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 mice 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). In the original paradigm, 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

130 conspecific mice were the same or different sexes. Our broad goal was to develop a paradigm
131 that would provide a useful first step for future investigations of social behavior across mouse
132 sexes, which are currently underrepresented in literature (22).
133
134
135
136 **Methods**
137
138 ***Mice***
139 Hybrid F1 offspring of both sexes resulting from pairing female 129S1/SvlmJ and male
140 C57BL/6J mice (hereafter experimental mice), and male and female C57BL/6J mice (hereafter
141 conspecifics), all ≥9 weeks old or older, were group-housed (2-5 per cage) within sex. All mice
142 had *ad libitum* access to food and water in rooms maintained on a 12:12 light/dark cycle with
143 lights on at 07:00 local standard time, and temperature maintained at 22 ± 2°C. All mice were
144 fed LabDiet 5001 rodent laboratory chow (LabDiet, Brentwood, MO) and were kept on 7090
145 Teklad Sani-chip bedding (Envigo, East Millstone, NJ) in cages containing Nestlets (Ancare,
146 Bellmore, NY) and huts (Bio-Serv, Flemington, NJ) for enrichment. Experiments were approved
147 by the Kent State University Institutional Animal Care and Use Committee, and adhered to the
148 National Research Council's Guide for the Care and Use of Laboratory Animals, 8th Ed.
149 undefined.
150
151 ***Social Conditioning Paradigm***
152
153 The entire paradigm spans four consecutive days. Procedures for each individual day are
154 outlined below in order.
155

156 *Pre-exposure (Day 0)*

157 Pre-exposure was used to help reduce the novelty of the transparent enclosure and/or the
158 conspecific to the experimental mouse, plus minimize any potential sex differences in
159 acquisition (48–51). On Day 0, experimental mice were placed in Coulbourn Instruments
160 chambers (17.8 cm D × 17.8 cm W × 30.5 cm H; Allentown, PA) with (Control A and SC mice)
161 or without (Control B-D mice) a conspecific (age-matched; either same sex or different sex
162 relative to experimental mouse, depending on if assigned to same sex or different sex
163 experiment) for pre-exposure (Fig. 1). Chambers comprised two opposing aluminum walls each
164 adjoining two clear acrylic walls. Conspecifics were put inside a transparent enclosure placed
165 within a corner of the social conditioning chamber. The transparent enclosure included 23 holes
166 (each 0.32 cm diameter) on each of the 2 sides facing the SC mouse to enable olfactory cue
167 exchanges. Previous work demonstrates that olfaction is the most important modality when
168 recognizing and interacting with novel conspecifics (see review (52)); tactile (whiskers) and
169 auditory modalities appear to only be recruited when recognizing cage mates (46) in male mice
170 (females were not studied). Chambers and enclosures were cleaned with 70% ethanol before
171 and after each session; chambers had visible illumination and one clear acrylic wall was marked
172 with a blue dotted pattern. These components contributed olfactory and visual cues to the
173 context, in addition to the tactile cue of the stainless steel grid floor for the experimental mouse.
174 During pre-exposure, experimental mice were allowed to explore the chamber and conspecific
175 enclosure for 5 min, then both mice were returned to their respective home cages.

176

177 *Social Conditioning (Day 1)*

178 On Day 1, experimental mice were placed in the same social conditioning chamber as Day 0
179 and received five, 1 s mild foot shocks (1.0 mA) in the presence of the same conspecific that
180 they encountered on Day 0 (Figure 1A). This was to have experimental mice form associations
181 between aversive foot shocks and their respective conspecific (Fig. 1). Conditioning lasted for 9

182 minutes; after a 2 minute baseline, shocks were then administered at 120, 210, 300, 390, and
183 480 s. Conspecifics did not receive foot shocks. Cameras mounted above the chambers were
184 used to record movements, and freezing behavior during conditioning was quantified using
185 FreezeFrame software (v. 5.201, Actimetrics, Wilmette, IL). Freezing behavior is indicative of
186 fear/aversive conditioning in mice; freezing was defined as the absence of movement except
187 breathing.
188
189 *Social Interaction Testing (Day 2)*
190 On Day 2, experimental mice were placed in a novel room containing open arenas (41.9 cm W
191 × 41.9 cm D × 39.6 cm H). Mice acclimated to the room in their home cage for 30 min prior to
192 behavior testing commencing. Each open arena had one empty PVC tube (8.9 cm outer
193 diameter) in a single corner of the square chamber with wire mesh (6.35 mm^2 openings)
194 covering a rectangular portion (11.4 cm W x 4.1 cm H) cut out of the tube bottom. Experimental
195 mice were placed in the corner of the arena opposite the PVC tube and could investigate freely
196 for 2.5 minutes (pre-test). Immediately after these 2.5 min, the conspecific that each
197 experimental mouse had previously been conditioned with on Day 1 was then placed in the PVC
198 tube in the corner. Experimental mice were allowed to continue investigating the arena for an
199 additional 5 min (post-test; Fig. 1). Because of the mesh at the bottom of the PVC tubes
200 containing conspecifics, exchanges of visual, auditory, and olfactory cues between experimental
201 mice and conspecifics were possible, but tactile interactions were minimized. Interaction
202 behavior was quantified as duration when at least 80% of an experimental mouse's body was in
203 the interaction zone, a 7 cm radius zone (~223 cm^2 – 240 cm^2, i.e., 14-15% of the open arena)
204 around the base of the PVC tube. This was quantified using ANY-maze software (v. 7.09
205 Stoelting Co., Wood Dale, IL). Post-test social interaction time was log-transformed
206 (Y=log(Y+0.001) to account for 3 mice with 0 s post-test social interaction) so that data would be
207 normally distributed for statistical analyses. Females were intentionally not assessed for estrus

208 cycle stage for five reasons: 1) to minimize mouse usage (53), we did not power our studies for
209 assessment of estrus; 2) with the goal of developing a social stress paradigm, we are seeking
210 effects robust enough to not depend upon estrus in intact, randomly cycling females; 3) we
211 intentionally focused here on fear and social behaviors, and did not measure sexual behaviors
212 (e.g., lordosis); 4) evidence that overall mouse behaviors are not affected by estrus stage
213 (54,55), (but see (56)); 5) cross-species evidence indicates vaginal lavage to determine estrus
214 cycle is stressful (57,58), and we sought to minimize stress confounds here.
215
216 *Context Testing (Day 3)*
217 On the last day of the social conditioning paradigm, experimental mice were placed in the social
218 conditioning chamber for behavior testing in the *absence* of any conspecific (Fig. 1). The
219 conspecific enclosure was still present to keep the context consistent with conditioning. All
220 other tactile, visual, and olfactory cues from Day 1 were present. Testing lasted 10 min and did
221 not involve any foot shocks. Freezing behavior was again quantified using FreezeFrame
222 software.
223
224 ***Treatment Groups***
225
226 The SC group involved mice that underwent social conditioning in the presence of a non-
227 shocked conspecific (either same sex or different sex relative to SC mouse, depending upon
228 experiment), with the hypothesis that SC mice would associate the conspecific with this aversive
229 experience. We planned four Control groups for this one SC group; each experiment (same sex
230 or different sex) had its own respective set of four Control groups for its respective SC group.
231 The goals of these were to control for: A) foot shock exposure; B) presence of conspecific
232 during pre-exposure & foot shock; C & D) exposure to conspecific temporally distal to foot shock
233 exposure. Control A involved procedures identical to those of the SC group, except a foot shock

