Journal of Visualized Experiments

Simple methods to analyze spatial learning and prosocial behavior in mice utilizing the Barnes maze and the Damsel-in-Distress paradigm

--Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE58008R1
Full Title:	Simple methods to analyze spatial learning and prosocial behavior in mice utilizing the Barnes maze and the Damsel-in-Distress paradigm
Keywords:	Barnes maze; spatial learning; memory; locomotion; exploration; Damsel-in-Distress; prosocial behavior; social responsiveness; empathy; motivation; ethanol; development
Corresponding Author:	Erin Clabough Hampden-Sydney College Hampden-Sydney, va UNITED STATES
Corresponding Author's Institution:	Hampden-Sydney College
Corresponding Author E-Mail:	ebdclabough@gmail.com
Order of Authors:	James D. Ingersoll
	Robert W Holland
	Michael T Moody
	Wesley Kuegler
	Charles P Murrah Jr.
	John D Winslow
	Neal M Reynolds
	Nathaniel De'Mar Lloyd
	John Brian Dugan
	Madalyn Hammock
	Katherine Houlé
	Erin Clabough
Additional Information:	
Question	**Response**
Please indicate whether this article will be Standard Access or Open Access.	Standard Access (US$2,400)
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	Gilmer Hall, 254 Via Sacra, Hampden Sydney, VA 23943

TITLE:

Analyzing Spatial Learning and Prosocial Behavior in Mice Using the Barnes Maze and Damsel-in-Distress Paradigms

AUTHORS AND AFFILIATIONS:

James Ingersoll[1], Robert Holland[1], Wesley Kuegler[1], Michael Moody[1], Charles Murrah Jr.[1], John Winslow[1], Neal Reynolds[1], Nathaniel Lloyd[1], Brian Dugan[1], Madalyn Hammock[1], Katherine Houlé[2], Erin Clabough[1]

[1]Department of Biology, Hampden-Sydney College, Hampden-Sydney, VA, USA
[2]College of Medicine at the Medical University of South Carolina, College of South Carolina, Charleston, SC, USA

Corresponding Author:

Erin B. D. Clabough
eclabough@hsc.edu
Tel: (434) 223-6172

Email Addresses of Co-authors:

Katherine Houlé	(houlek@musc.edu)
Brian Dugan	(duganj19@hsc.edu)
Robert Holland	(hollandr18@hsc.edu)
James Ingersoll	(ingersollj18@hsc.edu)
Wesley Kuegler	(kueglerw18@hsc.edu)
Nathaniel Lloyd	(lloydn20@hsc.edu)
Michael Moody	(moodymt20@hsc.edu)
Charles Murrah Jr.	(murrahc@hsc.edu)
Neal Reynolds	(reynoldsn18@hsc.edu)
John Winslow	(winslowj19@hsc.edu)
Madalyn Hammock	(hammockm18@hsc.edu)

KEYWORDS:

Barnes maze, spatial learning, memory, locomotion, exploration, Damsel-in-Distress, prosocial behavior, social responsiveness, empathy, motivation

SUMMARY:

This protocol measures spatial learning and memory using the Barnes maze. A novel Damsel-in-Distress paradigm is used to assess locomotor activity and prosocial behavior in mice.

ABSTRACT:

The Barnes maze is a reliable measure of spatial learning and memory that does not require food restriction or exposure to extremely stressful stimuli. The Barnes maze can also assess other mouse behaviors, such as general motivation to escape from the maze platform and exploratory behavior. The Barnes maze can measure whether a genetic mutation or environmental variable

can impact the acquisition and retention of spatial memories, as well as provide information about the search strategy employed by the mice. Here we use the Barnes maze to detect a memory deficit in adult mice following a single developmental ethanol exposure event. The newly described Damsel-in-Distress paradigm exposes a male mouse to a female mouse trapped in a chamber in the open center field of the arena. It provides an opportunity for the mouse to socially respond to the trapped female and exhibit prosocial behavior. The Damsel-in-Distress paradigm can also be used to examine mouse behavior in a novel arena and measure locomotor activity. Both the Barnes Maze and the Damsel-in-Distress protocols require minimal financial investment and most aspects of the tests can be constructed from common lab supplies. These flexible and accessible tools can also be used to detect behavioral changes over the course of development.

INTRODUCTION:

The purpose of this protocol is to measure spatial learning and memory in mice using the Barnes maze as well as social responsiveness and locomotion using the Damsel-in-Distress paradigm. Commonly used spatial learning and memory tests for rodents include the radial arm maze, which measures a mouse's ability to find hidden food in one arm of a multi-spoked apparatus, and the Morris water maze, which places a mouse in a large tub or pool of water and assesses how long it takes to find a hidden underwater platform. Typically, training for these paradigms spans multiple trials and allows the measuring of both learning acquisition rates and retention through short term and long-term memory trials.

Although the radial-arm maze and Morris water maze are reliable ways to test memory in rodents, they present complications for some researchers. Both mazes use deprivation or strong aversive stimuli as reinforcement[1]. The radial arm maze uses food deprivation to ensure that rodents are properly motivated to find a food reward. In the Morris water maze, the effects of stress induced by a forced swim may alter results in the mice[2].

The Barnes maze is an alternative spatial awareness task that requires rodents to learn the position of a hole in order to escape the bright, open maze surface[3]. Weak aversive stimulation (light or sound) is then applied to increase the likelihood that mice will escape from the platform. The Barnes maze does not require food deprivation, so the amount of animal preparation is less than in the radial arm maze. It can be used without conflict by researchers who are investigating behaviors or molecules associated with eating, hormonal regulation, or hypothalamic pathways.

The Barnes maze also has advantages over the Morris water maze. It is less stressful than the Morris water maze, causing less elevated levels of corticosterone in mice[4]. In addition, it is much simpler to assemble and requires less dedicated space during the testing procedure and storage.

The Damsel-in-Distress assay is a simple two-part experiment that can assess locomotor activity followed by prosocial behavior. The Damsel-in-Distress assay is designed to assess exploratory behaviors and social responsiveness of a male rodent in response to an entrapped female rodent. A commonly used method to assess sociability (as well as preference for social novelty) is the use

of Crawley's three-chamber sociability test, which assesses the free choice of a mouse to spend time near or away from other mice[5].

Similar to Crawley's three-chamber sociability test, the Damsel-in-Distress assay also measures free choice regarding how to spend time in the presence of another mouse, but it also provides measurements for deeper aspects of social functioning: 1) In the Damsel-in-Distress assay, the trapped female mouse is held in the center of an open field, so the male's potential aversion to an open field is pitted against his drive to socially explore or investigate a distressed female conspecific. 2) The Damsel-in-Distress model also provides a way to assess prosocial and empathetic behavior in response to the trapped mouse, which has not been frequently explored in mice.

Animal empathy is definitely observable and measurable, though not many paradigms exist for this purpose. For example, in rats, a trapped cage mate can induce a state of prosocial motivation, where rats will work to free the trapped animal. In fact, rats will choose to help the trapped animal even before opening a similar container containing a chocolate snack, then access the chocolate and share it with the newly freed rat[6].

Measures of empathy in mice typically involve the infliction of pain; indeed, mice who watch other mice in pain are more sensitive to pain themselves[7,8]. Restraint stress is a characterized phenomenon in rodents and has been coupled with shock to examine stress responses, as one measure of empathy in mice[9,10].

There are ethical considerations associated with induced pain or shock, so stress-inducing alternatives are needed. We developed the Damsel-in-Distress paradigm as a measure of empathetic behavior in the absence of either pain or shock treatment. The trapped mice in our Damsel-in-Distress protocol show overt signs of distress after only several moments as they are unable to turn around within the small container, yet they are unharmed while providing an opportunity for other mice to respond to their distress.

To begin the Damsel-in-Distress assessment, a male mouse is placed in a large novel arena containing a small empty central cylinder. Exploratory behaviors are recorded for several minutes, including how many sections of the arena are crossed and how much time is spent in the central open area. This method provides a quick and easy way to rule out locomotor deficits as a potential confounder in a learning situation that requires coordinated movement for successful completion. It also provides a basic measure of how much aversion to the open center field is present. Both measures could influence Barnes maze performance.

Following the initial exploration, the male is removed from the arena and a female mouse is placed in an enclosed, small clear central cylinder (similar to that used to collect blood from mice). Then, the original male mouse is reintroduced into the arena, and exploratory behavior is again scored. The Damsel-in-Distress paradigm assesses whether a mouse is interested in a social interaction based on changes in behavioral patterns when a trapped female is present (scored by time spent in the center square and the number of digging episodes), and whether the mouse

exhibits prosocial behavior towards the trapped female (scored by the amount of cylinder investigations and contact events with the trapped female). The Damsel-in-Distress assay can be used to measure a propensity for social novelty (similar to Crawley's three-chamber sociability test) depending on whether researchers trap a familiar or novel mouse.

Together, the Barnes maze and Damsel-in-Distress experiments allow the accurate evaluation of mouse learning capabilities and social responsiveness in the absence of extremely stressful stimuli. As with all behavioral assays, these experiments should be performed with great sensitivity towards the animal experience, minimizing the discomfort experienced by the animal.

