**Review Report Form 1**

English language and style

( ) Extensive editing of English language and style required
( ) Moderate English changes required
(x) English language and style are fine/minor spell check required
( ) I don't feel qualified to judge about the English language and style

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| --- | --- | --- | --- | --- |
|  | Yes | Can be improved | Must be improved | Not applicable |
| Does the introduction provide sufficient background and include all relevant references? | (x) | ( ) | ( ) | ( ) |
| Is the research design appropriate? | (x) | ( ) | ( ) | ( ) |
| Are the methods adequately described? | (x) | ( ) | ( ) | ( ) |
| Are the results clearly presented? | (x) | ( ) | ( ) | ( ) |
| Are the conclusions supported by the results? | ( ) | (x) | ( ) | ( ) |

Comments and Suggestions for Authors

The authors provided sufficient and careful responses to most of my technical comments. However, two items from the previous review remain to be addressed further.

1) The study on behavioral effects of donepezil in zebrafish must critically draw upon the only previous work that recently examined this drug CNS effects in zebrafish. This recent study by Giacomini et al 2020 (in press, J of Psychopharm), has tested 24-h DPZ in adult zebrafish, yielding anxiogenic-like effects of this drug, paralleled by analyses of AChE activity in the brain.  The authors noted difficulty in finding the recent study mentioned by the reviewer**. I am attaching here the details of this pertinent study DOI: 10.13140/RG.2.2.31376.64006 Giacomini et al. An acetylcholinesterase (AChE) inhibitor donepezil increases anxiety and cortisol levels in adult zebrafish.** Accordingly, the discussion needs to put the present study in the context of prior literature.

Thank you very much for providing more detailed information about the previous work in the acute exposure effect of DPZ in adult zebrafish. The authors already read the report thoroughly and carefully. As the reviewer suggested, the discussion regarding the result of the prior study and the current study was added to the manuscript, especially in the discussion part. The possible reason caused the difference in the present study results and previous work results were described in the manuscript.

2) This reviewer asked to provide statistical power calculationto ensure and justify that the study is not under-powered. The authors replied citing several other studies that have similar n's. However, this is not sufficient to address the question about this particular study. Please provide statistical power analysis for this study and justify in the methods section the selected sample size. Please consult expert biostatisticians, if necessary, to ensure the correctness of such analyses.

The authors appreciated the suggestion and understood that by citing several other studies that have similar n’s is not enough to address the question about this particular study. Thus, the authors tried their best to provide the statistical power calculation to ensure that the study is not underpowered. From the statistical power calculation with 90% confidence interval and a margin of error of 7 units, it was found that the sample size of 25 is needed, which was similar to n number applied in the current study. All of the explanation regarding the statistical power calculation was added to the manuscript, specifically in the Experimental Methodology part and supplementary data. The authors hoped that this revision is in agreement with the reviewer’s suggestion. If it is not, it will be very helpful for the authors if the reviewer showed some of the prior studies in this field that provided a statistical power calculation.

Submission Date 13 June 2020

Date of this review 13 Aug 2020 20:00:27

**Review Report Form 2**

English language and style

( ) Extensive editing of English language and style required
(x) Moderate English changes required
( ) English language and style are fine/minor spell check required
( ) I don't feel qualified to judge about the English language and style

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|  | Yes | Can be improved | Must be improved | Not applicable |
| Does the introduction provide sufficient background and include all relevant references? | (x) | ( ) | ( ) | ( ) |
| Is the research design appropriate? | (x) | ( ) | ( ) | ( ) |
| Are the methods adequately described? | (x) | ( ) | ( ) | ( ) |
| Are the results clearly presented? | (x) | ( ) | ( ) | ( ) |
| Are the conclusions supported by the results? | (x) | ( ) | ( ) | ( ) |

Comments and Suggestions for Authors

The authors have improved substantially the new version of the manuscript.

Submission Date 13 June 2020

Date of this review 07 Aug 2020 18:11:47

**Review Report Form 3**

English language and style

(x) Extensive editing of English language and style required
( ) Moderate English changes required
( ) English language and style are fine/minor spell check required
( ) I don't feel qualified to judge about the English language and style

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|  | Yes | Can be improved | Must be improved | Not applicable |
| Does the introduction provide sufficient background and include all relevant references? | ( ) | ( ) | (x) | ( ) |
| Is the research design appropriate? | (x) | ( ) | ( ) | ( ) |
| Are the methods adequately described? | ( ) | (x) | ( ) | ( ) |
| Are the results clearly presented? | ( ) | (x) | ( ) | ( ) |
| Are the conclusions supported by the results? | ( ) | (x) | ( ) | ( ) |

Comments and Suggestions for Authors

please see the attachment.

