Journal of Visualized Experiments

Elevated plus maze test combined with video tracking software to investigate anxiolytic effect of exogenous ketogenic supplements

--Manuscript Draft--

Article Type:	Methods Article - JoVE Produced Video
Manuscript Number:	JoVE58396R2
Full Title:	Elevated plus maze test combined with video tracking software to investigate anxiolytic effect of exogenous ketogenic supplements
Keywords:	Elevated plus maze test, Exogenous ketone supplements, Gavage, Ketogenic diet, Glucose, Anxiety
Corresponding Author:	Csilla Ari University of South Florida Tampa, Florida UNITED STATES
Corresponding Author's Institution:	University of South Florida
Corresponding Author E-Mail:	csari2000@yahoo.com
Order of Authors:	Csilla Ari
	Dominic P. D'Agostino
	David M. Diamond
	Mark Kindy
	Collin Park
	Zsolt Kovács
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.	University of South Florida, MDC Vivarium, 12901 Bruce B. Downs Blvd., Tampa, FL 33612

TITLE:

Elevated Plus Maze Test Combined with Video Tracking Software to Investigate the Anxiolytic Effect of Exogenous Ketogenic Supplements

AUTHORS AND AFFILIATIONS:

Csilla Ari[1], Dominic P. D'Agostino[2,3], David Diamond[1], Mark Kindy[4], Collin Park[1], Zsolt Kovács[5]

[1]Department of Psychology, Hyperbaric Neuroscience Research Laboratory, University of South Florida, Tampa, FL, United States of America

[2]Department of Molecular Pharmacology and Physiology, Metabolic Medicine Research Laboratory, Morsani College of Medicine, University of South Florida, Tampa, FL, United States of America

[3]Institute for Human and Machine Cognition, Ocala, FL, United States of America

[5]Savaria Department of Biology, ELTE Eötvös Loránd University, Savaria University Center, Szombathely, Hungary

Corresponding Author:

Csilla Ari (csari2000@yahoo.com)

Tel: (813) 240-9925

E-mail Addresses of the Co-authors:

Dominic P. D'Agostino (ddagosti@health.usf.edu)

David Diamond (ddiamond@usf.edu)

Mark Kindy (kindym@health.usf.edu)

Collin Park (crpark@mail.usf.edu)

Zsolt Kovács (kovács.zsolt@sek.elte.hu)

KEYWORDS:

Elevated plus maze test, exogenous ketone supplements, gavage, ketogenic diet, glucose, anxiety

SUMMARY:

Here, we present a protocol to investigate changes in the anxiety level of rodent animal models. The elevated plus maze (EPM) test, used together with a video tracking software, provides a reliable method to document the effect of various potential anxiolytic treatments in preclinical laboratory scenarios.

ABSTRACT:

The overall goal of this study is to describe the methodology of the elevated plus maze (EPM) test in combination with a video tracking software. The purpose of the method is to document the effect of various potential anxiolytic treatments on laboratory rodent models. The EPM test is based on the rodents' proclivity toward protected, enclosed dark spaces and unconditioned fear of open spaces and heights, and their innate intense motivation to explore novel environments. The EPM test is a widely used behavioral test for investigating the anxiolytic or anxiogenic responses of rodents given drugs that are known to affect behavior. Observation

demonstrating a decreased proportion of time spent on closed arms, an increased proportion of time spent on open arms, a reduced number of entries to closed arms, and an elevated number of entries to open arms measured by the EPM test may reflect reduced anxiety levels. Using this method, the effect of exogenous ketone supplements on anxiety-related behavior is tested in Sprague Dawley (SPD) rats. Exogenous ketone supplements are chronically fed to the rats for 83 days or subchronically and acutely orally gavaged, daily for 7 days, before conducting the EPM test. Behavioral data collection is performed using the SMART video tracking system by a blinded observer at the end of the treatments. The main findings indicate that the EPM test is an effective method to detect the ketone supplement-induced anxiolytic effect and can be considered a sensitive measure to assess changes in anxiety behavior associated with drug- or metabolic-based therapies.

INTRODUCTION:

The goal of this article is to describe the methodology of the EPM test in combination with a video tracking software in order to monitor changes in anxiety-related behavior and novel treatments in laboratory rodent models. The EPM test is a relatively simple behavioral assessment method, which was developed for investigation of quantifying anxiety behavior levels and anxiety responses of rats after the application of drug treatments[1]. Indeed, it has been demonstrated that the EPM test is a widely used and effective behavioral assay for the investigation of changes in the anxiety levels of rodents[1,2]. The applicability of the EPM test in rodents (mainly rats and mice) is based on their proclivity toward enclosed, dark spaces (approach), an unconditioned fear of open spaces/heights (avoidance), and their high level of innate motivation to explore novel environments. Consequently, the EPM test is a well-established methodology based on an approach-avoidance conflict[2,3].

The EPM is a plus-shaped apparatus consisting of four elevated arms, which has been described by Handley and Mithani[4] (**Figure 1**), and consists of two opposite arms that are open to the surroundings (open arms), whereas the two closed opposite arms (closed arms) are equipped with walls. After treatment, if increased time is spent on the open arms and/or an increased number of open arm entries compared to control (untreated) animals is detected on the EPM, this indicates an anxiolytic effect[2,3]. The most robust avoidance response has been demonstrated in the first 5 min after the start (placement of the rats in the intersection of the four arms of the EPM) of the EPM assay[5]; therefore, any behavior after a treatment is commonly recorded for 5 min on the EPM. As additional measures of an anxiety level, the number of head dips, rears (vertical standing of the rodent on two hind legs), fecal boli, as well as total arm entries (spontaneous motor activity) and different postures (stretching or freezing), can also be recorded on the EPM[2]. Thus, multiple behavioral parameters can be compiled to provide a comprehensive assessment of anxiety-related behavior.

In order to increase the validity of the results, two to three behavioral assays are commonly used together, such as the light-dark choice test, the social interaction test, and the EPM test, to measure the anxiety levels of different animal models[6]. The EPM assay performed alone on rodents is also a suitable method to investigate the anxiolytic or anxiogenic effect of different drugs[7]. The EPM test is sensitive not only to benzodiazepine-type anxiolytics (*e.g.*, diazepam)[8],

but also, among others, to amino acid, monoamine, peptidergic and nucleosidergic compounds (*e.g.*, N-methyl-D-aspartate (NMDA) antagonist AP7, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) antagonist CNQX, μ-opioid receptor agonist morphine, NPY1 antagonist BIBP3226, substance P, ghrelin, oxytocin, serotonin receptor agonists and antagonists such as 8-OH-DPAT and WAY-100635, and β1-adrenergic antagonist betaxolol)[9-12]. Consequently, the EPM assay on rodents is a suitable and sensitive method to investigate the influence of different treatments that influence brain areas involved in the anxiolytic effect (*e.g.*, the amygdala, hippocampus, and limbic areas) and mechanisms of action (*e.g.*, the serotonergic, GABAergic, and adenosinergic system) implicated in anxiety[2]. The agents tested in these EPM studies include exogenous ketone supplements that alter brain signaling in subtle ways that may require a sensitive method to detect behavioral changes.

In this article, we describe the EPM test used in combination with a video tracking software, which helps to eliminate experimental bias and facilitates the collection and analysis of behavioral alterations in response to novel anxiolytic treatments.

PROTOCOL:

The animal treatment and measuring procedures were performed in accordance with the University of South Florida Institutional Animal Care and Use Committee (IACUC) guidelines (Protocol #0006R). All efforts were made to reduce the number of animals used.

1. Preparations

NOTE: The protocol typically requires laboratory-bred rats or mouse for EPM testing. However, other animals, such as guinea pigs, have also been tested on EPMs[13]. It is important to consider the color contrast between the animals in the maze and the maze color when using video tracking. The contrast is less important for researchers watching animals live or *via* video. The settings of the video tracking software need to be configured to document that the animals are black or white on either a black or white maze. Problems with configuration settings can occur with a clear acrylic maze, but a matte grey maze can be optimal for both rodent colors.

1.1. Select animals for the experiment, considering the potential influencing factors, such as strain, sex, estrus cycle, and age, as well as body weight[2].

1.2. Based on the individual experiment, determine the number of animals per group for the test.

NOTE: The group size will be dependent on the effect size that is expected with the test treatment. Power analyses are generally done before the experiment is initiated to determine the minimum number of subjects to be included given the variability in animal's responses in any given task, as well as the number of experimental groups/conditions.

1.3. Design the experiment (in which a battery of different behavioral tests, such as the open-field test, EPM test, hole-board test, and forced-swimming test will be used) carefully.

NOTE: Pre-exposure of the rodents to a novel test environment (such as an open-field test) immediately before the EPM tests may change the behavior of the animals on the EPM[1,2].

1.4. Handle all animals in a similar way before the EPM test.

NOTE: It has been demonstrated that different stress factors, application of drugs (*e.g.*, injections), shipping stress, and handling can change the behavior and behavioral responses of rodents on the EPM[16]. Thus, habituation of the animals to an animal house (*e.g.*, after shipment, for 1 - 2 weeks before the EPM test), experimental conditions, and treatment procedures (*e.g.*, gavaging) are necessary. It is also important that the handling of the rodents and any experience with prior stressors, particularly immediately before testing, is consistent across animals and treatment groups.

1.5. Conduct the behavioral studies in nocturnal animals, such as rats and mice, using a reverse light cycle, so that the behavioral assessment can be performed when the animals are in their dark, active phase.

NOTE: The effects of different housing conditions and light cycle/circadian rhythms on behavior and their influence on EPM results were demonstrated previously[17], since the animals' hormones are regulated by the light cycle.

1.6. Use the same experimenters during the procedures and ask them to avoid perfume or soaps with a strong odor.

1.7. Ask the experimenters not to talk near the animal during the experiment or move objects near the EPM environment.

NOTE: It is critically important that the observer makes minimal movements and no noise when collecting behavioral data.

1.8. Clean the entire EPM after each trial to erase any smells of previous animals which might interfere with the exploration by the test animal.

1.9. (Recommended) Handle the animals for several days before the EPM test (picking it up gently by the torso and holding it for a minute or two) to acclimate them to the experimenter.

1.10. When placing the animals on the EPM, make sure to handle all animals in a consistent manner and place each rodent in the EPM in the same position facing the same arm (*e.g.*, in the center facing the open arm away from the experimenter).

2. Application of Exogenous Ketone Supplements

2.1. Measure the body weight of the animals before starting any treatments to determine the dosage calculation for the treatment (*e.g.*, intragastric gavage).

2.2. Familiarize the animals to the intragastric gavage method (adaptation period) using water by gavage for 5 d before ketone supplementation (standard rodent chow/standard diet [SD] + water gavage; *e.g.*, 2.5 g/kg body weight of water/day). Exclude the use of any animal that does not adapt to the intragastric gavage method.

2.3. Following the adaptation period, feed the animals chronically for 83 d and subchronically for 7 d with SD and gavage daily with either water (*e.g.*, 5 g/kg body weight/day; control group: *n* = 8), ketone supplements such as ketone ester (KE; 1,3-butanediol-acetoacetate diester; *e.g.*, 5 g/kg body weight/day; *n* = 8), ketone salt (KS; Na^+/K^+–beta-hydroxybutyrate [βHB] mineral salt; *e.g.*, 5 g/kg body weight/day; *n* = 8), or KS+ medium chain triglyceride (1:1 ratio, KSMCT; *n* = 8)[18-20].

NOTE: The animals that received intragastric gavage were tested on the EPM 1 h after treatment. Rats fed with standard rodent chow and gavaged with water (excluding ketone supplementation) served as control groups.

3. Anxiety Assay

3.1. EPM apparatus

3.1.1. Use the same apparatus across a study to standardize results. The EPM is a plus-shaped apparatus, which consists of four arms (*e.g.*, the arms may be 10 cm wide and 50 cm long): two opposite arms are opened, and the two closed opposite arms are equipped with high (*e.g.*, 30 cm) walls. The apparatus is elevated above the floor (*e.g.*, by 55 cm)[2].

NOTE: The most commonly used parameters are the accumulated time spent in the open arms and the number of entries into the open arms; however, the time spent in the closed arms and center, and the number of entries into the closed arms and center are measured, as well as the distance traveled in each area.

3.1.2. Light up the EPM by using indirect lighting (*i.e.*, direct the light source toward the ceiling instead of directly illuminating the EPM apparatus) and ensure all four arms are similarly illuminated (without shadows, see **Figure 2**).

NOTE: Changes in the level of light alter the behavior of the rodents on the EPM. Therefore, similar illumination is needed in consecutive experimental animals and days (*e.g.*, 2,800 lumens in the room)[2].

3.2. Video tracking system

NOTE: Use a video tracking system with a computer interface and a video camera for the data collection, which will automatically collect behavioral data in rats (**Figure 3**). For the video tracking system, a wide variety of standard analog cameras or user-defined image sources

(infrared cameras, camcorder, WIA-compliant USB camera, webcams, *etc.*) can be used. When analyzing the recorded video, the movement-tracking software supports all common video formats, such as .avi, .vob, .wmv, .asf, .mov, .qt, .mpg, .mpeg, .mp4, .3gp, and .mkv. If the video does not playback correctly, it might require a specific codec; additional video formats are supported if the corresponding codec is installed in the system. The movement-tracking software can also be used to analyze previously acquired videos and process the images from different sources, such as DVD/HD recorders, digital video files (.avi, .divx, .mpeg, *etc.*), webcams, DV cameras, and WIA-compatible imaging devices.

3.2.1. System setup

3.2.1.1. Plug the installation key of the movement-tracking software into a USB 2.0 port and launch the installation tool.

3.2.1.2. Fix the camera above the experimental area and ensure that it will stay immobile for the duration of the experiment.

3.2.1.3. Set up a new experiment in the movement-tracking software system by using the instruction manual. Select **New task**. Double-click on the icon of the protocol that the new experiment should follow (**Figure 3**, **Supplementary File 1**).

