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# Implementing Chronic Exercise After the Completion of Extinction Training in Mice Shows Promise in Reducing the Return of Fear in the Long Term

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**Abstract**

Post-traumatic stress disorder (PTSD) is a prevalent mental health condition that affects a wide variety of populations. One of the first-line treatments for this disorder is prolonged-exposure therapy (PE), a therapy that has produced inconsistent results with high rates of fear return over time. Fear conditioning, a process that relates to PTSD, has been studied in animals for many years. An additional component of this procedure, referred to as extinction training, is similar to the methods behind PE, and as such, different interventions can be tested in animals in hopes of finding ways to improve the success of the therapy in humans. In the past, researchers have tried to use exercise as an intervention, based on experiments demonstrating its ability to improve learning by strengthening the consolidation of memories. Chronic exercise implemented directly after fear conditioning, and before extinction, has been found to increase rather than to reduce the magnitude of the fearful response when tested after the passage of time. In our study, we sought to determine if altering the timing of chronic exercise would have a different effect. We ran three groups through a fear conditioning and extinction paradigm, with one group remaining sedentary for the entire experiment, a second group beginning to exercise directly after fear conditioning, and a third group beginning to exercise after the completion of extinction. Our results indicated a significant overall decrease in magnitude of the return of fear within the group that began exercising after extinction when compared to both the sedentary group and the group that began exercise after fear conditioning. These results show that there is promise in using this method for improving the long-term success of exposure-based therapies for PTSD. Continued adjustments to the procedure could lead to a more dependable method that could be employed in human populations.

## Introduction

Post-traumatic stress disorder (PTSD) is a mental health condition that manifests following the experience of a stressful, traumatic event. Roberts et al. (2011) report that 7.3% of Americans will have PTSD at some point in their lives. PTSD is most commonly associated with veterans of war; according to Wisco et al. (2014) there is an 8.3% lifetime prevalence in US veterans overall, with a lifetime prevalence of 19.4% found in female veterans and of 23.8% found in veterans aged 21-29. These results were based off of well-known PTSD assessments including the PTSD Checklist-Specific Stressor version (PCL-S), which assesses PTSD based on the criteria defined in the DMS-IV (Weathers FW, Litz BT, Herman DS, et al.) and the Trauma History Screen (Carlson EB, Smith SR, Palmieri PA, et al.). The most recent version of the DSM, the DSM-V, removed PTSD from the category of anxiety disorders and placed it into a new category called "Trauma and Stressor-related Disorders" (Pai et al., 2017). The criteria for PTSD diagnosis were also adjusted, beginning with the criterion of exposure to a traumatic event, in which trauma was more precisely defined as involving death, serious injury, or sexual violence (Pai et al., 2017). Additionally, the DSM-V adds the requirement of at least one symptom under the category of avoidance (Pai et al., 2017). These changes were made with the goal of defining trauma exposure more objectively, and leaving subjective responses to trauma exposure to the symptom categories (Pai et al., 2017). The PTSD checklist has since been updated to include these new changes and has been shown to have equal validity to that of the PCL-S (Hoge et al., 2014).

Though veterans are a population found to have high rates of PTSD, it can develop in anyone who has been involved directly in a traumatic experience or those who have observed a traumatic event (American Psychiatric Association, 2013, Pai et al., 2017). Wisco et al. (2014)

have shown an 87% prevalence of exposure to at least one traumatic event in the US, with traumas including deaths of a family member or friend. Traumas like these are often unavoidable, and therefore research into treatments for this disorder are critically needed.

One of the most common treatments for PTSD is exposure-based psychotherapy. The premise behind exposure therapy originates from the findings of Pavlov (1927). Pavlov trained dogs to salivate at the sound of a bell by repeatedly ringing the bell and then giving them food. The dogs learned to associate the sound of the bell with receiving food, resulting in the response of salivating at the sound of the bell only. Pavlov labeled the sound of the bell as the conditioned stimulus (CS) and salivation in response to the bell as the conditioned response (CR). When the dogs salivated in response to food only, Pavlov referred to the food as the unconditioned stimulus (US) and salivation as the unconditioned response (UR), due to the fact that salivation was an innate response to the presentation of food rather than a learned response like salivating at the sound of the bell. Pavlov furthered his work by reinforcing the CS with an unpleasant tasting food instead. He found that the dogs would then show a defensive response (the CR) to the CS, illustrating that the dogs could learn that something predicts an unpleasant or frightening stimulus. Pavlov referred to this phenomenon as fear conditioning. He then showed that if the CS is presented many times without it being followed by the unpleasant US, the dog would no longer show the defensive response (the CR in this case). This part of the procedure is known as extinction and has since been used as a model for exposure-based psychotherapy in individuals suffering from conditioned fear as a result of a traumatic experience.

During exposure-based psychotherapy, patients undergo extinction by being gradually exposed to a CS: a stimulus that reminds them of their trauma (the US) and that produces a fearful response (the CR). The goal of this type of therapy is to form a new memory in which the

CS is no longer associated with the US and therefore no longer causes the CR on its own. An example of this would be the fearful response that a person learns to associate with cars following a car accident. In this case, the car would be the CS and the CR would be the fearful response that the person has in the presence of the car. This fearful response is a natural, unconditioned response to a violent experience, and so the response is considered the UR in the absence of the car (or other stimulus that caused traumatic violence). The goal here would be to form a memory in which the car is no longer associated with the traumatic experience of the car crash and therefore no longer produces the fearful response on its own. The CS-US memory association and the extinction of this association through the formation of a new memory can be tested in animal models using fear conditioning, extinction training, and measures that test for the return of the CR. Pavlov demonstrated that fear returns after a period of time following extinction, which he referred to as spontaneous recovery. Pavlov took this result to mean that the initial memory associating the CS and the US was not eliminated during the extinction process. It still exists and after a period of time it is able to overcome the new memory formed during extinction. This return of the fearful memory can also be tested with reinstatement, a method in which the animals are presented with only the feared stimulus, and the time that it takes for the fear association to return is tested (Rescorla & Heth, 1975). When using these models to study exposure therapy, the goals are to find ways to reduce the magnitude of the fear that returns during spontaneous recovery as well as to lengthen the amount of time that it takes for the fear association to return during reinstatement.

This type of research is warranted due to the variation seen in the results of clinically applied prolonged exposure (PE) therapy. Foa and Kozak (1986) developed the method of prolonged exposure therapy based on the emotional processing theory, which applies the

experimental results of Pavlov to human populations. In 2007, Foa et al. employed 10-15 sessions of prolonged exposure (PE) in five victims of combat-related PTSD. Their results showed a 48% decrease in PTSD Symptom-Scale score. Powers et al. (2010) found that PE treated patients fared better than 86% of patients in control conditions. At the end of PE treatment in a population of veterans and soldiers, only 38.8% lost their PTSD diagnosis by the end of treatment and of those who did lose the diagnosis, 50.9% still had difficulty falling asleep, 60.7% had high irritability and anger, and 41.0% continued to feel distress surrounding trauma reminders (Schnurr et al., 2018). Due to the inconsistency of these results, methods for improving the success of exposure therapy should continue to be pursued. This variability is likely in part due to differences in the types and severities of traumas experienced, as well as genetic and environmental factors that differed between the participants. It is therefore beneficial to study this therapy in rodent models since we can control the environmental conditions and implement a traumatic experience consistently across all subjects. The biological and behavioral results of these experiments can then be more easily and accurately interpreted.

