Male and female mice respectively form stronger social aversive memories with same and different sex conspecifics

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Abstract

Mice offer a wealth of opportunities for investigating brain circuits regulating multiple behaviors, largely due to their genetic tractability. Social behaviors are of translational relevance, considering both mice and humans are highly social mammals, and disruptions in human social behavior are key symptoms of myriad neuropsychiatric disorders. Stresses related to social experiences are particularly influential in the severity and maintenance of neuropsychiatric disorders like anxiety disorders, and trauma and stressor-related disorders. Yet, induction and study of social stress in mice is disproportionately focused on males, influenced heavily by their natural territorial nature. Conspecific-elicited stress (i.e., defeat), while ethologically relevant, is quite variable and predominantly specific to males, making rigorous and sex-inclusive studies challenging. In pursuit of a controllable, consistent, high throughput, and sex-inclusive paradigm for eliciting social stress, we have discovered intriguing sex-specific social aversions that are dependent upon the sex of both experimental and conspecific mice. Specifically, we trained male and female F1 129S1/SvlmJ × C57BL/6J to associate (via classical conditioning) same or different sex C57BL/6J conspecifics with a mild, aversive stimulus. Upon subsequent testing for social interaction 24 h later, we found that males socially conditioned better to male conspecifics by exhibiting reduced social interaction, whereas females socially conditioned better to male conspecifics. Serum corticosterone levels inversely corresponded to social avoidance after different sex, but not same sex, conditioning, suggesting corticosterone-mediated arousal could influence cross sex interactions. While our paradigm has further optimization ahead, these current findings reveal why past pursuits to develop same sex female social stress paradigms may have met with limited success. Future research should expand investigation of utilizing male mouse conspecifics to instigate social stress across sexes.

Introduction

Social interaction behavior is a cross-mammalian phenomenon seen in humans, non-human primates, rats, hamsters, and mice, among other animals (1–6). Shifts in social interaction behavior can be informative of the physical and/or mental state of the mammal (7–9). In humans, disruptions in typical social interaction behavior are characteristic of numerous neuropsychiatric conditions including trauma and stress-related disorders, mood and anxiety disorders, schizophrenia, autism spectrum disorder, and substance use disorders (10).

Rodent studies examining social behavior and socially associated stressors within various contexts have advanced identification of neural pathways important for stress responsivity and social (dis)engagement, as well as assisted development of new therapeutic approaches and targets for neuropsychiatric disorders (6,11–20). However, social behavior research in the genetically tractable mouse species (*Mus musculus*) has been relatively limited in studying behavioral consequences (and thereby the underlying neurocircuitry and neurophysiological shifts) of negative, aversive, or otherwise stressful social interactions in *female* mice (21,22). This is largely due to most current social conditioning paradigms employing only male rodents (23–28) - something we ourselves are guilty of (14,29) - by capitalizing upon male-specific territorial aggression. A breadth of social stress techniques have been employed in hamsters, mice, and rats, including social instability (30,31), social transmission of stress (2,32), social isolation (33,34), social defeat (6,35,36), and social crowding (26,37). Of these, the most prevalent paradigm in mice is that of social defeat (both acute and chronic) (14,29,38–43). Aside from the inherent male-centric nature of social defeat stress, additional concerns of consistency and reproducibility arise from the inherently variable range of aversive social behaviors, from defensive postures to aggressive bites, that each experimental mouse perceives and experiences. Such variability - much beyond experimenter control - plus the single sex bias of social defeat stress left us seeking an improved paradigm. In particular, we sought to pair controllable, uniform, aversive stimuli with the presence of a social stimulus to study aversive social conditioning *across sexes* in mice using a high throughput approach (requiring 1 day, as opposed to multiple days or weeks).

To accomplish this, we modified a paradigm previously utilized in males (44–47). Manual administration of an aversive stimulus (mild foot shock) occurs selectively when a male mouse actively investigates a conspecific, with the goal of attenuating subsequent social engagement (44–47). Rather than using this operant-style approach, where a behavior exhibited by the experimental mouse directly affects the outcome, we instead wanted to develop a more classical conditioning approach that was less labor-intensive (i.e., did not require real-time monitoring for manual shock administration). In this way, experimental mice would not necessarily need to approach the social stimulus (conspecific) to develop an association between it and the aversive stimulus. Additionally, all mice would receive the same number of shocks, instead of variable numbers based upon behavior (44–47).

Using our modified version of this novel approach, we evaluated how employing this paradigm affected multiple measures across *both sexes* after being exposed to an assigned, previously never encountered, conspecific. Specifically, mice were socially conditioned (SC) with an aversive stimulus (mild foot shock) when in the presence of a novel conspecific, independent of investigative behavior. Then, mice were tested for social engagement in a novel environment in the presence of the same conspecific, followed by testing of behavior in the conditioning environment in the absence of any social stimulus. These behaviors were tested both under circumstances when the experimental and conspecific mice were the same sex and when they were different sexes. We hypothesized that SC mice would exhibit reduced social behavior

when tested with their assigned conspecific in a novel environment, regardless of if the SC and conspecific mice were the same or different sexes. Our broad goal was to develop a paradigm that would provide a useful first step for future investigations of social behavior across mouse sexes, which are currently underrepresented in literature (22).

Methods

Mice

Hybrid F1 offspring of both sexes resulting from pairing female 129S1/SvlmJ and male C57BL/6J x mice (hereafter experimental mice), and male and female C57BL/6J mice (hereafter conspecifics), all ≥9 weeks old or older, were group-housed (2-5 per cage) within sex. All mice had *ad libitum* access to food and water in rooms maintained on a 12:12 light/dark cycle with lights on at 07:00 local standard time, and temperature maintained at 22 ± 2°C. All mice were fed LabDiet 5001 rodent laboratory chow (LabDiet, Brentwood, MO) and were kept on 7090 Teklad Sani-chip bedding (Envigo, East Millstone, NJ) in cages containing Nestlets (Ancare, Bellmore, NY) and huts (Bio-Serv, Flemington, NJ) for enrichment. Experiments were approved by the Kent State University Institutional Animal Care and Use Committee, and adhered to the National Research Council's Guide for the Care and Use of Laboratory Animals, 8th Ed. undefined.

Social Conditioning Paradigm

The entire paradigm spans four consecutive days. Procedures for each individual day are outlined below in order.

Pre-exposure (Day 0)
Pre-exposure was used to help reduce the novelty of the transparent enclosure and/or the conspecific to the experimental mouse, plus minimize any potential sex differences in acquisition (48–51). On Day 0, experimental mice were placed in Coulbourn Instruments chambers (7 in D × 7 in W × 12 in H; Allentown, PA) with (Control A and SC mice) or without (Control B-D mice) a conspecific (age-matched; either same sex or different sex relative to experimental mouse, depending on if assigned to same sex or different sex experiment) for pre-exposure (Fig. 1). Chambers comprised two opposing aluminum walls each adjoining two clear acrylic walls. Conspecifics were put inside a transparent enclosure placed within a corner of the social conditioning chamber. The transparent enclosure included 23 holes (each 0.32 cm diameter) on each of the 2 sides facing the SC mouse to enable olfactory cue exchanges. Previous work demonstrates that olfaction is the most important modality when recognizing and interacting with novel conspecifics (see review (52)); tactile (whiskers) and auditory modalities appear to only be recruited when recognizing cage mates (46) in male mice (females were not studied). Chambers and enclosures were cleaned with 70% ethanol before and after each session; chambers had visible illumination and one clear acrylic wall was marked with a blue dotted pattern. These components contributed olfactory and visual cues to the context, in addition to the tactile cue of the stainless steel grid floor for the experimental mouse. During pre-exposure, experimental mice were allowed to explore the chamber and conspecific enclosure for 5 min, then both mice were returned to their respective home cages.

Social Conditioning (Day 1)
On Day 1, experimental mice were placed in the same social conditioning chamber as Day 0 and received five, 1 s mild foot shocks (1.0 mA) in the presence of the same conspecific that they encountered on Day 0 (Figure 1A). This was to have experimental mice form associations between aversive foot shocks and their respective conspecific (Fig. 1). Conditioning lasted for 9 minutes; after a 2 minute baseline, shocks were then administered at 120, 210, 300, 390, and 480 s. Conspecifics did not receive foot shocks. Cameras mounted above the chambers were used to record movements, and freezing behavior during conditioning was quantified using FreezeFrame software (v. 5.201, Actimetrics, Wilmette, IL). Freezing behavior is indicative of

fear/aversive conditioning in mice; freezing was defined as the absence of movement except breathing.

Social Interaction Testing (Day 2)
On Day 2, experimental mice were placed in a novel room containing open arenas (41.9 cm W × 41.9 cm D × 39.6 cm H). Mice acclimated to the room in their home cage for 30 min prior to behavior testing commencing. Each open arena had one empty PVC tube (8.9 cm outer diameter) in a single corner of the square chamber with wire mesh (6.35 mm^2 openings) covering a rectangular portion (11.4 cm W x 4.1 cm H) cut out of the tube bottom. Experimental mice were placed in the corner of the arena opposite the PVC tube and could investigate freely for 2.5 minutes (pre-test). Immediately after these 2.5 min, the conspecific that each experimental mouse had previously been conditioned with on Day 1 was then placed in the PVC tube in the corner. Experimental mice were allowed to continue investigating the arena for an additional 5 min (post-test; Fig. 1). Because of the mesh at the bottom of the PVC tubes containing conspecifics, exchanges of visual, auditory, and olfactory cues between experimental mice and conspecifics were possible, but tactile interactions were minimized. Interaction behavior was quantified as duration when at least 80% of an experimental mouse's body was in the interaction zone, a 7 cm radius zone (~223 cm^2 – 240 cm^2, i.e., 14-15% of the open arena) around the base of the PVC tube. This was quantified using ANY-maze software (v. 7.09 Stoelting Co., Wood Dale, IL). Post-test social interaction time was log-transformed ($Y=\log(Y+0.001)$ to account for 3 mice with 0 s post-test social interaction) so that data would be normally distributed for statistical analyses. Females were intentionally not assessed for estrus cycle stage for five reasons: 1) to minimize mouse usage (53), we did not power our studies for assessment of estrus; 2) with the goal of developing a social stress paradigm, we are seeking effects robust enough to not depend upon estrus in intact, randomly cycling females; 3) we intentionally focused here on fear and social behaviors, and did not measure sexual behaviors (e.g., lordosis); 4) evidence that overall mouse behaviors are not affected by estrus stage (54,55), (but see (56)); 5) cross-species evidence indicates vaginal lavage to determine estrus cycle is stressful (57,58), and we sought to minimize stress confounds here.