3.2.1.4. Enter details to label/describe the experiment in the **Experiment Info** dialog.

3.2.1.5. Specify the source of the video sequences to process.

3.2.1.6. Define the transformation rule for a correct distances measurement. The calibration process enables the movement-tracking software to be informed of the actual dimensions of the experimental area in order to obtain reliable values for distances and speeds.

3.2.1.7. Determine the regions of interest (zones) in the working area.

3.2.1.8. Adjust the parameters of the detection process.

3.2.1.8.1. In order for the movement-tracking software to precisely detect the position of the animal in the image, some detection adjustments must be set.

3.2.1.8.2. The tracking process requires a clear and well-contrasted image by using a fine adjustment of the general brightness and contrast parameters in the **Brightness & Contrast** section of the **Detection Settings** panel. As needed, adjust these settings for the whole image or for user-defined zones.

3.2.1.9. Put a rat into each arena to test the detection process.

3.2.1.10. Press the **Start Test** button to verify if the detection process can identify the subject correctly. Confirm the detection is activated by the appearance of a dot on the screen. The calibration process has to be done before starting the test.

3.2.1.11. Detection is considered confirmed when the only black dot shown in the player is the animal being tracked. The red tracking line needs to closely follow all the animal's displacements. Proper tracking is also confirmed with a white label listing the animal number and corresponding coordinates based on displacement. If such a detection is not obtained, adjust the **Threshold** and **Erosions** parameters for optimizing the detection and tracking process.

3.2.1.12. Adjust the **Threshold** and **Erosion** parameters to get a sharper and noise-free test image.

3.2.1.13. If the tracking path is correctly detected, press the **Stop Test** button (**Figure 4**). If these adjustments are going to be used for every new experimental file, press the **Save as default** button. Press the **Accept** button to save the new detection settings.

3.2.1.14. Set the time conditions of the trials.

3.2.1.15. If the experimental protocol requires the track acquisition process to start at the same time the subject is placed into the experimental area, it is possible to set up the remote unit that comes with the software or to use a wireless mouse.

NOTE: This option provides the possibility of remotely controlling the start and stop; thus, the recording session can be completed without the computer.

3.2.2. Setup of the Subjects in the system

3.2.2.1. Manage the experimentation subject's database. To create a database of experimental subjects, enter the **Subjects Database** manager by pressing the **Subjects** button in the **Experimentation Assistant** bar.

3.2.2.2. Press the **+** button to add new subjects to the database.

3.2.2.3. With the one subject option already selected, enter the subject's code.

3.2.2.4. Fill the rest of the subject's information in the **Subject Properties** section.

3.2.2.5. Press the **Create** button to add the new subject.

3.2.2.6. Define the experimentation plan. Use the **Scheduler** to define the different phases, sessions, trials, and subjects planned to be executed within the experimental project. The trial is selected automatically as "the next trial" to be executed. This property is shown as a green tick on the left side of the trial name.

3.2.3. Data acquisition by simultaneous recording and tracking

NOTE: When a live image source is selected, the **Player** panel provides an embedded recording module for easily capturing the video coming from the selected camera.

3.2.3.1. Prepare the movement-tracking software for data acquisition (calibration, zone definition, detection settings, time settings, scheduler).

3.2.3.2. Open the **Data acquisition** panel.

3.2.3.3. Start recording the video of the experiment without the animal by pressing the **Start recording** button available in the software.

3.2.3.4. Place the animal into the experimental area.

3.2.3.5. Start the data acquisition process by pressing the **Start** button on the **Time control** panel. The tracking process will be carried out simultaneous with the recording process. As needed, ask the experimenter to note down the behavioral variables manually, such as rears, head dips, and falls (**Figure 5**).

3.2.3.6. Collect the EPM data manually as well as by a blinded observer (separate the observer from the EPM by a curtain) in the testing room.

3.2.3.7. Wait until the end of the tracking-process recording or press the **Stop** button on the **Time control** panel.

3.2.3.8. Remove the animal from the experimental area. Stop the video-recording process by pressing the **stop** button available on the movement-tracking software player.

3.2.3.9. Prepare the experimental area for the next animal by washing and drying it. Repeat the cycle again.

3.2.4. Data analysis

3.2.4.1. To access the **Analysis** tool, press the **Analysis** button in the **Experimentation Assistant** bar.

3.2.4.2. To generate analysis reports of the finished trials, select the trials to analyze. Configure and select the analysis report. Set the time intervals to be analyzed. Generate and review the reports. Export the results to a spreadsheet or image formats (**Figure 6**).

3.3. EPM for the measurement of anxiety levels

3.3.1. Perform the EPM experiments under nonstress conditions (in a dimly lit and quiet room) after oral gavage.

NOTE: Make sure that the experiments are run in a close time interval (*e.g.*, between 1200 and 1400) because the circadian rhythm can influence the rodents' behavior on the EPM[15,17]. Avoid unnecessary movements and noise during the experiment.

3.3.2. Before the start of the test, make sure that the EPM is cleaned and dried and the video tracking system is ready to use.

3.3.3. Transfer the rats in their home cage to the experimental room 30 min prior to beginning the experiment.

3.3.4. Place a rat at the intersection of the four arms of the EPM, facing the open arm opposite of the experimenter.

3.3.5. Run the video tracking software, as well as manually record the behavior of the animal, for 5 min.

3.3.6. If the animal falls off the EPM, pick it up and place it back on the same point of the EPM where it fell off. Exclude the behavioral data of this animal from the analysis.

NOTE: A loud noise or movement may immobilize/freeze animals on open arms. If a loud noise is heard during the experiment, exclude the behavioral data of the animal undergoing the experiment at that moment from the analysis.

3.3.7. At the end of the 5 min test, stop the video tracking software and remove the animal from the EPM. Place it back into its home cage.

3.3.8. Before the next experiment/animal, clean the EPM with a disinfecting detergent (*e.g.*, Quatricide) followed by tap water. Dry the apparatus with paper towels.

4. Analyses of the Data Collected by the Video Tracking System

4.1. Based on the recorded data, analyze the amount of time spent in the open arms and in the closed arms; the number of entries made to the open arms, closed arms, and to the center zone; the latency to entry into the closed arms; the distance traveled in the open arms, closed arms, and in the center zone.

NOTE: The animal is considered to be in an area when all four paws are in that area.

4.2. Determine the effects of treatments on behavior by using an analysis of variance (ANOVA) with Fisher's least significant difference (LSD) test/Tukey's multiple comparisons test.

REPRESENTATIVE RESULTS:

The current experiment investigates the hypothesis that exogenous ketone supplementation administered either chronically (fed for 83 days) or subchronically (orally gavaged for 7 days) has an anxiolytic effect on two-month-old male Sprague-Dawley (SPD) rats (250 - 350 g). Chronic administration consisted of the following ketone supplements: low-dose ketone ester (LKE; 1,3-butanediol-acetoacetate diester, ~10 g/kg/day, LKE), high-dose ketone ester (HKE; ~25 g/kg/day, HKE), beta-hydroxybutyrate-mineral salt (bHB-S; ~25 g/kg/day, KS), and bHB-S + medium chain triglyceride (MCT; ~25 g/kg/day, KSMCT). For subchronic experiments, the following treatment groups were used: KE, KS, and KSMCT (5 g/kg/day). The control groups included SD or SD with water gavage (control). All data were represented as the mean ± the standard error of the mean (SEM). The results were considered significant when $p < 0.05$. The significance was determined by one-way ANOVA with Fisher's LSD test.

After chronic feeding, rats in the KSMCT group spent significantly more time in the open arms ($p = 0.0094$) compared to the control group. The time spent in the closed arms was significantly less in the LKE, KS, and KSMCT groups ($p = 0.0389$, 0.0077, and 0.0019, respectively), while the KS group spent significantly more time in the center ($p = 0.0239$) compared to the control (SD) group (**Figure 7A**)[18].

Rats in the KS and KSMCT groups traveled significantly longer distances in the open arms ($p = 0.036$ and 0.0165), while the rats in the LKE, KS and KSMCT groups showed significantly less distance traveled in the closed arms ($p = 0.0252$, 0.00041, and 0.0032, respectively), compared to the control group (SD) (**Figure 7B**). When compared to the control group, the KS and KSMCT groups had greater distance traveled in the center area ($p = 0.0206$ and 0.0482, respectively), while in the KSMCT group, the latency to the first entrance to the closed arms was significantly greater after chronic feeding ($p = 0.0038$)[18] (**Figure 7C**).

The time spent in the open arms was greater in the KE group ($p = 0.0281$) after 7 days of oral gavage, while in the KE, KS, and KSMCT groups, the time spent in the center decreased ($p = 0.0005$, < 0.0001, and = 0.023, respectively), compared to the control group (**Figure 8A**)[18]. In the KE and KS groups, the number of entries to the closed arms was significantly lower ($p = 0.0436$ and 0.0234, respectively) after 7 days of administration (**Figure 8B**), while the rats in the KS group also entered the center less frequently ($p = 0.0193$), compared to the control (SD) group.

FIGURE AND TABLE LEGENDS:

Figure 1: Elevated plus maze (EPM) used for testing rats. Each arm is 10 cm wide and 50 cm long, with two opposite arms opened with a raised edge. The two closed opposite arms are equipped with 30 cm-high walls. The runway height from the floor is 55 cm.

Figure 2: Examples of direct and indirect lighting. Ensure the light source is pointed toward the ceiling, while the direct light above the experimental area is blocked. It is important to use indirect light during EPM experiments in order to similarly illuminate all four arms without shadows.

Figure 3: The experimentation assistant bar of the movement-tracking software. It is designed to provide access to the main operations. The buttons correspond to the task within the typical experimentation process, while only the currently allowed tasks are active.

Figure 4: The subject track is marked with a red line following the animal's movement. By adjusting the threshold, the background can be decreased until only the animal is detected and tracked by the red line. The track is following the center of the mass of the subject, and the current position coordinates are indicated.

Figure 5: Elevated plus maze (EPM) with a Sprague Dawley (SPD) rat in the open arm. An example of the experimental set-up is demonstrated.

Figure 6: Accumulated movement track of the animal during a trial. As part of the data analysis, the collected trajectory trace of the subject in the tracking area can be displayed.

Figure 7: Behavioral responses of SPD rats in the EPM after 83 days of chronic feeding of exogenous ketone supplementation. These panels show representative results collected by the EPM and the movement-tracking system[18]. (**A**) The KSMCT group spent a greater percentage of time in the open arms, while the LKE, KS, and KSMCT groups spent less time in closed arms, compared to the control (SD) group. (**B**) The KS and KSMCT groups traveled more distance in the open arms, while the LKE, KS, and KSMCT groups traveled less distance in the closed arms, showing reduced anxiety compared to the control (SD) group. (**C**) The KSMCT group entered the closed arms later, indicating reduced anxiety compared to the control (SD) group. Abbreviations: SD = standard rodent chow + water (25 g/kg body weight (b.w.) of water/day); LKE = SD + LKE (1,3-butanediol-acetoacetate diester, 10 g/kg b.w./day); HKE = SD + HKE (25 g/kg b.w./day); KS = SD + beta-hydroxybutyrate-mineral salt (bHB-S; 25 g/kg b.w./day); KSMCT = SD + bHB-S + medium chain triglyceride (MCT; 25 g/kg b.w./day); SPD = Sprague-Dawley rat; EPM = elevated plus maze (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$). This figure has been modified from Ari *et al*.[18].

Figure 8: Behavioral responses of SPD rats after 7 days of oral gavage of exogenous ketone supplementation. Representative results were collected through the EPM test, using a movement-tracking software system[18]. (**A**) The KE group spent a greater percentage of time in the open arms, while the KE, KS, and KSMCT groups spent less time in the center (compared to the control [SD] group), thus indicating reduced anxiety. (**B**) Compared to the control (SD) group, less entries were detected in the closed arms from rats in the KE and KS groups. Abbreviations: SD = standard rodent chow + water (5 g/kg b.w. of water/day); KE = SD + ketone ester (1,3-butanediol-acetoacetate diester, 5 g/kg b.w./day); KS = SD + beta-hydroxybutyrate-mineral salt (bHB-S; 5 g/kg b.w./day); KSMCT = SD + bHB-S + MCT (5 g/kg b.w./day); SPD = Sprague-Dawley rat; EPM = elevated plus maze (* $p < 0.05$; *** $p < 0.001$; **** $p < 0.0001$). This figure has been modified from Ari *et al*.[18].

DISCUSSION:

In general, several commonly used tests, such as the light-dark choice test, the social interaction test, and the EPM test, are used to measure the anxiety level in different animal models. However, the EPM assay alone is a suitable method to investigate, for example, the effect of exogenous ketone supplements on rodents' anxiety levels[18,20].

The main advantage of the EPM method is that it relies on the rodents' instinctive proclivity toward dark, enclosed spaces, in addition to the unconditioned fear of heights and avoidance of open spaces. On the other hand, other methods used to study anxiety-like behavior are based on the behavioral responses to certain noxious stimuli, such as electric shock, food/water deprivation, loud noises, and exposure to predator odor[3]. These tests usually result in a conditioned response, while the EPM also represents a more humane alternative. Furthermore, the EPM can be a useful tool to study the involvement of different brain regions (*e.g.*, limbic regions, hippocampus) and the underlying mechanisms (*e.g.*, GABA, glutamate, serotonin, adenosine) of anxiety behavior[2].

When applying treatments that are quite stressful for the animals (*e.g.*, the oral gavage), it is important that all animals are handled the same way and by the same person, especially when assessing potential, subtle anxiolytic effects. If possible, introduction of the drug/compound in drinking water or *via* a palatable 'treat' may be a preferred method. To ensure that the same amount is administered to each animal, an oral gavage can be used. Based on the pharmacokinetic properties of the compound, it is usually advisable to test the animals on the EPM within 1 hour after gavaging. When selecting experimental subjects, it is important to consider their strain, sex, estrus cycle, and age, as well as body weight, according to the objectives and test substances[2]. In regard to age, when designing EPM studies and interpreting data, it is important to consider that the percentage of open arm entries linearly increases with age[21] and the aging-related changes in EPM behavior are strain-specific[22].

