Dear Dr. Ari,  
  
Your manuscript, JoVE58396 Investigation of anxiolytic effect of exogenous ketogenic supplements by elevated plus maze test, has been editorially and peer reviewed, and the following comments need to be addressed. Note that editorial comments address both requirements for video production and formatting of the article for publication. Please track the changes within the manuscript to identify all of the edits.  
  
After revising and uploading your submission, please also upload a separate rebuttal document that addresses each of the editorial and peer review comments individually. Please submit each figure as a vector image file to ensure high resolution throughout production: (.svg, .eps, .ai). If submitting as a .tif or .psd, please ensure that the image is 1920 pixels x 1080 pixels or 300 dpi.  
  
Your revision is due by **Jul 13, 2018**.  
  
To submit a revision, go to the [JoVE submission site](http://www.editorialmanager.com/jove) and log in as an author. You will find your submission under the heading "Submission Needing Revision".  
  
Best,  
  
Alisha DSouza, Ph.D.  
Senior Review Editor  
[JoVE](http://www.jove.com/)  
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**Editorial comments:**  
Changes to be made by the Author(s):  
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

Response: We proofread the manuscript to correct the spelling and grammar issues.

2. Unfortunately, there are a few sections of the manuscript that show text overlap with previously published work. Though there may be a limited number of ways to describe a technique, please use original language throughout the manuscript. Please see lines: 295-312, 328-340, 344-345.

Response: This has been addressed and original language has been added throughout the manuscript

3. The uploaded Author License Agreement (ALA) is not completed/signed. Please complete and sign ALA, then scan and upload it to your Editorial Manager account.

Response: ALA is signed and upload to my Editorial Manager account.

4. Please note that the uploaded file labeled as “Video Produced by Author: Less than 50 MB” is actually the manuscript file. Please correct this mistake.

Response: Mistake has been corrected.

5. Please number the figures in the sequence in which you refer to them in the manuscript text.

Response: Correction has been made to number the figures in the sequence referred to in text.

6. Figure 4: Please provide a figure with higher resolution.

Response: Higher resolution figure has been made and uploaded.

7. Please rephrase the Summary to clearly describe the protocol and its applications in complete sentences between 10-50 words: “Here, we present a protocol to …”

Response: Summary has been rewritten clearly.

8. Please rephrase the Introduction to include a clear statement of the overall goal of this method.

Response: A clear statement has been added to describe the overall goal of this method.

9. Please remove the brackets enclosing the subscripted reference number.

Response: Brackets have been removed.

10. Please include a space between all numbers and their corresponding units: 15 mL, 37 °C, 60 s; etc.

Response: Spaces have now been added to all numbers and their corresponding units

11. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.  
For example: Plexiglas, PLATFORM, Precision Xtra, etc.

Response: Commercial language has been removed and commercial products have been referenced in the Table of Materials and Reagents

12. Please revise the protocol text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

Response: Personal pronouns have been removed from the protocol text.

13. 1.5-1.7, lines 146-153, 3.1.2: Please revise the protocol to contain only action items that direct the reader to do something. The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “Note.” Please move the discussion about the protocol to the Discussion.

Response: Requested revisions have been made.

14. 3.2.1.2, 3.2.1.8, 3.2.1.11, 3.2.2.7, 3.2.3.6: Please write the text in the imperative tense.

Response: Requested revisions have been made.

15. 3.2.1.4: Please specify the details entered.

Response: Details have been specified.

16. 3.2.1.10: Please ensure that the protocol here can stand alone. As currently written, users must refer to another protocol and refer back and forth in order to complete this protocol. Please remove the references to the specific steps of the other manual. (this can stay as is if you do not wish to film the calibration steps).

Response: We do not wish to film the calibration steps

17. Lines 254-255: For in-text formatting, corresponding reference numbers should appear as numbered superscripts after the appropriate statement(s).

Response: Suggested revisions have been made to referenced numbers.

18. For computational steps, please provide software screenshots as supplementary files to match each step.

Response: Software screenshots have been added as supplementary files.

19. Please include single-line spaces between all paragraphs, headings, steps, etc.

Response: Formatting changes have been made to include single-line spaces.

20. There is a 2.75 page limit for filmable content. Please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.

Response: We have highlighted 2.75 pages (or less) of the protocol to identify essential steps of the protocol for the video.

21. Please ensure that the highlighted steps form a cohesive narrative with a logical flow from one highlighted step to the next. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense.