Context Testing (Day 3)
On the last day of the social conditioning paradigm, experimental mice were placed in the social conditioning chamber for behavior testing in the *absence* of any conspecific (Fig. 1). The conspecific enclosure was still present to keep the context consistent with conditioning. All other tactile, visual, and olfactory cues from Day 1 were present. Testing lasted 10 min and did not involve any foot shocks. Freezing behavior was again quantified using FreezeFrame software.

Treatment Groups

Timelines illustrating the SC group plus four accompanying Control groups are presented in Fig. 1. The SC group involved mice that underwent social conditioning in the presence of a non-shocked conspecific (either same sex or different sex relative to SC mouse, depending upon experiment), with the hypothesis that SC mice would associate the conspecific with this aversive experience. We planned four Control groups for this one SC group; each experiment (same sex or different sex) had its own respective set of four Control groups for its respective SC group. The goals of these were to control for: A) foot shock exposure; B) presence of conspecific during pre-exposure & foot shock; C & D) exposure to conspecific temporally distal to foot shock exposure. Control A involved procedures identical to those of the SC group, except a foot shock was never administered on Day 1 (Fig. 1). Control B was the same as the SC group procedure, except experimental mice never encountered the conspecific until testing on Day 2 (Fig. 1).

Controls C and D had experimental mice encountering conspecifics either 4 h before (Control C) or 4 h after (Control D) pre-exposure and social conditioning; in other words, conspecifics were not present in the conditioning chamber during these two periods. Instead, experimental mice were exposed to their conspecific in a separate room for 5 minutes or 9 minutes (the same amount of time as pre-exposure or social conditioning, respectively) 4 h before (Control C) or after (Control D) the experimental mice were exposed to the conditioning chamber. This exposure involved experimental mice and their respective conspecific being placed in a clean mouse cage separated with an acrylic divider to allow for visual, auditory, and olfactory exchange, but no tactile interaction. Mice in Control C or D conditions still experienced foot shock on Day 1, and encountered their assigned conspecific for testing on Day 2 (Fig. 1). This temporal separation of 4 h was to minimize consolidation of social encounters from interfering/intermingling with consolidation of the aversive foot shock encounter in the social conditioning chamber, while still making execution of these experiments feasible within a 12 h lights-on period (59–61) (see reviews (62,63)).

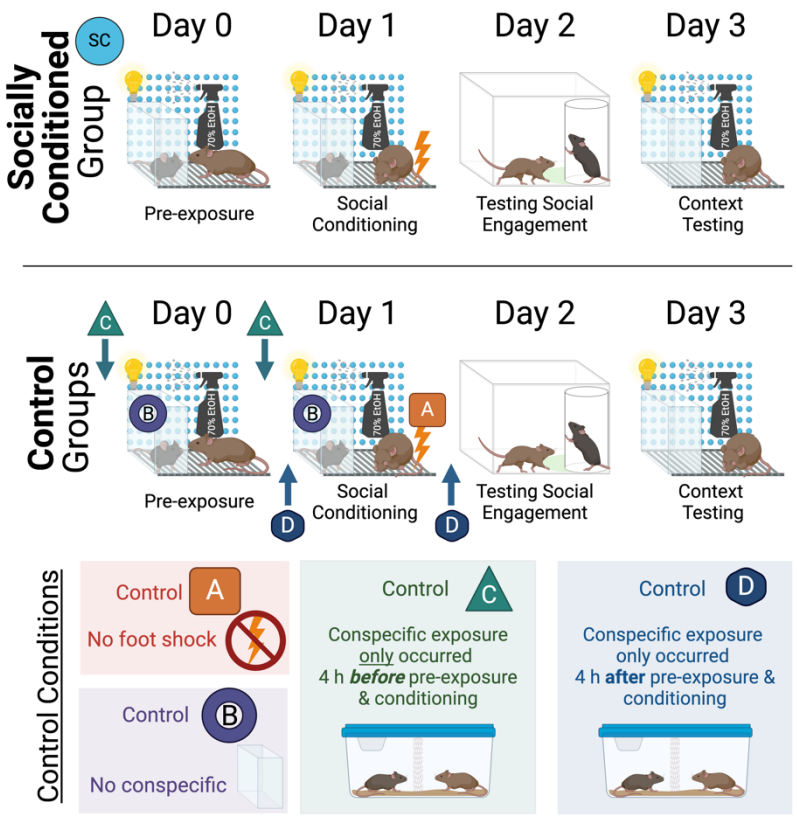

Figure 1. Study timeline and treatment groups. Five groups were used in this study for each experiment (same sex experiment, and different sex experiment): one socially conditioned (SC) group and four Control groups. Mice in the SC group (above horizontal line) underwent a 5 min pre-exposure (Day 0) in the social conditioning chamber and encountered their assigned conspecific (same sex or different sex relative to SC mouse, depending upon experiment) that was restricted to a clear plastic enclosure containing small holes for olfactory, visual, and auditory, but not tactile, exchanges. On Day 1, SC mice were returned to the social conditioning chamber and received five mild foot shocks in the presence of the same conspecific (conspecific did not receive foot shocks); this social conditioning procedure lasted 9 min. The

following day (Day 2), SC mice were tested for social engagement in a novel arena, first in the absence of the conspecific (2.5 min), then in the presence of the conspecific (5 min), the latter with the conspecific confined to a PVC tube with wire mesh to again allow visual, olfactory, and auditory, but not tactile, exchanges. Finally, the last day of the four day paradigm (Day 3) involved exposing SC mice to the social conditioning chamber in the absence of their conspecific to test for fear behavior, indexed by percent time spent freezing (i.e., absence of all movement except breathing). Details for the four Control groups are provided below the horizontal line; each Control group was composed of different mice. Control A mice underwent the same exact procedure as detailed for the SC group (above horizontal line), except no foot shock was administered on Day 1. Control B mice similarly experienced the same procedure as mice in the SC group (above horizontal line), save that Control B mice did not encounter any conspecific until Day 2 for testing of social engagement. Control C involved mice encountering their conspecific 4 h before pre-exposure and social conditioning, meaning no conspecific was present for Control C mice when they were in the social conditioning chamber for pre-exposure or social conditioning. To encounter their assigned conspecific, Control C mice were placed into a clean cage with a clear acrylic divider separating them from their conspecific. This divider allowed visual, olfactory, and auditory exchanges but no tactile interactions. On Day 0, Control C mice encountered their conspecific for 5 min; on Day 1, for 9 min; these were timed to match the length of conspecific exposure that mice in the SC group experienced. Control D mice were treated the same as Control C mice, except their encounters with their assigned conspecifics occurred 4 h *after*, rather than before, pre-exposure and social conditioning.

Serum Corticosterone

Thirty minutes after all experimental mice underwent contextual fear testing on Day 3, they were briefly anesthetized with isoflurane then rapidly decapitated for trunk blood collection. Blood clotted at room temperature for 10 minutes, then was spun at 3500 rpm for 1 h at 4°C. Serum was collected and stored at -80°C until corticosterone analyses could be performed. Serum corticosterone was measured using Enzo Life Sciences corticosterone enzyme-linked immunosorbent assay (ELISA) kits (Farmingdale, NY). Assays were run according to the manufacturer's instructions using their small volume protocol. Plates were read at 405 nm with correction at 580 nm. The sensitivity of the assay was 26.99 pg/mL.

Statistical Analyses

Data were graphed with GraphPad Prism 10.3.0 (442), Beta (GraphPad Software, San Diego, CA), and analyzed using IBM SPSS Statistics 28.0.0.0 (IBM, Armonk, NY), with the significance threshold set *a priori* at $p<0.05$. Non-significant trends ($p<0.10$) were mentioned only when the associated partial η^2 was >0.060. Data were graphed as the mean ± 95% confidence interval (CI). Details of identified outliers (greater than the mean ± 4 standard deviations) are provided in Supplemental Details. The same sex experiment and different sex experiment each included their own SC group plus accompanying four Control groups. Control A mice were analyzed across experiments. Within each experiment, the SC group and other three Control groups (all of which experienced foot shocks, i.e., shocked mice) were analyzed. Social conditioning and context fear expression time course were analyzed using 3-way repeated measures ANOVAs (shocked mice: time × treatment group × sex of experimental mouse) (Control A mice: time × experiment × sex of experimental mouse) and pairwise comparisons with Bonferroni correction. Greenhouse Geisser corrections were utilized for within-subjects analyses. Measurements of contextual fear expression average, social interaction, and serum corticosterone were analyzed with a 2-way ANOVA (shocked mice: treatment group × sex of experimental mouse) (Control A

mice: experiment × sex of experimental mouse) and pairwise comparisons with Bonferroni correction.

Results

Social Conditioning Acquisition

Social conditioning acquisition was examined in experimental mice in either the presence or absence of a conspecific. One experiment used same-sex conspecifics, and the other experiment used different sex conspecifics; each of these experiments included its own SC group and corresponding four Control groups.

Control A (no foot shock) mice
Control A mice were utilized to distinguish the effects of receiving mild foot shocks on subsequent social behavior. Figure 2A and 2C show social conditioning 'acquisition' on Day 1 for Control A mice with either a same sex conspecific (Figure 2A) or a different sex conspecific (Figure 2C). For acquisition, there was a significant three-way interaction of time × conspecific sex × experiment (Table 1). This appears to be driven primarily by inconsistent time course patterns across sexes, and mice in the different sex experiment exhibiting lower freezing levels at several timepoints compared to same sex experiment mice (Figure 2A, 2C).

