Passo Fundo, June 21, 2017

**Dr. Angelo Piato**

**Academic editor, Peer J**

June 21, 2017

Dear Angelo Piato,

We have been submitting the revised version of our manuscript entitled “Stress responses to conspecific visual cues of predation risk in zebrafish”, by Oliveira et al. for reconsideration for publication. We appreciate the opportunity to revise our manuscript. We also thank the two expert Referees and Editor for their time and positive evaluation of our study. We believe that the points raised by the referees have been adequately addressed in this revised version. Please find below a point-by-point reply to the Reviewers’ evaluation.

**Reviewer 1 (Anonymus)**

Basic reporting

Although the manuscript has been proof read by a native English speaking coleague, I do feel the document could use another proof reading to correct some sentence structures and remove grammatical errors.

***Reply:*** *We sent the manuscript to language revision by a fluent English-speaking colleague.*

For example - line 46-48 could use a rewrite. In addition, I am not sure if the word ‘subjugated’ is the proper term to use in this context.

***Reply:*** *We rewrite the sentence and changed the word ‘subjugated’. Please see lines 48-50.*

The manuscript seems to contain sufficient background information and is well supported with literature references. The manuscript is conform the correct layout of the journal (Single File, Figures embedded in text). The authors also supplied the raw data and statistics.

***Reply:*** *We thank the reviewer for this positive evaluation of our MS.*

The manuscript holds a plausible hypothesis, as the authors wish to explore to presence of visual eavesdropping when it comes to predator-induced behaviour. However, the authors suggest this information could be used in experiments towards Autism Spectrum Disorder for example. I would have liked to see a paragraph in the discussion that links back to how their findings would fit into such experiments (or if more experiments are needed to do so).

***Reply:*** *Thank you for this comment. We agree that our experiments are very preliminary to affirm its correlation with ASD. Thus, we deleted all mentions about this topic in the manuscript.*

Experimental design

The research provided in the manuscript is original and fits within the scope of the journal. The authors have clearly stated a research question in their Introduction. However, the relevance of their study feels lacking. There is mention of use in studies towards specific drugs and/or Autism Spectrum Disorder, but these examples are only mentioned. I would like to see how their study (visual eavesdropping of predatory-induced behaviour causes increased cortisol levels and predatory-induced behaviour in observer fish) fits into (future) studies on the mentioned topics.

***Reply:*** *Thank you for this comment. We agree that our experiments are very preliminary to affirm its correlation with ASD. Thus, we deleted all mentions about this topic in the manuscript.*

The experiment appears to be conducted in accordance with local ethical laws towards the handling of animals. However, the experiment seems lacking in the technical area. Whether this is due to insufficient elaboration on the methods, is unclear. I will therefore state my concerns with the experimental approach.[1] I understand that all experiments were conducted in triplicate (based upon the total number of fish used and the number of fish per group). Please state this clearly in the Material and Methods section.

***Reply:*** *Thank you for this comment. We corrected the sentence about this method. Please see line 125.*

[2] I am confused on how cortisol values were determined. The text states:“In each group, 10 fish were used at each sampling time, forming 5 pooled samples of 2 fish each.” What sampling times are the authors referring too? I understood that each group was only 10 fish in size, thus all fish in a group were used for cortisol analysis? Why did the authors pool the material of two fish into a single sample point?

*Reply: Thank you for this comment. In fact, our sentence was confused and we clarified it. . To extract and determine the whole-body cortisol we used all 10 fish of each experimental group of each replicate. There is a variation among cortisol “n” samples because whole-body cortisol analysis requires a minimum of 0.5g of tissue extract, so when fish did not reach this weight, we used pooled samples of two or three fish. We insert the “n” number in the figure legend. In relation to the sample time, it was the same for all animals collected for cortisol analysis, considering de initiation of exposure to a predator or to a conspecific fish or even to an empty tank.*

How many sample points are there in total per group? (5 sample points per replicate, 3 replicates = 15 data points?)

*Reply: Thank you for this comment. In fact, as depicted in the “Experimental design and procedures” subsection, we had just one single sample point: 60 minutes after the initiation of the contact among fish according to the respective group. All fishes of each group were collected at the same time, observing that the time of initiation of the collection and the finished of the procedure didn´t exceeded 30 seconds. This was made in order to avoid effects of manipulation on the cortisol secretion.*

[3] Regarding the video analysis. Why did the authors not split their video analysis in different time periods to get a better indication on habituation to the ‘predator’ tank? The authors did mention in their discussion, that the data on the behavioural response tothe gold fish might have been ‘polluted’ by an initial ‘false’ predator-induced behavioural response in the first 5 minutes. I strongly suggest re-analysing the data (since it was recorded) to show behavioural changes over time.

