Lessons learned while evaluating how pairing aversive experiences with same or different sex social stimulus mice affects subsequent social engagement

Abbreviated title: **Sex effects on social conditioning and behavior**

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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 translationally relevant, considering both mice and humans are highly social mammals, and human social behavior disruptions 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 has disproportionately focused on males, influenced heavily by their inherent territorial nature. Social target-instigated 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 method for social stress elicitation, we modified a paradigm to train male and female F1 129S1/SvlmJ × C57BL/6J mice to associate (via classical conditioning) same or different sex C57BL/6J targets with a mild, aversive stimulus. While further paradigm optimization is required, social interaction testing 24 h after conditioning indicates males socially conditioned better to male targets by exhibiting reduced social interaction, whereas females appeared not to form social stimulus associations. Serum corticosterone levels inversely corresponded to social avoidance after different sex, but not same sex, conditioning, suggesting corticosterone-mediated arousal influences cross-sex interactions. These rigorously controlled null outcomes align with past pursuits' limited success in creating a sex-inclusive social stress paradigm.

58 **Significance Statement**

59 Validated paradigms to study social stress in female mice, and across sexes, are needed. We
60 modified a published male mouse protocol by using classical conditioning to pair an aversive
61 stressor with a target. Our goal was to create a uniform, cross-sex, high-throughput social
62 stress technique to advance future research. Though our modified paradigm requires future
63 improvements, we did acquire evidence that males can be socially conditioned in this way, and
64 female same sex social engagement can be attenuated by a preceding non-social aversive
65 experience. These null findings, while not achieving our goal, provide useful information to
66 advance future sex-inclusive social stress investigations.

Introduction

Social interaction behavior is a cross-mammalian phenomenon seen in humans, non-human primates, rats, hamsters, and mice, among other animals (Bloomberg et al., 1994; Jasnow and Huhman, 2001; Kamps et al., 2001; Leung, 2011; Monfils and Agee, 2019; Lee et al., 2020). Social interaction behavior shifts can be informative of the physical and/or emotional state of the mammal (Ago et al., 2014; Wilson and Koenig, 2014; Venniro et al., 2018). In humans, typical social interaction behavior disruptions are characteristic of numerous neuropsychiatric conditions including trauma and stress-related disorders, mood and anxiety disorders, and autism spectrum disorder. (American Psychiatric Association, 2022).

Rodent studies examining social behavior and socially associated stressors within various contexts have advanced identification of regulating neural pathways, and assisted development of new therapeutic approaches (Jasnow and Huhman, 2001; Coccurello et al., 2009; Smith and Wang, 2012; Gilman et al., 2015; Caldwell et al., 2017; Bergamini et al., 2018; Donovan et al., 2020; Sahani et al., 2022; Venniro et al., 2022). However, social behavior research in the genetically tractable mouse species (*Mus musculus*) has been relatively limited in studying behavioral and neurophysiological consequences of aversive social interactions in *female* mice (Kondrakiewicz et al., 2019; Kuske and Trainor, 2021). This is largely due to capitalization of male-specific territorial aggression in most rodent social conditioning paradigms (Blanchard et al., 2001; Gilman et al., 2015; Latsko et al., 2016; Peña et al., 2019; Lopez and Bagot, 2021; Furman et al., 2022; Lyons et al., 2023; Pantoja-Urbán et al., 2024). A breadth of social stress techniques have been employed in hamsters, mice, and rats (Miyashita et al., 2006; McCormick et al., 2007; Bernberg et al., 2008; Kercmar et al., 2011; McCormick et al., 2013; Iñiguez et al., 2018; Sterley et al., 2018; Lee et al., 2020; Furman et al., 2022; Pan et al., 2023). Of these, the most prevalent paradigm in mice is that of social defeat (both acute and chronic) (Jasnow and

93 Huhman, 2001; Jasnow et al., 2015; Golden et al., 2011; Dulka et al., 2015; Gilman et al., 2015;
94 Latsko et al., 2016; Bonnefil et al., 2019; Wang et al., 2021). Aside from the male-centric nature
95 of social defeat, additional concerns of reproducibility arise from the inherently variable range of
96 aversive social experiences each 'defeated' experimental mouse encounters. Such variability,
97 mostly beyond experimenter control, plus the single sex bias of social defeat, left us wanting to
98 explore an improved paradigm. For this, we paired controllable, uniform, aversive
99 unconditioned stimuli with the presence of a social stimulus (conditioned stimulus; target) to
100 study aversive social conditioning *across sexes* in mice using a higher throughput approach
101 (requiring one conditioning day, rather than multiple days-weeks).
102
103 To accomplish this, we modified a paradigm previously utilized in male mice, involving manual
104 administration of an aversive stimulus (mild foot shock) selectively when a male mouse actively
105 investigated a target, with the goal of attenuating subsequent social engagement (Toth et al.,
106 2012; Zoicas et al., 2014; de la Zerda et al., 2022; Grossmann et al., 2024). Rather than
107 continuing this operant-style approach, where a mouse's behavior dictates an outcome, we
108 shifted to a classical conditioning approach in which shock delivery is standardized across
109 individual mice. We anticipated experimental mice would associate the presence of the target
110 with the aversive unconditioned stimulus. Additionally, all mice would receive the same number
111 of shocks, instead of variable numbers based upon behavior.
112
113 Here, we evaluated how this paradigm affected social engagement and fear behaviors *across*
114 *sexes* after mice were exposed to a novel target. Specifically, mice were socially conditioned
115 with an aversive stimulus (mild foot shock; stress) when in the presence of their assigned target,
116 independent of investigative behavior (Social Stimulus + Stress group). Then, mice were tested
117 for social engagement and fear behavior in a separate environment with their assigned target
118 present, followed by testing of freezing in the conditioning environment in the absence of any

target. The former test enabled evaluation of both social and fear behaviors, while the latter test assessed contextual memory sans social stimuli. These behaviors were tested under circumstances when the experimental and target mice were the same sex, and when they were different sexes. We hypothesized Social Stimulus + Stress mice would exhibit reduced social engagement (indexed as time spent directly adjacent to the target enclosure) when tested with their assigned target in a novel environment, regardless of if the Social Stimulus + Stress and target mice were the same or different sexes. Our broad goal was to develop a paradigm that would facilitate rigorous future investigations of social behavior across mouse sexes, which are currently underrepresented in literature (Kuske and Trainor, 2021).

Methods

Mice

Hybrid F1 offspring (i.e., 129SB6F1/J) of both sexes resulting from pairing female 129S1/SvlmJ (RRID:IMSR_JAX:002448) and male C57BL/6J (RRID:IMSR_JAX:000664) mice (hereafter experimental mice), and male and female C57BL/6J mice (hereafter targets), all ≥9 weeks old or older, were group-housed (two to five per cage) within sex. Experimental mice were hybrid F1 offspring of 129S1/SvlmJ and C57BL/6J mice because these two strains are the most prevalent among transgenic mice, and because hybrids provide broader generalizability (Kumar et al., 2021; Keady et al., 2022). Such hybrids are used for a variety of physiology and neuroscience research purposes (e.g., (Curley et al., 2010; Kelley et al., 2011; Agrimson et al., 2017; Niibori et al., 2020; Correia et al., 2024)). Targets were C57BL/6J mice housed in a separate room from experimental mice. C57BL/6J mice were used as targets to ensure they were entirely novel to experimental mice through separate housing; because C57BL/6Js are often used as the primary or background strain for testing social behaviors in mice (see reviews (Laman-