Like most mazes, differences in locomotor activity may influence performance on the Barnes maze, so researchers should also assess locomotor activity, especially when using the Barnes maze to assess learning in mice with mutations that may impair movement (such as those found in Huntington's disease mouse models, or those exposed to toxins that may induce hyperactivity or retard movement, such as ethanol). In addition, maze and chamber surfaces should be thoroughly cleaned and bedding changed between each animal to avoid scent cue confounds.

Importantly, all materials can be fabricated on site with minimal financial investment, and the small physical footprint of these assays means that these experiments can be replicated in almost any setting, allowing for great flexibility. This type of accessibility allows good science to be performed at smaller institutions with limited resources or in situations where pilot data needs to be collected quickly in the absence of substantial support.

PROTOCOL:

All methods described here were approved by the Institutional Animal Care and Use Committee (IACUC) of Hampden-Sydney College or Randolph-Macon College (where some work was previously performed).

1. Basic Housing of Mice

1.1 House the mice in plastic cages with solid bottoms and sides, and a layer of soft bedding and nesting material such as paper shredding. Use bedding that is composed of shredded corn cobs or wood shavings, and ensure that the bedding is changed regularly for sanitation.

1.2 Provide access to food and water *ad libitum*. The top of the plastic cage houses a food hopper. Dispense properly formulated mouse food pellets from the food hopper, and provide a water dispenser. Ensure that the cage top features an air filter to protect mice from outside contaminants.

1.3 Maintain the natural circadian rhythms of the mice by following a 12-hour light-dark cycle in the housing facility. Conduct behavioral testing at the same time of day, ideally during the animal's dark cycle, such as during the evening hours.

Note: Care should be taken to distinguish mice in the housing. Various methods for distinguishing mice are available, such as ear punches, ear tags, and tail markings.

2. Barnes Maze Testing: Construction

2.1 Obtain a circular wooden board that is 120 cm in diameter.

2.2 Cut 20 circular holes (4.5 cm in diameter) around the perimeter of the circle. Position each hole to be 2.5 cm away from the maze edge and 13 cm apart from neighboring holes.

2.3 Smooth the surfaces and paint them glossy white (a bright color is recommended).

2.4 Designate one side of the maze board for insertion of small cup hooks roughly 2-3 cm away from each of the 20 holes, and place two hooks for each hole (one on either side of each hole).

2.5 Use shallow black (plastic) disks for covering holes on side of maze with hooks. Use hooks to secure thick rubber bands in order to hold the disks onto the bottom of the maze.

2.6 Securely mount the Barnes maze 120 cm above the ground and away from other similarly tall objects like tables or chairs. A box or stool can be used to support the middle of the maze.

2.7 Place large white posters with one shape on each wall (triangle, circle, and cross) as extra-maze cues on 3 sides of the maze. Maintain the posters on the walls around the maze for each trial.

2.8 On last side of maze, set up a solid black curtain to hide observers so that data can be recorded accurately without the researchers being visible to mice on the maze.

2.9 Suspend a video camera over the arena with a bird's-eye view of the entire maze surface.

2.10 Clean every surface of the maze (black disks and target box included) with water then a 70% ethanol solution before and after each mouse trial.

2.11 Place a 100 W light source 25 cm over center of maze (turn on/off at the start/finish of trial) and be sure to have the disks and hooks facing the floor.

Note: All other overhead lights in the room should be turned off during testing. The addition of an ultrasonic noise maker hung next to 100 W light is recommended. Turn it on/off at the start/finish of each trial.

3. Barnes Maze Testing: Procedure

Note: Ensure that all the maze components are cleaned with water and 70% ethanol solution before and after each trial, allowing for time to dry completely before testing resumes. Be sure to have cleaning supplies ready as well as timers for the trials.

3.1 House all the mice in groups while using a reliable identification method. Keep mice outside of the testing room when they are not actively running on the maze. This is to ensure they are not subjected to the ultrasonic noisemaker prematurely.

3.2 Handle mice by gently picking up and holding them from the base of the tail, with the paws kept on one's hand.

3.3 For training, have the mice run on the maze daily once per day for 7 days in a row to assess learning/acquisition.

Note: A single long-term trial can be done on a later date to assess memory/retention. The current protocol trained adolescent mice beginning at postnatal day 32, and a long-term trial was performed in adults at 4 months of age.

3.4 Randomly assign each mouse to a target hole to use throughout the testing period. The holes can be labeled 1-20 on the bottom of the maze or on the outside of the maze perimeter, where they are not visible to the mouse in the maze.

3.5 Replace the assigned hole disk with a small black box (23 x 11 cm). Firmly attach it to the Barnes maze using rubber bands connected to the nearest hooks.

Note: The target box is shallow enough so that the mouse can easily step down into it or contains a step to ensure that the mouse does not have to jump down into it.

3.6 Place the mouse onto the center of the maze beneath a cup to acclimate for 30 seconds until the test begins. Begin video recording.

3.7 Turn on the light and ultrasonic noisemaker. Lift the cup via a string mechanism to avoid biasing the initial heading of the animal. Start a timer and sit behind the curtain to observe.

3.8 After the mouse enters the target hole, cover the hole with the same opaque cup used at the start of the trial and turn off the ultrasonic noisemaker. If the mouse has not entered the target after 5 minutes have elapsed, corral the mouse into the target hole.

3.8.1. Ensure the mouse enters the hole. When mouse is inside the target box, cover the hole and turn off the noisemaker. Allow the mouse to remain in the target box for 1 minute undisturbed.

3.9 Although the target hole will remain constant for each mouse throughout the training period, run mice in a random order each day to ensure that they are not following any cues/scents left by the previous mouse.

4. Barnes Maze Testing: Data Analysis

4.1 During the Barnes maze trials, record the following timed data: total time spent on maze, time to find target, and time to enter target hole if the target is entered.

4.2 Track the overall movement of the mouse to determine the number of errors (number of incorrect holes explored) before and after the target is found. Also record the distance from the target hole to the first hole explored (distance is measured in number of holes away), along with any notable grooming behavior.

4.3 Track the movements of each animal on a piece of paper with a diagram of the maze during each trial and use it to analyze search strategies, as well as determine the number of holes that were explored in the quadrant opposite the target. Use video analysis to confirm the paths.

5. Damsel-in-Distress Testing: Constructing the Restraint Chamber

5.1 Cut a clear cylinder to a length equal to ¾ of the length of the female mouse from the base of the tail to tip of the nose. Ensure that the dimensions of the cylinder are such that the mouse placed inside cannot turn around.

Note: We cut the cone end of a 50 mL conical tube down to make a 4 cm long tube for 1-month-old mice and an 8 cm long tube for 4-month-old mice.

5.2 Cap both ends of the cylinder such that one of the caps can be removed and reattached. Punch 3-4 holes, approximately 0.5 cm each, in each cap. Ensure that the holes are large enough to allow nose-touching between mice and breathing[12].

Note: Alternatively, a device for mouse restraint during blood collection can be used.

6. Damsel-in-Distress Testing: Preparing the Arena

6.1 Ensure that the arena is an opaque topless plastic square box of 38 x 38 cm with 19 cm walls. Fill it uniformly with corn bedding to a height of approximately 2.5 cm.

Note: The corncob bedding makes digging events easier to detect.

6.2. Suspend a video camera over the arena so that the entirety of the arena is in view.

7. Damsel-in-Distress Testing: Exploratory Behavior Measure

7.1 Place the closed and empty stress chamber into the center of the arena.

7.2 Begin recording with the suspended camera.

7.3 Select a male mouse. If the mice are marked for identification, note this male's identity; if not, mark this mouse so that it can be distinguished after returning to the cage.

7.4 Gently place the male mouse beneath a cup at the lower left corner in the arena. After 30 seconds, remove the cup via a string mechanism.

7.5 Allow the mouse to explore for 10 min, taking care to stay out of its field of view during recording. At the end of the 10 min, remove the mouse from the arena and return it to a holding cage for the next 5 min[12].

7.6 Stop recording and save the video file with an appropriate identifier.

8. Damsel-in-Distress Testing: Social Responsiveness Measure

8.1 Use the same arena and recording setup as in the exploratory behavior measure.

8.2 Select a female mouse. If the mice are marked for identification, note this female's identity; if not, mark this mouse so that it can be distinguished after it is returned to the cage.

Note: A littermate, cage mate, or novel mouse can be used, depending on the research question. If multiple trials are being performed, maintain the type of relationship across all trials.

8.3 Hold the female mouse gently by the base of the tail, or by neck restraint, if necessary, and lower it into the restraint chamber. Close the open end behind it and ensure that it is unable to turn around.

Note: Consider wearing bite-resistant gloves, as the female mouse will be resistant to entering the restraint chamber.

8.4 Begin recording with the suspended camera.

8.5 Place the restraint chamber with the trapped female mouse inside in the center of the empathy arena and allow the female to acclimate for 5 min. Take care to remain out of the female's field of view.

8.6 After the female mouse has been in the restraint chamber for 5 min, place the marked male mouse back into the empathy arena using the same process as before. Allow the male mouse an additional 5 min to explore the arena, again taking care to remain out of the field of view of the mice.

8.7 Stop recording and save the video file with an appropriate identifier.

8.8 At the end of the 5 min, remove both mice from the arena and place them into their cages. Replace the corn bedding and sanitize both the arena and the stress chamber with 70% ethanol[12].

9. Damsel-in-Distress Testing: Data Analysis

9.1 Tracking software can be used, but the video can also be analyzed manually. Once the video file is visible on the computer, overlay a transparent sheet over the screen and outline the square of the arena using a marker. Divide the arena square into nine equal compartments.

