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

Abbreviated title: **Conspecific sex affects social aversion memory** (46 characters, 50 character max)

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Abstract (250 word max)

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. Conspecific-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 conspecifics 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 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 influences cross sex interactions. These current outcomes 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. (250 words)

Significance Statement (120 word max)

Validated paradigms to study social stress in female mice, and across sexes, are needed. We modified a published male mouse protocol by using classical conditioning to pair an aversive stressor with a conspecific. Our goal was to create a uniform, cross-sex, high-throughput social stress technique to advance future research. Though our modified paradigm requires future improvements, we did acquire evidence that both males and females socially conditioned in this way exhibit stronger associations when a male conspecific is used. Future research seeking to induce social stress in female mice may meet with more success using male, rather than female, conspecifics. This work, while not achieving our goal, provides useful information to advance future sex-inclusive social stress investigations. (117 words)

Introduction (750 word max including citations; 741 words)

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; Kamps et al., 2001; Leung, 2011; Monfils and Agee, 2019; Lee et al., 2020) [author citations omitted for double-blind review]. Shifts in social interaction behavior 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, disruptions in typical social interaction behavior 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 (Coccurello et al., 2009; Smith and Wang, 2012; Caldwell et al., 2017; Bergamini et al., 2018; Donovan et al., 2020; Sahani et al., 2022; Venniro et al., 2022) [author citations omitted for double-blind review]. 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; Peña et al., 2019; Lopez and Bagot, 2021; Furman et al., 2022; Lyons et al., 2023; Pantoja-Urbán et al., 2024) [author citations omitted for double-blind review]. A breadth of social stress techniques has 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)

(Golden et al., 2011; Bonnefil et al., 2019; Wang et al., 2021) [author citations omitted for double-blind review]. Aside from the male-centric nature of social defeat, additional concerns of reproducibility arise from the inherently variable range of aversive social experiences each 'defeated' experimental mouse encounters. Such variability, mostly beyond experimenter control, plus the single sex bias of social defeat left us wanting to explore an improved paradigm. For this, we paired controllable, uniform, aversive unconditioned stimuli with the presence of a social stimulus (conspecific; conditioned stimulus) to study aversive social conditioning *across sexes* in mice using a higher throughput approach (requiring 1 conditioning day, rather than multiple days-weeks).

To accomplish this, we modified a paradigm previously utilized in male mice, involving manual administration of an aversive stimulus (mild foot shock) selectively when a male mouse actively investigates a conspecific, with the goal of attenuating subsequent social engagement (Toth et al., 2012; Zoicas et al., 2014; de la Zerda et al., 2022; Grossmann et al., 2024). Rather than continuing this operant-style approach, where a mouse's behavior dictates an outcome, we shifted to a classical conditioning approach in which shock delivery is standardized across individual mice. We anticipated experimental mice would associate the presence of the conspecific with the aversive unconditioned stimulus. Additionally, all mice would receive the same number of shocks, instead of variable numbers based upon behavior.

Here, we evaluated how this paradigm affected social engagement and fear behaviors *across sexes* after mice were exposed to a novel conspecific. Specifically, mice were socially conditioned (SC) with an aversive stimulus (mild foot shock) when in the presence of their assigned conspecific, independent of investigative behavior. Then, mice were tested for social engagement and fear behavior in a separate environment with their assigned conspecific present, followed by testing of freezing in the conditioning environment in the absence of any

136 conspecific. The former test enabled evaluation of both social and fear behaviors, while the
137 latter test assessed contextual memory sans social stimuli. These behaviors were tested under
138 circumstances when the experimental and conspecific mice were the same sex, and when they
139 were different sexes. We hypothesized SC mice would exhibit reduced social engagement
140 (indexed as time spent directly adjacent to the conspecific enclosure) when tested with their
141 assigned conspecific in a novel environment, regardless of if the SC and conspecific mice were
142 the same or different sexes. Our broad goal was to develop a paradigm that would facilitate
143 rigorous future investigations of social behavior across mouse sexes, which are currently
144 underrepresented in literature (Kuske and Trainor, 2021).
145
146
147 **Methods**
148
149 ***Mice***
150 Hybrid F1 offspring (i.e., 129SB6F1/J) of both sexes resulting from pairing female 129S1/SvlmJ
151 (RRID:IMSR_JAX:002448) and male C57BL/6J (RRID:IMSR_JAX:000664) mice (hereafter
152 experimental mice), and male and female C57BL/6J mice (hereafter conspecifics), all ≥9 weeks
153 old or older, were group-housed (2-5 per cage) within sex. Experimental mice were hybrid F1
154 offspring of 129S1/SvlmJ and C57BL/6J mice because these two strains are the most prevalent
155 among transgenic mice, and because hybrids provide broader generalizability (Kumar et al.,
156 2021; Keady et al., 2022). Such hybrids are used for a variety of physiology and neuroscience
157 research purposes (e.g., (Curley et al., 2010; Kelley et al., 2011; Agrimson et al., 2017; Niibori
158 et al., 2020; Correia et al., 2024)). Conspecifics were C57BL/6J mice housed in a separate
159 room from experimental mice. C57BL/6J mice were used as conspecifics to ensure they were
160 entirely novel to experimental mice through separate housing; because C57BL/6Js are often
161 used as the primary or background strain for testing social behaviors in mice (see reviews

162 (Laman-Maharg and Trainor, 2017; Varholick et al., 2021; Takahashi, 2025)); and because our
163 F1 breeding was limited to generation of experimental mice. All mice had *ad libitum* access to
164 food and water in rooms maintained on a 12:12 light/dark cycle with lights on at 07:00 local
165 standard time (i.e., Zeitgeber time 0), and temperature maintained at 22 ± 2°C. All mice were
166 fed LabDiet 5001 rodent laboratory chow (LabDiet, Brentwood, MO) and were kept on 7090
167 Teklad Sani-chip bedding (Envigo, East Millstone, NJ) in cages containing Nestlets (Ancare,
168 Bellmore, NY) and huts (Bio-Serv, Flemington, NJ) for enrichment. Experiments were approved
169 by [authors' university] Institutional Animal Care and Use Committee, and adhered to the
170 National Research Council's Guide for the Care and Use of Laboratory Animals, 8th Ed.
171 (National Research Council, 2011).
172
173 ***Social Conditioning Paradigm***
174
175 The entire paradigm spans four consecutive days. Procedures for each individual day are
176 outlined below in order. On Days 0 and 1, experimental mice underwent different experimental
177 manipulations according to their specific treatment group. On Days 2 and 3, all experimental
178 mice experienced identical testing conditions regardless of their treatment group (Fig. 1).
179
180 *Pre-exposure (Day 0)*
181 Pre-exposure was used to help reduce the novelty of the transparent enclosure and/or the
182 conspecific to the experimental mouse, plus minimize any potential sex differences in
183 acquisition (Siegfried et al., 1987; Wiltgen et al., 2001; Guzmán et al., 2009; Brown et al., 2011).
184 On Day 0, experimental mice were placed in Coulbourn Instruments chambers (17.8 cm D ×
185 17.8 cm W × 30.5 cm H; Allentown, PA) with (Shock Control and SC mice) or without (Social,
186 Pre-Contingency, and Post-Contingency Controls) a conspecific (age-matched; either same sex
187 or different sex relative to experimental mouse, depending on if assigned to same sex or

