Dear Reviewers,

We appreciate the time you took to read our manuscript. We are grateful for the review and we have made all the requested changes. Thanks to your careful review and feedback, we have made major revisions to the paper and it is now a much stronger work.

Individual responses are outlined below. Please let us know if there are outstanding issues.

Sincerely,

Erin Clabough

**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.

2. Figure 1A/1B/2: Please use SI abbreviations for time: h, min, s, etc.

*Response:* New figures were altered to to match with SI abbreviations.

3. Figure 1C: What are the units of distance on the y-axis?

*Response:* It was total number of holes investigated, but that figure has now been converted into two figures 2A and 3B.

4. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., “Do this,” “Ensure that,” etc.). 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.

*Response:* The text in the protocol section has been edited and all text that contains “can be” or “should be” has been removed and replaced by with imperative tense.

5. Please add more details to your protocol steps. Please ensure you answer the “how” question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action.

*Response:* More steps were added to better answer the “how” question of the experiment.

6. What type of mouse is used? Age/Strain?

*Response:* The age and strain of the mice are C57/Bl6J aged 1 month and 4 months. This is now included in the Representative Results section.

7. Please number all the steps. (1. Damsel in Distress Training, 2. Barnes Maze Construction).

*Response:* The number of steps were fixed throughout the protocol.

8. 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:* The important parts of the protocol were highlighted in the final edition of the paper.

9. 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:* The selected highlighted portions are in logical flow and include the important aspects of the protocol.

**Reviewers' comments:**  
  
**Reviewer #1:**   
Manuscript Summary:  
The authors describe a method to detect spatial learning and memory using Barnes maze. In addition general motivation and exploratory behavior are evaluated in an arena using the Damsel-in-distress paradigm ( empathy for a trapped mouse and prosocial motivation).The paper is well described. The techniques are described step by step in right sequence .  
  
Minor Concerns:  
There are some problems about the figures which not always correspond to what is written in the results or in the legends:

*Response:* Legends and figures have been reviewed and correspondence between legend and figure labels has been corrected. In addition, the figures have been elaborated upon to ensure clarity.

Figure 2:  
line 384-385 "The mouse may adopt a strategy to quickly find the hole. It may use a sequential hole-to-hole search in a ring-shaped fashion until the hole is found (Figure 2B). I think the figure to be cited is Fig. 2C

*Response:* The figure reference has been switched the content for line 384-385, but this section has been moved. The content has been revised so that the ring-shaped strategy is now found in Figure 4.

Line 386 "Alternatively, the mouse can target a general direction of the maze (Figure 2C), which indicates.." I think the figure to be cited is Fig. 2B

*Response:* The figure reference has been switched the content for line 386, but this section has been moved as well. Search strategy content is now in Figure 4.

FIGURE and TABLE LEGENDS:  
Figure 2:  
"C. The total number of holes investigated …" In the panel C Maze Completion is reported;  
"D. Errors are the number of hole investigation…". In D the authors reported "Distance to target from first hole"  
"F. The distance to target …" but holes investigated are reported  
"C. The total number of holes.." but I Maze Completion is reported

*Response:* The figures have been reorganized. Previous Figure 2 information is now spread throughout Figures 1-3. The error and distance to target information should now contain references to the correct figures and their purpose.

GENERAL  
-In the Damsel-in distress paradigm are the authors sure to evaluate a prosocial/empathic behavior ? Could not it just be a sexual call? Did the authors tested a trapped male mouse to verify this possibility?

*Response:* The mice who were trapped were visually in distress. They were reluctant to go into the small space of the tube. They all defecated/urinated, which is another sign of their distress. It is unlikely that they would be making sexual calls. However, male mice have been known to produce ultrasonic USV (with frequencies between 30 -110 kHz) when male mice are exposed to female mice or female urinary pheromones. Female mouse USVs haven’t been extensively studied as extensively as male USVs, but when present, they are found in female–female interactions and when the female is alone (particularly when their pups are removed) (see Portfors 2007 for a review).