9.2 Review the video data for the first 5 min of the initial exploratory measure. Record the number of compartments crossed (locomotor activity/exploratory behavior), time spent in the center square of the arena (open field aversion), number of digging and grooming episodes, and number of times the mouse touched the empty center restraint chamber.

Note: Scoring is not done for the second 5 min of the male's 10-minute initial exploration, nor for the 5 min the female is trapped in the central chamber in the absence of the male.

9.3 Review the video data for the 5 min of the social responsiveness measure immediately after the male is reintroduced into the arena with the trapped female. Record the same data as for the initial exploratory measure, but additionally record the number of times the male mouse touched noses with the trapped female mouse.

REPRESENTATIVE RESULTS:

The Barnes Maze

To illustrate how the Barnes maze can be used, we investigated whether a single early ethanol exposure caused a difference in learning over the course of mouse development. C57Bl6/J mice were either injected with a 2.5 g/kg ethanol solution (n = 8) or with saline (n = 6) twice, two hours apart, at postnatal day 6. We trained the animals on the Barnes maze during adolescence (P30), then performed a single long-term trial in adulthood (4 months). Our collected data can be divided into several main categories: 1) accuracy by time and error rate, 2) target entry, and 3) mapping the exploration path.

Accuracy by time and error rate:

Time measurements encompass several important aspects: The latency to reach the first hole (the time it takes for the mouse to investigate any first hole after starting the trial) will illustrate if the mouse is learning that it is supposed to go find the escape hole (**Figure 1A**). The latency to find the target hole (scored by an initial lowering of the nose across the plane of the target hole) is able to find the correct hole (**Figure 1B**). With each session, the time to run the training session should decrease, indicating that the animals are learning the maze.

The number of wrong hole investigations (termed errors) the mouse performs before locating the correct hole (**Figure 1C**) indicates if the animal is able to go relatively straight to the target escape hole. The total number of times a mouse explores an incorrect hole over the course of the trial gives a rudimentary measure of locomotor activity and exploratory behavior. (**Figure 1D**). All mice were successfully trained on the Barnes maze over a 7-day testing period during adolescence (represented here by daily sessions on postnatal days 32-39), indicated by a decrease in error rate and time to find the target hole. There was no statistical difference in learning between the ethanol and control animals over the course of the training period.

Target entry rate:

The latency to actually enter the target hole is commonly used as the criteria for successful maze completion, but likely does not give as much information as other aspects of the testing. Entry into the target can also be scored as a 0 or 1. If the mouse enters the target before the 5 min elapse, the testing is considered successful, but if the mouse does not enter the target, a binder or book should be used to corral the animal into the target so it experiences the safety of the chamber beneath. On the first training days, it should be expected to see low rates of entry into the target and animals that take the full 5 minutes on the maze. In subsequent days, the testing should take less time.

Making multiple errors after finding the target indicates low motivation to enter the target box and may also detect abnormal comfort with a situation that is typically aversive to mice (elevated platform, bright light, open field, ultrasonic noise). A large difference between errors made before finding the target and the total number of hole explorations during a trial indicates poor motivation to enter the target escape chamber (**Figure 2A**). If mice are not entering the target escape hole after discovery, bright lights or noise can be added to the testing situation as mildly noxious stimuli.

In our experiment, an ultrasonic noise emitter was added above the maze on test day 5 to introduce extra motivation to enter the target. Prior to the introduction of the ultrasonic noise, no saline treated mice had ever entered the target, but entrances spiked after the introduction of the noise. Significantly more mice (saline and ethanol combined) entered the target on Trial 6 compared to Trial 4 (*t*-test, $p = 0.0014$) (**Figure 2B**).

Path mapping:

Mapping the path of the mouse can help assess information retention and learning strategies, as well. The distance between the first explored hole and the target hole is a good indicator of memory for target location, as this tracks where the mouse goes first and how close they are to the target (**Figure 3A**). Expect this measure to vary widely at the beginning of training, but the first hole visited should move closer to the target as the mice learn the target location.

Although no statistical difference in learning was found between the ethanol and control animals over the course of the training period, when these mice were re-run as adults using a single long-term trial (at 4 months), the ethanol-injected animals had a harder time remembering where the target hole was located (**Figure 3B**)[12]. This difference in memory retention was not found in the

initial Barnes maze training, so using the maze again at a later time allowed us to pick up subtle differences in learning/memory, such as those found with a single developmental ethanol intoxication event. A representative diagram of the Barnes maze surface allows researchers to map the path the mouse took, including the number of errors and a record of the first hole visited (**Figure 3C**).

Time spent in the opposite quadrant corresponds to the amount of time a mouse explores one section of the maze over others, specifically the one opposite of the target. It is possible for a researcher to obtain and calculate all other measures as the mouse is running the maze, but this measure requires analysis at a later time. A mouse who is learning should decrease time spent exploring holes in the opposite quadrant. On the long-term adult trial, ethanol treated mice explored significantly more holes in the opposite quadrant from the target, indicating a failure to remember the correct general spatial location ($p = 0.03$) (**Figure 4A**).

It is important to break up this mapping measurements into errors made before and after actually finding the target. The number of errors before finding the target can reveal if learning is occurring, but it also can tell us about the mouse's search strategy. Instead of randomly visiting holes, the mouse may adopt a strategy to quickly find the target. During the long-term trial, all of the ethanol-treated animals used a sequential hole-to-hole search in a ring-shaped fashion until the hole was found, whereas significantly less saline treated mice employed this technique ($p = 0.004$) (**Figures 4B** and **4D**). Alternatively, mice can target a general direction of the maze (**Figure 4C**), which indicates that spatial learning is occurring and the animal is likely using external cues to head for the vicinity of the target hole.

Damsel-in-Distress

The Damsel-in-Distress arena was constructed and used to test the exploratory and social behavior of C57Bl6/J mice at both 1M ($n = 15$) and 4M ($n = 12$) of age. An empathy arena was utilized where the male mouse placed in a corner (**Figure 5A**). Mouse exploratory behavior is quantified as the number of squares crossed in 5 min in the novel arena. Our results show that older mice (4 month) cross more squares than younger mice (1 month) ($p < 0.0001$) (**Figure 5B**).

Next, the trapped female was placed in a cylinder in the middle of the arena (**Figure 5A**). Once the female is trapped in the chamber, exploratory behavior can again be quantified as the number of squares crossed. The presence of the trapped female impacts male exploratory behavior. Regardless of age, male mice explore less in the presence of a trapped female. Juvenile males explored the arena less (as measured by the number of squares crossed in the presence of a trapped female mouse than when alone (at 1 month: *t*-test, $p = 0.001$; mean male alone 46 ± 4.34 squares vs. with female 26.13 ± 3.42 squares). This difference was also apparent in adults (at 4 months: *t*-test, $p < 0.000001$; mean male alone 151.5 ± 5.31 squares vs. with female $103.08 \pm 0.3.59$ squares) (**Figure 5B**).

In addition, the amount of time a mouse spent in the middle square over a 5-minute period should also be assessed. Time spent in the center square should be compared in the presence and the absence of the female as a measure of social interest. Our results show that males spend

more time in the center square in the presence of the trapped female. Juvenile males spent more time exploring the center of the arena in the presence of a trapped female mouse than when alone (at 1 month: $p = 0.05$; mean male alone 13.14 ± 2.79 s vs. with female 33.42 ± 9.32 s). This difference was also seen in adults (at 4 months: $p = 0.001$; mean male alone 14.80 ± 1.89 s vs. with female 52.87 ± 8.9 s) (**Figure 5C**).

In addition, researchers should also quantify the number of digging events in the arena, which could be a measure of anxiety or prosocial behavior towards the trapped female. This measure was significantly different in the presence of the female for 1 month (1M) mice but not 4 month (4M) mice. Juvenile males spent more time digging during their time in the arena in the presence of a trapped female mouse than when alone (at 1 month: $p = 0.027$; mean male alone 0.4 ± 0.27 times vs. with female 3.2 ± 1.09 times). This difference was not seen in adults (at 4 months: $p = 0.65$; mean male alone 1.33 ± 0.54 times vs. with female 1 ± 0.49 times) (**Figure 5D**).

The number of times a male touches the restraint chamber containing the trapped female and the number of times the animals touched noses through the air holes are measures of prosocial behavior. In our results, male mice touched the tube containing the trapped female significantly more frequently as adults (4 months) than as juveniles (1 month) ($p = 0.00001$; mean 1 month 4.73 ± 0.95 times vs. 4 months 15.92 ± 1.64 times) (**Figure 5E**). Adult males also initiated more trapped female contact events. Male mice touched noses with the trapped female through the air holes significantly more frequently as adults (4 months) than as juveniles (1 month) ($p = 0.002$; mean 1 month 3.93 ± 1 times vs. 4 months 8.67 ± .94 times). (**Figure 5F**).

Ultrasonic recording equipment was used to listen for ultrasonic vocalizations above 20 kHz by female mice, and it was also used to listen for ultrasonic vocalizations by trapped male mice in a "bachelor-in-distress" reversal paradigm. No vocalizations were detected in either situation, which is expected, since previous research found no ultrasonic vocalizations mice during aversive stimuli exposure (such as physical restraint or electric shock)[11].

