing out this issue with the wording in the abstract, the abstract will be re-vised once we have collected the data from the proposed ontogeny experiment.

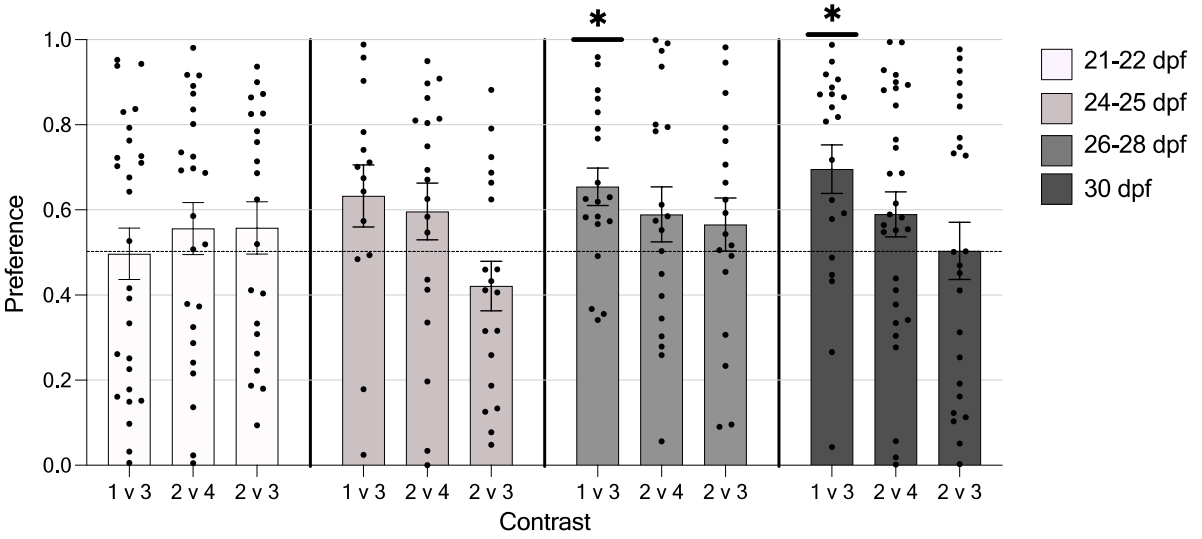
- **Related to the above, the discussion paragraph on 'fish showed a strong preference for the larger group only in the easiest contrasts (1 vs. 8 and 1 vs.3), although we observed a tendency to join the larger group in 2 vs. 5 and 2 vs. 3. One possibility for the lack of significance in these later contrasts may be the relatively small sample size used as fish successfully discriminated 2 vs. 5 and 2 vs. 3 in subsequent experiments where a greater sample size was employed. (Lines 336:340).' However, the fish were also older in these subsequent experiments (experiment 2: (27:33) and experiment 3 (30:33)? If only the older fish 30:33 were plotted in experiment 2, would they show successful discrimination in these contrasts? Would there be a large enough sample size to do this?**

This suggestion by reviewer 2 about age resulting in the preference for 2 v 3, and 2 v 5 seen in experiment 3, rather than increased n is interesting. The n number in our data from experiment 3 is not large enough to separate by age and see significance, however, by performing our repeated ontogeny experiment with contrasts such as 2 v 5 and 2 v 3 in an age range from 21-33 dpf we should be able to answer this question.

- **How were the stimuli fish placed in the apparatus in Experiment 2, Figure 1c? Were they randomly placed in the compartments or lined up from one side or the middle? Dots were used to demonstrate positioning in Figure 1e and it would be useful to also have an example for Figure 1c. This is of interest since in Experiment 3, the contrast is between the fish preferences when space is controlled and non-controlled for. What is the positioning in the non-space controlled experiments (randomly, clustered, etc…)?**

Below is the requested figure that has been added to figure 1, to replace panel c. The stimuli were always lined up from the middle as can see in the figure.