234 was never administered on Day 1 (Fig. 1). Control B was the same as the SC group procedure,
235 except experimental mice never encountered the conspecific until testing on Day 2 (Fig. 1).
236 Controls C and D had experimental mice encountering conspecifics either 4 h before (Control C)
237 or 4 h after (Control D) pre-exposure and social conditioning; in other words, conspecifics were
238 not present in the conditioning chamber during these two periods. Instead, experimental mice
239 were exposed to their conspecific in a separate room for 5 minutes or 9 minutes (the same
240 amount of time as pre-exposure or social conditioning, respectively) 4 h before (Control C) or 4
241 h after (Control D) the experimental mice were exposed to the conditioning chamber. This
242 exposure involved experimental mice and their respective conspecific being placed in a clean
243 mouse cage separated with an acrylic divider to allow for visual, auditory, and olfactory
244 exchange, but no tactile interaction. Mice in Control C or D conditions still experienced foot
245 shock on Day 1, and encountered their assigned conspecific for testing on Day 2 (Fig. 1). This
246 temporal separation of 4 h was to minimize consolidation of social encounters from
247 interfering/intermingling with consolidation of the aversive foot shock encounter in the social
248 conditioning chamber, while still making execution of these experiments feasible within a 12 h
249 lights-on period (59–61) (see reviews (62,63)).
250

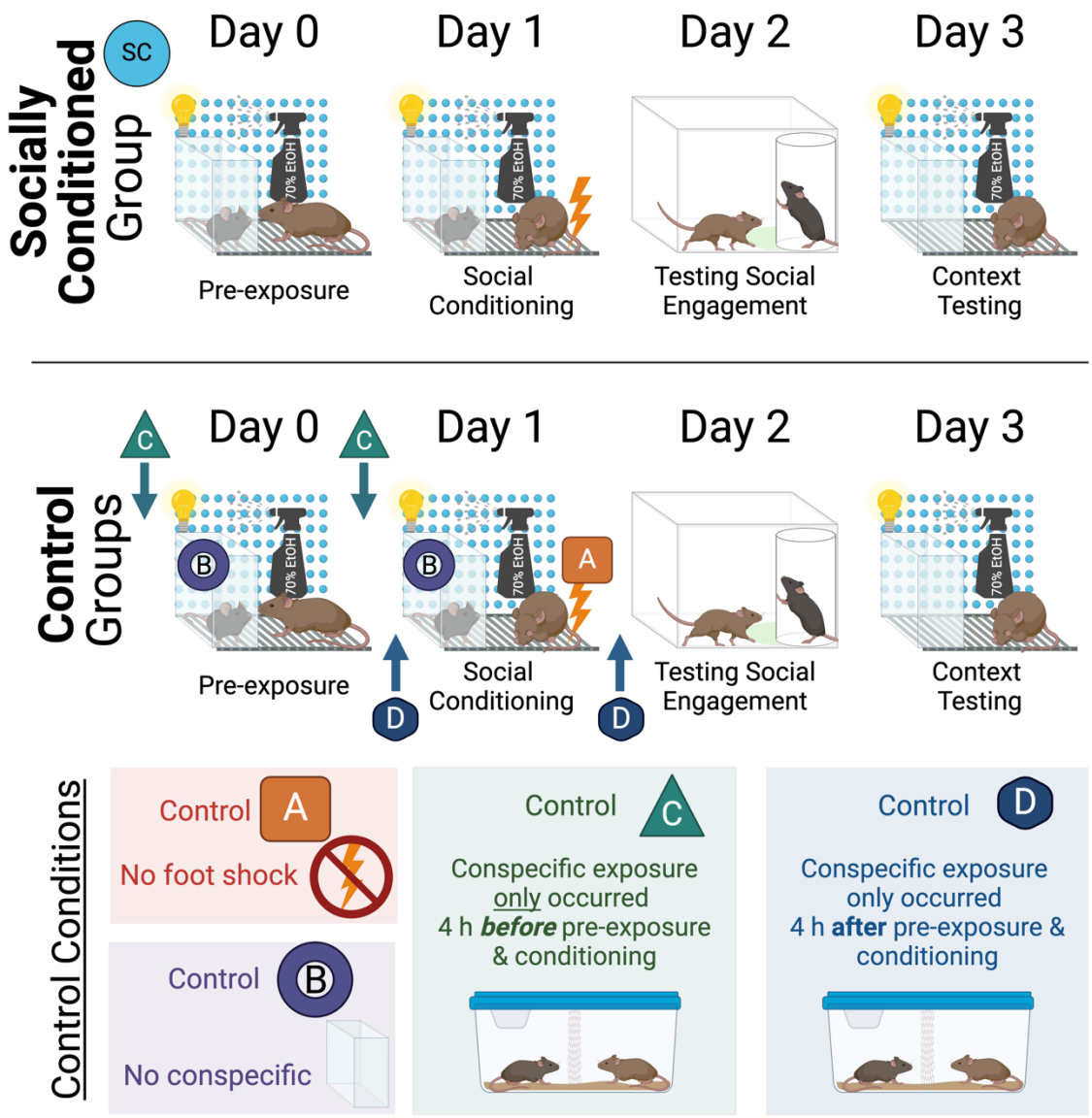


Figure 1. Study timeline and treatment groups. Five groups were used in this study for each experiment (same sex experiment, and different sex experiment): one socially conditioned (SC) group and four Control groups. Day 0 was for pre-exposure; Day 1 was when social conditioning occurred; Day 2 tested for social engagement; Day 3 tested for context-specific freezing behavior. 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),