Another behavior that may have clinically relevant outcomes and that has been highly studied in both humans and rodents is exercise. Research has shown that exercise impacts both physical and mental health (Ruegsegger & Booth, 2017). Physically, exercise has been shown to improve cardiovascular fitness (Blair et al., 1989, Myers et al., 2002) as well as to decrease risk of coronary heart disease (Morris et al., 1953) and type 2 diabetes (Booth et al., 2012). Koch et al. (2011) showed that chronic exercise increases the lifespan of rats, while Booth et al. (2012) found the same result in humans. Multiple research groups have reported improvements in both depression (Kratz et al., 2014, McKercher et al., 2014, Mura et al., 2014) and anxiety (Greenwood et al., 2012, Nashijima et al., 2013, Schoenfeld et al., 2013) as a result of chronic

exercise. The impacts of exercise on mental health disorders like these have led researchers to seek further understanding of the neurological changes caused by exercise and how it could be implemented therapeutically.

Another influence exercise has been shown to have is on learning and memory, specifically in learning and memory tasks that depend on the hippocampus (Fordyce and Farrar, 1991) and in tasks that depend on the amygdala (Greenwood et al., 2009). Based on prior research demonstrating the importance of the amygdala in cued fear conditioning (Maren, 2005), Falls et al. (2010) investigated the effects of two weeks of voluntary exercise on fear conditioning in mice. They exposed groups of mice to unlocked running wheels for either the two weeks prior to, or the two weeks following, fear conditioning (the pairing of a tone with a foot shock). A third group was given access to the unlocked wheels beginning two weeks after fear conditioning and ending after two weeks. They found that mice that had exercised directly after fear conditioning showed enhanced startle responses when compared to the other groups of mice and to a sedentary control group, as measured with a fear-potentiated startle test (Falls et al., 2010). These findings were interpreted to demonstrate that exercise augments consolidation of the memory formed when a tone is paired with a shock, as opposed to the encoding or retrieval of the memory.

According to Quirk et al. (2010), when extinction is performed following fear conditioning, competing memories are formed. Pavlov (1927) had demonstrated that a fear association can return after the passage of time even when it has been extinguished, which shows that extinction does not erase the initial memory formed from fear conditioning. Instead, both memory traces remain available, but the extinction memory is able to suppress the fear response for a period of time (Quirk et al., 2010). In 1992, Falls et al. found that injection of an NMDA

antagonist into the amygdala blocked extinction of fear, demonstrating that formation of the extinction memory is dependent on processes involved in long-term memory storage. Based on these ideas, research groups have searched for ways to increase the strength of the memory formed during extinction by using chronic exercise. Multiple experiments found that chronic exercise before conditioning had no impact on fear extinction when extinction was assessed immediately after conditioning (Greenwood et al., 2005 & Greenwood et al., 2003). It was hypothesized that this result was due to the fact that exercise-induced plasticity would not yet have had a chance to enhance the consolidation of the extinction memory. A later study by Greenwood et al (2009) looked at exercise and conditioning using a footshock as the conditioned stimulus in five groups of male rats. One group remained sedentary, two groups ran on wheels for either 1 or 6 weeks prior to fear conditioning, and two groups ran on wheels for either 1 or 6 weeks after fear conditioning. Findings were consistent with Neeper et al. (1995) in that wheel running increased plasticity factors in the hippocampus and the basolateral amygdala. However, neither exercise duration or timing was found to enhance fear extinction or to reduce spontaneous recovery (Greenwood et al., 2009). This variability and lack of success in using exercise to modulate fear extinction demonstrates the need for further studies to be performed.

We have chosen to perform our experiment in mice due to our ability to apply genetic tools in order to better understand the biological mechanisms underlying behavior. From the reviewed research, we found that chronic exercise has been implemented either before fear conditioning or after fear conditioning, with extinction implemented later. Since exercise has been found to modulate memory formation, and extinction forms a new memory association, we chose to focus on how we may be able to influence the memory formation of the extinction phase rather than that of the fear conditioning phase. One group of mice will have access to a

locked running wheel only, whereas a second group will have access to an unlocked wheel after conditioning and before extinction, and a third group will have access to an unlocked wheel only after the extinction phase is completed. It is hypothesized that chronic exercise following the extinction phase will strengthen the inhibitory memory formed during extinction and will in turn reduce the return of fear measured through spontaneous recovery and reinstatement tests. Fear response will be measured as percent time spent freezing in response to a noise that will become the CS.

## Materials and Methods

### *Subjects*

Six-week-old male C57BL6/J mice were obtained from Jackson Laboratories (Bar Harbor, Maine) and were housed for a 7-day acclimation period prior to experimental manipulation. Mice were maintained on a 12-h light/dark cycle, with lights on at 07:00 h, and with ad lib access to food and water. Fear conditioning and testing were carried out between 10:00 h and 18:00 h. All procedures were approved by the University of Vermont Animal Care and Use Committee.

### *Materials*

During all phases of the experiment, mice were housed in groups of four in standard acrylic cages 24 cm (*W*)x 45 cm (*D*) x 20 cm (*H*) located in an Association for Assessment and Accreditation of Laboratory Animal Care (AALAC) approved conventional animal facility. Fear conditioning and freezing tests were performed in four cubicles measuring 67.0 cm (*W*) x 50.8 cm (*D*) x 60.3 cm (*H*). Each cubicle encompassed a conditioning chamber 24.1 cm (*W*) x 25.4 cm (*D*) x 21.6 cm (*H*) constructed from metal walls on each side and clear acrylic walls on the front and back. The floor of each chamber consisted of a removable shock grid composed of 23

steel rods 3.2 mm in diameter and spaced 6.4 mm apart (Med-Associates, Georgia, VT) through which shock could be administered. The white noise (80 dB) stimulus (CS) was provided through a Radio Shack Super Tweeter located on top of the conditioning chamber. A GoPro 5 Sessions was mounted on a table in front of the cubicles so that all four conditioning chambers were in view of the camera at once. A Superpet mini run-a-round running wheel measuring 11.4 cm in diameter was used for the exercise phases of the experiment (Falls et al., 2010). The wheels were affixed to the tops of the cages with pieces of metal, screws, and bolts. Locked wheels were kept from spinning using zip ties and unlocked wheels were confirmed to be far enough off the floor of the cage and low enough from the ceiling of the cage to allow adequate spinning. Bicycle odometers and sensors were placed on top of the cages containing unlocked wheels in order to measure distance traveled by running mice.

*Group Assignments*

Following the 7-day acclimation period, six cages of four mice were randomly assigned to one of three groups describing the planned exposure to exercise: either the sedentary condition, the early exercise condition, or the late exercise condition. Two cages were assigned to each group. All six cages underwent the following procedures with video recordings taken during all tests and trainings. Mice were weighed every three days.

*Procedure*

Mice were brought to the conditioning chambers on the first day of experimentation. Mice were placed in the chambers and a pre-conditioning freezing test was given in order to assess the unconditioned effect of the white noise conditioned stimulus on freezing behavior. After a 2-min acclimation period, a 3-min white noise was presented followed by another 2-min period of no stimulus. Fear conditioning was conducted 24 h following the pre-conditioning

freezing test. Mice were placed in the conditioning chambers and were allowed a 2-min acclimation period. Five white noise + shock trials were then presented in which a 30-s white noise co-terminated with a 2-s, 0.4-mA foot shock. Trials were interspersed with variable intervals (3-min average ITI) consisting of no stimulus. Mice were returned to the conditioning chambers for a post-conditioning freezing test on the third day of experimentation. This session was identical to that given prior to fear-conditioning training.