When conducting an EPM test, there are potential problems that need to be addressed. Sometimes animals need to be excluded from the analysis due to outlier tendencies (*e.g.*, the animal never leaves the area where it was placed, almost falls off the apparatus, is distracted by a noise or event outside of the apparatus). Further complications with EPM testing may include treatments which cause sedation or hyperactivity because these types of effects need to be assessed *via* EPM parameters.

It is important to expose animals to the EPM test only once because decreased activity on the open arms and a decreased total time spent on the central platform were demonstrated on the second (repeated) exposure of rodents compared to the first exposure on the EPM[14,15]. Therefore, a single exposure of rodents to the EPM test is strongly recommended. However, if there is a minimum of three weeks between the first and second exposure to the EPM and the EPM set-up is moved to another room (different environment), the animals may be investigated by the EPM test more than once[2].

The EPM is available in different materials, sizes (*e.g.*, for mouse or rat), and colors, which needs to be considered when choosing study subjects. It is important to keep in mind that the odors

left by the previous animal on the apparatus may change the behavior of the subsequent animal. Therefore, we recommend using an EPM made of a material that is easy to clean, such as acrylic glass (not transparent), which does not retain odors after washing. Avoid EPM apparatus made of wood. Preferably, use a matte color that is different from the color of the animals tested on the EPM (*e.g.*, black if white animals are tested). The better the contrast between the animal and the enclosure, the better the detection of the animal and the higher the reliability and precision of the results obtained (distance covered, speed, tracking). EPM apparatus made from matte gray material are useful with white, black, and white and black animals.

A further advantage of the video tracking system is that in addition to the EPM, it offers a flexible and easy way to set it up with a wide variety of behavioral tests, such as water maze, open-field, plus/radial arm/T-Y mazes, place preference, forced swimming, and tail suspension tests.

In summary, the goal of this article is to describe the EPM test used in combination with a video tracking software to collect and analyze behavioral alterations in response to novel anxiolytic treatments. The possible applications of the EPM include the prescreening of newly developed pharmacological agents for the treatment of anxiety-related disorders. In addition to the anxiolytic and anxiogenic agents, the behavioral effect of different hormones and drugs of abuse can also be investigated. The influence of aging and exposure to various stressors can also be assessed. This study has concluded that when proper steps are taken, the use of the EPM has proven to be a sensitive method to assess behavioral changes associated with ketone supplementation[18,20].

ACKNOWLEDGMENTS:

This work was supported by an ONR Grant N000141310062 and a GLUT1D Foundation Grant #6143113500 (to Dominic P. D'Agostino), by the National Development Agency of Hungary (under Grant No. TIOP-1.3.1.-07/2-2F-2009-2008; to Zsolt Kovács) and by the Department of Veterans Affairs (to Mark Kindy). The authors wish to thank Quest Nutrition LLC for supporting ongoing research on this topic (to Csilla Ari).

DISCLOSURES:

D'Agostino, D.P., Kesl, S., Arnold, P. Compositions and Methods for Producing Elevated and Sustained Ketosis. International Patent # PCT/US2014/031237. University of South Florida.

Ari, C., D'Agostino, D.P., Exogenous ketone supplements for reducing anxiety-related behavior. Provisional patent #62289749. University of South Florida.

Dominic P. D'Agostino and Csilla Ari are co-owners of the company Ketone Technologies LLC.

These interests have been reviewed and managed by the University in accordance with its Institutional and Individual Conflict of Interest policies. All authors declare that there are no additional conflicts of interest.

REFERENCES:

1. Pellow, S., Chopin, P., File, S.E., Briley, M. Validation of open : closed arm entries in an elevated plus-maze as a measure of anxiety in the rat. *Journal of Neuroscience Methods.* **14** (3), 149-167, https://doi.org/10.1016/0165-0270(85)90031-7 (1985).

2. Walf, A., Frye, C. The use of the elevated plus maze as an assay of anxiety-related behavior in rodents. *Nature Protocols.* **2** (2), 322-328, doi: 10.1038/nprot.2007.44 (2007).

3. Barnett, S.A. *The rat: A study in behavior.* University of Chicago Press. Chicago, IL (1975).

4. Handley, S.L., Mithani, S. Effects of alpha-adrenoceptor agonists and antagonists in a maze-exploration model of 'fear'-motivated behaviour. *Naunyn Schmiedebergs Archives. In Pharmacology*. **327** (1), 1-5, https://doi.org/10.1007/BF00504983 (1984).

5. Montgomery, K.C. The relation between fear induced by novel stimulation and exploratory behavior. *Journal of Comparative Physiology and Psychology*. **48** (4), 254-260, http://dx.doi.org/10.1037/h0043788 (1955).

6. Sarkisova, K.Y., Midzianovskaia, I.S., Kulikov, M.A. Depressive-like behavioral alterations and c-fos expression in the dopaminergic brain regions in WAG/Rij rats with genetic absence epilepsy. *Behavioral Brain Research.* **144** (1-2), 211-226, https://doi.org/10.1016/S0166-4328(03)00090-1 (2003).

7. Jain, N., Kemp, N., Adeyemo, O., Buchanan, P., Stone, T.W. Anxiolytic activity of adenosine receptor activation in mice. *British Journal of Pharmacology.* **1116** (3), 2127-2133, https://doi.org/10.1111/j.1476-5381.1995.tb16421.x (1995).

8. Paslawski, T., Treit, D., Baker, G.B., George, M., Coutts, R.T. The antidepressant drug phenelzine produces antianxiety effects in the plus-maze and increases in rat brain GABA. *Psychopharmacology* (Berlin). **127** (1), 19-24, https://doi.org/10.1007/BF02805970 (1996).

9. Florio, C., Prezioso, A., Papaioannou, A., Vertua, R. Adenosine A1 receptors modulate anxiety in CD1 mice. *Psychopharmacology* (Berlin). **136** (4), 311-319, doi: 10.1007/s002130050572 (1998).

10. Engin, E., Treit, D. The effects of intra-cerebral drug infusions on animals' unconditioned fear reactions: a systematic review. *Progress in Neuro-Psychopharmacology & Biological Psychiatry.* **32** (6), 1399-1419, doi: 10.1016/j.pnpbp.2008.03.020. (2008).

11. Botton, P.H. *et al.* Aged mice receiving caffeine since adulthood show distinct patterns of anxiety-related behavior. *Physiology and Behavior*. **170**, 47-53, doi: 10.1016/j.physbeh.2016.11.030. (2017).

12. Hughes, R.N., Hancock, N.J., Henwood, G.A., Rapley, S.A. Evidence for anxiolytic effects of acute caffeine on anxiety-related behavior in male and female rats tested with and without bright light. *Behavioural Brain Research*. **271**, 7-15, doi: 10.1016/j.bbr.2014.05.038. (2014).

13. Rex, A., Marsden, C.A., Fink, H. Effect of diazepam on cortical 5-HT release and behaviour in the guinea-pig on exposure to the elevated plus maze. *Psychopharmacology* (Berlin). **110** (4), 490-496, https://doi.org/10.1007/BF02244657 (1993).

14. Almeida, S.S., Garcia, R.A., de Oliveira, L.M. Effects of early protein malnutrition and repeated testing upon locomotor and exploratory behaviors in the elevated plus-maze. *Physiology of Behaviour*. **54** (4), 749-752, https://doi.org/10.1016/0031-9384(93)90086-U (1993).

15. Bertoglio, L.J., Carobrez, A.P. Behavioral profile of rats submitted to session 1-session 2 in the elevated plus-maze during diurnal/nocturnal phases and under different illumination conditions. *Behavioural Brain Research*. **132** (2), 135-143, https://doi.org/10.1016/S0166-4328(01)00396-5 (2002).

16. Korte, S.M., De Boer, S.F. A robust animal model of state anxiety: fear-potentiated behaviour in the elevated plus-maze. *European Journal of Pharmacology*. **463** (1-3), 163-175, https://doi.org/10.1016/S0014-2999(03)01279-2 (2003).

17. Carobrez, A.P., Bertoglio, L.J. Ethological and temporal analyses of anxiety-like behavior: the elevated plus-maze model 20 years on. *Neuroscience & Biobehavioural Reviews*. **29** (8), 1193-1205, https://doi.org/10.1016/j.neubiorev.2005.04.017 (2005).

18. Ari, C. *et al.* Exogenous ketone supplements reduce anxiety-related behavior in Sprague-Dawley and Wistar Albino Glaxo/Rijswijk rats. *Frontiers in Molecular Neuroscience*. **9**, 137. doi: 10.3389/fnmol.2016.00137 (2016).

19. D'Agostino, D.P. *et al.* Therapeutic ketosis with ketone ester delays central nervous system oxygen toxicity seizures in rats. *American Journal of Physiology: Regulation Integration and Comparative Physiology*. **304** (10), R829-836, doi: 10.1152/ajpregu.00506.2012 (2013).

20. Kovács, Z., D'Agostino, D.P., Ari, C. Anxiolytic effect of exogenous ketone supplementation is abolished by adenosine A1 receptor inhibition in Wistar Albino Glaxo/Rijswijk rats. *Frontiers in Behavioral Neuroscience*. **12**, 29 doi: 10.3389/fnbeh.2018.00029 (2018).

21. Lynn, D.A., Brown, G.R. The ontogeny of anxiety-like behavior in rats from adolescence to adulthood. *Developmental Psychobiology.* **52** (8), 731-739, doi: 10.1002/dev. 20468 (2010).

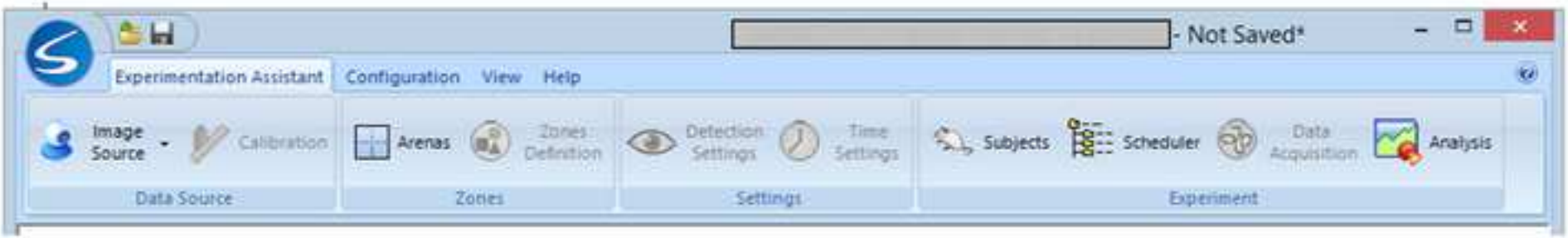
22. Ferguson, S.A., Gray, E.P. Aging effects on elevated plus maze behavior in spontaneously hypertensive, Wistar-Kyoto and Sprague-Dawley male and female rats. *Physiology of Behavior.* **85** (5), 621-628, doi: 10.1016/j.physbeh.2005. 06.009 (2005).

