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TITLE:

Fear Incubation Using an Extended Fear-Conditioning Protocol for Rats

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KEYWORDS:

emotional memory, fear conditioning, fear incubation, overtraining, freezing, context memory, cue memory.

SUMMARY:

We describe an extended fear-conditioning protocol that produces overtraining and fear incubation in rats. This protocol entails a single training session with 25 tone-shock pairings (i.e., overtraining) and a comparison of conditioned freezing responses during context and cue tests 48 h (short-term) and 6 weeks (long-term) after training took place.

ABSTRACT:

Emotional memory has been primarily studied with fear-conditioning paradigms. Fear conditioning is a form of learning through which individuals learn the relationships between aversive events and otherwise neutral stimuli. The most-widely utilized procedures for studying emotional memories entail fear conditioning in rats. In these tasks, the unconditioned stimulus (US) is a footshock presented once or several times across single or several sessions, and the

conditioned response (CR) is freezing. In a version of these procedures, called cued fear conditioning, a tone (conditioned stimulus, CS) is paired with footshocks (US) during the training phase. During the first test, animals are exposed to the same context in which training took place, and freezing responses are tested in the absence of shocks and tones (i.e., a context test). During the second test, freezing is measured when the context is changed (e.g., by manipulating the smell and walls of the experimental chamber) and the tone is presented in the absence of shocks (i.e., a cue test). Most cued fear conditioning procedures entail few tone-shock pairings (e.g., 1-3 trials in a single session). There is a growing interest in less common versions involving an extensive number of pairings (i.e., overtraining) related to the long-lasting effect called fear incubation (i.e., fear responses increase over time without further exposure to aversive events or conditioned stimuli). Extended fear-conditioning tasks have been key to the understanding of fear incubation's behavioral and neurobiological aspects, including its relationship with other psychological phenomena (e.g., post-traumatic stress disorder). Here, we describe an extended fear-conditioning protocol that produces overtraining and fear incubation in rats. This protocol entails a single training session with 25 tone-shock pairings (i.e., overtraining) and a comparison of conditioned freezing responses during context and cue tests 48 h (short-term) and 6 weeks (long-term) after training took place.

INTRODUCTION:

Memory is a psychological process encompassing different phases: information acquisition, consolidation (allows for the stability of acquired information), and retrieval (evidence for the consolidation process)[1]. During the consolidation phase, the establishment of new synaptic connections and modification of pre-existing connections occur. This suggests the necessity for a period of time during which molecular and physiological events responsible for these changes occur[1,2]. These physiological or molecular changes vary if the retrieved events are emotionally charged or not (i.e., emotional memory). For instance, research has shown that the lateral nucleus and basolateral amygdala complex are particularly relevant to emotional memory[3–5].

Emotional memory phenomena have been primarily studied with fear conditioning paradigms[5,6]. Fear conditioning is a form of learning through which individuals learn the relationships between aversive events and otherwise neutral stimuli[7]. Fear conditioning paradigms produce molecular, cellular, and structural changes in the amygdala. In addition, fear conditioning modifies the connectivity of the hippocampus during the consolidation and retrieval processes of emotional memory.

One of the most commonly used procedures for studying fear memories is classical (Pavlovian) conditioning in rats. This procedure typically uses footshock (US) as the aversive stimulus, which is delivered once or several times across one or several sessions. The conditioned response (CR) of rats exposed to this procedure is freezing behavior (i.e., "generalized immobility caused by a generalized tonic response of the animals' skeletal musculature except those muscles used in breathing"[7]). This response could be assessed on two types of tests: context and cue tests. For the context test, the subject undergoes a given number of footshocks during the training session, and then is removed from the experimental chamber for a defined time. During the test, the subject is returned to the same context in which the training took place and different measures

of freezing are collected in the absence of footshocks (e.g., duration, percentage or frequency of freezing episodes), and compared to baseline levels established during the training phase. For the second type of test, cue test, a stimulus (typically a tone) is paired with the footshocks during the training phase (i.e., conditional stimulus, CS). After training is completed, the animal is removed from the training context for a defined time and is subsequently placed in a modified context (e.g., a different experimental chamber that has different shapes of walls and different smell). The cue is then presented a given number of times, and freezing responses to the cue are measured and compared to baseline levels collected during training. The most common version of this paradigm uses 1 to 3 tone-shock pairings during a single training session, followed by context and cue tests conducted a number of hours or a few days later.

Other less frequently implemented fear conditioning procedures involve an extensive number of shock-cue pairings (i.e., trials), which have often been called overtraining procedures[8]. A growing interest in these tasks is related to their long-lasting and increased memory effects called fear incubation (i.e., conditioned fear responses increase over time in the absence of further exposure to aversive events or conditioned stimuli[9-11]. An example of such overtraining procedures entails a training phase of 100 tone-shock pairings distributed across 10 sessions, followed by context and cue tests conducted 48 h and 30 days later[11-12]. To avoid extensive training spread across several days, Maren (1998) reported that overtraining could be established and optimized in a single session with 25 pairings[8]. The incubation effect is evidenced in significantly higher levels of conditioned fear in rats tested 31 days after training, as compared to rats tested 48 h after. Extended fear-conditioning tasks have been key for the understanding of behavioral and neurobiological aspects underlying fear incubation, including its relationship with other psychological phenomena (e.g., delayed-onset post-traumatic stress disorder)[11–13].

Here, we describe an extended fear-conditioning protocol that induces overtraining and fear incubation in rats. Different to other paradigms that require several days of training[11], the current protocol is focused on a single training session[8], using 25 tone-shock pairings to produce higher conditioned freezing responses during context and cue tests conducted 6 weeks after training, as compared to tests conducted 48 h after.

PROTOCOL:

The following protocols and procedures were approved by the Institutional Animal Care and Use Committee of Konrad Lorenz University (IACUC-KL). The universal declaration of animal rights issued by International League of Animal Rights, Geneva, Switzerland (1989), and ethical principles of experimentation with animals issued by ICLAS were respected.

1. Subject preparation

1.1. Select male adult Wistar rats (n = 12). House them in groups of four per cage for three days of acclimatization, prior to the beginning of the training and testing protocol. Provide rats with free access to water throughout the experiment. Control the room temperature between 20 ° to 25 °C, under a 12 h light-dark cycle (lights on at 07:00 h).

NOTE: Rat strains had shown differential performance during fear conditioning. For instance, Schaap et al. (2013) reported that Wistar and Lewis strains showed longer durations of freezing behavior compared with Fawn Hooded and Brown Norway rats[12]. Thus, differences in pain and thermal threshold should be assessed to adjust the intensity and duration of shocks.

1.2. Maintain rats at 85% of their free-feeding weights (normal weight between 350-400 g) by giving restricted food access at the same hour every day. Weigh rats every day at the same hour during the light cycle. Calculate the ad lib weight (100% weight) for three days before the start of fear extended training.

NOTE: Animals used in the present experiment were tested on additional instrumental tests that are not reported. Food deprivation is based on those additional tests. This procedural variation is assumed as likely to expand the scope of the present procedure to fear conditioning procedures, as it suggests the potential for instrumental-fear combined tests. However, studies using only fear conditioning common tests will not require food deprivation as the previous condition.

1.3. Randomly assign subjects to one of the following groups: emotional testing 6 weeks after training (n = 6); emotional testing 48 h after training (n = 6).

1.4. Perform training and tests at similar hours, during the light phase of dark-light cycle. Assign the animals to the same experimental chamber and maintain the same order of animals during training and testing.

NOTE: At this point, a control step that could be implemented is counterbalancing the order of animals during training and testing phases. We recommend using this technique when multiples groups are assessed or different tasks are applied across experiments, to reduce a possible effect of task-order on behavior.

2. Apparatus setting and shock calibration

2.1. Clean all the internal surfaces of the experimental chamber and stainless-steel grid floor with 10% ethanol. Repeat before testing each animal.

2.2. Connect the equipment to a computer using a USB cable and start the freezing detection system equipment: the CPU, the controlling cabinet, the infrared light, the aversive stimulator/scrambler, and the shock-intensity calibrator.

NOTE: Although this protocol was executed using commercially available instruments (**Table of Materials**), equipment and software of different brands can be used. The apparatus consists of an internal acrylic square chamber (29.53 cm x 23.5 cm x 20.96 cm, called the experimental chamber) embedded in a box of wood and covered with plastic formic. The external doors allow the isolation of sound, noise or light (attenuating box doors). The camera is located laterally in

the internal part of the external door. The internal acrylic box with floor metal grids (36 stainless-steel rods, each one of 3 mm diameter and spaced 8 mm, center to center) allows footshock delivering. In one of the internal-lateral walls, a speaker is located to 6 cm from the floor to present an auditory cue.

2.3. Connect the red and black clips of the shock intensity calibrator (i.e., positive and negative connectors) to two any different rods on the grid floor. Connect the USB cable to the corresponding port of the computer. Make sure to connect the red and black clips to bars separated by another bar.