Following a 14-day exercise phase, mice underwent extinction training for five days. Extinction consisted of white noise-only trials as described in the pre and post-conditioning freezing tests. A second 14-day exercise phase was implemented, followed by one day of spontaneous recovery testing; mice were presented with the same white noise-only trial as in extinction. Reinstatement was performed 24 h after spontaneous recovery and consisted of five presentations of the shock stimulus. The same timeline was used for reinstatement as was used in fear conditioning, except without the 30-s white noise preceding the shock. All procedures were performed one cage at a time with the cages run in different orders over the entirety of the experiment.

*Exercise*

Running wheels were introduced into all cages following the post-conditioning freezing test. To examine the effect of exercise on extinction learning, mice in the sedentary group were given nonfunctioning, locked running wheels immediately after the post-conditioning freezing test. The wheels remained locked for both 14-day exercise phases of the experiment and throughout extinction, spontaneous recovery, and reinstatement. Mice in the early exercise group were given functioning, unlocked wheels for both exercise phases, extinction, spontaneous recovery, and reinstatement. Mice in the late exercise group were given locked wheels for the

first exercise segment and during extinction, and unlocked wheels for the second exercise segment and during spontaneous recovery and reinstatement tests. A timeline of the experiment is shown in the schematic in Figure 1. Wheel odometers were checked daily during both exercise phases of the experiment.

**Figure 1: Experimental Design and Timeline**

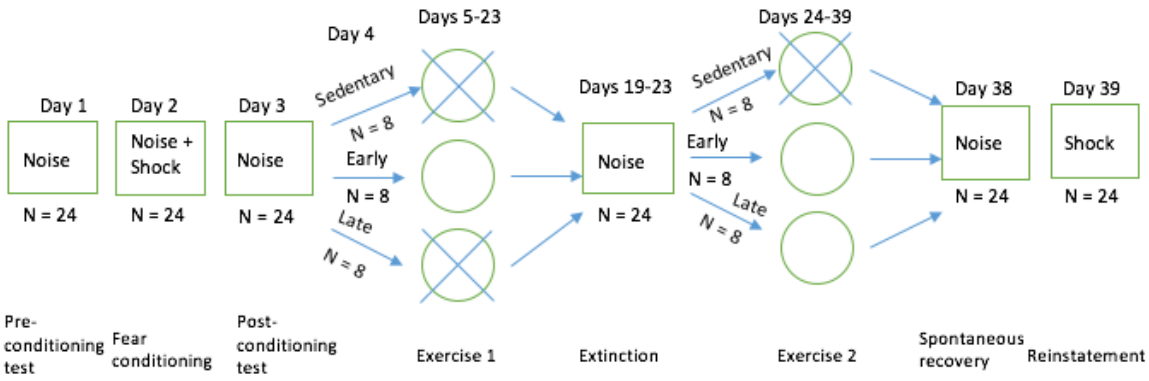



Figure 1: All mice underwent a pre-conditioning test on day 1 in which only the white noise was presented. Fear conditioning was conducted on day 2 and a post-conditioning test was given on day 3. Groups were randomly assigned on day 4 and wheels were placed on day 5. The sedentary and late exercise groups were given locked wheels for the first two-week exercise segment and the early exercise group was given an unlocked wheel for the same amount of time. Extinction of the noise and shock association was performed on days 19 through 23 and the second exercise segment was initiated on day 24 by replacing the locked wheel in the late exercise group with an unlocked wheel. After two weeks, all mice were tested in spontaneous recovery on day 38 and then in reinstatement on day 39.

*Scoring*

Freezing was scored by an unblinded observer in a location away from the laboratory. Videos taken using a GoPro 5 Sessions were downloaded onto a Macbook Air computer. Freezing was scored according to the 8-sec instantaneous time-sampling procedure developed by Fanselow and Bolles (1979). A computerized noise notified the observer of every 2-second passage of time, and at each noise the observer would score whether or not a particular mouse was found to be freezing. With four chambers in each video, the observer rotated from one chamber to the next every 2 seconds so that each mouse was observed every 8 seconds

(Fanselow and Bolles, 1979, Fanselow, 1980, Anagnostaras et al., 1999, 2000). A mouse was determined to be freezing when the observer saw an absence of movement in the mouse other than that required for respiration (Bolles and Riley, 1973, Bolles and Collier, 1976, Fanselow and Bolles, 1979). Though originally designed to measure freezing in rats, this procedure has also been shown to apply in mice (DeLorey et al., 1998, Anagostaras et al., 2003). The number of times each mouse was found to be freezing was tallied over the entire length of each experimental test. Percent time freezing was calculated by dividing the number of freezing observations of a mouse by the total number of observations for that mouse.

*Statistics*

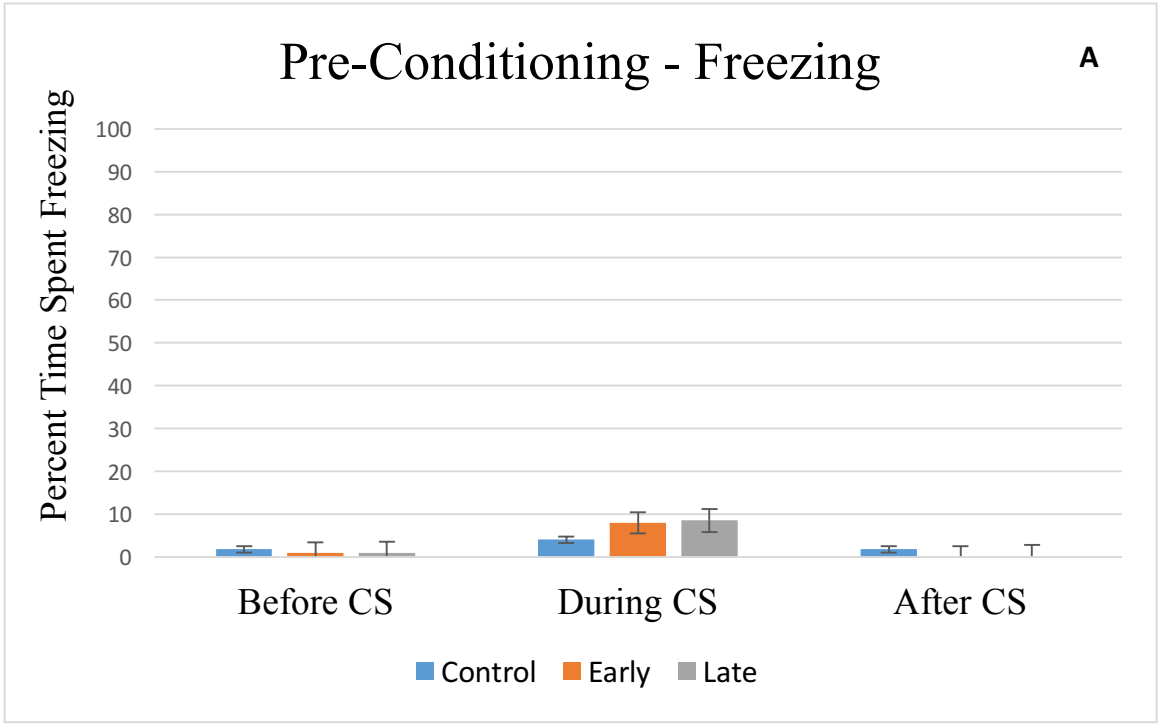
Data were analyzed using IBM SPSS Statistics. Percent time spent freezing was calculated for each mouse over the time period before the CS, during the CS, and after the CS for each experimental test. Box plots were produced in SPSS in order to identify outliers. One outlier was found in the pre-conditioning freezing data and another in the post-conditioning freezing data. Both values were excluded from statistical analysis. A one-way ANOVA was used to compare experimental group assignment to percent time freezing during the CS. An LSD Post-Hoc test was used for follow up in the event of statistical significance. Analyses were performed for the pre-conditioning, post-conditioning, extinction, and spontaneous recovery procedures. Due to time constraints, fear conditioning and reinstatement are to be analyzed in the coming months.