145 Maharg and Trainor, 2017; Varholick et al., 2021; Takahashi, 2025)); and because our F1
146 breeding was limited to generation of experimental mice. All mice had *ad libitum* access to food
147 and water in rooms maintained on a 12:12 light/dark cycle with lights on at 07:00 local standard
148 time (i.e., Zeitgeber time 0), and temperature maintained at 22 ± 2°C. All mice were fed LabDiet
149 5001 rodent laboratory chow (LabDiet, Brentwood, MO) and were kept on 7090 Teklad Sani-
150 chip bedding (Envigo, East Millstone, NJ) in cages containing Nestlets (Ancare, Bellmore, NY)
151 and huts (Bio-Serv, Flemington, NJ) for enrichment. Experiments were approved by Kent State
152 University's Institutional Animal Care and Use Committee, and adhered to the National
153 Research Council's Guide for the Care and Use of Laboratory Animals, 8th Ed. (National
154 Research Council, 2011).
155
156 ***Social Conditioning Paradigm***
157
158 *Timeline*
159 The entire paradigm spans four consecutive days. Procedures for each individual day are
160 outlined below in order. On Days 0 and 1, experimental mice underwent different experimental
161 manipulations according to their specific treatment group. On Days 2 and 3, all experimental
162 mice experienced identical testing conditions regardless of their treatment group (Fig. 1).
163
164 *Apparatus and Software*
165 On Days 0, 1, and 3, experimental mice were placed in Coulbourn Instruments chambers (17.8
166 cm D × 17.8 cm W × 30.5 cm H; Allentown, PA). Chambers comprised two opposing aluminum
167 walls each adjoining two clear acrylic walls. Targets were put inside a transparent enclosure
168 placed within a corner of the social conditioning chamber. The transparent enclosure included
169 23 holes (each 0.32 cm diameter) on each of the two sides facing the experimental mouse to
170 enable olfactory cue exchanges. Previous work demonstrates that olfaction is the most

171 important modality when recognizing and interacting with novel same-species targets (see
172 review (Sterley and Bains, 2021)); tactile (whiskers) and auditory modalities appear to only be
173 recruited when recognizing cage mates (de la Zerda et al., 2022) in male mice (females were
174 not studied). Chambers and enclosures were cleaned with 70% ethanol before and after each
175 session; chambers had visible illumination, and one clear acrylic wall was marked with a blue
176 dotted pattern. These components contributed olfactory and visual cues to the context, in
177 addition to the tactile cue of the stainless steel grid floor for the experimental mouse. Cameras
178 mounted above the chambers were used to record movements, and freezing behavior (≥0.25 s)
179 on Days 0, 1, and 3 was quantified using FreezeFrame software (v. 5.201, Actimetrics,
180 Wilmette, IL). Freezing – ceasing all movement save breathing – is an innate behavior
181 expressed by mice in response to a real or perceived threat, enabling threat assessment while
182 minimizing detection (see review (Blanchard and Blanchard, 1988)). All videos were manually
183 reviewed to ensure the software satisfactorily distinguished between freezing behavior and
184 mere absence of locomotion.
185
186 On Day 2, experimental mice were placed in a novel room (distinct from the room used for Days
187 0, 1, and 3) containing open arenas (41.9 cm W × 41.9 cm D × 39.6 cm H; Coulbourn
188 Instruments). Each open arena had one empty PVC tube (8.9 cm outer diameter) in a single
189 corner of the square chamber with wire mesh (0.25 × 0.25 cm openings) covering a rectangular
190 portion (11.4 cm W × 4.1 cm H) cut out of the tube bottom. Because of the mesh at the bottom
191 of the PVC tubes containing targets, exchanges of visual, auditory, and olfactory cues between
192 experimental mice and targets were possible, but tactile interactions were minimized. Social
193 interaction behavior was quantified as post-test duration when at least 80% of an experimental
194 mouse's body was in the interaction zone, a 7 cm radius zone (~223 cm^2 – 240 cm^2, i.e., 14-
195 15% of the open arena) around the base of the PVC tube. Freezing behavior, regardless of
196 arena location, lasting at least 250 ms during the post-test was also measured, then converted

197 into a percentage of the post-test time. Social and freezing behaviors were detected using ANY-
198 maze software (v. 7.09 Stoelting Co., Wood Dale, IL).
199
200 *Pre-exposure (Day 0)*
201 Pre-exposure was used to help reduce the novelty of the transparent enclosure and/or the
202 target to the experimental mouse, plus minimize any potential sex differences in acquisition
203 (Siegfried et al., 1987; Wiltgen et al., 2001; Guzmán et al., 2009; Brown et al., 2011). On Day 0,
204 experimental mice were placed in chambers with (Shock Control and Social Stimulus + Stress
205 mice) or without (Social, Pre-Contingency, and Post-Contingency Controls) a target (age-
206 matched; either same sex or different sex relative to experimental mouse, depending on if
207 assigned to same sex or different sex experiment) for pre-exposure (Fig. 1). During pre-
208 exposure, experimental mice were allowed to explore the chamber and target enclosure for five
209 min, then both mice were returned to their respective home cages. No aversive stimulus was
210 presented.
211
212 *Social Conditioning (Day 1)*
213 On Day 1, experimental mice were placed in the same social conditioning chamber as Day 0
214 and received five, 1 s mild foot shocks (1.0 mA) in the presence of the same target that they
215 encountered on Day 0 (Fig. 1). This was to have experimental mice form associations between
216 aversive foot shocks and their respective target (Fig. 1). Conditioning lasted for nine min; after
217 a two min baseline, shocks were then administered at 120, 210, 300, 390, and 480 s. Targets
218 did not receive foot shocks.
219
220 *Social Engagement Testing (Day 2)*
221 On Day 2, experimental mice intentionally underwent social engagement testing in a novel
222 environment with a novel target enclosure to ensure sensitivity of our social interaction measure

223 while minimizing potential contextual confounds; in other words, to isolate effects on social
224 engagement from those of context fear learning (the latter assessed on Day 3). Mice
225 acclimated to the room in their home cage for 30 min prior to behavior testing commencing.
226 Experimental mice were placed in the corner of the arena opposite the PVC tube and could
227 investigate freely for 2.5 min (pre-test). Immediately after these 2.5 min, the target that each
228 experimental mouse had previously been conditioned with on Day 1 was then placed in the PVC
229 tube in the corner. Experimental mice were allowed to continue investigating the arena for an
230 additional five min (post-test; Fig. 1). Proprietary incompatibilities and video file encoding
231 precluded analyses of freezing behavior during social interaction testing from being evaluated
232 with FreezeFrame software; this is why ANY-maze software was used instead here. Post-test
233 social interaction time was log-transformed (Y=log(Y+0.001) to account for three mice with zero
234 s post-test social interaction) so that data would be normally distributed for statistical analyses.
235 Females were intentionally not assessed for estrus cycle stage for five reasons: 1) to minimize
236 mouse usage (Russell and Burch, 1959), we did not power our studies for assessment of
237 estrus; 2) with the goal of developing a social stress paradigm, we are seeking effects robust
238 enough to not depend upon estrus in intact, randomly cycling females; 3) we intentionally
239 focused here on fear and social behaviors, and did not measure sexual behaviors (e.g.,
240 lordosis); 4) evidence that overall mouse behaviors are not affected by estrus stage
241 (Prendergast et al., 2014; Levy et al., 2023), (but see (Chari et al., 2020)); 5) cross-species
242 evidence indicates vaginal lavage to determine estrus cycle is stressful (Becegato et al., 2021;
243 Bahadır-Varol et al., 2022), and we sought to minimize stress confounds here.
244
245 *Context Testing (Day 3)*
246 On the last day of the paradigm, experimental mice were placed in the social conditioning
247 chamber for behavior testing in the *absence* of any target (Fig. 1). The target enclosure was still

