Sex differences in traumatic stress reactivity of rats with a history of alcohol drinking

Lucas Albrechet-Souza¹,², Connor L. Schratz¹, Nicholas W. Gilpin¹,²,³,⁴

¹Department of Physiology, School of Medicine, Louisiana State University Health Sciences Center, New Orleans, LA; ²Alcohol & Drug Center of Excellence, School of Medicine, Louisiana State University Health Sciences Center, New Orleans, LA; ³Neuroscience Center of Excellence, School of Medicine, Louisiana State University Health Sciences Center, New Orleans, LA; ⁴Southeast Louisiana VA Healthcare System (SLVHCS), New Orleans, LA.

Correspondence should be addressed to Lucas Albrechet-Souza

Email: ldesou@lsuhsc.edu

Address: 1901 Perdido St, Room 7242, New Orleans, LA 70112, USA

Acknowledgments: This study was supported by the National Institutes of Health grants R01AA023305, R01AA026531, and the Cohen Veterans Bioscience Foundation. This work was also supported in part by Merit Review Award #I01 BX003451 (NWG) from the United States (U.S.) Department of Veterans Affairs, Biomedical Laboratory Research and Development Service.

Conflict of Interest: Nicholas W Gilpin owns shares in Glauser Life Sciences Inc., a company with activities aimed at developing medications for treating mental health disorders; this affiliation had no direct association with the work presented here.
Abstract

**Background:** Alcohol misuse and post-traumatic stress disorder (PTSD) are highly comorbid and treatment outcomes are worse in individuals with both conditions. Although more men report experiencing traumatic events than women, the lifetime prevalence of PTSD is twice as high in females. Despite these data trends in humans, preclinical studies of traumatic stress reactivity have been performed almost exclusively in male animals.

**Methods:** This study was designed to examine sex differences in traumatic stress reactivity in alcohol-naïve rats and rats given intermittent access to 20% ethanol in a 2-bottle choice paradigm for 5 weeks. Rats were exposed to predator odor (bobcat urine) and tested for avoidance of the odor-paired context 24 hours later; unstressed Controls were never exposed to odor. Two days after stress, we measured physiological arousal using the acoustic startle response (ASR) test. We also measured anxiety-like behavior using the elevated plus-maze (EPM) and circulating corticosterone levels before and immediately after odor exposure.

**Results:** Male and female rats exposed to predator odor displayed blunted weight gain 24 hours post-stress, but only a subset of stressed animals exhibited avoidance behavior. Chronic intermittent alcohol drinking increased the proportion of Avoiders in males and predator odor exposure increased ASR in these animals. Predator odor stress reduced ASR in females relative to unstressed females and stressed males, regardless of alcohol drinking history. Bobcat urine exposure did not promote persistent anxiety-like behavior, but alcohol-experienced males exhibited reduced activity in the EPM in comparison to alcohol-experienced females.
Furthermore, predator odor increased circulating corticosterone levels in females relative to males and baseline.

**Conclusions:** We report robust sex differences in behavioral and endocrine responses to bobcat urine exposure in adult Wistar rats. Also, chronic moderate alcohol drinking increased traumatic stress reactivity in males but not females. Our findings emphasize the importance of considering sex as a biological variable in the investigation of traumatic stress effects on physiology and behavior.

**Keywords:** sex differences, predator odor, bobcat urine, alcohol, stress, trauma, anxiety, startle, arousal, corticosterone
**Background**

Post-traumatic stress disorder (PTSD) is a chronic psychiatric disease that is seen in some but not all individuals after experiencing a life-threatening traumatic event. Major diagnostic criteria for PTSD include re-experiencing the traumatic event, negative affective state, exaggerated startle responses and persistent avoidance of trauma-related cues [1]. Women are twice as likely to develop PTSD after trauma [2, 3] and women with trauma exposure and/or PTSD exhibit more sensitivity to and less tolerance of negative emotions [4, 5].

Alcohol use disorder (AUD) is one of the most common co-occurring conditions among individuals diagnosed with PTSD [6, 7]. Approximately one-third of individuals with lifetime PTSD also meet criteria for AUD [8]. Some populations, such as military personnel, are at high risk for AUD and PTSD comorbidities. For example, in a study of Iraq and Afghanistan veterans, 63% of those diagnosed with AUD also met criteria for PTSD [9]. Although men have a higher prevalence of AUD than women, and women have a higher prevalence of PTSD than men, any individual with either disorder is more likely to have the other [10]. Individuals with co-occurring AUD and PTSD exhibit a more complex and severe clinical profile than those diagnosed with either disorder alone [8, 11, 12]. Some people with PTSD use alcohol in an attempt to ameliorate debilitating symptoms such as anxiety and hyperarousal [13, 14]. However, there is a complex reciprocal relationship between stress and alcohol: while PTSD symptoms may lead an individual to drink more alcohol, alcohol use may exacerbate PTSD symptoms [15–17].
Exaggerated startle response is considered a hallmark symptom of PTSD [1] and is predictive of disease severity [18]. In a study of military service members referred for psychiatric evaluation for suicide-related concerns, the hyperarousal symptom cluster was the only significant predictor of subsequent suicide attempts [19]. Although previous studies have shown elevated autonomic responses to startling tones in trauma survivors [20–22], other investigations of startle responsivity in patients with PTSD have produced mixed results. For example, studies of Gulf War veterans with PTSD found both self-reported [23] and physiologically [24] exaggerated startle compared to non-PTSD veterans. On the other hand, Vietnam veterans with PTSD did not show increased startle [25] unless they were subjected to a stressful environment [26]. More extreme, some studies report that PTSD patients exhibit blunted motor reflex responses to acoustic stimuli [27, 28]. These last two studies used either all female subjects [28] or a majority of females [27], suggesting there may be a sex difference in the presentation of ASR in females.