[peer-review-8285449.v1.pdf](https://susy.mdpi.com/user/review/displayFile/12888830/YIU5cXEk?file=review&report=8285449)

Submission Date

Biomolecules re review.

The manuscript is overly long- something that can be addressed by use of more succinct language and removal of repetitive sections. The English needs considerable improvement to correct grammatical errors. For example there are several occasions of incorrect use of the definite article ‘the’; times when it is included when not necessary and others when omitted. The manuscript still needs amendment by a native English speaker.

Abstract.

Line 28 : I still find the term ‘circadian rhythm locomotor activity’ misleading. Nothing about the altered locomotor behaviour is linked to circadian *rhythm* indeed they did not assess circadian rhythm*.* They showed altered behavior at all times of the day/night and the extent to which behavior was affected did not differ during the light or dark period. If there was a greater effect of the DPZ in the light period than dark (or vice versa) that might reflect an interaction of DPZ with pathways regulating circadian rhythm/ sleep but from what I can tell, that was not the case. In my opinion the term ‘circadian rhythm locomotor behavior’ should not be used. If the authors are intending to assess effects on sleep patterns, then they should measure sleep latency and number of sleep bouts and report them. Here they measured circadian activity (not rhythm).

‘the phrase ‘higher reduction and variation’ is unclear.

The authors understood the reviewer’s opinion. Indeed, in the current experiment, only the locomotor activity measured during both and light cycles and it is not enough to confirm the sleep pattern of zebrafish. It is also true that measurement of sleep latency or sleep bouts is needed to show a more convincing result about the effects of DPS on sleep, as the reviewer stated. However, based on prior studies, the measurement of this endpoint can be used to help with circadian rhythm’s study, especially in the fish. More explanation about the role of the locomotor activity in determining zebrafish circadian rhythm and the limitation of the current study was explained more in the reviewer’s comment below later and in the discussion part in the manuscript. In addition, regarding the unclear phrase, it had been revised and the authors hope that it is clear enough now.

Line 30: which variations does the term ‘these variations’ refer to? Everything? Or the changes in social and aggressive behavior?

Further, all changes in behavior are associated with the change in hormones (as the changes occur concurrently) but changes in specific hormone level might underlie changes in specific aspects of behavior. I’d replace ‘might be associated with’ with ‘are associated with’..

The authors strongly agreed with the reviewer’s comment. Regarding the term ‘these variations’, the authors felt that it was not suitable to express the intended meaning as the reviewer stated. Thus, the sentence was revised and the authors hoped that it is clear enough. In addition, regarding the usage of the term “associated with”, the authors were totally understood and agreed with the reviewer’s explanation. Therefore, the term ‘might be associated with’ in the abstract was replaced with ‘are associated with’.

Line 33; replace ‘which led to’ with ‘might underlie’..

Thank you for the correction. The mentioned phrase was replaced with “might underlie” according to the reviewer’s suggestion.

Line 45: ‘this critical condition.’… is vague. Neural loss leads to loss of memory and executive function.

Thank you for the correction. The phrase “this critical condition” was revised as the reviewer suggested.

Line 47-48: Do they all increase levels of the same chemical messenger? If so state which (Ach), if they act on different systems then say ‘boost levels of chemical messengers involved’..You do not need the ‘with’ .. so the sentence should read ..’to manage patient behaviour’.

The authors appreciated the correction. Since the medications act on different systems, the sentence was revised according to the reviewer’s suggestion.

Line 51: similarly you do not need the ‘the’ before ‘cholinergic function’.

Thank you for the correction. The word “the” was removed as the reviewer suggested.

Line 50-52: I’d break this sentence in to 2. i.e. : DPZ, sold as the trade name of Aricept, is a reversible inhibitor of the enzyme acetylcholinesterase (AChE) and is one of the most widely used therapeutics. Inhibition of Ache helps to enhance cholinergic function by increasing the cortical amount of acetylcholine.

Thank you for the constructive suggestion. Thus, the sentence was divided into two sentences according to the reviewer’s suggestion.

Lines 50-109: the section should be checked by a native English speaker for grammer.

Thank you for the suggestion. The authors had tried their best to improve the manuscript’s English as the reviewer suggested.