188 different sex experiment) for pre-exposure (Fig. 1). Chambers comprised two opposing
189 aluminum walls each adjoining two clear acrylic walls. Conspecifics were put inside a
190 transparent enclosure placed within a corner of the social conditioning chamber. The
191 transparent enclosure included 23 holes (each 0.32 cm diameter) on each of the 2 sides facing
192 the SC mouse to enable olfactory cue exchanges. Previous work demonstrates that olfaction is
193 the most important modality when recognizing and interacting with novel conspecifics (see
194 review (Sterley and Bains, 2021)); tactile (whiskers) and auditory modalities appear to only be
195 recruited when recognizing cage mates (de la Zerda et al., 2022) in male mice (females were
196 not studied). Chambers and enclosures were cleaned with 70% ethanol before and after each
197 session; chambers had visible illumination and one clear acrylic wall was marked with a blue
198 dotted pattern. These components contributed olfactory and visual cues to the context, in
199 addition to the tactile cue of the stainless steel grid floor for the experimental mouse. During
200 pre-exposure, experimental mice were allowed to explore the chamber and conspecific
201 enclosure for 5 min, then both mice were returned to their respective home cages. No aversive
202 stimulus was presented.
203
204 *Social Conditioning (Day 1)*
205 On Day 1, experimental mice were placed in the same social conditioning chamber as Day 0
206 and received five, 1 s mild foot shocks (1.0 mA) in the presence of the same conspecific that
207 they encountered on Day 0 (Fig. 1). This was to have experimental mice form associations
208 between aversive foot shocks and their respective conspecific (Fig. 1). Conditioning lasted for 9
209 minutes; after a 2 minute baseline, shocks were then administered at 120, 210, 300, 390, and
210 480 s. Conspecifics did not receive foot shocks. Cameras mounted above the chambers were
211 used to record movements, and freezing behavior (≥0.25 s) during conditioning was quantified
212 using FreezeFrame software (v. 5.201, Actimetrics, Wilmette, IL). Freezing – ceasing all
213 movement save breathing – is an innate behavior expressed by mice in response to a real or

214 perceived threat, enabling threat assessment while minimizing detection (see review (Blanchard
215 and Blanchard, 1988)). All videos were manually reviewed to ensure the software satisfactorily
216 distinguished between freezing behavior and mere absence of locomotion.
217
218 *Social Engagement Testing (Day 2)*
219 On Day 2, experimental mice were placed in a novel room containing open arenas (41.9 cm W
220 × 41.9 cm D × 39.6 cm H). Social engagement testing intentionally occurred in a novel
221 environment with a novel conspecific enclosure to ensure sensitivity of our social interaction
222 measure while minimizing potential contextual confounds; in other words, to isolate effects on
223 social engagement from those of context fear learning (the latter assessed on Day 3). Mice
224 acclimated to the room in their home cage for 30 min prior to behavior testing commencing.
225 Each open arena had one empty PVC tube (8.9 cm outer diameter) in a single corner of the
226 square chamber with wire mesh (0.25 × 0.25 cm openings) covering a rectangular portion (11.4
227 cm W × 4.1 cm H) cut out of the tube bottom. Experimental mice were placed in the corner of
228 the arena opposite the PVC tube and could investigate freely for 2.5 minutes (pre-test).
229 Immediately after these 2.5 min, the conspecific that each experimental mouse had previously
230 been conditioned with on Day 1 was then placed in the PVC tube in the corner. Experimental
231 mice were allowed to continue investigating the arena for an additional 5 min (post-test; Fig. 1).
232 Because of the mesh at the bottom of the PVC tubes containing conspecifics, exchanges of
233 visual, auditory, and olfactory cues between experimental mice and conspecifics were possible,
234 but tactile interactions were minimized. Social interaction behavior was quantified as post-test
235 duration when at least 80% of an experimental mouse's body was in the interaction zone, a 7
236 cm radius zone (~223 cm^2 – 240 cm^2, i.e., 14-15% of the open arena) around the base of the
237 PVC tube. Freezing behavior, regardless of arena location, lasting at least 250 ms during the
238 post-test was also measured, then converted into a percentage of the post-test time. Social and
239 freezing behaviors were detected using ANY-maze software (v. 7.09 Stoelting Co., Wood Dale,

240 IL). Proprietary incompatibilities and video file encoding precluded analyses of freezing
241 behavior during social interaction testing from being evaluated with FreezeFrame software; this
242 is why ANY-maze software was used instead here. Post-test social interaction time was log-
243 transformed (Y=log(Y+0.001) to account for 3 mice with 0 s post-test social interaction) so that
244 data would be normally distributed for statistical analyses. Females were intentionally not
245 assessed for estrus cycle stage for five reasons: 1) to minimize mouse usage (Russell and
246 Burch, 1959), we did not power our studies for assessment of estrus; 2) with the goal of
247 developing a social stress paradigm, we are seeking effects robust enough to not depend upon
248 estrus in intact, randomly cycling females; 3) we intentionally focused here on fear and social
249 behaviors, and did not measure sexual behaviors (e.g., lordosis); 4) evidence that overall
250 mouse behaviors are not affected by estrus stage (Prendergast et al., 2014; Levy et al., 2023),
251 (but see (Chari et al., 2020)); 5) cross-species evidence indicates vaginal lavage to determine
252 estrus cycle is stressful (Becegato et al., 2021; Bahadır-Varol et al., 2022), and we sought to
253 minimize stress confounds here.
254
255 *Context Testing (Day 3)*
256 On the last day of the social conditioning paradigm, experimental mice were placed in the social
257 conditioning chamber for behavior testing in the *absence* of any conspecific (Fig. 1). The
258 conspecific enclosure was still present to keep the context consistent with conditioning. All
259 other tactile, visual, and olfactory cues from Day 1 were present. Testing lasted 10 min and did
260 not involve any foot shocks. Freezing behavior was again quantified using FreezeFrame
261 software.
262
263 ***Treatment Groups***
264

265 The SC group involved mice that underwent social conditioning in the presence of a non-
266 shocked conspecific (either same sex or different sex relative to SC mouse, depending upon
267 experiment), with the hypothesis that SC mice would associate the conspecific with this aversive
268 experience. We planned four Control groups for this one SC group; each experiment (same sex
269 or different sex) had its own respective set of four Control groups for its respective SC group.
270 The goals of these were to control for: 1) foot shock exposure, i.e., Shock Controls; 2) presence
271 of conspecific during pre-exposure & foot shock, i.e., Social Controls; 3) exposure to conspecific
272 temporally distal to foot shock exposure, such that social encounters still occurred in a manner
273 not contingent with foot shock nor the conditioning context, i.e., Pre- and Post-Contingency
274 Controls (Fig. 1). Shock Controls experienced procedures identical to those of the SC group,
275 except a foot shock was never administered on Day 1 (Fig. 1). Social Controls were treated the
276 same as the SC group, except experimental mice never encountered their assigned conspecific
277 until testing on Day 2 (Fig. 1). Pre- and Post-Contingency Controls involved experimental mice
278 encountering conspecifics either 4 h before (Pre-Contingency Controls) or 4 h after (Post-
279 Contingency Controls) pre-exposure (Day 0) and social conditioning (Day 1); in other words,
280 conspecifics were not present in the conditioning chamber during these two periods. Instead,
281 experimental mice were exposed to their conspecific in a separate room for 5 minutes or 9
282 minutes (the same amount of time as pre-exposure or social conditioning, respectively) 4 h
283 before (Pre-Contingency Controls) or 4 h after (Post-Contingency Controls) the experimental
284 mice were exposed to the conditioning chamber. This exposure involved experimental mice
285 and their respective conspecific being placed in a clean mouse cage separated with an acrylic
286 divider to allow for visual, auditory, and olfactory exchange, but minimizing tactile interaction,
287 mirroring the experiences of SC mice and Shock Controls within the social conditioning context
288 on Days 0-1. Mice in Pre- or Post-Contingency Control conditions still experienced foot shocks
289 on Day 1, and encountered their assigned conspecific for testing on Day 2 (Fig. 1). This
290 temporal separation of 4 h was to minimize consolidation of social encounters from

interfering/intermingling with consolidation of the aversive foot shock encounter in the social conditioning chamber, while still making execution of these experiments feasible within a 12 h lights-on period (Wanisch et al., 2008; Cai et al., 2016) [author citations omitted for double-blind review] (see reviews (Abel and Lattal, 2001; Sheppard et al., 2018)).