Table 1. Three-way repeated measures ANOVAs on social conditioning acquisition for Control A (no foot shock) mice across experiments and sexes.

Control A Mice - Fear Acquisition			
	F Statistic	**p value**	**Partial η^2**
Time	F(3.325,86.45)=0.334	0.821	0.013
Sex	F(1,26)=1.265	0.271	0.046
Experiment	F(1,26)=14.520	<0.001	0.358
Time × Sex	F(3.325,86.45)=1.836	0.141	0.066
Time × Experiment	F(3.325,86.45)=1.128	0.345	0.042
Sex × Experiment	F(1,26)=0.639	0.431	0.024
Time × Sex × Experiment	**F(3.325,86.45)=2.642**	**0.049**	**0.092**

Two days after social 'conditioning' (see Figure 1 for timeline), Control A mice were tested for their fear to the context in which they were 'conditioned'. Testing lasted for 10 minutes, and the time course data for testing Control A mice are in Figure 2B (same sex conspecific) and 2D (different sex conspecific). We observed no significant three-way interaction of time × sex × experiment (Table 2). There were likewise no significant two-way interactions of time × sex, time × experiment, or sex × experiment (Table 2). Main effects of sex and time were not significant, though a non-significant trend for time was noted (p=0.099; Table 2). A significant main effect of experiment was detected (Table 2), and pairwise comparisons support a pattern of female and male mice in the different sex experiment freezing less than mice in the same sex experiment (Figure 2B, 2D), as observed with 'training'.

Table 2. Three-way repeated measures ANOVA on context testing time course data for Control A (no foot shock) mice across experiments and sexes.

Control A Mice - Context Testing Time Course			
	F Statistic	**p value**	**Partial η^2**
Time	*F(7.884,205.0)=1.710*	*0.099*	*0.062*
Sex	F(1,26)=0.391	0.537	0.015
Experiment	**F(1,26)=16.16**	**<0.001**	**0.383**
Time × Sex	F(7.884,205.0)=0.599	0.776	0.023
Time × Experiment	F(7.884,205.0)=1.063	0.391	0.039

Sex × Experiment	F(1,26)=0.004	0.949	0.000
Time × Sex × Experiment	F(7.884,205.0)=0.963	0.465	0.036

Consistent with 'training' and time course testing of Control A mice, averaged freezing during minutes two through six of testing revealed no sex × experiment interaction, but a significant main effect of experiment (Table 3). Once more, different sex experiment mice exhibited less freezing than their counterparts in the same sex experiment (Figure 2E).

Table 3. Two-way ANOVA on context testing for Control A (no foot shock) mice across experiments and sexes.

Control A Mice - Context Testing Average			
	F Statistic	**p value**	**Partial η^2**
Sex	F(1,26)=0.050	0.825	0.002
Experiment	**F(1,26)=15.00**	**<0.001**	**0.366**
Sex × Experiment	F(1,26)=0.045	0.833	0.002

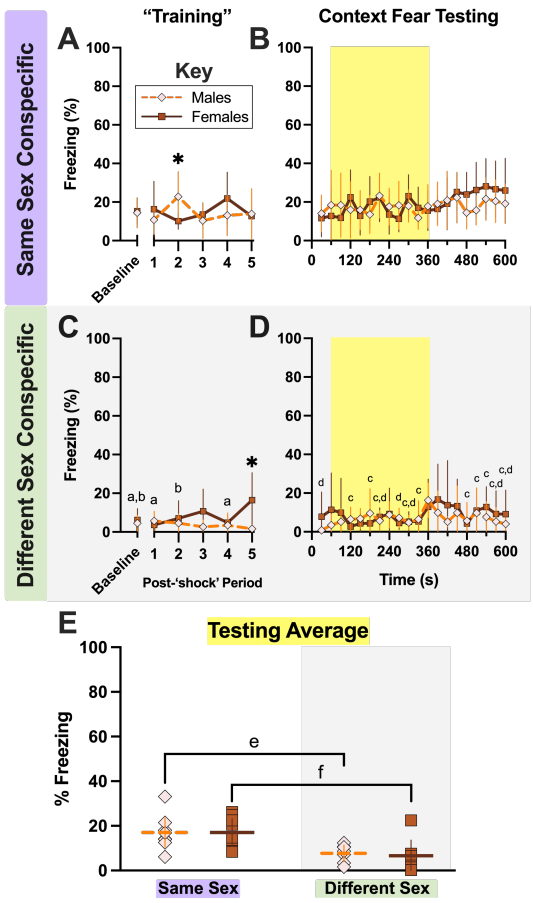

Figure 2. ***Day 1 social conditioning 'acquisition' and Day 3 context fear testing in Control A (no foot shock) mice***. Control A mice underwent 'acquisition' or 'training' for social conditioning after a two min baseline period on Day 1 (Panels A,C). Percent time freezing for the same 30 s periods that are graphed for SC and Control B-D mice (Figure 3) are indicated at time points 1-5 in Panels A,C. On Day 3, freezing behavior was quantified for 10 min when mice were re-exposed to the social conditioning chamber (Panels B,D). Average freezing behavior was evaluated between minutes two through six (yellow shading in Panels B,D) of the 10 min

test and graphed in Panels C,F. Mice either went through the Control A social conditioning process with same sex conspecifics (Panels A,B,E) or different sex conspecifics (Panels C,D,E). Male Control A mice are represented by diamonds and dashed orange lines; female Control A mice are represented by squares and solid brown lines. Same sex n=8, different sex n=7. *indicates p=0.017 (A), p=0.026 (Panel C) compared to other sex within the same experiment. [a]indicates p=0.026, p=0.026, p=0.007, left to right, comparing females in same sex (Panel A) versus different sex (Panel C) experiment 'training' at specified timepoints. [b]indicates p=0.016, p=0.002, left to right, comparing males in same sex (Panel A) versus different sex (Panel C) experiment 'training' at specified timepoints. [c]indicates p=0.008, p=0.020, p=0.018, p<0.001, p=0.027, p=0.001, p=0.037, p=0.033, p=0.020, p=0.027, left to right, comparing females in same sex (Panel B) versus different sex (Panel D) experiment testing at specified timepoints. [d]indicates p=0.024, p=0.006, p=0.030, p=0.011, p=0.030, p=0.046, left to right, comparing males in same sex (Panel B) versus different sex (Panel D) experiment testing at specified timepoints. [e]indicates p=0.016, [f]indicates p=0.008, comparing across experiments within sex for average testing freezing (Panel E). Data graphed as mean ± 95% CI.

Socially Conditioned and Control B-D mice

Social conditioning acquisition for mice that received mild foot shocks are illustrated in Figure 3A, 3B for mice in the same sex conspecific experiment, and in Figure 3C, 3D for mice in the different sex conspecific experiment. All mice expressed >40% freezing during at least one post-shock period during social conditioning. For acquisition in the same sex experiment, no three-way interaction of time × sex × group (of which there are four: socially conditioned mice or Control B-D mice) occurred (Table 4). We did not observe any significant two-way interactions either (Table 4). Nonetheless, significant main effects of time, sex, and group were found for mice in the same sex experiment (Table 4).

Table 4. Three-way repeated measures ANOVAs on fear acquisition for mice of both sexes with same sex conspecifics.

Same Sex Experiment – Fear Acquisition			
	F Statistic	**p value**	**Partial η^2**
Time	**F(4.05,223.0)=145.923**	**<0.001**	**0.726**
Sex	**F(1,55)=14.90**	**<0.001**	**0.213**
Group	**F(3,55)=5.322**	**0.003**	**0.225**
Time × Group	F(12.16,223.0)=1.014	0.437	0.052
Time × Sex	F(4.05,223.0)=1.766	0.136	0.031
Group × Sex	F(3,55)=1.833	0.143	0.093
Time × Group × Sex	F(12.16,223.0)=0.550	0.882	0.029

Pairwise comparisons indicated that socially conditioned males exhibited less freezing at two training timepoints relative to Control B mice (Figure 3A) in the same sex experiment. For all but the last post-shock period in the same sex experiment, female socially conditioned mice froze significantly more than male socially conditioned mice (Figure 3A, 3B).

Social conditioning acquisition for mice in the different sex experiment is shown in Figure 3C, 3D. No significant three-way interaction of time × sex × group was observed (Table 5), though a non-significant trend (p=0.065) was noted. While no sex × group interaction was detected, we did find significant time × group and time × sex interactions (Table 5).

Table 5. Three-way repeated measures ANOVAs on fear acquisition for mice of both sexes with different sex conspecifics.

Different Sex Experiment – Fear Acquisition			
	F Statistic	**p value**	**Partial η^2**
Time	F(4.264,243.0)=204.2	<0.001	0.782
Sex	F(1,57)=5.383	0.024	0.086
Group	F(3,57)=1.027	0.387	0.051
Time × Group	**F(12.79,243.0)=3.753**	**<0.001**	**0.165**
Time × Sex	**F(4.264,243.0)=2.752**	**0.026**	**0.046**
Group × Sex	F(3,57)=0.938	0.428	0.047
Time × Group × Sex	*F(12.79,243.0)=1.689*	*0.065*	*0.082*

Only the second post-shock period was different across sexes in SC mice in the different sex experiment (Figure 3C, 3D). For Control C mice, females displayed greater freezing for the first three post-shock periods than males in the different sex experiment (Figure 3C, 3D). All other between-group comparisons within each sex did not exhibit consistent patterns, and as with same sex experiment mice, different sex experiment mice concluded training at similar freezing levels. Combined with same sex experiment acquisition, these findings indicate that social exposure – whether concurrent or 4 h prior – can transiently augment social conditioning acquisition in female mice, depending upon the sex of the conspecific.