**Results**

Freezing percentage during the CS of the pre-conditioning freezing test was found to not differ significantly between the groups by the overall assessment of a one-way ANOVA ($F_{(2,20)}$ = 3.020, *n.s.*). Percent freezing during the CS was considered approaching significance ($p$ = 0.071),

however, so an LSD Post-Hoc test was run to look for statistical significant between particular groups. The outcome of this test showed that the control group did freeze significantly less than the late exercise group ($p = 0.037$) and that it was approaching a significant difference from the early exercise group ($p = 0.053$). The data is represented in Figure 2 on both an overall mean distribution as well as over time.

**Figure 2: Freezing Behavior During CS Test Prior to Fear Conditioning**



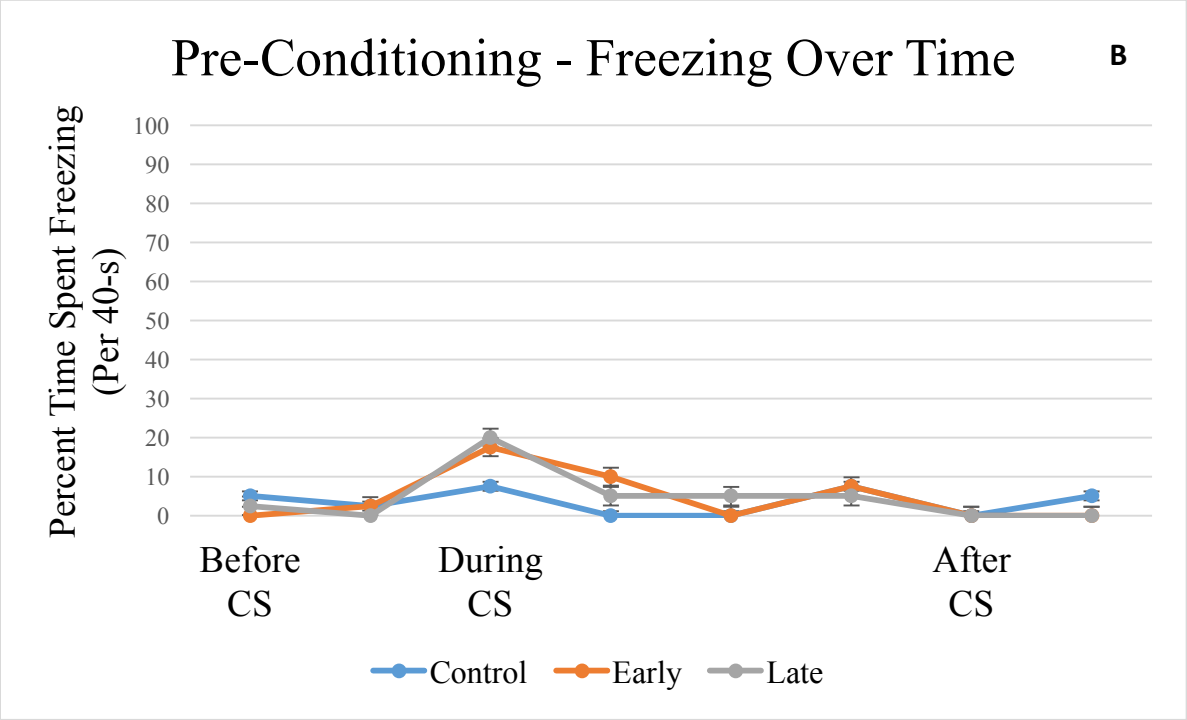



Figure 2: (A) Percent time freezing was compiled over the entire segment before, during, and after the white noise CS. The bar graph shows the means of each segment within each experimental group. The only statistically significant difference was found between the control group and the late exercise group, the former of which froze significantly less than the latter ($p = 0.037$). (B) The data was grouped into 40 second bins and is shown in a line graph over the three phases of the pre-conditioning freezing test. The highest percentage of freezing in seen in all groups to be right at the beginning of the CS.

Figure 3 shows the mean percent freezing before, during, and after the presentation of the CS. There were no statistical significant differences found in the mean freezing data during the CS between any of the groups ($F_{(2,20)} = 0.685$, *n.s.*). Freezing percentages were at a maximum at the beginning of the CS but remained relatively high throughout the rest of the CS. Low accuracy in the data can be seen from the wide standard error bars in the bar graph in Figure 3A. Accuracy is shown to be improved upon grouping the data into 40 second bins as shown in Figure 3B.