Line 109: We hypothesized that DPZ would slightly affect the zebrafish in the

absence of neurodegeneration, especially in their behavior. I think it reads better to say ‘we hypothesized that chronic DPZ treatment would affect zebrafish behavior in the absence of neurodegeneration’.

The authors appreciated the constructive suggestion. Thus, the sentence was revised according to the reviewer’s suggestion.

Results

Line 111: effects of DPZ **on…** (not in)

Thank you for the correction. The word “in” was corrected as the reviewer suggested.

Line 114-5: ‘First, we would like to test whether DPZ exposure has any beneficial or adverse

effects for short-term memory of normal adult zebrafish by using a T-maze test.’ I suggest ‘ First we tested the impact of DPZ on short term memory in healthy adult zf using a T maze assay (ref ref).

The authors appreciated the constructive suggestion. Thus, the sentence was revised according to the reviewer’s suggestion.

Line 119: training, not trainings

Thank you for the correction. The word “training” was corrected as the reviewer suggested.

Line 127 -130: However, significantly reduced time spent in punish-arm was observed in the DPZ-exposed fish on subsequent time points after the training when compared to the control group (P = 0.0063; F(1, 135) = 7.695) (Fig. 1E). Taken together, these results demonstrated that chronic exposure of DPZ in normal adult zebrafish had no adverse or beneficial effects on their learning or short-term memory. Surely, if it improved retention (ie had significant effect to reduce punishments at later time points), it has an effect on short term memory..??

Thank you for the correction. It is true that there was a slight improvement in the retention time. Thus, the authors decided to revise the sentence so now it states that the DPZ-treated fish showed a slight improvement in terms of short-term memory, which was shown by increased retention of the adverse stimulus. This conclusion was taken by considering the current and previous reviewer comments on this matter.

Line 130 ‘not any’, not ‘no any’

Thank you for the correction. The mentioned phrase was corrected as the reviewer’s suggestion.

Line 149-50 ‘Normally, zebrafish exhibits bottom-dwelling behavior when it is placed in the new environment and start exploring the area after a few minutes of adaptation.’ The grammar of this sentence is not correct. It is a mixture of single and plural. ‘zebrafish exhibits’ implies a single fish.. in which case you need an ‘s’ on starts and to have an ‘a’ before ‘zebrafish’. Alternatively use plural throughout : Zebrafish exhibit bottom-dwelling behavior when placed in a new environment and start exploring after… This type of English error is common throughout the manuscript and needs to be corrected before publication.

The authors appreciated the detailed correction regarding the English throughout the manuscript. The mistake mentioned by the reviewer above was corrected. In addition, the authors also had tried their best to correct this type of English error in the manuscript.

Line 161.. is this significant? ‘and total distance traveled in the top (P=0.0702;

F(2, 87)=2.739’ ? Either there is a typo or the statement that this is a significant difference needs changing.

Thank you for pointing out this matter. Actually, in the current novel tank test, the authors referred to the multiple comparisons test, which was Dunnett’s multiple comparisons test in this case, to draw out the conclusion than the two-way ANOVA test since this multiple comparison test gave better and more detail result regarding the interaction between each treatment group and control group than the two-way ANOVA test. Thus, the P value mentioned by the reviewer, even though it was not statistically significant, it was the P value calculated from two-way ANOVA test. Meanwhile, after the posthoc test was conducted later, the statistical differences were shown between the control group and each treatment group. However, to avoid confusion, the statistic test used as the reference to draw out the conclusion was added to the manuscript.

Line 180 : ‘that commonly used’ should be ‘that **is** commonly used’

Thank you for the correction. The mentioned phrase was corrected as the reviewer’s suggestion.

Line 181-3 The English of this sentence needs correcting. : Generally, butting or biting mirror behaviors as well as swim quickly back and forth are displayed by zebrafish when it was

placed in a tank with a mirror.

The authors appreciated the reminder. However, as suggested by the reviewer below, the sentence was removed from the manuscript since it was unnecessary.