FIGURE AND TABLE LEGENDS:

Figure 1: Barnes maze accuracy representative results by time and error rate. Mice were either injected with a 2.5 g/kg ethanol solution or with saline twice 2 hours apart at postnatal day 6. We trained the animals once daily for 7 days during adolescence, then performed a single long-term trial in adulthood[11]. (**A**) The latency to reach the first hole (the time it takes for the mouse to investigate any first hole after starting the trial) reveals if the mouse is learning to find the escape hole. (**B**) The latency to find the target hole (scored by an initial lowering of nose across the plane of the target hole) is able to find the correct hole. (**C**) The number of wrong hole investigations (termed errors) the mouse makes before locating the correct hole indicates if the animal is able to go relatively straight to the target escape hole. (**D**) The total number of times a mouse explores an incorrect hole over the course of the trial gives a rudimentary measure of locomotor activity and exploratory behavior. Error bars represent SEM[11].

Figure 2: Successful Barnes maze completion. The latency to actually enter the target hole is commonly used as the criteria for successful maze completion, but animals may not enter the

target hole after discovery. (**A**) A large difference between errors made before finding the target and the total number of hole explorations during a trial indicates poor motivation to enter the target escape chamber. In our experiment, an ultrasonic noise was added on Trial 5 to induce mice to enter the target. (**B**) Significantly more mice (saline and ethanol combined) entered the target on Trial 6 compared to Trial 4 (*t*-test, p = 0.0014). No ethanol mice kept exploring after finding the target by day 7. Error bars represent SEM.

Figure 3: Barnes maze motivation. Mapping mouse paths can provide information about motivation. (**A**) The distance between the first explored hole and the target hole is a good indicator of memory for target location, as this measure tracks where the mouse goes first and how far they are away from the target. (**B**) Although no statistical difference in learning was found between the ethanol and control animals over the course of the training period, when these mice were re-run as adults using a single long-term trial (at 4 months), the ethanol-injected animals had a harder time remembering where the target hole was located. (**C**) A representative diagram of the Barnes maze surface allows researchers to map the path the mouse took, including the number of errors and a record of the first hole visited. This mouse went straight to the target within 16 seconds, but never entered it over the course of 5 minutes and explored 41 holes afterwards. This behavior indicates low motivation to enter the target. Error bars represent SEM.

Figure 4: Barnes maze search strategy. Mapping mouse paths can uncover search strategies and provide information about learning strategy. (**A**) On the long-term adult trial, ethanol treated mice explored significantly more holes in the opposite quadrant from the target, indicating a failure to remember the correct general spatial location (*p = 0.03). (**B**) During the long-term trial, all of the ethanol-treated animals used a sequential hole-to-hole search in a ring-shaped fashion until the hole was found, whereas significantly less of the saline treated mice employed this technique (**p = 0.004) (**B, D**). Trial day 6 results are pictured for a (**C**) saline treated mouse and (**D**) ethanol treated mouse. Mice can target a general direction of the maze (**C**), which indicates that spatial learning is occurring and the animal is likely using external cues to head for the vicinity of the target hole. Typically, quadrant scores are much better for mice using this strategy. (**D**) The sequential hole-to-hole search is a ring strategy, where the mouse goes to any edge of the maze and looks in each hole to find the target. Quadrant scores are poor with great variation when mice use the ring strategy. Error bars represent SEM.

Figure 5. Damsel-in-distress. (**A**) The Damsel-in-Distress arena is divided into 9 squares. The male mouse is placed in a corner and male behavior is observed. The female mouse, trapped in a cylinder container, is placed in the central square of the arena. (**B**) Exploratory behavior within the arena increases between adolescence (1 month = 1M) and adulthood (4 months = 4M). Overall, male mice explored less in the presence of a trapped female. In addition, juvenile mice explored less than adult mice in the presence of the trapped female. (**C**) Social responsiveness can also be measured by the time a mouse spends in the center square where the trapped mouse is held. Both juvenile and adult males spent more time in the center square in the presence of the trapped female. (**D**) Juvenile males spent more time digging during their time in the arena in the presence of a trapped female mouse than when alone. (**E**) Male mice touched the tube containing the trapped female significantly more frequently as adults than as juveniles. (**F**) Adult

males also initiated more trapped female contact events than juveniles. Error bars represent SEM (***$p < 0.0001$, **$p \leq 0.001$, *$p \leq 0.05$).

DISCUSSION:

The Barnes maze and the Damsel-in-distress experiments are inexpensive, quick, and relatively easy ways to evaluate spatial learning, locomotor activity, and prosocial behavior in mice. Other advantages include the absence of overt stressors, pain, or food restriction for the animal. Like most learning/memory paradigms, a disadvantage of the Barnes maze is the number of trials required for the animals to learn where the target hole is located and enter.

Data collection:

Data should be consistently gathered when observing the mouse's activities and behaviors in both paradigms. For the Barnes maze, this includes recording the time the first hole is reached, the number of errors/wrong holes it explores, the time at which it reaches the target hole, and the time at which it enters into the target hole. In addition, researchers should map the path of each mouse on the maze. Data can be tracked using a paper map of the Barnes maze at the same time that the mouse is running the maze, and the timing and hole numbers can be confirmed using video footage. When tracking the animal, it may be helpful to change pen colors once every minute to keep track of potentially overlapping hole explorations. Computer-assisted tracking systems are available to ensure accuracy and reliability, such as idTracker, Ctrax, or tracktor, and a few are free or open-source.

For the Damsel-in-Distress assay, recorded data should include how many squares the male enters, the time it takes for the male to reach the female, how much time the male spends in the center square when the trapped female is present compared to only the empty cylinder, how many contact events there are with the cylinder, the number of times the animals touched noses through the chamber, and how many times digging events occurred overall and in the center square.

Constants in behavioral testing:

As with all behavioral tests, it is critical that conditions are kept constant between and within animal trials to ensure that environmental variables do not confound the results of the study. Visual, auditory, or olfactory stimuli in the experimental environment can cause behavioral changes in the mice, whether intended or not[13]. As such, care should be taken to shield the mice from distracting external stimuli[14]. Both the arena used for the Damsel-in-Distress scenario and the Barnes maze should be cleaned after each trial with water and sanitized with 70% ethanol to ensure that no lingering indicators of previous mice influence a later trial. In addition, bedding should be changed after each Damsel-in-Distress assay. Data collection should always be recorded on video to allow the experimenters to review the data and ensure accurate analysis. It can be a challenge for the researcher to remain hidden behind a curtain or otherwise out of sight while collecting the data, but this is important to maintain a constant, distraction-free setting for the mice.

Barnes maze paradigm design and modifications:

There are multiple published paradigms in use for the Barnes maze. Barnes maze training protocols typically range from 7-15 days, but the protocol can be shortened. Sometimes truncating the number of training sessions to make learning the task harder can isolate differences that longer protocols miss[15]. It can be used with only an overhead light (as in the original Barnes study), or other elements can be added (such as a brighter light, a fan, or a noise) in order to make the mice more motivated to enter the escape chamber[3,16]. If you observe poor motivation to enter the target box during a pilot study, the addition of these elements should be considered during the actual training.

Entry into the correct target hole will likely occur less rapidly without these elements, but researchers should be aware that these additions may be stress-inducing stimuli. The presence or absence of aversive stimuli can be manipulated to assess motivation to enter the target hole. Our study showed that prenatal ethanol had an effect on the likelihood that a mouse would enter the escape hole during the training period when only the bright light was used, and this phenomenon was distinct from actually learning where the target hole was located.

As seen in days 1-4 of our protocol, the mice were not very motivated to enter the hole, though they seemed to find it easily enough. However, when the ultrasonic noise was placed above the maze on day 5, scores improved on most Barnes measures, including the likelihood that mice would enter the target chamber after finding it. The noise appeared to dramatically reduce the number of extraneous hole investigations on trial day 6 and 7, and by day 7 no ethanol mice kept exploring after finding the target (**Figure 2A**). Though it is possible this is a natural consequence of learning the maze, the animals did appear more agitated after the introduction of the ultrasonic noise and less likely to explore the maze. In order for the chamber to remain a rewarding situation for the mice, the researchers must also be careful to turn off the aversive stimuli (cover the hole and turn off the noise) and allow the animal to remain undisturbed in the chamber for the allotted amount of recovery time before removing the animal.

The design of the Barnes maze can have an impact on the learning and memory assessment. The current protocol uses extra-maze cues. In addition to the asymmetry of the testing room, posters of four simple black-on-white-background shapes (square, triangle, circle, cross) were mounted on the walls of the room as visual cues. The Barnes maze can be solved with or without the distal cues on the walls of the testing room or can be solved using a serial search strategy. Because of this, using only a “first hole distance from target” measurement may miss relevant differences in the way that the mouse solves the maze and may not differentiate spatial from non-spatial abilities[17]. Multiple analysis measures should be used, such as also examining the number of holes explored in the opposite square or the number of errors before the target. The pattern of search strategy can also be analyzed.

Different inbred strains of mice can have variable performance on the Barnes maze, possibly due to differences in visual acuity. If a strain of mice with poor visual acuity is chosen for use in the Barnes maze, the addition of a small wall around the edge of the maze may increase thigmotaxis (wall-hugging behavior) and increase the use of a non-visual search strategy. Males had better learning performance than females in DBA/2J and C3H/HeJ strains, and although only males were

used in the current protocol, C57/Bl6 mice have not been found to display gender differences in memory on the Barnes maze[18].