Preclinical studies using animal models to recapitulate PTSD-like behavioral deficits allow investigation of the biological mechanisms underlying traumatic stress effects, but most preclinical research has been conducted in male animals, potentially neglecting issues specific to female subjects [29, 30]. Predator exposure and predator scent are psychological stressors commonly used as animal models of PTSD. Rodents are exposed to predator odor in a variety of ways, including indirect exposure to a predator (cat), predator urine (bobcat, fox), predator feces or litter, or trimethylthiazoline (TMT) – a synthetic compound isolated from fox feces [31–34]. Although predator stress has been shown to elicit lasting increases in freezing and avoidance behaviors [35, 36], the effects of predator odor stress on ASR are not consistent. For example,
male rats exhibit potentiated ASR magnitude during exposure to TMT [37], but three exposures to cat odor failed to promote persistent changes on startle reactivity [38]. Prior work from our laboratory showed small increases in ASR at low decibel levels after bobcat urine exposure in alcohol-naïve male rats [39].

The current experiments were designed to test sex differences in traumatic stress reactivity in rats with and without a history of chronic voluntary alcohol consumption exposed to a bobcat urine stress model developed in our laboratory [40]. The urine of carnivorous species contains 2-phenylethylamine, a trace amine produced by the breakdown of the amino acid phenylalanine [41], that activates trace amine-associated receptor 4 (TAAR4) in the rodent olfactory cortex and produces avoidance behavior in rodents [41]. The main hypothesis of this study was that bobcat urine exposure would increase acoustic startle reactivity in male and female rats and that this effect would be exaggerated in rats that exhibit avoidance of trauma-paired cues and in rats with a history of alcohol drinking.

Methods

Animals

Eight-week-old male and female Wistar rats (total N = 110) (Charles River, Raleigh, NC) were housed in same-sex pairs in a humidity- and temperature-controlled (22 °C) vivarium on a 12-hours reversed light-dark cycle (lights off at 7 a.m.). Animals had ad libitum access to food and water throughout the experiments and were handled daily for 1 week before the initiation of experimental protocols. All behavioral testing occurred in the dark phase. Female rats were freely
cycling and assigned to treatment groups without regard to estrous cycle stage. At the end of the experiments, rats were sacrificed by decapitation under isoflurane anesthesia.

**Intermittent access 2-bottle choice alcohol homecage drinking**

Paired-housed adult male and female Wistar rats were given access to alcohol and water in three 24-hour sessions per week for 5 weeks prior to stress, as previously described [42]. Briefly, rats were weighed and given access to 1 bottle of 20% v/v ethanol and 1 bottle of water approximately 3 hours after the start of the dark cycle on Mondays, Wednesdays and Fridays. After 24 hours, the alcohol bottle was replaced with a second water bottle that was available for the next 24 hours. Over the weekends, rats had unlimited access to 2 water bottles after the alcohol bottle was removed on Saturday. Bottles were weighed 24 hours after alcohol presentation. The position of the alcohol bottle was alternated across sessions to control for side preferences. Blood was collected from the tail 2 hours after the start of alcohol access during the last drinking session to determine blood alcohol concentration. Blood samples were centrifuged at 1900xg for 14 minutes, after which plasma was collected and immediately analyzed using an Analox AM1 analyzer (Analox Instruments).

**Predator odor stress**

Rats were tested in a 5-day conditioned place aversion procedure [40] that began after the acclimatization period in alcohol-naïve rats or 24 hours after the last drinking session in alcohol-experienced rats. On the first day, rats were allowed 5 minutes of free exploration of the apparatus (3-chamber pre-test session), which consisted of three large chambers (36 cm length
× 30 cm width × 34 cm height) with different types of floor texture (circles, grid or rod floor) and patterned walls (circles, white or stripes), separated by a small triangular connecting chamber. The apparatus was thoroughly cleaned between animals with Quatricide® PV in water at a concentration of 1:64 (Pharmacal Research Labs, Waterbury, CT). For each rat, the chamber that exhibited the most deviant time score of the three (i.e., highly preferred or highly avoided) was excluded from all future sessions for that rat. On day 2, the rat was allowed 5 minutes to explore the two non-excluded conditioning chambers (pre-exposure session). Rats were assigned to predator odor stress or unstressed Control groups that were counterbalanced for the magnitude of baseline preference for one chamber versus the other (i.e., groups were assigned such that mean pre-existing preference for each of the two chambers was approximately zero for Stress and Control groups). For rats in the Stress group, an unbiased and counterbalanced design was used to determine which chamber (i.e., more preferred or less preferred) would be paired with predator odor for each rat. On day 3, each rat was placed in one of the two chambers with the guillotine door shut without odor for 15 minutes (neutral exposure). On day 4, rats were weighed and placed in the other chamber with the guillotine door shut and a sponge soaked with 3 ml of bobcat urine (Lynx rufus; Maine Outdoor Solutions, Hermon, ME) placed under the floor for 15 minutes (odor exposure). Control rats were treated identically to odor-exposed rats, but the sponges did not contain bobcat urine. On day 5, rats were weighed and allowed to explore the two chambers for 5 minutes (post-exposure session). All testing was conducted under indirect dim illumination (one 60W white light facing the wall providing approximately 10 lux in the apparatus) and all sessions were recorded and time spent in each chamber was scored by a treatment-blind observer. Avoidance was quantified as a difference score calculated as time
spent in odor-paired chamber on day 5 minus time spent in the same chamber on day 2. Rats that displayed >10-s decrease in time spent in the odor-paired context were classified as Avoiders; all other bobcat urine-exposed rats were classified as Non-Avoiders.

**Acoustic startle response (ASR) test**

Two days after exposure to predator odor stress (i.e., day 6 of the protocol), rats were tested for acoustic startle reactivity. Rats were placed in a Plexiglas tube attached to an accelerometer inside a dark, soundproof chamber (SR-Lab, San Diego Instruments, CA) and allowed to acclimate for 5 minutes (75-dB background noise) before the test session, as previously described [39]. This background white noise was present throughout the session. The chamber and Plexiglas tube were cleaned with Quatricide between each animal. Before testing, an S-R calibrator tube was used to calibrate the chambers. The test session consisted of 30 trials with startle stimuli of three different decibel levels: a 750-ms burst of 95 dB, 105 dB or 115 dB white noise was presented 10 times each, separated by a 30-s fixed inter-trial interval. The maximum startle response (Vmax, arbitrary units) was recorded during the first 100 milliseconds of each trial.