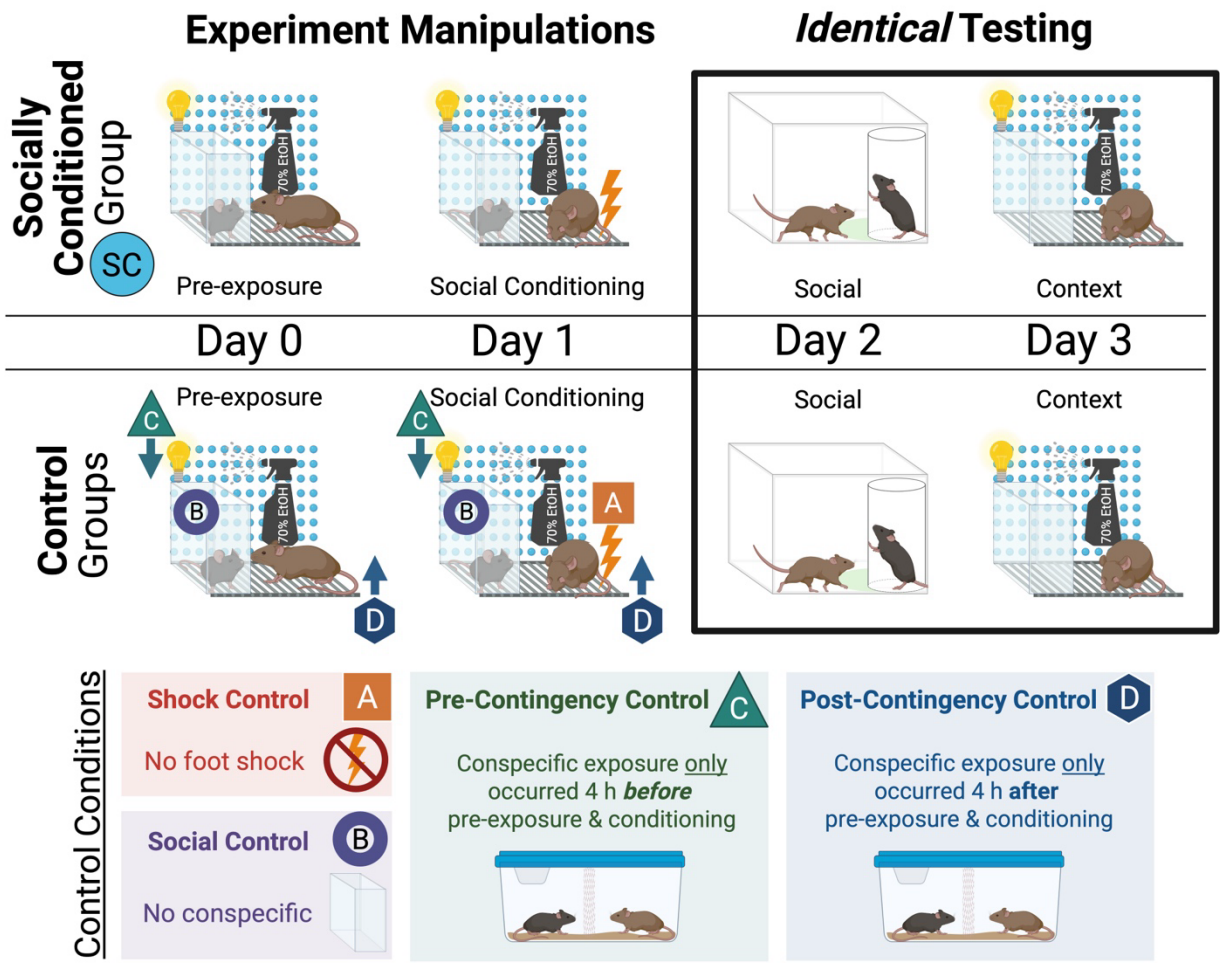

Figure 1. Study timeline and treatment groups. Five groups were used in this study for each experiment (same sex experiment, and different sex experiment): one socially conditioned (SC) group and four Control groups. Day 0 was for pre-exposure; Day 1 was when social conditioning occurred; Day 2 tested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

302 conspecific in the social conditioning context, where olfactory, visual, and auditory cues could
303 be exchanged while tactile interactions were minimized. No aversive stimulus was applied on
304 Day 0, but on Day 1, experimental mice and their assigned conspecific were returned to this
305 context. SC (but not conspecific) mice then experienced five mild foot shocks to associate their
306 conspecific with that aversive experience. SC mice were then tested for social engagement
307 with that same conspecific on Day 2, followed by testing for context fear behavior on Day 3 in
308 the absence of their assigned conspecific. Each Control group (below horizontal lines) was
309 composed of different mice. Shock Control (A) mice underwent the same exact procedure as
310 detailed for the SC group, except no foot shock was administered on Day 1. Social Control (B)
311 mice similarly experienced the same procedure as mice in the SC group, save that Social
312 Control mice did not encounter any conspecific until Day 2 for testing of social engagement.
313 Pre-Contingency Control mice encountered their conspecific 4 h before pre-exposure and social
314 conditioning, meaning no conspecific was present for Pre-Contingency Control mice when they
315 were in the social conditioning chamber for pre-exposure or social conditioning. To encounter
316 their assigned conspecific, Pre-Contingency Control mice were placed into a clean cage with a
317 clear acrylic divider separating them from their conspecific. This divider allowed visual,
318 olfactory, and auditory exchanges with the conspecific but minimized tactile interactions, the
319 same as for SC mice and Shock Controls when in the social conditioning context. On Day 0,
320 Pre-Contingency Control mice encountered their conspecific for 5 min; on Day 1, for 9 min;
321 these were timed to match the length of conspecific exposure that mice in the SC group
322 experienced. Post-Contingency Control mice were treated the same as Pre-Contingency
323 Control mice, except the former's encounters with their assigned conspecifics occurred 4 h
324 *after*, rather than before, pre-exposure and social conditioning.
325
326 ***Serum Corticosterone***
327

328 Thirty minutes after all experimental mice underwent contextual fear testing on Day 3, they were
329 briefly anesthetized with isoflurane then rapidly decapitated for trunk blood collection. Blood
330 clotted at room temperature for 10 minutes, then was spun at 3500 rpm for 1 h at 4°C. Serum
331 was collected and stored at -80°C until corticosterone analyses could be performed. Serum
332 corticosterone was measured using Enzo Life Sciences corticosterone enzyme-linked
333 immunosorbent assay (ELISA) kits (Farmingdale, NY). Assays were run according to the
334 manufacturer's instructions using their small volume protocol. Plates were read at 405 nm with
335 correction at 580 nm. The sensitivity of the assay was 26.99 pg/mL. After serum corticosterone
336 levels were interpolated from each plate's standard curve, they were log-transformed to account
337 for the typical skewness of serum corticosterone (Teilmann et al., 2014; Uarquin et al., 2016)
338 [author citations omitted for double-blind review]. Log-transformed serum corticosterone is
339 hereafter referred to as "cort".
340
341 ***Statistical Analyses***
342
343 Data were graphed with GraphPad Prism 10.3.0 (442), Beta (GraphPad Software, San Diego,
344 CA), and analyzed using IBM SPSS Statistics 28.0.0.0 (IBM, Armonk, NY), with the significance
345 threshold set *a priori* at $p<0.05$. Non-significant trends ($p<0.10$) were mentioned only when the
346 associated partial η^2 (η_p^2) was ≥0.060. Data were graphed as the mean ± standard error of the
347 mean (SEM). Details of identified outliers (greater than the mean ± 4 standard deviations) are
348 provided in the Supplemental Dataset. The same sex experiment and different sex experiment
349 each included their own SC group plus accompanying four Control groups. Experiments were
350 analyed separately. Social conditioning acquisition was analyzed using a 3-way repeated
351 measures (RM) general linear model (GLM; time × treatment group × sex of experimental
352 mouse) and pairwise comparisons with Bonferroni correction. Greenhouse Geisser corrections
353 were utilized for within-subjects analyses. Measurements of contextual fear expression average,

354 log-transformed post-test social interaction time, percent time freezing during social interaction
355 testing, and cort were analyzed with a 2-way GLM (treatment group × sex of experimental
356 mouse) and pairwise comparisons with Bonferroni correction.
357
358
359 **Results**
360
361 ***Social Conditioning Acquisition***
362
363 Social conditioning acquisition was examined in experimental mice in either the presence or
364 absence of a conspecific. One experiment used same-sex conspecifics, and the other
365 experiment used different sex conspecifics; each of these experiments included its own SC
366 group plus four corresponding Control groups (Fig. 1). Shock Control mice never received any
367 foot shocks during social 'conditioning', to distinguish the effects of receiving mild foot shocks on
368 subsequent social and fear behaviors. The influence of encountering *any* novel conspecific
369 prior to Day 2 and 3 testing was assessed with Social Control mice, which did not have a
370 conspecific present during either pre-exposure or 'social' conditioning. To assess how social
371 interaction with a novel conspecific prior to Day 2 and 3 testing, but temporally separate from
372 pre-exposure and 'social conditioning', Pre-Contingency Control mice encountered their
373 assigned conspecific 4 h *prior* to pre-exposure and 'social conditioning'. Post-Contingency
374 Control mice evaluated the same thing as Pre-Contingency Control mice, except Post-
375 Contingency Control mice encountered their assigned conspecific 4 h *after* pre-exposure and
376 'social conditioning'. Social conditioning acquisition is illustrated in Fig. 2A, 2B for mice in the
377 same sex conspecific experiment, and in Fig. 2C, 2D for mice in the different sex conspecific
378 experiment. All mice receiving a foot shock expressed >40% freezing during at least one post-
379 shock period during social conditioning.