Rats have been found to emit USVs when exposed to inescapable aversive stimuli, but previous reports found no USVs in mice during aversive stimuli exposure (such as physical restraint or electric shock) (Portfors 2007). Instead, BALB/c mice emitted USV exclusively during nonaggressive social interactions and when performing mating behaviors (Gourbal 2004).

However, other studies have shown that adult male C57Bl/6J mice emit some USVs during non-social exploration of a novel environment or when subjected to restraint stress (in a condition very similar to the one used in the current paper) (Chabout et al,2012), although these calls were greatly reduced in number compared to those emitted in social conditions. In fact, mice emit *less* USVs when experiencing stressful conditions (including bright light, novel environment, and standing water)(Mun et al, 2015), and researchers have suggested that the number of USVs decreases as mice move to a negative emotional state (Chabout et al,2012). Though females were not tested in either of these described studies, it remains unlikely that females would vocalize during the restraint stress.

Because female mice typically do not emit USV when in the presence of a male, and mice in general emit no or less USVs in stressful conditions, we did not expect our trapped females to elicit USV “distress calls” towards males. We tested for the presence of USVs in the trapped mouse after 10 minutes of restraint stress in both the presence and absence of an observing male mouse. Three female and male mice were individually trapped for 9 minutes prior to being recorded for one minute each. We were unable to detect USVs (characterized as anything over 20 Hz) in trapped female mice nor trapped male mice using a Stanford Research Systems Model SR785 with a self-contained spectrum analyzer. In our test conditions, the microphone was placed just outside the restraint container on the side that the trapped mouse was facing. There were several air holes in the container, but it is possible that the plastic blocked the vocalizations, but our equipment was able to detect the control emissions from the Barnes maze aversive ultrasonic stimulus.

We have now included 1-2 sentences in the paper about the ultrasonic vocalizations.

-In a previous work the authors reported that ethanol-exposed mice appeared to be less motivated to complete the Barnes maze at 1 month , but were able to successfully learn the maze. However, deficits in long-term spatial memory retrieval were observed in ethanol-exposed mice when the Barnes maze recall was measured at 4Months. No significant differences were found in open field behavior or social responsiveness at 1M or 4M of age.  
The results of the present work appear different. May the authors explain such difference?

*Response:* The present work shows the same results as the previous work (Houle et al 2016). The ethanol treated animals displayed a deficit in memory retention on the Barnes maze as adults. This work is depicted here in a similar manner. We present the ethanol as a variable that can change learning/memory.

The Damsel-in-Distress measure revealed no significant differences between ethanol and saline treated mice. Instead, we portray these results in a more developmental way, focusing on the difference between juvenile and adult mice. In the D-in-D, age is the variable that impacts behavior, which is perhaps more appropriate for an assay that has not been widely published. We have reworked the paper and the figures to ensure clarity here.

**Reviewer #2:**  
Manuscript Summary:  
The manuscripts presents two rodent paradigms that require no food deprivation or electric shock but still assess motivation, locomotor activity and learning / memory.  
The authors introduce a new paradigm, the Damsel-in-Distress procedure. However, the paper provides no validation. Even if this is JOVE, some validation would be highly beneficial, esp since you restrain a mouse which requires ethical permission, whereas the 3-chamber social test is without any major restrainment and preferred by animal welfare. It is further unclear what the connection is between the Barnes maze (learning) and the D-i-D which shall measure prosocial behaviour / empathy.  
The connection could be if the Barnes maze is used to validate the D-i-D with respect to exploratory behaviour, stress levels  
but the two protocols are quite different, i.e. learning quick way to a safe hole = open arena, solitary behaviour vs spontaneous prosocial behaviour (no learning)  
I recommend to focus solely on the D-i-D and maybe compare it to the 3-chamber protocol

*Response:* We are open to making this into two *JoVE* papers, though I think the reformatting in the current paper makes the paper flow in a much more logical format. The two protocols do differ in their approach to animal behavior, but the unifying factor for us was that we present a very low cost, do-it-yourself accessible approach to animal behavior assessment.