**Figure 3: Freezing Behavior During CS Test After Fear Conditioning**

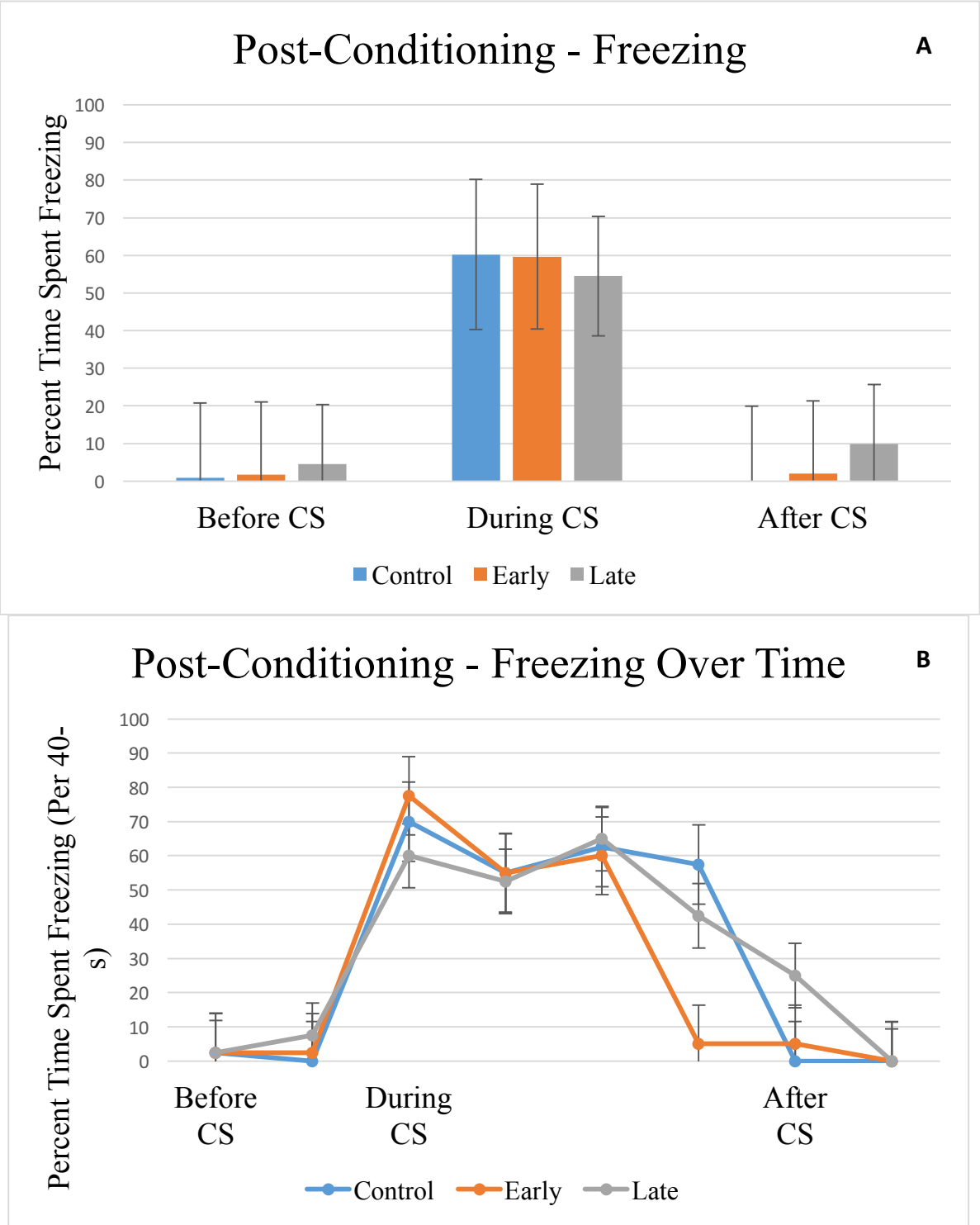
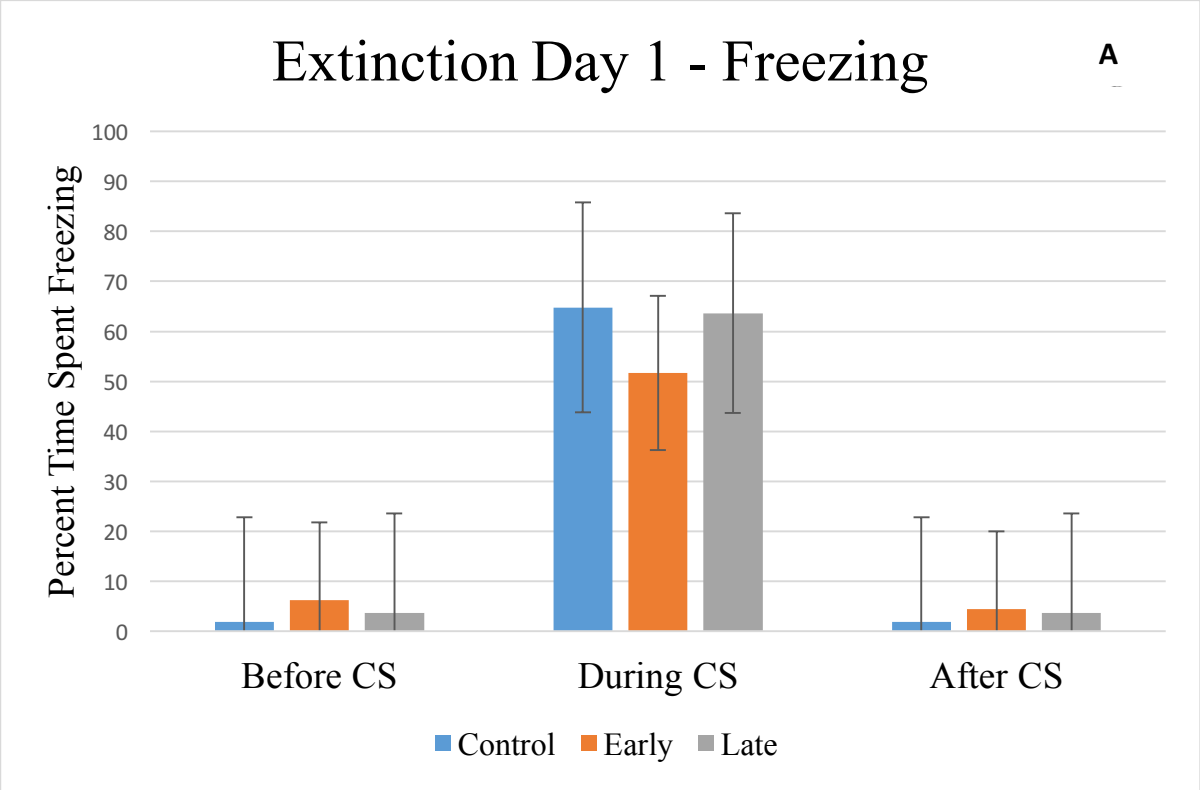
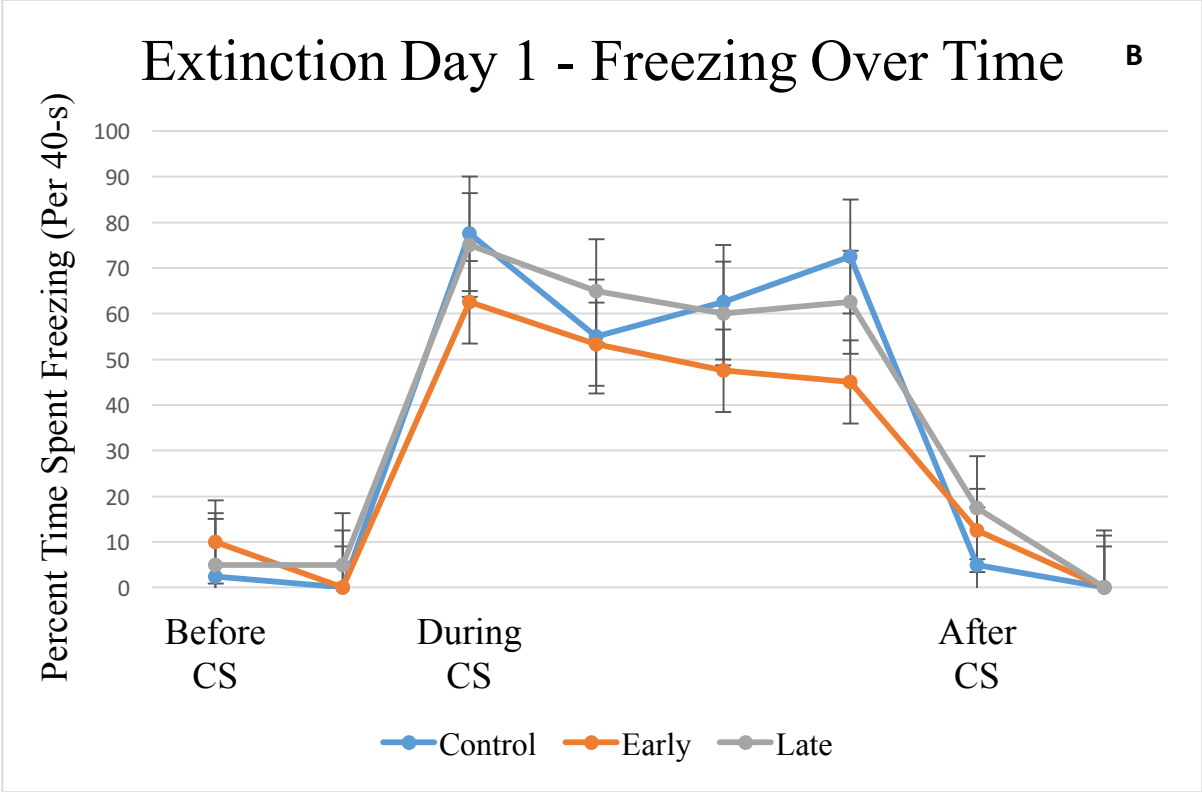