**Elevated plus-maze (EPM) test**

The EPM test was used to test anxiety-like behavior on day 21 (17 days after exposure to bobcat urine). The EPM was a black Plexiglas apparatus consisting of two closed arms (50 cm x 10 cm x 40 cm) and two open arms (50 cm x 10 cm) attached to metal legs elevating the maze 50 cm above the ground. All testing was conducted under dim illumination (approximately 10 lux in the open arms) during the dark phase of the light-dark cycle. Rats were placed individually in the
center of the maze facing a closed arm and allowed 5 minutes of free exploration. Behavior was recorded with a video camera positioned above the maze. The EPM was cleaned thoroughly between subjects using Quatricide. Video scoring was done by an observer blind to the conditions; we measured percent time spent in the open arms ((open/open+closed) × 100) and number of closed and open arm entries. One arm entry was defined as all four paws entering the arm.

**Plasma Corticosterone Levels**

A separate cohort of alcohol-naïve male and female rats was individually transferred from the home cage to a clean cage and exposed to bobcat urine for 15 minutes. Bobcat urine (~3 ml) was added to a sponge that was placed beside the cage. Tail blood was collected before and immediately after exposure to predator odor stress in EDTA-covered tubes and samples were centrifuged at 1900×g for 20 minutes. Plasma was stored at −80 °C and analyzed in duplicate for corticosterone levels using a DetectX ELISA kit (Arbor Assays, Ann Arbor, MI) according to the manufacturer’s instructions.

**Statistical analysis**

Data are reported as mean ± SEM, except where otherwise indicated. All statistics were run using Prism 8 (GraphPad, La Jolla, CA). Alcohol drinkers and alcohol-naïve animals were not tested in parallel, therefore, they are not analyzed together. Change in time spent in predator odor-paired chamber, body weight gain, percent time spent in the open arms of the EPM and closed arm entries were analyzed with two-way analysis of variance (ANOVA) – the variables in all cases were
sex and stress condition. Measures of Vmax were normalized by body weight in kilograms. Three-way repeated measures ANOVAs were performed – the variables were sex, stress condition and decibel level, followed by two-way ANOVAs to test stress and sex effects on startle reactivity at each decibel level. Alcohol consumption and plasma corticosterone were analyzed with two-way repeated measures ANOVAs – the variables were sex and sessions. Fisher’s exact test was used to analyze the proportion of Avoiders and Non-Avoiders in each sex in alcohol-naïve and alcohol-experienced rats. Student’s unpaired t-tests were used to compare the magnitude of avoidance in male and female Avoiders, and the startle response and percent time spent in the open arms of the EPM between Avoiders and Non-Avoiders in each sex in alcohol-naïve and alcohol-experienced rats. In cases of significant ANOVA effects, post hoc comparisons were performed using Tukey's multiple comparisons test. Values of P < 0.05 were considered statistically significant.

Results

Predator odor stress reactivity in alcohol-naïve rats

Independent of sex, Avoiders exhibited significantly greater avoidance of the predator odor-paired chamber at 24 hours post-exposure (F (1, 44) = 78.51, P < 0.0001) relative to Non-Avoiders (Fig. 2a). There was no significant difference in the magnitude of avoidance between male and female Avoiders (Fig. 2a; t = 1.86, P = 0.08), and the proportion of animals that met Avoider criteria was similar in both sexes (Fig. 2b; P > 0.05). Likewise, both male and female rats exposed to predator odor exhibited significantly reduced body weight gain relative to unstressed Controls.
24 hours post-stress (day 5 minus day 4) (Fig. 2c; stress effect: F (1, 44) = 6.99, P = 0.01) but not 4 days post-stress (stress effect: F (1, 44) = 1.12, P = 0.29; data not shown).

Because we did not find significant differences in the ASR between Avoider and Non-Avoider males nor between Avoider and Non-Avoider females, these animals were pooled into a single group, designated Stress, and compared to unstressed Controls (Fig. 2d). A three-way repeated measures ANOVA yielded a significant main effect of decibel level (F (2, 116) = 147.50, P < 0.0001), a decibel level × sex interaction effect (F (2, 116) = 4.45, P = 0.01) and a sex × stress interaction effect (F (1, 116) = 7.69, P = 0.01) on ASR. To determine whether predator odor stress affected startle reactivity differently in male and female rats, ASR data for each decibel were analyzed with a two-way ANOVA. At 90 dB, we found a significant main effect of sex (F (1, 58) = 5.45, P = 0.02) and a sex × stress interaction effect (F (1, 58) = 10.59, P = 0.002) on ASR. Tukey’s post-hoc comparisons revealed that control females exhibited higher startle reactivity than control males (P = 0.01) and that stressed females showed lower startle reactivity relative to control females (P = 0.01) in response to a 90 dB stimulus. At 105 dB, the two-way ANOVA revealed a significant sex × stress interaction effect (F (1, 58) = 4.11, P = 0.047) on ASR. Tukey’s post-hoc comparison revealed that stressed females exhibited lower startle reactivity in response to a 105 dB stimulus than control females (P = 0.02). At 115 dB, the two-way ANOVA revealed a significant sex × stress interaction effect (F (1, 58) = 6.02, P = 0.02) on ASR. Tukey’s post-hoc comparison revealed that stressed males exhibited higher startle reactivity in response to a 115 dB stimulus than stressed females (P = 0.0008).
On day 21, rats were tested for anxiety-like behavior in the EPM. Again, we did not find significant differences between Avoider and Non-Avoider males nor between Avoider and Non-Avoider females on percent time spent in the open arms of the EPM; thus, these animals were pooled into a single group, designated Stress, and compared to unstressed Controls. A two-way ANOVA revealed no significant effect of sex (F (1, 59) = 0.73, P > 0.05) or stress (F (1, 59) = 0.04, P > 0.05) on percent time spent in the open arms of the EPM (Fig. 2e). General locomotor performance was assessed by counting closed-arm entries in the EPM. Neither sex (F (1, 59) = 0.59, P > 0.05) nor stress (F (1, 59) = 3.65, P > 0.05) affected number of closed arms entries (Fig. 2f).