NOTE: This section describes the shock intensity calibration process using a specific brand equipment mentioned in the **Table of Materials**. However, the calibration process can be performed using different brands of equipment. It is recommended to calibrate the intensity of the shock in three sectors of the grid floor to verify that it is consistent. In addition, always remove fecal and urine residues from the grid floor to avoid interference with a shock dispenser.

2.4. Start the shock-intensity calibrator software (**Table of Materials**). Choose an intensity of 1.0 mA in the application by clicking on the range arrow. Then, change the Run/Stop switch to Run.

NOTE: We propose 1.0 mA based on internal studies with rodent models and literature studies that report a range from 0.75 mA as adequate to 1.5 mA as a high intensity for studies of fear conditioning[33-35].

2.5. Switch on the aversive stimulator or the equipment used to deliver the footshocks and look at the shock intensity displayed on the panel of the application. If needed, adjust the intensity to 1.0 mA using the knob on the aversive stimulator.

NOTE: Aversive stimulator should be set to “OUT” to appropriately test, calibrate, and run the experiment.

3. Freezing detection system calibration

3.1. Close the experimental chamber and sound-attenuating box doors. Do not introduce the animal at this point, as it will be placed into the chamber after the freezing detection system calibration has been completed. Check that the light intensity inside the box is between 20 and 30 lux.

3.2. Start the freezing detection system software and open the **Experiment setup** dialogue window. Enter the details of each subject (such as subject identification number, date and group) and load the file titled “Training protocol VFC.pro” (available at https://osf.io/4nkfq/?view_only=0640852a88544b239549462f9c21175b).

NOTE: Context and cue tests use a different program configuration; thus, make sure to use the correct file on each test. At this point the correct file corresponds to “Training protocol VFC.pro”.

Remember that during test phases the file corresponding will be different to training session.

3.3. Choose the corresponding camera(s) and check the **Save Video** option (if needed). Set the **Motion Threshold** to 100, and **Min Freeze Duration** to 30 frames.

NOTE: This Motion Threshold value is based on the size of the species used (based on number of pixels). Minimum Freeze Duration value is recommended by the manufacturer. These values are used to ensure proper recognition of the animals in the chamber.

3.4. Verify that the live feed from the chosen camera(s) appears on the screen, together with the motion threshold graph and the timeline of the different stimuli that are presented during the training (e.g., sound and shock).

NOTE: Using a different brand, the equipment setup should offer the possibility to measurement the movements of animal to detect an "index" of motion that should allow for comparisons on the amount of time the animal is moving or freezing. Another possibility is using a software that with only the video source (during or after the experiment) can detect the amount of time in motion or freezing, such as free software ImageFZ[13], open-source toolbox in Matlab[14] , or a free classifier of animal behavior as JAABA[15].

3.5. Click the **Calibrate** option three times, while checking that the Motion Index remains below 100 (threshold). Then, set the equipment to **lock** by clicking on the corresponding button on the screen.

NOTE: This section describes a freezing detection system calibration process using a specific brand equipment mentioned in the **Table of Materials**. As was mentioned before, the calibration process can be done using different brands of equipment (for a review of different options in equipment and software see Anagnostaras et al. 2010)[16].

4. Extended fear conditioning training

4.1. Transport the rats in their home cages, covered with a cloth, from the animal care facility to the behavioral training room in the laboratory. Avoid exposure to noise or stress-generating conditions during the transport of animals to the behavioral training room. If several animals are transported at the same time, only bring the animals to be tested and maintain other rats in a holding room to enhance experimental control. Gently handle the animals for 2 min before starting the training.

NOTE: In the protocol, the animals were handled each day for 2 minutes before behavioral training. Following handling, animals were introduced in the experimental chamber. We recommended to manipulate animals to make rats habituate to the researcher.

4.2. Introduce the rat to the experimental chamber. Handle it gently by the base of its tail and place it on the middle of the chamber. Close the experimental chamber and sound-attenuating

box doors.

4.3. Start the session by clicking on the **Record** button. Let the rat acclimate to the chamber for 3 min. This 3 min period is the standard recommended by the equipment manufacturer and serves as a baseline and habituation time to the chamber.

4.4. Deliver twenty-five tone-shock pairings (trials) with a 60 s Inter-Trial Interval (ITI), starting on minute 3 of the session. Present the tone (conditioned stimulus – CS; 90 dB SPL, 2000 Hz, 50-ms Rise Time) during the last 10 s of each ITI, and the shock (unconditioned stimulus – US) during the last 2 s of each ITI.

NOTE: Activation of the **Record** button is conditional on cameras being properly calibrated and locked.

4.5. Remove the rat from the experimental chamber when the 28 min session is over. Return animals to the respective home cage. Transport the rats in their home cages covered with a cloth from the behavioral training room to the animal care facility.

4.6. Repeat freezing detection system calibration (steps 3.1-3.5) and fear conditioning (steps 4.1 and 4.3) to train all the subjects.

NOTE: We strongly recommend recalibrating the detection system for each animal to ensure that the software maintains the same parameters when it processes information on freezing detection.

4.7. Resting period: During this period, have the animals rest in their home cages and house individually. Monitor the weight of the animals twice per week during the 6 weeks of the incubation period. Gently manipulate each animal for two min while they are weighted.

5. Context test – single 10 min session

5.1. After the training phase, expose the animals to the first memory test called the *context test*. During this 10 min phase, expose the rats to the same context in which training took place but no cues or shocks occur. Transport the rats in their covered home cages (e.g., with a cloth) from the animal care facility to the behavioral training room. Keep in mind that animals were divided into groups, thus one of them is tested 48 h after the training phase and another group is tested 6 weeks after training (**see Figure 1**).

5.2. Clean all the internal surfaces of the experimental chamber and stainless-steel grid floor with 10% ethanol. Repeat before testing each animal.

5.3. Repeat freezing detection system calibration (steps 3.1 to 3.5). Open the **Experiment setup** dialogue window and load the file named “Context test protocol.pro”, which is available from https://osf.io/4nkfq/?view_only=0640852a88544b239549462f9c21175b

NOTE: This file contains the setup for this experimental phase that consists of no shocks or tones.

5.4. Introduce the animal to the experimental chamber. Handle it gently by the base of its tail and place it on the middle of the chamber. Close the experimental chamber and sound-attenuating box doors.

5.5. Start the session by clicking on the **Record** button. During this single 10 min context-test session, no stimuli are presented (shock neither sound).

5.6. Remove the subject from the experimental chamber when the 10 min session is over. Return the animals to their respective cages and transport the rats in their home cages covered with a cover from the behavioral training room to the animal care facility. Repeat steps 5.2-5.5 to test all the subjects.

6. Cue test single – 13 min session

6.1. One day after the context test, have animals undergo the second test of memory called the cue test single. During this phase, the rats will be in a different context of training and without cue or shock during 13 min. Transport the rats in their home cages covered with a cover from the animal care facility to the behavioral training room. Test a group 72 h after training, and test another group 6 weeks and one day after training (**Figure 1**).

NOTE: A different system of transportation (from the animal care facility to the experimental room) could be implemented to differentiate more the context and cue tests. Since the animals were transported to the training session and context test in their home cages, a different transport cage, bedding and/or cover could be used during transportation to the cue test.

6.2. Clean all the internal surfaces of the experimental chamber and stainless-steel grid floor with 10% ethanol. Repeat before testing each animal.

6.3. To change the visual context, insert the plastic surrounding wall of the experimental chamber.

6.4. To change olfactory context, apply 1% acetic acid to a cotton-tipped swab, and place it in the metal tray below the grid floor[17–19].

6.5. Repeat the freezing detection system calibration (steps 3.1-3.5). Load the file named file "Cue test protocol.pro" file, which is available from https://osf.io/4nkfq/?view_only=0640852a88544b239549462f9c21175b

NOTE: This file contains the setup for this experimental phase, which consists of delivery of the same tones presented during the training phase (CS), but in the absence of shocks (US).

6.6. Introduce the animal to the experimental chamber. Handle it gently by the base of its tail and place it on the middle of the chamber. Close the experimental chamber and sound-attenuating box doors.

6.7. Start the session by clicking on the **Record** button. During the single 13 min cue test session, the CS stimulus (tone) is presented 10 times, starting on minute 3 of the session.

NOTE: The first 3 min correspond to the baseline of this session, followed by 10 cue test trials (that is 10 s each) delivered with 50 s ITIs in the absence of shocks. The delivery of tones is automatic, via using the previously loaded file.

6.8. Remove the animal from the experimental chamber when the 13 min session is over. Return animals to the respective cage and transport them covered to the animal care facility. Repeat steps 6.2 through 6.5 to test all the subjects.

7. Data analysis

7.1. Obtain the general activity index (i.e., motion index) that is derived from the video stream using the freezing detection system software. This software automatically transforms the motion index to provide the percentage of freezing time per session and the number of freezing episodes. Set the freezing threshold to the default Minimum Freeze Duration setting of the system (1 s = 30 frames).