Line 183: what does the author mean by ‘This phenomenon indicates the reflection and boldness of zebrafish.’ ? Biting the mirror does not indicate the ‘reflection’ of the zebrafish. It is assumed to be a response to the reflected image. The biting is interpreted as indicating aggression and also related to the boldness of the fish. I’d suggest that the whole section ‘Generally, butting or biting mirror behaviors as well as swim quickly back and forth are displayed by zebrafish when it was

placed in a tank with a mirror. This phenomenon indicates the reflection and boldness of zebrafish. In our study, a mirror biting test was performed to detect the aggressiveness of zebrafish after chronic exposure of DPZ.’ is unnecessary. The test is established and described

elsewhere. The manuscript is overly long and the authors’ English makes writing succinctly problematic. A description of how they measured aggression using ‘biting time’ should be in the methods section so is not needed here

Thank you for the correction and the suggestion. The authors admitted that there was a mistake in the mentioned sentence. In addition, the authors also agreed with the reviewer’s suggestion regarding the removal of the section as it was established and described elsewhere. Thus, the section was removed and the description of how the aggression was measured was added to the methods section.

line 190 How does the effect on locomotion here compare with the effects on locomotion seen in other assays? The locomotion was altered during the ‘circadian rhythm’ analysis but not in the tank diving? are there consistent locomotor effects or different ones in different assays? A point for the discussion at least.

Thank you for the question. Actually, even though both of these tests measured the locomotor activity of zebrafish, there is a fundamental difference that differed these tests to each other. This difference is lying in the condition of the test tank from the test fish’s perspective. In the novel tank test, the test tank was exposed to the test fish as a novel environment while in the circadian locomotor test, the test fish was already adapted to the environment since the test was conducted in a whole day.

line 283: The English needs correcting ‘In a human physiological study, an increase in sleep efficiency and shortens in sleep latency were observed after DPZ administration. Replace shortens with shortening. Also, please state whether the human study was in healthy individuals or AD patients. Effects of Donepezil Exposure in Circadian Rhythm Locomotor Activity of

Zebrafish There were no effects on circadian rhythm, indeed the authors did not assess

circadian rhythm. DPZ treated fish showed a reduction in locomotion and increased zig-zag behavior in both light and dark phases. If the authors wish to relate their findings to the human study then they should report a measure of sleep latency… I.e. define when the fish can be considered asleep, and indicate how long it took to get to that state in treated and un-treated fish . Similarly a measure of sleep efficiency..perhaps number of ‘sleep bouts’ during the dark

phase. A general finding that locomotion is reduced in both light and dark does not address the question they are posing… does chronic DPZ affect sleep parameters in fish?. In my opinion the use of the term ‘circadian rhythm locomotor activity’, is misleading as it implies an effect on circadian rhythm which they did not assess. At the very least remove the word ‘rhythm’ and just

have circadian locomotor activity.

The authors thanked the reviewer for pointing out this matter and the authors really understood the reviewer’s point of view. Regarding the English’s correction in line 283, the word ‘shortens’ was changed to ‘shortening’ and more information about the prior study in humans was also added. In addition, in terms of the circadian rhythm locomotor activity, indeed, the measurement of locomotor activity only is not enough to confirm the sleep pattern of zebrafish. However, it does not mean that the measurement of this endpoint did not have any significant meaning on circadian rhythm’s study, especially in the fish, since many prior studies showed that this endpoint is one of many ways to evaluate circadian rhythm in zebrafish. Moreover, locomotor activity is already shown to be useful for studying the physiology of the adult zebrafish circadian system. More explanation about the role of the locomotor activity in determining zebrafish circadian rhythm and the limitation of the current study was explained more in the reviewer’s comment below later and in the discussion part in the manuscript. In addition, the authors agreed with the reviewer’s suggestion by removing the word ‘rhythm’ and just have circadian rhythm locomotor activity.

Line 351 Here, we would like to observe whether there are significant changes in

several biomarker expressions caused by DPZ exposure and validate the corresponding

mechanism that controls these behavioral alterations by performing biochemical assays on some

important biomarkers I suggest you ‘examined the expression of biomarkers to gain insight into the molecular mechanisms underlying the observed behavioural changes.’ It is much more succinct and more grammatically correct. Also, you did not ‘validate’ the mechanisms. You would have to directly test whether increasing or decreasing the expression level of a particular biomarker resulted in the same behavioural change or prevented the changes seen in the presence of DPZ. You did not do that here; you merely looked for an association. Therefore you cannot

say you validated or even that you tried to validate.

The authors appreciated the correction with the constructive suggestion from the reviewer. As the reviewer suggested, the referred sentence was changed to the one that the reviewer mentioned above. In addition, the authors strongly agreed with the misused word ‘validate’ in the manuscript since in the current study, the authors did not validate the mechanisms, instead, the authors investigated them. Thus, the word ‘validate’ in the manuscript was changed to avoid confusion.