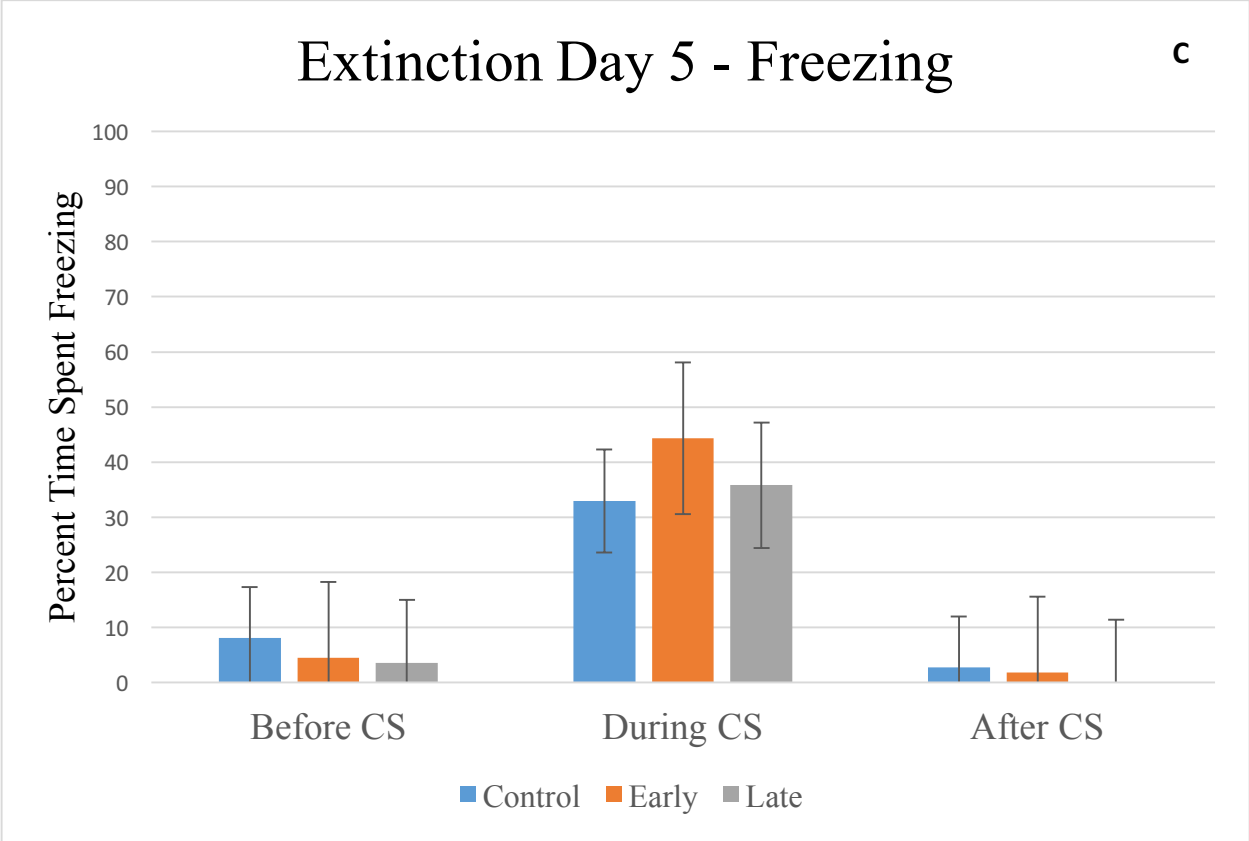

Figure 3: (A) Mean percentages of each group are represented in a bar graph. No statistical differences were found between the mean percentages of each group during the CS ($F_{(2,20)} = 0.685$, *n.s.*). The standard error bars illustrate low accuracy of the data when it was compiled over time in this way. (B) The post-conditioning freezing data were spread out over time in 40-second bins. The smaller standard error bars indicate higher accuracy in the data when it is distributed this way. Freezing is at a maximum early in the CS for all groups but remains high throughout the CS.

The data from the first and last days of extinction training are displayed in Figure 4. Differences between group mean percentages were not statistically significant during the CS for either the first day ($F_{(2,21)} = 1.618$, *n.s.*) or the last day ($F_{(2,21)} = 1.248$, *n.s.*). Standard error was higher when means were grouped over the entire section and lower when the data was spread out into 40-second bins. Freezing percentage was consistently high during the CS on the first day of extinction but showed a clear maximum in the beginning of the CS followed by a steep decline throughout the rest of the CS in all groups on the last day of extinction.

**Figure 4: Freezing Behavior During Extinction Training**




Extinction Day 1 - Freezing Over Time
B
Percent Time Spent Freezing (Per 40-s)
100
90
80
70
60
50
40
30
20
10
0
Before CS
During CS
After CS
Control
Early
Late


Extinction Day 5 - Freezing
C
Percent Time Spent Freezing
100
90
80
70
60
50
40
30
20
10
0
Before CS
During CS
After CS
Control
Early
Late