7.2. Use the additional custom-made program (file available from https://osf.io/4nkfq/?view_only=0640852a88544b239549462f9c21175b) to obtain:

7.2.1. Use the program to determine the percentage of freezing during the first three minutes of the training session (i.e., baseline freezing, since no shocks or tones were presented before or during that 3 min period) and during the first three minutes of the cue test session.

7.2.2. Use the program to determine the percentage of freezing for each of eight 3 min bins of the training session.

7.2.3. Use the program to determine the percentage of freezing during the cue presentations (i.e., freezing during the tones) and no-cue periods (intertrial intervals; ITIs), for both training and cue-test sessions.

7.3. To obtain these data, open the freezing detection system software.

7.3.1. Select **File** | **Reports** | **Batch Component summary**.

7.3.2. Select the file with extension .CMP available from https://osf.io/4nkfq/?view_only=0640852a88544b239549462f9c21175b) .

7.3.3. Name the output file and change Motion Threshold to a 100. Then, click **OK**.

7.3.4. Select the files to be analyzed (extension.RAW). These files are automatically generated from the freezing detection system software when the session is over and correspond to the raw data of each session. Initially, the files are saved in the desktop of the computer, but they can be stored in a custom folder (e.g., named Fear conditioning) to facilitate their subsequent identification and opening when they need to be analyzed.

7.3.5. Open the output files (extension .CSV). They can be edited in a spreadsheet software for further analysis. This file contains the results of freezing during the experimental session.

NOTE: To obtain the total percentage of freezing, divide the time that the subject spent immobile over the total session time. The number of freezing episodes can be calculated counting the number of freezing events through the session. In both cases, it is necessary to define a motion threshold based on a minimum freeze duration. This is the temporal criterium that defines whether a Freeze Episode is recorded. Automated systems of recording can use certain amount of fps as a measure of minimum freeze duration. For instance, with a sample rate of 30 fps, a minimum freeze duration of 15 frames will record as freezing an instance of immobility that last for 30 s.

7.4. Calculate the average duration of each freezing episode for each session (training and both tests) by dividing the total freezing duration (in seconds) over the total number of freezing episodes.

REPRESENTATIVE RESULTS

Variations in percentage of freezing time during different stages of the training session were analyzed for all subjects (n = 12) using a dependent *t* test (**Table 1**). Animals were active and explored the experimental chamber during the first three minutes of the training session (first day of the protocol), time during which no tones or shocks were delivered (i.e., baseline (BL). As shown in **Figure 2A**, percentage of freezing time during the subsequent 25 tone-shock pairings (*M* = 48.88; *SE* = 4.37) was significantly higher than during BL (*M* = 14.65; *SE* = 4.05), which is assumed as an indication of fear acquisition.

[Place **Figure 2** here]

An analysis of the freezing response throughout acquisition was conducted by segmenting the training session in eight 3 min bins (**Figure 2B**). These data show that the mean time allocated to this response reaches asymptote near or at 180 s during the first three tone-shock trials (i.e., Bin 1). This finding has been considered in previous research an indication of overtraining[11]. Repeated-measures ANOVA revealed consistent significant differences between baseline and all subsequent bins, with large effect sizes (**Table 1** and **Table 2**).

A mixed ANOVA was conducted to test differences in percentage of freezing during the task, having *phases* (BL, training, context test, and cue test) as the within-subject factor and group (48

h and 6 weeks) as the between-subjects factor (**Table 1**). Percentage of freezing of all animals during the training period was significantly higher than during the baseline period (see **Figure 2C**). No significant differences were observed between percentage of freezing during the memory tests and the training period (*p*s > .05).

No significant differences between the two groups (48 h and 6 weeks) were observed in the percentage of freezing during BL, training, and cue test (*p*s > .115; see **Figure 2C**). Conversely, animals tested 6 weeks after training showed significantly higher percentages of freezing during the context test than animals tested at 48 h, with a large effect size (see **Figure 2C**). Overall, **Figure 2C** shows that freezing during long-term delayed context and cue tests (i.e., 6 weeks after training) was overall significantly higher than during the training session. The opposite declining trend was observed in the group of animals that were tested 48 h after training. However, these differences in the group of 48 h were not statistically significant (*p*s > .05). Finally, although the freezing level showed differences across different phases, they could be considered low compared to other protocols. One explanation could be due to methodological differences inherent between laboratories or the threshold used as motion index during calibration process, making comparison of data among laboratories difficult.

The conditioned freezing response of the two groups of subjects during the context test was further explored via analysis of other measures, namely average *activity* (i.e*., motion index*), *total freezing time* and *freezing time per episode*. A one-way ANOVA was used to test differences across these variables (**Table 1**). Activity of subjects that were tested 6 weeks after training was significantly lower than that of animals tested 48 h after conditioning session (**Figure 3A**). Accordingly, total freezing time of animals tested shortly after training was significantly lower than that of animals tested 6 weeks after (**Figure 3B**). Lastly, an analysis of the average duration of each freezing episode indicated that animals tested 6 weeks after training displayed longer freezing episodes than animals tested 48 h after training (**Figure 3C**). Altogether, these findings indicate a fear incubation effect.

[Place **Figure 3** here]

A further examination of performance during the cue test session was conducted via analyses of (a) percentages of freezing during baseline periods (BL Training and BL Cue Test) and during the entire 10 min cue test (ten 10 s tone presentations and ten ITIs of 50 s - **Figure 4A**), (b) average freezing time specifically during the 10 s presentations of the cue (tone), for both Training and Cue Test sessions (**Figure 4B**), and (c) average freezing time (in seconds) during the 50 s intertrial intervals (ITIs; i.e., no-tone periods only – **Figure 4C**). A mixed ANOVA was used to analyze each of these dependent measures, assuming *phases* (BL Training, BL Cue Test, and Cue Test) as the within-subjects factor and *groups* (48 h and 6 weeks) as the between-subjects factor (**Table 1**). As shown on **Figure 4A**, the group of rats tested 6 weeks after training significantly increased their percentage of freezing during the baseline of the Cue Test session (BL Cue Test; first 3 min of the session) and during the 10 min Cue Test, as compared to BL training (i.e., prior to any exposure to tone and shocks). No analogous difference between BL training and BL Cue was observed for the group of rats tested after 48 h (*p* > .05). For both groups of rats, the percentage

of freezing during the 10 min Cue Test was higher than during the corresponding baseline period of that same session (BL Cue), which suggests a retrieval effect. No differences were observed between the groups of rats on percentage of freezing across the different periods (*ps* > .05).

Figure 4B shows a comparison of mean freezing time (in seconds) specifically during the 10 s tone presentations across Training (tone-shock pairings) and Cue Test (only tone presentations). Only rats tested 6 weeks after training significantly increased the amount of time freezing during the cue.

Lastly, as shown on **Figure 4C**, only the group of rats tested 48 h after training significantly decreased the freezing time during the ITIs from the Training session to the Cue Test. No differences in freezing time during the ITIs were observed across the two groups of rats (*ps* >.05).

[Place **Figure 4** here]

[Place **Table 1** here]

[Place **Table 2** here]

FIGURE LEGENDS:

Figure 1: Timeline of the experiment.

Figure 2: Training phase of an extended cued fear conditioning protocol. Data is shown as the mean (bars) and the SEM (error bars) of the freezing response. (**A**) shows mean percentage of freezing of all subjects (n = 12) during the first 3 min of the training session, during which no shocks or tone were presented (baseline, BL), and the remaining 25 min of the session (25 tone-shock trials, with intertrial interval, ITI, of 60 s); *** = different from BL ($p < .001$). (**B**) shows average freezing time of all the animals (n = 12) during the 3 min baseline period (BL, no shocks or tones delivered) and subsequent 3 min bins of the training session; *** = different from all the remaining bins ($p < .001$). (**C**) shows mean percentage of freezing of each group of rats (testing 48 h after training; testing 6 weeks after training) during the baseline (BL, first 3 min of the training session), training period (25 tone-shock pairings), context test session, and cue test session; * = different from testing after 48 h ($mean\ diff_{Context} = -34.95$, $SE = 14.99$, $p = < .05$, *Cohen´s d* = 1.34); *a* = different from training period ($mean\ diff_{Training48h} = 42.51$; $SE = 7.28$; $p < .05$; *Cohen´s d* = 3.03); *b* = different from training period ($mean\ diff_{Training6Weeks} = 25.94$; $SE = 7.28$; $p < .05$; *Cohen´s d* = 1.77), context test ($mean\ diff_{Context6Weeks} = 50.36$; $SE = 10.58$; $p < .01$; *Cohen´s d* = 3.13), and cue test ($mean\ diff_{Cue6Weeks} = 55.86$; $SE = 10.25$; $p < .01$; *Cohen´s d* = 2.47).