380

381 For acquisition in the same sex experiment, no three-way interaction of time × sex × group (of
382 which there are five: SC mice plus four Control treatment groups) occurred (Table 1). We
383 observed a significant two-way interaction between time × group, and a non-significant trend for
384 group × sex was noted (p=0.070; Table 1). A significant main effect of sex was also found for
385 mice in the same sex experiment (Table 1). Pairwise comparisons indicated that same sex
386 conspecific experiment SC males exhibited less freezing at post-shock periods 1-4 compared to
387 SC females in the same experiment (Fig. 2A, 2B). In contrast, sex differences within the four
388 same sex Control groups were not observed at any timepoint in the same sex experiment, with
389 the sole exception being Post-Contingency Control females freezing more than Post-
390 Contingency Control males at post-shock period 3 (Fig. 2A, 2B). Within males, SC plus Social,
391 Pre-Contingency, and Post-Contingency Controls in the same sex experiment froze significantly
392 more than Shock Control males at post-shock periods 4 and 5. At post-shock period 3, Social,
393 Pre-Contingency, and Post-Contingency Controls were freezing more than Shock Control
394 males, but SC males weren't (Fig. 2A). SC males exposed to same sex conspecifics during
395 acquisition temporarily exhibited less freezing at post-shock periods 3 and 4 specifically
396 compared to Social Control males, which had no conspecifics present during acquisition (Fig.
397 2A). Similar to males, female SC mice and Social, Pre-Contingency, and Post-Contingency
398 Controls in the same sex experiment all exhibited significantly greater freezing compared to
399 Shock Control mice at post-shock periods 3-5, as expected (Fig. 2B). At post-shock period 2,
400 SC mice and Social and Post-Contingency Controls displayed higher freezing compared to
401 Shock Control mice, whereas Pre-Contingency Control mice did not (p=0.268; Fig. 2B).
402

403 **Table 1.** Two-way RM GLM on learning of social conditioning for mice of both sexes with same
404 sex conspecifics.

Same Sex Experiment – Social Conditioning Acquisition			
	F Statistic	**p value**	**η_p^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). Only the second post-shock period was different across sexes in SC mice in the different sex experiment (Fig. 2C, 2D), with females exhibiting greater freezing than males. Social Control female mice in the different sex experiment exhibited higher freezing than Social Control males solely at the third post-shock period. For Pre-Contingency Control mice, females displayed greater freezing for the first three post-shock periods than males in the different sex experiment (Fig. 2C, 2D). SC males exhibited an initial higher level of freezing at post-shock period one compared to Shock, Social, and Pre-Contingency Controls, but not Post-Contingency Controls (Fig. 2C), but this was not sustained as a pattern at later post-shock periods. Females at post-shock period one in the SC and Pre-Contingency Control groups froze more than Shock Control females, partially mirroring the males' initial acquisition response (Fig. 2D). At post-shock period two, male Post-Contingency Control mice were the only group that froze more than male Shock Controls (Fig. 2C). The same time point in females in the different sex experiment revealed a more divergent pattern continuing what was observed in that sex at post-shock period one.

421 Specifically, different sex experiment SC and Pre-Contingency Control females continued to
422 freeze more than female Shock Controls, plus the two former exhibited significantly greater
423 freezing than female Social Controls (Fig. 2D). However, this clustering pattern of Pre-
424 Contingency Control and SC females, and Shock and Social Control females, disappeared from
425 post-shock period three onwards. Post-Contingency Control mice briefly exhibited less freezing
426 than Pre-Contingency Control mice at post-shock period three, but this was the only such
427 occurrence in females (Fig. 2D). Social, Pre-Contingency, and Post-Contingency Controls and
428 SC mice of both sexes in the different sex experiment froze significantly more than same sex
429 Shock Control mice at post-shock periods 3-5 (Fig. 2C, 2D). Combined with same sex
430 experiment acquisition, these findings indicate that social exposure – whether concurrent with or
431 temporally distal from an aversive unconditioned stimulus – can have transient impacts on the
432 freezing behavior exhibited by experimental mice across sexes. All conditioning procedures
433 here, though, culminated in similar final freezing levels across sexes of both experimental mice
434 and their conspecifics. This facilitates subsequent comparisons of the different conditioning
435 manipulations on subsequent social and freezing behaviors by minimizing potential acquisition
436 confounds.
437
438 **Table 2.** Two-way RM GLM on learning of social conditioning for mice of both sexes with
439 different sex conspecifics.

Different Sex Experiment – Learning Social Conditioning			
	F Statistic	**p value**	**η_p^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

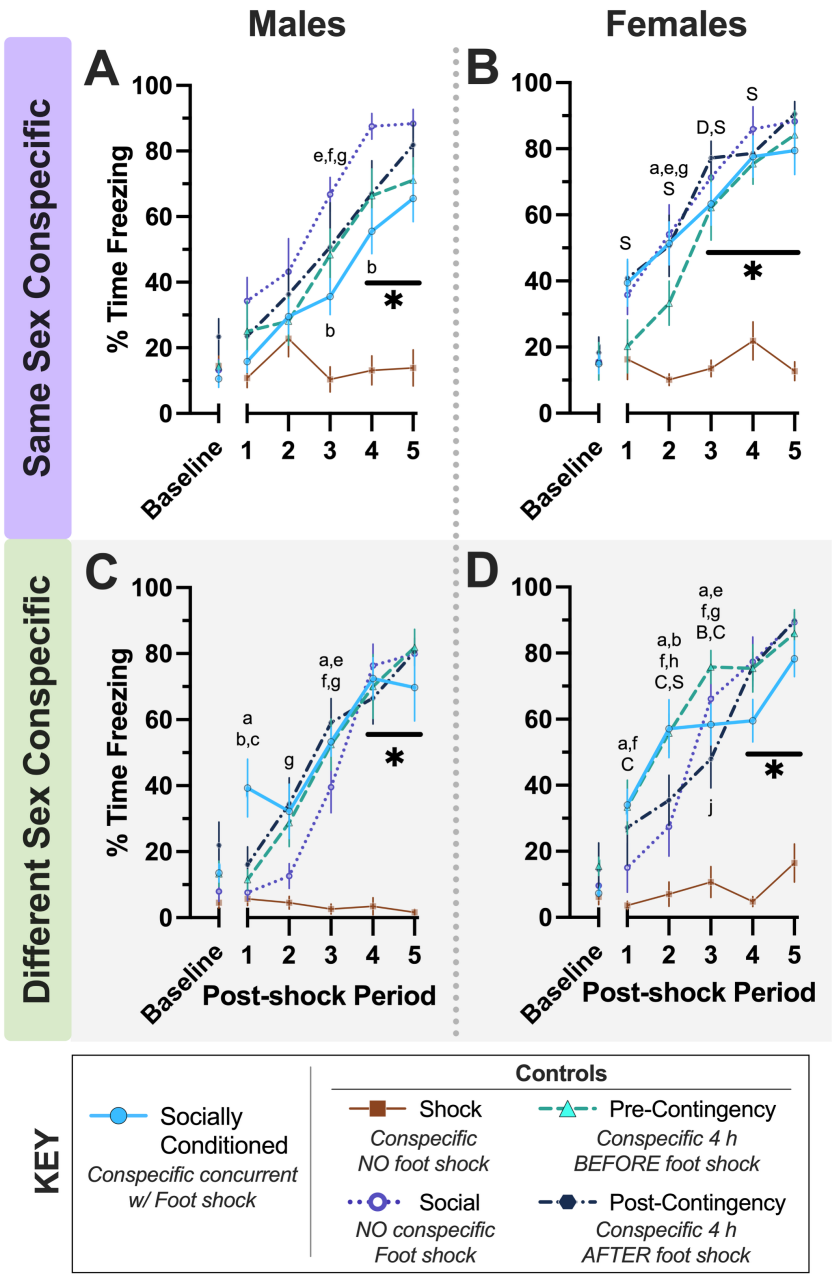

Figure 2. ***Day 1 acquisition during social conditioning procedure***. Percent time freezing during social conditioning acquisition for mice in same sex (Panels A, B) and different sex