**Predator odor stress reactivity in rats with a history of alcohol drinking**

Before exposure to predator odor stress, pair-housed rats were given intermittent access to alcohol 20% v/v and water for 5 weeks in the homecage. Because rats were pair-housed, we did not measure intake for individual animals but instead for each cage. We chose this procedure to avoid exposing animals to social isolation stress. A two-way repeated measures ANOVA showed that males consumed significantly higher quantities of alcohol than females over the weeks (Fig 3c; F (1, 15) = 15.49, P = 0.001). However, males and females did not exhibit different blood alcohol levels (BALs) analyzed 2 hours after the beginning of the last alcohol drinking session (t = 0.21, P = 0.83; data not shown). Independent of sex, Avoiders with an alcohol drinking history exhibited significantly greater avoidance of the predator odor-paired chamber at 24 hours post-exposure (F (1, 18) = 28.79, P < 0.0001) relative to Non-Avoiders with an alcohol drinking history (Fig. 3a). There was no significant difference in the magnitude of avoidance between male and female Avoiders (Fig. 3a; t = 0.76, P = 0.48). Although the difference in the proportion of Avoiders
and Non-Avoiders in male and female rats did not reach statistical significance (P = 0.39) due to sample size (given a 95% confidence level and 80% power, the recommended sample size would be n = 95), the proportion of alcohol-drinking males that met Avoider criteria was 27% higher than alcohol-drinking females (Fig. 3b) and 25% higher than alcohol-naive males (compare to data in Fig. 2b).

In males and females with an alcohol drinking history, Avoider and Non-Avoider did not exhibit differences in ASR, so these animals were pooled into a single group, designated Stress, and compared to unstressed Controls (Fig. 3d). A three-way repeated measures ANOVA yielded a significant main effect of decibel level (F (2, 58) = 130.00, P < 0.0001), a decibel level × sex interaction effect (F (2, 58) = 7.42, P = 0.001) and a sex × stress interaction effect (F (1, 58) = 5.62, P = 0.02) on ASR. To determine whether predator odor stress affected startle reactivity differently in male and female rats with a history of alcohol drinking, ASR data for each decibel were analyzed with a two-way ANOVA. At 90 dB, we found a significant sex × stress interaction effect (F (1, 29) = 7.02, P = 0.01) on ASR. Tukey’s post-hoc comparison revealed that stressed males exhibited higher startle reactivity in response to a 90 dB stimulus than control males (P = 0.03). At 105 dB, the two-way ANOVA revealed a significant sex × stress interaction effect (F (1, 29) = 5.94, P = 0.02) on ASR. Tukey’s post-hoc comparisons revealed that stressed males exhibited higher startle reactivity in response to a 105 dB stimulus relative to control males (P = 0.04) and also relative to stressed females (P = 0.004). At 115 dB, the two-way ANOVA revealed a significant main effect of sex (F (1, 29) = 5.23, P = 0.03) on ASR. Tukey’s post-hoc comparison revealed that
stressed males exhibited higher startle reactivity in response to a 115 dB stimulus than stressed females (P = 0.01).

On day 21, rats were tested for anxiety-like behavior in the EPM. We did not find significant differences between Avoider and Non-Avoider males nor between Avoider and Non-Avoider females on percent time spent in the open arms of the EPM; thus, these animals were pooled into a single group, designated Stress, and compared to unstressed Controls. A two-way ANOVA revealed that regardless of stress condition, males with a history of alcohol drinking spent less time in the open arms of the EPM relative to females (Fig. 3e; F (1, 30) = 11.86, P = 0.002), likely driven by the apparent stress-induced increase in time spent in the open arms of the EPM in females. Males with a history of alcohol drinking also exhibited fewer closed arm entries compared to females (Fig. 3f; F (1, 30) = 4.96, P = 0.03).

**Predator odor stress effects on plasma corticosterone**

Predator odor stress produced sexually dimorphic changes in circulating corticosterone levels (Fig. 4). A two-way repeated measures ANOVA yielded a significant main effect of sex (F (1, 9) = 17.43, P = 0.002), time (F (1, 9) = 62.40, P < 0.0001) and a sex × time interaction effect (F (1, 9) = 20.91, P = 0.001) on plasma corticosterone measured before and immediately after a 15-min exposure to bobcat urine. Tukey’s *post-hoc* comparison revealed that, after bobcat urine exposure, circulating corticosterone levels were significantly higher in stressed females relative to stressed males (P = 0.0001) and also relative to their own baseline (P = 0.001).
Discussion

We report that alcohol-naïve male and female rats exposed to predator odor displayed blunted weight gain 24 hours post-stress even though only a subset of stressed animals exhibited avoidance behavior. A similar percentage of alcohol-naïve males and females were classified as Avoiders after stress, but the proportion of Avoiders was much higher in alcohol-experienced males relative to females with a history of alcohol drinking and alcohol-naïve males. Predator odor exposure enhanced startle reactivity in alcohol-experienced males only, but regardless of alcohol drinking history, stress reduced startle reactivity in females. Predator odor stress did not produce anxiety-like behavior, but alcohol-experienced males spent less time in the open arms than females and exhibited less locomotor activity than females in the EPM. Furthermore, bobcat urine exposure significantly increased circulating corticosterone levels in females relative to males and baseline. These results are important because alcohol use exacerbates PTSD symptoms in humans [15–17] and risk factors for co-morbid PTSD and AUD may differ based on sex [12].

Predator odor stress produces behavioral, molecular, and physiological alterations that recapitulate some PTSD symptoms [40, 43]. In line with previous studies, bobcat urine exposure elicited significant avoidance in a subset of animals [35, 44]. A similar proportion of alcohol-naïve male and female rats exposed to predator odor were classified as Avoiders (males: 42%; females: 37%), with similar magnitude of bobcat urine-paired context avoidance 24 hours post-stress. After chronic voluntary ethanol consumption, a much higher percentage of males (but not females) were classified as Avoiders (males: 67%; females: 40%). A systematic review of the
comorbidity between PTSD and alcohol misuse found associations between alcohol consumption and the avoidance/numbing and hyperarousal PTSD symptom clusters [45]. We previously reported that male Avoider rats exhibit persistent increases in alcohol self-administration and that avoidance behavior predicts post-stress escalation of alcohol drinking [35].