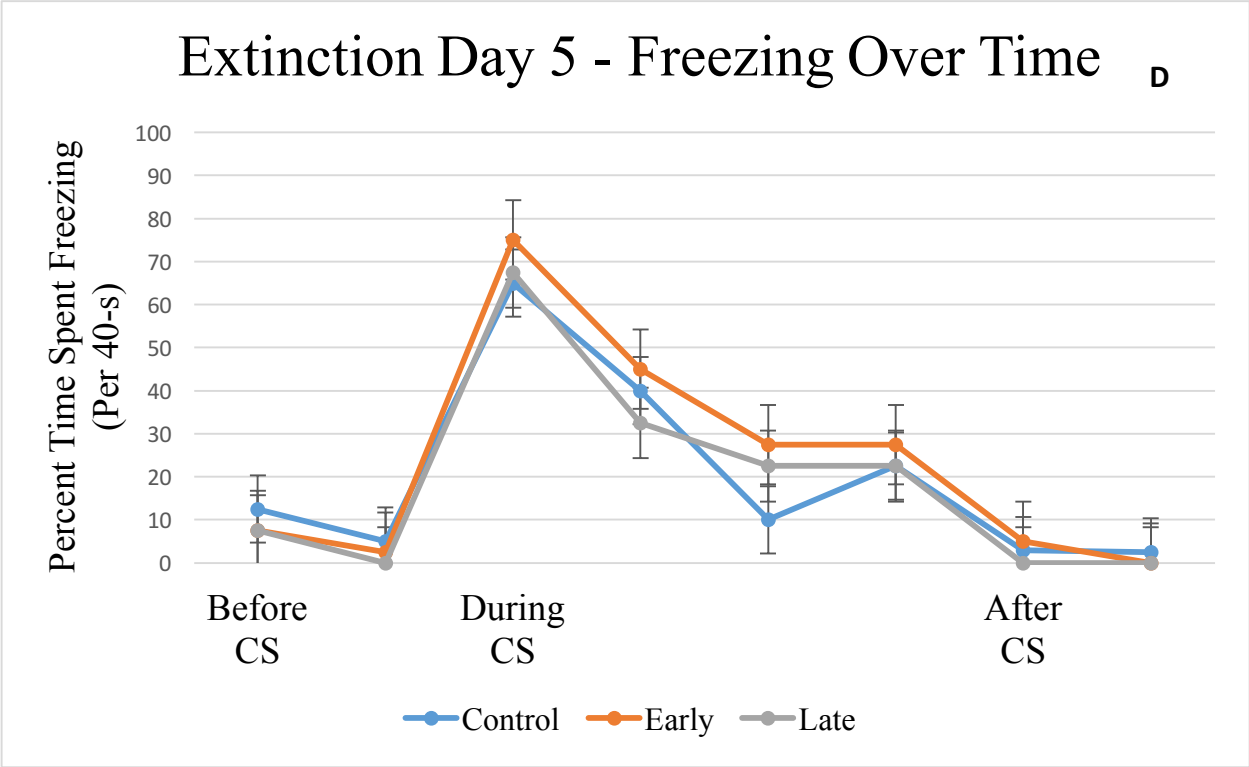



Figure 4: (A) Mean percent time spent freezing is shown before, during, and after the CS on the first day of extinction training. The early exercise group appeared to spend less time freezing than the other groups, but the differences were not statistically significant ($F_{(2,21)} = 1.618$, *n.s.*). (B) The mean percentages remained consistently high for all groups over the course of the CS, as grouped into 40 second bins. Standard error was reduced with this grouping relative to the bar graph in Figure 4A. (C) No statistically significant differences were found between the mean percentages during the CS on the last day of extinction when the means were compiled over the entirety of each segment ($F_{(2,21)} = 1.248$, *n.s.*). (D) The mean percent time freezing data were grouped into 40 second bins throughout the trial. The highest percent time freezing was found at the start of the CS and it then quickly declined throughout the rest of the CS in all groups.

The data collected from the spontaneous recovery test on the second to last day of the experiment is shown in Figure 5. A one-way ANOVA found statistical significance between the three groups ($F_{(2,21)} = 5.535$, $p = 0.012$). A follow-up LSD Post-Hoc test revealed that the late exercise group froze for significantly less time than both the control group ($p = 0.010$) and the early exercise group ($p = 0.008$).

**Figure 5: Freezing Behavior During Spontaneous Recovery**

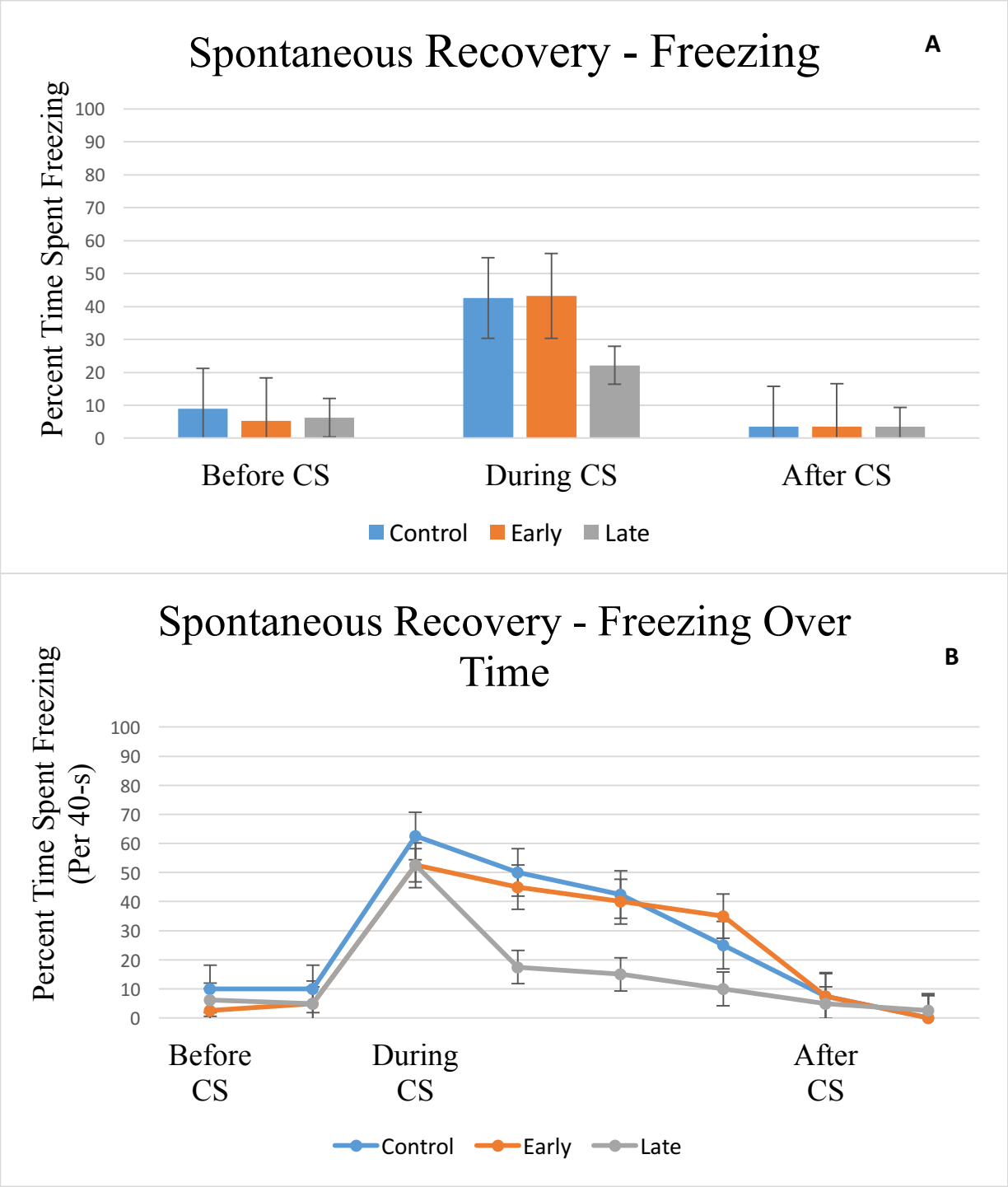



Figure 5: (A) Group means for percent time spent freezing are displayed. The late group froze for a significantly lower percent of the time than the other two groups during the CS ($F_{(2,21)} = 5.535$, $p = 0.012$). (B) The percent time freezing data was grouped into 40 second bins and illustrated on a line graph over the entire spontaneous recovery trial. All groups froze for a maximum percent of the time at the onset of the CS and then declined in freezing behavior throughout the rest of the CS. The late exercise group had a faster decline in percent time freezing than did the control and early exercise groups.

**Discussion**

The baseline freezing test performed prior to fear conditioning showed low scores of freezing as expected. The mice had yet to learn that the white noise CS would be followed by a footshock, and so they had no reason to be afraid other than that from the new context of the conditioning chambers and the surprise of the noise being turned on. A statistical significance was found in that the control group froze less than the other groups, however since all groups had been under the same conditions at this point, this difference could only be due to chance. The freezing test that took place the day after fear conditioning showed very different trends. All of the group freezing means were much higher during the white noise CS in this post-conditioning freezing test, demonstrating that the mice had learned that a footshock, the US, follows the CS. This freezing behavior in response to the CS can be deemed the conditioned response (CR) since the white noise on its own does not produce this response, as shown in the pre-conditioning data, but that it does once it has become paired with the unpleasant US. We can interpret this data to show that the mice formed a memory in which the CS predicts the US, and as such they responded to the CS with the CR of freezing.