258 except no foot shock was administered on Day 1. Control B mice similarly experienced the
259 same procedure as mice in the SC group (above horizontal line), save that Control B mice did
260 not encounter any conspecific until Day 2 for testing of social engagement. Control C involved
261 mice encountering their conspecific 4 h before pre-exposure and social conditioning, meaning
262 no conspecific was present for Control C mice when they were in the social conditioning
263 chamber for pre-exposure or social conditioning. To encounter their assigned conspecific,
264 Control C mice were placed into a clean cage with a clear acrylic divider separating them from
265 their conspecific. This divider allowed visual, olfactory, and auditory exchanges but no tactile
266 interactions. On Day 0, Control C mice encountered their conspecific for 5 min; on Day 1, for 9
267 min; these were timed to match the length of conspecific exposure that mice in the SC group
268 experienced. Control D mice were treated the same as Control C mice, except their encounters
269 with their assigned conspecifics occurred 4 h *after*, rather than before, pre-exposure and social
270 conditioning.
271
272 ***Serum Corticosterone***
273
274 Thirty minutes after all experimental mice underwent contextual fear testing on Day 3, they were
275 briefly anesthetized with isoflurane then rapidly decapitated for trunk blood collection. Blood
276 clotted at room temperature for 10 minutes, then was spun at 3500 rpm for 1 h at 4°C. Serum
277 was collected and stored at -80°C until corticosterone analyses could be performed. Serum
278 corticosterone was measured using Enzo Life Sciences corticosterone enzyme-linked
279 immunosorbent assay (ELISA) kits (Farmingdale, NY). Assays were run according to the
280 manufacturer's instructions using their small volume protocol. Plates were read at 405 nm with
281 correction at 580 nm. The sensitivity of the assay was 26.99 pg/mL.
282
283 ***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 the Supplemental Dataset. 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 (RM) 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 (SC and Control B-D 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

309 Social conditioning acquisition was examined in experimental mice in either the presence or
310 absence of a conspecific. One experiment used same-sex conspecifics, and the other
311 experiment used different sex conspecifics; each of these experiments included its own SC
312 group and corresponding four Control groups.
313
314 *Control A (no foot shock) mice*
315 Control A mice were utilized to distinguish the effects of receiving mild foot shocks on
316 subsequent social behavior. Figure 2A and 2C show social conditioning 'acquisition' on Day 1
317 for Control A mice with either a same sex conspecific (Figure 2A) or a different sex conspecific
318 (Figure 2C). For acquisition, there was a significant three-way interaction of time × conspecific
319 sex × experiment (Table 1). This appears to be driven primarily by inconsistent time course
320 patterns across sexes, and mice in the different sex experiment exhibiting lower freezing levels
321 at several timepoints compared to same sex experiment mice (Figure 2A, 2C).
322
323 **Table 1.** Three-way RM ANOVAs on Control A mice's social conditioning acquisition across
324 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**

325 **Bold** indicates highest interaction or main effect with p<0.05
326
327 Two days after social 'conditioning' (see Figure 1 for timeline), Control A mice were tested for
328 their fear to the context in which they were 'conditioned'. Testing lasted for 10 minutes, and the
329 time course data for testing Control A mice are in Figure 2B (same sex conspecific) and 2D

330 (different sex conspecific). We observed no significant three-way interaction of time × sex ×
331 experiment (Table 2). There were likewise no significant two-way interactions of time × sex, time
332 × experiment, or sex × experiment (Table 2). Main effects of sex and time were not significant,
333 though a non-significant trend for time was noted (p=0.099; Table 2). A significant main effect
334 of experiment was detected (Table 2), and pairwise comparisons support a pattern of female
335 and male mice in the different sex experiment freezing less than mice in the same sex
336 experiment (Figure 2B, 2D), as observed with 'training'.
337
338 **Table 2.** Three-way RM ANOVA on Control A mice's context testing time course across
339 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

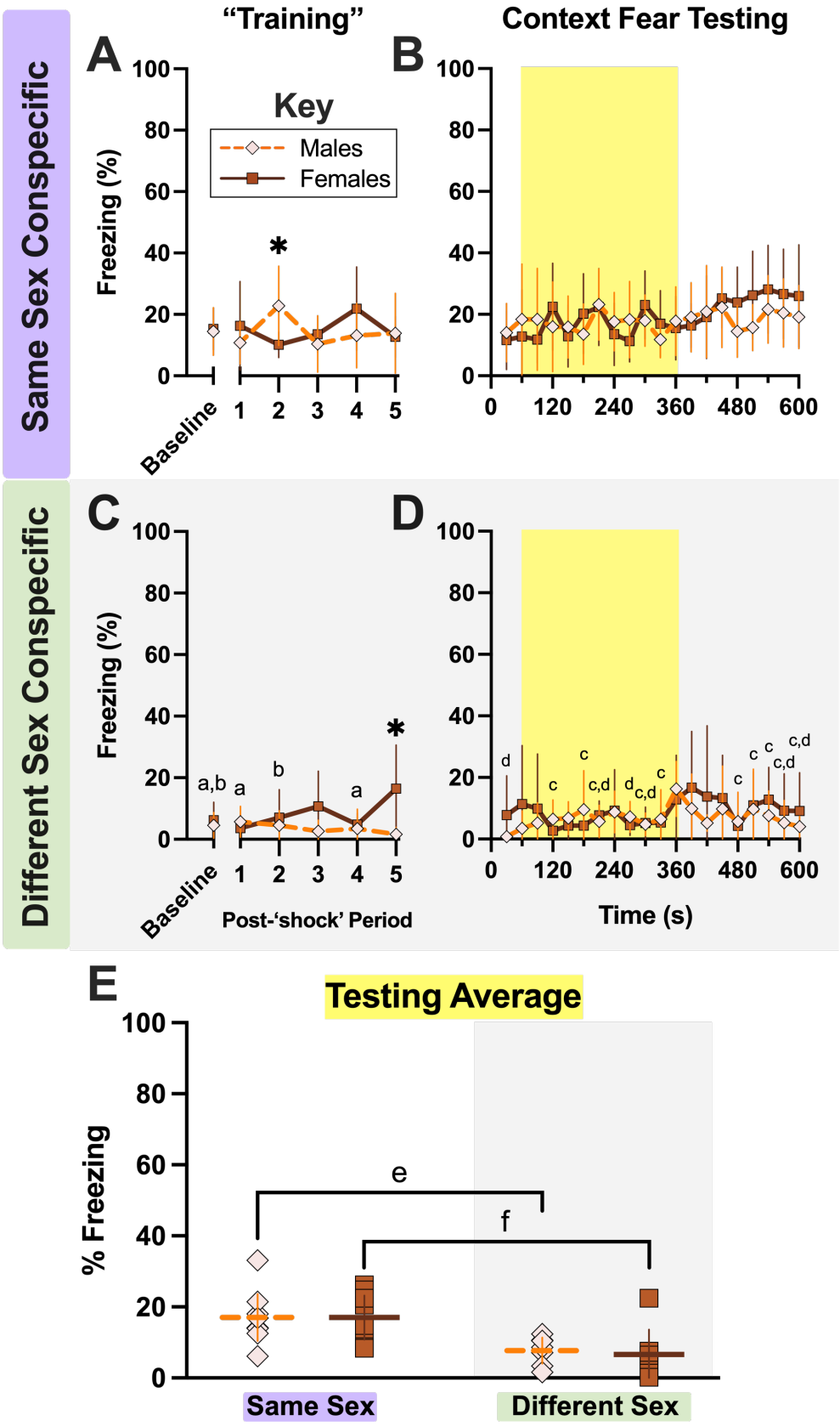
340 **Bold** indicates highest interaction or main effect with p<0.05; *italics* indicates p<0.10 and partial η^2 >0.060
341
342 Consistent with 'training' and time course testing of Control A mice, averaged freezing during
343 minutes two through six of testing revealed no sex × experiment interaction, but a significant
344 main effect of experiment (Table 3). Once more, different sex experiment mice exhibited less
345 freezing than their counterparts in the same sex experiment (Figure 2E).
346
347 **Table 3.** Two-way ANOVA on Control A mice's context testing average across experiments and
348 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