248 present to keep the context consistent. All other tactile, visual, and olfactory cues from Days 0
249 and 1 were present. Testing lasted 10 min and did not involve any foot shocks.
250
251 ***Treatment Groups***
252
253 The Social Stimulus + Stress group involved mice that underwent social conditioning in the
254 presence of a non-shocked target (either same sex or different sex relative to Social Stimulus +
255 Stress mouse, depending upon experiment), with the hypothesis that Social Stimulus + Stress
256 mice would associate the target with this aversive experience. We planned four Control groups
257 for this one Social Stimulus + Stress group; each experiment (same sex or different sex) had its
258 own respective set of four Control groups for its respective Social Stimulus + Stress group. The
259 goals of these were to control for: 1) foot shock exposure, i.e., Shock Controls; 2) presence of
260 target during pre-exposure & foot shock, i.e., Social Controls; 3) exposure to target temporally
261 distal to foot shock exposure, such that social encounters still occurred in a manner not
262 contingent with foot shock nor the conditioning context, i.e., Pre- and Post-Contingency Controls
263 (Fig. 1). Shock Controls experienced procedures identical to those of the Social Stimulus +
264 Stress group, except a foot shock was never administered on Day 1 (Fig. 1). Social Controls
265 were treated the same as the Social Stimulus + Stress group, except experimental mice never
266 encountered their assigned target until testing on Day 2 (Fig. 1). Pre- and Post-Contingency
267 Controls involved experimental mice encountering targets either four h before (Pre-Contingency
268 Controls) or four h after (Post-Contingency Controls) pre-exposure (Day 0) and social
269 conditioning (Day 1); in other words, targets were not present in the conditioning chamber
270 during these two periods. Instead, experimental mice were exposed to their target in a separate
271 room for five min or nine min (the same amount of time as pre-exposure or social conditioning,
272 respectively) four h before (Pre-Contingency Controls) or four h after (Post-Contingency
273 Controls) the experimental mice were exposed to the conditioning chamber. This exposure

involved experimental mice and their respective target being placed in a clean mouse cage separated with an acrylic divider to allow for visual, auditory, and olfactory exchange, but minimizing tactile interaction, mirroring the experiences of Social Stimulus + Stress mice and Shock Controls within the social conditioning context on Days 0-1. Mice in Pre- or Post-Contingency Control conditions still experienced foot shocks on Day 1, and encountered their assigned target for testing on Day 2 (Fig. 1). This temporal separation of four 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 (Wanisch et al., 2008; Cai et al., 2016; Lynch et al., 2017) (see reviews (Abel and Lattal, 2001; Sheppard et al., 2018)).

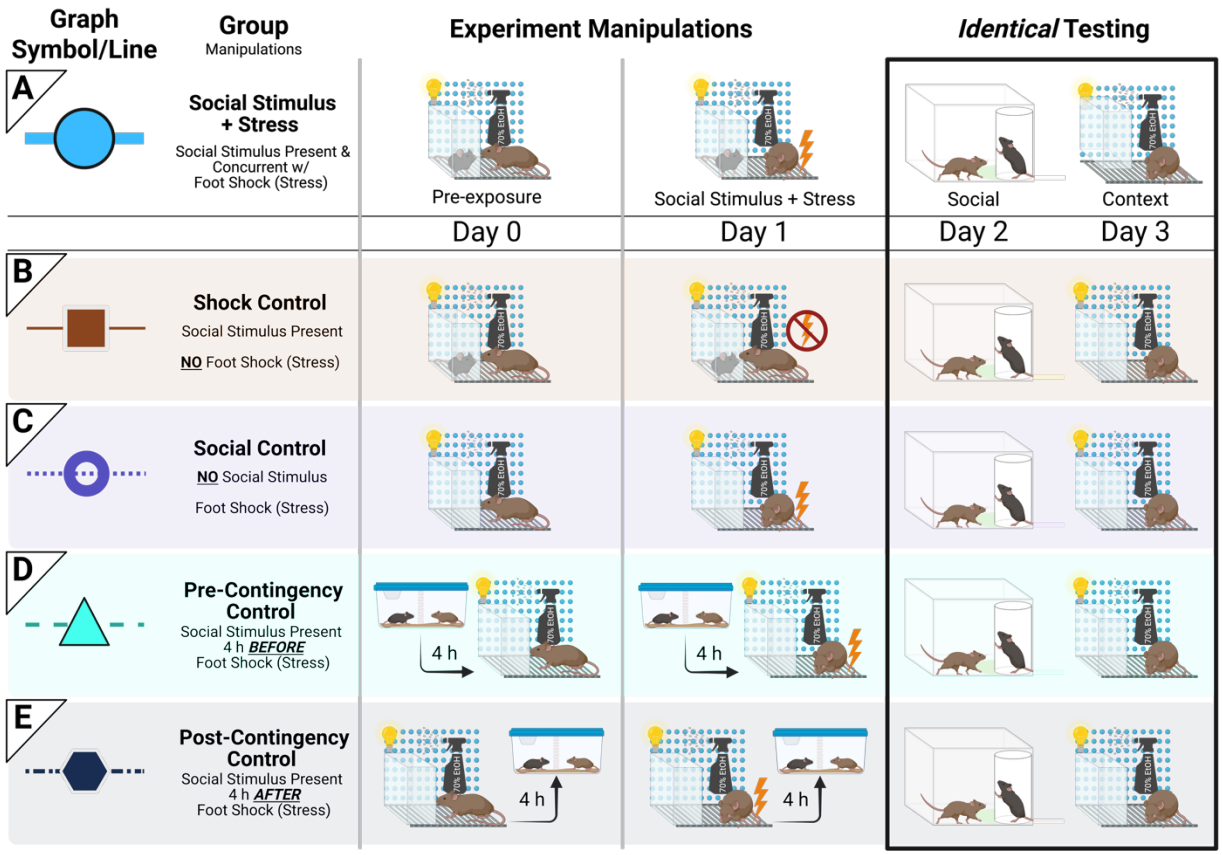

Figure 1. Study timeline and treatment groups. Five groups were used in this study for each experiment (same sex experiment and different sex experiment): one socially conditioned (Social Stimulus + Stress) group and four Control groups. Day 0 was for pre-exposure; Day 1 was when social conditioning occurred; Day 2 tested for social engagement; Day 3 tested for context-specific freezing behavior. On Day 0, Social Stimulus + Stress mice (A; above horizontal lines) encountered their assigned target in the social conditioning context, where olfactory, visual, and auditory cues could be exchanged while tactile interactions were minimized. No aversive stimulus was applied on Day 0, but on Day 1, experimental mice and their assigned target were returned to this context. Social Stimulus + Stress (but not target) mice then experienced five mild foot shocks to associate their target with that aversive experience. Social Stimulus + Stress mice were then tested for social engagement with that same target on Day 2, followed by testing for context fear behavior on Day 3 in the absence of their assigned target. Each Control group (below horizontal lines) was composed of different mice. Shock Control (B) mice underwent the same exact procedure as detailed for the Social Stimulus + Stress group, except no foot shock was administered on Day 1. Social Control (C) mice similarly experienced the same procedure as mice in the Social Stimulus + Stress group, save that Social Control mice did not encounter any target until Day 2 for testing of social engagement. Pre-Contingency Control (D) mice encountered their target four h before pre-exposure and social conditioning, meaning no target was present for Pre-Contingency Control mice when they were in the social conditioning chamber for pre-exposure or social conditioning. To encounter their assigned target, Pre-Contingency Control mice were placed into a clean cage with a clear acrylic divider separating them from their target. This divider allowed visual, olfactory, and auditory exchanges with the target but minimized tactile interactions, the same as for Social Stimulus + Stress mice and Shock Controls when in the social conditioning context. On Day 0, Pre-Contingency Control mice encountered their target for five min; on Day 1, for

nine min; these were timed to match the length of target exposure that mice in the Social Stimulus + Stress group experienced. Post-Contingency Control (E) mice were treated the same as Pre-Contingency Control mice, except the former's encounters with their assigned targets occurred four h *after*, rather than before, pre-exposure and social conditioning.

Serum Corticosterone

Thirty min 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 min, then was spun at 3500 rpm for one 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 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. After serum corticosterone levels were interpolated from each plate's standard curve, they were log-transformed to account for the typical skewness of serum corticosterone (Teilmann et al., 2014; Uarquin et al., 2016; Weber et al., 2023).