The exploratory behaviors found in the D-i-D and the Barnes maze are quite different and the motivations to explore are also very different, so a comparison of the exploration would also presumably be different. We reran many of the statistics in both the Barnes maze and the D-i-D data to provide more statistical documentation for our findings. We also added more information in the introduction about the difference between Crawley’s 3 chamber protocol and the D-i-D. The D-in-D evaluates different aspects of social behavior, including a measure of prosocial behavior in the context of distress.

Major Concerns:  
the paper needs to be restructured. First Barnes maze (advantages over radial maze / Morris water maze etc) as you have it but then first why (advantages) the Damsel-in-Distress paradigm (re-order this section) before describing the how. In this order you should also have the protocol and result section and not have first the D-i-D paradigm as first in the protocol.

*Response:* We have reorganized the paper introduction to make the Barnes maze information come first, followed by the D-i-D information throughout the paper.

It would be also a great advantage if you validated the D-i-D by using the same animals in the Barnes maze, measure their exploratory behaviour and validate it in the D-i-D == exploration in round Barnes maze similar to exploration in squared / rectangular box with bedding.  
See also comments above

*Response:* The D-i-D and Barnes Maze are very different assays (D-i-D is in a box with walls, while the Barnes maze is an open circle raised off the ground). The differences in the open space, combined with the different motivations to both explore and to inhibit exploration, make it unlikely that exploratory behaviors would match, though it is possible that the sheer amount of locomotor activity would correlate between them as a possible measure of hyperactivity.

Minor Concerns:  
please use rodents instead of subjects

rodents esp mice should not be kept by the tail but paws should be on one's hand; when placing the mouse in a maze, use a cup and lift it (via a string mechanism), that avoids the mouse first running away from the direction where it either sees / smells the human or got hold on base of the tail == biases the initial heading of the animal! It is important to avoid this and should by no means be shown in the videos

*Response:* Subjects was switched to rodents in the paper. The orientation of handling the mice was examined and changed.

**Reviewer #3:**  
Manuscript Summary:  
Ingersoll et al. describe two separate protocols for mouse behavioural training: one for Barnes maze and a 'Damsel-in-Distress' paradigm.  
The Barnes maze is a well-known test for spatial learning, and there is even a JoVE protocol paper on it from Rosenfeld and Ferguson (2014). The Damsel-in-Distress paradigm is quite similar to Crawley's three-chamber sociability test, thus it is unclear what unique advantage it offers. Therefore it is unclear why this protocol is preferable over others and the authors do not provide any comparisons.  
In addition, very limited representative results are included, especially for Damsel-in-Distress.

*Response:* In order to provide more representative results, we reran many of the statistics in both the Barnes maze and the D-i-D data to provide more statistical documentation for our findings. We also rewrote the justification information to provide more of a clear idea for how these assays would be appropriate.

Major Concerns:  
1. The Barnes maze is a widely used paradigm, with a well-known protocol. There are many articles on PubMed, and a well-explained video article in JoVE. The only novel addition seems to be the ultrasonic noise. In this regard, a control experiment should show that the finding that more mice entered the target in trials after introducing the ultrasound noise was indeed because of the noise and not learning by time (lines 364-367).

*Response:* A follow-up control experiment would indeed make sense here, but we no longer have access to these particular animals. I have previously published using the Barnes maze in the absence of the ultrasonic noise using a 9-day training protocol in mixed strain C57BL6/129Sv mice (Clabough and Zeitlin, 2006). The addition of the noise was able to shorten the protocol so the training could be completed in 7 days instead of 9 days. In our revision, we have depicted the data differently in order to show in each figure exactly when the noise was added.

2. The Damsel-in-Distress is a modified sociability test. While potentially interesting, it is unclear why it should be preferred over similar tests for sociability (like Crawley's three-chamber test).

*Response:* We added more information in the introduction about the difference between Crawley’s three-chamber protocol and the D-i-D. The D-in-D evaluates different aspects of social behavior, including a measure of prosocial behavior in the context of distress.