349 **Bold** indicates highest interaction or main effect with $p<0.05$

350


Same Sex Conspecific
Different Sex Conspecific
A
"Training"
B
Context Fear Testing
Key
Males
Females
Freezing (%)
Baseline
1
2
3
4
5
0
120
240
360
480
600
C
D
a,b
a
b
a
c
c,d
d
Post-'shock' Period
Time (s)
E
Testing Average
% Freezing
e
f
Same Sex
Different Sex

351

352 **Figure 2.** ***Day 1 social conditioning 'acquisition' and Day 3 context fear testing in Control***
353 ***A mice***. Control A (no foot shock) mice underwent 'acquisition' or 'training' for social
354 conditioning after a two min baseline period on Day 1 (Panels A,C). Percent time freezing for
355 the same 30 s periods that are graphed for SC and Control B-D mice (Figure 3) are indicated at
356 time points 1-5 in Panels A,C. On Day 3, freezing behavior was quantified for 10 min when mice
357 were re-exposed to the social conditioning chamber (Panels B,D). Average freezing behavior
358 was evaluated between minutes two through six (yellow shading in Panels B,D) of the 10 min
359 test and graphed in Panels C,F. Mice either went through the Control A social conditioning
360 process with same sex conspecifics (Panels A,B,E) or different sex conspecifics (Panels C,D,E).
361 Same sex n=8, different sex n=7. *indicates p=0.017 (A), p=0.026 (Panel C) compared to other
362 sex within the same experiment. [a]indicates p=0.026, p=0.026, p=0.007, left to right, comparing
363 females in same sex (Panel A) versus different sex (Panel C) experiment 'training' at specified
364 timepoints. [b]indicates p=0.016, p=0.002, left to right, comparing males in same sex (Panel A)
365 versus different sex (Panel C) experiment 'training' at specified timepoints. [c]indicates p=0.008,
366 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
367 right, comparing females in same sex (Panel B) versus different sex (Panel D) experiment
368 testing at specified timepoints. [d]indicates p=0.024, p=0.006, p=0.030, p=0.011, p=0.030,
369 p=0.046, left to right, comparing males in same sex (Panel B) versus different sex (Panel D)
370 experiment testing at specified timepoints. [e]indicates p=0.016, [f]indicates p=0.008, comparing
371 across experiments within sex for average testing freezing (Panel E). Data graphed as mean ±
372 95% CI.

373

374 *Socially Conditioned and Control B-D mice*

375 Social conditioning acquisition for mice that received mild foot shocks are illustrated in Figure
376 3A, 3B for mice in the same sex conspecific experiment, and in Figure 3C, 3D for mice in the

377 different sex conspecific experiment. All mice expressed >40% freezing during at least one
378 post-shock period during social conditioning. For acquisition in the same sex experiment, no
379 three-way interaction of time × sex × group (of which there are four: socially conditioned mice or
380 Control B-D mice) occurred (Table 4). We did not observe any significant two-way interactions
381 either (Table 4). Nonetheless, significant main effects of time, sex, and group were found for
382 mice in the same sex experiment (Table 4).
383
384 **Table 4.** Three-way RM ANOVAs on fear acquisition for mice of both sexes with same sex
385 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

386 **Bold** indicates highest interaction or main effect with p<0.05
387
388 Pairwise comparisons indicated that socially conditioned males exhibited less freezing at two
389 training timepoints relative to Control B mice (Figure 3A) in the same sex experiment. For all
390 but the last post-shock period in the same sex experiment, female socially conditioned mice
391 froze significantly more than male socially conditioned mice (Figure 3A, 3B).
392
393 Social conditioning acquisition for mice in the different sex experiment is shown in Figure 3C,
394 3D. No significant three-way interaction of time × sex × group was observed (Table 5), though a
395 non-significant trend (p=0.076) was noted. While no sex × group interaction was detected, we
396 did find significant time × group and time × sex interactions (Table 5).
397

398 **Table 5.** Three-way RM ANOVAs on fear acquisition for mice of both sexes with different sex

399 conspecifics.

Different Sex Experiment – Fear Acquisition			
	F Statistic	**p value**	**Partial η^2**
Time	F(4.342,251.8)=203.7	<0.001	0.778
Sex	F(1,58)=5.464	0.023	0.086
Group	F(3,58)=1.098	0.357	0.054
Time × Group	**F(13.03,251.8)=3.548**	**<0.001**	**0.155**
Time × Sex	**F(4.342,251.8)=3.321**	**0.009**	**0.054**
Group × Sex	F(3,58)=0.950	0.423	0.047
Time × Group × Sex	*F(13.03,251.8)=1.636*	*0.076*	*0.078*

400 **Bold** indicates highest interaction or main effect with $p<0.05$; *italics* indicates $p<0.10$ and partial η^2 >0.060

401

402 Only the second post-shock period was different across sexes in SC mice in the different sex

403 experiment (Figure 3C, 3D). For Control C mice, females displayed greater freezing for the first

404 three post-shock periods than males in the different sex experiment (Figure 3C, 3D). All other

405 between-group comparisons within each sex did not exhibit consistent patterns, and as with