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 (μ) ± standard error of the mean. Details of identified outliers (greater than the mean ± four standard deviations (σ)) are

337 provided in the Supplemental Dataset. The same sex experiment and different sex experiment
338 each included their own Social Stimulus + Stress group plus accompanying four Control groups.
339 Experiments were analyzed separately. Social conditioning acquisition was analyzed using a
340 three-way repeated measures general linear model (time × treatment group × sex of
341 experimental mouse). Greenhouse Geisser corrections were utilized for within-subjects
342 analyses. Measurements of contextual fear expression average, log-transformed post-test
343 social interaction time, percent time freezing during social interaction testing, and log-
344 transformed corticosterone were each analyzed with a two-way general linear model (treatment
345 group × sex of experimental mouse). Our primary outcomes of interest for this study were
346 freezing during social conditioning acquisition, social engagement behavior, and freezing during
347 context testing. Accordingly, our *a priori* hypotheses and planned contrasts were:

348 - **Hypothesis 1**: Freezing during the final time point of social conditioning acquisition
349 would be minimal in Shock Control mice compared to all other groups; and female
350 experimental mice would exhibit higher levels of freezing than males.
351 - **Hypothesis 2**: In female more than male experimental mice, log-transformed social
352 engagement would be decreased in Social Stimulus + Stress mice compared to all four
353 Control groups.
354 - **Hypothesis 3**: Freezing during testing in the social conditioning chamber would be
355 minimal in Shock Control mice compared to all other groups; and female experimental
356 mice would exhibit higher levels of freezing than males.

357 These planned contrasts were selectively analyzed without correction alongside our omnibus
358 analyses. For our secondary outcomes that either were used to assess the possibility of
359 confounds (percent time freezing during social interaction testing) or were exploratory (log-
360 transformed serum corticosterone), and as follow-ups to our planned contrasts on our primary
361 outcomes, we performed *post hoc* pairwise comparisons with Bonferroni correction. The results
362 of these *post hoc* tests for our primary outcomes are reported in Supplemental Figures S1-S3.

363

364

365 **Results**

366

367 ***Social Conditioning Acquisition***

368

369 Social conditioning acquisition is illustrated in Fig. 2A, 2B for mice in the same sex target
370 experiment, and in Fig. 2C, 2D for mice in the different sex target experiment. All mice receiving
371 a foot shock expressed >40% freezing during at least one post-shock period during social
372 conditioning.

373

374 For acquisition in the same sex experiment, no three-way interaction of time × sex × group
375 occurred (Table 1). We observed a significant two-way interaction between time × group, and a
376 non-significant trend for group × sex was noted (p=0.070; Table 1). A significant main effect of
377 sex was also found for mice in the same sex experiment (Table 1). Our *a priori* planned
378 contrast for **Hypothesis 1** within the same sex experiment ($F_{(1,69)}$=1.140 p=0.289, partial
379 η^2=0.016) did not support our anticipated observation (Fig. 2A, B). Graphs of these data
380 showing the outcomes of pairwise comparisons are located in Supplemental Figure S1A, B.

381

382 Table 1. Two-way repeated measures general linear model on learning of social conditioning
383 for mice of both sexes with same sex targets.

Same Sex Experiment – Social Conditioning Acquisition			
	F Statistic	**p value**	**partial η^2**
Time	$F_{(4.18,288)}$=135.9	<0.001	0.663
Group	$F_{(4,69)}$=45.09	<0.001	0.723

Sex	$F_{(1,69)}$=13.54	<0.001	0.164
Time × Group	$F_{(16.7,288)}$=9.518	<0.001	0.356
Time × Sex	$F_{(4.18,288)}$=1.714	0.144	0.024
Group × Sex	$F_{(4,69)}$=2.275	0.070	0.117
Time × Group × Sex	$F_{(16.7,288)}$=0.787	0.706	0.044

Social conditioning acquisition for mice in the different sex experiment is shown in Fig. 2C, 2D. A significant three-way interaction of time × sex × group was observed (Table 2). Similar to the same sex experiment, however, our planned contrast did not reach significance for **Hypothesis 1** ($F_{(1,70)}$=0.537 p=0.466, partial η^2=0.008). Indeed, no significant pairwise comparisons indicated within-group significant sex differences at the final social conditioning acquisition time point, either for the same sex or different sex experiments (Supplemental Figure S1).

Combined with same sex experiment acquisition (Supplemental Figure S1), these findings indicate that social exposure – whether concurrent with or temporally distal from an aversive unconditioned stimulus – can have transient impacts on the freezing behavior exhibited by experimental mice across sexes. All conditioning procedures here, though, culminated in similar final freezing levels across sexes of both experimental mice and their targets. This facilitates comparisons of the different conditioning manipulations on subsequent social and freezing behaviors by minimizing potential acquisition confounds.

Table 2. Two-way repeated measures general linear model on learning of social conditioning for mice of both sexes with different sex targets.

Different Sex Experiment – Learning Social Conditioning			
	F Statistic	**p value**	**partial η^2**

Time	$F_{(4.35,304)}=184.2$	<0.001	0.725
Group	$F_{(4,70)}=38.88$	<0.001	0.690
Sex	$F_{(1,70)}=6.694$	0.012	0.087
Time × Group	$F_{(17.4,304)}=12.67$	<0.001	0.420
Time × Sex	$F_{(4.35,304)}=3.317$	0.009	0.045
Group × Sex	$F_{(4,70)}=0.892$	0.474	0.048
Time × Group × Sex	$F_{(17.4,304)}=1.658$	0.048	0.087

402

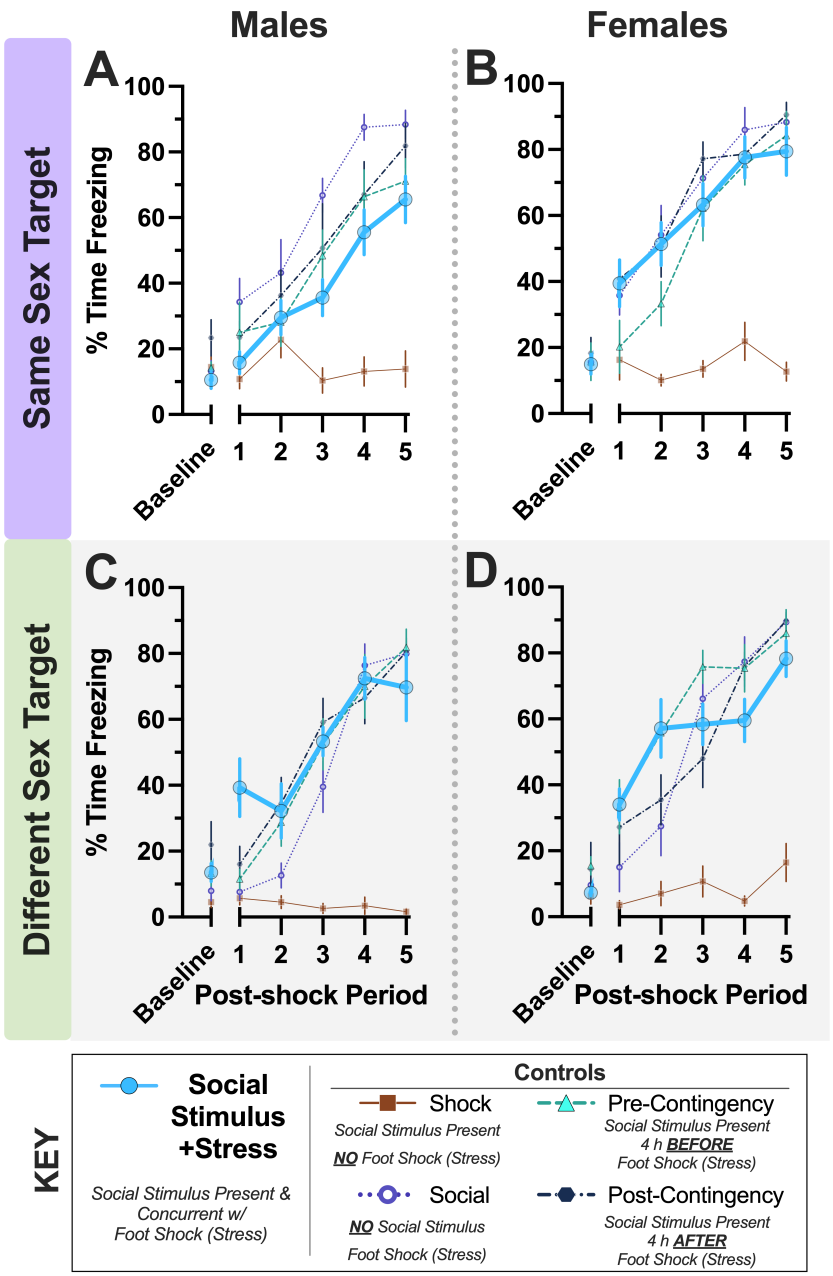

Figure 2. ***Day 1 acquisition during social conditioning procedure***. Percent time freezing during social conditioning acquisition for mice in same sex (Panels A, B) and different sex (Panels C, D) experiments for male (A, C) and female (B, D) mice. Numbers of mice graphed within Panels A-D in order: Social Stimulus + Stress (n=8, 9, 9, 8); Shock Control (8, 8, 7, 7); Social Control (n=8 for all); Pre-Contingency Control (n=7, 8, 8, 9); Post-Contingency Control (n=7, 8, 8, 8). Average freezing for the first two min, 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). Data graphed as mean ± standard error of the mean.