Figure 3. Effects of an extended cued fear conditioning protocol on freezing response of rats. Data are showed as the mean (bars) and the SEM (error bars) of the freezing response. (**A**) shows activity (i.e., motion index) of each group of subjects (testing 48 h after training; testing 6 weeks after training) during the context test; * = different from *6 weeks*. (**B**) shows the average total

freezing time (in seconds) of each group of subjects during the context test; * = different from *6 weeks*. (**C**) shows the average duration of each freezing episode (in seconds) for each group of subjects during the context test; * = only different from *6 weeks*.

Figure 4. Effects of an extended cued fear conditioning protocol on freezing response during the cue test. Data are showed as the mean (bars) and the SEM (error bars) of the freezing response. (**A**) shows percentage of freezing of each group of subjects (testing *48 h* after training; testing *6 weeks* after training) during the first 3 min of the training session (BL, baseline), during the first 3 min of the cue test session (BL Cue) and during the 10 min of the cue test (Cue Test); *a* = different from Cue test after *48 h* (*mean* $diff_{BLTraining\text{-}Cue48h}$ = 32.84; *SE* = 10.25; *p* <.05; *Cohen´s d* = 1.52); *b* = different from BL Cue Test (*mean* $diff_{BLCue\text{-}BL6Weeks}$ = 33.98; *SE* = 8.36; *p* <.05; *Cohen´s d* = 1.59) and Cue Test (*mean* $diff_{Cue\text{-}BL6Weeks}$ = 55.86; *SE* = 10.25; *p* <.05; *Cohen´s d* = 2.47); *c* = different from Cue Test after *48 h* (*mean* $diff_{BLCue\text{-}Cue48h}$ = 18.99; *SE* = 5.17; *p* <.05; *Cohen´s d* = .67); *d* = different from Cue Test after *6 weeks* (*mean* $diff_{BLCue\text{-}Cue6Weeks}$ = 21.87; *SE* = 5.17; *p* <.05; *Cohen´s d* = .88). (**B**) shows the average freezing time (in seconds) during cue (tone) of each group of subjects during Training and the Cue Test; * = different from *6 weeks* during test period of Cue Test (*mean* $diff_{Training\text{-}Cue6Weeks}$ = -3.14; *SE* = 1.37; *p* <.05; *Cohen´s d* = 1.64). (**C**) shows the average freezing time (in seconds) during the intertrial intervals (ITI) of the Training session (10 tone-shock pairings) and the Cue Test (10 tone-only presentations) across the two groups of rats (48 h and 6 weeks); *** = different from Training for group of rats tested 48 h after training (*mean* $diff_{Training\text{-}Cue48h}$ = 506.16; *SE* = 95.08; *p* <.001; *Cohen´s d* = 2.48).

Table 1. Statistics used in the data analysis. For **Figure 2A**, mean percentage of freezing of all subjects (n = 12) during the first 3 min of the training session (corresponding to baseline, BL) was compared to the percentage of freezing during the remaining 25 min of the session (25 tone-shock trials) showing a significant difference and a high effect size (*Cohen's d* = 2.34). For **Figure 2B**, a comparison was performed across bins of 3 minutes showing a significant difference in a Repeated Measures (BL and 8 bins) ANOVA test. For **Figure 2C**, comparisons between the mean percentage of freezing of each group of rats during the baseline (BL, first 3 min of the training session), training period (25 tone-shock pairings), context test session, and cue test session were conducted via a Mixed ANOVA with between-subjects factor the group (48 h or 6 weeks) and as within-subjects factor the phases (BL, Training, Context and Cue). Differences in phases and group, but not in the interaction Phases*Group were found. **Figure 3A – 3B** shows data on activity (panel 3A, motion index), freezing (panel 3B, mean freezing in seconds) and duration of episodes (panel 3C, mean freezing episodes in seconds). These data were analyzed using a One-way ANOVA, which indicated differences between groups in all measurements. Finally, for **Figure 4A – 4C** a Mixed ANOVA was performed for each panel (A, B and C), having as between-subjects factor the group (48 h or 6 weeks) and within-subjects factor the phases (BL, Training, Context and Cue).

Table 2. Mean difference, standard error and effect size for 3 min bins in Figure 2B. This table shows the comparisons between the baseline Bin and each of the subsequent bins (**Figure 2B**). Mean difference, standard error, and *p*-value and Cohen's d are reported as an index of the size of these differences (effect size).

DISCUSSION:

The present extended fear-conditioning protocol is an efficient and valid approach to assess emotional memory across short (48 h) and long-term periods (6 weeks). Thus, the protocol allows to study overtraining and fear incubation phenomena in rats. Among the different advantages of this protocol are the following. It offers two types of memory tests, namely context and cue, that allow to identify the differential effect of two delays (48 h and 6 weeks) across context and cue manipulations. Second, the task entails a single 28 min training session, which in turn produces long-term effects that extend by several weeks. This advantage is remarkable, considering that some versions of extended fear conditioning need at least 100 shocks across 10 sessions of training[11]. Third, the protocol offers several measurement alternatives, which are calculated automatically. In addition, there is mounting pharmacological, physiological, and anatomical evidence that supports the validity of this paradigm for assessing emotional memory phenomena[15,16].

Compared to other fear-conditioning paradigms with brief training sessions (i.e., few trials), extended protocols that result in overtraining effects have received less attention. However, extended fear-conditioning tasks have been key to the understanding of fear incubation's underlying behavioral and neurobiological processes, including its relationship with other psychological phenomena (e.g., delayed-onset post-traumatic stress disorder)[11–13]. The present fear-conditioning protocol reliably produces fear incubation. This is demonstrated with higher freezing times and lower motion indexes in animals assessed 6 weeks after training, as compared to animals tested 48 h after training. In addition, this effect could be observed differentially in each of the types of test; specifically, longer freezing episodes during the context test 6 weeks after training and increments in freezing during cue presentations 6 weeks after training. Related to this latter effect (i.e., increments in freezing during cue presentations 6 weeks after training), it is possible to discard the possibility of novelty of the experimental situation (i.e., new context), considering that baseline freezing levels during that same session were significantly lower than during the subsequent cue presentations.

Although a trend towards fear learning was evident in both groups (i.e., differences between 3 min baseline and training), animals that were tested after 48 h (context) and 72 h (cue) did not exhibit significant differences in freezing level during both tests. This absence of effect can be considered a limitation of the protocol, which is associated with a high behavioral variability for the 48 h group (see **Figure 2C**). A methodological change that can be implemented in order to reduce the variability and improve the procedure is to carry out the context and cue test 24 h after training, something that is common in some fear conditioning procedures.

The present protocol could be applied in clinical research[23]. The strong memory trace and incubation effect that result from its implementation may allow to test the effects of medications regularly used for treatment of psychological and psychiatric pathologies (e.g., anxiolytic or mood regulators treatments[24]) on emotional memory phenomena (e.g., fear extinction)[25–27]. The protocol thus could allow to measure the influence of medications on the memory trace across different time frames, including biological correlates such as neurotransmitters and molecules

related to memory maintenance[28,29]. The protocol could also be of relevance for research with a translational perspective, which has proposed that fear paradigms could be useful to test preclinical models of behavioral therapies[30] and comparative studies on fear across species[21,22]. Lastly, from a neurobiological view, the present protocol is a robust model to study brain mechanisms, communications between structures, networks or neuronal ensembles involved in long-term acquisition, consolidation and storage of emotional memory, or effects of incubation during development[32].

Some other aspects of the protocol are worth discussing. Food deprivation was used throughout the experiment. This decision was adopted because other behavioral tests based on food rewards (e.g., operant or instrumental techniques)[33–35] can be integrated with minimum changes, making the present protocol a more versatile technique. For instance, we have successfully integrated this protocol with wheel-based exercise protocols and T-maze memory tasks. Another aspect is related to the group size (n=6) implemented in this protocol. Though it was a relatively small sample, and larger samples are certainly recommended, the size of the incubation effect compensates for this limitation (see **Table 1**). This could be considered an advantage of this protocol, especially regarding animal committees' recommendations based on the reduction principle. A limitation of the protocol was that minimal or no exposure to footshocks and time-course of fear incubation were not evaluated. An additional control group with the before conditions could increase the rigor of the experimental design.

Final recommendations for the best implementation and results of this protocol include correct cleaning of the experimental chamber, especially the grid floor, calibration of the shock intensities prior to training each subject (e.g., feces and urine often reduce the reliability of the shock intensity across different areas of the chamber) and freezing detection system calibration (reliability of the freezing measures depends on proper setting of motion threshold and minimal freezing duration).