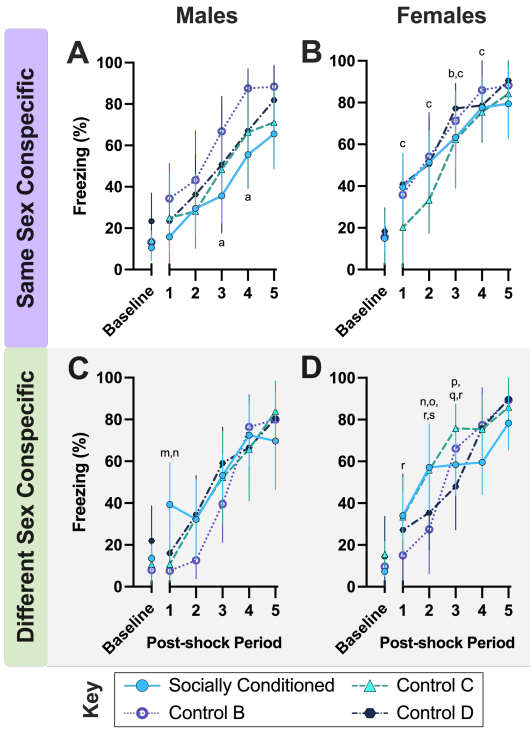

Figure 3. ***Day 1 acquisition during social conditioning procedure***. Socially conditioned (SC) mice are represented by filled circles and solid lines, with data for the same sex experiment in A and B (male n=8; female n=9), and the different sex experiment in C and D (male n=9; female n=8). Control B mice are indicated by open circles and dotted lines; same sex experiment data are in A and B (male n=8; female n=8), and different sex experiment data in C and D (male n=8; female n=8). Control C data are shown with triangles and dashed lines for the same sex (A, males n=7; B, females n=9) and different sex (C, male n=8; D, female n=9) experiments. Control D mice are graphed with hexagons and dashed/dotted lines for same sex (A, male n=7; B, female n=8) and different sex (C, male n=8; D, female n=8) experiments.

Average freezing for the first two minutes, prior to commencement of acquisition, is plotted on the x-axis as baseline. The average percent freezing for each 30 second period following each of the five mild foot shocks are thereafter plotted along x-axis (Post-shock Periods 1-5). *Same sex experiment*: [a]indicates p=0.037, p=0.006 (left to right, panel A) when comparing within males and between SC and Control B groups. [b]indicates p=0.022 (Panel B) when comparing between sexes and within Control D group. [c]indicates p=0.011, p=0.047, p=0.012, p=0.016 (left to right, Panel B) when comparing between sexes within the SC group. *Different sex experiment*: [m]indicates p=0.048 (Panel C) when comparing within males and between Control C and SC groups. [n]indicates p=0.015 (Panel C) or p=0.043 (Panel D) when comparing within sex and between Control B and SC groups. [o]indicates p=0.048 (Panel D) when comparing within females and between Control B and C groups. [p]indicates p=0.036 (Panel D) when comparing within females and between Control C and D groups. [q]indicates p=0.011 (Panel D) when comparing between sexes and within Control B group. [r]indicates p=0.034, p=0.036, p=0.023 (left to right, Panel D) when comparing between sexes and within Control C group. [s]indicates p=0.019 (Panel D) when comparing between sexes and within SC group. Data graphed as mean ± 95% CI.

Social Interaction

Twenty four hours after social conditioning, mice were tested for social engagement using the social interaction test. Three mice did not interact at all with their conspecific, and thus were excluded from social interaction analyses (1 different sex Control A female; 1 same sex Control B female; 1 same sex SC male; see Supplemental Details). Figure 4A illustrates social interaction data for Control A mice across experiments. There was no significant sex × experiment interaction, but there was a significant main effect of experiment (Table 6). This was driven by males in the different sex experiment interacting more with female than male conspecifics (Figure 4A).

Table 6. Two-way ANOVA on social interaction for Control A mice across experiments and sexes.

Control A Mice – Log-transformed Social Interaction			
	F Statistic	**p value**	**Partial η^2**
Sex	F(1,23)=0.429	0.519	0.018
Experiment	**F(1,23)=8.529**	**0.008**	**0.271**
Sex × Experiment	F(1,23)=2.773	0.109	0.108

Social conditioning in the same sex experiment did not result in a sex × group interaction, but a main effect of group was found (Table 7). Pairwise comparisons indicated that SC males exhibited less social interaction than SC females (Figure 4B).

Table 7. Two-way ANOVA on social interaction for mice of both sexes with same sex conspecifics.

Same Sex Experiment – Log-transformed Social Interaction			
	F Statistic	**p value**	**Partial η^2**
Sex	F(1,52)=0.438	0.511	0.008
Group	**F(3,52)=2.923**	**0.042**	**0.144**
Sex × Group	F(3,52)=1.870	0.146	0.097

In contrast to the same sex experiment, there were no significant interactions nor main effects in the different sex experiment (Table 8). A non-significant trend for group was noted (p=0.066,

Table 8). Pairwise comparisons demonstrated that females exhibited significantly reduced social interaction, both compared to female Control B mice and to SC male mice (Figure 4C). Combined, these findings indicate that the social behavior of females is more affected by aversive associations with males, whereas males are more socially affected by aversive associations with their own sex.

Table 8. Two-way ANOVA on social interaction for mice of both sexes with different sex conspecifics.

Different Sex Experiment – Log-transformed Social Interaction			
	F Statistic	**p value**	**Partial η^2**
Sex	F(1,56)=0.405	0.527	0.007
Group	*F(3,56)=2.538*	*0.066*	*0.120*
Sex × Group	F(3,56)=1.678	0.182	0.082

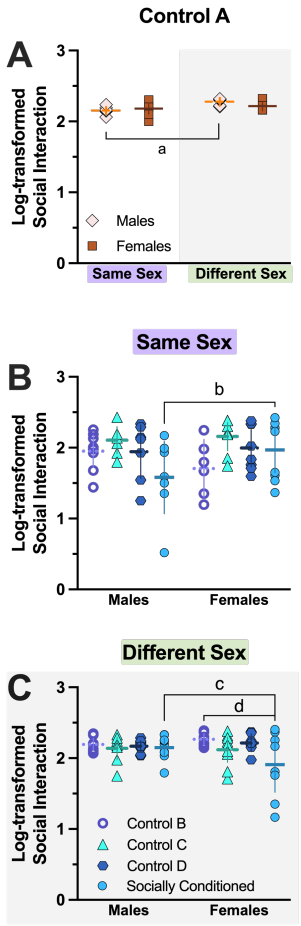

Figure 4. ***Day 2 social interaction behavior following social conditioning***. Log-transformed social interaction data are shown for mice in Control A groups (Panel A), and in the same (Panel B) and different (Panel C) sex experiments. For Panel A, male data are shown with diamonds and dashed orange lines, female data with squares and solid brown lines. For Panels B-C, data from left to right are: Control B mice, open circles and dotted lines; Control C mice, triangles and dashed lines; Control D mice, hexagons and dashed/dotted lines; socially conditioned mice, filled circles and solid lines. Numbers of mice graphed within each panel, left

to right: A) n=7, 8, 6, 6; B) n=8, 7, 7, 7, 6, 8, 8, 9; C) n=8, 8, 8, 9, 7, 9, 7, 8. [a]p=0.004, [b]p=0.038, [c]p=0.029, [d]p=0.017. Data graphed as mean ± 95% CI.

Social Conditioning Context Fear Testing

After Day 2's social interaction evaluation of mice's association between their assigned conspecific and the aversive stimulus they experienced on Day 1, we next assessed their fear response to the social conditioning context on Day 3. This was accomplished by quantifying percent of time spent freezing (Table 9, Figure 5).

In the same sex experiment, no significant three-way interaction was detected, nor were significant two-way interactions of group × sex or time × sex (Table 9). A non-significant trend for time × group was noted (p=0.057). Time was found to have a significant main effect (Table 9), as is typical for context fear expression testing, with continued exposure to the social conditioning context in the absence of the conditioned stimulus resulting in gradual fear extinction. In the different sex experiment, no significant three- nor two-way interactions were observed. Along with a main effect of time (Table 10), a significant effect of group was also found (Table 10). Pairwise comparisons revealed significant differences between groups at specific timepoints (Figure 5). The most consistent of these for the same sex experiment involved Control C males in the same sex experiment exhibiting accelerated extinction processes relative to females (Figure 5A, 5B). In the different sex experiment, the most robust time course difference was SC males displaying less freezing behavior relative to Control D males (Figure 5D).

Because of the natural extinction process that can occur during testing, we also specifically examined behavioral expression of social conditioning context fear during minutes two through six of testing, to best capture fear expression with minimal confounds from extinction processes (61,64,65). With this approach, freezing behavior did not significantly differ between any groups in the same sex experiment (Figure 5C), though a non-significant trend for group was noted (Table 11; p=0.089). While no group × sex interaction occurred for the different sex experiment, a main effect of group was observed (Table 12). After pairwise comparisons were evaluated, the significant difference detected in the different sex experiment was the same as that observed in the time course for males (Figure 5D); SC male mice displayed significantly less freezing relative to Control D male mice (Figure 5F). Integrated with the results from social interaction testing, these findings indicate that conditioned shifts in social engagement are distinct from conditioned fear to the social conditioning context.

Table 9. Three-way repeated measures ANOVAs on social conditioning context fear testing for mice of both sexes with same sex conspecifics.

Same Sex Experiment – Fear Expression Time Course			
	F Statistic	**p value**	**Partial η^2**
Time	**F(11.50,609.6)=9.646**	**<0.001**	**0.154**
Sex	F(1,53)=2.707	0.106	0.049
Group	F(3,53)=1.836	0.152	0.094
Time × Group	*F(34.51,609.6)=1.426*	*0.057*	*0.075*
Time × Sex	F(11.50,609.6)=1.590	0.094	0.029
Group × Sex	F(3,53)=0.487	0.692	0.027
Time × Group × Sex	F(34.51,609.6)=1.085	0.342	0.058

Table 10. Three-way repeated measures ANOVAs on social conditioning context fear testing for mice of both sexes with different sex conspecifics.