(Panels C, D) experiments for male (A, C) and female (B, D) mice. Numbers of mice graphed within Panels A-D in order: Socially conditioned (SC; 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 minutes, prior to commencement of acquisition, is plotted on the x-axis as baseline. The average percent freezing for each 30 second period following each of the five mild foot shocks are thereafter plotted along x-axis (Post-shock Periods 1-5). Alphabetically, within experiment, sex, and time point: [a]indicates (Shock Control vs. SC) p<0.001, p=0.007, p<0.001, p=0.025, p<0.001, p<0.001. [b]indicates (Social Control vs. SC) p=0.026, p=0.005, p=0.009, p=0.038. [c]indicates (Pre-Contingency Control vs. SC) p=0.032. [e]indicates (Shock Control vs. Social Control) p<0.001, p<0.001, p=0.003, p<0.001. [f]indicates (Shock Control vs. Pre-Contingency Control) p=0.004, p<0.001, p=0.024, p<0.001, p<0.001. [g]indicates (Shock Control vs. Post-Contingency Control) p=0.002, p=0.002, p=0.047, p<0.001, p=0.002. [h]indicates (Social Control vs. Pre-Contingency Control) p=0.043. [j]indicates (Pre-Contingency Control vs. Post-Contingency Control) p=0.028. *****indicates p<0.001 Social, Pre-Contingency, and Post-Contingency Controls and SC mice vs. Shock Controls. Alphabetically, within experiment, group, and time point: [B]indicates (Social Controls male vs. female) p=0.005. [C]indicates (Pre-Contingency Controls male vs. female) p=0.019, p=0.006, p=0.012. [D]indicates (Post-Contingency Controls male vs. female) p=0.012. [S]indicates (SC male vs. female) p=0.007, p=0.031, p=0.006, p=0.012, p=0.012. Data graphed as mean ± SEM.

Social Interaction Testing

Mice were tested for social engagement and freezing behavior using the social interaction test 24 h after social conditioning concluded. Three mice did not interact at all with their conspecific, and thus were excluded from social interaction analyses, but were retained for freezing behavior

analyses (1 different sex Shock Control female; 1 same sex Social Control female; 1 same sex SC male; see Supplemental Dataset).

Social conditioning in the same sex experiment did not result in a sex × group interaction on social engagement, but a main effect of group was found (Table 3). Pairwise comparisons indicated that SC males exhibited less social interaction than SC females, Shock Control males, and Pre-Contingency Control males in the same sex experiment (Fig. 3A).

Table 3. Two-way GLM on social interaction for mice of both sexes with same sex conspecifics.

Same Sex Experiment – Log-transformed Social Interaction			
	F Statistic	**p value**	**η_p^2**
Group	$F_{(4,65)}=4.357$	0.003	0.211
Sex	$F_{(1,65)}=0.535$	0.467	0.008
Group × Sex	$F_{(4,65)}=1.746$	0.151	0.097

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 Control females compared to males (Fig. 3C). SC and Social Control females froze more than Shock Control females. Similarly, Pre and Post--Contingency Control females exhibited less fear behavior than Social 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).

489

490 **Table 4.** Two-way GLM on percent time freezing during social interaction for mice of both

491 sexes with same sex conspecifics.

Same Sex Experiment – % Freezing During Social Interaction			
	F Statistic	**p value**	**η_p^2**
Group	$F_{(4,67)}$=10.55	<0.001	0.387
Sex	$F_{(1,67)}$=5.244	0.025	0.073
Group × Sex	$F_{(4,67)}$=0.730	0.575	0.042

492

493 As with the same sex experiment, there was no significant interaction nor a main effect of sex in

494 the different sex experiment, but there was a significant group effect on social engagement

495 (Table 5). Pairwise comparisons demonstrated that, opposite to the same sex experiment, SC

496 females in the different sex experiment exhibited significantly reduced social interaction, both

497 compared to female Social Control mice and to SC male mice (Fig. 3B). Combined, these

498 findings indicate that the social engagement of female mice is more affected by temporally

499 concurrent aversive associations with males, whereas males are more socially affected by

500 temporally concurrent aversive associations with their own sex.

501

502 **Table 5.** Two-way ANOVA on social interaction for mice of both sexes with different sex

503 conspecifics.

Different Sex Experiment – Log-transformed Social Interaction			
	F Statistic	**p value**	**η_p^2**
Group	$F_{(4,66)}$=2.903	0.028	0.150
Sex	$F_{(1,66)}$=0.740	0.393	0.011
Group × Sex	$F_{(4,66)}$=1.474	0.220	0.082

504

505 Unlike the same sex experiment, neither an interaction nor main effects of sex and group were
506 observed with freezing behavior during social interaction testing for the different sex experiment
507 (Table 6). Moreover, no significant pairwise comparisons were observed (Fig. 3D). The
508 predominant consistency across experiments for freezing was that expression of fear was
509 minimal during the post-test of social interaction, as compared to freezing quantified in the
510 conditioning context (Figs. 2, 4A-B).

511

512 **Table 6.** Two-way ANOVA on percent time freezing during social interaction for mice of both
513 sexes with different sex conspecifics.

Different Sex Experiment – % Freezing During Social Interaction			
	F Statistic	**p value**	**η_p^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

