# Effects of Chronic Variable Stress Across Developmental Stages in Mice

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# EFFECTS OF CHRONIC VARIABLE STRESS ACROSS DEVELOPMENTAL STAGES IN MICE

by

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ABSTRACT
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Posttraumatic Stress Disorder (PTSD) is a response to trauma exposure that involves a number of symptoms that can be highly impairing to affected individuals. Only a subset of those exposed to traumatic events will develop the disorder, which is conceptualized as developing via conditional fear. Research into factors predisposing for PTSD is needed. Furthermore, little work has been done to investigate predisposing factors in children more specifically. This research tests the effects of stress exposure on subsequent fear learning, across developmental stages in mice, as a model for PTSD. Juvenile and adult male mice were exposed to chronic variable stress (CVS) for a period of 7d and their behavior was examined immediately thereafter. Both juvenile and adult mice exposed to CVS showed exaggerated anxiety behavior, as indicated by decreased exploratory behavior on the elevated plus-maze. While adult mice exposed to CVS displayed enhancements in long-term context fear learning, juvenile mice failed to display this pattern. Findings suggest differences in stress effects across developmental stages and provide further evidence supporting dissociation of the anxiety and fear pathways in the rodent brain. While PTSD does occur in childhood, onset is more common in adulthood, which may be reflective of differential developmental schedules in the fear and anxiety pathways.

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## Introduction

Over 700,000 children in the United States are victims of maltreatment each year (U.S. Department of Health & Human Services, 2008), and evidence suggests that chronic stress and exposure to traumatic events in early life predispose individuals to the subsequent development of mental disorders. Childhood adversity has been linked to the formation of a variety of psychiatric illnesses in a multitude of investigations. Molnar, Buka and Kessler (2001), for example, have demonstrated a link between childhood sexual abuse and increased rates of depression, substance use and Posttraumatic Stress Disorder (PTSD) in adulthood. Similarly, MacMillan and colleagues (2001) showed that childhood physical abuse was related to increased rates of both depression and anxiety disorders. Such findings are quite common in the literature (e.g., McCauley et al., 1997; Mullen, Martin, Anderson, Romans, & Herbison 1996; Young, Abelson, Curtis, & Nesse 1997) and support the hypothesis that disruptions to normal childhood development can have long lasting effects on mental health.

Research has shown that exposure to childhood maltreatment predisposes victims to the development of PTSD during adulthood (e.g., Molnar et al., 2001; Bremner, Southwick, Johnson, Yehuda, & Charney, 1993; Zaidi & Foy, 1993). The lifetime prevalence rate of PTSD in adults living in the United States is estimated at nearly 8% of the general population (American Psychiatric Association, 2000). PTSD is an anxiety disorder that may develop following exposure to a strongly traumatic event, and is characterized by persistent psychiatric symptoms including re-experiencing of the trauma, avoidance of stimuli associated with the event, and increased arousal (American

Psychiatric Association, 2000). Recent research suggests that anxiety disorders such as PTSD may be expressions of dysfunctions in the stress response (Risbrough & Stein, 2006), which while essential in responding to acute challenges, can become problematic when activated for extended periods of time (Campbell, Lin, DeVries & Lambert, 2003). Factors distinguishing individuals susceptible to development of the disorder following exposure are currently under investigation, and one hypothesis posits that inflated conditional fear underlies the formation of PTSD in these individuals. This theory proposes that the traumatic event (an unconditional stimulus, US) generates an unconditional response (UR), characterized by arousal and fear, and also is associated with the contextual and other cues (conditional stimuli, CS) present during the traumatic event. Subsequently, this theory hypothesizes that cues similar to the CS can trigger a response similar to the UR, the conditional response (CR), even in the absence of any US. According to this framework, individual susceptibility may be marked by: 1) more intense generalized fear reactions to cues mirroring the CS, 2) stronger CS-US associations, and 3) difficulty dissociating the CS and US even after repeated cue exposure in the absence of additional trauma. Investigations aimed at understanding how individuals develop the inflated fear conditioning responses that have predisposed them to PTSD development upon trauma exposure are many, and current findings in animals suggest that fear learning is significantly enhanced by previous exposure to stress (Cordero, Venero, Nyika, Kruyt, & Sandi, 2003; Rau, DeCola, & Fanselow, 2005). These findings, in conjunction with data indicating that human subjects exposed to previous traumatic events are predisposed to the development of PTSD following trauma exposure, suggest that former life stress may predispose individuals to the later

development of psychopathology, perhaps by sensitizing those individuals to fear learning.

Developmental stage is one factor that may mediate the effects of stress exposure on fear learning and thus the predisposition to PTSD development. Responses of children and adults to traumatic events or life stressors can vary greatly due to fundamental differences in cognitive and emotional development (Salmon & Bryant, 2002). Both encoding of aversive events and subsequent resolution of the stressful incident can be challenging for children (Salmon & Bryant, 2002). These difficulties suggest that stress exposure may be more distressing for children than adults, and we therefore hypothesized that the effects of stress on subsequent fear learning would be more extreme in pre-pubescent mice exposed to stress than adult mice exposed to similar experiences.

Investigations aimed at understanding the relationship between childhood adversity and psychopathology in the human population are limited in that they are unable to accurately control for consistency across subject environments. Furthermore, they often rely on self-reports for information concerning maltreatment history, which can be inaccurate and thereby decrease result validity. In the specific context of better understanding the effects of chronic stress on subsequent trauma exposure, a true experimental design in human subjects would be highly unethical. Therefore, animals present an ideal solution to an otherwise impossible investigation. The advantages of using an animal model include the possibility of basic behavioral experiments but also, and more importantly, the eventual possibility of exploring the physiological mechanisms of PTSD susceptibility.