Although only a subset of rats exposed to predator odor displayed avoidance behavior, all stressed male and female rats exhibited signs of physiological stress. A previous study from our laboratory demonstrated that all male rats exposed to predator odor (i.e., Avoiders and Non-Avoiders) exhibit anxiety-like behavior measured 2 days and 5 days post-stress [46]. The current data build on that work by reporting that all alcohol-naïve stressed male and female rats displayed blunted weight gain 24 hours after predator odor exposure (we did not evaluate changes in body weight in rats with a history of alcohol drinking). Moreover, prior work from our laboratory showed that male rats exposed to bobcat urine exhibit a non-significant general increase in startle reactivity [39]. In the present study, we confirmed and extended these findings by testing females and animals with a history of chronic voluntary alcohol drinking.

The startle response is an operational measure of threat anticipation linked to fear circuit activation in humans and animals [47, 48]. Although alterations in arousal or reactivity are commonly reported in humans with PTSD [1], empirical evidence for exaggerated startle response is mixed [49, 50]. A meta-analysis that compared adults with and without PTSD indicated only modest increases in baseline startle reactivity in PTSD patients [51]. One potential reason for these moderate associations is that stress may differentially alter startle reactivity in
subgroups (i.e., males versus females) of PTSD patients with distinct trauma-related pathology or trauma histories [52]. For example, after exposure to a terrorist attack, women reported higher levels of re-experiencing symptoms and exaggerated startle response than men [53]. In contrast, in female victims of childhood corporal punishment and partner aggression, higher PTSD symptom scores were related to lower startle reflex [28].

In this study, only male rats with a history of alcohol consumption showed stress-induced increases in startle reactivity after bobcat urine exposure. In agreement with these findings, previous studies have reported that individuals with comorbid PTSD and alcohol misuse exhibit more severe PTSD symptoms [54, 55]. Startle reactivity was tested 6 days after the last drinking session (i.e., not during intoxication). Furthermore, it is unlikely that animals in this study consumed quantities of alcohol sufficient to produce alcohol dependence or withdrawal. Contrary to prior reports [56], we found that male rats consumed more alcohol than females, and neither males nor females significantly escalated ethanol intake during the 5 weeks of intermittent 20% ethanol drinking. It is worth mentioning that our animals were pair-housed throughout the experiment, whereas in most studies using this protocol rats were housed individually [42, 57]. While the procedure used here precludes correlations between alcohol intake and startle reactivity in individual animals, it has the benefit of avoiding potential confound effects of social isolation.

Bobcat urine exposure reduced startle reactivity in females, regardless of alcohol drinking history. It is important to note that we did not measure baseline ASR to counterbalance the
groups before the test session, but it is unlikely that random assignment of animals would select for rats with different startle reactivity. Although contrary to our initial hypothesis, prior work from others has reported lower startle reactivity in female rats after inescapable tail shock stress [58], an effect that was blocked by a systemic IL-1β injection in intact females, but not in ovariectomized females [59].

Our group previously reported that bobcat urine exposure increased anxiety-like behavior in male rats 2 days and 5 days later [46]. Here, we show that bobcat urine exposure did not alter anxiety-like behavior on day 21, i.e. 17 days post-stress, in male or female alcohol-naïve rats. Males with a history of alcohol drinking spent less time in the open arms of the elevated plus-maze and exhibited reduced general activity on the EPM relative to alcohol-drinking females. A similar reduction in general activity on the EPM was described in Long-Evans male rats after a 6-week intermittent-access to 20% ethanol in comparison to females exposed to the same procedure [56]. The higher open arm times in females may reflect sex differences in stress/fear coping strategies rather than “less anxiety-like behavior”. A previous study using factor analysis suggested that the behavior of female rats in the EPM is characterized more by activity than anxiety [60]. Also, females are four times more likely than males to display fear in the form of rapid movements instead of freezing in traditional models of Pavlovian fear conditioning [61].

Predator odor exposure increased corticosterone levels in females relative to unstressed controls and stressed males. Our group has previously shown that male Avoiders exhibit attenuated corticosterone response immediately following exposure to bobcat urine [46]. Differently from
this study, the animals used here to evaluate endocrine responses were exposed to bobcat urine in a clean cage instead of in the 3-chamber apparatus, therefore they were not indexed as Avoiders or Non-Avoiders. Similar to our results, female rats exposed to acute restraint stress exhibit higher adrenocorticotropic hormone (ACTH) and corticosterone responses than males, in addition to significantly higher c-fos mRNA expression in the paraventricular nucleus of the hypothalamus (PVN) [62]. Conversely, TMT exposure elicits similar increase in circulating corticosterone in both male and female Wistar rats [63], indicating that distinct predator odors (i.e., natural olfactory stimuli or synthetic olfactory stimulus) can produce different responses. Future studies will determine whether higher corticosterone levels in females after bobcat urine exposure mediate stress-induced suppression of startle reactivity. Interestingly, suppression of ASR following combined cat exposure and saline injection in male rats can be blocked by substituting the injected saline with a glucocorticoid receptor antagonist [64].

**Conclusions**

We report robust sex differences in behavioral and endocrine responses to bobcat urine exposure in adult Wistar rats. Males and females exposed to predator odor displayed blunted weight gain 24 hours post-stress, but only a subset of stressed animals exhibited avoidance behavior. Chronic moderate alcohol drinking increased traumatic stress reactivity in males but not females. Predator odor stress reduced startle reactivity in females relative to unstressed females and stressed males, regardless of alcohol drinking history. Furthermore, females exhibited higher increases in circulating corticosterone concentrations immediately following predator odor stress compared to males.
**Perspectives and significance**

Sex differences in traumatic stress responses are among the most widely reported phenomena in epidemiological and clinical studies. To the best of our knowledge, the findings reported here are the first to provide evidence that a history of chronic moderate alcohol drinking differentially modulates predator odor stress reactivity in male and female rats. Our data support the notion that females rather respond differently to trauma and open doors for future work aimed at testing the neurobiology underlying sex differences in traumatic stress reactivity.