Different Sex Experiment – Fear Expression Time Course			
	F Statistic	**p value**	**Partial η^2**
Time	**F(9.434,518.9)=5.590**	**<0.001**	**0.092**
Sex	F(1,55)=0.030	0.862	0.001
Group	**F(3,55)=3.149**	**0.032**	**0.147**
Time × Group	F(28.30,518.9)=0.863	0.672	0.045
Time × Sex	F(9.434,518.9)= 0.575	0.826	0.010
Group × Sex	F(3,55)=0.844	0.476	0.044
Time × Group × Sex	F(28.30,518.9)=0.483	0.990	0.026

Table 11. Two-way ANOVA on social conditioning context fear testing for mice of both sexes with same sex conspecifics.

Same Sex Experiment – Fear Expression Average			
	F Statistic	**p value**	**Partial η^2**
Sex	F(1,53)=1.136	0.291	0.021
Group	*F(3,53)=2.288*	*0.089*	*0.115*
Group × Sex	F(3,53)=0.338	0.798	0.019

Table 12. Two-way ANOVA on social conditioning context fear testing for mice of both sexes with different sex conspecifics.

Different Sex Experiment – Fear Expression Average			
	F Statistic	**p value**	**Partial η^2**
Sex	F(1,55)=0.000	0.993	0.000
Group	**F(3,55)=4.438**	**0.007**	**0.195**
Group × Sex	F(3,55)=1.416	0.248	0.072

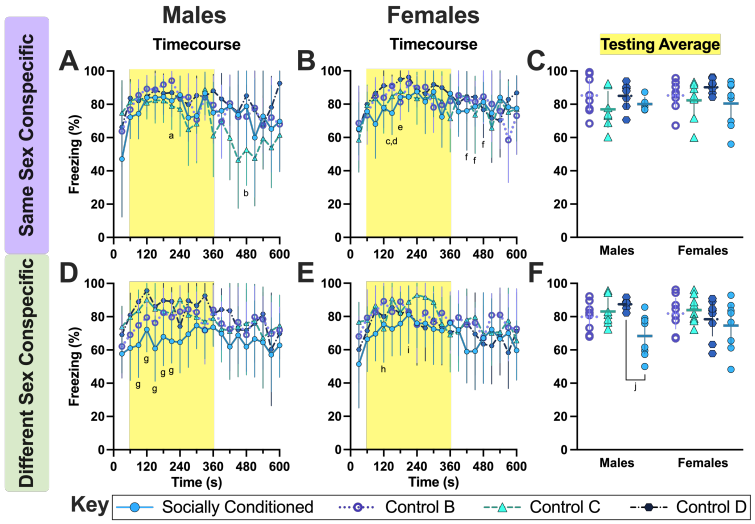

Figure 5. Day 3 social conditioning context fear behavior as time courses and averages. Time course (A, B, D, E) and average (C, F) context fear behavior data are shown for mice in all groups in the same sex (top row) and different sex (bottom row) experiments. For all Panels, groups are graphed as: Control B mice, open circles and dotted lines; Control C mice, triangles and dashed lines; Control D mice, hexagons and dashed/dotted lines; socially conditioned (SC) mice, filled circles and solid lines. Respectively, numbers of mice in top row: males – 8, 7, 7, 6;

females – 8, 8, 8, 9 mice. Numbers of mice in bottom row: males – 8, 7, 7, 8; females – 8, 9, 8, 8. [a]indicates p=0.038 between Control B and C male mice at indicated time point (Panel A). [b]indicates p=0.011 between Control C and D male mice at indicated time point (Panel A). [c]indicates p=0.042 between Control B and SC female mice in the same sex experiment at indicated time point (Panel B). [d]indicates p=0.047 between Control D and SC female mice in the same sex experiment at indicated time point (Panel B). [e]indicates p=0.041 between Control B mice mice of both sexes within the same sex experiment at indicated time point (Panel B). [f]indicates p=0.024, p=0.024, p=0.003 (left to right, Panel B) between Control C mice of both sexes within the same sex experiment at indicated time points. [g]indicates p=0.023, p=0.046, p=0.040, p=0.048, p=0.019 (left to right, Panel D) between Control D and SC male at indicated time points. [h]indicates p=0.037 between Control C mice of both sexes within the different sex experiment at indicated time point. [i]indicates p=0.038 between SC mice of both sexes within the different sex experiment at indicated time point. [j]indicates p=0.006. Data graphed as mean ± 95% CI.

Serum Corticosterone

No significant differences were detected in log-transformed corticosterone levels in Control A mice of either experiment (Table 13, Figure 6A).

Table 13. Two-way ANOVA on corticosterone for Control A mice across experiments and sexes.

Control A Mice – Log-transformed Corticosterone			
	F Statistic	**p value**	**Partial η^2**
Sex	F(1,26)=1.868	0.183	0.067
Experiment	F(1,26)=2.024	0.167	0.072
Sex × Experiment	F(1,26)=0.044	0.836	0.002

In parallel, same sex log-transformed corticosterone levels exhibited no interaction nor main effects, and no significant pairwise comparison results (Table 14, Figure 6B).

Table 14. Two-way repeated measures ANOVAs on corticosterone for mice of both sexes with same sex targets.

Same Sex Experiment – Log-transformed Corticosterone			
	F Statistic	**p value**	**Partial η^2**
Sex	F(1,54)=0.236	0.629	0.004
Group	F(3,54)=1.465	0.234	0.075
Sex × Group	F(3,54)=1.425	0.245	0.073

Different sex log-transformed corticosterone levels, while not exhibiting a significant sex × group interaction, did display a significant main effect of sex (Table 15). Pairwise comparisons indicated that this was primarily driven by SC males exhibiting significantly lower cort than SC females (Figure 6C), the inverse of what was observed for different sex social interaction (Figure 5C).

Table 15. Two-way repeated measures ANOVAs on corticosterone for mice of both sexes with different sex targets.

Different Sex Experiment – Log-transformed Corticosterone			
	F Statistic	**p value**	**Partial η^2**

Sex	**F(1,58)=8.683**	**0.005**	**0.130**
Group	F(3,58)=0.787	0.506	0.039
Sex × Group	F(3,58)=1.101	0.356	0.054

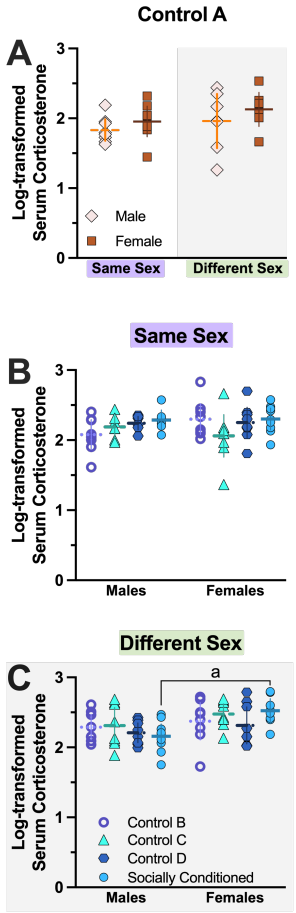

Figure 6. ***Day 3 corticosterone levels following social conditioning context fear expression testing***. Log-transformed corticosterone data are shown for mice in Control A groups (Panel A), and in the same (Panel B) and different (Panel C) sex experiments. For Panel A, male data are shown with diamonds and dashed orange lines, female data with squares and solid brown lines. For Panels B-C, data from left to right are: Control B mice, open circles and dotted lines; Control C mice, triangles and dashed lines; Control D mice, hexagons and dashed/dotted lines; socially conditioned mice, filled circles and solid lines. Numbers of mice graphed within each panel, left to right: A) n=8, 8, 7, 7; B) n=8, 7, 7, 7, 8, 8, 8, 9; C) n=8, 8, 8, 9, 8, 9, 8, 8. [a]p=0.004. Data graphed as mean ± 95% CI.

Discussion

Our goal in executing these experiments was to begin establishing a paradigm for inducing consistent, reproducible socially paired stress in mice of both sexes. While our results indicate we have not yet fully optimized this paradigm, we nevertheless have discovered sex-specific patterns of behavioral and physiological stress responsivity that are useful to fields incorporating sex as a biological variable and/or seeking to employ cross-sex social stressors in mice.

In the absence of any explicit stressor, our Control A mice displayed distinctive responses to social stimuli depending both upon the sex of the Control A mouse and the sex of their assigned conspecific. Freezing – ceasing all movement save breathing – is an innate behavior expressed by mice in response to a real or perceived threat, enabling threat assessment while minimizing detection (see review (66)). Despite all mice having already encountered both their assigned conspecific and the social conditioning context a day prior to freezing measurements during 'acquisition', freezing remained higher in mice of both sexes in the same sex experiment versus the different sex experiment. Though these freezing levels were considerably less than the ~80% freezing exhibited by mice after experiencing five mild foot shocks at the conclusion of acquisition, the discrepancy in basal freezing levels by Control A mice across experiments is consistent and visible. This observation indicates that, in the presence of a potential sexual partner, threat assessment is suppressed. The inverse has been demonstrated in female rats; that is, fear attenuates sexual behaviors, at least in part through amygdala and hypothalamus estrogen receptor signaling (67). Indirect evidence in male rats housed with females post-context fear conditioning suggests that encounters with different sexes may suppress threat assessment. These male rats subsequently exhibited attenuated fear expression, involving dopamine receptor signaling in the hippocampus (68). However, a different group studying mice reported that ejaculation by males was required for retention of extinguished social fear conditioning (47). Continued evaluations are needed to determine whether our observations – under conditions where copulation is impossible – that male exposure to a female can diminish threat assessment behaviors are reproducible. An alternative explanation for our findings is that locomotor activity was elevated, but additional studies would be necessary to determine this. That only Control A males, but not Control A females, exhibited elevated social interaction time with female conspecifics versus male conspecifics suggests this possibility is unlikely. Corticosterone levels in Control A mice were relatively low as expected, given these mice were never exposed to a mild foot shock. Similarly, neither sex nor experiment affected corticosterone levels, suggesting no enduring stress was disproportionately experienced after repeated same- or different-sex conspecific encounters across the four day study timeline.