Animal models of chronic life stress date back approximately thirty years to Katz and colleagues (1981), who first presented the Chronic Variable Stress (CVS) procedure in an attempt at simulating chronic, unpredictable life stress. The CVS procedure originally involved exposure to a variety of stressors such as footshock, changes in housing conditions, and forced swim, over a period of two to three weeks (Katz et al., 1981). A modified version of this procedure was used here. The CVS paradigm lasted 7 days, a period of time that has been shown in previous research to effectively increase anxiety levels and disrupt fear learning in a variety of adult animals (Zurita, Martijena, Cuadra, Brandao, & Molina, 2000; Tauchi, Zhang, D'Alessio, Seeley, & Herman, 2008; Sanders, Stevens, & Boeh, 2010). The specific stressors employed (swim, restraint, cold, vibration, isolation, crowding, and noise) are also commonly used in the literature (e.g., Zurita et al., 2000; Lepsch et al., 2005; Cullinan, Kcrmarik, Pokorney, & Gloss, 2005; Sanders et al., 2010).

To first confirm the stress effects of our CVS procedures and investigate any differences in sensitivity levels of juvenile and adult mice to stress exposure, both age groups were tested for anxiety on the elevated plus-maze following the CVS procedures. Open arm avoidance on the elevated plus-maze is suppressed by anxiolytic drugs, and exacerbated by anxiogenic drugs, and the paradigm has become a well-validated animal model of clinical anxiety (Pellow, Chopin, File, & Briley, 1985).
In the animal research setting, Pavlovian conditioning has become an accepted representative model of clinical fear, and human research thus far has replicated findings from existing animal models (Phelps & LeDoux, 2005; Delgado, Olsson, & Phelps, 2006). As detailed above, fear conditioning models of PTSD hypothesize a

fundamental role for fear learning in its development. Investigations aimed at understanding the ways in which chronic stress may render individuals more sensitive to the formation of subsequent psychopathology may thus accurately do so by examining the direct effects of stress on fear conditioning.

While previous research examining the effects of CVS on anxiety and fear learning in adult rats has revealed increases in anxious behaviors and sensitized fear conditioning (Zurita et al., 2000; ), only one study has investigated the effects of our CVS procedure on fear learning in adult mice (Sanders et al., 2010). This research examined the effects of the 7d CVS procedure in adult male and female mice, and provided evidence that these stress procedures caused significant changes to fear conditioning responses. While fear learning results varied by gender, this study provided preliminary evidence suggesting that the 7d CVS paradigm to be used in the present study successfully elicits a stress response in adult mice and alters subsequent fear learning behaviors (Sanders et al., 2010). For ease of experimentation, the current investigation aimed to investigate a homogeneous sample, eliminating variability in findings due to gender, therefore all subjects employed were male.

The present study aimed first to confirm the stress effects of our 7d CVS procedures on fear conditioning in adult male mice. Furthermore, this research hoped to extend the model for use in juvenile male mice. Most importantly, this investigation attempted to demonstrate the critical role of developmental stage in the enhancing effects of CVS on fear learning.

Chronic stress experienced during childhood has been linked to an increased risk of psychiatric illness, specifically to PTSD, during adulthood in a number of studies (e.g.,

Molnar et al., 2001; MacMillan et al., 2001). Very little work, however, has explored prior life stress as a risk factor for PTSD following a traumatic event experienced during the pre-pubescent years, and within that limited area of research, none has explored the mechanisms by which this association forms. This study proposed to fill that gap in the literature by examining the effects of chronic juvenile stress on subsequent childhood fear learning behaviors, in an attempt to better understand how childhood stress may increase childhood susceptibility to PTSD development. With regard to stress effects on fear learning we hypothesized that both juvenile and adult mice exposed to chronic stress would exhibit enhanced fear acquisition, which presumably suggests an increased susceptibility to PTSD. Furthermore, due to our hypothesis that juveniles would experience more distress in response to stress than adults, we also hypothesized that juveniles would exhibit greater stress-enhancement of fear learning than adults. This finding would suggest that chronic stress exposure increases PTSD susceptibility to subsequent trauma experienced within the same developmental period more profoundly in childhood than in adulthood.

Findings from this research could shed light on the importance of developmental stage in mediating the effects of stress exposure on risk of later psychopathology. The expected findings, that juvenile mice would display more anxiety and enhanced fear learning following stressor exposure, would provide the field with additional evidence that stress is not experienced uniformly over different developmental periods. Implications for the field could include increased awareness regarding the importance of preventing child maltreatment, and should our findings reveal stress effects on fear learning, we may have evidence that supports commencing psychological interventions

for maltreated children directly following exposure to chronic stress despite the absence of any immediate psychopathology. These interventions should be focused on reducing fear learning sensitization and could take place via behavioral or pharmacological means.

## Methods

### Subjects

16 juvenile (23d) and 16 adult (58d) male mice served as subjects. C57B1/6 strain mice were purchased from Charles-River (Portage, MI). Animals were housed in boxes of four in the Marquette University Vivarium with free access to food and water, under a 12:12h light: dark cycle (lights on 7:00 am). All experimental procedures occurred during the light portion of the cycle. Procedures were conducted under protocol AR237, approved by the Marquette University IACUC and in accordance with the U.S. Public Health Service "Policy on Humane Care and Use of Laboratory Animals."

One mouse expired prior to the beginning of procedures. The remaining subjects were split into four groups: Stressed Juveniles (n=8), Control Juveniles (n=8), Stressed Adults (n=8), and Control Adults (n=7).

### Procedures

Mice in the Stressed Juvenile (n=8) and Stressed Adult (n=8) groups were subjected to the Chronic Variable Stress (CVS) procedure for a period of 7d. Two stressors from a total battery of seven (Table 1) were applied each day in a semi-randomized fashion, one in the a.m. and one in the p.m., such that each stressor was presented twice over the course of the 7d period. Two of the stressors were applied overnight, from the afternoon of the designated day until the following morning. Animals were tailmarked with a

Sharpie marker prior to the a.m. stressor exposure each day, and stressor exposure required transportation to a separate laboratory room. During the 7d period, Control Juveniles and Control Adults remained in their homecages and were exposed to the same tailmarking and transportation schedules as the experimental animals.