**Declarations**

**Ethics approval**

All procedures were approved by the Institutional Animal Care and Use Committee of the Louisiana State University Health Sciences Center and were in accordance with the National Institute of Health Guidelines.

**Consent for publication**

Not applicable.

**Availability of data and materials**

All data are available from the corresponding author upon request.

**Competing interests**
NWG owns shares in Glauser Life Sciences, a company with interest in developing therapeutics for mental health disorders. There is no direct link between those interests and the work contained herein.

**Funding**

This study was supported by the National Institutes of Health grants R01AA023305, R01AA026531; by U.S. Department of Veterans Affairs grant I01BX003451; and by Cohen Veterans Bioscience.

**Authors' contributions**

LA-S conceived and planned the study, performed the experiments, analyzed data and wrote the paper. CLS performed the experiments. NWG conceived and planned the study, supervised the project and contributed to the final version of the manuscript. All authors read and approved the final manuscript.

**Acknowledgments**

The authors thank Curtis Vande Stouwe for his technical support.
References

1. American Psychiatric Association. Diagnostic and statistical manual of mental disorders. 2013. 5th ed. Arlington, VA: American Psychiatric Publishing.

2. Breslau N, Peterson EL, Schultz LR. A second look at prior trauma and the posttraumatic stress disorder effects of subsequent trauma: a prospective epidemiological study. Arch Gen Psychiatry. 2008;65:431–7.

3. Kessler RC, Sonnega A, Bromet E, Hughes M, Nelson CB. Posttraumatic stress disorder in the national comorbidity survey. Arch Gen Psychiatry. 1995;52:1048–60.

4. Miles SR, Menefee DS, Wanner J, Tharp AT, Kent TA. The relationship between emotion dysregulation and impulsive aggression in veterans with posttraumatic stress disorder symptoms. J Interpers Violence 2016; 31:1795–816.

5. Vujanovic AA, Rathnayaka N, Amador CD, Schmitz JM. Distress tolerance: Associations with posttraumatic stress disorder symptoms among trauma-exposed, cocaine-dependent adults. Behav Modif. 2016;40:1–24.

6. Gilpin NW, Weiner JL. Neurobiology of comorbid post-traumatic stress disorder and alcohol-use disorder. Genes Brain Behav. 2017;16:15–43.

7. Kessler RC, Chiu WT, Demler O, Merikangas KR, Walters EE. Prevalence, severity, and comorbidity of 12-month DSM-IV disorders in the National Comorbidity Survey Replication. Arch Gen Psychiatry. 2005;62:617–27.

8. Blanco C, Xu Y, Brady K, Pérez-Fuentes G, Okuda M, Wang S. Comorbidity of posttraumatic stress disorder with alcohol dependence among U.S. adults: results from National Epidemiological Survey on Alcohol and Related Conditions. Drug Alcohol Depend. 2013;132:630–8.

9. Seal KH, Cohen G, Waldrop A, Cohen BE, Maguen S, Ren L. Substance use disorders in Iraq and Afghanistan veterans in VA healthcare, 2001-2010: Implications for screening, diagnosis and treatment. Drug Alcohol Depend. 2011;116:93–101.

10. Smith NDL, Cottler LB. The epidemiology of post-traumatic stress disorder and alcohol use disorder. Alcohol Res. 2018;39:113–20.

11. Leeies M, Pagura J, Sareen J, Bolton JM. The use of alcohol and drugs to self-medicate symptoms of posttraumatic stress disorder. Depress Anxiety. 2010;27:731–6.

12. Straus E, Haller M, Lyons RC, Norman SB. Functional and psychiatric correlates of comorbid post-traumatic stress disorder and alcohol use disorder. Alcohol Res. 2018;39:121–9.
13. Back SE, Brady KT, Sonne SC, Verduin ML. Symptom improvement in co-occurring PTSD and alcohol dependence. J Nerv Ment Dis. 2006;194:690–6.

14. McFall ME, Mackay PW, Donovan DM. Combat-related posttraumatic stress disorder and severity of substance abuse in Vietnam veterans. J Stud Alcohol. 1992;53:357–63.

15. Brown PJ, Stout RL, Gannon-Rowley J. Substance use disorder-PTSD comorbidity. Patients' perceptions of symptom interplay and treatment issues. Subst Abuse Treat. 1998;15:445–8.

16. Dworkin ER, Wanklyn S, Stasiewicz PR, Coffey SF. PTSD symptom presentation among people with alcohol and drug use disorders: Comparisons by substance of abuse. Addict Behav. 2018;76:188–94.

17. Read JP, Wardell JD, Colder CR. Reciprocal associations between PTSD symptoms and alcohol involvement in college: a three-year trait-state-error analysis. J Abnorm Psychol. 122:984–97.

18. Able ML, Benedek DM. Severity and Symptom Trajectory in Combat-Related PTSD: a Review of the Literature. Curr Psychiatry Rep. 2019;21:58.

19. Stanley IH, Rogers ML, Hanson JE, Gutierrez PM, Joiner TE. PTSD symptom clusters and suicide attempts among high-risk military service members: A three-month prospective investigation. J Consult Clin Psychol. 2019;87:67–78.

20. Shalev AY, Orr SP, Peri T, Schreiber S, Pitman RK. Physiologic responses to loud tones in Israeli patients with posttraumatic stress disorder. Arch Gen Psychiatry. 1992;49:870–5.

21. Shalev AY, Peri T, Orr SP, Bonne O, Pitman RK. Auditory startle responses in help-seeking trauma survivors. Psychiatry Res. 1997;69:1–7.

22. Shalev AY, Peri T, Brandes D, Freedman S, Orr SP, Pitman RK. Auditory startle response in trauma survivors with posttraumatic stress disorder: a prospective study. Am J Psychiatry. 2000;157:255–61.

23. Southwick SM, Morgan CA 3rd, Darnell A, Bremner D, Nicolaou AL, Nagy LM, Charney DS. Trauma-related symptoms in veterans of Operation Desert Storm: a 2-year follow-up. Am J Psychiatry. 1995;152:1150–5.