Social Interaction Testing

Social conditioning in the same sex experiment resulted in a sex × group interaction on social engagement (Table 3). Moreover, our planned contrast supported **Hypothesis 2** for the same sex experiment (Fig. 3A; $F_{(1,68)}=7.037$ $p=0.010$, partial $\eta^2=0.094$), though our contrast estimate was negative. This informed us that our proposed directionality of the sex × group interaction was not supported by the data. In evaluating means for our planned contrast, we found that log-transformed social engagement values for Social Stimulus + Stress males ($\mu=1.008$, $\sigma=1.698$, $n=8$) were indeed decreased compared to all male Controls ($\mu=2.036$, $\sigma=0.269$, $n=29$). However, the mean for Social Stimulus + Stress females ($\mu=1.813$, $\sigma=0.991$, $n=32$) was actually higher than that for all pooled female Controls ($\mu=1.968$, $\sigma=0.417$, $n=9$), likely due to some Social Control females with low social engagement values (Fig. 3A). Pairwise comparisons (Supplemental Figure S2A, B) corroborate the low mean for Social Control females, plus indicate that Social Stimulus + Stress males displayed less social engagement than Social Stimulus + Stress females, reiterating that our paradigm was not successful in its goal.

Table 3. Two-way general linear model on social interaction for mice of both sexes with same sex targets.

Same Sex Experiment – Log-transformed Social Interaction			
	F Statistic	**p value**	**partial η^2**
Group	$F_{(4,68)}=2.967$	0.025	0.149

Sex	$F_{(1,68)}$=0.004	0.951	0.000
Group × Sex	$F_{(4,68)}$=3.097	0.021	0.154

Fear behavior, indexed as percent time freezing during the social interaction post-test, was also evaluated. While the goal of our modified paradigm was to ultimately decrease social engagement following conditioning, we also assessed the level of freezing expressed in the novel social testing arena. In the same sex experiment, no sex × group interaction occurred, but main effects of both group and sex were observed (Table 4). Pairwise comparisons indicated that freezing was higher in Social Stimulus + Stress females versus Shock Control females, while the same comparison in males revealed no differences (Fig. 3C). In a somewhat inverted pattern compared to Controls during same sex social interaction, Social Control females exhibited more freezing during social interaction testing than Social Control males, Shock Control females, and Pre- and Post-Contingency Control females (Fig. 3C). Overall, though, freezing levels during social interaction testing were minimal as compared to those occurring during acquisition (Fig. 2) and context testing (Fig. 4A-B).

Table 4. Two-way general linear model on percent time freezing during social interaction for mice of both sexes with same sex targets.

Same Sex Experiment – % Freezing During Social Interaction			
	F Statistic	**p value**	**partial η^2**
Group	$F_{(4,68)}$=11.45	<0.001	0.402
Sex	$F_{(1,68)}$=6.252	0.015	0.084
Group × Sex	$F_{(4,68)}$=1.207	0.316	0.066

449 Contrasting with the same sex experiment, there was no significant interaction nor any main
450 effects in the different sex experiment for social engagement (Table 5). Further distinguishing
451 the different sex experiment with the same sex experiment, our **Hypothesis 2** planned contrast
452 for the different sex experiment did not achieve significance (Fig. 3B; $F_{(1,67)}=0.033$ $p=0.856$,
453 partial $\eta^2=0.000$). Pairwise comparisons demonstrated that Social Stimulus + Stress did not
454 generate any sex differences in social engagement (Supplemental Figure S2B). The only sex
455 effect observed was in Shock Controls, with females demonstrating less social engagement
456 than males, though this appears driven by a single Shock Control female (Supplemental Figure
457 S2B). Combined, these findings indicate that the social engagement of female mice with other
458 females is more affected by prior aversive experiences in the *absence* of any social stimuli,
459 whereas males are more socially affected by temporally concurrent aversive associations with
460 their own sex.
461
462 **Table 5.** Two-way general linear model on social interaction for mice of both sexes with
463 different sex targets.

Different Sex Experiment – Log-transformed Social Interaction			
	F Statistic	**p value**	**partial η^2**
Group	$F_{(4,67)}=0.726$	0.577	0.042
Sex	$F_{(1,67)}=1.756$	0.190	0.026
Group × Sex	$F_{(4,67)}=1.172$	0.331	0.065

464
465 Unlike the same sex experiment, neither an interaction nor main effects of sex and group were
466 observed with freezing behavior during social interaction testing for the different sex experiment
467 (Table 6). Moreover, no significant pairwise comparisons were observed (Fig. 3D). The
468 predominant consistency across experiments for freezing was that expression of fear was

469 minimal during the post-test of social interaction, as compared to freezing quantified in the

470 conditioning context (Figs. 2, 4A-B).

471

472 **Table 6.** Two-way general linear model on percent time freezing during social interaction for

473 mice of both sexes with different sex targets.

Different Sex Experiment – % Freezing During Social Interaction			
	F Statistic	**p value**	**partial η^2**
Group	$F_{(4,67)}=0.698$	0.596	0.040
Sex	$F_{(1,67)}=3.414$	0.069	0.048
Group × Sex	$F_{(4,67)}=0.491$	0.743	0.028