Damsel-in-Distress paradigm design and modifications:
For the Damsel-in-Distress experiment, a major limitation is that there is no way for the exploratory mouse to free the female mouse, or "damsel," from the tube in the center of the arena. Redesigning the chamber to contain a release mechanism would be an interesting future modification. A disadvantage of the Damsel-in-Distress is the absence of comparative studies using the paradigm, as it is a new assessment for measuring prosocial behavior.

The Damsel-in-Distress could be further modified to assess sociability (defined as the inclination to spend time with another mouse), preference for social novelty (the inclination to investigate a novel mouse compared to a familiar mouse), mating or aggressive behaviors (trapping a female in estrus or a novel male in the chamber), and evolutionary biology issues, such as the impact of shared genetic background on altruistic behavior tendencies (the response to a trapped littermate compared to a trapped non-littermate mouse)[19].

ACKNOWLEDGMENTS:
We at Hampden-Sydney College give acknowledgement and appreciation to Sean Walden, Zach Leitner, Hunter Lee, and Anton Kheirani for their involvement in testing the protocols for the Barnes maze and Damsel-in-Distress experiments. We would also like to thank James Foster at Randolph-Macon College for the construction of the Barnes maze and the Randolph-Macon College Department of Biology for providing testing space.

DISCLOSURES:
The authors have nothing to disclose.

REFERENCES
1) Sunyer, B., Patil, S., Höger, H., Lubec, G. Barnes maze, a useful task to assess spatial reference memory in the mice. *Protocol Exchange.* 2007, (2016).
2) Vorhees, V., Williams, M.T. Assessing Spatial Learning and Memory in Rodents. *ILAR Journal.* **55**(2), 310–332 (2014).
3) Barnes, C.A. Memory deficits associated with senescence: A neurophysiological and behavioral study in the rat. *Journal of Comparative and Physiological Psychology*. **93** (1), 74-104 (1979).
4) Harrison, F.E., Hosseini, A.H., McDonald, M.P. Endogenous anxiety and stress responses in water maze and Barnes maze spatial memory tasks. *Behavioural Brain Research.* **198** (1), 247–251 (2009).
5) Silverman, J.L., Yang, M., Lord, C., Crawley, J.N. Behavioural phenotyping assays for mouse models of autism. *Nature Reviews Neuroscience*. **11** (7), 490 (2010).
6) Bartal, I.B.A., Decety, J., Mason, P. Empathy and pro-social behavior in rats. *Science*. **334** (6061), 1427-1430 (2011).
7) Langford, D.L., *et al.* Social modulation of pain as evidence for empathy in mice. *Science*. **312** (5782), 1967-1970 (2006).

8) Smith, M.L., Hostetler, C.M., Heinricher, M.M., Ryabinin, A.E. Social transfer of pain in mice. *Science Advances*. **2** (10), e1600855 (2016).
9) Melia, K.R., Ryabinin, A.E., Schroeder, R., Bloom, F.E., Wilson, M.C. Induction and habituation of immediate early gene expression in rat brain by acute and repeated restraint stress. *Journal of Neuroscience*. **14** (10), 5929-5938 (1994).
10) Watanabe, S. Empathy and reversed empathy of stress in mice. *Public Library of Science (PloS) One*. **6**(8), e23357 (2011).
11) Portfors, C. V. Types and functions of ultrasonic vocalizations in laboratory rats and mice. *Journal of the American Association for Laboratory Animal Science*. **46** (1), 28-34 (2007).
12) Houlé, K., Abdi, M., Clabough, E.B.D. Acute ethanol exposure during late mouse neurodevelopment results in long term deficits in memory retrieval, but not in social responsiveness. *Brain and Behavior.* (2017).
13) Sales, G.D., Wilson, K.J., Spencer, K.E., Milligan, SR. Environmental ultrasound in laboratories and animal houses: A possible cause for concern in the welfare and use of laboratory animals. *Laboratory Animals.* **22,** 369–375 (1988).
14) Crabbe, J.C., Walhsten, D., Dudek, B.C. Genetics of mouse behavior: Interactions with laboratory environment. *Science.* **248,** 1670 - 1672 (1999).
15) Attar, A., Liu, T., Chan, W.T.C., Hayes, J., Nejad, M., Lei, K., Bitan, G. A shortened Barnes maze protocol reveals memory deficits at 4-months of age in the triple-transgenic mouse model of Alzheimer's disease. *PloS One*. **8** (11), (2013).
16) Inman-Wood, S.L., Williams, M.T., Morford, L.L., Vorhees, C.V. Effects of prenatal cocaine on Morris and Barnes maze tests of spatial learning and memory in the offspring of C57BL/6J mice. *Neurotoxicology and Teratology*. **22** (4), 547-557 (2000).
17) Harrison, F.E., Reiserer, R.S., Tomarken, A.J., McDonald, M.P. Spatial and nonspatial escape strategies in the Barnes maze. *Learning and Memory*. **13** (6), 809-819 (2006).
18) O'Leary, T.P., Savoie, V., Brown, R.E. Learning, memory and search strategies of inbred mouse strains with different visual abilities in the Barnes maze. *Behavioural Brain Research*. **216** (2), 531-542 (2011).
19) Kaidanovich-Beilin, O., Lipina, T., Vukobradovic, I., Roder, J., Woodgett, J.R. Assessment of social interaction behaviors. *Journal of Visualized Experiments.* **48,** (2011).