514

515

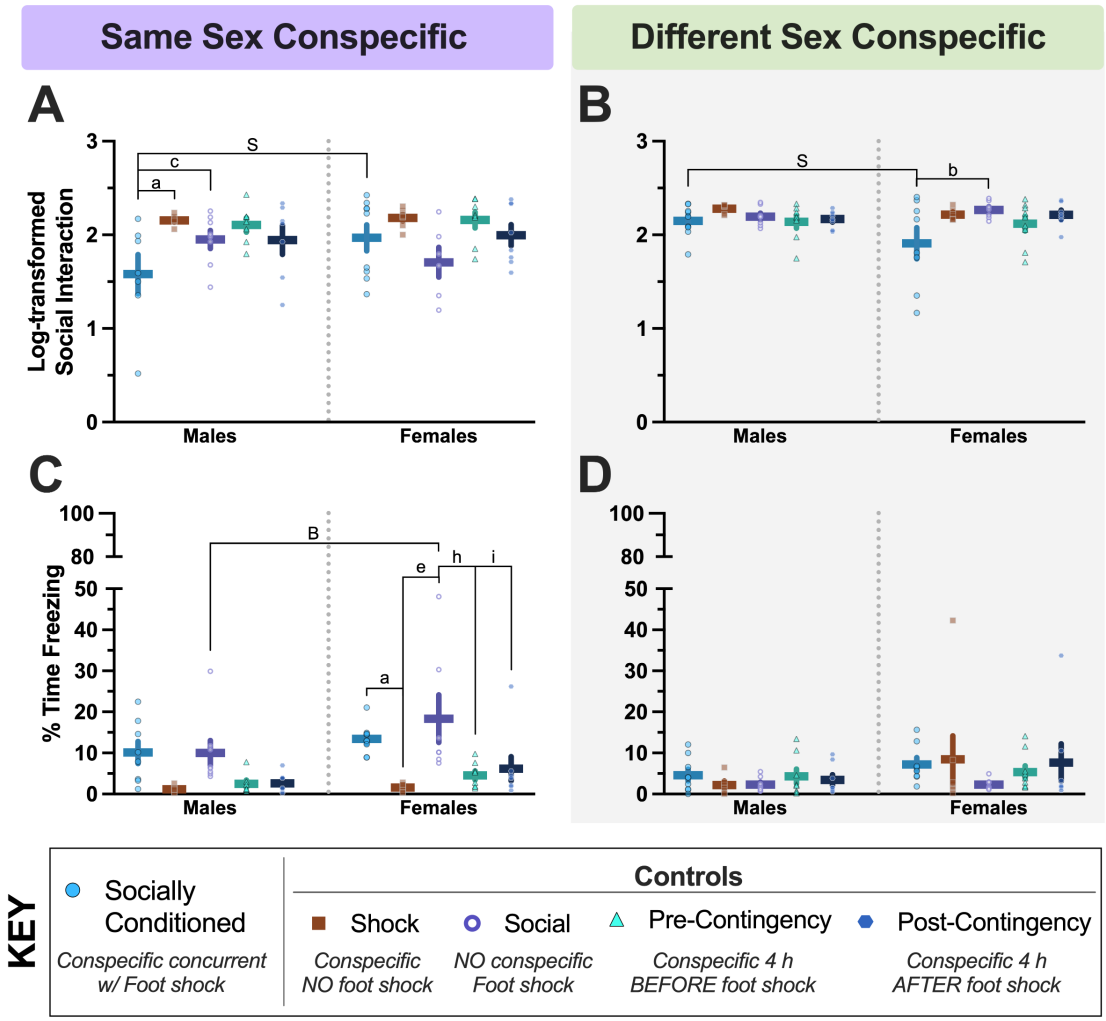

Figure 3. ***Day 2 social interaction and fear behaviors following social conditioning***. Log-transformed social interaction (Panels A, B) and percent time freezing during post-test social interaction (Panels C, D) data are shown for mice in the same (A, C) and different (B, D) sex experiments. Numbers of mice graphed within each panel, left to right: A) n=7, 7, 8, 7, 7, 9, 8, 6, 8, 8; B) n=9, 6, 8, 8, 8, 8, 6, 7, 9, 7; C) n=8, 7, 8, 7, 7, 9, 8, 7, 8, 8; D) n=9, 6, 8, 8, 8, 8, 7, 7, 9, 7. Y axes for C, D were split to facilitate clearer visualization of the low freezing levels exhibited during social engagement testing. Left to right, top to bottom: $^{a}p=0.016$, $^{c}p=0.037$, $^{S}p=0.021$, $^{S}p=0.019$, $^{b}p=0.013$, $^{B}p=0.019$, $^{a}p=0.005$, $^{e}p<0.001$, $^{h}p=0.002$, ^{i}indicates (Social Control vs. Pre-Contingency Control) p=0.008. Data graphed as mean ± SEM.

527 ***Social Conditioning Context Fear Testing***

528

529 After Day 2's social interaction evaluation of mice's association between their assigned
530 conspecific and the aversive stimulus they experienced on Day 1, we next assessed their fear
531 response to the social conditioning context on Day 3. This was accomplished by quantifying
532 percent of time spent freezing in the conditioning context, in the absence of any conspecific.
533 Because of the natural extinction process that can occur during testing, we specifically
534 examined behavioral expression of social conditioning context fear during minutes two through
535 six of testing, to best capture fear expression with minimal confounds from extinction processes
536 [author citations omitted for double-blind review]. This testing further allowed us to determine if
537 social engagement testing on Day 2 might have any carry-over effects on contextual fear
538 expression on Day 3 (Tables 7-8, Fig. 4A-B).

539

540 For the same sex experiment, no two-way interaction between group × sex was found, but a
541 significant main effect of group emerged (Table 7). Mostly, this was due to Shock Control mice,
542 as expected, freezing significantly less than all mice in the same sex experiment that
543 experienced foot shocks during conditioning (Fig. 4A). Male SC mice exhibited significantly less
544 average freezing in the conditioning context compared to female SC mice (Fig. 4A).

545

546 **Table 7.** Two-way GLM on both sexes' context fear testing average with same sex
547 conspecifics.

Same Sex Experiment – Fear Expression Average			
	F Statistic	**p value**	**η_p^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

In the different sex experiment, no significant group × sex interaction was found, nor was a significant effect of sex (Table 8). A significant group effect was similarly observed for the different sex experiment, again attributable to the low freezing levels by Shock Control mice across sexes (Fig. 4B). Male SC mice also froze significantly less than Post-Contingency Control males (Fig. 4B). 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. In parallel, freezing behavior was relatively minimal during social interaction testing compared to testing in the conditioning context, suggesting both that freezing behavior did not confound social interaction outcomes and that mice were able to sufficiently discriminate between contexts. Curiously, interaction of male SC mice with familiar conspecifics in a novel environment on Day 2 might lead to mild reductions in contextual fear expression on Day 3.

Table 8. Two-way GLM on both sexes' context fear testing average with different sex conspecifics.

Different Sex Experiment – Fear Expression Average			
	F Statistic	**p value**	**η_p^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

Serum Corticosterone

567 Thirty minutes after Day 3 testing concluded, blood was collected for subsequent analyses of
568 serum corticosterone levels. Cort levels of mice in the same sex experiment exhibited no
569 significant interaction of group × sex, but a main effect of group was observed (Table 9, Fig.
570 4C). Pairwise comparisons showed that, across sex, SC mice in the same sex experiment
571 exhibited elevated cort levels compared to their respective same sex Shock Control mice (Fig.
572 4C). While this was not surprising, male Pre- and Post-Contingency Control mice also exhibited
573 higher cort levels than Shock Control mice, whereas Social Control mice did not (Fig. 4C).
574 Conversely, female Social Control mice had higher cort levels than Shock Control mice, but
575 female Pre- and Post-Contingency Control mice did not (Fig. 4C). Given no conspecific was
576 present during Day 3 testing, these findings suggest enduring and sex-specific physiological
577 differences following social conditioning, despite minimal behavioral shifts. Specifically, any
578 social interaction with their assigned conspecific on Day 1, whether concurrent or temporally
579 separate with foot shock exposure, elevated male cort levels but attenuated female cort levels
580 after context testing on Day 3 *in the absence of any conspecifics*. Only SC females did not align
581 with this pattern, exhibiting heightened cort levels on Day 3 similar to those of Social Control
582 females (Fig. 4C).
583
584 **Table 9.** Two-way GLM on cort for mice of both sexes with same sex conspecifics.

Same Sex Experiment – Log-transformed Serum Corticosterone			
	F Statistic	**p value**	**η_p^2**
Group	$F_{(4,68)}=7.098$	<0.001	0.295
Sex	$F_{(1,68)}=0.813$	0.370	0.012
Group × Sex	$F_{(4,68)}=1.207$	0.316	0.066

585

586 Cort levels of mice in the different sex experiment, while not exhibiting a significant sex × group
587 interaction, did display significant main effects of both group and sex (Table 10). In contrast to
588 the same sex experiment, pairwise comparisons indicated that within each sex, levels of cort did
589 not differ across groups, including relative to Shock Control mice (Fig. 4D). The only difference
590 that reached significance was male SC mice having lower cort levels compared to female SC
591 mice (Fig. 4D). Combined with cort data from the same sex experiment (Fig. 4C), these findings
592 illustrate how same sex conspecific interactions on the same day as an aversive learning
593 experience can elicit sex-specific and enduring hormone changes. Moreover, these lasting
594 physiological shifts are not mirrored after different sex conspecific interactions. This indicates
595 the combination of conspecific and experimental sex, when temporally concurrent or adjacent to
596 a fear learning event, influences endocrine responses to context days later.
597
598 **Table 10.** Two-way GLM on cort for mice of both sexes with different sex conspecifics.