Figure1

Click here to access/download;Figure;Figure_1.jpg

Figure 3

Click here to access/download;Figure;Figure_3.jpg



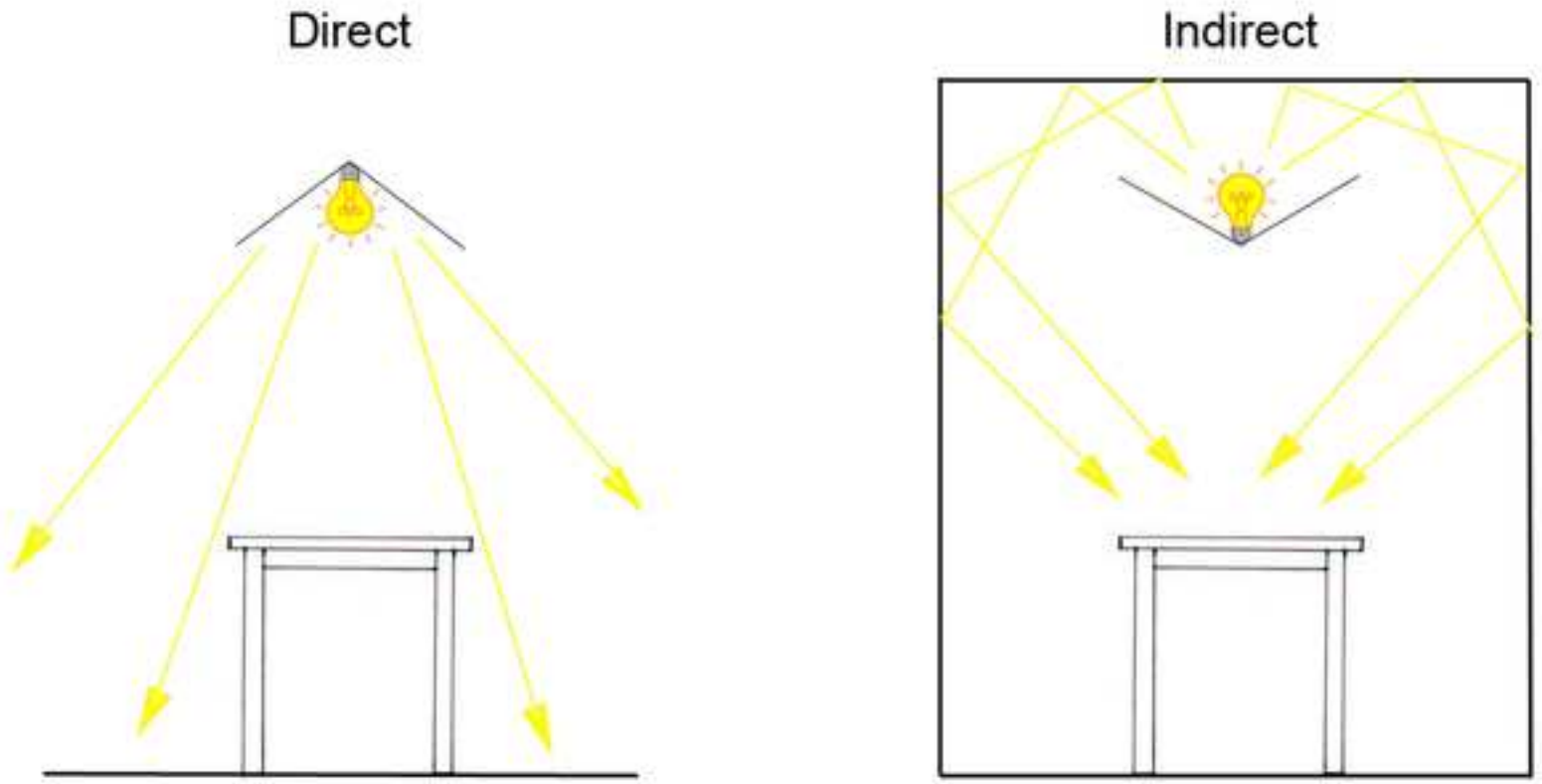


Figure4

Click here to access/download;Figure;Figure_4.jpg

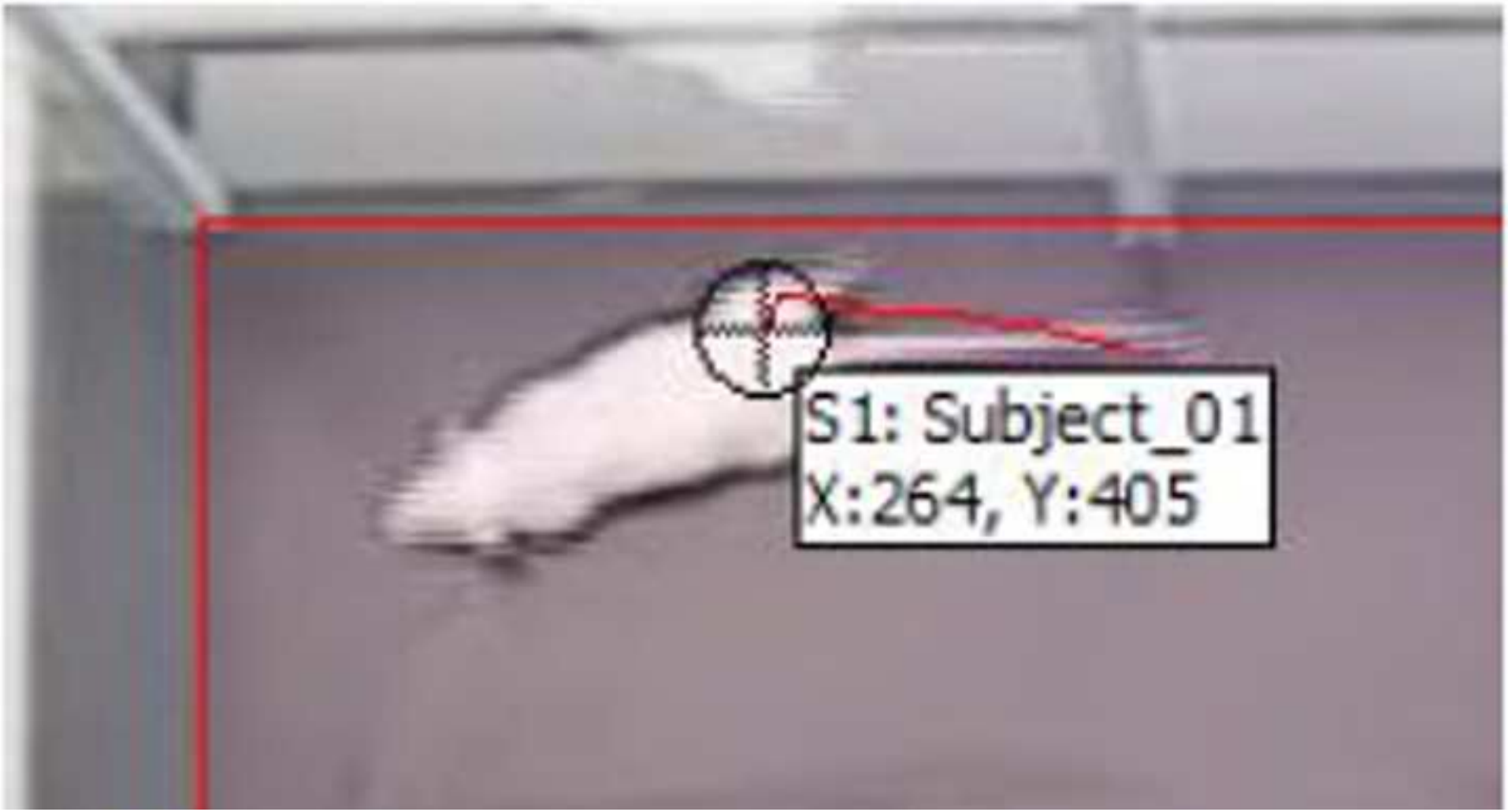


Figure5

Click here to access/download;Figure;Figure_5.jpg

Figure6

Click here to access/download;Figure;Figure_6.jpg

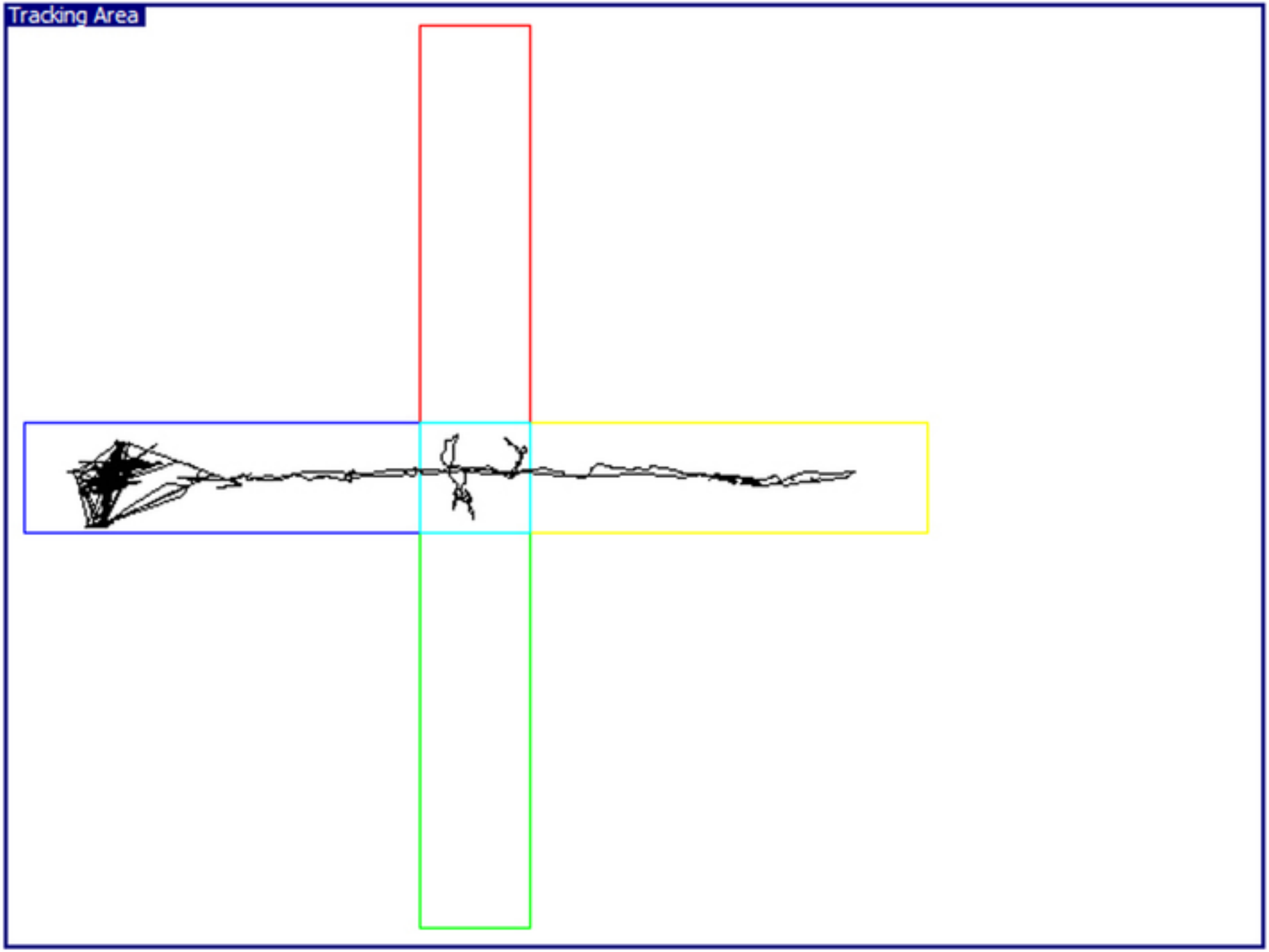


Figure7

Click here to access/download;Figure;Figure_7.jpg

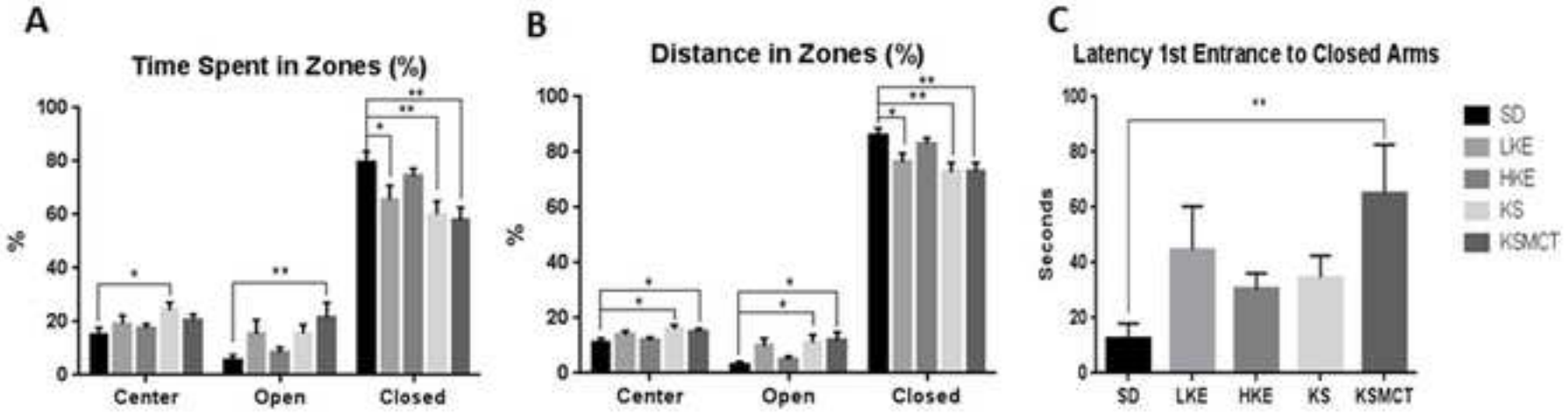


Figure8

Click here to access/download;Figure;Figure_8.jpg

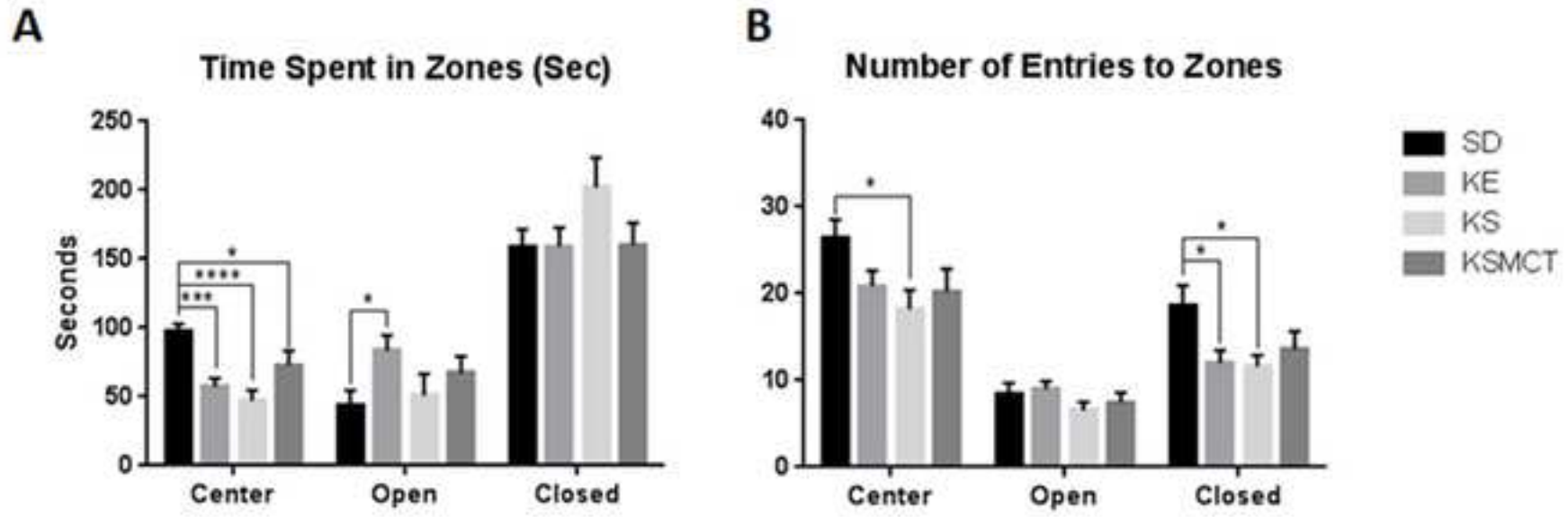


Table of Materials

Click here to access/download;Table of Materials;JoVE_Materials.xlsx

Name of Material/ Equipment	Company	Catalog Number
	Coulbourn Instruments	H10-35-EPM
	Harvard Apparatus	SMART 3.0

Comments/Description

Elevated Plus Maze for mice and rats

SMART Video Tracking Software

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: Elevated plus maze test combined with video tracking software to investigate antianxiolytic effect of exogenous ketogenic supplements

Author(s): Csilla Ari, Dominic P. D'Agostino, David Diamond, Mark Kindy, Collin Park, Zsolt Kovács

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.