*Reply: Thank you for this comment. In fact, as depicted in the “Experimental design and procedures” subsection, we recorded and evaluated the entire period of 60 minutes. To prevent this confusion, we deleted our observation of zebrafish reaction in the initial 5-minutes period. In fact, this is an observation made by only one member of the research group and needs to be better explored. This will be focused in the future studies on this topic.*

Validity of findings

The study seems novel in its findings and adds to the previous reports of eavesdropping in zebrafish. I have a few major concerns with the statistics in this study and I would like the authors to elaborate more on their choice of statistical analysis. [1] Please include the results for ANOVA and/or Kruskal Wallis tests in the figures (or legends: p-value, df, F-value). Also include the total number of data points per group.[2] Why did the authors chose to display S.E.M. and not S.D. in the graphs? And have the authors considered presenting their data as individual dots or box-plots (instead of a bar graph). This would provide a lot more information on the spread and distribution of the samples.[3] Seeing how one of the analysis required a Kruskal Wallis, were there signs of two groups within the population (see point 2 on individual data points).[4] Why did the authors analyse their data with an ANOVA/Kruskal Wallis test? Would other statistical approaches not be better suited to analyse the data-sets? By using an ANOVA, the authors also compare groups that have no need for comparison (for example: the RF fish in the Empty Tank experiment, compares to SF fish in the Predator Experiment). By adding these comparisons, the power of the statistical test goes down. In addition, when comparing all groups like the authors have done, would a two-way ANOVA not be the correct analysis to use (there are two variables that are being compared: Group of Zebrafish and Contents of Experiment Tank). I think the authors should ask themselves what it is they wish to show with their data and match the statistical analysis to that specific question. To me, it appears they wish to show the following: Is there a change in behaviour and physiology in SF fish when exposed to a predator or a non-predator fish [Does our experimental setup work? -> analyse SF control, predator and non-predator].

*Reply: Thank you for this comment. We inserted the statistics values in the text of the results section, but in figures captions maintain only the indications of P values of each comparison using the asterisks keys. Regarding points [2] and [3] we changed the panel A of the figure, since data were not normal. Thus, we used the non-parametric test (Kuskal Wallis) and changed the figure to represent as individual dots and median ± interquartile range. In the panel B, since the data distribution was normal, we used the One-Way ANOVA and opted to maintain the mean ± S.E.M. and the bars. Regarding the point [4] we did not used a two-way ANOVA since we not aimed to evaluate the interaction between the presence of a predator or not and the empty compartments were used only in the additional experiments, proceeded only as a control of our experimental aquaria setup and not a treatment to be analysed and compared. Based on these controls we feel secure that our experimental setup works. Therefore, we opted to maintain the present statistical analysis.*

[5] I suggest changing the way statistical significance is displayed in the figures. As it is now, it is very confusing. Use letters to indicate significantly different groups (different letter). Use capital letters and lowercase letters to seperate groups within a single figure (for example ‘AAB’ for an ANOVA done across the RF fish and ‘abb’ for an ANOVA done across the SF fish).

*Reply: Thank you for this comment. The figure layout was really unclear. We changed the figure to include letters as statistical indication of significance. We hope it is clearer.*

The conclusions presented in the manuscript are not always backed up by the data. This may in part be due to the lack of clear research questions that are answered by the data (see previous statement on statistical analysis). In this regard, it would be good to split the discussion into sections (one section for each question answered by the data and statistical analysis).

*Reply: Thank you for this comment. We disagreed with the writing style of itemized discussion, since the text lacks its cohesion and readability.*

In its current form, I have some concerns with the following conclusions:

LINE 251-252. the authors state that RF fish in the non-predator experimental conditions had no changes in cortisol and behaviour. However, this appears to be only true for cortisol values? Their behaviour appears significantly different from the control group?LINE 269-271: But RF fish DID change their behaviour (compared to controls). However, cortisol levels were not different

*Reply: Thank you for this comment. Our former text was wrong; we changed this sentence to clarify this issue: “In the non-predator treatment, both sender and receiver fish had an augmented time spent near the tank bottom when compared with the control group (non-stimulus fish). Even though this response was smaller when compared with the predator exposure treatment, being the response of the SF- non predator higher when compare with his respective RF.”*

LINE 265-268: Split the video data? Analyse first 5 minutes and last 5 minutes to show changes in behaviour during the 1h exposure period? See previous comments on Experimental Design.