Response: Suggested revisions have been made to highlighted text.

22. Please include all relevant details that are required to perform the step in the highlighting. For example: If step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be highlighted.

Response: All relevant details have been included in all highlighted steps.

23. Please include a figure or a table in the Representative Results showing the effectiveness of your technique backed up with data. Please revise to explain the Representative Results in the context of the technique you have described, e.g., how do these results show the technique, suggestions about how to analyze the outcome, etc.

Response: Representative Results showing the technique now discuss how the data is analyzed to support the behavioral outcome measures.

24. As we are a methods journal, please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:  
a) Critical steps within the protocol  
b) Any modifications and troubleshooting of the technique  
c) Any limitations of the technique  
d) The significance with respect to existing methods  
e) Any future applications of the technique

Response: The suggested edits have been made in the Discussion with emphasis on utility and application of this method

25. References: Please do not abbreviate journal titles.  
  
Response: Revisions have been made to include full title of journal.

**Reviewers' comments:**  
Editor's Note: Please note that the reviewers raised some significant concerns regarding your method and your manuscript. For each peer review comment, please provide either (1) a description of how the comment was addressed within the manuscript or (2) a rebuttal describing why the comment was not addressed if you feel it was incorrect or out of the scope of this work for publication in JoVE.  
  
**Reviewer #1:**  
The submitted manuscript is a methodological paper on the use of the elevated plus maze test to assess anxiety in laboratory rodents. The authors provided a detailed description of this behavioral technique including the use of video tracking system and data analysis, alongside with representative results. Elevated plus maze test can be considered a gold standard for investigation of anxiety-related behaviors in rodent models. Although the method is well known, visual demonstration will help researchers apply it in a standardized way and avoid making simple mistakes, which is particularly important for those new in the field of behavioral science. Importantly, the video tracking systems are becoming more and more advanced. The ability to use video-tracing software in the most efficient way possible (e.g. the proper adjustment of detection settings) is crucial for investigators. It minimizes the manual labor,, thus reducing the influence of investigator on the data and ensuring achievement of more reliable and comparable results. In the reviewed manuscript the Authors use SMART software. In my opinion, it would be great if the Authors would bring more attention to the use of the video tracking system and not so much to the behavioral technique itself. It can be done in the terms of general principles of video tracing in the EPM and/or the use of particular software.

Response: In order to emphasize the video tracking system we added it to the title and highlighted its importance in the text.  
  
What are the advantages of SMART system in comparison to e.g. commonly known freeware - ImageJ, which application is described in the older JoVe video on EPM? What are the possible difficulties that the users meet? I believe that focusing more on the video tracking system will greatly improve the manuscript and increase its value to readers. I will get back to this point in specific comments.

Response The manuscript has been modified to emphasize the video tracking system and its advantages.  
  
The manuscript is well-structured, consistent and well-written, but requires some modification and clarification before publication. There are at least two major changes that need to be made. First of all, it is very important to clearly state what is the goal of this article. It should be included in the introduction as well as in the abstract.

The aim of the study indicated in the abstract is actually the aim of the research from which the representative results come from. This should not be the case. If this is a methodological paper on EMP the aim should not be confused with the aim of the study from which the representative results come from. So please correct the aim of the study in the abstract.

Response: The manuscript has been modified to emphasize the goal of the article as the description of the video tracking system together with EPM.  
  
Another part that needs major improvement is the discussion. This section should be focused on the protocol and not the representative results. I would recommend removing the first paragraph which discusses the representative results and construct the discussion according to the JoVE's instructions for authors. The points that should be included in the discussion but are missing are: critical steps in the protocol, modifications and troubleshooting of the method and limitations of the method.

Response: The manuscript has been modified according to the suggestions of the reviewer.  
  
Please see detailed comments below.  
  
INTRODUCTION:  
As already mentioned please include the goal of the article in the last paragraph of the introduction.

Response It was included.

Some parts of the second paragraph are confusing:  
- Lines 62-64: Please clarify the sentence: "After treatment, increased time spent on the open arms and/or increased number of open arm entries compared to control (untreated) animals can be determined by EPM, which changes in behavior may mirror anti-anxiety behavior on EPM"

Response: This sentence was modified.

- Lines 66-67: Please remove "and changes in behavior" from the sentence "Therefore, behavior and changes in behavior after different treatment is commonly recorded for 5 min on EPM.