474

475

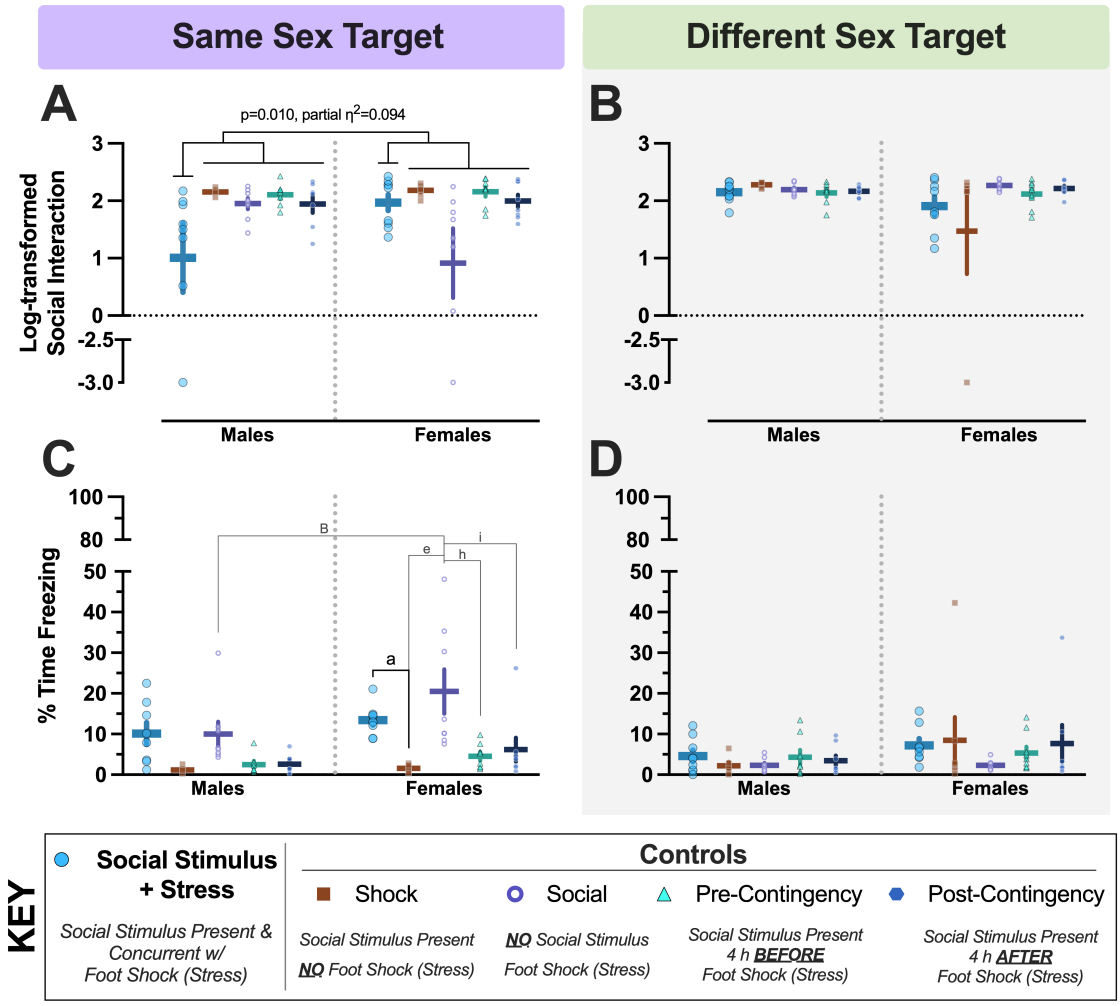

Figure 3. ***Day 2 social interaction and fear behaviors following social conditioning***. Log-transformed social interaction (Panels A, B) and percent time freezing during post-test social interaction (Panels C, D) data are shown for mice in the same (A, C) and different (B, D) sex experiments. Numbers of mice graphed within each panel, left to right: A) n=8, 7, 8, 7, 7, 9, 8, 8, 8, 8; B) n=9, 6, 8, 8, 8, 8, 7, 7, 9, 7; C) n=8, 7, 8, 7, 7, 9, 8, 8, 8, 8; D) n=9, 6, 8, 8, 8, 8, 7, 7, 9, 7. Y axes for A, B were split to visualize data points for three total mice across three separate groups that exhibited no social interaction. Y axes for C, D were split to facilitate clearer visualization of the low freezing levels exhibited during social engagement testing. An *a priori* planned contrast evaluating if log-transformed social interaction was reduced in Social Stimulus + Stress mice across sexes compared to all four Control groups within the same

experiment was significant for mice in the same sex experiment (Panel A; $F_{(1,68)}=7.037$ $p=0.010$, partial $\eta^2=0.094$). Results of pairwise comparisons with Bonferroni correction, left to right in Panel C: [B]indicates (Social Controls male vs. female) $p=0.004$; [a]indicates (Shock Control vs. Social Stimulus + Stress) $p=0.008$; [e]indicates (Shock Control vs. Social Control) $p<0.001$; [h]indicates (Social Control vs. Pre-Contingency Control) $p<0.001$; [i]indicates (Social Control vs. Pre-Contingency Control) $p=0.001$. Data graphed as mean ± standard error of the mean.

Social Conditioning Context Fear Testing

Because of the natural extinction process that can occur during testing, we 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 (Lynch et al., 2017; Beaver et al., 2023; Weber et al., 2023). This testing further allowed us to determine if social engagement testing on Day 2 might have any carry-over effects on contextual fear expression on Day 3 (Tables 7-8, Fig. 4A-B).

For the same sex experiment, no two-way interaction between group × sex was found, but a significant main effect of group emerged (Table 7). Given omnibus statistics did not report an interaction, it is not surprising that our planned contrast for **Hypothesis 3** in the same sex experiment likewise was not significant (Fig. 4A; $F_{(1,69)}=0.733$ $p=0.395$, partial $\eta^2=0.011$). This was further supported by pairwise comparisons (Supplemental Figure S3A), which indicated that male Social Stimulus + Stress mice exhibited significantly less average freezing in the conditioning context compared to female Social Stimulus + Stress mice (Fig. 4A). Shock Control mice of both sexes exhibited reduced fear behavior compared to all other groups, as expected (Fig. 4A).

513 **Table 7.** Two-way general linear model on both sexes' context fear testing average with same
514 sex targets.

Same Sex Experiment – Fear Expression Average			
	F Statistic	**p value**	**partial η^2**
Group	$F_{(4,69)}$=108.5	<0.001	0.863
Sex	$F_{(1,69)}$=2.859	0.095	0.040
Group × Sex	$F_{(4,69)}$=0.676	0.611	0.038

515

516 In the different sex experiment, no significant group × sex interaction was found, nor was a
517 significant effect of sex (Table 8). A significant group effect was similarly observed for the
518 different sex experiment (Fig. 4B). As with the same sex experiment, the lack of a significant
519 omnibus interaction was reflected in the non-significant outcome of our planned contrast for
520 **Hypothesis 3** ($F_{(1,67)}$=0.037 p=0.848, partial η^2=0.001). Pairwise comparisons once more
521 supported low freezing in Shock Controls across sex relative to all other groups, plus indicated
522 that male Social Stimulus + Stress mice froze significantly less than Post-Contingency Control
523 males (Supplemental Figure S3B).
524
525 Integrated with the results from social interaction testing, these findings indicate that conditioned
526 shifts in social engagement are distinct from conditioned fear to the social conditioning context.
527 In parallel, freezing behavior was relatively minimal during social interaction testing compared to
528 testing in the conditioning context, suggesting both that freezing behavior did not confound
529 social interaction outcomes and that mice were able to sufficiently discriminate between
530 contexts. Curiously, interaction of male Social Stimulus + Stress mice with familiar targets in a
531 novel environment on Day 2 might lead to mild reductions in contextual fear expression on Day
532 3.

533

534 **Table 8.** Two-way general linear model on both sexes' context fear testing average with

535 different sex targets.

Different Sex Experiment – Fear Expression Average			
	F Statistic	**p value**	**partial η^2**
Group	$F_{(4,67)}$=155.9	<0.001	0.903
Sex	$F_{(1,67)}$=0.008	0.929	0.000
Group × Sex	$F_{(4,67)}$=1.220	0.311	0.068

536

537 ***Serum Corticosterone***

538

539 Log-transformed corticosterone levels of mice in the same sex experiment exhibited no

540 significant interaction of group × sex, but a main effect of group was observed (Supplemental

541 Table S1, Supplemental Figure S3C). Pairwise comparisons showed that, across sex, Social

542 Stimulus + Stress mice in the same sex experiment exhibited elevated log-transformed

543 corticosterone levels compared to their respective same sex Shock Control mice (Supplemental

544 Figure S3C). While this was not surprising, male Pre- and Post-Contingency Control mice also

545 exhibited higher log-transformed corticosterone levels than Shock Control mice, whereas Social

546 Control mice did not (Supplemental Figure S3C). Conversely, female Social Control mice had

547 higher log-transformed corticosterone levels than Shock Control mice, but female Pre- and

548 Post-Contingency Control mice did not (Supplemental Figure S3C). Given no target was present

549 during Day 3 testing, these findings suggest enduring and sex-specific physiological differences

550 following social conditioning, despite minimal behavioral shifts. Specifically, any social

551 interaction with their assigned target on Day 1, whether concurrent or temporally separate with

552 foot shock exposure, elevated male log-transformed corticosterone levels but attenuated female