Despite some interim deviations in fear acquisition across groups receiving mild foot shocks (Controls B-D; SC mice) in each experiment, all mice ultimately ended their acquisition at similar freezing levels. Males exhibited greater variability, as indexed by 95% CIs, across groups in both experiments than females. This aligns with evidence that male rodent data are just as (69–72) or more variable (54,73,74) than female data. Such relative consistency in ultimate terminal freezing levels during social conditioning acquisition is important when interpreting subsequent differences in social engagement, social conditioning context fear, and corticosterone levels in each experiment; these are not confounded by social conditioning acquisition discrepancies.

Most telling from our social interaction findings are the opposing patterns of reduced social interaction across sexes in each experiment. In the same sex experiment, socially conditioned males exhibited reduced social engagement compared to females; in the different sex experiment, socially conditioned females displayed attenuated social exploration versus males. These results indicate that encoding of socially aversive experiences depends upon both the

sex of the mouse experiencing the aversive event and upon the sex of the associated conspecific. Such sex-specific behavior patterns correspond to evidence that accumbal dopamine signaling dynamics in mice are sex-dependent, both upon the sex of the studied mouse and the sex of their assigned conspecific (75). Our findings align with the success of using social defeat to stress male mice. Additionally, they indicate that future studies seeking to suppress female mice's social interaction behavior through a socially associated stimulus will meet with more success if using male, rather than female, conspecifics. Indeed, others have met with challenges when trying to elicit behavioral shifts in female mice, even after 4 weeks of stress that involved only female conspecifics (76). One group reported that female mice consistently prefer a socially paired food reinforcer, even if the pairing was with a same sex conspecific that had just previously undergone acute stress (mild foot shock) (77). Because males' food preference was not studied, and neither did females encounter stressed different sex conspecifics, it remains unclear if these effects are sex-specific, and if they would generalize to different sex interactions. Additionally, future studies will need to determine how socially conditioning female mice to male conspecifics subsequently affects interactions with female conspecifics, and vice versa.

Regarding social conditioning context fear exhibited by mice when tested in the absence of any conspecifics, these did not align with changes in social interaction. For the purposes of developing a sex-inclusive social conditioning paradigm, this is preferable. Our goal was for mice to associate the aversive mild foot shock with their assigned conspecific, and any social buffering of social conditioning context fear could counteract our goal. This is why our use of conspecifics of a different strain, that were never housed even in the same room as all Control and SC mice, ensured they had never previously been encountered by the mice tested here. Given the critical contribution of attachment to social buffering (see review (78)), our data indicate we can be reasonably confident there were no social buffering effects upon social conditioning context fear in same sex experiment mice. In the different sex experiment, we did find only in SC males that their freezing was attenuated, but only relative to Control D mice. This could be related to our postulated threat assessment reduction relating to Control A mice, in that males in the vicinity of a possible sexual partner could experience impaired encoding of context cues.

Considering corticosterone measurements were taken thirty minutes after social conditioning context fear testing, we were interested to discover that these levels were inverted with respect to social interaction in the different sex experiment. In other words, socially conditioned males exhibited increased interaction with female conspecifics and lower corticosterone levels, as compared to socially conditioned females displaying reduced interaction with male conspecifics and elevated corticosterone levels. Further, corticosterone levels did not appear to correspond to expressions of social conditioning context fear, despite this test being most temporally proximal to blood collection. Thus, the different sex social conditioning procedure employed here appears sufficient to elicit both behavioral and physiological responses. However, though corticosterone levels appear overall elevated in same sex experiment mice, there were not group- or sex-specific differences. Most striking, corticosterone levels in same sex SC mice looked nearly identical, meaning this physiological stress marker did not map onto the reductions in male versus female social interaction behavior in that experiment. This could be that the same sex experiment's stress induction was not as enduring as that in the different sex experiment, or it might mean that different sex social conditioning affects more neurobiological processes than same sex social conditioning.

We may have been overzealous in our inclusion of controls. In our efforts to account for temporally proximal social encounters with non-cage mates (Controls C and D), we potentially

muddied the waters when it comes to interpreting how social conditioning affects behavior and circulating corticosterone. That said, we were surprised by what our Control A mice revealed, and think that this information helps provide context for interpreting our social conditioning findings. Nevertheless, the overall absence of social interaction differences between Controls B-D and SC mice (save for different sex females) indicates that this protocol requires additional optimization to achieve its overarching goal. Evidence for social transmission of stress in rodents suggests an alternative interpretation (79). Instead, it could be that because the conspecific for each SC mouse did not experience the same stress (mild foot shock), this in turn affects the SC mouse's perception of their aversive experience (51), likely in a sex-specific manner. Even if so, the present findings still generate useful information about environmental threat assessment in social contexts, and highlight heretofore unrecognized sex-specific social learning influences under aversive conditions.

Another limitation is that we could not obtain time course corticosterone measurements without introducing additional confounding stressors to repeatedly sample blood. Daily blood sampling would reveal if our corticosterone observations are attributable to the initial conditioning day, the social interaction or social conditioning context fear tests, and/or if these differences are transient or persistent. These experiments were all performed in sexually naïve mice, so we recognize that sexually experienced mice (for example (80)) of both sexes might respond, behaviorally and physiologically, in a manner distinct from what we report here. Earlier practices of dividing mice into 'susceptible' and 'resilient' groups following social stress have more recently, and justifiably, fallen out of favor, given such responses are artificially elicited, context-specific, and neither phenotype can be universally deemed 'better' or 'worse' (27,81). We therefore evaluated all socially conditioned mice as a single group. Yet, possible bimodal distribution of social interaction behavior looks likely in all but the different sex male SC mice. Increasing numbers to establish definitive thresholds with sufficient power for phenotypic grouping might prove useful, particularly with the same sex setup. Finally, our studies used a brief protocol spanning only four days, intended for high throughput and maximal comparison to other social stress literature. We therefore cannot speak to whether these outcomes persist for weeks or months, timeframes that are ethologically relevant for social learning and memory.

Here, we have reported a novel foray into sex-inclusive efforts to develop a controllable social stress paradigm in mice. These experiments have provided utilizable insights regarding how mice of both sexes associate a same- or different-sex conspecific with an aversive experience. We report that these associations seem strongest in males after same sex encounters, and in females after different sex encounters. Such distinctive responses are probably evolutionarily favorable. Given the behaviorally exhibited associations could inversely correspond to circulating corticosterone levels under different sex conditions, we propose that future studies focused on socially stressing females utilize male conspecifics for optimal outcomes.

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References

1. Bloomberg L, Meyers J, Braverman MT. The Importance of Social Interaction: A New Perspective on Social Epidemiology, Social Risk Factors, and Health. Heal Educ Behav. 1994;21(4):447–63.

2. Lee J, Raycraft L, Johnson AW. The dynamic regulation of appetitive behavior through lateral hypothalamic Orexin and Melanin Concentrating Hormone expressing cells. Physiol Behav. 2020;113234.

3. Monfils MH, Agee LA. Insights from social transmission of information in rodents. Genes, Brain Behav. 2019;18(1):e12534.

4. Kamps D, Royer J, Dugan E, Kravits T, Gonzalez-Lopez A, Garcia J, et al. Peer Training to Facilitate Social Interaction for Elementary Students with Autism and Their Peers. Except Child. 2001;68(2):173–87.

5. Leung L. Loneliness, social support, and preference for online social interaction: the mediating effects of identity experimentation online among children and adolescents. Chin J Commun. 2011;4(4):381–99.

6. Jasnow AM, Huhman KL. Activation of GABAA receptors in the amygdala blocks the acquisition and expression of conditioned defeat in Syrian hamsters. Brain Res. 2001;920(1–2):142–50.

7. Ago Y, Tanaka T, Ota Y, Kitamoto M, Imoto E, Takuma K, et al. Social crowding in the night-time reduces an anxiety-like behavior and increases social interaction in adolescent mice. Behav Brain Res. 2014;270:37–46.

8. Wilson CA, Koenig JI. Social interaction and social withdrawal in rodents as readouts for investigating the negative symptoms of schizophrenia. Eur Neuropsychopharmacol. 2014;24(5):759–73.

9. Venniro M, Zhang M, Caprioli D, Hoots JK, Golden SA, Heins C, et al. Volitional social interaction prevents drug addiction in rat models. Nat Neurosci. 2018;21(11):1520–9.

10. American Psychiatric Association. Diagnostic and statistical manual of mental disorders 2022;(5th ed., text rev.). https://doi.org/10.1176/appi.books.9780890425787

11. Bergamini G, Mechtersheimer J, Azzinnari D, Sigrist H, Buerge M, Dallmann R, et al. Chronic social stress induces peripheral and central immune activation, blunted mesolimbic dopamine function, and reduced reward-directed behaviour in mice. Neurobiol Stress. 2018;8:42–56.

12. Caldwell HK, Aulino EA, Rodriguez KM, Witchey SK, Yaw AM. Social Context, Stress, Neuropsychiatric Disorders, and the Vasopressin 1b Receptor. Front Neurosci. 2017;11:567.

13. Veney SL, Caldwell HK. Neuroendocrinology of Behavior and Emotions, Environmental and Social Factors Affecting Behavior. Mastercl Neuroendocr. 2024;109–29.

14. Gilman TL, DaMert JP, Meduri JD, Jasnow AM. Grin1 deletion in CRF neurons sex-dependently enhances fear, sociability, and social stress responsivity. Psychoneuroendocrino. 2015;58:33–45.