- **For Figure 2, the x axis is not contrast but age group? However, since the text mentions the other two contrasts and how there is no significant discrimation across age groups there, I am curious to see the data. Would it be possible to modify Figure 2 as a grouped bar plot to show all contrasts in the experiments?**



As requested by reviewer two, here is the modified version of figure 2 showing the ontogeny preference for 1 v 3, 2 v 4 and 2 v 3 in the open shoal assay. This figure will be repeated using the new set up and a greater age range of fish in the repeated ontogeny experiment.

- **Is there an overall effect of contrast in the controlled space experiment (Line 267)? For consistently, please report Z and p values for 2 vs 4 (line 273).**

There was no overall effect of contrast in the controlled experiment ($\chi^2(1) = 0.313$, $p = 0.855$). Now we have analysed the One sample Wilcoxon signed rank tests in graphpad prism we do not get Z statistics, however, we have added the p values for 2 v 4 in line 239. 'One sample Wilcoxon signed rank tests showed a statistically significant preference for the larger group when compared to chance only in the non-controlled groups for 2 vs. 5 ($p = 0.025$) and 2 vs. 3 ($p = 0.013$) but not 2 vs. 4 ($p > 0.99$) (Figure 2b)'.

- **For experiment 4, the procedural methods indicated that an additional contrast was presented (3 vs 9 to examine increases in numerical distance). However, this contrast appears to be missing from the statistics and figure? The 3 v 9 contrast would be interesting given the prediction that limits of the subitizing range influences number discrimation and the discussion on ratio and distance effects.**

We thank the reviewer for noticing this mistake in line 245, the contrast 3 v 9 is not possible in our experimental set up and was mistyped, it should have been 3 v 6. We have now corrected this 'when the numerical distance was increased from 1 to 3 (1 : (1 vs. 2), 2 : (2 vs. 4) or 3 : (3 vs. 6))'.

Appendix B

We thank the editor and reviewers for their comments on our new manuscript we resubmitted. See below our response to the reviewers' comments on the resubmitted manuscript. The changes made are listed with line and page number, and have been tracked in the manuscript for reviewers ease.

Referee: 3

Comments to the Author(s).
The Authors replied to the comments almost, but not entirely, satisfactory. I also wonder why their considerations were not inserted in the revised manuscript. Thus, I will revise the manuscript again once we will receive the revised document.
The main aspects that should be better integrated in the current version are the following:

We apologise that the reviewer did not receive the marked-up text. We were told to submit this as a new manuscript.

- the use of individual and opaque compartments aligned one next to the other in a linear space does not offer, in my opinion, a satisfactory control for quantitative information. Even if the amount of movement in each compartment is limited and possibly similar, it preserves the fact that the movement of three fish is larger than the movement of two fish, additionally three fish occupy three slots, thus occupying a larger space than two fish.
Moreover, the volume surface and other physical quantitative variables that co-vary with number, are still larger in the larger group.
Thus, I find correct the sentence "Here we investigated quantitative skills of juvenile zebrafish using a 'group size preference" (lines 255 and 256). These are quantitative skills and they should be described as such in the whole manuscript, including the title.

We have now accordingly modified the manuscript throughout as suggested by the reviewer.

- Although fascinating, these results are puzzling. The lack of discrimination in the 2 vs. 4 comparison while they discriminate not only between two small quantities 1 vs. 2 but also for sets which comprise a small and a large quantity 3 vs. 6. A deeper discussion though is needed (please see the previous revision).

We have now expanded the discussion regarding the failure of discrimination of 2 vs. 4 as per previous comments from the reviewer: (Page 10 and 11, lines 300- 315 and page 11 lines 315-329 (where we discuss ATOM)).

We have now explained in more detail why neither the noise nor the threshold hypothesis can explain the consistent failure in 2 vs 4 (line 373- 378 page 12)

"One more remark: if fish used other non-numerical cues (e.g. contour length) they should have been able to distinguish 2 vs 4 as well as 2 vs 3 and 2 vs 5. The consistent failure in discriminating only 2 vs 4 thus excludes that fish were only using non-numerical information, but rather suggests that fish relied on some other kind information, namely, numerical information."
I miss the logic behind this affirmation, in fact an alternative explanation can be that in all

cases they are using quantitative information (but these experiments, lacking for control for possible physical quantitative information do not allow to conclude that). Moreover, using a frequentist statistical analysis does not allow to conclude anything about null results. If the Authors are interested in discussing "non-significant" results a Bayesian approach is more appropriate.