Table 1

*Chronic Variable Stress Procedure Stressors*

| | |
|---|---|
| Cold | Placed in cold room at 4°C for 30 min. |
| Vibratio | Cage placed on shaker for 30 min. |
| Swim | Placement in room-temperature water for 5 min. |
| Isolation | Each animal placed in separate cage overnight. |
| Crowdin | Two homecages of animals placed in single cage overnight. |
| Restraint | Placement in wire mesh restrainers for 30 min. |
| Noise | Placement in bucket 40 cm below ultrasound emitter for 10 min |

All subsequent testing was conducted in rooms separate from that in which stress procedures were administered.

On d8, all mice were tailmarked and transported to the laboratory where they were tested on an elevated plus-maze. The elevated plus-maze consisted of a plus-shaped maze with two opposing arms (open and closed), raised off the floor. Each mouse was placed at the center of the maze facing an open arm and was allowed to explore the maze for a period of 5 min.

On d9, all mice were tailmarked, transported to the laboratory and trained in a Pavlovian fear conditioning procedure. Animals were allowed 2 min of exploration

before the presentation of any stimuli. After 2 min, they were exposed to three tone-shock pairings. Each tone was approximately 28s in duration, 2800 Hz in frequency, and 85dB in intensity. Shocks were administered at 0.7mA for a duration of 2s. The conditioning procedure timeline is detailed in Figure 1.

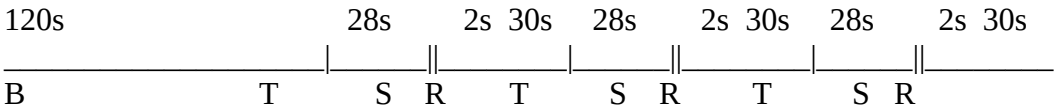


*Figure 1*. Fear conditioning procedure timeline. Mice were exposed to a 120s stimulus-free baseline period (B). A 28s tone was then administered (T), followed by a 2s shock (S) and a 30s rest period (R). The tone-shock-rest cycle was then repeated twice more.

On d10, following tailmarking and transportation, a context fear test was conducted on all mice. Animals were placed in the conditioning chambers but were not exposed to any stimuli for a period of 5 min.

On d11, all mice underwent a tone test after tailmarking and transportation. Mice were placed in novel chambers distinct from those used during training, to avoid contamination of tone fear by context fear. After a period of 2 min, they were exposed to a 3 min tone identical to that used during conditioning.

Detailed information on the timeline of experimental procedures during the life stages of each group of mice is presented in Table 2.

Table 2

*Procedural Timeline*

| Procedure | Ages of Juvenile Groups | Ages of Adult Groups |
|---|---|---|
| CVS or Homecage | 23d-30d | 58d-65d |
| Elevated plus-maze | 31d | 66d |
| Fear conditioning | 32d | 67d |
| Context fear testing | 33d | 68d |
| Tone fear testing | 34d | 69d |

All maze, fear conditioning and fear testing procedures were video-recorded. Fear conditioning and testing procedures were double scored by researchers blind to group membership. Maze procedures were scored by a single researcher also blind to group membership. Video records were digitized at 1Hz. During the elevated plus-maze testing (d8) time spent on the open arms in seconds, was measured as an index of anxiety for each mouse. During Pavlovian fear conditioning (d9), as a measure of general activity level the cage was bisected and the number of cage crossovers was assessed as the number of times the mouse's whole body (excepting the tail), crossed the midline during the 2 min baseline period.

Measurements of short-term fear learning were gathered during the final tone and final rest period of the fear conditioning session (short-term tone fear and short-term context fear, respectively; d9). Long-term fear learning was assessed during the long-term context and tone tests (d10, d11). All fear learning was calculated as percent of

time spent freezing. Experimenters counted the number of samples, per minute, in which each animal made any movement and freezing was quantified as the percentage of samples in which no movement was detected. Due to the impossibility of directly measuring 'fear,' freezing behavior was used as a quantitative measure of the construct of learned fear. The use of freezing as a measure of learned fear is common in the literature and is thought to represent defensive responding to threatening stimuli.

**Apparatus**

The Vibration condition of the CVS procedures was completed with a Dubnoff metabolic shaking incubator (GCA Precision Scientific, Chicago, IL). The Noise condition was conducted with a Pest Chaser Ultrasonic Repeller (Lititz, PA).

The elevated plus-maze consisted of a plus-shaped maze made of urethane-sealed wood with one open arm, and one closed, raised 50 cm off the floor. Testing was completed with minimal illumination provided by a nightlight located approximately 4 feet from the base of the maze. If a mouse fell off one of the open arms, it was quickly picked up and returned to the center of the maze. The maze was cleaned with alcohol, and thoroughly dried after each mouse was tested.

Pavlovian fear conditioning and long-term context testing were performed in the same context. This context consisted of four identical chambers (30cm x 24cm x 21cm; Med Associates Inc., St. Albans, VT). The ceilings and back walls of the chambers were constructed of opaque white plastic, and the sides of aluminum. One sidewall of each chamber held a speaker through which the tone stimuli were delivered. The door was constructed of clear polycarbonate plastic, and the chamber floors were constructed of removable grids and waste pans. Grid floors were composed of 36 stainless steel rods

(3mm diameter, spaced 8mm apart center to center), and made contact with a circuit board though which shocks were delivered. Prior to each session chambers were cleaned with a 1% acetic acid solution and dried thoroughly. A thin film of solution was placed in the waste pan of each chamber before the session. The conditioning room was lit with 8 overhead 100-W incandescent bulbs lit for each session, and background white noise (60dB) was administered by a standard HEPA air filter during each session. Background noise and tone stimuli were calibrated with a RadioShack dB meter (A Scale) prior to testing. Additionally, shock intensity was confirmed with a storage oscilloscope (B&K Precision Corporation, Yorba Linda, CA) and a 10KΩ resistor in each testing chamber prior to each conditioning session.