Different Sex Experiment – Log-transformed Serum Corticosterone			
	F Statistic	**p value**	**η_p^2**
Group	$F_{(4,70)}=3.841$	0.007	0.180
Sex	$F_{(1,70)}=8.708$	0.004	0.111
Group × Sex	$F_{(4,70)}=0.709$	0.589	0.039

599
600

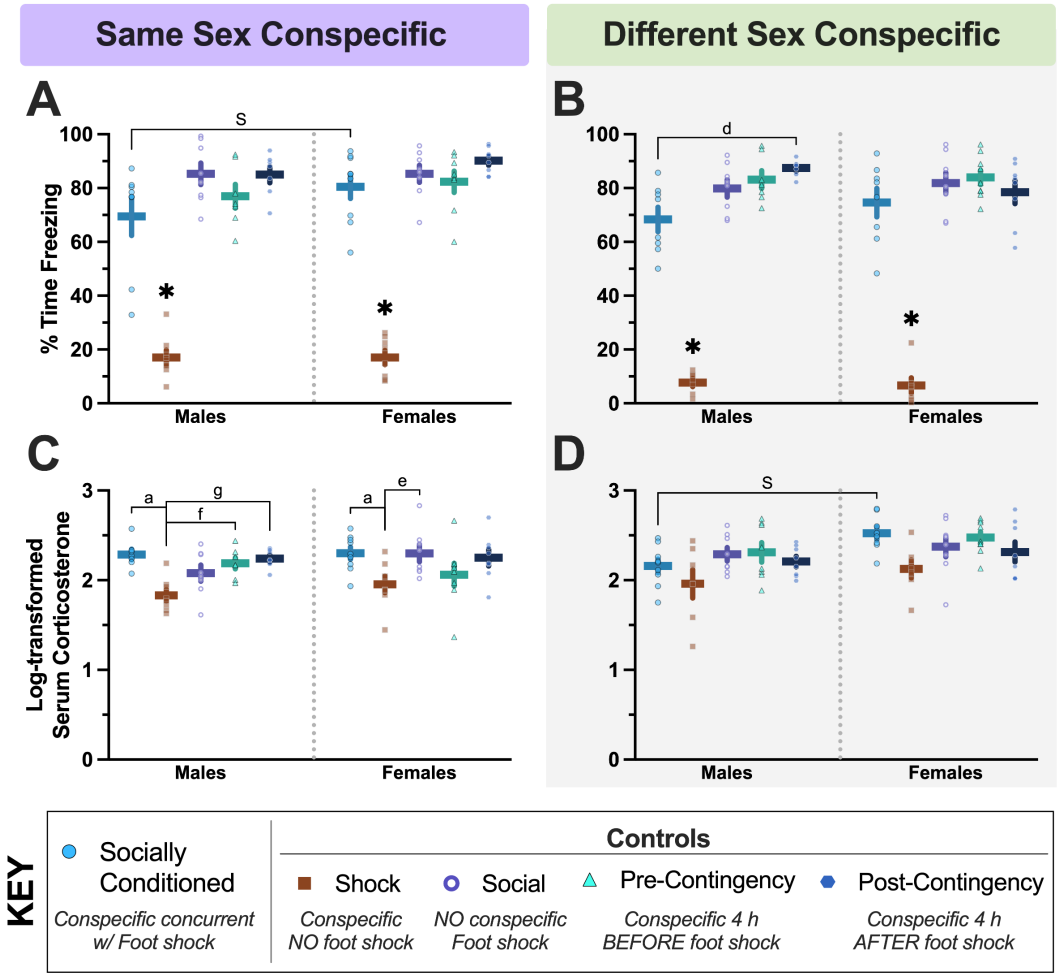

Figure 4. Day 3 social conditioning context fear expression averages and subsequent cort levels. Average context fear behavior (Panels A, B) and cort (Panels C, D) data are shown for mice in all groups in the same sex (A, C) and different sex (B, D) experiments. Numbers of mice graphed within Panels A-D 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; C) n=7, 8, 8, 7, 7, 9, 8, 8, 8, 8; D) n=9, 7, 8, 8, 8, 8, 7, 8, 9, 8. Left to right, top to bottom: ^{S}p=0.049, ^{d}indicates (Pre-Contingency Control vs. SC) p=0.004, ^{a}p=0.004, ^{f}p=0.045, ^{g}p=0.012, ^{a}p=0.033, ^{e}p=0.044, ^{S}p=0.007. *p<0.001 Social, Pre-Contingency, and Post-Contingency Controls and SC mice vs. Shock Control. Data graphed as mean ± SEM.

612 **Discussion** (3000 word max, including citations; 2317 words)

613

614 Our goal in executing these experiments was to begin establishing a paradigm for inducing
615 consistent, reproducible socially paired stress in mice across sexes. While our results indicate
616 this specific paradigm does not yet accomplish our goal, we want to share our approach and
617 findings so that others can utilize this information to efficiently prioritize future efforts in this
618 domain. Moreover, despite a mostly negative outcome towards our overarching goal, we
619 nevertheless uncovered sex-specific patterns of behavioral and physiological stress responsivity
620 that are useful to fields incorporating sex as a biological variable, and/or seeking to employ
621 different sex social stressors in mice.

622

623 All mice that underwent social conditioning with mild foot shocks (Social, Pre-Contingency, and
624 Post-Contingency Controls plus SC mice) ultimately ended their acquisition stage at similar
625 freezing levels. Subsequent outcomes in behavioral testing for social engagement and
626 contextual fear expression, as well as for serum corticosterone, could, therefore, be interpreted
627 with minimal concern for acquisition confounds. Conversely, this consistency might have
628 contributed to our negative findings. Most of the statistically significant differences between
629 Social, Pre-Contingency, and Post-Contingency Controls and SC mice occurred during the first
630 three post-shock periods, both in the same sex and different sex experiments. These well might
631 have been flukes. Alternatively, they might indicate that our social conditioning protocol resulted
632 in a ceiling effect. Additional studies would need to parse this out.

633

634 Our social interaction findings were not as robust as anticipated. For our same sex experiment,
635 we found that male SC mice engaged less during social testing compared to Shock Control
636 mice, and compared to Social Control mice that never encountered a conspecific during
637 training. Additionally, male SC mice exhibited less social engagement than female SC mice in

638 same sex experiment, whereas the converse was true for the different sex experiment. This
639 reveals that male mice likely form stronger associations when aversive experiences occur in
640 conjunction with the presence of another male mouse, whereas female mice appear to
641 associate male mice more strongly when their presence is concurrent with aversive stimuli.
642 Such a sex difference in response to conspecific sex could help explain some of the challenges
643 in developing social stress paradigms using female laboratory mice (*Mus musculus*). Relatedly,
644 this could indicate that, in the presence of a potential sexual partner, threat assessment is
645 suppressed. The inverse has been demonstrated in female rats; that is, fear attenuates sexual
646 behaviors, at least in part through amygdala and hypothalamus estrogen receptor signaling
647 (Moëne et al., 2019). Indirect evidence in male rats housed with females post-context fear
648 conditioning suggests that encounters with different sexes may suppress threat assessment.
649 These male rats subsequently exhibited attenuated fear expression, involving dopamine
650 receptor signaling in the hippocampus (Bai et al., 2009). However, a different group studying
651 mice reported that ejaculation by males was required for retention of extinguished social fear
652 conditioning (Grossmann et al., 2024). Continued evaluations are needed to determine whether
653 our observations – under conditions where copulation is impossible – that male exposure to a
654 female can diminish threat assessment behaviors are reproducible. Evidence for social
655 transmission of stress in rodents suggests an alternative interpretation (Brandl et al., 2022).
656 Instead, it could be that because the conspecific for each SC mouse did not experience the
657 same stress (mild foot shock), this in turn affects the SC mouse's perception of their own
658 aversive experience (Guzmán et al., 2009), likely in a sex-specific manner.
659
660 These sex-specific behavior patterns correspond to evidence that accumbal dopamine signaling
661 dynamics in mice are sex-dependent, both upon the sex of the studied mouse and the sex of
662 their assigned conspecific (Dai et al., 2022). Our findings align with the success of using social
663 defeat to stress male mice. Additionally, they indicate that future studies seeking to suppress

664 female mice's social interaction behavior through a socially associated stimulus will meet with
665 more success if using male, rather than female, conspecifics. Indeed, others have encountered
666 challenges when trying to elicit behavioral shifts in female mice, even after 4 weeks of stress
667 that involved only female conspecifics (Díez-Solinska et al., 2023). One group reported that
668 female mice consistently prefer a socially paired food reinforcer, even if the pairing was with a
669 same sex conspecific that had just previously undergone acute stress (mild foot shock)
670 (Kietzman et al., 2022). Because males' food preference was not studied by the researchers,
671 and neither did their females encounter stressed different sex conspecifics, it remains unclear if
672 these effects are sex-specific, and if they would generalize to different sex interactions. Future
673 studies will also need to determine how socially conditioning female mice to male conspecifics
674 subsequently affects interactions with female conspecifics, and vice versa.
675
676 One day after social engagement testing, mice were tested for fear expression in the social
677 conditioning context in the absence of any conspecifics. As expected, Shock Control mice
678 across experiments exhibited little freezing, given they never experienced a foot shock in this
679 environment. Indeed, Shock Controls drove most of the group differences across sexes and
680 experiments. This indicates social conditioning did not have a consistent effect upon fear
681 expression, measured during minutes two through six to minimize extinction confounds [author
682 citations omitted for double-blind review]. The only semi-consistent group difference in fear
683 expression was between male SC and Post-Contingency Control mice in the different sex
684 experiment. This suggests that when male mice are in the vicinity of a possible sexual partner,
685 they could experience impaired encoding of context cues. However, the absence of significant
686 differences of male SC mice relative to Social and Pre-Contingency Controls argues against this
687 interpretation.
688