24. Morgan CA 3rd, Grillon C, Southwick SM, Davis M, Charney DS. Exaggerated acoustic startle reflex in Gulf War veterans with posttraumatic stress disorder. Am J Psychiatry. 1996;153:64–8.

25. Grillon C, Morgan CA, Southwick SM, Davis M, Charney DS. Baseline startle amplitude and prepulse inhibition in Vietnam veterans with posttraumatic stress disorder. Psychiatry Res. 1996;64:169–78.
26. Grillon C, Morgan CA 3rd, Davis M, Southwick SM. Effects of experimental context and explicit threat cues on acoustic startle in Vietnam veterans with posttraumatic stress disorder. Biol Psychiatry. 1998;44:1027–36.

27. Ornitz EM, Pynoos RS. Startle modulation in children with posttraumatic stress disorder. Am J Psychiatry. 1989;146:866–70.

28. Medina AM, Mejia VY, Schell AM, Dawson ME, Margolin G. Startle reactivity and PTSD symptoms in a community sample of women. Psychiatry Res. 2001;101:157–69.

29. Lebron-Milad K, Milad MR. Sex differences, gonadal hormones and the fear extinction network: implications for anxiety disorders. Biol Mood Anxiety Disord. 2012;2:3.

30. Shansky RM. Sex differences in PTSD resilience and susceptibility: challenges for animal models of fear learning. Neurobiol Stress. 2015;1:60–5.

31. Cohen H, Zohar J, Matar M. The relevance of differential response to trauma in an animal model of posttraumatic stress disorder. Biol Psychiatry. 2003. 53:463–73.

32. Cohen H, Matar MA, Richter-Levin G, Zohar J. The contribution of an animal model toward uncovering biological risk factors for PTSD. Ann N Y Acad Sci. 2006;1071:335–50.

33. Corley MJ, Caruso MJ, Takahashi LK. Stress-induced enhancement of fear conditioning and sensitization facilitates extinction-resistant and habituation-resistant fear behaviors in a novel animal model of posttraumatic stress disorder. Physiol Behav. 2012;105:408–16.

34. Kondoh K, Lu Z, Ye X, Olson DP, Lowell BB, Buck LB. A specific area of olfactory cortex involved in stress hormone responses to predator odours. Nature. 2016;532:103–6.

35. Edwards S, Baynes BB, Carmichael CY, Zamora-Martinez ER, Barrus M, Koob GF, Gilpin NW. Traumatic stress reactivity promotes excessive alcohol drinking and alters the balance of prefrontal cortex-amygdala activity. Transl Psychiatry. 2013;3:e296.

36. Takahashi LK, Nakashima BR, Hong H, Watanabe K. The smell of danger: a behavioral and neural analysis of predator odor-induced fear. Neurosci Biobehav Rev. 2005;29:1157–67.

37. Endres T, Apfelbach R, Fendt M. Behavioral changes induced in rats by exposure to trimethylthiazoline, a component of fox odor. Behav Neurosci. 2005;119:1004–10.

38. Mackenzie L, Nalivaiko E, Beig MI, Day TA, Walker FR. Ability of predator odour exposure to elicit conditioned versus sensitised post traumatic stress disorder-like behaviours, and forebrain deltaFosB expression, in rats. Neuroscience. 2010;169:733–42.

39. Roltsch EA, Baynes BB, Mayeux JP, Whitaker AM, Baiamonte BA, Gilpin NW. Predator odor stress alters corticotropin-releasing factor-1 receptor (CRF1R)-dependent behaviors in rats. Neuropharmacology. 2014;79:83–9.
40. Albrechet-Souza L, Gilpin NW. The predator odor avoidance model of post-traumatic stress disorder in rats. Behav Pharmacol. 2019;30:105–14.

41. Ferrero DM, Lemon JK, Fluegge D, Pashkovski SL, Korzan WJ, Datta SR, Spehr M, Fendt M, Liberles SD. Detection and avoidance of a carnivore odor by prey. Proc Natl Acad Sci U S A. 2011;108:11235–40.

42. Simms JA, Steensland P, Medina B, Abernathy KE, Chandler LJ, Wise R, Bartlett SE. Intermittent access to 20% ethanol induces high ethanol consumption in Long-Evans and Wistar rats. Alcohol Clin Exp Res. 2008;32:1816–23.

43. Cohen H, Kozlovsky N, Alona C, Matar MA, Joseph Z. Animal model for PTSD: from clinical concept to translational research. Neuropharmacology. 2012;62:715–24.

44. Schreiber AL, Lu YL, Baynes BB, Richardson HN, Gilpin NW. Corticotropin-releasing factor in ventromedial prefrontal cortex mediates avoidance of a traumatic stress-paired context. Neuropharmacology. 2017;113:323–30.

45. Debell F, Fear NT, Head M, Batt-Rawden S, Greenberg N, Wessely S, Goodwin L. A systematic review of the comorbidity between PTSD and alcohol misuse. Soc Psychiatry Psychiatr Epidemiol. 2014;49:1401–25.

46. Whitaker AM, Gilpin NW. Blunted hypothalamo-pituitary adrenal axis response to predator odor predicts high stress reactivity. Physiol Behav. 2015;147:16–22.

47. Davis M, Falls WA, Campeau S, Kim M. Fear-potentiated startle: a neural and pharmacological analysis. Behav Brain Res. 1993;58:175–98.

48. Grillon C. Models and mechanisms of anxiety: evidence from startle studies. Psychopharmacology. 2008;199:421–37.

49. Acheson DT, Geyer MA, Risbrough VB. Psychophysiology in the study of psychological trauma: where are we now and where do we need to be? Current Topics in Behavioral Neurosciences. 2014;21:157–83.

50. Zoladz PR, Diamond DM. Current status on behavioral and biological markers of PTSD: a search for clarity in a conflicting literature. Neurosci Biobehav Rev. 2013;37:860–95.