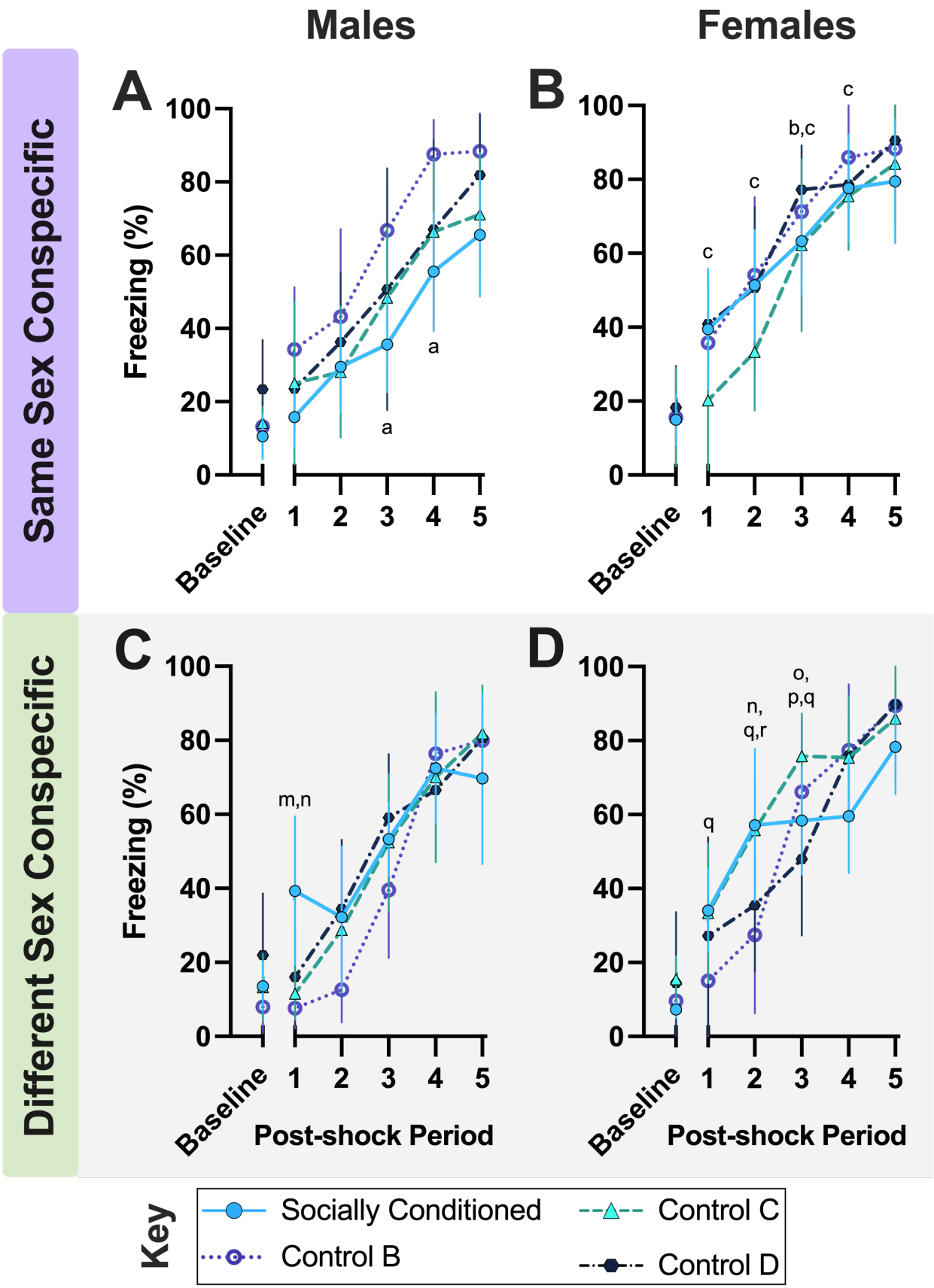
406 same sex experiment mice, different sex experiment mice concluded training at similar freezing

407 levels. Combined with same sex experiment acquisition, these findings indicate that social

408 exposure – whether concurrent or 4 h prior – can transiently augment social conditioning

409 acquisition in female mice, depending upon the sex of the conspecific.

410


Males
Females
Same Sex Conspecific
Different Sex Conspecific
A
B
C
D
Freezing (%)
Baseline
1
2
3
4
5
Post-shock Period
a
a
c
c
b,c
c
m,n
q
n,
q,r
o,
p,q
Key
Socially Conditioned
Control B
Control C
Control D

411

412 **Figure 3.** ***Day 1 acquisition during social conditioning procedure***. Male (Panels A, C) and
413 female (Panels B, D) mouse numbers are, respectively: Socially conditioned (SC; n=8, 9, 9, 8);
414 Control B (n=8 for all); Control C (n=7, 8, 8, 9); Control D (n=7, 8, 8, 8). Same sex experiment
415 data are in Panels A,B; different sex experiment data in Panels C,D. Average freezing for the
416 first two minutes, prior to commencement of acquisition, is plotted on the x-axis as baseline. The
417 average percent freezing for each 30 second period following each of the five mild foot shocks
418 are thereafter plotted along x-axis (Post-shock Periods 1-5). *Same sex experiment*: [a]indicates
419 p=0.037, p=0.006 (left to right, Panel A) when comparing within males and between SC and
420 Control B groups. [b]indicates p=0.022 (Panel B) when comparing between sexes and within
421 Control D group. [c]indicates p=0.011, p=0.047, p=0.012, p=0.016 (left to right, Panel B) when
422 comparing between sexes within the SC group. *Different sex experiment*: [m]indicates p=0.042
423 (Panel C) when comparing within males and between Control C and SC groups. [n]indicates
424 p=0.014 (Panel C) or p=0.046 (Panel D) when comparing within sex and between Control B and
425 SC groups. [o]indicates p=0.033 (Panel D) when comparing within females and between Control
426 C and D groups. [p]indicates p=0.010 (Panel D) when comparing between sexes and within
427 Control B group. [q]indicates p=0.032, p=0.012, p=0.019 (left to right, Panel D) when comparing
428 between sexes and within Control C group. [r]indicates p=0.020 (Panel D) when comparing
429 between sexes and within SC group. Data graphed as mean ± 95% CI.

430

431 ***Social Interaction***

432

433 Twenty four hours after social conditioning, mice were tested for social engagement using the
434 social interaction test. Three mice did not interact at all with their conspecific, and thus were
435 excluded from social interaction analyses (1 different sex Control A female; 1 same sex Control
436 B female; 1 same sex SC male; see Supplemental Dataset). 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

Bold indicates highest interaction or main effect with $p<0.05$

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

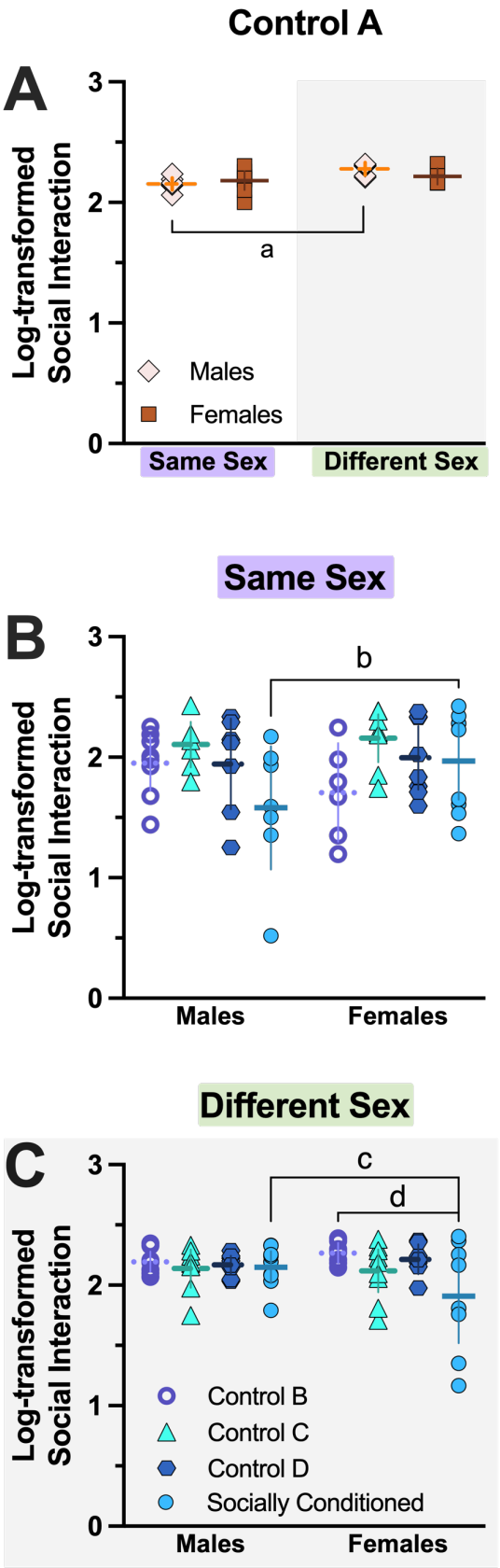
Bold indicates highest interaction or main effect with $p<0.05$

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).