Figure 1

Click here to download Figure Fig 1 JOVE.png

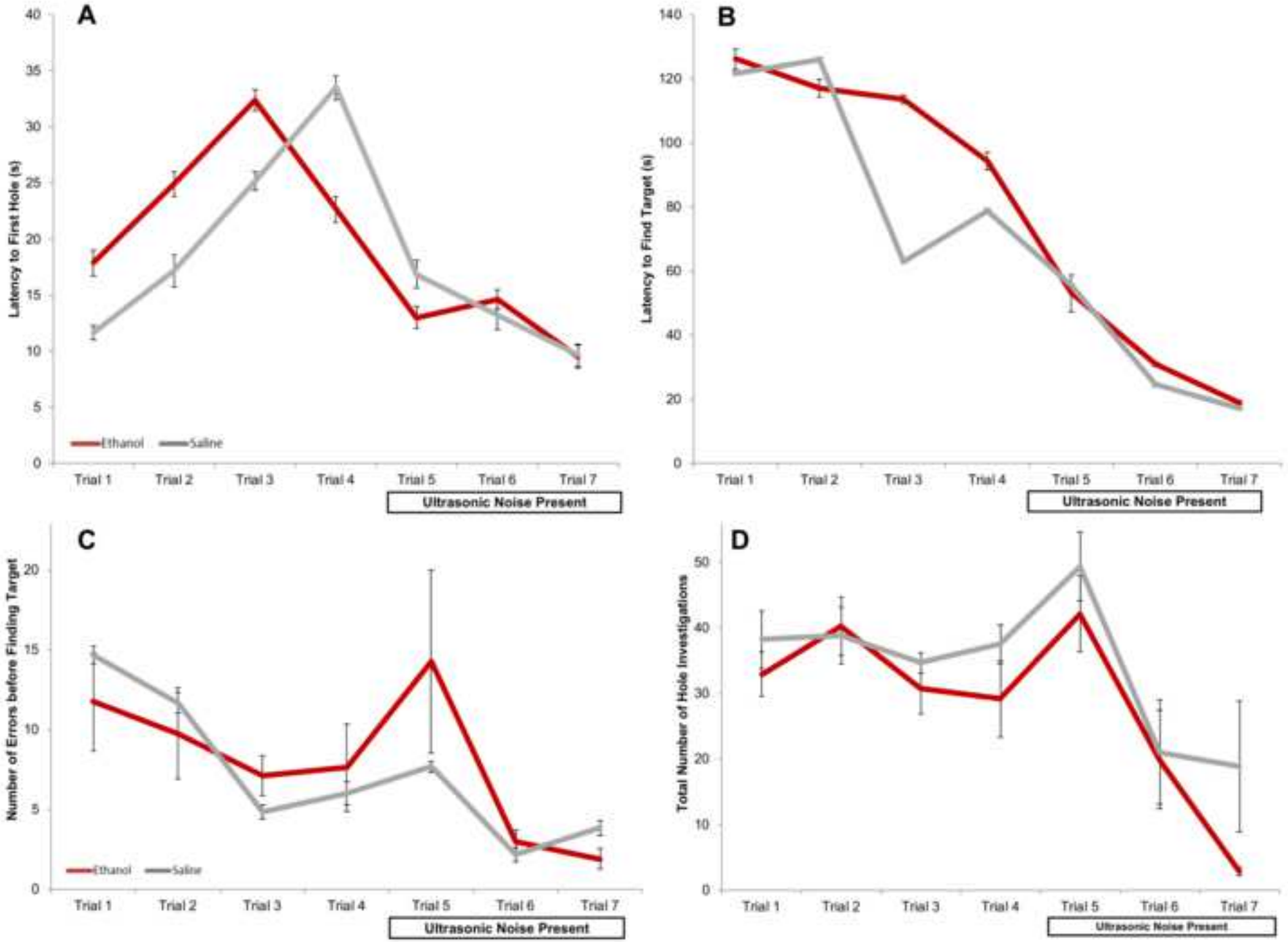


Figure 2

Click here to download Figure Fig2 JOVE.png

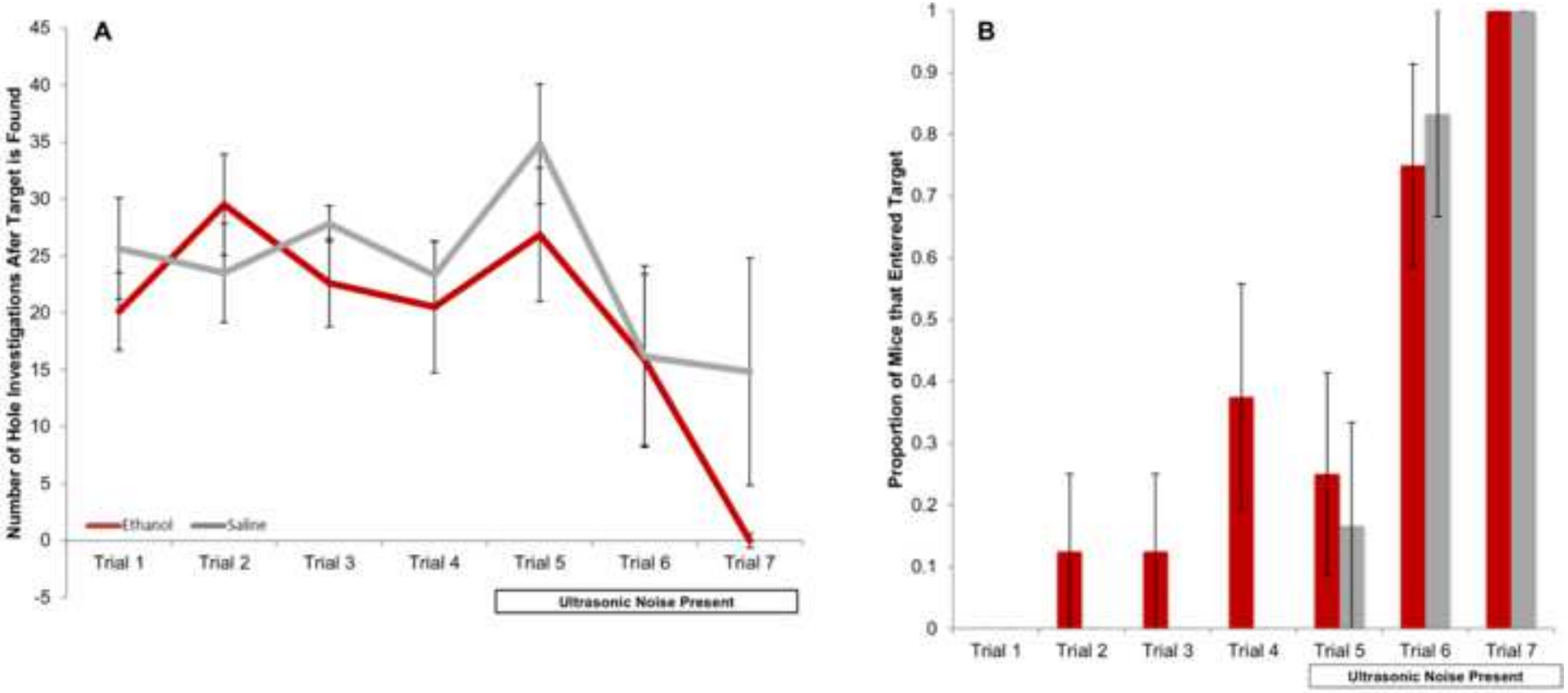


Figure 3

Click here to download Figure Fig 3 JOVE.png

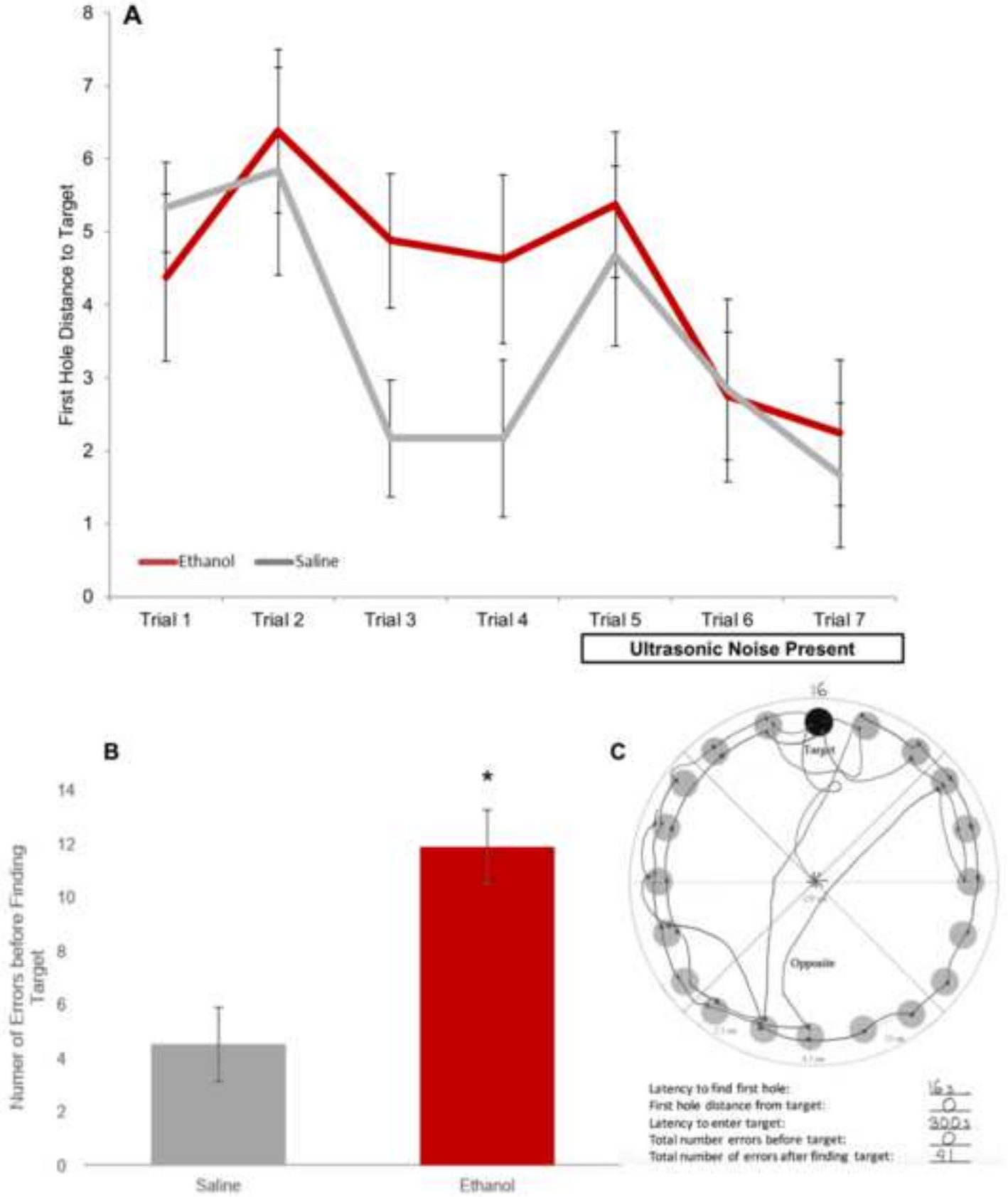


Figure 4

Click here to download Figure Fig4 JOVE.png

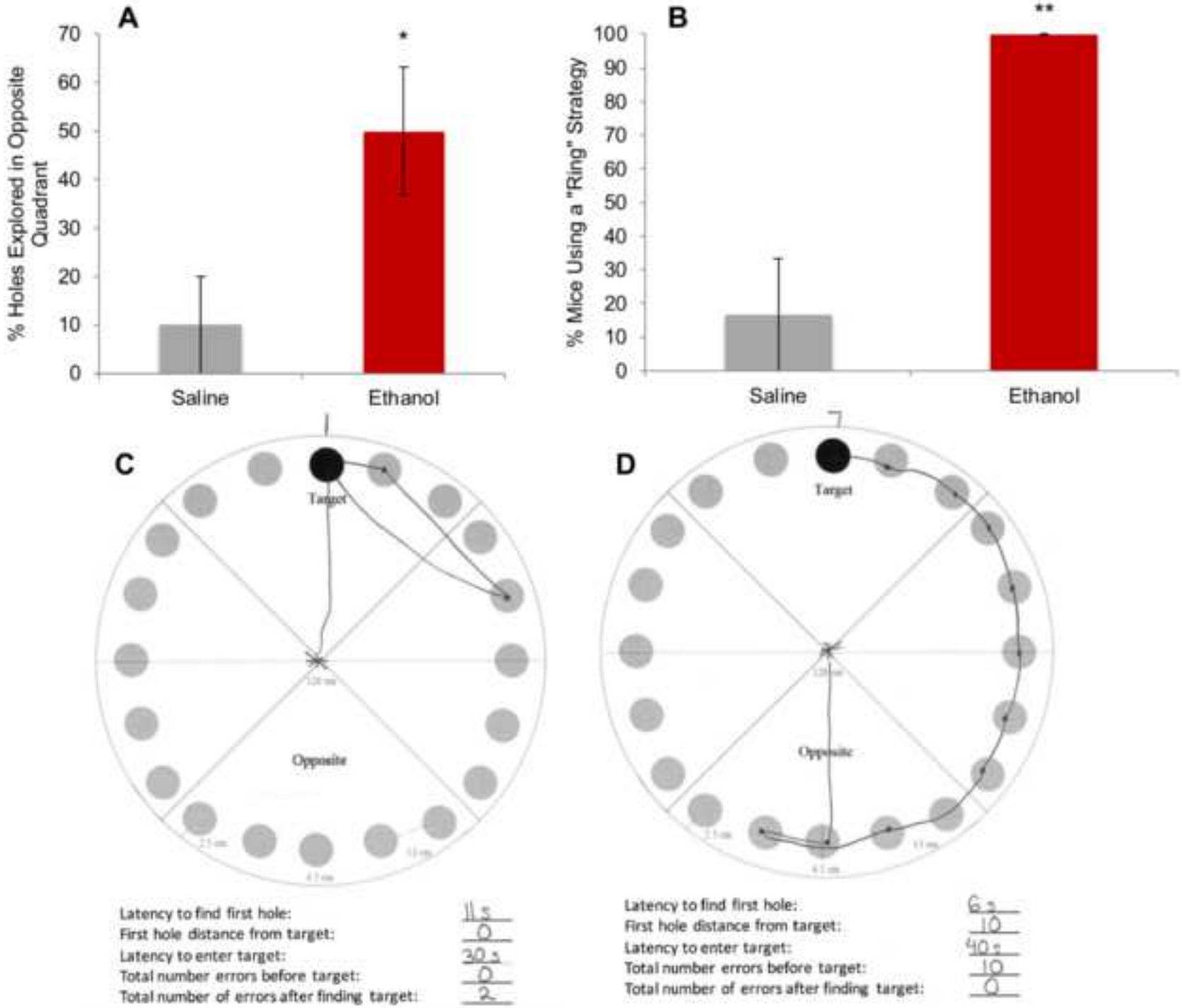
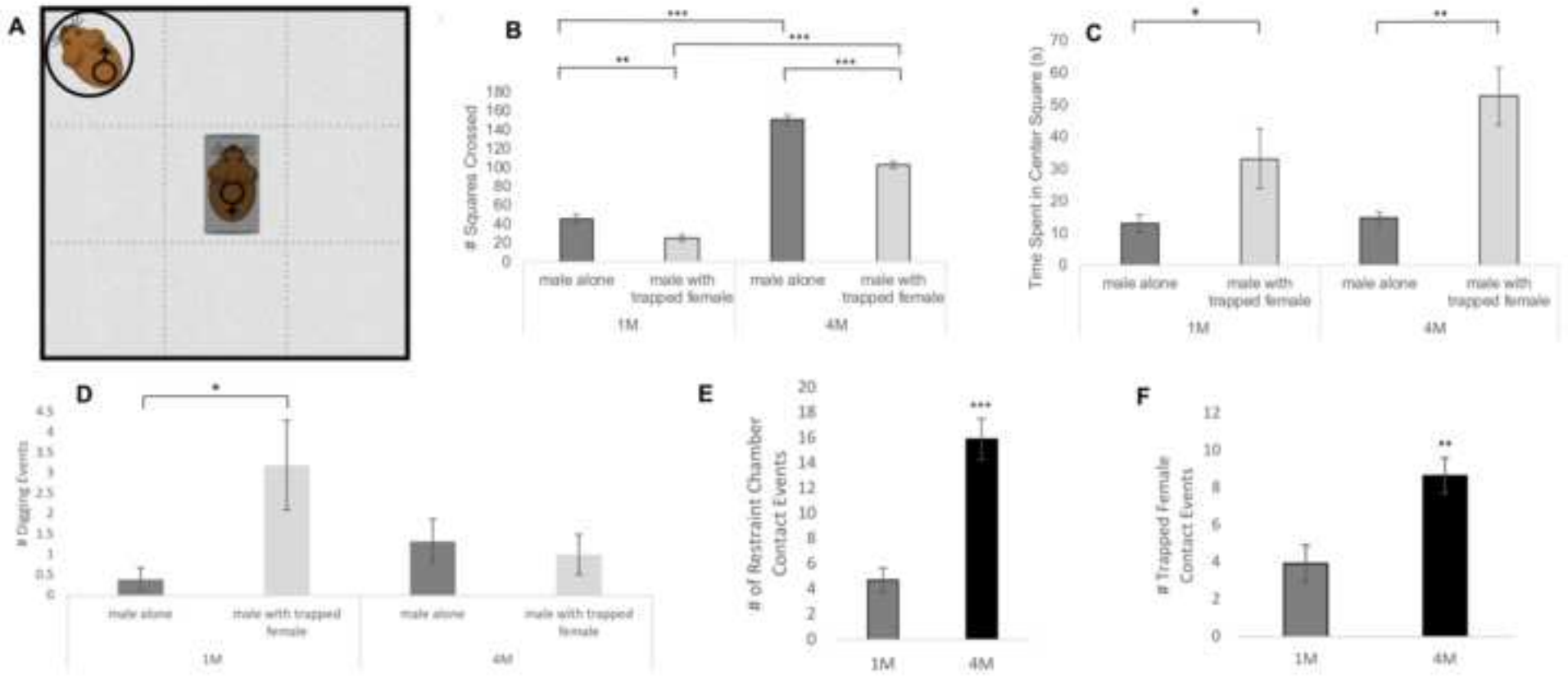


Figure 5

Click here to download Figure Fig5 JOVE.png



Materials Table

Click here to download Table of Materials Clabough JoVE Materials.xls.xlsx

Name of Material/ Equipment	Company
Damsel-in-Distress	
50 mL conical tube	Fisher Scientific
Rubber bands	Sprano Brand
hammer	Grainger
nail (size 8D)	Grainger
opaque, topless plastic box	AcmePlastics
video camera (smartphone)	N/A
bite-resistant gloves	Kent Scientific
transparency sheet	Staples
Barnes Maze	
Petri Dishes	Corning
Plywood (3/4 in.)	LP Building Products
Spray Paint	Krylon
Cup Hooks (5/8 in.)	Ace Hardware
Poster Board	Creatology
Light Bulbs	Phillips
Rubber bands	Sprano Brand
Ultrasonic noisemaker	PestChaser

Catalog Number

14-432-22
n/a
6R252
4NFE3
CUT-TO-SIZE-ACRYLIC-CAST-BLACK-SHEET-2025
N/A
GLVDYN02
954145

353025
22487
1274937
5360615
n/a
n/a
n/a
M753SN

Comments/Description

Any brand of 50 mL conical tube will work
Size 62, used to keep caps held to plywood
Any standard hammer will work
Similarly-sized nails should work just as well
Opaque plastic, cut to size (30 cm L x 19 cm W x 3-6 cm H). Step may be added to ensure no more than a 3.5 cm e
Any camera-equipped smartphone will work
Any brand offering appropriate protection
Any brand of clear plastic sheet will work, used for scoring

Spray painted and used as covers for Barnes maze holes
To contruct Barnes maze
Used to paint petri dish caps black, white paint used to paint plywood
2 used on either side of ventral hole surfaces; rubberband wraps around hooks to hold cap flat
Used at edges of maze as extra cues
100W light bulb, used to during the trials
Size 62, used to keep caps held to plywood
Used as aversive stimuli

ntrance depth for the mouse.

jove
1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT

full, unfettered access to the facilities of the Author or of the Author's institution as necessary to make the Video, whether actually published or not. JoVE has sole discretion as to the method of making and publishing the Materials, including, without limitation, to all decisions regarding editing, lighting, filming, timing of publication, if any, length, quality, content and the like.