*Reply: Thank you for this comment. As answered above, we record and evaluated the entire period of 60 minutes. In the future experiments we will split the videos in pre-determined periods. We recognize that this issue could be a limitation of our work, but this is not lessening the relevance of our initial assessment.*

LINE 265-271: The authors state that the changes in behaviour and cortisol might be due to ‘misinterpretation’ of the goldfish by the SF fish. The authors refer to other cues (such as smell and sound) that could have informed the zebrafish that the gold fish is not a predator and thus the absence of these cues may have caused the change in behaviour and physiology. However, it should be noted that the authors only analysed position within the tank (scoring time spent at the bottom) of the zebrafish. It is possible that the SF fish display other types of behaviour that informs the RF fish of the presence of another non-predatory fish. The lack of a cortisol response in the RF group suggests there is a difference in the behaviour of the SF predator and non-predator group. Perhaps examining the recordings may give more insight.

*Reply: Thank you for this comment. We did not evaluated other behavioural parameters since the videos were manually analysed. Maybe if we have used a behavioural analysing software, we can track more parameters. However, since fish were evaluated in groups of ten and the more accessible and used software (AnyMaze®) analyse only individual fish, we opted to evaluate only the relative time spent at the tank bottom only with visual/manual assessment. In addition, the experimental aquaria setup its very difficult to drawn and analyse in the AnyMaze software. In the future projects, we will try to acquire a software to analyse groups of fish (Noldus Ethovision for example). We also recognize that this issue is a limitation of our work but, as the stated for the periods, this not lessen the relevance of our initial assessment.*

LINE 272-275: Please elaborate what is meant by ‘others visual stimulus’. Although true that their behaviour and physiology did change in response to a gold fish, there is no evidence that similar changes occur when exposed to ‘other visual stimulus’ - this response may be specific for exposure to other fish species?

*Reply: Thank you for this comment. We insert in the text, some examples of visual stimuli, as “May be others visual stimulus,(e.g. humans presence, other fish species or even other animals) can be interpreted like a threatening, having effects on the behavioral and hormonal patterns in zebrafish and consequently influencing experiments' results.”*

LINE 277-282: I agree with this conclusion. Experiments that alter group behaviour in fish should be done out of sight of other experimental groups of fish. These data indicate effects on behaviour and physiology as a result of eavesdropping.

*Reply: Thank you for this comment.*

**Reviewer 2 (MichailPavlidis)**

Comments for the Author

The manuscript brings an interesting data-set that adds to the recent findings on eavesdropping in zebrafish. However, I do feel that the authors have not gotten the most out of the results that they have found. Specifically the approach in analysing the behavioural data as well as performing statistical analysis on their data could provide more (and important) information that will strenghten the manuscript. I have advice a major revision of the manuscript, as I feel some new data needs to be generated (fairly easy to do, as the raw material is available) and the analysis of the data needs to be expanded. In addition, the manuscript as a whole would become better when the finds from the data are split into seperate research questions. I have provided my thoughts and suggestions on how to improve the manuscript in each of the section boxes above.

*Reply: Thank you for this comment.We expend all our best efforts to answer all reviewer’s issues.*

Basic reporting

There are a lot of typing, syntax and grammar errors, throughout the text. The authors are encouraged to get editing help from someone with full professional proficiency in English.

*Reply: Thank you for this comment. We sent the MS to language revision by a fluent English-speaking colleague!*

Introduction should be improved by focusing on the subject (i.e. general and specific cues for predator detection; visual cues and anti-predator behavior) and by adding relevant literature on the field (e.g. Wisenden et al., 2004. Animal Behavior, 67: 59-67; Holms &McCormic, 2010; Dunlop-Hayden &Rehage, 2011. Behaviour, 148:795-823; O’Conor et al., 2015. Behavioural Processes, 121: 21-29 etc.)

*Reply: Thank you for this comment. these topics were covered and supported by strong literature in the text of introduction. However, we agree with your comment that some points were not covered and thus inserted a paragraph into introduction, as:“Prey-predator interaction occurs throughout the animal kingdom (Cresswel, 2010) with peculiar and general characteristics in every interaction. We can say that this interaction occurs in every direction (prey to predator, predator to prey, prey to prey and even predator to predator)(Barcellos et al., 2014, Mullan et al., 2015; Dunlop-Hayden &Rehage, 2011). The prey can access the predator trough a diversity of signals that can be visual, olfactory, acous-tic, vibration, (Barcellos et al., 2014, Barreto et al., 2003). In fish, we have a vast literature citing these types of perceptions (Wisendem et al., 2004, Barcellos et al., 2011) of a predator or even of a conspecific fish treated by a predator (Jordão, 2000). The different combination of this different signals of the presence of a predator, or even about diverse ways of communication about predator threatening, between conspecifics prey, have different effects in anti-predator maneuvers (O`Connor et al., 2015).*”

Experimental design

The submission is in line with the aims and scope of the Journal. The research question is clearly defined and Figure 1 (schematic representation of the experimental conditions) is very useful to understand and evaluate the experimental procedure. It is recommended to add information on the size and sex of fish used, as well as on the dimensions of the experimental chambers – in relation to the stocking density (number of fish per L).