This protocol could be tested with other strains of rats or other rodents (e.g., mice or Mongolian gerbils), broadening the scope of applications. In those cases, it is important to adjust shock intensity and motion and duration thresholds. The shock intensity used in fear conditioning protocols with mice typically ranges 0.4 mA to 1.5 mA, 0.75 mA being an often reported effective intensity[16,36–38] and 1.5 mA a high intensity[39]. The Mongolian gerbil is a rodent model less frequently chosen for fear conditioning research; however, Mongolian gerbils have been successfully used to model circadian rhythms in mammals[40]. Accordingly, the current protocol could be implemented to study potential relations between circadian rhythms and emotional memory, both of them relevant in pathologies such as depression, anxiety or alteration of mood[41,42]. In the case of gerbils, an effective shock intensity range for this and analogous aversive conditioning protocols is between 1.0 and 4.0 mA[43–46]. Lastly, it is important to note that motion and duration thresholds should be adjusted depending on the species chosen[47]. These thresholds are within the limits established on the movement tracking software above which the animal behavior is registered as movement and below which the software registers freezing. In aversive conditioning studies with mice and gerbils, effective motion and duration thresholds reported have been 25 and 30 fps (i.e., minimum 1 s immobility), respectively[30,35].

To ensure adequate control of aversive stimulation (footshocks), all sectors of the grid floor must deliver the same intensity. It is recommended to calibrate the intensity of the shock in three sectors of the grid floor to verify that it is consistent. This prevents animals from learning to reduce exposure to the shocks by moving to a place in the box that emits a lower intensity. In case the calibration shows that the metal grid is not delivering the same intensity in all sectors, remove the grid from the floor, clean the rods, and replace in the chamber. The grid floor must be properly inserted into the chamber to ensure the best electric transmission from the aversive stimulation device to the grid floor.

The focus and aperture of the freezing detection system camera is calibrated by the manufacturer. However, if additional calibration is required, loosen the setscrew on the focus ring, adjust until achieve a clear image and then tighten the setscrew on the focus ring. The manufacturer recommends locking the lens opening in the maximum open position. To achieve this setting, make sure that the white point of the opening ring is aligned with the number 1.4 on the lens barrel. It is recommended to consult the manufacturer's manual. Note that if adjusting the focus of the camera, calibrate of the camera using the corresponding software must occur. Camera calibration requires adjustment of the brightness, gain, and shutter. It is recommended to consult the manufacturer's manual for precise instructions on the camera calibration process.

In conclusion, the protocol allows to test emotional memory across short and long-term periods and generates long-term fear incubation. This fear-incubation effect is generated via a single-session overtraining, which shows effects 6 weeks later in context and cue tests, which suggests a strong emotional memory trace. This protocol is an efficient and valid approach to explore the components of the emotional memory in rats.

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DISCLOSURES:
The authors have nothing to disclose.

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Figure 1

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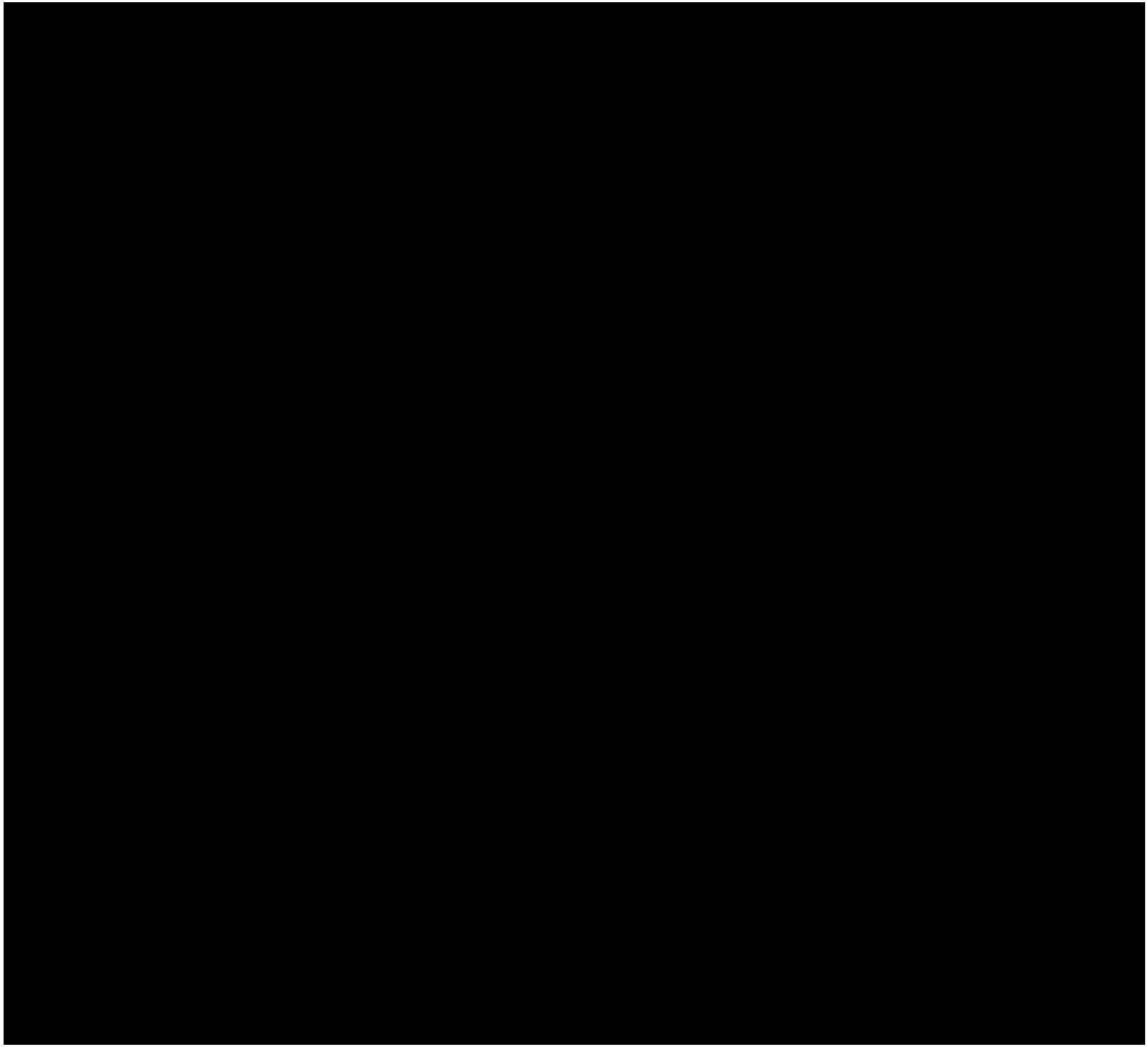