11. Indemnification. The Author agrees to indemnify JoVE and/or its successors and assigns from and against any and all claims, costs, and expenses, including attorney's fees, arising out of any breach of any warranty or other representations contained herein. The Author further agrees to indemnify and hold harmless JoVE from and against any and all claims, costs, and expenses, including attorney's fees, resulting from the breach by the Author of any representation or warranty contained herein or from allegations or instances of violation of intellectual property rights, damage to the Author's or the Author's institution's facilities, fraud, libel, defamation, research, equipment, experiments, property damage, personal injury, violations of institutional, laboratory, hospital, ethical, human and animal treatment, privacy or other rules, regulations, laws, procedures or guidelines, liabilities and other losses or damages related in any way to the submission of work to JoVE, making of videos by JoVE, or publication in JoVE or elsewhere by JoVE. The Author shall be responsible for, and shall hold JoVE harmless from, damages caused by lack of sterilization, lack of cleanliness or by contamination due to the making of a video by JoVE its employees, agents or independent contractors. All sterilization, cleanliness or decontamination procedures shall be solely the responsibility of the Author and shall be undertaken at the Author's expense. All indemnifications provided herein shall include JoVE's attorney's fees and costs related to said losses or damages. Such indemnification and holding harmless shall include such losses or damages incurred by, or in connection with, acts or omissions of JoVE, its employees, agents or independent contractors.

12. Fees. To cover the cost incurred for publication, JoVE must receive payment before production and publication the Materials. Payment is due in 21 days of invoice. Should the Materials not be published due to an editorial or production decision, these funds will be returned to the Author. Withdrawal by the Author of any submitted Materials after final peer review approval will result in a US$1,200 fee to cover pre-production expenses incurred by JoVE. If payment is not received by the completion of filming, production and publication of the Materials will be suspended until payment is received.