Discussion;

The first paragraph is too long/ uncecessary. Classically one just states the aim of the study and then discusses the results. e.g. The aim was to test the hypothesis that chronic exposure to DPZ leads to changes in behavior in healthy individuals using zebrafish as a model, and to explore the underlying biochemical processes affected. Then discuss the sig findings in relation to the literature.. As mentioned previously, you did not validate anything so the use of that word is incorrect. The sentence ‘As zebrafish is becoming an important in vivo model organism

for research on neurodegenerative disorders, a requirement of assessing learning, memory,

and preference is high to validate the results in the paradigm.’ is not necessary. This information has been given in the introduction. I question the validity of the statement ‘In the present study, 2.5 ppm of DPZexposed fish with no neurodegenerative disorder did not show any significant difference to control fish in terms of short term memory and cognitive performance like it was displayed in the earlier studies on Alzheimer’s disease patients [37,38]. ‘If ‘However, the treated fish showed increased retention of the adverse stimulus.’ Surely the treatment had an effect on short term memory? (see also line 407)

Thank you for the correction. As the reviewer suggested, the first paragraph in the discussion section was combined together with the second paragraph since it was too long and some information was already mentioned in the introduction part, so it was not necessary. Furthermore, regarding the effect of DPZ in zebrafish cognitive performance and short-term memory, the authors decided to revise the sentence as mentioned in another comment above.

line 458-471, the discussion of effects of DPZ on sleep in relation to the measures reported is inappropriate. If you find a difference in a measure related to sleep then all good, but at the moment from what I can tell you report a general reduction in locomotion across both light and dark with no effects on rhythm and no measure of sleep latency or sleep bouts / efficiency reported.

The authors strongly agreed with the reviewer’s point. It is true that in the current experiment, only the locomotor activity was measured during both and light cycles. In addition, as the reviewer stated, the measurement of sleep latency or sleep bouts is needed to show a more convincing result about the effects of DPS on sleep, even though it is already well known that the locomotor activity is one of the many ways to evaluate the circadian rhythm in zebrafish. Therefore, to make the discussion part more appropriate, some sentences were revised as the reviewer’s suggestion.

del Pozo, A., Sánchez-Férez, J. A., & Sánchez-Vázquez, F. J. (2011). Circadian rhythms of self-feeding and locomotor activity in zebrafish (Danio rerio). *Chronobiology international*, *28*(1), 39-47.

Hurd, M. W., Debruyne, J., Straume, M., & Cahill, G. M. (1998). Circadian rhythms of locomotor activity in zebrafish. *Physiology & behavior*, *65*(3), 465-472.

López‐Olmeda, J. F., Madrid, J. A., & Sánchez‐Vázquez, F. J. (2006). Light and temperature cycles as zeitgebers of zebrafish (*Danio rerio*) circadian activity rhythms. *Chronobiology international*, *23*(3), 537-550.

Wolter, M. E., & Svoboda, K. R. (2020). Doing the locomotion: Insights and potential pitfalls associated with using locomotor activity as a readout of the circadian rhythm in larval zebrafish. *Journal of Neuroscience Methods*, *330*, 108465.

Method:

Line 616. Please indicate how you define and measure ‘biting’. The fish can approach as wishing to explore or to attack the perceived conspecific. If attacking (defined as biting) they show particular behaviours. Please indicate how you defined and measured biting

Thank you for the suggestion. In the current study, the ‘biting’ was defined as the movement of the fish when they traced their reflection as they swim quickly up and down or back and forth. This was measured by counting the duration of the fish stayed in the ‘mirror biting zone’, which was defined as a zone with a radius of 5 cm from the mirror, and contacted the mirror, regardless of its intention, as described in the previous study. In addition, the information regarding the biting definition and measurement was added to the manuscript.

Line 629: you say you were assessing effects on sleep patterns but you do not indicate how you defined sleep or what parameters were measured. General activity in light and dark periods does not indicate latency to sleep or number of sleep bouts. The manuscript can be shortened by not repeating information that is given in the method section in the results. In the results section you could state ‘to measure X we used Y test (ref ref).’ Then state what is and is not significant. The

methods section should indicate what the outcome measures are for each test.

Thank you for the correction. The authors strongly agreed with the reviewer’s suggestion. Thus, the methods section was revised according to the reviewer’s instruction by removing some repeating information that is also given in the results section.

13 June 2020

Date of this review

17 Aug 2020 02:41:35