***Reply:*** *Thank you for this comment. As answered above, we inserted these information in the text. Please see lines115-166 and 120-125.*

The authors should explain why they include only one parameter (time spent near the tank bottom) for behavioral quantification of fear behavior and did not use other parameters too, like shoal cohesion, faster swimming with spontaneous rapid turns, increased frequency and length of freezing bouts etc. (see Egan et al., Behav. Brain Res., 2010).

*Reply: Thank you for this comment. As answered above we did not evaluated other behavioural parameters since the videos were manually analysed. Since fish were evaluated in groups of ten and the more accessible and used software (AnyMaze®) analyse only individual fish, we opted to evaluate only the relative time spent at the tank bottom only with visual/manual assessment. In addition, the experimental aquaria setup (L-shaped aquarium) it’s very difficult to drawn and analyse in the AnyMaze software.*

Validity of the findings

There is a major concern on the statistical analysis. The authors should apply logarithmic transformation of cortisol data to check for the normality criterion. In addition, multivariate analysis of variance (MANOVA) should be applied and/or Two-way ANOVA to quantify/specify the difference among the experimental groups [Factor 1: fish status (sender – receiver); Factor 2: Trial (Predator, Non-predator, Non stimulus).

*Reply: Thank you for this comment. For cortisol comparison, we used the non-parametric test (Kuskal Wallis) and changed the figure to represent as individual dots and median ± interquartile range. In the panel B, since the data distribution was normal, we used the One-Way ANOVA and opted to maintain the mean ± S.E.M. and the bars. We did not used a two-way ANOVA since we not aimed to evaluate the interaction between the presence of a predator or not and the empty compartments were used only in the additional experiments, proceeded only as a control of our experimental aquaria setup and not a treatment to be analysed and compared. Thus, we opted to maintain the present statistical analysis.*

The authors should provide an explanation on why:

(a) receiver – non-predator exposed fish have statistically lower whole-body-cortisol concentrations than sender-non-predator fish).

*Reply: Thank you for this comment. In fact, we did not have an empirical bases to this explanation. The cortisol value of SF exposed to non-predator fish was higher, but the behaviour (time in the tank bottom) was similar. Thus, since RF receive only visual cues, this similar behaviour cannot elicit a cortisol response.*

(b) non-stimulus fish have quite higher cortisol concentrations from the basal values reported in other published studies on zebrafish.

*Reply: Thank you for this comment. These discrepancies may be due to different experimental aquaria (L-shaped), dimensions and the management of the opaque partition. Another reasons as previous history, strain, origin and management at pet stores, may be caused this different values.*

Finally, the authors should check the appropriateness of ANOVA for percentage comparisons of the behavioral parameter “time spent near the tank bottom)…

*Reply: Thank you for this comment. Since we used a mean of different percentile data, we feel secure that our data meets the ANOVA criteria. Thus, we opted to maintain the statistical analyses used.*

…and to add date on the number of fish that were located near by the bottom of the tank per session.

*Reply: Thank you for this comment. In fact we only count the time when three fish are near to the bottom, however, since fish actively swan, we not specify the exact number of fish.*

Comments for the Author

The authors use the terms "sender" and "receiver" fish. Is this an established / common accepted term or just a neologism?

**Reply:***Reply: Thank you for this comment. They are well-established terms and this nomenclature was similar to used in our previous previous works (Barcellos et al., 2014. Sci. Rep. 4: 5076. Barcellos et al.,2011. Physiol. Behav. 103: 372-375). Even though in previous works we used the terms donor fish for actually we consider sender fish, and receptor fish for actually we consider receiver fish. In these context we assumed this terms because donator fish communicated his conspecific about threatening, releasing chemical cues in to the water. In present study this “donation” doesn´t occur.*

Overall, the authors believe that all points raised by the expert reviewers and editor have been thoroughly addressed in this revised MS. We hope that this revised MS can now be accepted for publication in your Journal.

Sincerely,

Leonardo Barcellos