Through extinction training we then tried to present the CS enough times alone that the mice would form a new memory in which the CS did not predict the US. The first day of extinction produced trends similar to that of the post-conditioning freezing test, with high percent freezing means during the CS. These high freezing rates were expected since the CS hadn't been presented enough times to form a new memory and therefore the CS-US association memory was still strong enough to produce the CR. At this point, the control group and the late exercise groups hadn't done any exercise, but the early exercise group had had two weeks with an unlocked wheel. Though not statistically significant, the early exercise group had an overall

lower mean percent freezing score during the CS than the other groups. This was unexpected since the idea of exercise modulating memory consolidation would lead to the early exercise group having a stronger memory of the CS-US association formed during fear conditioning which should result in more time spent freezing. On the 5$^{th}$ day of extinction, mean percent time spent freezing switched its trend such that the early exercise group froze for a higher average percentage of time during the CS than the other groups, though this difference was still not statistically significant. These results are difficult to interpret and to apply meaning to due to the fact that the cohorts were very small. We cannot say for sure if this would be the trend seen in a larger cohort or if it turned out this way due to chance; therefore, it would be important to repeat the experiment with a larger number of subjects in order to make more certain assumptions about the behavioral responses during extinction. We can, however, note the difference in the appearance of the timeline of the behavioral response during the 5$^{th}$ day of extinction when compared to the 1$^{st}$ day of extinction. Freezing during the CS was consistently high on day 1, whereas on day 5 it was high at the start of the CS but rapidly declined throughout the rest of the CS. This trend can be interpreted to show that a new memory had formed in which the CS did not predict the US, and so would result in less of a CR, but that the memory formed earlier in which the CS did predict the US was still strong enough to overcome the new memory, at least in the beginning of the CS.

Upon testing spontaneous recovery, the control group hadn't had any exercise, the early exercise group had four weeks and five days of exercise, and the late exercise group had two weeks of exercise beginning after extinction. The trends shown in Figure 5 and the accompanying statistics support our hypothesis that the late exercise group would freeze for less time than the other two groups. By waiting to begin exercise until after the completion of

extinction training, we were able to influence the consolidation of the memory formed during extinction in which the CS does not predict the US. The new memory trace was made stronger by chronic exercise implemented during its consolidation window. Figure 5A shows this result as a mean over the entire duration of the CS, but from Figure 5B it can be seen that the result is not so straightforward. All three groups have a maximum percent time freezing score at the onset of the CS that is quite similar. The differences between the groups are more specifically seen throughout the rest of the CS, in which all values decline, but those of the late exercise group decline faster than those of the other two groups. This suggests that the initial memory in which the CS predicts the US was stronger than the new memory at the onset of the CS, but that as the CS went on, the new memory was better able to compete with and suppress the old one. Exercise initiated after extinction was able to make a significant difference in the strength of the new memory over time, but the CR was still present early on. The standard error bars within the figures illustrate higher accuracy in the results when they are grouped temporally, supporting this interpretation.

The results of this experiment were limited by the use of an unblinded, inexperienced, observer. The experiment was planned initially with the intent of using fear-potentiated startle as a measure of fear but technological difficulties caused the procedure to be reworked using freezing. A computerized scoring system was unavailable for freezing and so hand-scoring of the videos was the only option. Additionally, the quality of the view of the animals in the videos varied depending on where the mouse chose to be within its chamber. Of the four conditioning chambers within view of the camera, the two closest to the ground each had a corner in which a mouse could be hidden from view by the metal side of the chamber. There were a few cases in which mice were hidden and as such a best guess had to be made for whether or not there was

movement. In the top two chambers, if mice were facing the back of the chamber, it could be difficult to see if their heads were moving. Head movement was often the one indication of a lack of freezing, as whenever the CS was present in trials after fear conditioning, the mice would either be moving in very quick bursts, standing still with some head movement, or still with no movement (freezing). Differentiating between the latter two was sometimes difficult depending on where the mouse was located and its orientation. The front clear acrylic wall of the chambers also caught the light and produced shadows that further obscured the clarity of the animal. It will be important to have a blinded and more experienced observer score the videos in order to confirm the data presented as well as to better position the camera if the experiment is to be repeated.

Our results support the work showing increased fear responses to only the CS after pairing with a feared US in a fear conditioning paradigm (Pavlov, 1927). In contrast to results reported by Falls et al. (2010), we did not see enhanced fear responses in the early exercise group compared to the other two groups when measured on the first day of extinction. By the fifth day of extinction, however, our early exercise group was producing higher fear responses when compared with the other groups, which does support the findings by Falls et al. (2010) showing that exercise directly following fear conditioning strengthens the memory association between the CS and the fearful US. This difference was not statistically significant but rather can only be seen observationally when looking at the results grouped into a single mean. These inconsistencies show that repetition of this study will be important in order to make more confident conclusions. In accordance with results reported by Greenwood, et al., (2009) in rats, chronic exercise implemented directly after fear conditioning did not reduce fear return during spontaneous recovery.

One problem with the model used in most fear conditioning studies in rodents is that it is thought to not adequately reflect PTSD if only a few shocks or only shorter, milder shocks are given during conditioning. It may not be able to cause as significant of a fear response as that induced by more severe traumatic human experiences. Norrholm et al (2011) found that more severe stressor exposure can impair extinction of the fear memories significantly more. This result is more consistent with how PTSD manifests in humans (Norrholm et al., 2011). Falls et al. (2010) used 5, 0.25-s 0.4-mA footshocks in mice and Greenwood et al. (2009) used 3, 2-s 0.8-mA footshocks in rats during fear conditioning. Our study used the same number and intensity of shocks as Falls et al. (2010) and the same longer duration shock (2-s) as Greenwood et al. (2009) in mice, and so therefore employed a more severe stressor. It could be because of this that we still saw high levels of freezing at the onset of the CS on the last day of extinction. Five days of extinction was not enough for the memory separating the US from the CS to become stronger than the memory associating the two for the severity of the US that was used.

Upon repetition of this study, it would be important to extend extinction training to the point at which the response to the CS more closely resembles that of the pre-conditioning data. Since the response was not reduced to this level by the end of extinction, it makes it difficult to compare with spontaneous recovery. Though there was an effect of chronic exercise initiated after extinction training on the return of fear in spontaneous recovery, the effect had a clear temporal element. Exercise following extinction strengthened the new memory overall, but not enough to keep the CR from recurring completely. Perhaps if extinction had continued for another few days, the new memory would have become strong enough on its own for exercise to have more of an impact on reducing the CR at the onset of the CS during spontaneous recovery. Clinically, the current model would translate to a much shorter period of exposure therapy than is

actually implemented. If we extend extinction training to better represent the timeline of exposure therapy in humans (usually around one 90-minute session per week for about 10 weeks), and still initiate chronic exercise at the completion of the training, we may see a more significant difference in the strength of the new memory that could better translate to application in humans (Kip et al., 2013). Within the context of research on treatments for post-traumatic stress disorder, these results show promise in the use of chronic exercise when implemented at the completion of exposure-based therapy to reduce the magnitude of the return of fear over time.

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