Figure 2

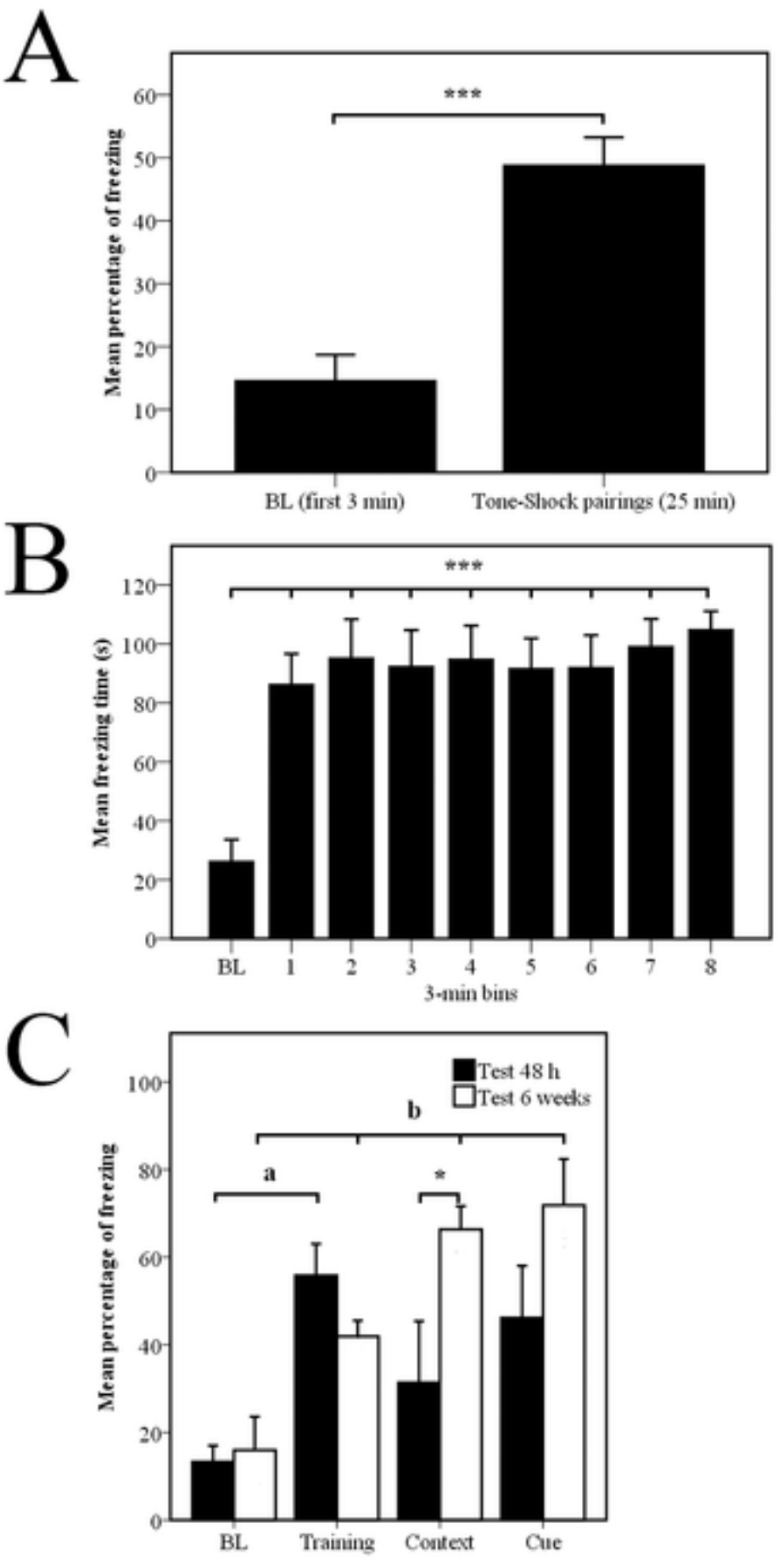


Figure 3

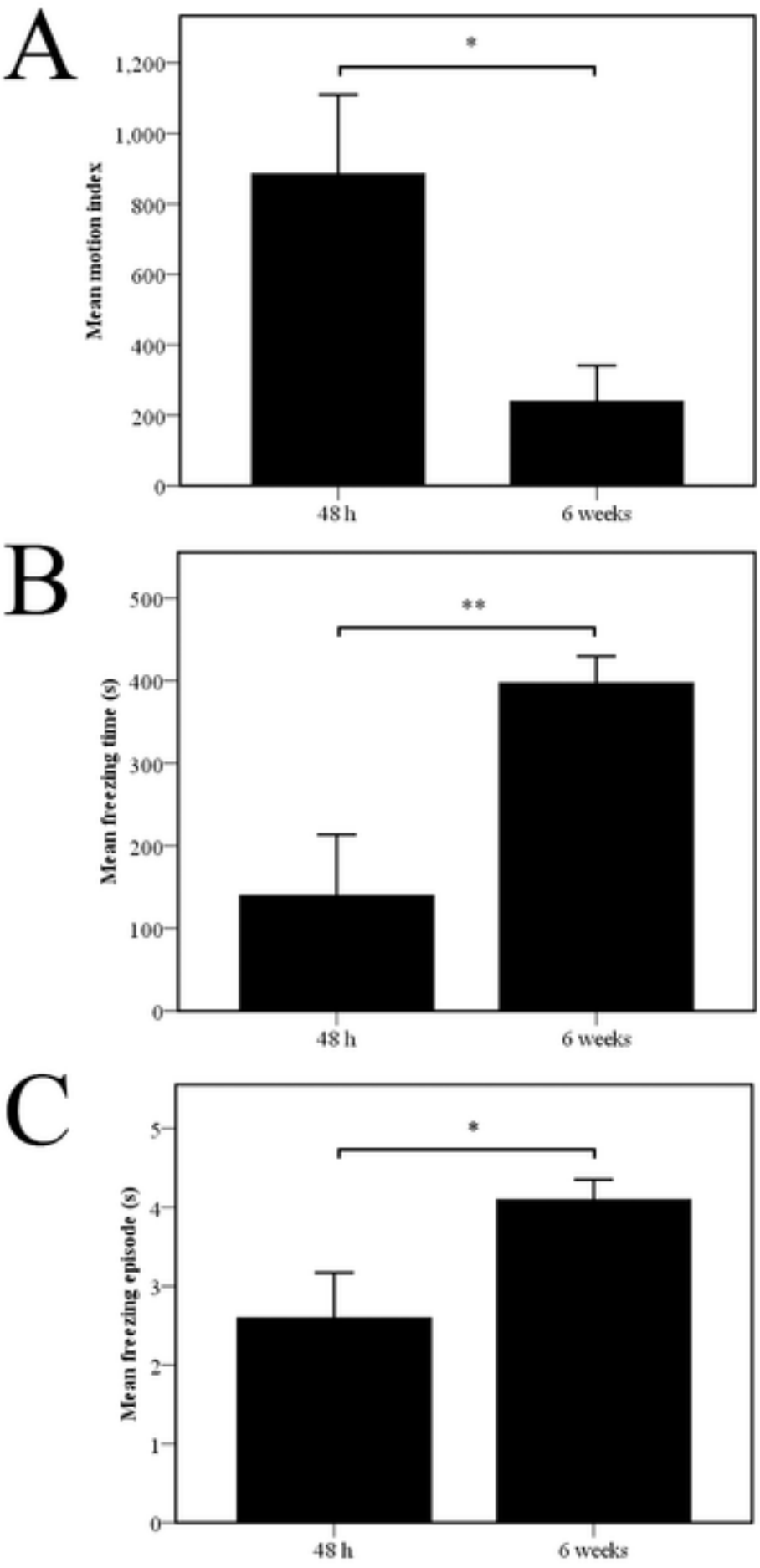
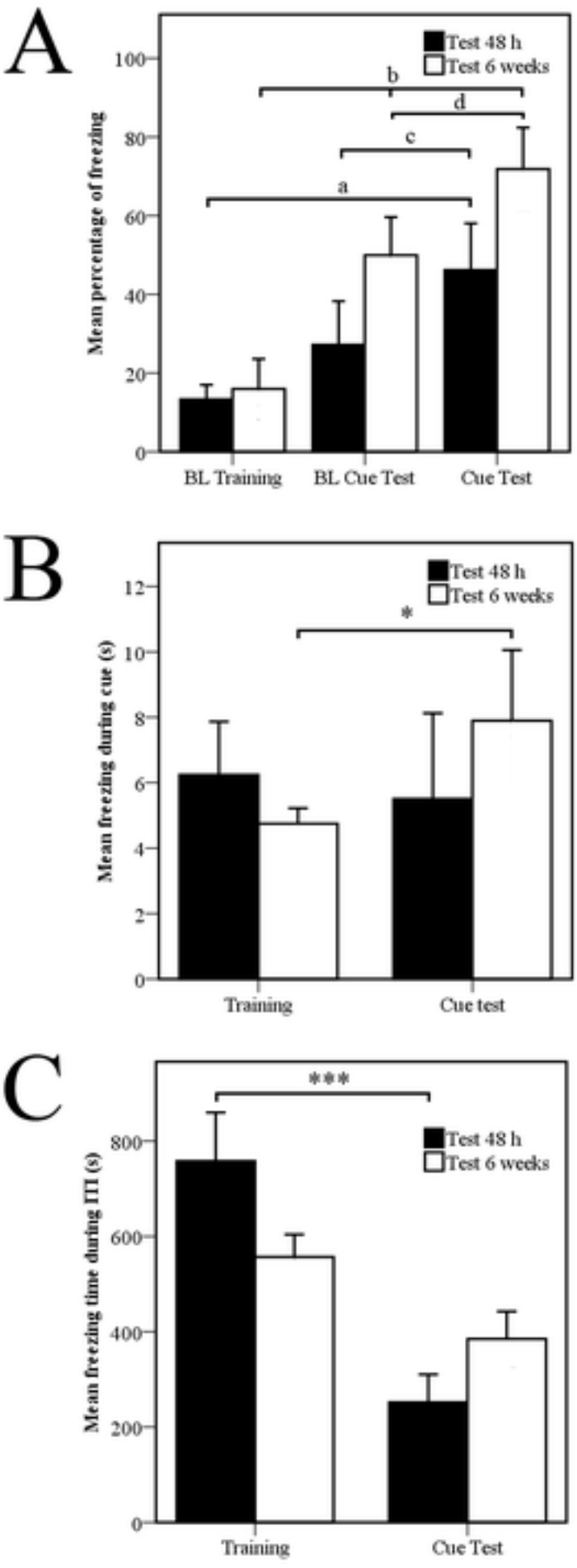


Figure 4



Table

Statistic Test	Figure	Phases
Dependent *t* Test	2A	t (11) = -6.21, p < .001, d = 2.34
		3-min bins
Repeated Measures ANOVA	2B	F (3.75, 41.32) = 11.19, p < .001, η_p^2 = .50.
		Phases
Mixed ANOVA	2C	F (3, 30) = 14.21, p <.001, ηp^2 =.58
One-Way ANOVA	3A	F (1, 10) = 6.91, p <.05, ηp^2 =.40
One-Way ANOVA	3B	F (1, 10) = 10.30, p <.05, ηp^2 =.50
One-Way ANOVA	3C	F (1, 10) = 5.83, p <.05, ηp^2 =.36
Mixed ANOVA	4A	F (2, 20) = 29.28, p <.001, ηp^2 =.74
Mixed ANOVA	4B	F (1, 10) = 1.53, p >.05, ηp^2 =.13
Mixed ANOVA	4C	F (1, 10) = 25.43, p <.001, ηp^2 =.71