Response: Part was removed.

- Line 68: The mentioned factors i.e. "number of head dips, rears, fecal boli" are measures of anxiety so it can not be said that they are monitored "parallel with investigation of anxiety level"

Response: The sentence has been modified.

PROTOCOL:  
- Page 2, line 109: Point 1.5. can be moved to discussion.

Response: This text was moved to discussion.

- Lines 146-153 : Please consider moving this paragraph to discussion. Also, do you have any experience with a grey material? It may be worth to mention that EPM apparatus made from matte gray material is useful with white, black and white and black animals.

Response: The paragraph was moved to discussion and grey material was mentioned.

- Page 4, line 180: What are possible sources of video to process? Does the SMART software allow to analyse previously acquired video? This is an useful knowledge in the process of planning experiments.

Response: This information was added to section 3.2.

- Page 4, line 190: I would not suggest using the object instead of animal to test the detection settings. It is important to ensure that the system recognizes moving subject well.

Response: The sentence was modified.

- Page 5, line 247: It may be worth to mention what kind of behaviors are counted manually.

Response: Manual data collection was added to 3.2.3.6

- Page 6, line 284: There is no need to include the chapter "5. Blood analyses and weight measurement" in the manuscript, especially that the blood analysis data are not mentioned in the results. Moreover this  
section does not mention the methodology of weight measurement as it is indicated in the chapter's title.-

Response: Chapter 5 has been deleted.

DISCUSSION:  
  
I strongly recommend to rewrite the discussion, adjust it to the journal requirements and make it more coherent. From the second paragraph of the discussion one may conclude that the fact that the behavior of the animals in the EPM is age-dependent is a disadvantage of this test.  
Moreover, the EPM does not use noxious stimuli to test anxiety-related responses, which is of course an advantage of this method, but the same is true for other methods based on approach-avoidance behaviors (e.g. light dark box, open field test). The last paragraph of the discussion is not supported by any citation or adequate results.

Response: The discussion has been rewritten in accordance to the journal requirements and to make it more coherent. The disadvantages and advantages of the testing have been discussed along with citation support for statements made in the last paragraph.

**Reviewer #2**:  
  
Manuscript Summary:  
This paper is about ketone supplements on anxiety-like responses of rats. In some ways, this seems like a Methods paper, but there are details missing. The same is true if we interpret this as an empirical article. This should be clarified in the revision of this paper. Other feedback is as follows.  
  
Major Concerns:  
  
This paper requires extensive editing by a native English speaker.

Response: Paper has been proofread by native English speaker and revise and clarified in many areas.   
  
Paragraph structure and paper structure: Is it best that a standard description of the EPM, which has been described many times in the literature, lead this paper? Or perhaps the idea about studying the brain mechanisms and/or manipulations to use to study anxiety-like responses more important and should begin the paper? In terms of paragraph structure, paragraphs should begin with topic sentences that introduce the reader to what the paragraph will be about and end with concluding statements.

Response: The introduction was modified to highlight that this is a methodology paper.

In between these statements, there should be the details that the authors are conveying. Please be sure to reconsider both the paragraph and entire paper structure here.

Response: Additional details have been added throughout, when appropriate, to further clarify statements.   
  
This statement needs to be expanded upon and explained: "In addition, parallel with investigation of anxiety level, number of head dips, rears, fecal boli, as well as closed arms entries and total arm entries (spontaneous motor activity) and different postures (stretched- or freezing- attend) can also be measured on EPM (2)."

Response: The introduction section was expanded upon this statement.  
  
It is unclear why this statement is included: "In spite of the high validity of EPM, to increase validity of results, 2-3 behavioral assays are commonly used together, such as the light-dark choice test, social interaction test and EPM test to measure anxiety level in different animal models (6)."

Response: The sentence was modified to clarify the meaning.   
  
What is the relevance here?: "EPM is sensitive not only to benzodiazepine-type anxiolytics (e.g., diazepam) (8), but also among others to amino acid, monoamine, peptidergic and nucleosidergic compounds (e.g., NMDA antagonist AP7, AMPA antagonist CNQX, μ-opioid receptor agonist morphine, NPY1 antagonist BIBP3226, Substance P, Ghrelin, Oxytocin, serotonin receptor agonists and antagonists such as 8-OH-DPAT and WAY-100635, β1-adrenergic antagonist Betaxolol, caffeine) (9-12)."