We have modified the discussion indicating that the fish were unable to perform the discrimination based on numerical information alone and discussed the issue of other continuous variables (lines 300-305).

- Lines 77-78 "These findings suggest that the systems involved in numerosity representation in fish do not operate separately from other cognitive mechanisms."
This sentence is vague, please explain how this data show that the numerosity representation in fish do not operate separately from them. (If it is unfeasible in the abstract this concept need to be considered in the discussion).

We have now amended the sentence in the abstract (line 77-79 page 3) indicating that our studies are consistent with work of others in other species and expanded the discussion of this point (lines 388-394 page 13).

Referee: 1

Comments to the Author(s).
"The reviewer mentions the possibility that our setup did not allow for satisfactory control of occupancy."

I meant that the null result obtained may be due to the specificities of the apparatus employed in which the individual fish in the two sets compared could not see one another. I asked you to take this into account in the discussion.

The reviewer suggested that the use of individual and opaque compartments aligned one next to the other possibly provides a poor setting for the choice among what should be perceived as two groups: basically, the physical and visual separation among individual fish may have altered the nature of this specific task.

We acknowledge the experimental set up does not fully replicate an ecological context as, in nature, fish can interact with each other.

We have now added a sentence in the revised MS about the possibility that the experimental set up, despite being successfully used in other studies, may have affected fish performance (line 286-295 Page 10).

"According to the occupancy model, each element in a set is hypothesised to have a region of influence around it and perceived numerosity is based on overlapping regions of influence of neighbouring elements. To date, clustering (total area of the regions of influence) has been found to decrease perceived numerosity in humans (Allik & Tuulmets, 1991, Percept Psychophys ; Beltramini et al., 2016, Atten. Percept. Psychophys) but its impact on quantitative abilities in animals has been poorly investigated (Beltramini et al., 2018, J. Comp. Psychol.)."

I guess you meant Bertamini et al., not Beltramini.

Thank you for identifying this typo, we have now corrected it.

"Despite the present study did not control for occupancy, we believe it is unlikely that fish relied on this cue as it cannot explain the result obtained in 2 vs 3 contrast when the overall space was controlled. In fact, according to the model, the clustering would have caused stronger underestimation of the perceived numerosity in the group with three fish than in the group with 2 spatially distant fish. In this scenario, it would have been almost impossible to identify the shoal with more fish as the total area of the regions of influence in the larger shoal would have been perceived similar to the total area on the other side thus leading to a random choice. However, the significant preference we observed for the 3-fish group indicates that the subjects clearly perceived that shoal as the larger one."

I am not sure such effect would be effective for very small numerosities such as 2 vs. 3. In that case your line of reasoning would not hold.

According to Kramer et al. (PLoS ONE, 2011), occupancy seems to be effective also for small quantities. However, we have now added occupancy as a possible non-numerical information that may have been used to discriminate between the two shoals (line 296-315, Page 10 and 11)

"One more remark: if fish used other non-numerical cues (e.g. contour length) they should have been able to distinguish 2 vs 4 as well as 2 vs 3 and 2 vs 5. The consistent failure in discriminating only 2 vs 4 thus excludes that fish were only using non-numerical information, but rather suggests that fish relied on some other kind information, namely, numerical information."

Neither am I very convinced by this argumentation.

In all I simply feel the authors have missed the point in my previous review and consequently have failed to take it into account in their revised manuscript.

We have now softened the discussion based on yours and Reviewer's 3 concern about this point. We have modified the discussion indicating that the fish were unable to perform the discrimination based on numerical information alone and discussed the issue of other continuous variables (lines 296-315, Page 10 and 11). We have also revised the whole manuscript focusing on quantitative skills rather than numerical abilities.