Group	Phases X Group
F (3, 30) = 4.63, p <.05, ηp^2 =.31	F (1, 10) = 2.06, p >.05, ηp^2 =.17
F (2, 20) = 2.33, p >.05, ηp^2 =.18	F (1, 10) = 2.14, p >.05, ηp^2 =.17
F (1, 10) = 3.98, p <.05, ηp^2 =.28	F (1, 10) = .23, p >.05, ηp^2 =.02
F (1, 10) = 6.17, p <.05, ηp^2 =.38	F (1, 10) = .22, p >.05, ηp^2 =.02

Table 2

Comparison	Mean difference	Standard error	p value	Cohen´s d
Bin baseline vs bin 1	-60.075*	12,243	< .05	1.95
Bin baseline vs bin 2	-69.053*	16,220	< .05	1.89
Bin baseline vs bin 3	-66.197*	13,706	< .05	1.91
Bin baseline vs bin 4	-68.595*	11,969	< .05	2.08
Bin baseline vs bin 5	-65.475*	10,991	< .05	2.15
Bin baseline vs bin 6	-65.795*	13,509	< .05	2.06
Bin baseline vs bin 7	-72.900*	12,231	< .05	2.53
Bin baseline vs bin 8	-78.633*	8,692	< .001	3.37

Table of Materials

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

Name of Material/ Equipment	Company	Reference
Acetic acid (ethanoic acid)		
Aversive Stimulation Current Package	MED Associates Inc	ENV-420
Contextual test protocol.pro		
Cue test protocol.pro		
Curved Wall Insert	MED Associates Inc	VFC-008-CWI
Data processing.zip		
NIR/White Light Control Box	MED Associates Inc	NIR-100
Pellets	BioServ	F0165
Quick Change Floor/Pan Unit for Mouse	MED Associates Inc	ENV-005FPU-M
Small Tabletop Cabinet and Power Supply	MED Associates Inc	SG-6080D
Standalone Aversive Stimulator/Scrambler (115 V / 60 Hz)	MED Associates Inc	ENV-414S
Standard Fear Conditioning Chamber	MED Associates Inc	VFC-008
Training protocol VFC.pro		
Video Fear Conditioning Package for Rat	MED Associates Inc	MED-VFC-SCT-R

Comments/Description

https://pubchem.ncbi.nlm.nih.gov/compound/acetic_acid

https://www.med-associates.com/product/aversive-stimulation-current-test-package/
https://osf.io/4nkfq/?view_only=0640852a88544b239549462f9c21175b.
https://osf.io/4nkfq/?view_only=0640852a88544b239549462f9c21175b.

https://www.med-associates.com/product/curved-wall-insert/
https://osf.io/4nkfq/?view_only=0640852a88544b239549462f9c21175b.

http://www.bio-serv.com/pdf/F0165.pdf

https://www.med-associates.com/product/quick-change-floorpan-unit-for-mouse/

https://www.med-associates.com/product/small-tabletop-cabinet-and-power-supply-120v-60-hz/

https://www.med-associates.com/product/standalone-aversive-stimulatorscrambler-115-v-ac-60-hz/

https://www.med-associates.com/product/standard-fear-conditioning-chamber/
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https://www.med-associates.com/product/nir-video-fear-conditioning-system-for-rat/

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Signature:	
Date:	July 6th 2019

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Troy, AL, USA, April 30, 2020

Re: Manuscript
Fear incubation using an extended fear-conditioning protocol for rats

Dr. Vineeta Bajaj
Senior Review Editor
Journal of Visualized Experiments (JoVE)

Dear Dr. Bajaj:

We would like to thank you and the reviewers for the feedback we have received. We are convinced that it has importantly improved the clarity and scope of our paper.

In the attached file we address each of the comments and describe the corresponding changes to the manuscript. We have highlighted the sections that were edited/added in the revised version.

Sincerely,

Camilo Hurtado-Parrado, **PhD** (on behalf of the authors).
Associate Professor, Department of Psychology
Troy University
353 Hawkins Hall
Troy, AL
36082

---------- Editor´s comments ----------

1. The editor has formatted the manuscript to match the journal's style. Please retain and use the attached version for revision.

We made all modifications on the format, as requested.

2. Please address all the specific comments marked in the text both for the video and the text manuscript.

We worked on all comments from the reviewers, for both video and text.

3. Please proofread the manuscript before submission.

We edited and proofread the manuscript, as requested.

---------- Editor´s comments on video ----------

1. Please shorten the interview section both in the introduction and conclusion.

We reduced the duration of the introduction and conclusion sections, as requested.

2. 0:41-1:01: Please remove the background noise from the interview section both in the introduction and in the conclusion section.

We made the modification, as requested.

3. 2:53: Please format the degree unit as 20 ˚C. Please include a single space between the number the units as shown here.

Done, as requested.

4. Please use h for hour throughout the video and the figures. Please include a single space between the number and the unit e.g., 2 h.

We made the modification, as requested.

5. Please do not show the commercial term Med associates in the video.

We made the modification, as requested.

6. 11:08: Please reword the discussion subheading to Conclusion instead. Please shorten this section to half. Presently this is more than 4:00 min.

We made the modification, as requested.

7. 10:08-10:51 - The narration audio here is much louder in the right stereo channel, compared to the left stereo channel. The voice should be balanced equally between the left and right channels.

We made the modification, as requested.

Reviewer 1

The authors have responded to reviewer comments, and the manuscript now includes some more information that will be useful for replication.

We appreciate the comments made by the reviewer.

Reviewer 2

The authors significantly improved the manuscript and followed the suggestions of the reviewer. I recommend the paper for publication.

We appreciate the comment made by the reviewer.

typo line 426: Fig. 7A

Typographic error amended.

typo line 507 (spanish question mark)

This fragment was excluded from the text.

Reviewer 3

This is a re-submission by Acevedo-Triana and colleagues described the methodology for expression of fear incubation using an extended fear conditioning protocol. The authors have now mostly described the methods and statistics in adequate detail to allow for replication of this work.

We appreciate the comment made by the reviewer

My main concern that was expressed in the original review, with respect to the lack of rigor in the experimental design, specifically in control conditions still stands. At the very least, please acknowledge in the discussion section that rats with minimal or no exposure to footshock, the time-course of fear incubation etc have not been assessed here.

We added the suggestion of reviewer. Please see lines 636-638.

-Please specify the weights of the Wistar rats used for this experiment.

We specified the range of weights of rats. Please see line 138.

-Please state specifically that the rats were trained during the light cycle.

We added the clarification. Please see line 153.

-Please specify details of how often and how the rats were handled during the incubation period.

We added the clarification. Please see lines 259-261.

Reviewer 4

My concerns with the earlier version have been satisfied.

We appreciate the comments made by the reviewers.

Reviewer 5

The authors described an extended fear conditioning protocol that produces fear incubation. Since fear incubation may be related to delayed post-traumatic stress disorder, this protocol will be useful in neuroscience and psychiatry fields. Overall, this protocol is well written. I have a few minor concerns.

1. The authors should describe brightness level in the experimental chamber.

We added the required information. Please see lines 211-213.

2. The authors should describe size of the experimental chamber.

We added the required information. Please see lines 172-179.

3. Although the authors used extended fear conditioning (25 tone-shock pairings), the freezing level in the context and cue tests looks low or similar compared to the standard protocol. Why?

We agree with the reviewer, and added a possible explanation. Please see lines 553-568 and lines 453-457.

Reviewer 6

The protocol describes a new paradigm using an overtraining method of fear conditioning to assess fear incubation in rats. The authors showed that after 1 session of overtraining rats with 25 tone-shock pairings, rats freeze more to the conditioned context at 6 weeks compared to 48 hours after training.

1) The paradigm described is interesting and useful because there is a growing interest in research investigating fear incubation. However, there is a novelty to this protocol, as a PubMed search for articles using this type of protocol could not be found nor publications from the authors utilizing this protocol. Because of this novelty, the authors need to give more background information for the development of this protocol and describe the pros/cons of this protocol compared to other existing overtraining fear conditioning protocols used to assess fear incubation. In addition, authors should address whether publications are pending or reference any publications using this protocol that may not have found on PubMed to support the validity of the protocol.

We used several references to build our protocol. Effectively, we reviewed some references and found lengthy protocols, such as Pickens (2013) with 100 pairings and Maren (1998) with considerably shorter versions. We decided to use the protocol used by Maren (1998) with 25 pairing. Unfortunately, in the previous version of our manuscript, we missed including the reference, and appreciate the reviewer's comment that pointed it out. Please see lines 106-109.