458 Combined, these findings indicate that the social behavior of females is more affected by
459 aversive associations with males, whereas males are more socially affected by aversive
460 associations with their own sex.
461
462 **Table 8.** Two-way ANOVA on social interaction for mice of both sexes with different sex
463 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

464 *Italics* indicates $p<0.10$ and partial $\eta^2 >0.060$
465


Control A
A
Log-transformed Social Interaction
3
2
1
0
a
Males
Females
Same Sex
Different Sex
Same Sex
B
Log-transformed Social Interaction
3
2
1
0
b
Males
Females
Different Sex
C
Log-transformed Social Interaction
3
2
1
0
c
d
Control B
Control C
Control D
Socially Conditioned
Males
Females

466

467 **Figure 4.** ***Day 2 social interaction behavior following social conditioning***. Log-
468 transformed social interaction data are shown for mice in Control A groups (Panel A), and in the
469 same (Panel B) and different (Panel C) sex experiments. Numbers of mice graphed within each
470 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,
471 [b]p=0.038, [c]p=0.029, [d]p=0.017. Data graphed as mean ± 95% CI.

472

473 ***Social Conditioning Context Fear Testing***

474

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

479

480 In the same sex experiment, no significant three-way interaction was detected, nor were
481 significant two-way interactions of group × sex or time × sex (Table 9). A non-significant trend
482 for time × group was noted (p=0.073). Time was found to have a significant main effect (Table
483 9), as is typical for context fear expression testing, with continued exposure to the social
484 conditioning context in the absence of the conditioned stimulus resulting in gradual fear
485 extinction. A significant main effect of sex was also found, likely driven by lower freezing levels
486 across time in Control C and SC mice (Table 9, Figure 5A, 5B). A non-significant trend for
487 group (p=0.056, Table 9) was also noted. In the different sex experiment, no significant three-
488 nor two-way interactions were observed. Along with a main effect of time (Table 10), a
489 significant effect of group was also found (Table 10). Pairwise comparisons revealed significant
490 differences between groups at specific timepoints (Figure 5). The most consistent of these for
491 the same sex experiment involved Control C males in the same sex experiment exhibiting

accelerated extinction processes relative to Control C females, and SC males displaying attenuated freezing behavior compared to SC 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 resulted in a significant main effect of group in the same sex experiment (Table 11, Figure 5C), though pairwise comparisons did not reveal any significant between-group differences. 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 RM ANOVAs on both sexes' context fear testing with same sex conspecifics.

Same Sex Experiment – Fear Expression Time Course			
	F Statistic	**p value**	**Partial η^2**
Time	**F(11.34,623.4)=9.498**	**<0.001**	**0.147**
Sex	**F(1,55)=5.052**	**0.029**	**0.084**
Group	*F(3,55)=2.671*	*0.056*	*0.127*
Time × Group	*F(34.00,623.4)=1.388*	*0.073*	*0.070*
Time × Sex	F(11.34,623.4)=1.743	0.059	0.031
Group × Sex	F(3,55)=0.898	0.448	0.047
Time × Group × Sex	F(34.00,623.4)=1.064	0.372	0.055

Bold indicates highest interaction or main effect with $p<0.05$; *italics* indicates $p<0.10$ and partial $\eta^2 >0.060$

Table 10. Three-way RM ANOVAs on both sexes' context fear testing 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

Bold indicates highest interaction or main effect with $p<0.05$

Table 11. Two-way ANOVA on both sexes' context fear testing average with same sex conspecifics.

Same Sex Experiment – Fear Expression Average			
	F Statistic	**p value**	**Partial η^2**
Sex	F(1,55)=3.137	0.082	0.054
Group	**F(3,55)=3.603**	**0.019**	**0.164**
Group × Sex	F(3,55)=0.338	0.636	0.030

Bold indicates highest interaction or main effect with $p<0.05$

Table 12. Two-way ANOVA on both sexes' context fear testing average 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