Long-term tone testing was performed in a distinct context. This context consisted of four identical chambers with the same outer dimensions and construction as the conditioning chambers, but with opaque white plastic floors in place of grids and waste pans. Lighting in this context was dim (8 x 40 W incandescent bulbs overhead). The inside spaces of the chambers were rendered hemi-cylindrical by addition of a flexible white plastic insert to each chamber. Chambers in this context were cleaned with a 10% Simple Green solution (Huntington Harbour, CA) before each session. Background noise was set at 50dB and was provided by a standard HEPA air filter. Background noise, tone stimuli and shock intensity were confirmed following the above procedures for training and context testing.

For all fear conditioning and testing sessions, stimuli were controlled by a PC running MedAssociates software (MedAssociates Inc., St. Albans, VT).

## Results

Data analyzed for fear conditioning and testing measures represented the average scores obtained by the two raters. A series of 2x2 factorial ANOVAs were conducted (with age and stress as independent variables) for the following dependent measures: open-arm time in the elevated plus-maze, pre-training crossovers, short-term context and tone freezing, and long-term context freezing. The tone test results were analyzed using a mixed model ANOVA with stress treatment and age group as the between-groups factors, and temporal period (baseline or tone) as the repeated measure. All analyses were conducted in PASW 17.0 (PASW, 2009) and statistical significance was established at $p < .05$ for all tests.

**Elevated plus-maze open arm time.** A 2x2 independent groups factorial ANOVA was conducted to determine the effects of age and stress on time spent on the open arms of an elevated plus-maze (Figure 2). The main effect of stress was significant, $F(1, 27) = 12.47$, $p < .05$, observed power= 0.93, with animals in the control condition spending more time on open arms than those in the stressed condition. The main effect of age, and the interaction effect between age and stress were not significant (all $F < 1$). One control adult mouse fell off of the maze during testing; time was adjusted to reflect that lost during return of the mouse to the maze.

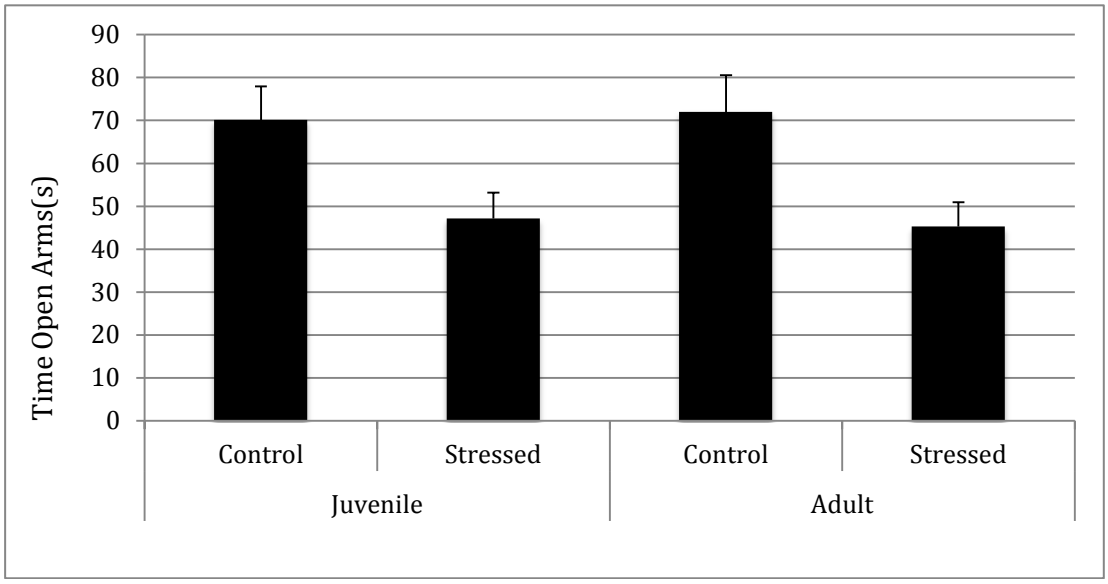


*Figure 2*. Behavioral observations during the elevated plus-maze session. Mean (+SEM) time spent on open arms. Values represent the time, in seconds, the mouse spent on the open arm of the maze. Stressed animals were significantly less exploratory than Control animals.

**Pre-training cage crossovers**. The effects of age and stress on mean number of cage crossovers completed during the baseline period preceding fear conditioning were also investigated with a 2x2 independent groups factorial ANOVA (Figure 3). A main effect of age was uncovered, $F$ (1, 27)= 5.69, $p$= 0.024, observed power= 0.63, wherein juvenile mice crossed the midline fewer times than adult mice. The main effect of stress and the interaction effect between age and stress failed to reach significance, all $F < 1$. Inter-rater reliability on this measure was high (Cronbach's alpha= 0.99, Intraclass correlation coefficient= 0.99).