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

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:	Csilla Ari
Department:	Department of Psychology
Institution:	University of South Florida
Article Title:	Elevated plus maze test combined with video tracking software to investigate antianxiolytic effect of exogenous ketogenic supplements
Signature:	Csilla Ari D'Agostino
Date:	7/20/2018

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

Dear Dr. Ari,

Your manuscript, JoVE58396 Investigation of anxiolytic effect of exogenous ketogenic supplements by elevated plus maze test, has been editorially and peer reviewed, and the following comments need to be addressed. Note that editorial comments address both requirements for video production and formatting of the article for publication. Please track the changes within the manuscript to identify all of the edits.

After revising and uploading your submission, please also upload a separate rebuttal document that addresses each of the editorial and peer review comments individually. Please submit each figure as a vector image file to ensure high resolution throughout production: (.svg, .eps, .ai). If submitting as a .tif or .psd, please ensure that the image is 1920 pixels x 1080 pixels or 300 dpi.

Your revision is due by **Jul 13, 2018**.

To submit a revision, go to the JoVE submission site and log in as an author. You will find your submission under the heading "Submission Needing Revision".

Best,

Alisha DSouza, Ph.D.
Senior Review Editor
JoVE
617.674.1888
Follow us: Facebook | Twitter | LinkedIn
About JoVE

_____

Editorial comments:
Changes to be made by the Author(s):
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

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

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

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

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

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

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

Response: Mistake has been corrected.

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

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

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

Response: Higher resolution figure has been made and uploaded.

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

Response: Summary has been rewritten clearly.

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

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

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

Response: Brackets have been removed.

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

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

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

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

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

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

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

Response: Requested revisions have been made.

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

Response: Requested revisions have been made.

15. 3.2.1.4: Please specify the details entered.

Response: Details have been specified.

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

Response: We do not wish to film the calibration steps

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

Response: Suggested revisions have been made to referenced numbers.

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

Response: Software screenshots have been added as supplementary files.

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

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

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

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

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

Response: Suggested revisions have been made to highlighted text.

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

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

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

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

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

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

25. References: Please do not abbreviate journal titles.

Response: Revisions have been made to include full title of journal.

Reviewers' comments:
Editor's Note: Please note that the reviewers raised some significant concerns regarding your method and your manuscript. For each peer review comment, please provide either (1) a description of how the comment was addressed within the manuscript or (2) a rebuttal describing why the comment was not addressed if you feel it was incorrect or out of the scope of this work for publication in JoVE.

Reviewer #1:

The submitted manuscript is a methodological paper on the use of the elevated plus maze test to assess anxiety in laboratory rodents. The authors provided a detailed description of this behavioral technique including the use of video tracking system and data analysis, alongside with representative results. Elevated plus maze test can be considered a gold standard for investigation of anxiety-related behaviors in rodent models. Although the method is well known, visual demonstration will help researchers apply it in a standardized way and avoid making simple mistakes, which is particularly important for those new in the field of behavioral science. Importantly, the video tracking systems are becoming more and more advanced. The ability to use video-tracing software in the most efficient way possible (e.g. the proper adjustment of detection settings) is crucial for investigators. It minimizes the manual labor,, thus reducing the influence of investigator on the data and ensuring achievement of more reliable and comparable results. In the reviewed manuscript the Authors use SMART software. In my opinion, it would be great if the Authors would bring more attention to the use of the video tracking system and not so much to the behavioral technique itself. It can be done in the terms of general principles of video tracing in the EPM and/or the use of particular software.

Response: In order to emphasize the video tracking system we added it to the title and highlighted its importance in the text.

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

Response The manuscript has been modified to emphasize the video tracking system and its advantages.

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

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

Response: The manuscript has been modified to emphasize the goal of the article as the description of the video tracking system together with EPM.

Another part that needs major improvement is the discussion. This section should be focused on the protocol and not the representative results. I would recommend removing the first paragraph which discusses the representative results and construct the discussion according to the JoVE's instructions for authors. The points that should be included in the discussion but are missing are: critical steps in the protocol, modifications and troubleshooting of the method and limitations of the method.

Response: The manuscript has been modified according to the suggestions of the reviewer.

Please see detailed comments below.

INTRODUCTION:
As already mentioned please include the goal of the article in the last paragraph of the introduction.

Response It was included.

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

Response: This sentence was modified.

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

Response: Part was removed.

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

Response: The sentence has been modified.

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

Response: This text was moved to discussion.

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

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

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

Response: This information was added to section 3.2.

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

Response: The sentence was modified.

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

Response: Manual data collection was added to 3.2.3.6

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

Response: Chapter 5 has been deleted.

DISCUSSION:

I strongly recommend to rewrite the discussion, adjust it to the journal requirements and make it more

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

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

Reviewer #2:

Manuscript Summary:
This paper is about ketone supplements on anxiety-like responses of rats. In some ways, this seems like a Methods paper, but there are details missing. The same is true if we interpret this as an empirical article. This should be clarified in the revision of this paper. Other feedback is as follows.

Major Concerns:

This paper requires extensive editing by a native English speaker.

Response: Paper has been proofread by native English speaker and revise and clarified in many areas.

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

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

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

Response: Additional details have been added throughout, when appropriate, to further clarify statements.

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

Response: The introduction section was expanded upon this statement.

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

Response: The sentence was modified to clarify the meaning.

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

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

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

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

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

Under Preparations:

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

Response: The importance of these factors was further explained in the discussion section.

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

Response: Section 1.3 was corrected to justify the sample size used in the experiment.

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

Response: The text has been revised and section 1.7 was corrected.

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

Response: Notes on contrast were added to section 1.1. to describe this important consideration.

Why were several sections in yellow highlight?

Response: Those parts are identified as sections to be video recorded by JOVE.

In the Abstract, authors need additional details about this particular report. For example, this is in certain types of subjects, using the EPM and SMART system. The way it is written now it suggests a more general methods type paper using the EPM, which is not exactly what this paper is about.

Response: The abstract was modified.

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

Response: The additional details were included in the protocol section.

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

In this specific experiment we used rats fed with standard rodent chow gavaged with water (control), without any ketone supplement. Treatment results are compared to control.

Reviewer #3:

Manuscript Summary:
Overall, this paper clearly presents an EPM technique aimed at testing treatments for anti-anxiety. However, some of the advice given is not appropriate for all types of drug/compound testing. For example, the oral gavage treatment is quite stressful for the animals and thus should be avoided, especially when assessing potentially subtle anxiolytic effects. Introduction of the drug/compound in drinking water or via a palatable 'treat' is a better method (though it may be necessary to yoke control animals' drinking or consumption to achieve comparable amounts.

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

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

Response: The manuscript was modified not to include the blood collection/analyses based on suggestions from the editor and other reviewers and only the methodology around the EPM combined with video tracking software was emphasized.

Major Concerns:
Methods:
The authors need to add a section on 'experimenter' guidelines. For instance, experimenters shouldn't wear strong perfume or soaps, or talk near the animal during the experiment, or move objects near the EPM environment, taking care to clean all of the EPM to erase smells of previous animals which might interfere with exploration of the test animal. It also may be useful to describe handling of animals (picking up gently by the torso and holding for a minute or two) several days before EPM .

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

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

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

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

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

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

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

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

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

How are blood glucose/ketone analysed, statistically?

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

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

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

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

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

Editorial comments:

1) Please see the attached word doc. In-text comments have been made; these require your attention. Please address the comments by editing your manuscript/figures. Please maintain the current format and track all your edits.

The article was edited based on the comments in the text.

2) If you are re-using figures from a previous publication, please obtain explicit permission to re-use the figure from the previous publisher (this can be in the form of a letter from an editor or a link to the editorial policies that allows you to re-publish the figure). Please upload the text of the re-print permission (may be copied and pasted from an email/website) as a Word document to the Editorial Manager site in the "Supplemental files (as requested by JoVE)" section. Please also cite the figure appropriately in the figure legend, i.e. "This figure has been modified from [citation]."

The following link gave information on the re-use of already published figures:
https://zendesk.frontiersin.org/hc/en-us/articles/201945842-Do-I-need-permission-to-reproduce-figures-or-text-previously-published-in-Frontiers-

In most cases, adaptation and reuse of figures is permitted provided that the authors and original source are appropriately credited and that no third-party licenses apply (please see the citation on the article on-line page).

Frontiers does not provide any formal permissions for reuse.

Reviewers' comments:

Reviewer #1:
The authors have addressed all of my comments and concerns. I think that the manuscript has greatly improved and contains a lot of useful information about EPM test. I suggest to avoid the repetition of the goal of the study in the first and the last paragraph of the introduction.

The last paragraph was modified.

Reviewer #3:
Manuscript Summary:
This article describes the elevated plus maze (EPM) procedure, and tests various ketogenic treatments for anxiolytic effects in Sprague Dawley rats.

Major Concerns:
It's not clear why Figure 8 (chronic feeding) includes % times spent in arms while Figure 9 displays overall amount of time spent in the open arms. Unless there is a strong reason for not presenting a component of EPM data, display the same data (% time, distance, entries) for each type of treatment (chronic and sub-chronic). otherwise it looks as if data wasn't presented due to inconsistencies in results. Also, be specific when reporting results. Example: the KSMCT group spent a greater percentage of time in the open arms (Fig 8a)

In the referenced article only the results were presented that showed significant difference. Since we use only published results as demonstration in this article, we can present only what was published already in the referenced article. We think that the two figures represent different possibilities for data collection with the demonstrated method, without much repetition.

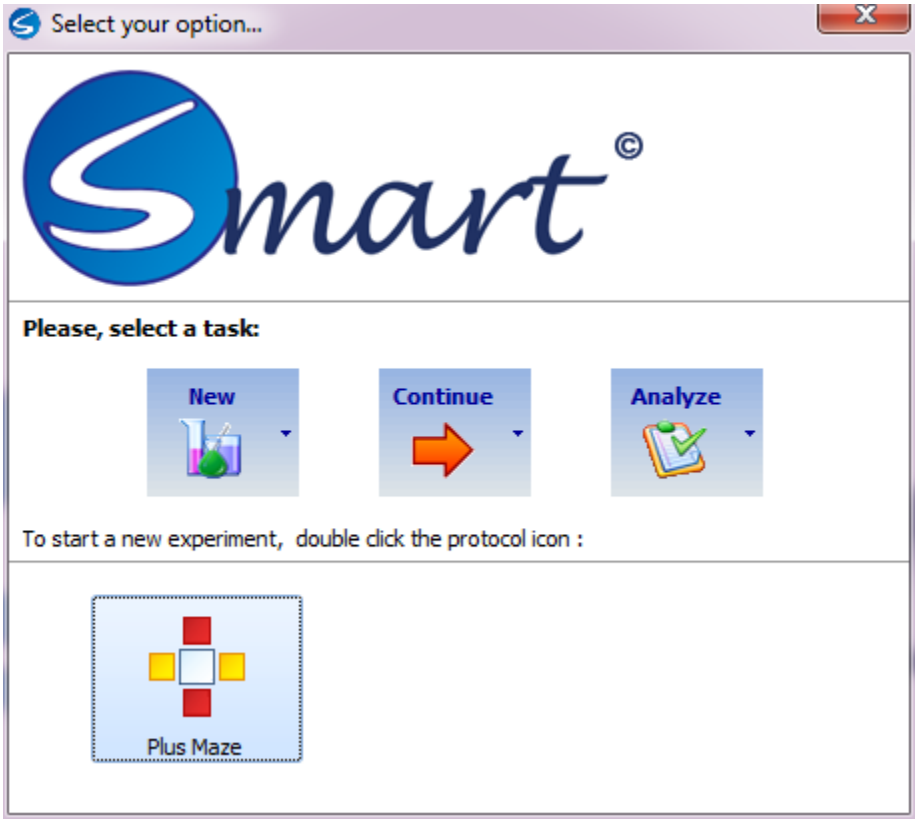
The figure legends were corrected to make them more specific.

Minor Concerns:
note that oral gavage stress may have influenced the unusually low % time in open arms in the control rats. In the future it may be useful to check % open arm times of rats not gavaged to ascertain if the procedure is causing undue stress

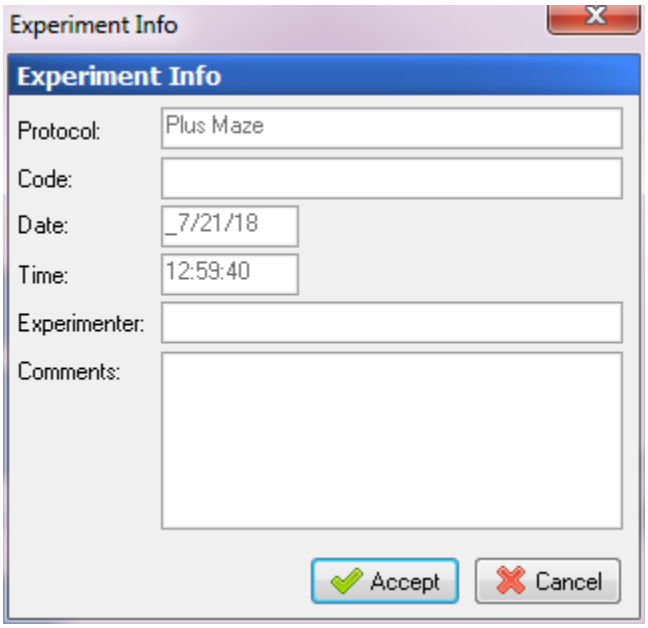
Thank you for the helpful suggestion, we will do that in the future.