In the D-in-D, in order to interact socially, the mouse must venture into the open center of the arena, which is normally aversive to the animal. Even if the animal does venture into the center, our task allows for very limited social interaction, so the social drive must be sufficiently strong to overpower the open field aversion without much reward.

If a researcher is interested in social interaction, the number of times the mouse touches the container, or touches noses (olfactory investigation) can be measured. The element of distress alters what the D-i-D is measuring compared to the Crawley 3 chambered measure.

If a researcher is interested in prosocial behavior, the amount of digging episodes before and after the introduction of the trapped animal can be measured. One reason to dig would be to “help” the trapped animal, but digging can sometimes be seen as a measure of anxiety as well. An interesting adaptation would be to modify the container so the mouse can release the trapped female if desired, and this is mentioned in the text.

3. Were the distress calls of the trapped female recorded? Those can heavily influence the results.

*Response:* Because female mice typically do not emit USV when in the presence of a male, and mice in general emit no or less USVs in stressful conditions, we did not expect our trapped females to elicit USV “distress calls” towards males. We tested for the presence of USVs in the trapped mouse after 10 minutes of restraint stress in both the presence and absence of an observing male mouse. Three female and male mice were individually trapped for 9 minutes prior to being recorded for one minute each. We were unable to detect USVs (characterized as anything over 20 Hz) in trapped female mice nor trapped male mice using a Stanford Research Systems Model SR785 with a self-contained spectrum analyzer. In our test conditions, the microphone was placed just outside the restraint container on the side that the trapped mouse was facing. There were several air holes in the container, but it is possible that the plastic blocked the vocalizations, but our equipment was able to detect emissions from the Barnes maze aversive ultrasonic stimulus.

4. Many computer-assisted tracking systems are available and a few are free/open source. These are more accurate and reliable than manual tracking by the experimenter; therefore, the latter should be avoided.

*Response:* That is valuable information to include and we have added this into the paper under Data Collection.

5. Fig. 2C does not seem to show a 'more direct search pattern' as the authors claim (line 434). More generally, the figures carry very little convincing power that these protocols should be preferred over the many available similar options.

*Response:* Figure 4 now contains information about the search strategies. The figure does show a direct search pattern since the exploration is limited to the correct quadrant, but this particular animal didn’t enter the target immediately.

We reorganized both the analysis and the figures in order to make the benefits of these paradigms more apparent.

Minor Concerns:  
1. In Damsel-in-Distress, the drawing of the task is not informative.

*Response:* The drawing serves as an introduction to the D-i-D arena as a new assay, allowing an easy way to visualize where the male and female are placed.

2. In Barnes maze, the locomotor activity could be better quantified, e.g. measuring the time spent by investigating the holes instead of just counting the number of holes visited.

*Response:* Nearly all of the time on the Barnes maze was spent investigating the holes. Animals were almost never in the center field. We have added a few more figures to the Barnes analysis to show hole explorations both before and after finding the target hole, as well as a discussion of motivation to enter (which was an issue). Mice shouldn’t be sitting in the false holes grooming instead of searching for the target, and this behavior was often seen before the absence of the ultrasonic noise, particularly in the ethanol treated mice, but not afterward.

3. The authors should provide more information on the animals used (number, strain, etc.).

*Response:* All animals were C57/Bl6J mice. The Barnes maze used n=8 ethanol, n=6 saline mice. The Damsel-in-Distress used n=15 mice at 1 month and n=12 mice at 4 months of age. This information has been added into the paper.

**References:**

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Gourbal BEF, Barthelemy M, Petit G, Gabrion C. 2004. Spectrographic analysis of the ultrasonic vocalisations of adult male and female Balb/c mice. Naturwissenschaften 91:381–385.

Mun, H. S., Lipina, T. V., & Roder, J. C. (2015). Ultrasonic vocalizations in mice during exploratory behavior are context-dependent. *Frontiers in behavioral neuroscience*, *9*, 316.

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