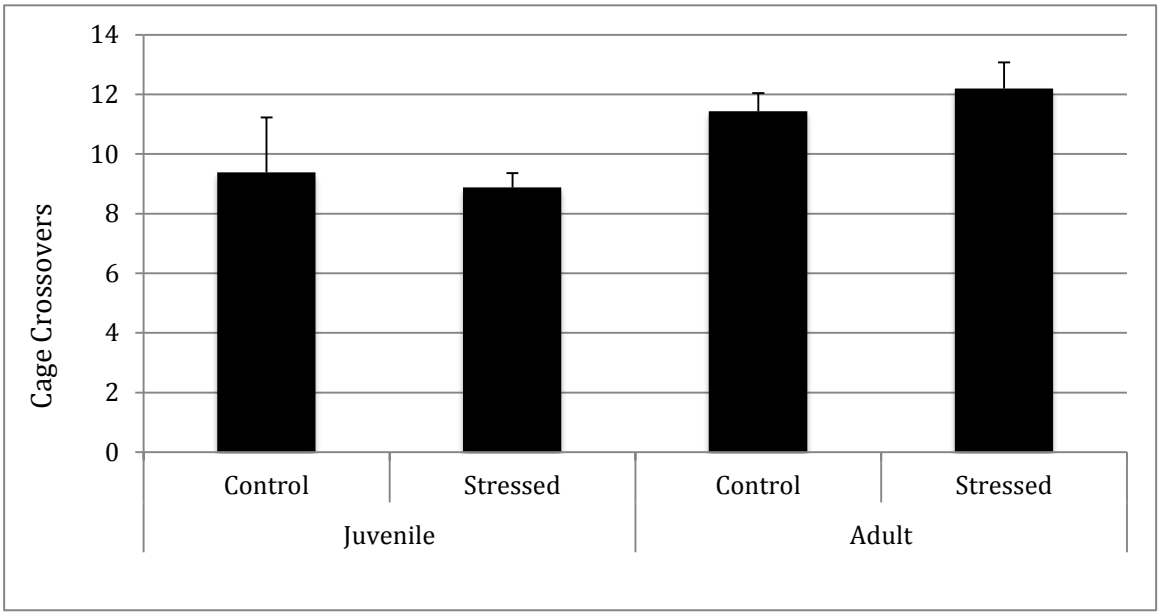


*Figure 3*. Behavioral observations during the training session. Mean (+SEM) number of cage crossovers during the first 2 min of the training session. Values indicate the number of times the mouse crossed the midline of the testing chamber. Adult mice were significantly more active than juvenile mice.

**Short-term tone fear freezing.** Short-term tone fear freezing was also explored with a 2x2 independent groups factorial ANOVA (Figure 4). The main effects of age and stress, and the interaction effect between the two variables were not significant, all $F <$ 1. Inter-rater reliability on this measure was high (Cronbach's alpha= 0.97, Intraclass correlation coefficient= 0.97).

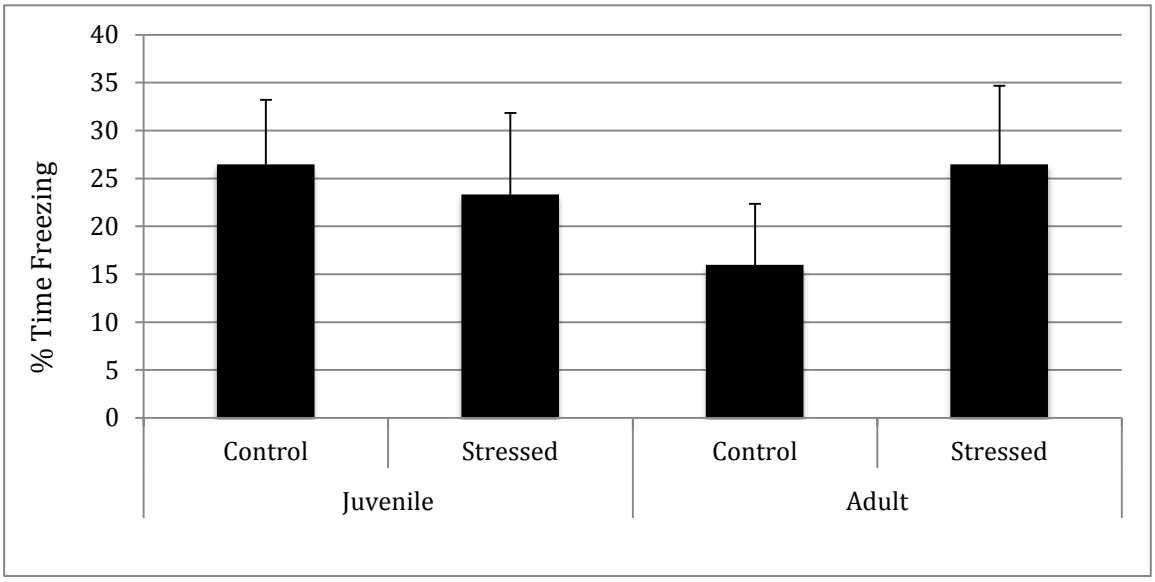


*Figure 4*. Behavioral observations during the training session. Mean (+SEM) freezing response during the final tone of the training session. Values represent the average percentage of video samples scored as devoid of movement.

**Short-term context fear freezing.** Effects of age and stress on short-term context fear freezing also were examined with a 2x2 independent groups factorial ANOVA (Figure 5). The main effect of age was significant, $F$ (1,27)= 8.5, $p$ = 0.007, observed power= 0.803, wherein adults displayed more freezing behavior than juveniles. The main effect of stress, and the interaction effect between the two variables were not significant, respectively, $F < 1$, $F$ (1, 27)= 2.48, $p$= 0.127. Inter-rater reliability on this measure was low (Cronbach's alpha= 0.46, Intraclass correlation coefficient= 0.46).