689 A sex difference in context fear expression was observed between SC mice in the same sex
690 experiment, but not in the different sex experiment. This is the only directionally comparable
691 outcome with social engagement, in that male SC mice in the same sex experiment both
692 exhibited less context fear and less social engagement versus female SC mice. This could be
693 interpretated in at least two ways. Male SC mice might have found their male conspecific more
694 salient than the context, thereby leading them to form stronger associations between the mild
695 foot shocks and their conspecific as compared to female SC mice. Alternatively, the presence
696 of the male conspecific may have led to social buffering of the mild foot shocks for male SC
697 mice, whereas this process did not occur for female SC mice in the same sex experiment.
698 Because our goal was for mice to associate the aversive mild foot shock with their assigned
699 conspecific, any social buffering of social conditioning context fear could counteract our goal.
700 This is why our use of conspecifics of a different strain, that were never housed in the same
701 room as experimental mice, ensured they had never previously been encountered (even via
702 olfaction) by the mice tested here. Given the critical contribution of attachment to social
703 buffering (see review (Kiyokawa and Hennessy, 2018)), we can be reasonably confident that
704 there were no social buffering effects upon social conditioning context fear in same sex
705 experiment mice. Thus, we are inclined toward the first interpretation.
706
707 Serum was collected thirty minutes after context fear testing, 24 h after experimental mice's last
708 encounter with their assigned conspecific. We were interested to discover that sex-specific
709 group differences in cort levels in the same sex experiment appeared to be best explained by
710 Day 1 group conditions, rather than behavior on Days 1-3. For example, male Shock Control
711 mice's cort levels were lower than Pre- and Post-Contingency Controls and SC groups – the
712 three conditions where both conspecific exposure and foot shocks occurred within an 8 h
713 window on Day 1. Social Control male mice only experienced foot shocks, and did not
714 encounter a conspecific, on Day 1. Shock Control males similarly only encountered

conspecifics, and did not experience foot shocks on Day 1; and male Social Control cort levels did not differ from Shock Control males. Like males in the same sex experiment, female SC mice had elevated cort relative to Shock Control females. In contrast, however, with this experiment's females, only Social Control females had higher cort levels than Shock Control females. Pre- and Post-Contingency Control females' cort was not different from Shock Controls, and the former groups encountered conspecifics 4 h before or after, respectively, experiencing foot shocks. Collectively, this suggests social encounters temporally distal from the foot shock, but still occurring within the same day, might augment males' and mitigate females' cort responses to a stressful experience *two days later*. In contrast, coinciding foot shocks and conspecific exposure in SC mice obscured this sex difference in endocrine response.

Unlike the same sex experiment, the different sex experiment mice's cort levels did not map onto groups in either sex, despite a significant group effect being detected. Also distinct from the same sex experiment is that cort levels of SC mice in the different sex experiment had a sex difference. Specifically, female SC mice had greater cort levels than male SC mice. This inversely maps onto their social engagement on Day 2, in that female SC mice engaged less with their male conspecifics than male SC mice did with their female conspecifics. However, average context fear expression was not different between these two groups on Day 3. This cort level difference could, therefore, be an enduring endocrine effect of encountering their assigned conspecifics on Day 2. That said, the absence of any inverted patterns between social engagement and cort levels across all Controls in the different sex experiment makes this possibility seem less plausible than what we observed with cort levels in the same sex experiment.

740 To ensure we rigorously evaluated this modified paradigm's hypothesized utility as a higher
741 throughput approach for more consistent induction of social stress across sexes, we included
742 four separate controls. Had we only included one control group, e.g., Shock Controls, we might
743 have incorrectly concluded that our modified paradigm successfully reduced social engagement
744 in females, if not across sexes. Through the inclusion of four control groups, however, we have
745 concluded that our findings do not support our hypothesis, and that we cannot endorse this
746 paradigm in its current state. Instead, our data indicate that this protocol requires additional
747 optimization to achieve its overarching goal. Nevertheless, we are sharing our approach and
748 findings so that others with similar procedure goals can learn from our observations, and
749 improve upon our study's limitations. If we had been able to continue our investigations, we
750 would have next reduced the mild foot shock amplitude, the number of foot shocks, or some
751 combination thereof. Given the group differences in freezing that emerged early during our
752 social conditioning, milder and/or less aversive stimuli could uncover enduring effects of group
753 conditions. To begin though, it was important here that all our groups attained similar starting
754 points to minimize confounds for testing interpretations. We would also advise other
755 researchers to consider optimizing their apparatus and software, if possible, to facilitate
756 consistent analyses of interaction and freezing, if their conditioning/stress component generates
757 freezing. We used one software to administer foot shocks while simultaneously recording and
758 quantifying freezing, and another software to track animal location and identify time spent within
759 specific arena locations, each in separate apparatus. Both software programs excel at their
760 purposes, but each have unique – and unfortunately incompatible – video formats plus
761 requirements regarding lighting, contrast, and background for optimal behavior/location
762 detection.
763
764 Another limitation is that we could not evaluate cort levels over time without introducing
765 additional confounding stressors to repeatedly sample blood. Daily blood sampling would

766 reveal if our cort observations were attributable to specific days, how cort levels mapped on to
767 each day's behavior measure, and/or if cort differences are transient or persistent. Our
768 experiments were all performed in sexually naïve mice. We recognize that sexually
769 experienced mice (for example (Newman et al., 2019)) of both sexes might respond,
770 behaviorally and physiologically, in a manner distinct from what we report here. Earlier
771 practices of dividing mice into 'susceptible' and 'resilient' groups following social stress have
772 more recently fallen out of favor, given categorization of those responses are context-specific,
773 require substantial rodent numbers for optimal data confidence, and neither phenotype can be
774 universally deemed 'better' or 'worse' (Koolhaas et al., 2017; Lyons et al., 2023). We therefore
775 evaluated all SC mice as a single group. Still, bimodal distribution of social engagement in SC
776 mice looks possible in all but the different sex SC males. Increasing numbers to establish
777 definitive thresholds with sufficient power for phenotypic grouping could prove informative,
778 particularly for the same sex experiment. The same limitations as listed above would still apply,
779 however. Finally, our studies used a brief protocol spanning only four days, intended for high
780 throughput and maximal comparison to other social stress literature. We therefore cannot
781 speak to whether these outcomes persist for weeks or months, timeframes that are ethologically
782 relevant for social learning and memory. Once a short-term protocol has been optimized,
783 duration of effects would be a critical next step in determining validity of the paradigm.
784
785 Here, we have reported a novel foray into sex-inclusive efforts to develop a controllable, less
786 labor-intensive, and higher throughput social stress paradigm in mice. These experiments have
787 provided utilizable insights regarding how mice of both sexes associate a same- or different-sex
788 conspecific with an aversive experience. We report that these associations seem strongest in
789 males after same sex encounters, and in females after different sex encounters. Such
790 distinctive responses are probably evolutionarily favorable. While our investigation has
791 generated new information, our modified paradigm did not accomplish what we anticipated. We

792 share our negative findings here to encourage and assist other labs to achieve their own
793 positive outcomes, and to spare them (and mice) the time and effort of duplicating the present
794 work. Development of a sex-inclusive social stress paradigm for mice remains a worthwhile
795 pursuit, as such would be a boon across multiple disciplines. Perhaps the most salient
796 contribution our study provides to the broader literature is that future studies focused on socially
797 stressing females might utilize male conspecifics for optimal outcomes.

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