Bold indicates highest interaction or main effect with $p<0.05$

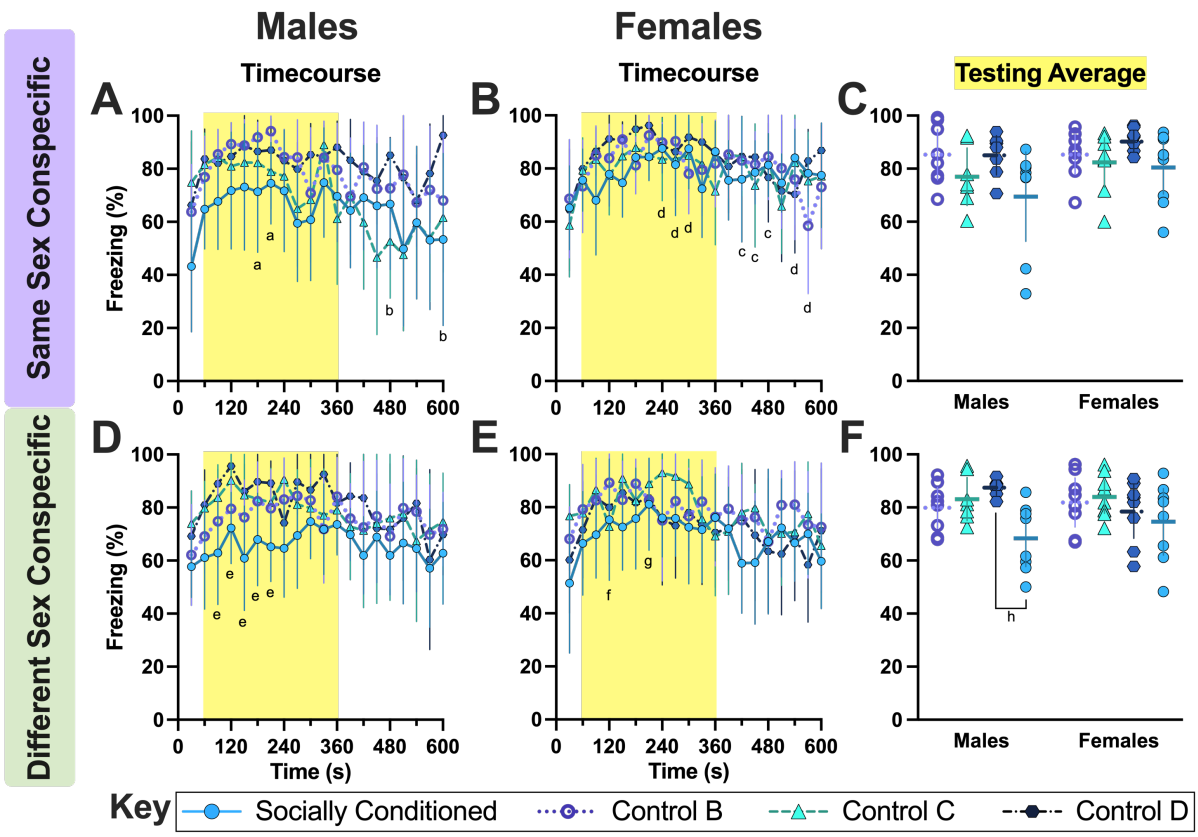


Figure 5. Day 3 social conditioning context fear behavior as time courses and averages. Time course (A, B, D, E) and average (C, F) context fear behavior data are shown for mice in all groups in the same sex (top row) and different sex (bottom row) experiments. Respectively, numbers of mice in top row: males – 8, 7, 7, 8; 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=0.025, p=0.009 (left to right, Panel A) between Control B and SC male mice in the same sex experiment at indicated time points. [b]indicates p=0.020, p=0.021 (left to right, Panel A) between Control C and D male mice at indicated time points (Panel A). [c]indicates p=0.031, p=0.026, p=0.005 (left to right, Panel B) between Control C mice of both sexes within the same sex experiment at indicated time points. [d]indicates p=0.049, p=0.025, p=0.011, p=0.045, p=0.039 (left to right, Panel B) between SC mice of both sexes within the same sex experiment at indicated time points. [e]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. [f]indicates p=0.037 between Control C mice of both sexes within the

540 different sex experiment at indicated time point. [g]indicates p=0.038 between SC mice of both
541 sexes within the different sex experiment at indicated time point. [h]indicates p=0.006. Data
542 graphed as mean ± 95% CI.

543

544 ***Serum Corticosterone***

545

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

548

549 **Table 13.** Two-way ANOVA on corticosterone for Control A mice across experiments and
550 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

551

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

554

555 **Table 14.** Two-way repeated measures ANOVAs on corticosterone for mice of both sexes with
556 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

557

558 Different sex log-transformed corticosterone levels, while not exhibiting a significant sex × group
559 interaction, did display a significant main effect of sex (Table 15). Pairwise comparisons

560 indicated that this was primarily driven by SC males exhibiting significantly lower cort than SC

561 females (Figure 6C), the inverse of what was observed for different sex social interaction

562 (Figure 5C).

563

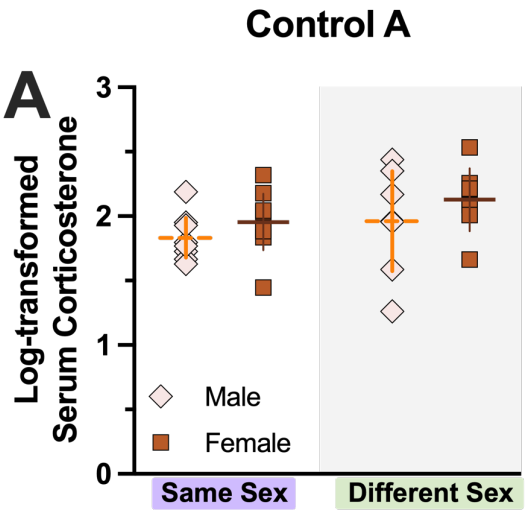
564 **Table 15.** Two-way repeated measures ANOVAs on corticosterone for mice of both sexes with

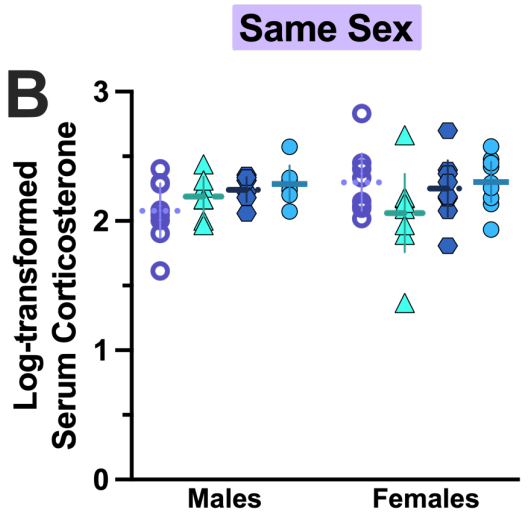
565 different sex targets.

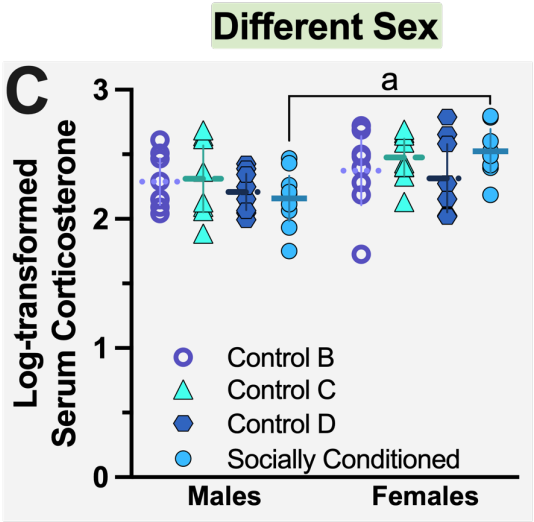
Different Sex Experiment – Log-transformed Corticosterone			
	F Statistic	**p value**	**Partial η^2**
Sex	**F(1,58)=8.683**	**0.005**	**0.130**
Group	F(3,58)=0.787	0.506	0.039
Sex × Group	F(3,58)=1.101	0.356	0.054

566 **Bold** indicates highest interaction or main effect with $p<0.05$

567


Control A
A
Log-transformed
Serum Corticosterone
3
2
1
0
Male
Female
Same Sex
Different Sex


Same Sex
B
Log-transformed
Serum Corticosterone
3
2
1
0
Males
Females


Different Sex
C
a
Log-transformed
Serum Corticosterone
3
2
1
0
Control B
Control C
Control D
Socially Conditioned
Males
Females

568

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. 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 Control A 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