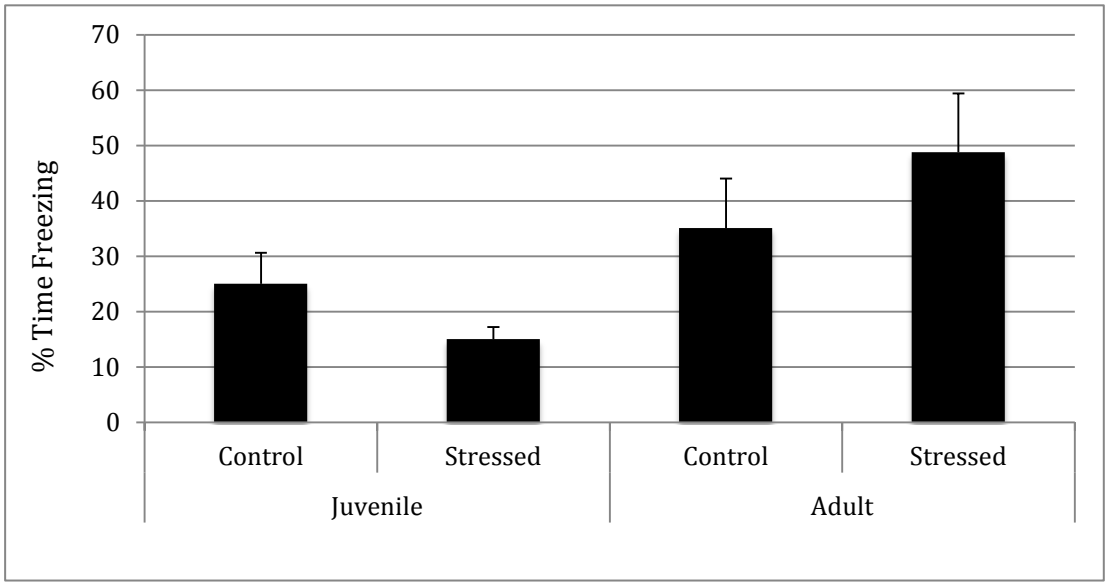


Figure 5. Behavioral observations during the training session. Mean (+SEM) freezing response during the final rest period (following the final shock) of the training session. Values represent the average percentage of video samples scored as devoid of movement. Adults displayed more freezing behavior than juveniles.

**Long-term context fear freezing.** Finally, a 2x2 independent groups factorial ANOVA was conducted to determine if age and stress affected long-term context fear freezing (Figure 6). The interaction effect of age and stress was significant, $F$ (1, 27)= 4.34, $p$ = 0.047, observed power= 0.52. Juveniles in the control condition exhibited more freezing than those in the stressed condition, while adults in the control condition exhibited less freezing behavior than those in the stressed condition. The main effects of age and stress were not significant, all $F < 1$. Additional independent groups t-tests examining differences between the stressed and control mice within each age group were conducted. Within the adult mice, results approached significance, $t$ (13)= 1.97, $p$ = 0.070. Within juvenile mice, results were not significant, $t < 1$. Inter-rater reliability on this measure was high (Cronbach's alpha= 0.99, Intraclass correlation coefficient= 0.99).

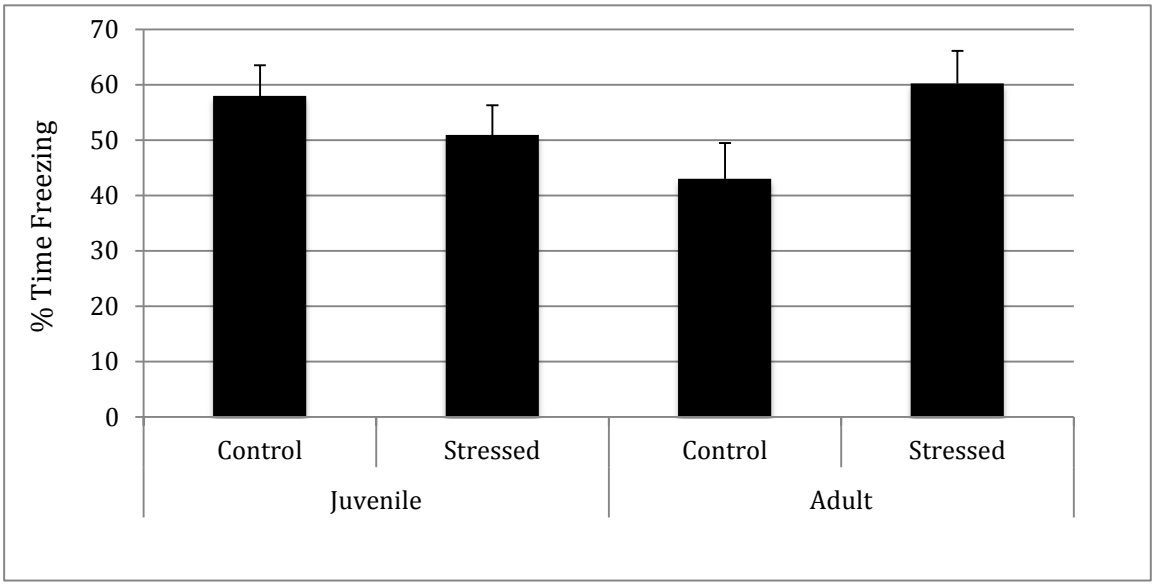


*Figure 6*. Behavioral observations during the context test. Mean (+SEM) freezing response during the context test, conducted 24h after training. Values represent the average percentage of video samples scored as devoid of movement. Juvenile controls displayed more freezing than stressed juveniles while adult controls displayed less freezing behavior than stressed adults. Differences between adult controls and stressed animals approached significance.

**Long-term tone fear freezing.** A mixed model ANOVA was conducted to analyze the effects of age and stress on long-term tone fear freezing across temporal period (baseline and tone presentation; Figure 7). There was no significant interaction among age, stress and temporal period, $F$ (1, 27)= 2.60, $p$ = 0.119. No significant interactions were found between age and temporal period, or between stress and temporal period, all $F < 1$. The main effects of age and stress were not significant, all $F < 1$. The main effect of temporal period was significant, $F$ (1, 27)= 55.03, $p$ = 0.000, observed power= 1.0. All groups showed robust tone fear responses by virtue of an increase in freezing behavior with tone onset. Inter-rater reliability on this measure was high during both the baseline (Cronbach's alpha= 0.98, Intraclass correlation coefficient= 0.98) and tone presentation (Cronbach's alpha= 0.99, Intraclass correlation coefficient= 0.99) periods.