Response: The relevance of this section is to mention some examples of compounds previously tested in order to provide guidance on what is the potential utility/sensitivity of this equipment. For example, to provide insight into understanding EPM results in the context of mechanisms or agents that have well-established behavioral effects.

One way to consider this is that "anxiety" (or "anxiety-like" behavior when we are referring to behavior in animals) is mediated by many brain targets. Alternatively, this could mean that EPM is not a precise measure. It is likely the former interpretation, but it is important for the authors to describe this to their readers. This might be useful for the authors to describe, especially as it leads them to their hypothesis about ketone supplements.

Response: Thank you for the suggestion. We agree that it is important to state the hypothesis up front and to emphasize that nutritional ketosis (induced with supplementation) likely works through numerous mechanisms in synergy, which would be expected when you are elevating energy metabolites that change the neuropharmacology and signaling of the brain.

Newman JC, Verdin E. β-Hydroxybutyrate: A Signaling Metabolite. Annu Rev Nutr. 2017 Aug 21;37:51-76. doi: 10.1146/annurev-nutr-071816-064916. Review. PubMed PMID: 28826372.

Under Preparations:  
  
Under section 1.2., that there are robust differences in animals' behavior based upon individual differences (e.g. possibly due to strain) or current condition (e.g. estrous cycle phase) is a major consideration for experimenters. As it is now, these are listed without any explanation and should be further explained.

Response: The importance of these factors was further explained in the discussion section.  
  
Under section 1.3, what is the justification for using 8-10 animals per group? This might be valid if there are only two treatment conditions, but the number of subjects per group may have to increase with additional treatment conditions. Power analyses are generally done before the experiment is initiated to determine the minimum number of subjects to be included given the variability in animal's responses in any given task as well as the number of experimental groups/conditions.

Response: Section 1.3 was corrected to justify the sample size used in the experiment.   
  
Under section 1.7., behavioral studies in nocturnal animals, such as rats and mice, are better suited for environments with a reverse light cycle, meaning that lights are on at night so that researchers can assess behavior when animals are in their dark, active phase. This also relates to point 1.2. above and the fact that animals' hormones are regulated by light cycle and influence behavior in the EPM.

Response: The text has been revised and section 1.7 was corrected.  
  
In Discussing the EPM set-up, it would be helpful to note that the contrast between the animals in the maze and the maze color is especially important for video-tracking. This is less important for researchers watching animals live or via video. Also, there are many products now that are good and precise in situations with less than ideal contrast (e.g. black and white Long-Evans rats can be reliably tracked by some programs when the settings are configured to note that the animals are black and white on either a black or white maze). The authors should mention problems with usage of a clear Plexiglas maze.

Response: Notes on contrast were added to section 1.1. to describe this important consideration.   
  
Why were several sections in yellow highlight?

Response: Those parts are identified as sections to be video recorded by JOVE.  
  
In the Abstract, authors need additional details about this particular report. For example, this is in certain types of subjects, using the EPM and SMART system. The way it is written now it suggests a more general methods type paper using the EPM, which is not exactly what this paper is about.

Response: The abstract was modified.  
  
The authors include some details about the results in their study, but without necessary details (such as the hypothesis of the study, number of subjects per group, details about the subjects, the statistical tests used, and the inferential statistics) this cannot be interpreted. Providing p values only are not useful.

Response: The additional details were included in the protocol section.  
  
If this is to be a Methods paper (which I think to be the case given the journal this is submitted to), the authors need to describe the methods more fully. For instance, how does the reader know that there is validity in this test without positive and negative control groups?

In this specific experiment we used rats fed with standard rodent chow gavaged with water (control), without any ketone supplement. Treatment results are compared to control.   
 **Reviewer #3:**  
  
Manuscript Summary:  
Overall, this paper clearly presents an EPM technique aimed at testing treatments for anti-anxiety. However, some of the advice given is not appropriate for all types of drug/compound testing. For example, the oral gavage treatment is quite stressful for the animals and thus should be avoided, especially when assessing potentially subtle anxiolytic effects. Introduction of the drug/compound in drinking water or via a palatable 'treat' is a better method (though it may be necessary to yoke control animals' drinking or consumption to achieve comparable amounts.

Response: Thank you for pointing out this important factor to consider. The palatability of the substance has been an issue when adding the compound to water or food-integration. The gavage was also used since a relatively large dose of this compound is needed to achieve the desired effects. For this reason we used gavage for these experiments to evaluate effects of acute adminstration. We included your recommendation in the manuscript and also the steps that need to be taken to minimize gavage-related stress.