If you would like your personal information to be removed from the database, please contact the publication office.

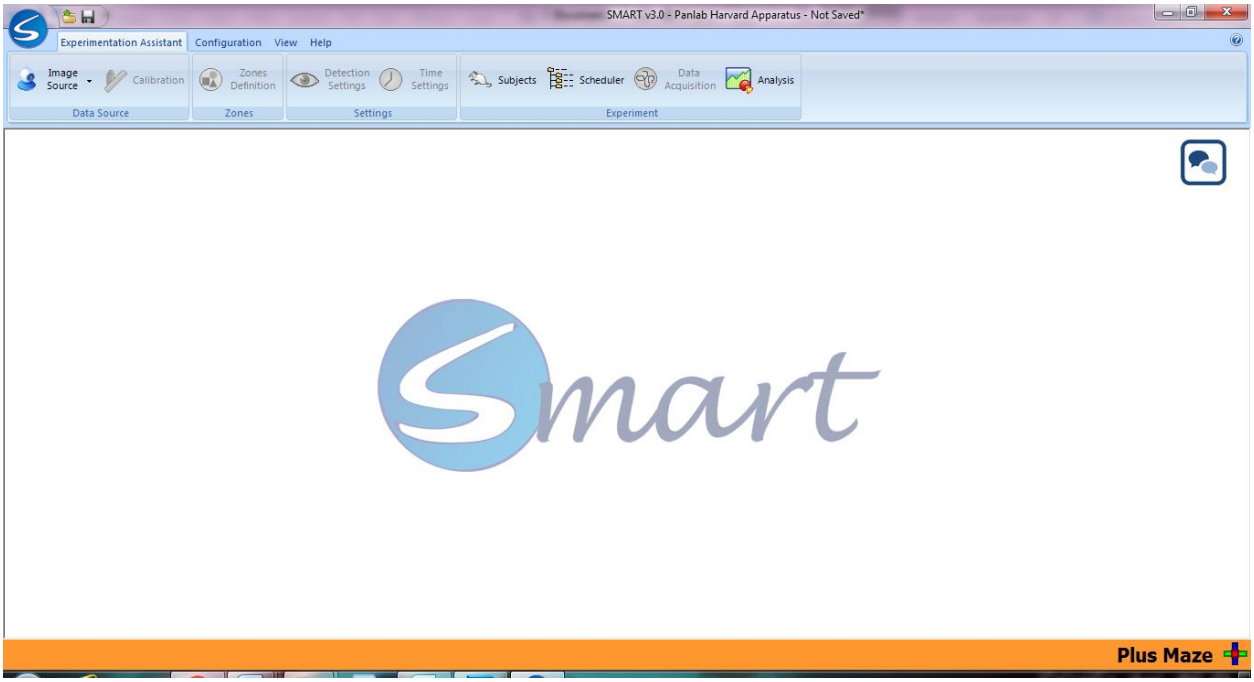
3.2.1.3.

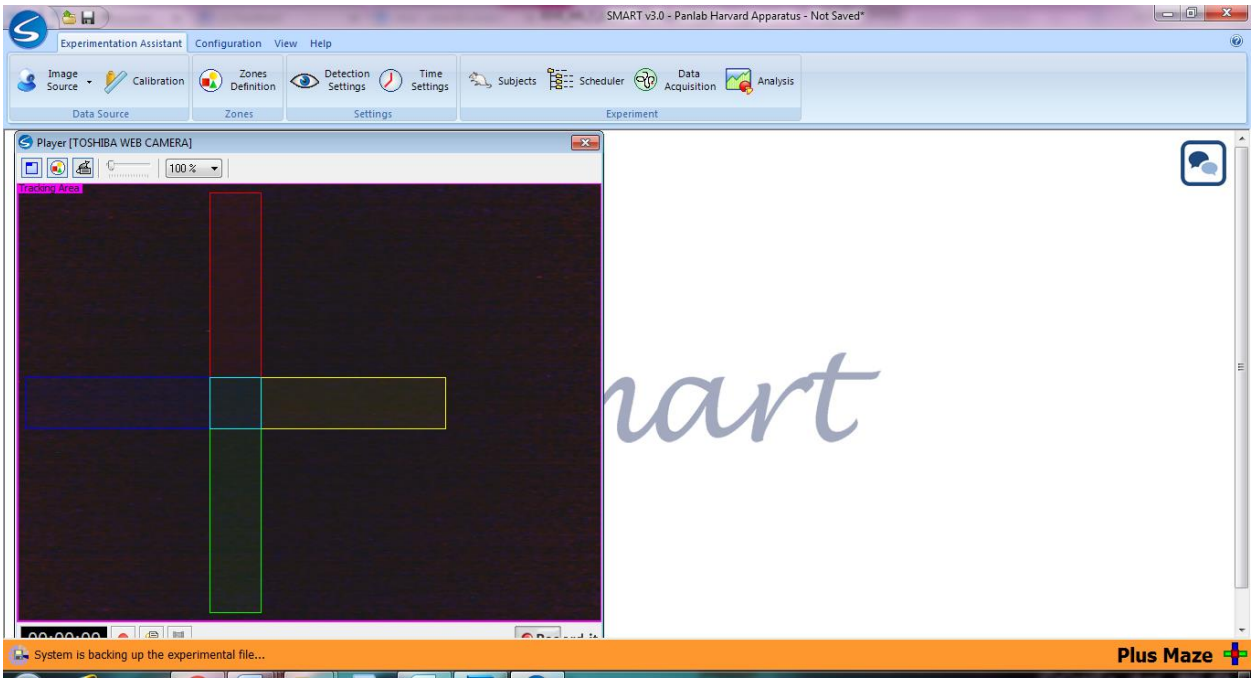


3.2.1.4.

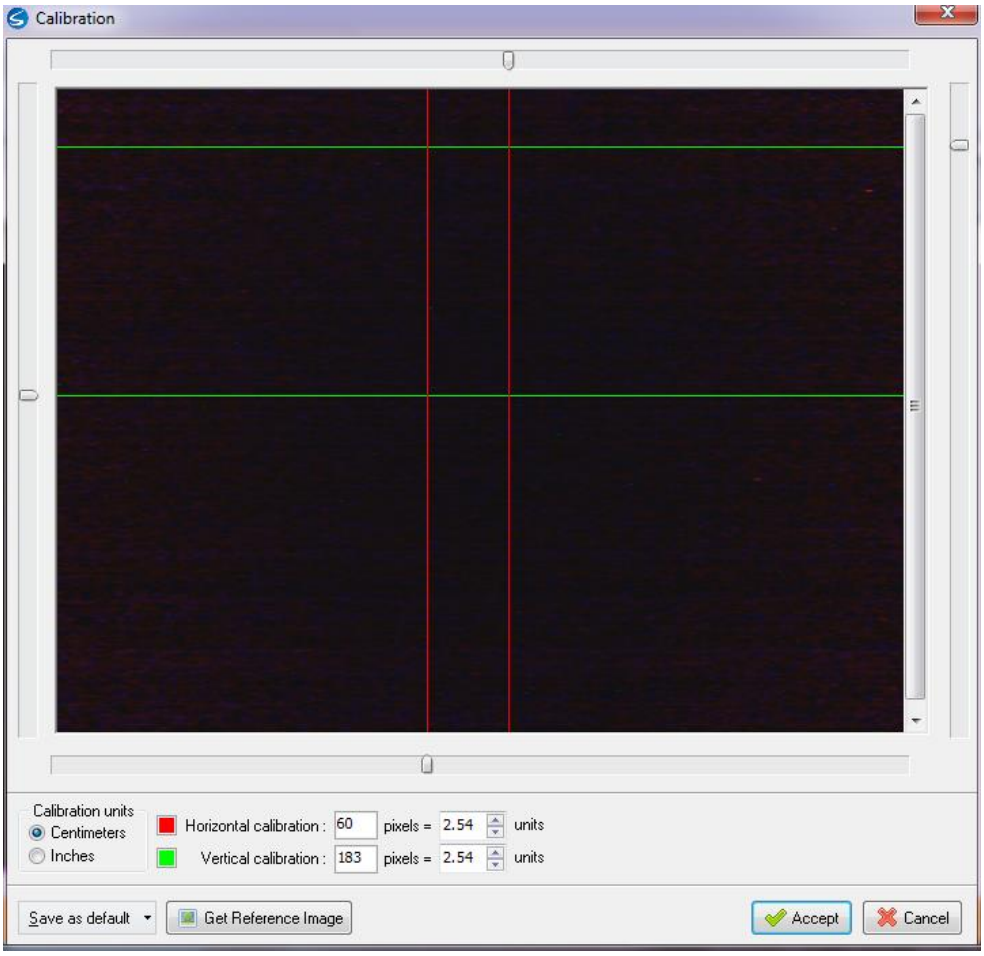


3.2.1.5.

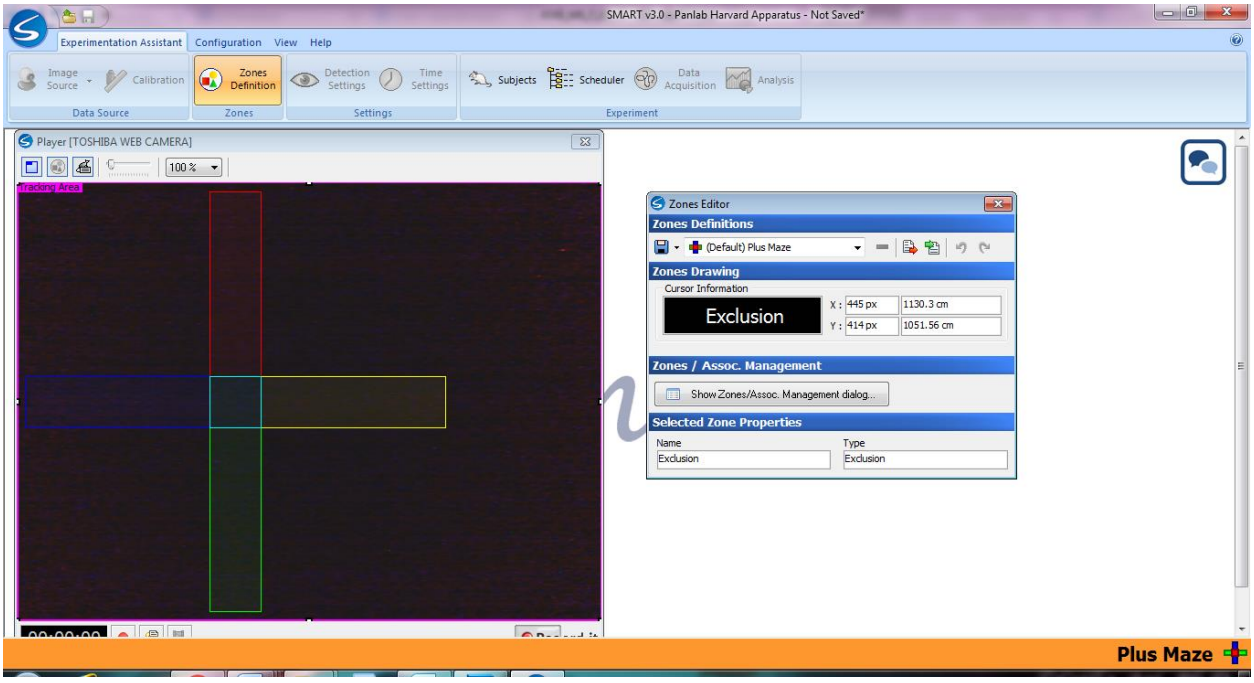

SMART v3.0 - Panlab Harvard Apparatus - Not Saved*
Experimentation Assistant
Configuration
View
Help
Image Source
Calibration
Zones Definition
Detection Settings
Time Settings
Subjects
Scheduler
Data Acquisition
Analysis
Data Source
Zones
Settings
Experiment
Plus Maze


SMART v3.0 - Panlab Harvard Apparatus - Not Saved*
Experimentation Assistant
Configuration
View
Help
Image Source
Calibration
Zones Definition
Detection Settings
Time Settings
Subjects
Scheduler
Data Acquisition
Analysis
Data Source
Zones
Settings
Experiment
Player [TOSHIBA WEB CAMERA]
100 %
Tracking Area
System is backing up the experimental file...
Plus Maze

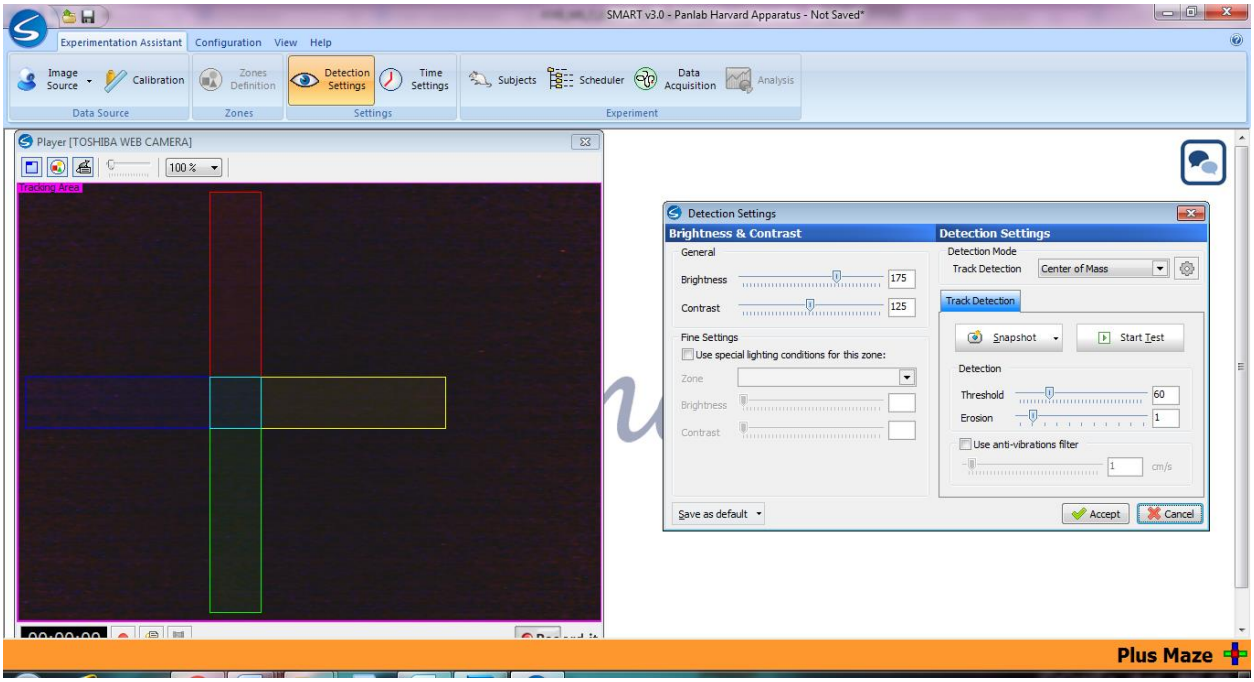
3.2.1.6.