2) The overall protocol is difficult to follow. It is more written for an investigator that has the same exact system in their lab and if not, following the protocol becomes difficult. Each step is too detailed and does not give enough information to researchers that may have an alternate setup. This should be kept in mind when writing this protocol.

We have added some NOTES in each step to help generalize the protocol to different users and equipment, we have added information about measurements and information to that same aim.

3) A representative schematic of the protocol (conditioning, context test, and cued test) and photos would be beneficial.

We agree with the reviewer, and added a graphical representation of the study's design. Please see Figure 1.

4) The incubation effects in the cued test were not as robust as the context test. Could it be possible that the context of the cued test may have been too similar to the conditioning and it blunted some of the effects that could have been seen between 48 hours and 6 week groups? In addition, results in figure 3 shows baseline freezing levels in the cued test looks higher than baseline freezing during training. Could the animals be responding to the context before the cues are even tested? Is it possible to change the floor in addition to smell and walls for the cued test?

We consider this lack of effect in the cue test a limitation of the protocol, which we acknowledge and explain as resulting from variability in the 48 h group.

5) The data analysis section is too dependent on the authors' exact methods. This section should include alternate methods for people without Med Associates. For example, describe what parameters should be analyzed if using an alternate setup, what should be scored for freezing, what are the behavioral endpoints people should examine in their results.

We added descriptions aimed at improving generalization to other settings and equipment, including clarifying the measurements and the contents/functions of the processing files. Please see lines 408-415.

6) The authors state on lines 349-350 that there was declining trend of freezing % in the 48 hour group in the context and cued test compared to training freezing. This statement is slightly deceiving, as it sounds more as if the training did not work. If the baseline freezing levels measured during the first 3min of training is being used as the control, then context and cued test freezing should be compared to this control. Therefore, both 48 h and 6 wk groups showed high levels of freezing compared to control baselines, demonstrating learning and memory of the tone-shock pairings. However, the 48 h group did not show statistically significant increases in freezing in the context and cued tests compared to control freezing levels. This should be addressed by the authors. Did the paradigm not work for the 48 hour group, was there too much variability, what was the issue that rats in the 48 h group did not show elevated freezing after conditioning to the context or cue, but the 6 wk group did?

We addressed this absence of statistical significance in the discussion section. We consider it a limitation of our protocol, which is associated with a high behavioral variability in the 48-h group. We made some suggestions to improve the procedure regarding this issue. Please see lines 605-611.

7) In line with comment 6, for context and cued test results, 48 hr and 6 wk freezing levels should be compared to the control, which is described as the first 3 min of the training session. Therefore, figure 2 and 3 should include those levels and statistics compared to those controls as well.

As shown in Figure 4a, baseline measures can be included in the analyses when percentages are used for the comparison. With Figure 3a,b,c and 4,b,c it is not appropriate to include baseline measures because the variables are not proportional (seconds and motion index).

8) Statistics on the figures are confusing to follow. Generally, asterisks and letters are placed above the treated groups to compare to control, not above the control.

We agree with the reviewer, and we have amended the significance information in the figures to avoid confusion. Please see Figures 2-4.

9) Table 1 and 2 are very confusing and difficult to follow. More descriptions of the factors are needed.

We added additional information to improve clarity. Please see lines 553-574.

10) Discussion should address why fear incubation was seen in the context test but not necessarily in the cued test.

We discuss this topic in the discussion. Please see lines 605-611.

11) Are there any publications available that may not have been found on PubMed using this protocol to support the validity of this paradigm?

As was mentioned before, we used information from other protocols and we conducted a study with exercise to stimulate neurogenesis and evaluated its relationship with fear incubation. This study is under review.

12) Have the authors looked at a time point between 48h and 6 weeks? If so, does the incubation effect increase as time increases from conditioning day as seen in the Pickens model?

Unfortunately, we didn't conduct measurements at any point between 48 h and 6 weeks, mainly to avoid effects of reconsolidation.

Introduction - The introduction adequately addresses fear conditioning and fear incubation. However, background of overtraining is unsatisfactory. For fear incubation, they discuss the Pickens method of 100 tone-shock pairing to assess fear incubation, but the methods they use in one session of 25 tone-shock pairing. It is not well understood why and how their method was developed.

We added information to clarify the numbers of sessions. Please see lines 106-108 and lines 115-116.

Introduction - It would be useful to include reference to the Stephen Maren's 1998 paper (J Neurosci, Overtraining Does Not Mitigate Contextual Fear Conditioning Deficits Produced by Neurotoxic Lesions of the Basolateral Amygdala), which describes asymptotic freezing behavior during overtraining and what is the minimum tone-shock pairings needed for overtraining.

We appreciate the reviewer's feedback and suggestion. We actually used this reference to build our protocol, and as such, we have added to the present manuscript. Please see lines 106-108 and lines 115-116.

-Possibility of usage of other strains of rats should be addressed. Is there a benefit to using Wistar rats over other common strains?

We agree with the reviewer, and have added the clarification in a NOTE. Please see lines 133-136.

-Include a note to why the rats need to be food restricted in a fear conditioning protocol. It should be mentioned that users using the protocol need necessary IACUC approval for food restriction. Will not food restricting rats affect the protocol or results in any way?

We agree and added the clarification in a NOTE. Please see lines 143-148.

-Counterbalancing of animals during testing, using the same exact box for conditioning and context for each rat, and testing rats at the same time of day should be explained.

We agree and added the clarification in a NOTE. Please see lines 157-160.

-Include procedures for animal handling before testing begins, time of day testing (light/dark phase).

We included the clarification, as suggested. Please see line 153 and lines 252-257.

-How long do the rats need to be acclimated to the facility after delivery?

We included the clarification, as suggested. Please see lines 267-268.

-It would be beneficial to describe the components of all the important parts to operate this protocol. Med Associates equipment components should be better explained in these sections. Also, further describe what is needed to execute the protocol.

We edited all protocol and included different NOTEs to enable generalization to different users. By other hand following indications of JoVE papers, we previously received comments to avoid specific indications with a particular equipment.

-Context of conditioning and context test versus cue test should be adequately described.

We added a better description of the context of the training and tests. Please see lines 250-368.

-Animal transportation should be described for each test. Transportation route for cued test should differ from conditioning to further differentiate context for the cued test.

We included the clarification, as requested. Please see lines 252 -257, lines 296 – 301 and lines 333-336.

-Calibration should have their own section. In that section, shock calibration and freezing detection calibration should be described.

We separated the sections, as suggested. We added to section 2 the clarification about shock calibrations. Line 162

-Notes for any possibly issues that may occur with calibration and how to troubleshoot should be included.

We edited some NOTEs to avoid confusion, and edited and improved the section of Troubleshooting. Please see lines 690-712.

-Describe which animals to bring in during testing. Most protocols only bring in the rats to be tested and leave the remaining rats in a holding room to be not affected.

We clarify the information on the transportation of animals. We only moved the animals that were immediately tested and maintained the other rats in a separate holding room to avoid possible affectations. Please see lines 252-257.

-Rats should not be handled by the tail. If this statement is necessary, be specific and state "base of the tail" and never pick up by the end of the tail.

We added the clarification, as requested. We appreciate the reviewer’s comment. Please see lines 263, 318 and 355.

-Include the step, return animals to cage and vivarium

We clarified the transportation system. Please see lines 252-257.

-step 5.1 and 6.1 should be rewritten

We edited the corresponding sections. Please see lines 296-301 and lines 327-329.

-On line 307, should be Figure 1A and not 7A.

We agree with the reviewer, and figures were relabeled. Please see lines 426.

-Where is the data described in lines 304-306?

We added the necessary information. Please see lines 399-403.

-For lines 474-476, reference should be included.

We included the related reference. Please see lines 616.

Veterinary comments

. Please clarify in the text if housing 4 rats per cage is meeting ILAR Guide requirement? If not, what kind of guidelines do you use for housing multiple rats?

We did not follow directly the ILAR guide to housing, instead we follow the requirements of the IACUC of our institution related with housing recommendations. However, it is important to clarify that animals were housed in groups of 4 only for 5 days previous to the start of the training protocol, which required individual housing, since they were maintained at 85% of their weight. Clarification of individual housing was made. Please see lines 133-145.

Under 1.2. Please clarify why you need to maintain rats at 85% of their free-feeding weight for 6 weeks study by giving restricted food access? - - If the animals start losing weight, will you assume feeding animals to ad libitum again? --- How much percentage of body weight loss when you resume ad libitum feeding again?

We added the clarification related to caloric restriction. The procedure to reach the 85% was calculated on the bases of the free-food-consumption weight during five days before fear training. Please see lines 143-146.

Under 6.4. Please add into the text on how you change of the olfactory complex by using 1% of acetic acid to the metal tray below the grid floor?

We added in the manuscript that acetic acid is the standard procedure to change the olfactory context in fear conditioning of rodents, but we did not conduct further measurements below the grid floor. Please see lines 344-345.