The results of the EPM in this particular study are not well explained in terms of the differences expected by the specific ketone forms vs the MCT and thus this also needs to be elaborated on further in the discussion section. Particularly it is of interest to readers to understand how the measurement of ketones in blood may be used to support dose effect potential of ketones indicating the extent of the anxiolytic effect.

Response: The manuscript was modified not to include the blood collection/analyses based on suggestions from the editor and other reviewers and only the methodology around the EPM combined with video tracking software was emphasized.  
  
Major Concerns:  
Methods:  
The authors need to add a section on 'experimenter' guidelines. For instance, experimenters shouldn't wear strong perfume or soaps, or talk near the animal during the experiment, or move objects near the EPM environment, taking care to clean all of the EPM to erase smells of previous animals which might interfere with exploration of the test animal. It also may be useful to describe handling of animals (picking up gently by the torso and holding for a minute or two) several days before EPM .

Response: Thank you for the suggestion. These important experimenter guidelines have been added within the 1.8 section.

It's not clear how useful it is to give instructions on how to use the SMART software when there are many types of tracking programs, where this instruction will not be useful. One suggestion is to list the main software suites available for tracking, and also list on-line sources of instruction manuals for each of the software packages.

Response: The list of possible software and their online instruction manuals are now listed in discussion section.

The timing of EPM after gavage is not clearly stated and is a very important consideration for effects of a ketone treatment. Also, KSMCT was not described as a treatment (is the MCT at the same dose as the ketone esters and salt treatments? Is it expected that the MCT will produce the same blood concentrations of ketones as the other treatments?

Response: The timing after gavage and the KSMCT group were added to the methods section. Blood measurements were removed from this manuscript to emphasize the EPM method.

The method of EPM is described quite well for each parameter (entry into open arms, number of entries into open arms) but it may also be useful to explain what parameters are mostly commonly used, e.g. accumulated time in the open arms.

Response: Thank you for the helpful suggestions, it was added to the discussion.

Also mention when animals are excluded from analysis due to outlier tendencies (e.g. never leaves the area where it was placed, almost falls off the apparatus, is distracted by a noise of event outside of the apparatus)

Response: Thank you for the helpful suggestions, these options were added to the discussion.

How are blood glucose/ketone analysed, statistically?

Response: Description of blood measurements were removed since other reviewers suggested focusing only on the EPM methodology.

Results and Discussion:  
It's important to discuss other parameters that might indicate issues with EPM testing, such as treatments which cause sedation or hyperactivity, and how these types of effects might be assessed via EPM parameters. It's also important to note changes in weight after prolonged gastric feeding (especially with caloric treatments)

Response: These important potential issues were now added to the discussion.

Minor Concerns:  
The manuscript is poorly copy-edited. Below are some examples of poor grammar and unclear language  
P1 Ln 48: don't use 'for example' in abstract, just state main findings Corrected.  
P1 Ln 59: confusing sentence, seems to be missing a verb. Break it up? Corrected.  
P1 ln 61: add : instead of 'open' add 'open to the surroundings' Corrected.  
P1. Ln 64: omit this phrase: which changes in behavior may mirror anti-anxiety behavior on EPM. Deleted.  
P1. Ln 67 omit 'different', add 'a' Corrected.  
P1 ln 71 omit 'In spite of the high validity of EPM,' Deleted.  
P2 ln 102: 'Use minimum 8-10 animals per a group.' Group size will be dependent on effect size you expect to see with the test treatment, thus it is unwise to state a minimum size needed for groups. The sentence has been modified.  
P2 ln 107: use 'tests' not 'test'. Corrected.  
P2 ln 137 replace 'fed' with 'feed', omit 'for example'. Corrected.  
P2 ln 160: replace 'lit' with 'light'. Corrected.  
P4 ln 166, why is this highlighted yellow? To indicate which part of the text will be filmed by JOVE.  
P4 ln 201: add 'button' after 'Save'. Added.  
P5 ln 230: add 'until' after 'Wait'. Added.  
P6 ln 265: replace fall with 'falls' . Corrected.  
P6 ln 301 : no need to add SPD before rats. It's clear no other rat breed was used. SPD was removed.

Response: We sincerely appreciate the valuable feedback and have incorporated the suggestions and revisions.