553 log-transformed corticosterone levels after context testing on Day 3 *in the absence of any*
554 *targets*. Only Social Stimulus + Stress females did not align with this pattern, exhibiting
555 heightened log-transformed corticosterone levels on Day 3 similar to those of Social Control
556 females (Supplemental Figure S3C).
557
558 Log-transformed corticosterone levels of mice in the different sex experiment, while not
559 exhibiting a significant sex × group interaction, did display significant main effects of both group
560 and sex (Supplemental Table S2). In contrast to the same sex experiment, pairwise
561 comparisons indicated that within each sex, levels of log-transformed corticosterone did not
562 differ across groups, including relative to Shock Control mice (Supplemental Figure S3D). The
563 only difference that reached significance was male Social Stimulus + Stress mice having lower
564 log-transformed corticosterone levels compared to female Social Stimulus + Stress mice
565 (Supplemental Figure S3D). Combined with log-transformed corticosterone data from the same
566 sex experiment (Supplemental Figure S3C), these findings illustrate how same sex target
567 interactions on the same day as an aversive learning experience can elicit sex-specific and
568 enduring hormone changes. Moreover, these lasting physiological shifts are not mirrored after
569 different sex target interactions. This indicates the combination of target and experimental sex,
570 when temporally concurrent or adjacent to a fear learning event, influences endocrine
571 responses to context days later.
572
573

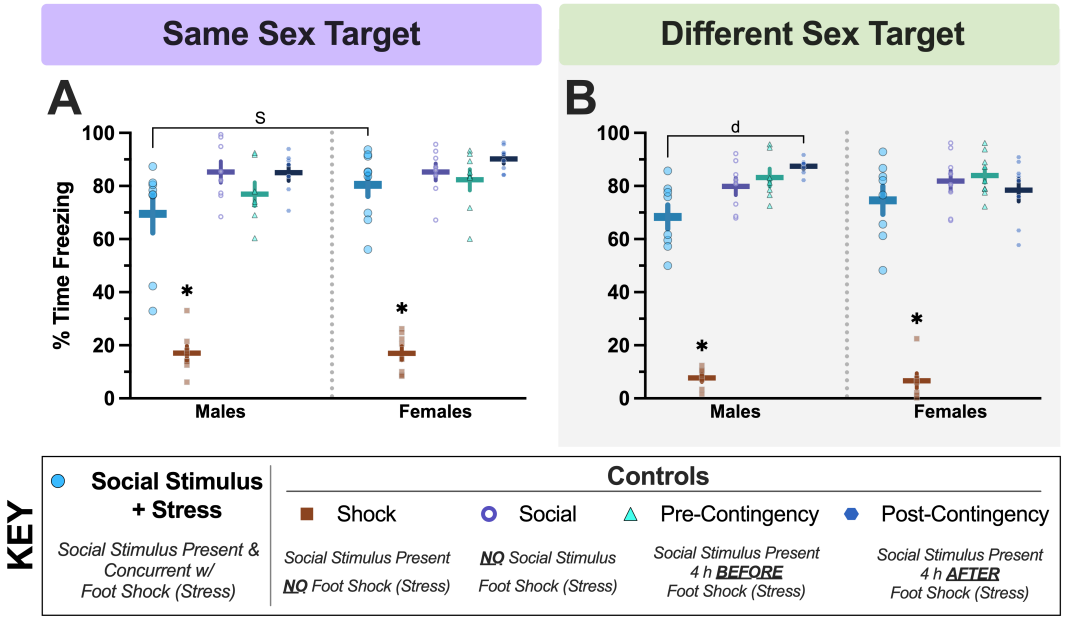

Figure 4. Day 3 social conditioning context fear expression averages. Average context fear behavior (Panels A, B) data are shown for mice in all groups in the same sex (A) and different sex (B) experiments. Numbers of mice graphed within Panels A-B in order, left to right: A) n=8, 8, 8, 7, 7, 9, 8, 8, 8, 8; B) n=8, 7, 8, 7, 7, 8, 7, 8, 9, 8. Left to right: ^{S}p=0.049, ^{d}indicates (Pre-Contingency Control vs. Social Stimulus + Stress) p=0.004. *p<0.001 Social, Pre-Contingency, and Post-Contingency Controls and Social Stimulus + Stress mice vs. Shock Control. Data graphed as mean ± standard error of the mean.

Discussion

Our goal in executing these experiments was to begin establishing a paradigm for inducing consistent, reproducible socially paired stress in mice across sexes. While our results indicate this specific paradigm does not yet accomplish our goal, we want to share our approach and findings so that others can utilize this information to efficiently prioritize future efforts in this domain. Moreover, despite a mostly negative outcome towards our overarching goal, we

591 nevertheless uncovered sex-specific patterns of behavioral and physiological stress responsivity
592 that are useful to fields incorporating sex as a biological variable, and/or seeking to employ
593 different sex social stressors in mice.
594
595 All mice that underwent social conditioning with mild foot shocks (Social, Pre-Contingency, and
596 Post-Contingency Controls plus Social Stimulus + Stress mice) ultimately ended their
597 acquisition stage at similar freezing levels. Subsequent outcomes in behavioral testing for
598 social engagement and contextual fear expression, as well as for serum corticosterone, could,
599 therefore, be interpreted with minimal concern for acquisition confounds. Conversely, this
600 consistency might have contributed to our negative findings. Alternatively, they might indicate
601 that our social conditioning protocol resulted in a ceiling effect. Additional studies would need to
602 parse this out.
603
604 Our social interaction findings were not as robust as anticipated. For our same sex experiment,
605 we found that male Social Stimulus + Stress mice engaged less during social testing compared
606 to Shock Control mice, and compared to Social Control mice that never encountered a target
607 during training. Additionally, male Social Stimulus + Stress mice exhibited less social
608 engagement than female Social Stimulus + Stress mice in same sex experiment, whereas
609 social engagement was unaffected across sexes in the different sex experiment. This reveals
610 that male mice likely form stronger associations when aversive experiences occur in conjunction
611 with the presence of another male mouse, whereas female mice appear to reduce social
612 engagement after experiencing non-social aversive stimuli. Such a sex difference could help
613 explain some of the challenges in developing social stress paradigms using female laboratory
614 mice (*Mus musculus*). Relatedly, this could indicate that, in the presence of a potential sexual
615 partner, threat assessment is suppressed. The inverse has been demonstrated in female rats;
616 that is, fear attenuates sexual behaviors, at least in part through amygdala and hypothalamus

617 estrogen receptor signaling (Moëne et al., 2019). Indirect evidence in male rats housed with
618 females post-context fear conditioning suggests that encounters with different sexes may
619 suppress threat assessment. These male rats subsequently exhibited attenuated fear
620 expression, involving dopamine receptor signaling in the hippocampus (Bai et al., 2009).
621 However, a different group studying mice reported that ejaculation by males was required for
622 retention of extinguished social fear conditioning (Grossmann et al., 2024). Continued
623 evaluations are needed to determine whether our observations – under conditions where
624 copulation is impossible – that male exposure to a female can diminish threat assessment
625 behaviors are reproducible. Evidence for social transmission of stress in rodents suggests an
626 alternative interpretation (Brandl et al., 2022). Instead, it could be that because the target for
627 each Social Stimulus + Stress mouse did not experience the same stress (mild foot shock), this
628 in turn affects the Social Stimulus + Stress mouse's perception of their own aversive experience
629 (Guzmán et al., 2009), likely in a sex-specific manner.
630
631 These sex-specific behavior patterns correspond to evidence that accumbal dopamine signaling
632 dynamics in mice are sex-dependent, both upon the sex of the studied mouse and the sex of
633 their assigned target (Dai et al., 2022). Our findings align with the success of using social defeat
634 to stress male mice. Additionally, they indicate that future studies seeking to suppress female
635 mice's social interaction behavior through a socially associated stimulus will meet with more
636 success if using an approach distinct from ours, which produced null results. Indeed, others
637 have encountered challenges when trying to elicit behavioral shifts in female mice, even after
638 four weeks of stress that involved only female targets (Díez-Solinska et al., 2023). One group
639 reported that female mice consistently prefer a socially paired food reinforcer, even if the pairing
640 was with a same sex target that had just previously undergone acute stress (mild foot shock)
641 (Kietzman et al., 2022). Because males' food preference was not studied by the researchers,
642 and neither did their females encounter stressed different sex targets, it remains unclear if these