15. Garbarino VR, Santos TA, Nelson AR, Zhang WQ, Smolik CM, Javors MA, et al. Prenatal metformin exposure or organic cation transporter 3 knock-out curbs social interaction preference in male mice. Pharmacol Res. 2019;140:21–32.

16. Sahani V, Hurd YL, Bachi K. Neuroscience of Social Stress. Curr Top Behav Neurosci. 2022;54:483–515.

17. Venniro M, Marino RAM, Chow JJ, Caprioli D, Epstein DH, Ramsey LA, et al. The Protective Effect of Social Reward on Opioid and Psychostimulant Reward and Relapse: Behavior, Pharmacology, and Brain Regions. J Neurosci. 2022;42(50):9298–314.

18. Coccurello R, D'Amato FR, Moles A. Chronic social stress, hedonism and vulnerability to obesity: Lessons from Rodents. Neurosci Biobehav Rev. 2009;33(4):537–50.

19. Donovan M, Mackey CS, Platt GN, Rounds J, Brown AN, Trickey DJ, et al. Social isolation alters behavior, the gut-immune-brain axis, and neurochemical circuits in male and female prairie voles. Neurobiol Stress. 2020;13:100278.

20. Smith AS, Wang Z. Salubrious effects of oxytocin on social stress-induced deficits. Horm Behav. 2012;61(3):320–30.

21. Kondrakiewicz K, Kostecki M, Szadzińska W, Knapska E. Ecological validity of social interaction tests in rats and mice. Genes, Brain Behav. 2019;18(1):e12525.

22. Kuske JX, Trainor BC. Neuroscience of Social Stress. Curr Top Behav Neurosci. 2021;54:95–124.

23. Blanchard RJ, McKittrick CR, Blanchard DC. Animal models of social stress: effects on behavior and brain neurochemical systems. Physiol Behav. 2001;73(3):261–71.

24. Lopez J, Bagot RC. Defining Valid Chronic Stress Models for Depression With Female Rodents. Biol Psychiatry. 2021;90(4):226–35.

25. Pantoja-Urbán AH, Richer S, Mittermaier A, Giroux M, Nouel D, Hernandez G, et al. Gains and Losses: Resilience to Social Defeat Stress in Adolescent Female Mice. Biol Psychiatry. 2024;95(1):37–47.

26. Furman O, Tsoory M, Chen A. Differential chronic social stress models in male and female mice. Eur J Neurosci. 2022;55(9–10):2777–93.

27. Lyons DM, Ayash S, Schatzberg AF, Müller MB. Ecological validity of social defeat stressors in mouse models of vulnerability and resilience. Neurosci Biobehav Rev. 2023;145:105032.

28. Peña CJ, Nestler EJ, Bagot RC. Environmental Programming of Susceptibility and Resilience to Stress in Adulthood in Male Mice. Front Behav Neurosci. 2019;13:40.

29. Latsko MS, Farnbauch LA, Gilman TL, Lynch JF, Jasnow AM. Corticosterone may interact with peripubertal development to shape adult resistance to social defeat. Horm Behav. 2016;82:38–45.

30. McCormick CM, Merrick A, Secen J, Helmreich DL. Social Instability in Adolescence Alters the Central and Peripheral Hypothalamic-Pituitary-Adrenal Responses to a Repeated Homotypic Stressor in Male and Female Rats. J Neuroendocr. 2007;19(2):116–26.

31. McCormick CM, Green MR, Cameron NM, Nixon F, Levy MJ, Clark RA. Deficits in male sexual behavior in adulthood after social instability stress in adolescence in rats. Horm Behav. 2013;63(1):5–12.

32. Sterley TL, Baimoukhametova D, Füzesi T, Zurek AA, Daviu N, Rasiah NP, et al. Social transmission and buffering of synaptic changes after stress. Nat Neurosci. 2018;21(3):393–403.

33. Bernberg E, Andersson IJ, Gan L ming, Naylor AS, Johansson ME, Bergström G. Effects of social isolation and environmental enrichment on atherosclerosis in ApoE−/− mice. Stress. 2008;11(5):381–9.

34. Kercmar J, Büdefeld T, Grgurevic N, Tobet SA, Majdic G. Adolescent social isolation changes social recognition in adult mice. Behav Brain Res. 2011;216(2):647–51.

35. Pan Y, Mou Q, Huang Z, Chen S, Shi Y, Ye M, et al. Chronic social defeat alters behaviors and neuronal activation in the brain of female Mongolian gerbils. Behav Brain Res. 2023;448:114456.

36. Iñiguez SD, Flores-Ramirez FJ, Riggs LM, Alipio JB, Garcia-Carachure I, Hernandez MA, et al. Vicarious Social Defeat Stress Induces Depression-Related Outcomes in Female Mice. Biol Psychiatry. 2018;83(1):9–17.

37. Miyashita T, Yamaguchi T, Motoyama K, Unno K, Nakano Y, Shimoi K. Social stress increases biopyrrins, oxidative metabolites of bilirubin, in mouse urine. Biochem Biophys Res Commun. 2006;349(2):775–80.

38. Pizarro JM, Lumley LA, Medina W, Robison CL, Chang WE, Alagappan A, et al. Acute social defeat reduces neurotrophin expression in brain cortical and subcortical areas in mice. Brain Res. 2004;1025(1–2):10–20.

39. Jasnow AM, Shi C, Israel JE, Davis M, Huhman KL. Memory of social defeat is facilitated by cAMP response element-binding protein overexpression in the amygdala. Behav Neurosci. 2005;119(4):1125.

40. Dulka BN, Lynch JF, Latsko MS, Mulvany JL, Jasnow AM. Phenotypic responses to social defeat are associated with differences in cued and contextual fear discrimination. Behav Process. 2015;118:115–22.

41. Bonnefil V, Dietz K, Amatruda M, Wentling M, Aubry AV, Dupree JL, et al. Region-specific myelin differences define behavioral consequences of chronic social defeat stress in mice. eLife. 2019;8:e40855.

42. Wang W, Liu W, Duan D, Bai H, Wang Z, Xing Y. Chronic Social Defeat Stress Mouse Model: Current View on Its Behavioral Deficits and Modifications. Behav Neurosci. 2021;135(3):326–35.

43. Golden SA, Covington HE, Berton O, Russo SJ. A standardized protocol for repeated social defeat stress in mice. Nat Protoc. 2011;6(8):1183–91.

44. Toth I, Neumann ID, Slattery DA. Social Fear Conditioning: A Novel and Specific Animal Model to Study Social Anxiety Disorder. Neuropsychopharmacology. 2012;37(6):1433–43.

45. Zoicas I, Slattery DA, Neumann ID. Brain Oxytocin in Social Fear Conditioning and Its Extinction: Involvement of the Lateral Septum. Neuropsychopharmacology. 2014;39(13):3027–35.

46. Zerda SH de la, Netser S, Magalnik H, Briller M, Marzan D, Glatt S, et al. Social recognition in laboratory mice requires integration of behaviorally-induced somatosensory, auditory and olfactory cues. Psychoneuroendocrinology. 2022;143:105859.

47. Grossmann CP, Sommer C, Fahliogullari IB, Neumann ID, Menon R. Mating-induced release of oxytocin in the mouse lateral septum: Implications for social fear extinction. Psychoneuroendocrinology. 2024;166:107083.

48. Wiltgen BJ, Sanders MJ, Behne NS, Fanselow MS. Sex Differences, Context Preexposure, and the Immediate Shock Deficit in Pavlovian Context Conditioning With Mice. Behav Neurosci. 2001;115(1):26–32.

49. Brown KL, Kennard JA, Sherer DJ, Comalli DM, Woodruff-Pak DS. The context preexposure facilitation effect in mice: A dose–response analysis of pretraining scopolamine administration. Behav Brain Res. 2011;225(1):290–6.

50. Siegfried B, Frischknecht HR, Riggio G, Waser PG. Preexposure to a Nonaggressive Opponent Prevents Low-Intensity, Social-Conflict Analgesia in Mice. Behav Neurosci. 1987;101(3):423–6.

51. Guzmán YF, Tronson NC, Guedea A, Huh KH, Gao C, Radulovic J. Social modeling of conditioned fear in mice by non-fearful conspecifics. Behav Brain Res. 2009;201(1):173–8.

52. Sterley TL, Bains JS. Social communication of affective states. Curr Opin Neurobiol. 2021;68:44–51.

53. Russell W, Burch R. The Principles of Humane Experimental Technique. Nature [Internet]. 1959 Nov 1;184(4700):1675–6. Available from: https://doi.org/10.1038/1841675b0

54. Levy DR, Hunter N, Lin S, Robinson EM, Gillis W, Conlin EB, et al. Mouse spontaneous behavior reflects individual variation rather than estrous state. Curr Biol. 2023;33(7):1358-1364.e4.

55. Prendergast BJ, Onishi KG, Zucker I. Female mice liberated for inclusion in neuroscience and biomedical research. Neurosci Biobehav Rev. 2014;40(Endocrinology 153 2012):1–5.

56. Chari T, Griswold S, Andrews NA, Fagiolini M. The Stage of the Estrus Cycle Is Critical for Interpretation of Female Mouse Social Interaction Behavior. Front Behav Neurosci. 2020;14:113.

57. Bahadır-Varol A, Esen EC, Eren-Koçak E. Repeated collection of vaginal smear causes stress in mice. Arch Neuropsychiatry. 2022;59(4):325–9.

58. Becegato M, Meurer YSR, Paiva-Santos MA, Lima AC, Marinho GF, Bioni VS, et al. Impaired discriminative avoidance and increased plasma corticosterone levels induced by vaginal lavage procedure in rats. Physiol Behav. 2021;232:113343.

59. Wanisch K, Wotjak CT, Engelmann M. Long-lasting second stage of recognition memory consolidation in mice. Behav Brain Res. 2008;186(2):191–6.