Calibration
Calibration units
Centimeters
Inches
Horizontal calibration :
60
pixels =
2.54
units
Vertical calibration :
183
pixels =
2.54
units
Save as default
Get Reference Image
Accept
Cancel

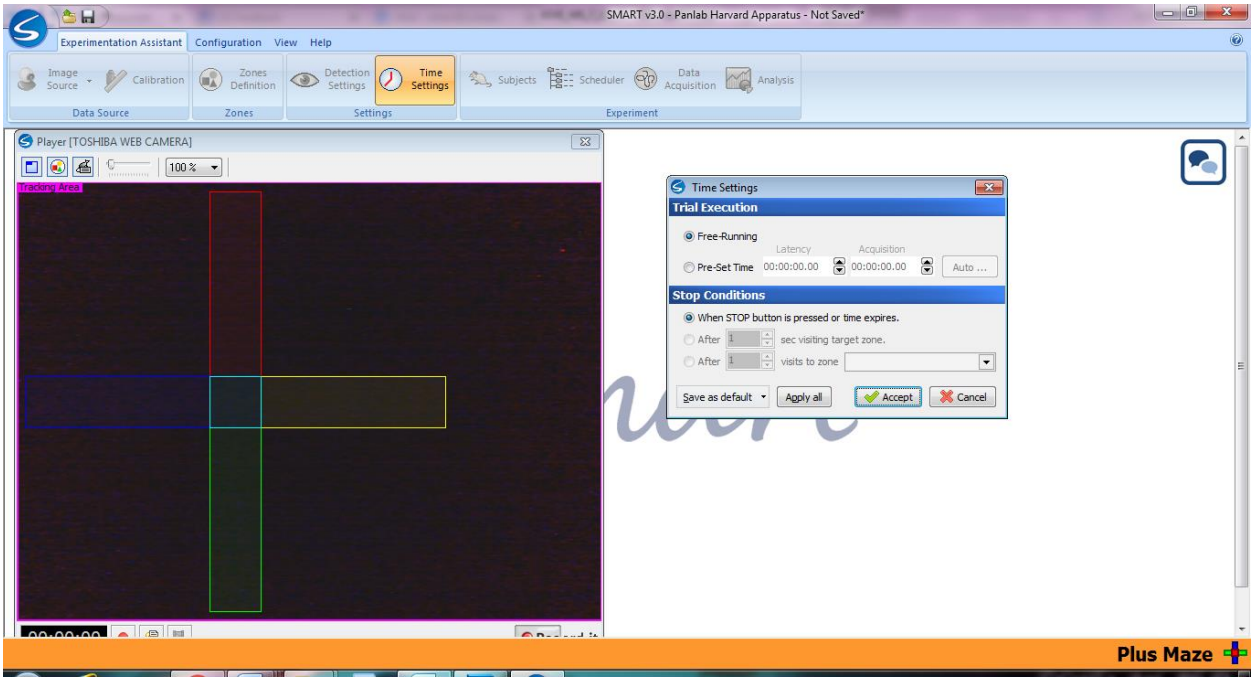
3.2.1.7.


SMART v3.0 - Panlab Harvard Apparatus - Not Saved*
Experimentation Assistant
Configuration
View
Help
Image Source
Calibration
Zones Definition
Detection Settings
Time Settings
Subjects
Scheduler
Data Acquisition
Analysis
Data Source
Zones
Settings
Experiment
Player [TOSHIBA WEB CAMERA]
100 %
Tracking Area
Zones Editor
Zones Definitions
(Default) Plus Maze
Zones Drawing
Cursor Information
Exclusion
X : 445 px
1130.3 cm
Y : 414 px
1051.56 cm
Zones / Assoc. Management
Show Zones/Assoc. Management dialog...
Selected Zone Properties
Name
Exclusion
Type
Exclusion
Plus Maze

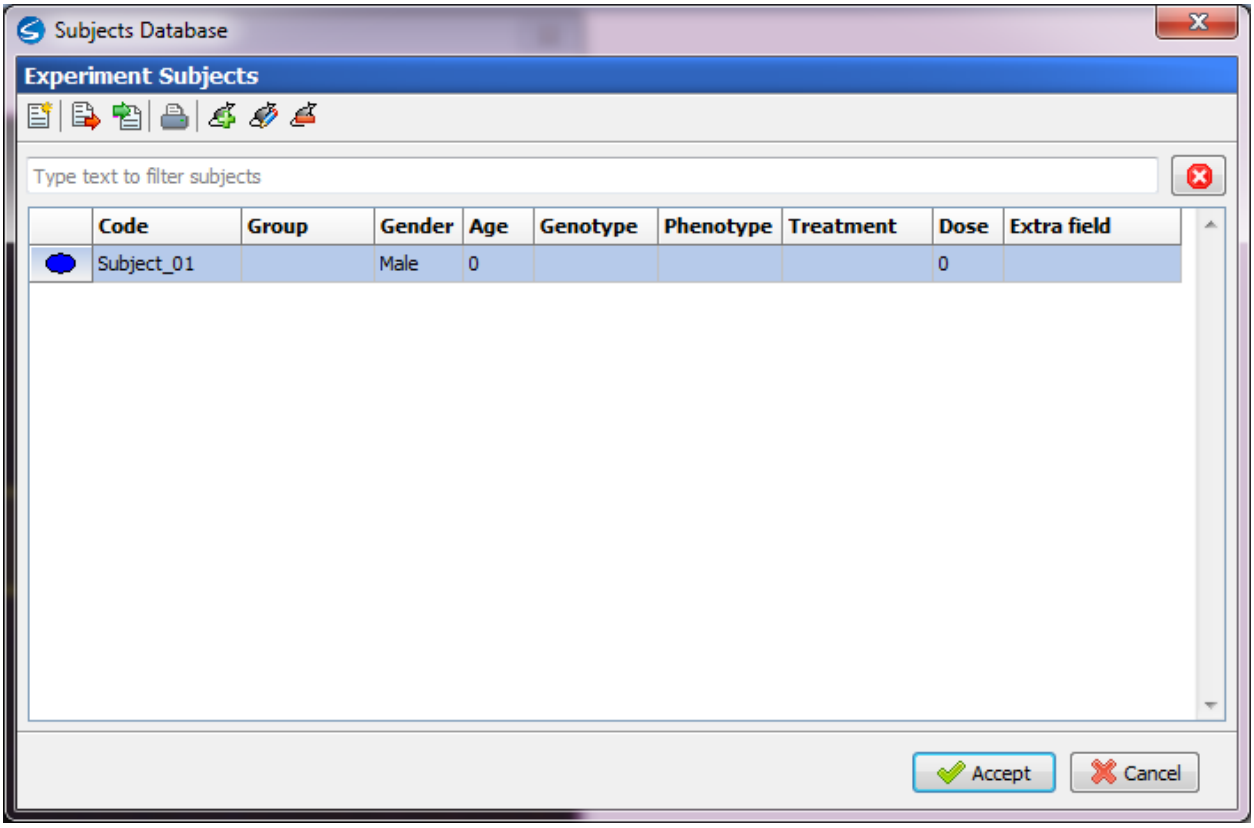
3.2.1.8.


SMART v3.0 - Panlab Harvard Apparatus - Not Saved*
Experimentation Assistant
Configuration
View
Help
Image Source
Calibration
Zones Definition
Detection Settings
Time Settings
Subjects
Scheduler
Data Acquisition
Analysis
Data Source
Zones
Settings
Experiment
Player [TOSHIBA WEB CAMERA]
Tracking Area
Detection Settings
Brightness & Contrast
Detection Settings
General
Brightness
Contrast
Fine Settings
Use special lighting conditions for this zone:
Zone
Detection Mode
Track Detection
Center of Mass
Track Detection
Snapshot
Start Test
Detection
Threshold
Erosion
Use anti-vibrations filter
Save as default
Accept
Cancel
Plus Maze

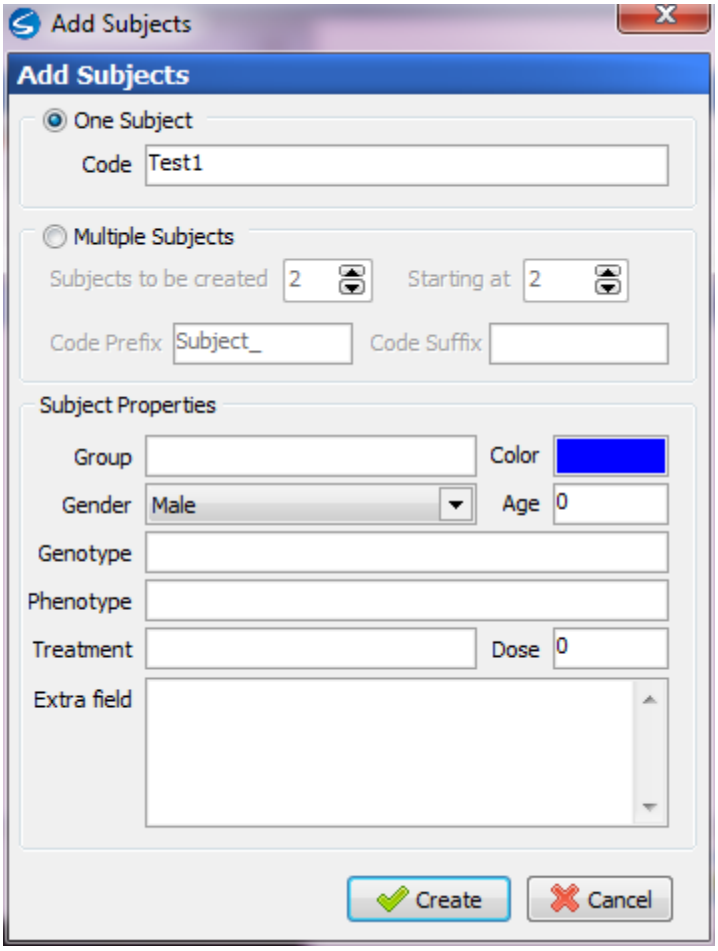
3.2.1.14.


SMART v3.0 - Panlab Harvard Apparatus - Not Saved*
Experimentation Assistant
Configuration
View
Help
Image Source
Calibration
Zones Definition
Detection Settings
Time Settings
Subjects
Scheduler
Data Acquisition
Analysis
Data Source
Zones
Settings
Experiment
Player [TOSHIBA WEB CAMERA]
Tracking Area
Time Settings
Trial Execution
Free-Running
Latency
Acquisition
Pre-Set Time
Auto ...
Stop Conditions
When STOP button is pressed or time expires.
After
sec visiting target zone.
visits to zone
Save as default
Apply all
Accept
Cancel
Plus Maze

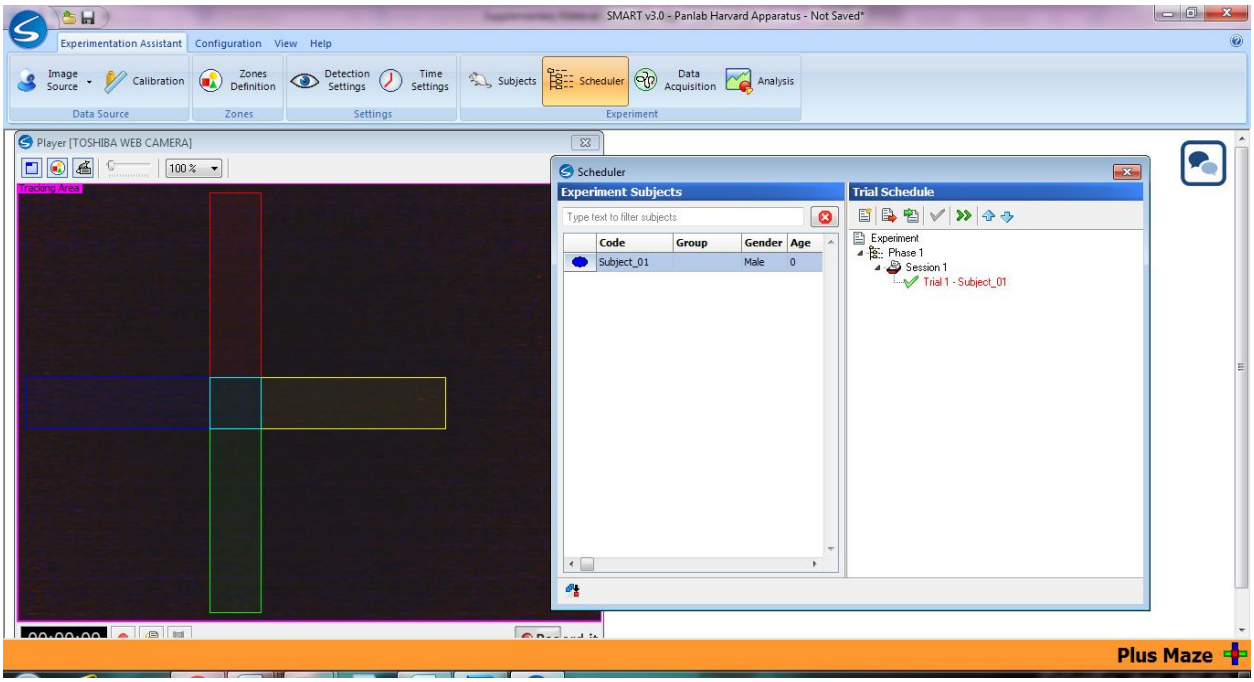
3.2.2.1.