643 effects are sex-specific, and if they would generalize to different sex interactions. Future studies
644 will also need to determine how socially conditioning female mice to male targets subsequently
645 affects interactions with female targets, and vice versa.
646
647 One day after social engagement testing, mice were tested for fear expression in the social
648 conditioning context in the absence of any targets. A sex difference in context fear expression
649 was observed between Social Stimulus + Stress mice in the same sex experiment, but not in
650 the different sex experiment. This is the only directionally comparable outcome with social
651 engagement, in that male Social Stimulus + Stress mice in the same sex experiment both
652 exhibited less context fear and less social engagement versus female Social Stimulus + Stress
653 mice. This could be interpreted in at least three ways. Male Social Stimulus + Stress mice
654 might have found their male target more salient than the context, thereby leading them to form
655 stronger associations between the mild foot shocks and their targets as compared to female
656 Social Stimulus + Stress mice. Another possibility is that when mice are in the vicinity of a
657 possible sexual partner, they could experience comparably impaired encoding of context cues,
658 though as discussed in the preceding three paragraphs, literature investigating such target-sex
659 based encoding effects is limited. Alternatively, the presence of the male target may have led to
660 social buffering of the mild foot shocks for male Social Stimulus + Stress mice (Guzmán et al.,
661 2009; Brandl et al., 2022), whereas this process did not occur for female Social Stimulus +
662 Stress mice in the same sex experiment. Because our goal was for mice to associate the
663 aversive mild foot shock with their assigned target, any social buffering of social conditioning
664 context fear could counteract our goal. This is why our use of targets of a different strain, that
665 were never housed in the same room as experimental mice, ensured they had never previously
666 been encountered (even via olfaction) by the mice tested here and therefore at baseline there
667 was no familiarity. Given the critical contribution of attachment to social buffering (see review
668 (Kiyokawa and Hennessy, 2018)), we can be reasonably confident that there were no social

669 buffering effects upon social conditioning context fear in same sex experiment mice. Thus, we
670 are inclined toward the first interpretation.
671
672 Serum was collected 30 minutes after context fear testing, 24 h after experimental mice's last
673 encounter with their assigned target. We were interested to discover that sex-specific group
674 differences in log-transformed corticosterone levels in the same sex experiment appeared to be
675 best explained by Day 1 group conditions, rather than behavior on Days 1-3. For example,
676 male Shock Control mice's log-transformed corticosterone levels were lower than Pre- and
677 Post-Contingency Controls and Social Stimulus + Stress groups – the three conditions where
678 both target exposure and foot shocks occurred within an eight h window on Day 1. Social
679 Control male mice only experienced foot shocks, and did not encounter a target, on Day 1.
680 Shock Control males similarly only encountered targets, and did not experience foot shocks on
681 Day 1; and male Social Control log-transformed corticosterone levels did not differ from Shock
682 Control males. Like males in the same sex experiment, female Social Stimulus + Stress mice
683 had elevated log-transformed corticosterone relative to Shock Control females. In contrast,
684 however, with this experiment's females, only Social Control females had higher log-
685 transformed corticosterone levels than Shock Control females. Pre- and Post-Contingency
686 Control females' log-transformed corticosterone was not different from Shock Controls, and the
687 former groups encountered targets four h before or after, respectively, experiencing foot shocks.
688 Collectively, this suggests social encounters temporally distal from the foot shock, but still
689 occurring within the same day, might augment males' and mitigate females' log-transformed
690 corticosterone responses to a stressful experience *two days later*. In contrast, coinciding foot
691 shocks and target exposure in Social Stimulus + Stress mice obscured this sex difference in
692 endocrine response.
693

694 Unlike the same sex experiment, the different sex experiment mice's log-transformed
695 corticosterone levels did not map onto groups in either sex, despite a significant group effect
696 being detected. Also distinct from the same sex experiment is that log-transformed
697 corticosterone levels of Social Stimulus + Stress mice in the different sex experiment had a sex
698 difference. Specifically, female Social Stimulus + Stress mice had greater log-transformed
699 corticosterone levels than male Social Stimulus + Stress mice. This does not map onto their
700 social engagement on Day 2, nor their average context fear expression on Day 3. This log-
701 transformed corticosterone level difference could, therefore, be an enduring endocrine effect of
702 encountering their assigned targets on Day 2.
703
704 To ensure we rigorously evaluated this modified paradigm's hypothesized utility as a higher
705 throughput approach for more consistent induction of social stress across sexes, we included
706 four separate controls. Had we only included one Control group, e.g., Shock Controls, we might
707 have incorrectly concluded that our modified paradigm successfully reduced social engagement
708 in females, if not across sexes. Through the inclusion of four Control groups, however, we have
709 concluded that our findings do not support our hypothesis, and that we cannot endorse this
710 paradigm in its current state. Instead, our negative findings indicate that this protocol requires
711 additional optimization to achieve its overarching goal. Nevertheless, we are sharing our
712 approach and findings so that others with similar procedure goals can learn from our
713 observations, and improve upon our study's limitations. If we had been able to continue our
714 investigations, we would have next reduced the mild foot shock amplitude, the number of foot
715 shocks, or some combination thereof. Given the group differences in freezing that emerged
716 early during our social conditioning, milder and/or less aversive stimuli could uncover enduring
717 effects of group conditions. To begin though, it was important here that all our groups attained
718 similar starting points to minimize confounds for testing interpretations. We would also advise
719 other researchers to consider optimizing their apparatus and software, if possible, to facilitate

720 consistent analyses of interaction and freezing, if their conditioning/stress component generates
721 freezing. We used one software to administer foot shocks while simultaneously recording and
722 quantifying freezing, and another software to track animal location and identify time spent within
723 specific arena locations, each in separate apparatus. Both software programs excel at their
724 purposes, but each have unique – and unfortunately incompatible – video formats plus
725 requirements regarding lighting, contrast, and background for optimal behavior/location
726 detection.
727
728 Another limitation is that we could not evaluate log-transformed corticosterone levels over time
729 without introducing additional confounding stressors to repeatedly sample blood. Daily blood
730 sampling would reveal if our log-transformed corticosterone observations were attributable to
731 specific days, how log-transformed corticosterone levels mapped on to each day's behavior
732 measure, and/or if log-transformed corticosterone differences are transient or persistent. Our
733 experiments were all performed in sexually naïve mice. We recognize that sexually
734 experienced mice (for example (Newman et al., 2019)) of both sexes might respond,
735 behaviorally and physiologically, in a manner distinct from what we report here. Earlier
736 practices of dividing mice into 'susceptible' and 'resilient' groups following social stress have
737 more recently fallen out of favor, given categorization of those responses are context-specific,
738 require substantial rodent numbers for optimal data confidence, and neither phenotype can be
739 universally deemed 'better' or 'worse' (Koolhaas et al., 2017; Lyons et al., 2023). We therefore
740 evaluated all Social Stimulus + Stress mice as a single group. Still, bimodal distribution of
741 social engagement in Social Stimulus + Stress mice looks possible in all but the different sex
742 Social Stimulus + Stress males. Increasing numbers to establish definitive thresholds with
743 sufficient power for phenotypic grouping could prove informative, particularly for the same sex
744 experiment. The same limitations as listed above would still apply, however. Finally, our
745 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. Once a short-term protocol has been optimized, duration of effects would be a critical next step in determining validity of the paradigm.

Here, we have reported a novel, albeit unsuccessful, foray into sex-inclusive efforts to develop a controllable, less labor-intensive, and higher throughput social stress paradigm in mice. These experiments have provided utilizable insights regarding how mice of both sexes associate a same- or different-sex target with an aversive experience. We report that these associations seem strongest in males after same sex encounters, whereas females appeared not to form social stimulus associations. Such distinctive responses are probably evolutionarily favorable. While our investigation has generated new information, our modified paradigm did not accomplish what we anticipated. We share our negative findings here to encourage and assist other labs to achieve their own positive outcomes, and to spare them (and mice) the time and effort of duplicating the present work. Development of a sex-inclusive social stress paradigm for mice remains a worthwhile pursuit, as such would be a boon across multiple disciplines.

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