594 acquisition, the discrepancy in basal freezing levels by Control A mice across experiments is
595 consistent and visible. This observation indicates that, in the presence of a potential sexual
596 partner, threat assessment is suppressed. The inverse has been demonstrated in female rats;
597 that is, fear attenuates sexual behaviors, at least in part through amygdala and hypothalamus
598 estrogen receptor signaling (67). Indirect evidence in male rats housed with females post-
599 context fear conditioning suggests that encounters with different sexes may suppress threat
600 assessment. These male rats subsequently exhibited attenuated fear expression, involving
601 dopamine receptor signaling in the hippocampus (68). However, a different group studying
602 mice reported that ejaculation by males was required for retention of extinguished social fear
603 conditioning (47). Continued evaluations are needed to determine whether our observations –
604 under conditions where copulation is impossible – that male exposure to a female can diminish
605 threat assessment behaviors are reproducible. An alternative explanation for our findings is that
606 locomotor activity was elevated, but additional studies would be necessary to determine this.
607 That only Control A males, but not Control A females, exhibited elevated social interaction time
608 with female conspecifics versus male conspecifics suggests this possibility is unlikely.
609 Corticosterone levels in Control A mice were relatively low as expected, given these mice were
610 never exposed to a mild foot shock. Similarly, neither sex nor experiment affected
611 corticosterone levels, suggesting no enduring stress was disproportionately experienced after
612 repeated same- or different-sex conspecific encounters across the four day study timeline.
613
614 Despite some interim deviations in fear acquisition across groups receiving mild foot shocks
615 (Controls B-D; SC mice) in each experiment, all mice ultimately ended their acquisition at similar
616 freezing levels. Males exhibited greater variability, as indexed by 95% CIs, across groups in
617 both experiments than females. This aligns with evidence that male rodent data are just as (69–
618 72) or more variable (54,73,74) than female data. Such relative consistency in ultimate terminal
619 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.

645 Regarding social conditioning context fear exhibited by mice when tested in the absence of any
646 conspecifics, these did not align with changes in social interaction. For the purposes of
647 developing a sex-inclusive social conditioning paradigm, this is preferable. Our goal was for
648 mice to associate the aversive mild foot shock with their assigned conspecific, and any social
649 buffering of social conditioning context fear could counteract our goal. This is why our use of
650 conspecifics of a different strain, that were never housed in the same room as all Control and
651 SC mice, ensured they had never previously been encountered by the mice tested here. Given
652 the critical contribution of attachment to social buffering (see review (78)), our data indicate we
653 can be reasonably confident there were no social buffering effects upon social conditioning
654 context fear in same sex experiment mice. In the different sex experiment, we did find only in
655 SC males that their freezing was attenuated, but only relative to Control D mice. This could be
656 related to our postulated threat assessment reduction relating to Control A mice, in that males in
657 the vicinity of a possible sexual partner could experience impaired encoding of context cues.
658
659 Considering corticosterone measurements were taken thirty minutes after social conditioning
660 context fear testing, we were interested to discover that these levels were inverted with respect
661 to social interaction in the different sex experiment. In other words, socially conditioned males
662 exhibited increased interaction with female conspecifics and lower corticosterone levels, as
663 compared to socially conditioned females displaying reduced interaction with male conspecifics
664 and elevated corticosterone levels. Further, corticosterone levels did not appear to correspond
665 to expressions of social conditioning context fear, despite this test being most temporally
666 proximal to blood collection. Thus, the different sex social conditioning procedure employed
667 here appears sufficient to elicit both behavioral and physiological responses. However, though
668 corticosterone levels appear overall elevated in same sex experiment mice, there were not
669 group- or sex-specific differences. Most striking, corticosterone levels in same sex SC mice
670 looked nearly identical, meaning this physiological stress marker did not map onto the

671 reductions in male versus female social interaction behavior in that experiment. This could be
672 that the same sex experiment's stress induction was not as enduring as that in the different sex
673 experiment, or it might mean that different sex social conditioning affects more neurobiological
674 processes than same sex social conditioning.
675
676 We may have been overzealous in our inclusion of controls. In our efforts to account for
677 temporally proximal social encounters with non-cage mates (Controls C and D), we potentially
678 muddied the waters when it comes to interpreting how social conditioning affects behavior and
679 circulating corticosterone. That said, we were surprised by what our Control A mice revealed,
680 and think that this information helps provide context for interpreting our social conditioning
681 findings. Nevertheless, the overall absence of social interaction differences between Controls
682 B-D and SC mice (save for different sex females) indicates that this protocol requires additional
683 optimization to achieve its overarching goal. Evidence for social transmission of stress in
684 rodents suggests an alternative interpretation (79). Instead, it could be that because the
685 conspecific for each SC mouse did not experience the same stress (mild foot shock), this in turn
686 affects the SC mouse's perception of their aversive experience (51), likely in a sex-specific
687 manner. Even if so, the present findings still generate useful information about environmental
688 threat assessment in social contexts, and highlight heretofore unrecognized sex-specific social
689 learning influences under aversive conditions.
690
691 Another limitation is that we could not obtain time course corticosterone measurements without
692 introducing additional confounding stressors to repeatedly sample blood. Daily blood sampling
693 would reveal if our corticosterone observations were attributable to the initial conditioning day,
694 the social interaction test, or social conditioning context fear test, and/or if these differences are
695 transient or persistent. These experiments were all performed in sexually naïve mice, so we
696 recognize that sexually experienced mice (for example (80)) of both sexes might respond,

697 behaviorally and physiologically, in a manner distinct from what we report here. Earlier
698 practices of dividing mice into 'susceptible' and 'resilient' groups following social stress have
699 more recently, and justifiably, fallen out of favor, given such responses are artificially elicited,
700 context-specific, and neither phenotype can be universally deemed 'better' or 'worse' (27,81).
701 We therefore evaluated all SC mice as a single group. Yet, possible bimodal distribution of
702 social interaction behavior in SC mice looks likely in all but the different sex SC males.
703 Increasing numbers to establish definitive thresholds with sufficient power for phenotypic
704 grouping might prove useful, particularly with the same sex setup. Finally, our studies used a
705 brief protocol spanning only four days, intended for high throughput and maximal comparison to
706 other social stress literature. We therefore cannot speak to whether these outcomes persist for
707 weeks or months, timeframes that are ethologically relevant for social learning and memory.
708
709 Here, we have reported a novel foray into sex-inclusive efforts to develop a controllable social
710 stress paradigm in mice. These experiments have provided utilizable insights regarding how
711 mice of both sexes associate a same- or different-sex conspecific with an aversive experience.
712 We report that these associations seem strongest in males after same sex encounters, and in
713 females after different sex encounters. Such distinctive responses are probably evolutionarily
714 favorable. Given the behaviorally exhibited associations could inversely correspond to
715 circulating corticosterone levels under different sex conditions, we propose that future studies
716 focused on socially stressing females utilize male conspecifics for optimal outcomes.
717
718 **Acknowledgements**
719 This work was supported by R15 MH118705 to AMJ and TLG, and by Kent State University.
720 We gratefully acknowledge the mice used in this study, the unrivaled veterinary care by Stan
721 Dannemiller, DVM, MS, DACLAM, and the dedicated work of our vivarium caretakers. Figure 1
722 was created with BioRender.com (Toronto, ON).

723

724 **Availability of data**

725 The dataset supporting the conclusions of this article is included with the article as an additional

726 file, *Supplemental Dataset*.

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