Subjects Database
Experiment Subjects
Type text to filter subjects
Code
Group
Gender
Age
Genotype
Phenotype
Treatment
Dose
Extra field
Subject_01
Male
0
0
Accept
Cancel

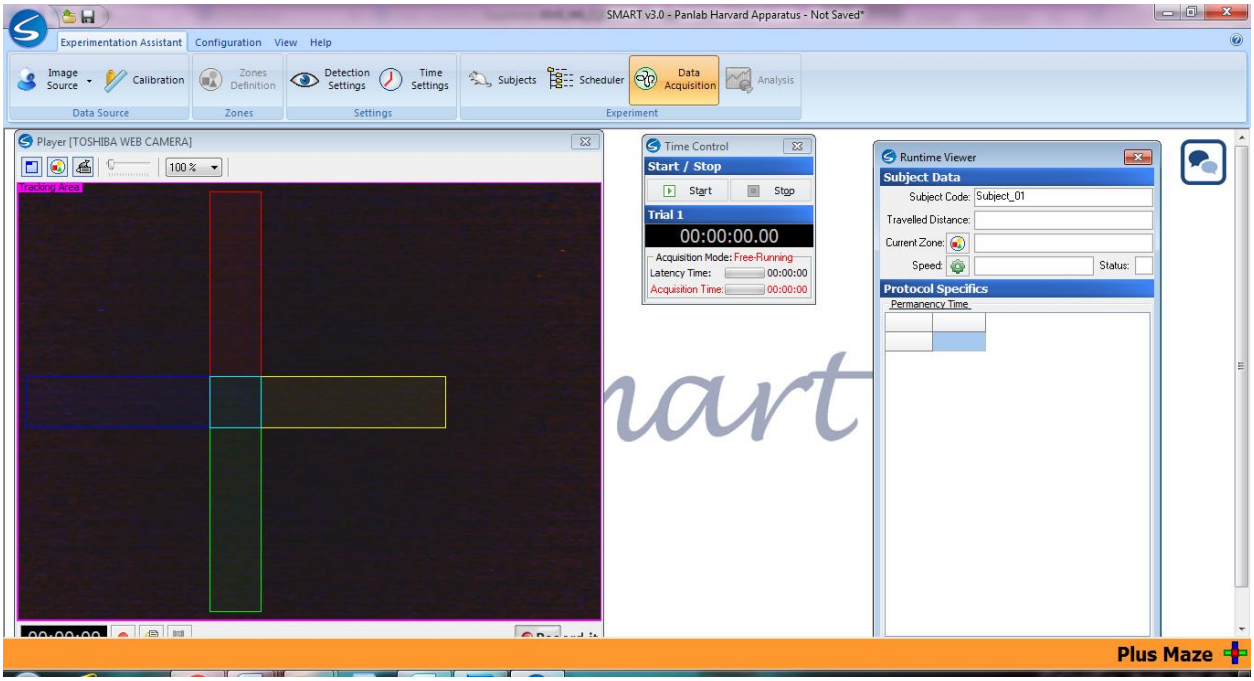
3.2.2.5.


Add Subjects
Add Subjects
One Subject
Code
Test1
Multiple Subjects
Subjects to be created
2
Starting at
2
Code Prefix
Subject_
Code Suffix
Subject Properties
Group
Color
Gender
Male
Age
0
Genotype
Phenotype
Treatment
Dose
0
Extra field
Create
Cancel

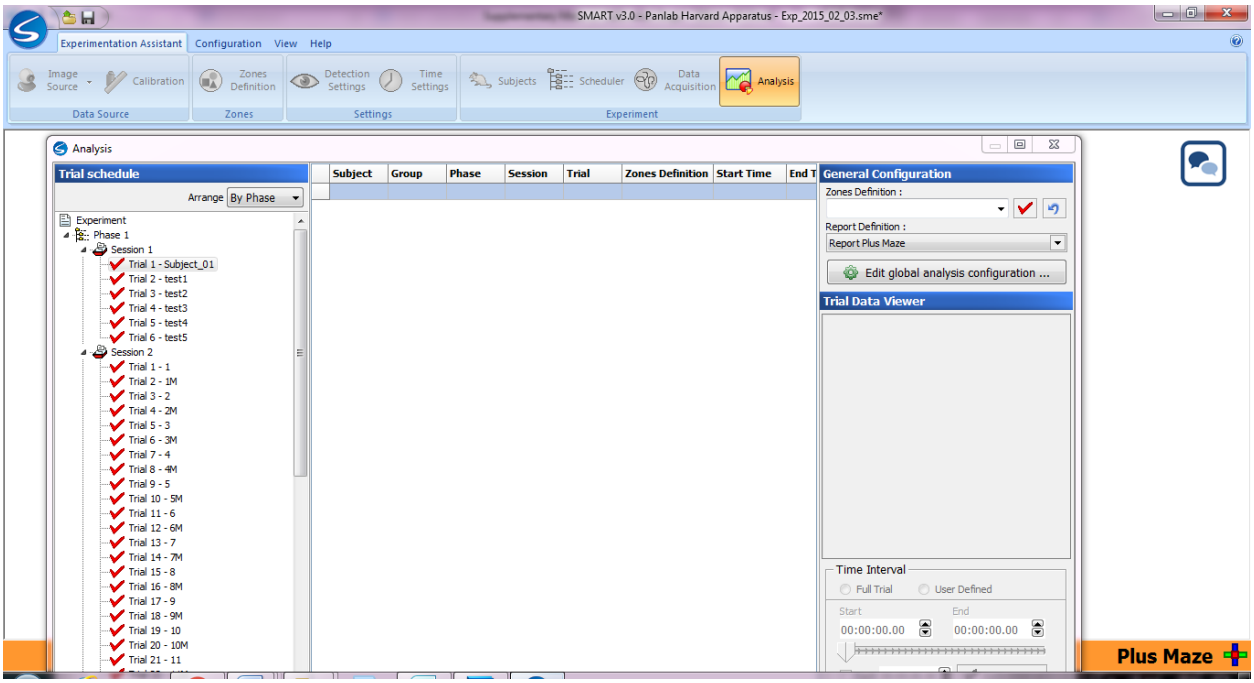
3.2.2.6.


SMART v3.0 - Panlab Harvard Apparatus - Not Saved*
Experimentation Assistant
Configuration
View
Help
Image Source
Calibration
Zones Definition
Detection Settings
Time Settings
Subjects
Scheduler
Data Acquisition
Analysis
Data Source
Zones
Settings
Experiment
Player [TOSHIBA WEB CAMERA]
Tracking Area
Scheduler
Experiment Subjects
Type text to filter subjects
Code
Group
Gender
Age
Subject_01
Male
0
Trial Schedule
Experiment
Phase 1
Session 1
Trial 1 - Subject_01
Plus Maze

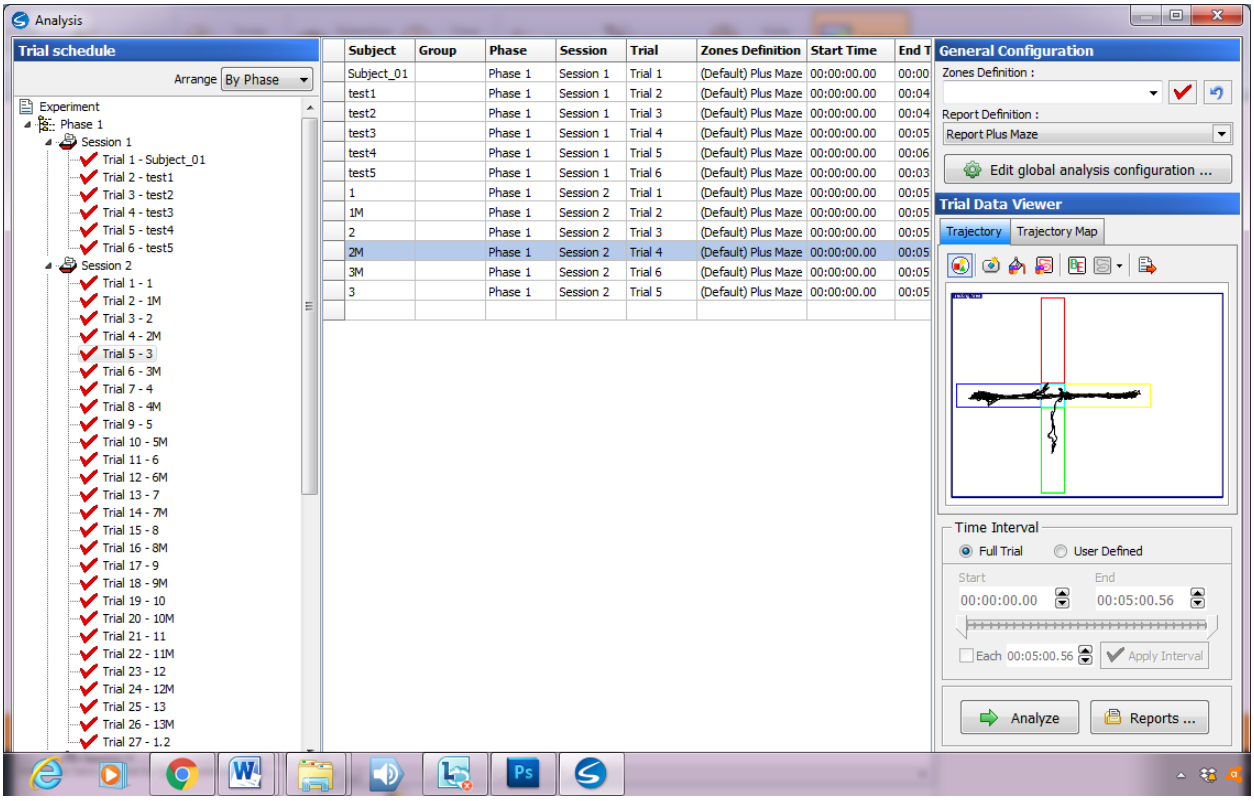
3.2.3.2.



3.2.4.1.



3.2.4.2.


Analysis
Trial schedule
Arrange By Phase
Experiment
Phase 1
Session 1
Trial 1 - Subject_01
Trial 2 - test1
Trial 3 - test2
Trial 4 - test3
Trial 5 - test4
Trial 6 - test5
Session 2
Trial 1 - 1
Trial 2 - 1M
Trial 3 - 2
Trial 4 - 2M
Trial 5 - 3
Trial 6 - 3M
Trial 7 - 4
Trial 8 - 4M
Trial 9 - 5
Trial 10 - 5M
Trial 11 - 6
Trial 12 - 6M
Trial 13 - 7
Trial 14 - 7M
Trial 15 - 8
Trial 16 - 8M
Trial 17 - 9
Trial 18 - 9M
Trial 19 - 10
Trial 20 - 10M
Trial 21 - 11
Trial 22 - 11M
Trial 23 - 12
Trial 24 - 12M
Trial 25 - 13
Trial 26 - 13M
Trial 27 - 1.2
Subject Group Phase Session Trial Zones Definition Start Time End T
Subject_01 Phase 1 Session 1 Trial 1 (Default) Plus Maze 00:00:00.00 00:00
test1 Phase 1 Session 1 Trial 2 (Default) Plus Maze 00:00:00.00 00:04
test2 Phase 1 Session 1 Trial 3 (Default) Plus Maze 00:00:00.00 00:04
test3 Phase 1 Session 1 Trial 4 (Default) Plus Maze 00:00:00.00 00:05
test4 Phase 1 Session 1 Trial 5 (Default) Plus Maze 00:00:00.00 00:06
test5 Phase 1 Session 1 Trial 6 (Default) Plus Maze 00:00:00.00 00:03
1 Phase 1 Session 2 Trial 1 (Default) Plus Maze 00:00:00.00 00:05
1M Phase 1 Session 2 Trial 2 (Default) Plus Maze 00:00:00.00 00:05
2 Phase 1 Session 2 Trial 3 (Default) Plus Maze 00:00:00.00 00:05
2M Phase 1 Session 2 Trial 4 (Default) Plus Maze 00:00:00.00 00:05
3M Phase 1 Session 2 Trial 6 (Default) Plus Maze 00:00:00.00 00:05
3 Phase 1 Session 2 Trial 5 (Default) Plus Maze 00:00:00.00 00:05
General Configuration
Zones Definition :
Report Definition :
Report Plus Maze
Edit global analysis configuration ...
Trial Data Viewer
Trajectory
Trajectory Map
Time Interval
Full Trial
User Defined
Start
End
00:00:00.00
00:05:00.56
Each 00:05:00.56
Apply Interval
Analyze
Reports ...