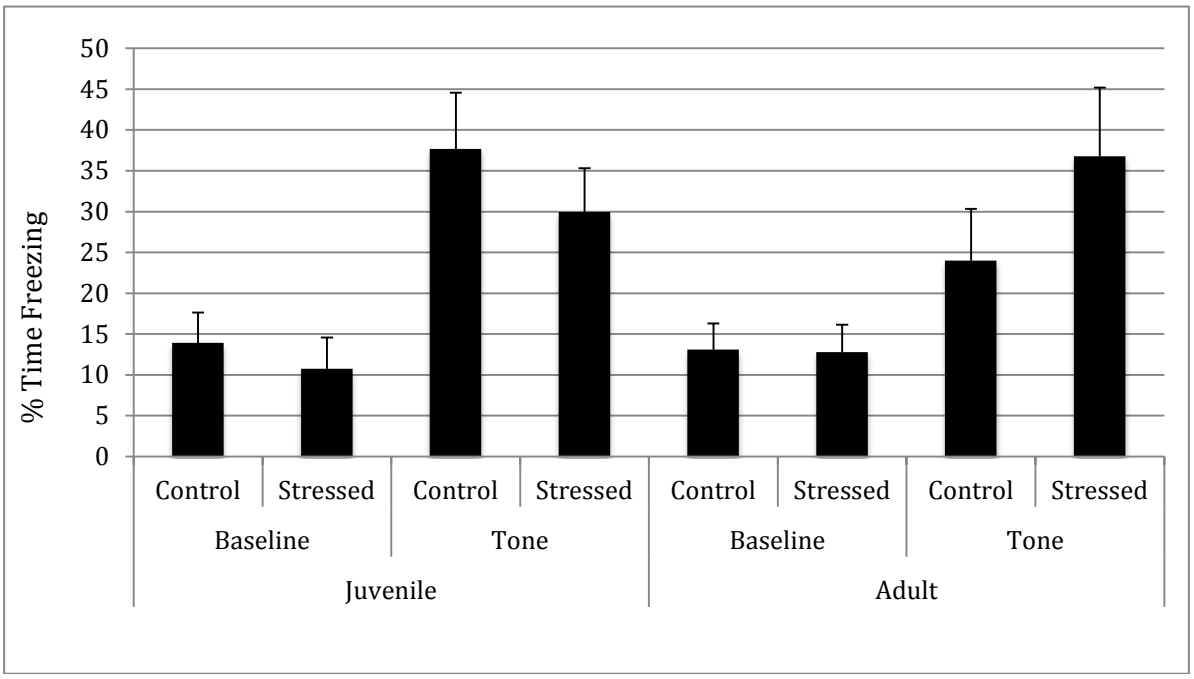


*Figure 7.* Behavioral observations during the tone test. Mean (+SEM) freezing response during the tone test, conducted 48h after training. Values represent the mean percentage of video samples scored as devoid of movement, taken during the 2 min baseline and 3 min tone exposure periods.

## Discussion

The current findings suggest that exposure to CVS heightens anxious behavior, as measured by the EPM, across developmental stages. We suggest that stressful experiences increase unconditional fear responses, causing a marked inflation in general anxiety levels. These results align with prior research investigating the effects of CVS on adult male rats, which also demonstrated anxiogenic behaviors on the EPM following stress exposure (Zurita et al., 2000). These results do not support our hypothesis that developmental level mediates the stress-anxiety relationship; both

juvenile and adult mice appear to have experienced increases in baseline anxiety levels subsequent to CVS exposure.

The failure of our cage crossover results to align with findings on the EPM remains difficult to understand. While exploratory behavior was significantly reduced in animals exposed to chronic stress on the EPM, this behavior was unaffected by stress exposure as measured during the cage crossover paradigm. Recent investigations of our CVS procedures have also replicated this failure of stress exposure inhibition of exploratory behavior prior to fear conditioning as measured by cage crossovers (Sanders et al., 2010). One possibility for the discrepancy in exploratory behaviors between contexts may be that the EPM environment is experienced as more threatening than that of the chambers used during crossover measurements, and thus triggers more unconditional fear (anxiety) in the mice. Furthermore, the restricted dimensions of the chambers used for cage crossover measurements may have limited the range of movement possible by the mice, thereby affecting our findings. Age differences observed within the cage crossover measurements, which suggested that adult mice displayed more exploratory behavior, may simply be a result of the size differences across the developmental periods. Due to the larger stature of the adult mice, it is conceivable that cage crossover was more likely to occur simply because of the small physical dimensions of the chamber.

Also in opposition with our a priori hypotheses, juvenile mice did not experience increases in conditional fear responses following stress exposure above and beyond that experienced by adult mice. In fact, while adult mice displayed the expected pattern of context fear learning sensitization after CVS exposure, with stressed mice exhibiting

heightened long-term context fear learning, juvenile mice exposed to chronic stress failed to display evidence of sensitization during the context test.

Increases noted in the fear learning responses of adult mice exposed to stress are limited to the context condition. Recent studies investigating the effects of our CVS procedures have suggested that while stress exposure in adult females enhances long-term tone fear conditioning, exposure in adult male mice amplifies long-term context fear learning, leaving tone fear learning largely unaffected (Sanders, et al., 2010). Sex differences in the effects of stress on subsequent fear learning have been attributed to its differential impact on the hippocampus, a structure known to play an integral role in the fear learning process (Bowman, Beck, & Luine, 2003; Galea et al., 1997; McLaughlin, Baran, & Conrad, 2009; McLaughlin, Gomez, Baran, & Conrad, 2007). This evidence suggests that our a priori hypothesis was incorrect in assuming that both types of long-term fear would be affected by CVS exposure in the adult male mice used here. The current study serves as a crucial confirmatory investigation of the effects of CVS on fear learning in adult male mice uncovered in previous work.