60. Cai DJ, Aharoni D, Shuman T, Shobe J, Biane J, Song W, et al. A shared neural ensemble links distinct contextual memories encoded close in time. Nature. 2016;534(7605):115–8.

61. Lynch JF, Winiecki P, Gilman TL, Adkins JM, Jasnow AM. Hippocampal GABAB(1a) Receptors Constrain Generalized Contextual Fear. Neuropsychopharmacol. 2017;42(4):914–24.

62. Abel T, Lattal KM. Molecular mechanisms of memory acquisition, consolidation and retrieval. Curr Opin Neurobiol. 2001;11(2):180–7.

63. Sheppard PAS, Koss WA, Frick KM, Choleris E. Rapid actions of oestrogens and their receptors on memory acquisition and consolidation in females. J Neuroendocr. 2018;30(2).

64. Weber BL, Nicodemus MM, Hite AK, Spalding IR, Beaver JN, Scrimshaw LR, et al. Heterotypic Stressors Unmask Behavioral Influences of PMAT Deficiency in Mice. Int J Mol Sci. 2023;24(22):16494.

65. Beaver JN, Weber BL, Ford MT, Anello AE, Ruffin KM, Kassis SK, et al. Generalization of contextual fear is sex-specifically affected by high salt intake. PLOS ONE. 2023;18(7):e0286221.

66. Blanchard DC, Blanchard RJ. Ethoexperimental Approaches to the Biology of Emotion. Annu Rev Psychol. 1988;39(1):43–68.

67. Moëne OL, Stavarache M, Ogawa S, Musatov S, Ågmo A. Estrogen receptors α and β in the central amygdala and the ventromedial nucleus of the hypothalamus: Sociosexual behaviors, fear and arousal in female rats during emotionally challenging events. Behav Brain Res. 2019;367:128–42.

68. Bai H, Cao J, Liu N, Xu L, Luo J. Sexual behavior modulates contextual fear memory through dopamine D1/D5 receptors. Hippocampus. 2009;19(3):289–98.

69. Becker JB, Prendergast BJ, Liang JW. Female rats are not more variable than male rats: a meta-analysis of neuroscience studies. Biol Sex Differ. 2016;7(1):34.

70. Smarr B, Rowland NE, Zucker I. Male and female mice show equal variability in food intake across 4-day spans that encompass estrous cycles. PLoS ONE. 2019;14(7):e0218935.

71. Kazavchinsky L, Dafna A, Einat H. Individual variability in female and male mice in a test-retest protocol of the forced swim test. J Pharmacol Toxicol Methods. 2019;95:12–5.

72. Beery AK. Inclusion of females does not increase variability in rodent research studies. Curr Opin Behav Sci. 2018;23:143–9.

73. Smarr BL, Grant AD, Zucker I, Prendergast BJ, Kriegsfeld LJ. Sex differences in variability across timescales in BALB/c mice. Biol Sex Differ. 2017;8(1):7.

74. Smarr B, Kriegsfeld LJ. Female mice exhibit less overall variance, with a higher proportion of structured variance, than males at multiple timescales of continuous body temperature and locomotive activity records. Biol Sex Differ. 2022;13(1):41.

75. Dai B, Sun F, Tong X, Ding Y, Kuang A, Osakada T, et al. Responses and functions of dopamine in nucleus accumbens core during social behaviors. Cell Rep. 2022;40(8):111246.

76. Díez-Solinska A, Azkona G, Muñoz-Culla M, Beitia-Oyarzabal G, Goñi-Balentziaga O, Gómez-Lazaro E, et al. The role of sociability in social instability stress: Behavioral, neuroendocrine and monoaminergic effects. Physiol Behav. 2023;270:114306.

77. Kietzman HW, Trinoskey-Rice G, Blumenthal SA, Guo JD, Gourley SL. Social incentivization of instrumental choice in mice requires amygdala-prelimbic cortex-nucleus accumbens connectivity. Nat Commun. 2022;13(1):4768.

78. Kiyokawa Y, Hennessy MB. Comparative studies of social buffering: A consideration of approaches, terminology, and pitfalls. Neurosci Biobehav Rev. 2018;86:131–41.

79. Brandl HB, Pruessner JC, Farine DR. The social transmission of stress in animal collectives. Proc Royal Soc B. 2022;289(1974):20212158.

80. Newman EL, Covington HE, Suh J, Bicakci MB, Ressler KJ, DeBold JF, et al. Fighting Females: Neural and Behavioral Consequences of Social Defeat Stress in Female Mice. Biol Psychiatry. 2019;86(9):657–68.

81. Koolhaas JM, Boer SF de, Buwalda B, Meerlo P. Social stress models in rodents: Towards enhanced validity. Neurobiology Stress. 2017;6:104–12.

Supplemental Details

Specifics of outlier determinations are provided below. To reach criterion as an outlier, a single data point must have exceeded four standard deviations (SDs) ± mean in the absence of that single suspected outlier. Confirmed outliers are marked by red highlighting. Three mice did not interact at all with their social target, and thus were excluded from social interaction analyses (1 different sex Control A female; 1 same sex Control B female; 1 same sex Socially Conditioned male); these mice are indicated by yellow highlighting.

Different Sex	Female	Control A	
Social Conditioning Baseline Freezing (%)			
Mouse ID	**Data**	**Outlier Determination**	
SB096	60.747	*6.220*	*Avg*
SB097	3.229	*6.300*	*SD*
SB163	9.553	*56.624*	*Avg + 8 SD*
SB164	10.259		
SB165	17.223		
SB166	0.000		
SB167	1.840		
SB168	1.438		

NOTE: SB096 excluded from all subsequent measures

Same Sex	Male	Social Conditioning Group	
Average Testing Freezing (%)			
Mouse ID	**Data**	**Outlier Determination**	
SB019	87.333	*80.128*	*Avg*
SB021	78.431	*3.990*	*SD*
SB037	80.459	*44.218*	*Avg - 9 SD*
SB053	81.160		
SB054	76.585		
SB059	76.799		
SB066	42.309		

Different Sex	Male	Control C	
Average Testing Freezing (%)			
Mouse ID	**Data**	**Outlier Determination**	
SB107	81.244	*83.166*	*Avg*
SB108	72.553	*8.795*	*SD*
SB109	37.761	*39.189*	*Avg - 5 SD*
SB123	95.672		

SB152	76.618
SB153	94.453
SB154	78.569
SB169	83.053

Different Sex	Male	Control D	
Average Testing Freezing (%)			
Mouse ID	**Data**	**Outlier Determination**	
SB090	88.963	*87.426*	*Avg*
SB091	87.096	*3.049*	*SD*
SB092	88.510	*59.987*	*Avg - 9 SD*
SB093	91.733		
SB126	85.100		
SB127	57.868		
SB128	82.205		
SB129	88.378		

Different Sex	Male	Social Conditioning Group	
Average Testing Freezing (%)			
Mouse ID	**Data**	**Outlier Determination**	
SB075	11.304	*68.336*	*Avg*
SB083	78.920	*12.742*	*SD*
SB084	50.024	*17.367*	*Avg - 4 SD*
SB105	59.566		
SB106	75.898		
SB134	85.705		
SB135	57.283		
SB148	61.651		
SB149	77.639		

Same Sex	Female	Control B	
Log-Transformed Social Interaction			
Mouse ID	**Data**	**Outlier Determination**	
SB020	1.196	*1.707*	*Avg*
SB023	2.246	*0.390*	*SD*
SB033	1.672	*0.146*	*Avg - 4 SD*
SB034	1.797		
SB049	0.080		

SB060	1.352	
SB078	-3.000	No social interaction - excluded
SB079	1.981	

Same Sex	Male	Control A	
Log-Transformed Social Interaction			
Mouse ID	**Data**	**Outlier Determination**	
SB017	2.191	*2.155*	*Avg*
SB018	2.145	*0.054*	*SD*
SB035	2.140	*1.504*	*Avg - 12 SD*
SB036	2.060		
SB050	2.148		
SB051	1.486		
SB057	2.162		
SB058	2.237		

Same Sex	Male	Social Conditioning Group	
Log-Transformed Social Interaction			
Mouse ID	**Data**	**Outlier Determination**	
SB019	2.171		
SB021	1.591		
SB037	-3.000	No social interaction - excluded	
SB038	1.933		
SB053	1.354	*1.757*	*Avg*
SB054	1.502	*0.320*	*SD*
SB059	0.519	*0.478*	*Avg - 4 SD*
SB066	1.990		

Different Sex	Female	Control A
Log-Transformed Social Interaction		
Mouse ID	**Data**	**Outlier Determination**
SB096	2.239	Removed due to baseline fear
SB097	-3.000	No social interaction - excluded
SB163	2.262	
SB164	2.160	
SB165	2.321	
SB166	2.221	
SB167	2.169	

SB168	2.164

Different Sex	Female	Control B	
Log-Transformed Social Interaction			
Mouse ID	**Data**	**Outlier Determination**	
SB102	2.367	*2.267*	*Avg*
SB103	2.272	*0.092*	*SD*
SB132	1.790	*1.808*	*Avg - 5 SD*
SB133	2.183		
SB140	2.145		
SB142	2.388		
SB157	2.300		
SB158	2.212		

Different Sex	Female	Control D	
Log-Transformed Social Interaction			
Mouse ID	**Data**	**Outlier Determination**	
SB104	2.372	*2.214*	*Avg*
SB114	2.187	*0.134*	*SD*
SB119	2.240	*1.678*	*Avg - 4 SD*
SB120	1.569		
SB159	1.976		
SB160	2.154		
SB161	2.360		
SB162	2.210		

Different Sex	Male	Control A	
Log-Transformed Social Interaction			
Mouse ID	**Data**	**Outlier Determination**	
SB076	2.208	*2.279*	*Avg*
SB098	2.222	*0.050*	*SD*
SB099	1.912	*1.930*	*Avg - 7 SD*
SB143	2.302		
SB144	2.306		
SB172	2.315		
SB173	2.319		