13. Transfer, Governing Law. This Agreement may be assigned by JoVE and shall inure to the benefits of any of JoVE's successors and assignees. This Agreement shall be governed and construed by the internal laws of the Commonwealth of Massachusetts without giving effect to any conflict of law provision thereunder. This Agreement may be executed in counterparts, each of which shall be deemed an original, but all of which together shall be deemed to me one and the same agreement. A signed copy of this Agreement delivered by facsimile, e-mail or other means of electronic transmission shall be deemed to have the same legal effect as delivery of an original signed copy of this Agreement.

A signed copy of this document must be sent with all new submissions. Only one Agreement required per submission.

CORRESPONDING AUTHOR:

Name: Erin Clabough

Department: Biology

Institution: Hampden Sydney College

Article Title: a simple method of analyzing spatial learning and memory in mice

Signature: [signature]

Date: Feb 10, 18

Please submit a signed and dated copy of this license by one of the following three methods:

1) Upload a scanned copy of the document as a pfd on the JoVE submission site;
2) Fax the document to +1.866.381.2236;
3) Mail the document to JoVE / Attn: JoVE Editorial / 1 Alewife Center #200 / Cambridge, MA 02139

For questions, please email submissions@jove.com or call +1.617.945.9051

jove
JOURNAL OF VISUALIZED EXPERIMENTS
1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT

Title of Article: A simple method of analyzing spatial learning and episodic memory in mice

Author(s): Erin Clabough

Item 1 (check one box): The Author elects to have the Materials be made available (as described at http://www.jove.com/author) via: ☑ Standard Access ☐ Open Access

Item 2 (check one box):

☑ The Author is NOT a United States government employee.

☐ The Author is a United States government employee and the Materials were prepared in the course of his or her duties as a United States government employee.

☐ The Author is a United States government employee but the Materials were NOT prepared in the course of his or her duties as a United States government employee.

ARTICLE AND VIDEO LICENSE AGREEMENT

1. Defined Terms. As used in this Article and Video License Agreement, the following terms shall have the following meanings: "**Agreement**" means this Article and Video License Agreement; "**Article**" means the article specified on the last page of this Agreement, including any associated materials such as texts, figures, tables, artwork, abstracts, or summaries contained therein; "**Author**" means the author who is a signatory to this Agreement; "**Collective Work**" means a work, such as a periodical issue, anthology or encyclopedia, in which the Materials in their entirety in unmodified form, along with a number of other contributions, constituting separate and independent works in themselves, are assembled into a collective whole; "**CRC License**" means the Creative Commons Attribution-Non Commercial-No Derivs 3.0 Unported Agreement, the terms and conditions of which can be found at: http://creativecommons.org/licenses/by-nc-nd/3.0/legalcode; "**Derivative Work**" means a work based upon the Materials or upon the Materials and other pre-existing works, such as a translation, musical arrangement, dramatization, fictionalization, motion picture version, sound recording, art reproduction, abridgment, condensation, or any other form in which the Materials may be recast, transformed, or adapted; "**Institution**" means the institution, listed on the last page of this Agreement, by which the Author was employed at the time of the creation of the Materials; "**JoVE**" means MyJove Corporation, a Massachusetts corporation and the publisher of *The Journal of Visualized Experiments*; "**Materials**" means the Article and / or the Video; "**Parties**" means the Author and JoVE; "**Video**" means any video(s) made by the Author, alone or in conjunction with any other parties, or by JoVE or its affiliates or agents, individually or in collaboration with the Author or any other parties, incorporating all or any portion of the Article, and in which the Author may or may not appear.

2. Background. The Author, who is the author of the Article, in order to ensure the dissemination and protection of the Article, desires to have the JoVE publish the Article and create and transmit videos based on the Article. In furtherance of such goals, the Parties desire to memorialize in this Agreement the respective rights of each Party in and to the Article and the Video.

3. Grant of Rights in Article. In consideration of JoVE agreeing to publish the Article, the Author hereby grants to JoVE, subject to **Sections 4** and **7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Article in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Article into other languages, create adaptations, summaries or extracts of the Article or other Derivative Works (including, without limitation, the Video) or Collective Works based on all or any portion of the Article and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. If the "Open Access" box has been checked in **Item 1** above, JoVE and the Author hereby grant to the public all such rights in the Article as provided in, but subject to all limitations and requirements set forth in, the CRC License.

jove
1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT

4. Retention of Rights in Article. Notwithstanding the exclusive license granted to JoVE in **Section 3** above, the Author shall, with respect to the Article, retain the non-exclusive right to use all or part of the Article for the non-commercial purpose of giving lectures, presentations or teaching classes, and to post a copy of the Article on the Institution's website or the Author's personal website, in each case provided that a link to the Article on the JoVE website is provided and notice of JoVE's copyright in the Article is included. All non-copyright intellectual property rights in and to the Article, such as patent rights, shall remain with the Author.

5. Grant of Rights in Video – Standard Access. This **Section 5** applies if the "Standard Access" box has been checked in **Item 1** above or if no box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby acknowledges and agrees that, Subject to **Section 7** below, JoVE is and shall be the sole and exclusive owner of all rights of any nature, including, without limitation, all copyrights, in and to the Video. To the extent that, by law, the Author is deemed, now or at any time in the future, to have any rights of any nature in or to the Video, the Author hereby disclaims all such rights and transfers all such rights to JoVE.

6. Grant of Rights in Video – Open Access. This **Section 6** applies only if the "Open Access" box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby grants to JoVE, subject to **Section 7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Video in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Video into other languages, create adaptations, summaries or extracts of the Video or other Derivative Works or Collective Works based on all or any portion of the Video and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. For any Video to which this Section 6 is applicable, JoVE and the Author hereby grant to the public all such rights in the Video as provided in, but subject to all limitations and requirements set forth in, the CRC License.

7. Government Employees. If the Author is a United States government employee and the Article was prepared in the course of his or her duties as a United States government employee, as indicated in **Item 2** above, and any of the licenses or grants granted by the Author hereunder exceed the scope of the 17 U.S.C. 403, then the rights granted hereunder shall be limited to the maximum rights permitted under such statute. In such case, all provisions contained herein that are not in conflict with such statute shall remain in full force and effect, and all provisions contained herein that do so conflict shall be deemed to be amended so as to provide to JoVE the maximum rights permissible within such statute.

8. Likeness, Privacy, Personality. The Author hereby grants JoVE the right to use the Author's name, voice, likeness, picture, photograph, image, biography and performance in any way, commercial or otherwise, in connection with the Materials and the sale, promotion and distribution thereof. The Author hereby waives any and all rights he or she may have, relating to his or her appearance in the Video or otherwise relating to the Materials, under all applicable privacy, likeness, personality or similar laws.

9. Author Warranties. The Author represents and warrants that the Article is original, that it has not been published, that the copyright interest is owned by the Author (or, if more than one author is listed at the beginning of this Agreement, by such authors collectively) and has not been assigned, licensed, or otherwise transferred to any other party. The Author represents and warrants that the author(s) listed at the top of this Agreement are the only authors of the Materials. If more than one author is listed at the top of this Agreement and if any such author has not entered into a separate Article and Video License Agreement with JoVE relating to the Materials, the Author represents and warrants that the Author has been authorized by each of the other such authors to execute this Agreement on his or her behalf and to bind him or her with respect to the terms of this Agreement as if each of them had been a party hereto as an Author. The Author warrants that the use, reproduction, distribution, public or private performance or display, and/or modification of all or any portion of the Materials does not and will not violate, infringe and/or misappropriate the patent, trademark, intellectual property or other rights of any third party. The Author represents and warrants that it has and will continue to comply with all government, institutional and other regulations, including, without limitation all institutional, laboratory, hospital, ethical, human and animal treatment, privacy, and all other rules, regulations, laws, procedures or guidelines, applicable to the Materials, and that all research involving human and animal subjects has been approved by the Author's relevant institutional review board.

10. JoVE Discretion. If the Author requests the assistance of JoVE in producing the Video in the Author's facility, the Author shall ensure that the presence of JoVE employees, agents or independent contractors is in accordance with the relevant regulations of the Author's institution. If more than one author is listed at the beginning of this Agreement, JoVE may, in its sole discretion, elect not take any action with respect to the Article until such time as it has received complete, executed Article and Video License Agreements from each such author. JoVE reserves the right, in its absolute and sole discretion and without giving any reason therefore, to accept or decline any work submitted to JoVE. JoVE and its employees, agents and independent contractors shall have

Responses to Reviewers

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:

Chabout, J., Serreau, P., Ey, E., Bellier, L., Aubin, T., Bourgeron, T., & Granon, S. (2012). Adult male mice emit context-specific ultrasonic vocalizations that are modulated by prior isolation or group rearing environment. *PloS One*, *7*(1), e29401.

Clabough, E. B., & Zeitlin, S. O. (2006). Deletion of the triplet repeat encoding polyglutamine within the mouse Huntington's disease gene results in subtle behavioral/motor phenotypes in vivo and elevated levels of ATP with cellular senescence in vitro. *Human molecular genetics*, *15*(4), 607-623.

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.

Portfors, C. V. (2007). Types and functions of ultrasonic vocalizations in laboratory rats and mice. *Journal of the American Association for Laboratory Animal Science*, *46*(1), 28-34.