Taken together, our results suggest that while juvenile mice exposed to chronic stress experienced heightened anxiety levels akin to those experienced by adult mice, they failed to become sensitized to context fear learning as the adults did. The dissociation of anxiety and fear responses may be reflective of different neural mechanisms underlying stress responses in mice. Current research indicates that the amygdala may be at the crux of the defense system involved in the acquisition and expression of conditional fear (Davis, 1992; Davis, 1995; LeDoux, 1987). The amygdala receives sensory information via its lateral and basolateral nuclei, which subsequently project to the central nucleus of

the amygdala. The central nucleus of the amygdala in turn projects to a number of brainstem and hypothalamic sites known to affect signals of fear (Davis, 1992); lesions of the central nucleus of the amygdala have been shown to disrupt fear-potentiated startle to visual and auditory conditional stimuli (Hitchcock & Davis, 1986; Hitchcock & Davis, 1987), and electrical stimulation of the amygdala generates many of the behaviors associated with fear such as freezing, corticosteroid release, and increased vigilance (Lang, Davis &, Ohman, 2000). One can postulate that this system was affected by CVS in the stressed adults, leading to changes in conditional fear responses. While much of the fear conditioning literature has, over the years, used the terms 'anxiety' and 'fear' somewhat interchangeably, and assumed that the neurobiological underpinnings of the two are very similar, if not identical, Davis (2006) has recently begun delineating the neural pathways involved in these two phenomenon. The bed nucleus of the stria terminalis (BNST) is considered part of the 'extended amygdala' due to its similarity to the central nucleus of the amygdala in terms of its morphology, content and connections (Alheid, deOlmos, & Beltramino, 1995); however, lesions of this nucleus fail to inhibit conditional freezing and fear-potentiated startle responses (Hitchcock & Davis, 1991; LeDoux, Iwata, Cicchetti, & Reis, 1988), suggesting that it may not be involved in conditioning to explicit cues. Recently, Davis (2006) has suggested that the central nucleus of the amygdala is integral to "stimulus-specific fear responses," while the BNST is the key to more sustained responses to threat, which he labels as anxiety. He provides evidence that while lesions of the central nucleus of the amygdala disrupt fear conditioning responses to explicit cues, lesions of the BNST inhibit long-term sensitization of the startle reflex to unconditional threatening stimuli

(Hitchcock & Davis, 1986; Hitchcock & Davis, 1987; Gewirtz, McNish, & Davis, 1998). Interestingly, research on the development of the mouse brain suggests that neurons intended for the BNST are generated between prenatal days 12 and 15 (Crepps, 1974), and literature exploring the rat brain indicates that BNST neurons congregate approximately 6-10d postnatally (Jacobson, Davis, & Gorski, 1985), while investigations of the development of the central nucleus of the amygdala in the mouse brain suggests that it is not established until approximately 35d after birth (Mouse Atlas of Gene Expression, n.d.). We propose that it is these neurobiological differences in the development of the fear and anxiety pathways that underlie the behavioral discrepancies found here. The anxiety responses of both adult and juvenile mice may reflect full development of the BNST, while the lack of long-term fear conditioning sensitization to context following stress exposure in juveniles may be evidence of the underdeveloped state of the fear conditioning system, namely the central nucleus of the amygdala. While juvenile mice did display evidence of successful fear conditioning, suggesting they are capable of stimulus-specific fear learning, they failed to exhibit any significant effects of stress exposure on the process of fear conditioning. This implies a failed connection between the stress and fear learning systems during the juvenile stage, which we propose is continuing to develop during this developmental period.

In addition to findings of amygdala involvement in the anxiety and fear responses of mice, the amygdala has been implicated in the acquisition and expression of conditional fear in humans (LaBar, Gatenby, Gore, LeDoux, & Phelps, 1998), and researchers have found increased amygdalar activity in humans with high trait anxiety (Indovina, Robbins, Nunez-Elizalde, Dunn, & Bishop, 2011). Additionally, while prevalence

estimates for generalized anxiety disorder (GAD) in pre-pubertal children range from 0.2% to 11%, estimates for anxiety disorders more commonly conceptualized as developing from the fear learning process, such as PTSD and specific phobias, have estimates that fall at less than 1% (Cartwright-Hatton, McNicol, & Doubleday, 2006). In contrast, lifetime prevalence rates for those diagnoses in adults are relatively even, with estimates falling at 8% for PTSD, 7-11% for specific phobias, and 5% for GAD (American Psychiatric Association, 2000). If we are to understand GAD as an unconditional fear response (anxiety), and PTSD and simple phobias as conditional fear responses then changes in the patterns of anxiety disorder presentation across development may be representative of a similar developmental trajectory for the neural substrates underlying these phenomena in the human brain. While certainly preliminary in nature, it is possible that exposure to high levels of stress during the pre-pubescent years may increase subsequent anxiety yet fail to alter subsequent conditional fear responses because of the differential maturation rates of anxiety and fear circuits.

One crucial limitation to the present study is its focus on behavior; no physiological measures of the stress response were collected and no neuroanatomical investigations were completed. While the behavioral differences noted here following exposure to stress in juveniles and adults hints at potential neural patterns, no conclusive statements can be made in this regard. Future work is needed to explore the proposed neurobiological developmental differences as they relate to the differences in behavior observed here.

Future studies are also needed to extend this model to humans. While prevalence data allows for postulation regarding the anxiety and fear responses of children and

adults, and leads us back to the anxiety-fear differentiation hypothesis, additional work is needed to confirm these hypotheses.

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*Note*: The Mouse Atlas of Gene Expression is a project developed by Canada's Michael Smith Genome Sciences Centre and supported by Genome Canada, the British Columbia Cancer Agency, British Columbia Cancer Foundation, and the National Cancer Institute (USA). The Mouse Atlas of Gene Expression strives to establish a comprehensive atlas of gene expression over development in the mouse.

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