51. Pole N. The psychophysiology of posttraumatic stress disorder: a meta-analysis. Psychol Bulletin. 2007;133:725–46.

52. McTeague LM, Lang PJ, Laplante MC, Cuthbert BN, Shumen JR, Bradley MM. Aversive imagery in posttraumatic stress disorder: trauma recurrence, comorbidity, and physiological reactivity. Biol Psychiatry. 2010;67:346–56.

53. Birkeland MS, Blix I, Solberg Ø, Heir T. Gender Differences in Posttraumatic Stress Symptoms after a Terrorist Attack: A Network Approach. Front Psychol. 2017;8:2091.
54. McCarthy E, Petrakis I. Epidemiology and management of alcohol dependence in individuals with post-traumatic stress disorder. CNS Drugs. 2010;24:997–1007.

55. Sells JR, Waters AJ, Schwandt ML, Kwako LE, Heilig M, George DT. Characterization of comorbid PTSD in treatment-seeking alcohol dependent inpatients: Severity and personality trait differences. Drug Alcohol Depend. 2016;163:242–6.

56. Nelson NG, Suhaidi FA, Law WX, Liang NC. Chronic moderate alcohol drinking alters insulin release without affecting cognitive and emotion-like behaviors in rats. Alcohol. 2018;70:11–22.

57. Carnicella S, Ron D, Barak S. Intermittent ethanol access schedule in rats as a preclinical model of alcohol abuse. Alcohol. 2014;48:243–52.

58. Beck KD, Servatius RJ. Stress-induced reductions of sensory reactivity in female rats depend on ovarian hormones and the application of a painful stressor. Horm Behav. 2005;47:532–9.

59. Beck KD, Servatius RJ. Interleukin-1beta as a mechanism for stress-induced startle suppression in females. Ann N Y Acad Sci. 2006;1071:534–7.

60. Fernandes C, González MI, Wilson CA, File SE. Factor analysis shows that female rat behaviour is characterized primarily by activity, male rats are driven by sex and anxiety. Pharmacol Biochem Behav. 1999;64:731–8.

61. Gruene TM, Flick K, Stefano A, Shea SD, Shansky RM. Sexually divergent expression of active and passive conditioned fear responses in rats. Elife. 2015;4 pii:e11352.

62. Babb JA, Masini CV, Day HE, Campeau S. Sex differences in activated corticotropin-releasing factor neurons within stress-related neurocircuitry and hypothalamic-pituitary-adrenocortical axis hormones following restraint in rats. Neuroscience. 2013;234:40–52.

63. Homiack D, O’Cinneide E, Hajmurad S, Barrileaux B, Stanley M, Kreutz MR, Schrader LA. Predator odor evokes sex-independent stress responses in male and female Wistar rats and reduces phosphorylation of cyclic-adenosine monophosphate response element binding protein in the male, but not the female hippocampus. Hippocampus. 2017;27:1016–29.

64. Adamec R, Strasser K, Blundell J, Burton P, McKay DW. Protein synthesis and the mechanisms of lasting change in anxiety induced by severe stress. Behav Brain Res. 2006;167:270–86.
Figure Legends

**Fig. 1** Schematic representation of the experimental design. Male and female rats underwent the conditioned place aversion paradigm using bobcat urine. Avoidance behavior was measured on day 5 (24 hours post-stress). Controls were never exposed to predator odor. Rats with a history of alcohol consumption (intermittent access 2-bottle choice, 5 weeks) were exposed to the same procedure starting 24 hours after the last drinking session. Acoustic startle reactivity was evaluated on day 6 (2 days post-stress) and anxiety-like behavior was tested on day 21 (17 days post-stress).

**Fig. 2** Traumatic stress response in alcohol-naïve rats. **a** Change in time spent in predator odor-paired chamber in male and female rats indexed as Avoiders or Non-Avoiders 24 hours post-stress. **b** Avoidance distribution (Avoiders × Non-Avoiders) in male and female rats. **c** Weight gain in male and female rats measured 24 hours after exposure to predator odor. **d** Acoustic startle response in male and female rats measured 48 hours after exposure to predator odor stress. **e** Percent time spent in the open arms of the elevated plus-maze in male and female rats on day 21 (17 days post-stress). **f** Frequency of entries into the closed arms of the elevated plus-maze in male and female rats on day 21 (17 days post-stress). Data presented as mean ± SEM. * denotes P < 0.05 between indicated groups; # denotes P < 0.05 between sexes.

**Fig. 3** Traumatic stress response in rats given intermittent access to 20% ethanol in a 2-bottle choice paradigm for 5 weeks. **a** Change in time spent in predator odor-paired chamber in male
and female rats indexed as Avoiders or Non-avoiders 24 hours post-stress. b Avoidance distribution (Avoiders × Non-Avoiders) in male and female rats. c Weekly alcohol consumption per cage in same-sex paired-housed male and female rats. d Acoustic startle response in male and female rats measured 48 hours after exposure to predator odor stress. e Percent time spent in the open arms of the elevated plus-maze in male and female rats on day 21 (17 days post-stress). f Frequency of entries into the closed arms of the elevated plus-maze in male and female rats on day 21 (17 days post-stress). Data presented as mean ± SEM. * denotes P < 0.05 between indicated groups; # denotes P < 0.05 between sexes.

**Fig. 4** Plasma corticosterone levels measured at baseline (BL) and immediately following 15 minutes exposure to bobcat urine (Stress) in male and female rats. Data presented as mean ± SEM. * denotes P < 0.05.
Figure 1

Day 1
3-chamber pre-test

Day 2
pre-exposure session

Day 3
neutral exposure

Day 4
odor exposure

Day 5
post-exposure session

Day 6
acoustic startle

Day 21
elevated plus-maze
Figure 2

(a) Δ Time in Odor-Paired Context

(b) Avoidance Distribution

(c) Weight gain 24h post-stress

(d) Acoustic Startle

(e) %Time Open Arms

(f) Closed Arms Entries
Figure 3

(a) Δ Time in Odor-Paired Context

(b) Avoidance Distribution

(c) Alcohol Consumption/Cage

(d) Acoustic Startle

(e) % Time Open Arms

(f) Closed Arms Entries
Figure 4

Plasma Corticosterone

* * *

Males
Females

ng/ml

BL Stress BL Stress

0 500 1000 1500 2000